CARDIOMETABOLIC DISEASE IN SOUTH ASIANS

RISK FACTORS AND THERAPEUTIC STRATEGIES

Laura Janssen

Cardiometabolic disease in South Asians

Risk factors and therapeutic strategies

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1. ETIOLOGY OF CARDIOMETABOLIC DISEASE IN SOUTH ASIANS

The position of cardiometabolic disease in global health

Clustering of cardiometabolic risk factors, *e.g.* (visceral) adiposity, glucose intolerance, atherogenic dyslipidemia and hypertension, may culminate in clinical diseases including non-alcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D) and cardiovascular disease (CVD). The occurrence of these cardiometabolic diseases has risen progressively during the past decades, thereby imposing a major and costly burden on the worldwide health-care system. Moreover, CVD is the leading global cause of death, with 18 million annual deaths by ischemic heart disease and stroke (1). Notably, people from South Asian descent, who form one quarter of the total world population, are particularly susceptible to develop T2D and CVD. The main aim of this thesis is to elucidate yet unknown factors that could contribute to the disadvantageous metabolic phenotype in South Asians, in search for new treatment strategies against cardiometabolic diseases.

Migration patterns of Dutch Hindostani in the last century

In this thesis we use the terms 'South Asians' and 'Hindostani' interchangeably, as all subjects who have participated in our studies were Dutch Hindostani. The word 'Hindostani' represents descendants of 'Hindostan', which is the Hindi term for British-India. British-India used to be an English colony on the Indian subcontinent until **1947**, whereafter it was divided into the countries currently known as India and Pakistan. Other countries of the Indian subcontinent include Bangladesh, Bhutan, Nepal and Sri Lanka. Subjects descending from India who participate in studies conducted in Western countries, such as the United Kingdom, United States and Canada, predominantly represent offspring from educated Indian migrants and are commonly referred to as 'Asian-Indians'. In contrary, the Dutch Hindostani mainly represent offspring from Indian contract workers in Surinam (2).

Surinam is located in South America and was colonized by the Dutch until **1975**. Surinam thrived on plantations, especially sugarcane, coffee and cocoa, but expected a shortage of fieldworkers after slavery was abolished in **1863**. Other Caribbean islands and mainlands that were colonized by the British and French at that time had therefore already started contracting British-Indians as fieldworkers. In **1872** the Dutch followed by signing the Dutch-British Treaty Pact (in Dutch '*Koelietraktaat*'); an agreement stating the rights and obligations of indentured laborers. A massive migration followed with over 34,000 British-Indians arriving in Surinam between **1873** and **1916** (3). The Dutch-British Treaty Pact was terminated approximately 50 years after its initiation, whereafter one third of the British-Indians contract workers returned home and two thirds remained in Surinam. Aside from this British-Indian majority, the Surinamese

population consisted of a minority of Javanese inhabitants (contract workers descending from the Indonesian Island of Java), Creol and Marron inhabitants (descendants of former African slaves) and to a lesser extent Chinese inhabitants (4). A large migration wave consisting of almost 40,000 Surinamese inhabitants to the Netherlands occurred when Suriname became independent in **1975**. A second migration wave consisting of over 15,000 people occurred around the **1980**s due to political reasons. Lastly, a smaller third migration wave took place during the early **1990**s, which is believed to be due to poor economic circumstances (4).

Estimating the number of Dutch Hindostani and their offspring living in the Netherlands is not clear cut, since Statistics Netherlands (in Dutch '*Centraal Bureau voor de Statistiek*') registers immigrants based on nationality and not ethnicity. Based on the available data it is suggested that approximately 340,000 Surinamese live in the Netherlands, who are mostly located in Amsterdam, followed by Rotterdam, the Hague, Almere and Utrecht (**Figure 1**). In an attempt to further identify these Surinamese immigrants into ethnic subgroups, Statistics Netherlands classified subjects based on their family names. These analyses point towards 150,000 subjects being Hindostani, which is 1% of the total Dutch population. This includes people of both first and second generations Hindostani, *i.e.* contract workers and their children, respectively. There are also an estimated 10,000 third generation Hindostani living in the Netherlands, *i.e.* grandchildren of contract workers, but as they are born in the Netherlands they have the Dutch nationality (5).

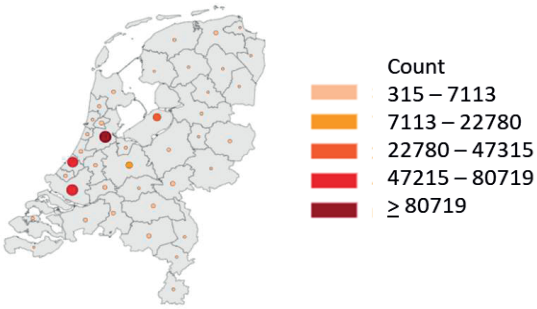

Figure 1. First and second generation Surinamese (Hindostani, Creoles, Javanese and Chinese) living in the Netherlands on January 1st 2019. Adapted from Statistics Netherlands (6).

Of note, from a socioeconomical point of view, Hindostani living in the Netherlands are successfully integrated, with a generally high level of education and employment. The Dutch Hindostani do remain strongly connected to each other, which has led to the establishment of a large and deeply rooted Hindostani community with various active organizations in the Netherlands (2). Fascinatingly, South Asians both in India

and after migration to Western countries have a disadvantageous metabolic phenotype compared to that of white Caucasians. Albeit various research groups around the world have committed to exploring this topic, additional dedicated studies are necessary to fully grasp the underlying genetic and environmental factors leading to the high rate of cardiometabolic disease in South Asians.

Thin-fat phenotype and dysregulated energy balance in South Asians

The aforementioned susceptibility of South Asians to develop T2D and CVD is likely due, at least in part, to their relatively high amount and unfavorably distributed body fat, which is present already from a young age on. We will discuss factors contributing to this unfavorable metabolic phenotype in South Asians further below in this chapter.

When energy intake is equal to expenditure, the energy balance is considered neutral and body weight is kept constant. When energy intake however exceeds expenditure, the energy balance turns positive and results in weight gain. After a prolonged period of time this leads to excessive weight gain in terms of obesity. This excess energy is mainly stored in the form of triglycerides in white adipose tissue (WAT). WAT consists of depots just beneath the skin ('cutis'), *i.e.* subcutaneous WAT, and smaller compartments surrounding internal organs ('viscera'), *i.e.* visceral WAT. On the long-term, a positive energy balance results in both cellular hypertrophy (enlargement of white adipocytes) and hyperplasia (recruitment of new white adipocytes from precursor cells), thereby expanding WAT depots (7). Enlarged WAT depots, especially visceral, then become more and more saturated with lipids, followed by an influx of immune cells causing local insulin resistance. This leads to an overflow of lipids towards nonfatty tissues, *e.g.* the liver, skeletal muscles, heart, kidneys and pancreas, resulting in the lipotoxic formation of 'ectopic fat' (8).

Excessive storage of triglycerides in visceral WAT and the formation of ectopic fat in particular have detrimental consequences for metabolic health, as both are positively associated with insulin resistance and atherosclerosis, and their progression into T2D and CVD, respectively (9, 10). Interestingly, the predominant WAT storage pool for lipids varies between individuals. For example, there is a preference for visceral over subcutaneous lipid deposition in males *vs* females and in aged *vs* young people, which may partly explain the independent predictive value of sex and age on cardiometabolic disease (11). In addition, fat distribution is strongly affected by genetics, and the effect of genetic variants on fat distribution differs between sexes (12). Possibly, genetic variants also contribute to an unfavorable fat distribution pattern in South Asians, with perhaps detrimental consequences for cardiometabolic health.

Intriguingly, from a young age on, people from South Asian descent have a relatively high amount of fat mass, as reflected by a higher ratio of fat mass over lean mass, compared with white Caucasians. This phenomenon is already present at birth, with

South Asian neonates having a small gesture and low body weight compared with white Caucasian neonates, as characterized by a low muscle mass and high fat mass; the 'thin-fat' body phenotype. These relatively high amounts of body fat in South Asians are mainly located in their trunk rather than their extremities, as repeatedly shown by a higher subscapular skinfold thickness in South Asian neonates born in India compared with white Caucasian neonates (13, 14). This difference in body fat distribution remains preserved in following generations of South Asian neonates born in Surinam (15) and the United Kingdom (16), and to some extent in the Netherlands (17). Moreover, magnetic resonance imaging (MRI) analyses showed that the amounts of visceral and deep subcutaneous adipose tissue are larger in South Asian compared with white Caucasian neonates (18). This relatively higher amount of body fat and unfavorable fat distribution pattern is maintained throughout life and during progression to obesity, as demonstrated in South Asian children (19-21) and adults (22-27) in the United Kingdom, United States and Canada. Furthermore, the amount of ectopic fat is higher in South Asians compared with white Caucasians, as measured by lipid deposition in skeletal muscle (intramyocellular lipid; IMCL) (28-30), and in the liver resulting in NAFLD (31-34). The congenital high amount of body fat in South Asians, especially located in visceral and ectopic depots, is likely partly responsible for the high risk and progressive nature of cardiometabolic disease in this population throughout life. An important question however remains what the underlying cause of the unfavorable fat distribution pattern is in South Asians.

Various evolutionary hypotheses aim to answer this key question. The essence of these hypotheses is based on the gain of specific traits that increase chances of survival under difficult environmental conditions, for example by accumulation of adipose tissue as energy storage after having experienced famine (35, 36). Although beneficial in times of a negative energy balance, this may lead to the development of adiposity and cardiometabolic consequences under more prosperous and food-rich conditions, such as upon urbanization in India and after migration to Western countries (37). The contribution of genetics to the increased risk of cardiometabolic disease in South Asians is not yet known, since the still limited studies to date have not yielded pronounced and consistent differences in relevant genetic variants in South Asians compared with white Caucasians (35, 38).

Especially in populations that are predisposed to cardiometabolic disease, a healthy lifestyle is of utmost importance to maintain a neutral energy balance and thereby prevent obesity and its cardiometabolic complications. Various lifestyle aspects embedded in the South Asian culture may contribute to a positive energy balance. For example, traditional South Asian dishes typically include dense foods that are high in caloric content, refined carbohydrates, saturated fats and trans-fats (especially from cooking oils and deep-frying), and low in unsaturated fats and fibers (39). Moreover, consuming

fairly large portions of such foods during social engagements and religious gatherings is part of the traditional South Asian culture (40). In addition to excessive intake of unhealthy foods, South Asians in India generally show sedentary behavior (41), and studies conducted in Western countries also show lower physical activity levels in South Asians compared with white Caucasians (42-44). Since physical activity increases muscle mass, little physical activity may contribute to the low fat-free mass observed in adolescent and adult South Asians. Since fat-free mass is the most important component of body composition determining energy expenditure (45), low physical activity levels may thereby contribute to a low energy expenditure in South Asians. Indeed, healthy young South Asians have a lower resting energy expenditure compared with white Caucasians (46). Notably, in this study resting energy expenditure remained significantly lower in South Asians after correction for fat-free mass, also suggesting a role for additional unknown factors. One factor might be energy-combusting brown adipose tissue (BAT), which was lower in South Asians compared with white Caucasians in this study (46). We thus propose that less amounts of active BAT could contribute to a positive energy balance and thereby negatively affect cardiometabolic health in South Asians.

Tissue-specific insulin resistance and type 2 diabetes in South Asians

Both native and migrant South Asians are at particularly high risk to develop T2D (47, 48). Moreover, South Asians show a more rapid progression of prediabetes to overt diabetes (49-51), develop T2D already at a lower BMI (52, 53) and do so on average 5 to 10 years earlier compared with white Caucasians (49). Importantly, in South Asians, glycated hemoglobulin (HbA1c) levels also increase more rapidly and the onset of micro-vascular complications, *e.g.* retinopathy and nephropathy, is accelerated compared with white Caucasians (49). The American Diabetes Association has therefore advised to lower the BMI threshold for diabetes screening, from 25 kg/m^2 applied for whites, to 23 kg/m^2 for Asian Americans (54). The primary defect in T2D is generally resistance of metabolic organs, mainly liver, skeletal muscle and WAT, to the effects of insulin. Insulin resistance leads to compensatory insulin hypersecretion by the pancreas, which eventually deteriorates β-cell function and thereby impairs insulin secretion, which is further aggravated by lipid accumulation in the pancreas. Circulating insulin levels are indeed higher in South Asian children and adults compared with white Caucasians, and coincide with lower insulin sensitivity (35, 49). Notably, insulin levels are also already higher in umbilical cord blood of South Asian compared with white Caucasian neonates (13, 55, 56). These data suggest that the foundation for the high risk of T2D in South Asians is laid already upon birth, probably even in utero, and remains preserved throughout life.

A relatively high amount of fat mass *vs* fat-free mass is notorious to promote insulin resistance (10, 57, 58). Since such a body composition is characteristic for South Asians, as previously described in this chapter, this likely contributes for a large part to the high

rate of insulin resistance and T2D in this population. Insulin sensitivity however also remains lower in South Asians after correction for the amount of body fat (30, 59, 60). Other factors than the relative amount of body fat therefore also seem to play a role in the high degree of insulin resistance in South Asians, such as their fat distribution pattern. The formation of ectopic fat in particular negatively affects insulin sensitivity. More specifically, hepatic lipid deposition is detrimental for glucose homeostasis, and a clear negative effect of NAFLD on glucose metabolism and insulin sensitivity has been shown (10), also in South Asians (31, 32, 61). Ectopic lipid deposition in skeletal muscle may play only a limited role in the high degree of insulin resistance in South Asians, since no correlation between IMCL accumulation and insulin sensitivity in this ethnicity has been shown to date (30, 62). It may however also be the specific location of lipid deposition within the skeletal muscle rather than the total amount of IMCL that affects myocellular insulin sensitivity (10). To date, only one study demonstrated reduced gene expression and protein levels of components of the insulin signaling pathway in skeletal muscle in South Asians (60). To summarize, there is ample data suggesting that the relatively large amount of body fat and especially its ectopic deposition in the liver contributes to the high degree of insulin resistance in South Asians, whereas evidence for the involvement of skeletal muscle is scarce.

The metabolic characteristics of adipocytes also affect their lipid storage capacity and insulin sensitivity. The lipolysis rate is increased in hypertrophic subcutaneous adipocytes, resulting in an overspill of fatty acids towards visceral WAT compartments and nonfatty tissues, especially in the context of inflammation caused by infiltrating immune cells. Additionally, a large size of abdominal adipocytes is associated with insulin resistance and T2D (63). Subcutaneous abdominal adipocytes are indeed larger in South Asians compared with white Caucasians (32, 64, 65), and negatively associate with the glucose disposal rate in South Asians as demonstrated previously by the euglycemic-hyperinsulinemic clamp technique (64). Furthermore, a high amount of small and less differentiated adipocytes is considered an unhealthy form of WAT expansion and is associated with an impaired intracellular lipid storage capacity and insulin resistance (66, 67). There is indeed evidence pointing towards less matured subcutaneous adipocytes in South Asians compared with white Caucasians (65, 68). These studies suggest that unfavorable adipocyte characteristics and a potentially infiltrated immune cell pattern also contribute to the high degree of insulin resistance in South Asians. Further research investigating intracellular signaling pathways in metabolic tissues that may be altered in South Asians are needed. One interesting pathway that is involved in adipogenesis and insulin resistance is Wnt signaling, which is mainly known for its osteoanabolic effects by promoting differentiation of precursor cells into osteoblasts rather than adipocytes (69). Since impairing mutations in the Wnt signaling pathway are linked to both adiposity (70) and diabetes in humans (71), it would be interesting to investigate whether Wnt

signaling is reduced in South Asians and could thereby contribute to fat accumulation and insulin resistance in this population.

In addition to the key role of WAT, the previously mentioned lifestyle factors may be implicated in the high rate of insulin resistance and its progression to T2D in South Asians. Importantly, physical activity and cardiorespiratory fitness levels, *i.e.* the supply of oxygen to skeletal muscle during physical activity, are inversely associated with insulin resistance, and are indeed lower in South Asians compared with white Caucasians (24, 60). Promoting physical activity could therefore be a successful treatment strategy to improve cardiometabolic health in South Asians, and regular exercise was indeed shown to reduce diabetes risk (72), also in South Asians (73). Underlying mechanisms include stimulating non-insulin-mediated glucose uptake by skeletal muscle and improving cardiorespiratory fitness, as well as improving whole-body insulin sensitivity, also independent from weight loss (72, 74). In addition to stimulating physical activity and exercise, promoting healthy dietary habits has been shown to reduce diabetes risk, also in South Asians (73). This includes limiting caloric intake, increasing the intake of healthy dietary components such as fibers and unsaturated fatty acids, and minimizing the intake of saturated fats, added sugars and processed foods (75, 76), which could be particularly relevant for those consuming typical South Asian foods.

Atherosclerotic cardiovascular disease in South Asians

In addition to their exceptionally high risk of developing T2D, South Asians are at increased risk to develop atherosclerotic CVD compared with other ethnic groups, including other Asians and whites (38, 47). Similar to T2D, CVD occurs earlier in South Asians compared with white Caucasians, with a first myocardial infarction presenting on average 10 years earlier in South Asians (77). Moreover, once CVD has established, South Asians have a higher hospitalization rate and mortality risk compared with whites (38). T2D is a well-known risk factor for CVD (78), and the high occurrence of T2D in South Asians is therefore likely an important mediator of their increased CVD risk (77, 79, 80). Furthermore, the aforementioned adiposity in South Asians likely plays a role, as this does not only negatively affect glucose regulation but also promotes other classical CVD risk factors including hypertension and dyslipidemia (81, 82). This holds true especially for the visceral WAT depot, as the waist-to-hip ratio is strongly independently positively associated with the risk of myocardial infarction in South Asians (77, 83). Furthermore, adiposity promotes CVD by directly affecting the cardiovascular system via secreting pro-inflammatory cytokines. Interestingly, there is a fat depot directly surrounding the heart, *i.e.* epicardial fat, which is located between the myocardium and the visceral layer of the pericardium, that secretes a variety of adipokines, cytokines and other factors that promote atherosclerosis and thereby negatively impact cardiovascular function (82). Notably, the volume of this fat depot is higher in South Asians compared with

white Caucasians (84). Epicardial adipose tissue might therefore be involved in the pronounced atherosclerosis in South Asians, and therefore it would be highly interesting to investigate whether this fat depot indeed possesses more pro-inflammatory and pro-atherogenic characteristics in South Asians compared with white Caucasians. Collecting biopsies from this region is however difficult for ethical reasons.

In addition to adiposity, one of the most important classical risk factors for CVD is dyslipidemia (85-87). South Asians are particularly susceptible to develop dyslipidemia, in particular high triglyceride levels and low HDL-cholesterol levels (88-90), and often show a trend towards increased LDL-cholesterol levels (88). Small dense LDL-particles are enriched in triglycerides and are strong predictors of atherosclerotic CVD (91, 92). Therefore, assessing the quality and quantity of LDL particles rather than measuring total LDL-cholesterol levels has gained much interest in terms of predicting CVD. Specifically, levels of apoB, a protein present on atherogenic lipoproteins, are strongly positively associated with the risk of and mortality from atherosclerotic CVD (91). Such atherogenic dyslipidemia is also generally present in South Asians, as multiple studies have shown that South Asians have more small dense LDL particles (93), higher apoB levels (94) and a higher apoB/apoA-I ratio (94-96) compared with white Caucasians. Furthermore, lipoprotein(a) (Lp(a)), a lipoprotein structurally similar to LDL but containing an additional apo(a) (97), is an independent risk factor for CVD (98). Also in South Asians, Lp(a) levels are often higher compared with whites (85, 99-102) and are strongly positively associated with CVD risk (103, 104). Since the Lp(a) concentration is strongly genetically determined (105), genetic variants probably explain the increased Lp(a) levels in South Asians. Altogether, atherogenic dyslipidemia may contribute for an important part to the susceptibility of South Asians to develop CVD. Interestingly, the susceptibility of LDL to aggregate has recently been described as a novel risk factor for CVD (106), but differences in LDL aggregation between ethnicities are currently unknown.

The lifestyle factors described previously in this chapter also likely contribute to the high CVD risk in South Asians. A sedentary lifestyle and unhealthy diet stimulate development and progression of CVD via promoting classical risk factors such as adiposity, diabetes and dyslipidemia. Targeting these lifestyle factors by increasing physical activity levels and implementing a healthy diet reduces CVD risk and mortality, also independently from their beneficial effects on glucose regulation, albeit future studies specifically including South Asians are warranted (47, 78). Not unimportantly, tobacco smoking accelerates atherosclerosis via various mechanisms, and active smoking even doubles 10-year CVD mortality and reduces life expectancy with an average of 10 years (47). Also in South Asians smoking is the most important risk factor for myocardial infarction (38), and quitting smoking is the most cost-effective evidence-based lifestyle change to attenuate CVD risk (47). Since tobacco products are frequently used in the

South Asian culture (38), quitting smoking is particularly important to lower the risk and burden of CVD in South Asians.

The combined risk factors for T2D and CVD in South Asians described in this paragraph are summarized in **Figure 2**.

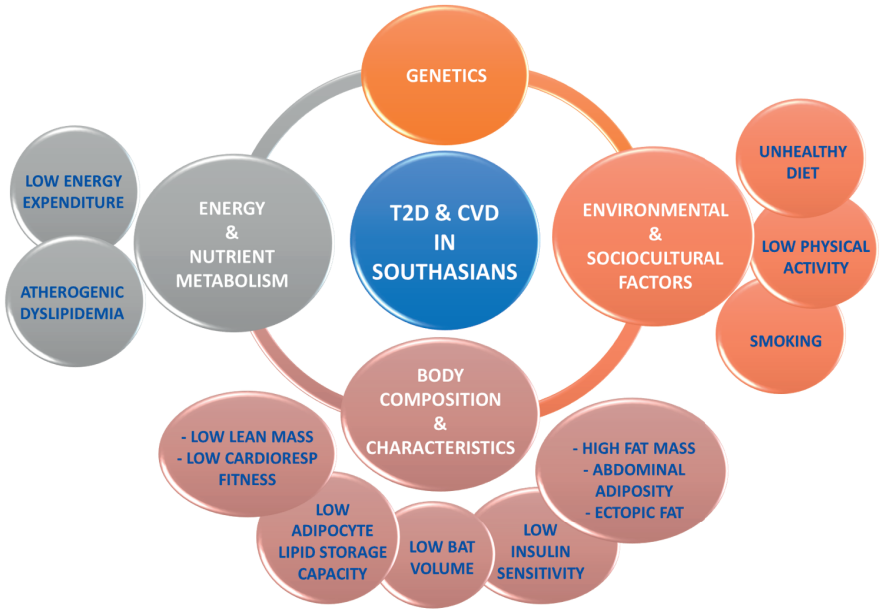

Figure 2. Risk factors for type 2 diabetes (T2D) and cardiovascular disease (CVD) in South Asians

2. TARGETING CARDIOMETABOLIC DISEASE IN SOUTH ASIANS: BROWN ADIPOSE TISSUE ACTIVATION

Brown adipose tissue physiology

Lowering body weight by inducing a negative energy balance is an effective strategy to reduce obesity and its cardiometabolic complications. A negative energy balance can be achieved by lowering food intake, which is however notoriously difficult to adhere to, especially in the current society with 24/7 access to excessive amounts of unhealthy foods, unfortunately often resulting in weight regain on the long term. Increasing energy expenditure, for example by performing exercise, can therefore aid in successfully maintaining a healthy body weight. In addition, stimulating energy-combusting BAT activity is a promising treatment strategy to further enhance energy expenditure. BAT and WAT form the two main distinct types of adipose tissue in humans. As described previously in this chapter, WAT is abundantly represented in humans and stores excess energy contained in fatty acids and glucose in the form of triglycerides. In contrary to

WAT, BAT is present in much lower amounts in humans, with an estimated average of 50-150 mL in adults (107). BAT is located mainly in the neck area and around the aorta in adult humans (108), likely in order to efficiently distribute its generated heat throughout the body. Whereas white adipocytes have one unilocular lipid droplet and sparse mitochondria, brown adipocytes are characterized by multilocular (multiple small) lipid droplets and many mitochondria that express the unique protein uncoupling protein-1 (UCP-1). In addition, scattered within WAT, so-called 'beige' or 'brite' adipocytes exist which have an intermediate and flexible phenotype. Under basal conditions these adipocytes morphologically resemble white adipocytes, however under (cold) stimulated conditions they gain a brown-like phenotype with multilocular lipid droplets and increased numbers of mitochondria high in UCP-1 expression (109).

BAT burns fatty acids and glucose into heat in order to maintain core body temperature under cold conditions (110). Firstly, when cold is sensed by transient receptor potential channels in nerve endings in the skin, a signal is forwarded to the hypothalamic temperature regulating center. This promotes sympathetic outflow, which stimulates the release of noradrenalin by nerve endings that densely innervate brown adipocytes. Noradrenalin subsequently binds to β-adrenergic receptors (β-ARs) on these brown adipocytes, inducing an intracellular signaling cascade that promotes hydrolysis of triglycerides stored within the intracellular lipid droplets. The fatty acids that are then released enter the mitochondria to stimulate UCP-1-mediated uncoupled respiration, thereby preventing ATP generation and resulting in dissipation of energy as heat (110). Upon prolonged stimulation, the intracellular lipid stores of brown adipocytes become depleted and need to be replenished. Replenishment occurs via the uptake of circulating fatty acids and glucose by BAT, the latter probably mainly for de novo lipogenesis (107). BAT takes up fatty acids mainly via lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride-rich lipoproteins (TRLs) (111). The uptake of TRL-derived fatty acids by metabolic tissues, such as BAT and WAT, is under strict regulation of several factors, including the family of angiopoietin-like proteins (ANGPTLs) of which the LPL inhibitor ANGPTL4 is the most studied. Mouse studies established that cold exposure regulates *Angptl4* expression in a tissue-specific manner, *i.e.* being increased in WAT and decreased in BAT. As a result, during cold exposure, TRLs are shuttled away from WAT towards heat-producing BAT for LPL-mediated hydrolysis and subsequent uptake of fatty acids (112). Furthermore, the novel family members ANGPTL3 and ANGPTL8 are linked to T2D and CVD (113). How these ANGPTLs exactly affect not only lipid distribution but also glucose regulation however still warrants further investigation. For example, the effect of cold exposure on ANGPTL3 and ANGPTL8 levels in humans is yet unknown, which would be especially interesting to investigate in insulin-resistant subjects such as South Asians. Stimulating BAT thermogenesis is a potentially promising treatment strategy to target obesity, hyperlipidemia and hyperglycemia, via increasing energy expenditure and

stimulating the uptake of circulating lipids and glucose, respectively (114). We propose that this holds true especially for populations with a high prevalence of cardiometabolic disease, such as South Asians.

Brown adipose tissue activation to improve cardiometabolic health

Only a decade ago it was discovered that adult humans have metabolically active BAT that takes up glucose during cold exposure, as measured by ^{18}F-fluorodeoxyglucose ([^{18}F]FDG) positron emission tomography computed tomography (PET/CT) scan (115-117). Since then, much research has focused on determining BAT substrate uptake preferences (107) and optimizing BAT visualization methods (118). For example, MRI is a promising imaging method which can differentiate BAT from WAT based on its lower amount of intracellularly stored triglycerides and higher amount of water, *i.e.* the 'fat fraction'. The fat fraction lowers when active BAT utilizes its triglycerides for thermogenic purposes (119). Since humans possess large amounts of WAT and only little BAT, in contrast to rodents, it is of specific interest to study the possibility of stimulating beige adipocytes to gain a brown-like phenotype to enhance their thermogenic capacity, *i.e.* 'browning'. The ultimate goal is to discover strategies with the potential to activate BAT that could thereby improve cardiometabolic health (114).

From the beginning of this BAT era, clinical trials have focused on cold exposure as a potent method to activate and recruit BAT, mostly in healthy young subjects, and to a lesser extent in overweight or obese older subjects with (120) or without T2D (121). Since cold acclimation is however not a particularly attractive treatment option for humans, studies have also focused on other lifestyle interventions, such as adjusting dietary composition (114). Of all the investigated dietary components, most evidence pointing towards browning has been derived from the use of polyphenols. Polyphenols consist of a group of natural occurring compounds that are present in amongst others fruits and vegetables, whole grains and chocolate (122). Interestingly, various polyphenols enhance browning and improve metabolism in mice. Moreover, in humans, the polyphenol subgroups of capsinoids, *i.e.* analogues of capsaicine that are naturally present in red chili peppers, and green tea catechins also increase energy expenditure, promote lipid oxidation, and enhance BAT activity as mostly demonstrated by [^{18}F]FDG-PET/CT scan (114). These results were however not always consistent (123), and it remains to be studied whether dietary compounds in feasible amounts for human consumption also improve cardiometabolic health on the long term. In addition to cold exposure and dietary compounds, promoting physical activity has been a treatment strategy of interest aiming to stimulate BAT activity and browning of WAT. Numerous studies in rodents indicate that exercise stimulates browning of WAT. The effect of exercise on BAT in rodents is however controversial, which also holds true for the effect of exercise on thermogenic tissues in humans (114).

Parallel to studies investigating the effect of lifestyle interventions on BAT activity and browning of WAT, the urge for more effective treatments has stimulated the search for pharmacological agents with such properties. A key paper from Cypess *et al.* (124) showed that a single dose of the β_3-AR agonist mirabegron increases energy expenditure and [^{18}F]FDG uptake by BAT in healthy young subjects. Mirabegron is prescribed in clinical practice to patients with overactive bladder disease due to its relaxation effect on smooth muscle tissue. The dosage prescribed in clinical practice is however 4 times lower (50 mg) than was needed to show effects on energy expenditure and BAT activity (200 mg) in this proof-of-principle study (124). This may explain, at least in part, why other β_3-AR agonists in previous clinical trials did not clearly improve the metabolic phenotype (125-127). Promising, a recent study from this same group showed that treatment of healthy young women for a prolonged period (4 weeks) with mirabegron in a dose of 100 mg per day increased metabolically active BAT, as measured by [^{18}F]FDG-PET/CT, and resting energy expenditure, and even improved markers for whole-body insulin sensitivity, pancreatic β-cell insulin secretion and glucose disposal (128). These data further support that β-AR agonism has the potential to improve energy metabolism, possibly in part via increasing nutrient uptake by BAT. It remains to be elucidated how β-AR agonism exactly affects the various pathways in lipid metabolism and whether it truly increases lipid uptake and thermogenesis by BAT in humans. Furthermore, it would be relevant to determine whether the effect of β-AR agonism is different in cardiometabolically compromised patients, for example in South Asians. Another promising treatment strategy that improves cardiometabolic health possibly in part via BAT activation, is agonism for the glucagon-like peptide-1 (GLP-1) receptor. GLP-1 is an incretin hormone that is produced and released into the blood by intestinal L-cells after food intake, and subsequently stimulates pancreatic insulin secretion to lower postprandial blood glucose levels. Once entering the blood, GLP-1 is degraded within minutes by the enzyme dipeptidyl peptidase-4 (DPP-IV) (129), which has led to the development of DPP-IV inhibitors that increase endogenous GLP-1 levels. In fact, both GLP-1 receptor agonists and DPP-IV inhibitors are successfully used in clinical practice to lower blood glucose levels in patients with T2D. Interestingly, these compounds exert many beneficial metabolic effects in addition to lowering blood glucose levels, including a reduction in body weight, lowering of lipid levels, increase in lipid utilization and possibly also an increase in energy expenditure (129-132). Notably, we (133) and others (130) have shown that central agonism of the GLP-1 receptor in mice stimulates sympathetic outflow towards BAT and increases glucose and lipid uptake by BAT, which is accompanied by a reduction in body weight and blood glucose and lipid levels. BAT activation therefore likely mediates part of these beneficial effects on cardiometabolic parameters. Whether GLP-1 receptor agonism also stimulates BAT activity in humans is however still unknown, which could be especially relevant for South Asians. The various

lifestyle factors and pharmacological compounds described in this paragraph that aim to activate BAT and thereby improve cardiometabolic health are summarized in **Figure 3**.

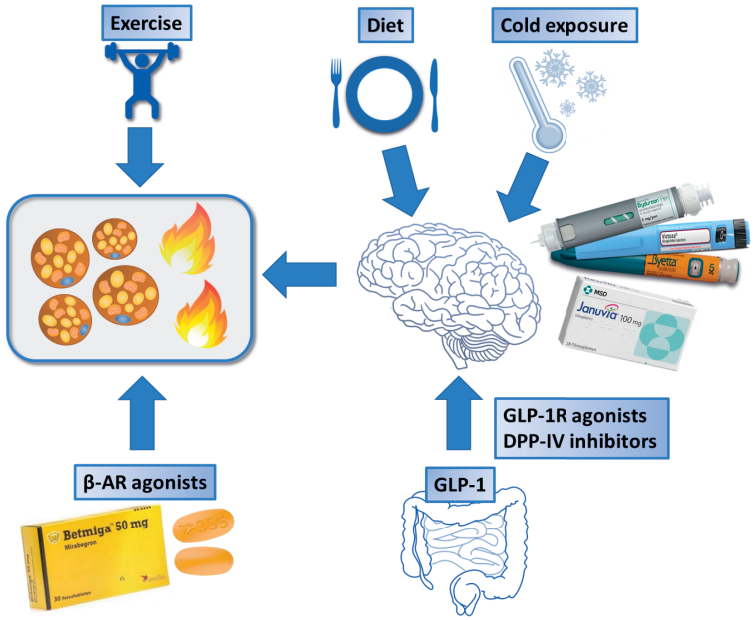

Figure 3. Lifestyle factors and the promising pharmacological agents of beta-adrenergic receptor (β-AR) agonists, glucagon-like peptide-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 (DPP-IV) inhibitors as treatment strategies to activate BAT and brown WAT.

OUTLINE OF THIS THESIS

In **this chapter** we described the etiology of the cardiometabolic epidemic that the world is currently facing. Notably, people from South Asian descent are particularly susceptible to develop T2D and CVD compared with other ethnicities including whites, and suffer to a disproportionally large extent from morbidity and mortality due to these diseases. Aside from having many classical cardiometabolic risk factors, South Asians also possess additional risk factors that may explain at least part of their high cardiometabolic risk. Treatment of T2D and CVD is of utmost importance due to their burden on global health care, and therapies should primarily be aimed at preventing their onset by targeting cardiometabolic risk factors. One currently widely investigated and potentially promising treatment strategy to improve energy metabolism is stimulating BAT thermogenesis. In the studies described in this thesis, we therefore firstly aimed to further unravel underlying mechanisms contributing to T2D and CVD particularly

in South Asians. We then aimed to explore the potential of different pharmacological agents to activate BAT in South Asians compared with white Caucasians.

Firstly, in **chapter 2** we investigated the effect of cold exposure on a subset of ANGPTLs. ANGPTLs are proteins involved in lipid trafficking between metabolic tissues. In this chapter we aimed to explore cold-induced changes in plasma levels of ANGPTLs and their relation with lipid and glucose levels and BAT activity. To investigate how age and unfavorable metabolic characteristics, *e.g.* adiposity and insulin resistance, affect the cold-induced response in ANGPTLs, we also compared these observations between healthy young lean and middle-aged overweight prediabetic subjects. Moreover, since we previously observed a lower cold-induced increase in serum free fatty acids in South Asians compared with white Caucasians, possibly indicating less sympathetic outflow in this ethnicity, we also compared the effect of cold on ANGPTLs between South Asians and white Caucasians. We then focused on Wnt signaling, a cell signaling transduction route which is mainly known for its role in oncogenesis and embryonic development. Since mutations in Wnt genes and blood levels of Wnt family proteins have also been linked to glucose regulation and diabetes, with evidence suggesting interaction between Wnt and insulin signaling in white adipocytes, in **chapter 3** we studied whether Wnt signaling is impaired in South Asians compared with white Caucasians. To this end we assessed expression of Wnt signaling genes in skeletal muscle and subcutaneous abdominal WAT biopsies and plasma levels of the Wnt inhibitor sclerostin, as well as the relation of these parameters with measures for insulin signaling in overweight prediabetic South Asian and white Caucasian men. **In chapter 4**, we continued aiming to unravel underlying mechanisms that could contribute to the excess CVD risk in South Asians. It was recently shown that LDL particle aggregation predicts future cardiovascular events in patients with CVD. We therefore assessed LDL aggregation susceptibility in healthy young South Asians and compared this with white Caucasians, as a possible predictor for future CVD.

We then shifted our focus in **chapters 5** and **6** towards investigating the ability of pharmacological agents to improve cardiometabolic health by stimulating BAT activity in South Asians and white Caucasians. More specifically, in **chapter 5**, we performed a placebo-controlled randomized trial evaluating the effect of the β_3-AR agonist mirabegron on BAT activity and energy expenditure compared with cold exposure in South Asian and white Caucasian men. We measured the effect of mirabegron on BAT activity by means of its fat fraction with MRI. We additionally investigated the effect of mirabegron and cold exposure on serum lipidomics, aiming to gain mechanistic insight into the changes in different lipid species during BAT activation. Next, in **chapter 6** we studied the effect of GLP-1 receptor agonism on BAT activity. We hypothesized that GLP-1 receptor agonism stimulates BAT activity in humans, since it was shown in mice that central agonism for the GLP-1 receptor stimulates thermogenesis and increases lipid and glucose uptake by BAT. We therefore evaluated the effect of the GLP-1 receptor ago-

nist exenatide on BAT activity and energy expenditure in healthy young South Asian and white Caucasian men, in addition to its effects on body composition and blood glucose and lipid levels. In this study we visualized BAT by measuring its glucose uptake via [^{18}F] FDG-PET/CT scan as well as by assessing its fat fraction with MRI. Lastly, in **chapter 7** the results of our studies are put in the context of the available literature to date and future perspectives for our field of research are discussed.

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2

Short-Term Cooling Increases Plasma ANGPTL3 and ANGPTL8 in Young Healthy Lean Men but not in Middle-Aged Men with Overweight and Prediabetes

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ABSTRACT

Angiopoietin-like proteins (ANGPTLs) regulate triglyceride (TG)-rich lipoprotein distribution via inhibiting TG hydrolysis by lipoprotein lipase in metabolic tissues. Brown adipose tissue combusts TG-derived fatty acids to enhance thermogenesis during cold exposure. It has been shown that cold exposure regulates ANGPTL4, but its effects on ANGPTL3 and ANGPTL8 in humans have not been elucidated. We therefore investigated the effect of short-term cooling on plasma ANGPTL3 and ANGPTL8, besides ANGPTL4. Twenty-four young, healthy, lean men and 20 middle-aged men with overweight and prediabetes were subjected to 2 hours of mild cooling just above their individual shivering threshold. Before and after short-term cooling, plasma ANGPTL3, ANGPTL4, and ANGPTL8 were determined by ELISA. In young, healthy, lean men, short-term cooling increased plasma ANGPTL3 (+ 16%, $p < 0.05$), ANGPTL4 (+ 15%, $p < 0.05$), and ANGPTL8 levels (+ 28%, $p < 0.001$). In middle-aged men with overweight and prediabetes, short-term cooling only significantly increased plasma ANGPTL4 levels (+ 15%, $p < 0.05$), but not ANGPTL3 (230 ± 9 vs. 251 ± 13 ng/mL, $p = 0.051$) or ANGPTL8 (2.2 ± 0.5 vs. 2.3 ± 0.5 µg/mL, $p = 0.46$). We show that short-term cooling increases plasma ANGPTL4 levels in men, regardless of age and metabolic status, but only overtly increases ANGPTL3 and ANGPTL8 levels in young, healthy, lean men.

INTRODUCTION

Increased plasma triacylglycerol (TG) levels are an independent risk factor for cardiovascular disease [1]. TG is either derived from dietary lipids or synthesized by the liver and white adipose tissue (WAT) from glucose and carried in the circulation within TG-rich lipoproteins (TRLs). TRLs can be hydrolysed by lipoprotein lipase (LPL) on endothelial cells to provide underlying oxidative tissues with fatty acids (FA) as fuel during increased energy demands (fasting or exercise) or to increase lipid storage in WAT during nutrient excess [2].

As energy needs can rapidly change, the LPL-mediated clearance of TRL-derived TG is under strict regulation of several factors including lipoprotein-associated apolipoproteins (e.g., APOC2 and APOC3) and angiopoietin-like proteins (ANGPTLs) [3]. ANGPTLs consist of a family of multifunctional glycoproteins, of which ANGPTL3, ANGPTL4, and ANGPTL8 inhibit LPL activity and work in concert to regulate lipoprotein metabolism [4]. Loss-of-function mutations in either one of these proteins are associated with a favourable lipid profile including lower TG levels in humans [5–8]. Moreover, deficiency for either one of these proteins in mouse models results in lower plasma TG levels, whereas overexpression leads to hypertriglyceridemia [9–13].

A recently identified novel player in TG metabolism is brown adipose tissue (BAT). Mouse studies have shown that activating BAT reduces plasma TG [14], mainly via LPL-mediated processing of TRLs [15] and alleviates dyslipidemia and atherosclerosis [16]. Therefore, BAT activation is currently widely investigated as potential treatment strategy aiming to improve cardiometabolic diseases in humans [17]. The main function of BAT is to generate heat by combustion of intracellular lipids to maintain core body temperature, and the LPL-dependent FA influx into activated brown adipocytes is required to replenish these intracellular lipid stores. The most potent physiological stimulus of BAT activation is cold exposure, which results in sympathetic activation of β-adrenergic receptors on brown adipocytes [18].

Cold exposure was previously shown to affect ANGPTL4 expression in adipose tissue in mice and to increase circulating ANGPTL4 levels in men [19,20]. The effects of cold exposure on ANGPTL3 and ANGPTL8 in humans have not been established as yet. We, therefore, investigated the effect of short-term cooling on plasma ANGPTL3 and ANGPTL8, in comparison with ANGPTL4, in relation to changes in lipid metabolism in young, healthy, lean men as well as middle-aged men with overweight and prediabetes

MATERIAL AND METHODS

Study Design and Participants

In this study, blood samples of two clinical trials were used; one consisting of a cohort of young, healthy, lean men [21] (Dutch Trial Register 2473) and one consisting of a cohort of middle-aged men with overweight and prediabetes [22] (Clinicaltrials.gov NCT02291458). Both studies were approved by the Medical Ethical Committee of the LUMC and conducted in accordance with the principles of the revised Declaration of Helsinki (2013) and the Medical Research Involving Human Subjects Act (WMO). All subjects signed written informed consent prior to participation.

The study setup of the young, healthy, lean cohort was described in detail elsewhere [21]. In short, the study investigated the effect of cold exposure on brown adipose tissue and energy metabolism in 24 healthy, lean (BMI < 25 kg/m^2) men, aged 18–28 years, of white Caucasian (N = 12) and South Asian (N = 12) descent. Subjects were included between March 2013 and June 2013. After an overnight fast, body fat percentage was measured by dual-energy X-ray absorptiometry (iDXA, GE Healthcare, UK). A blood sample was collected at thermoneutrality and at the end of an individualized water-cooling protocol, and here analyzed for plasma ANGPTL3, ANGPTL4, ANGPTL8, and serum lipids. The individualized cooling protocol consisted of approximately 2 hours mild cooling between 2 water-perfused blankets; 1 above and 1 beneath the study subject (Blanketrol III, Cincinnati Sub-Zero Products, Cincinnati, OH, USA). The individualized cooling protocol started at 32 °C and the water temperature was gradually decreased until shivering occurred, after which the temperature was increased by 3–4 °C to stop shivering. Hereafter, an [^{18}F]FDG-PET/CT scan was performed to quantify BAT volume and glucose uptake.

An elaborate description of the study setup of the middle-aged overweight prediabetic cohort can be found elsewhere [22]. Briefly, 20 middle-aged (40–55 years) white Caucasian (N = 10) and South Asian (N = 10) men with overweight or obesity (BMI 25–35 kg/m^2) and prediabetes were included in a randomized double-blind cross-over study, evaluating the effect of *L*-arginine on brown adipose tissue and energy metabolism. Subjects were included between October 2014 and June 2015. Prediabetes was defined according to ADA criteria as having either fasting plasma glucose levels between 5.6–6.9 mmol/L or plasma glucose levels 2 hours after an oral glucose tolerance test between 7.8–11.1 mmol/L [23]. Two South Asian subjects used simvastatin 40 mg once daily. While subjects received either L-arginine or placebo for 6 weeks, in the current study only data after ingestion of the placebo were used. Placebo tablets consisted of a mixture of pregelatinized maize starch, microcrystalline cellulose, and magnesium stearate. Placebo supplements were divided over 3 gifts: after breakfast, lunch, and dinner. On the following day after placebo intake, after 4 hours of fasting in the morning, a blood

sample was collected under thermoneutral conditions. Hereafter, an individualized mild cooling protocol lasting approximately 2 hours was initiated, after which another blood sample was collected. The individualized cooling protocol consisted of gradually lowering the water temperature until just above the shivering point of the subjects, here being wrapped in a water-perfused suit (ThermaWrap Universal 3166, MTRE Advanced Technologies, Yavne, Israel). In the current study, we assessed these blood samples for serum lipids and plasma ANGPTL3, ANGPTL4, and ANGPTL8 as well as plasma glucose and insulin. The following day after the cooling experiment, body fat percentage was determined with DXA (Discovery A, Hologic, Bedford, MA, USA).

In both the young, healthy, lean cohort and middle-aged overweight prediabetic cohort, subjects were instructed not to exercise more than 3 times per week and to refrain from exercise prior to the experimental day. In both study cohorts, subjects were also instructed to not change their dietary habits and to consume a standardized evening meal prior to the experimental day.

Serum and Plasma Analyses

Plasma samples of both the young, healthy, and lean and middle-aged overweight prediabetic cohorts were analysed for ANGPTL3 [24] and ANGPTL8 [25] by in-house developed ELISAs. For the ANGPTL8 ELISA, in brief, antibodies against multiple synthesized ANGPTL8 peptides were chosen from different parts of the ANGPTL8 protein molecule. Rabbit R355 antibodies against the ANGPTL8 amino acid region 54–68 were combined with the horseradish peroxidase-labelled capture antibody against the ANGPTL8 peptide region 182–196 (rabbit R360) [25]. Plasma samples were also analysed for ANGPTL4 with a commercial ELISA assay (R&D Systems, Minneapolis, MN, USA). The intra- and inter-assay coefficients of variation for ANGPTL3 and ANGPTL4 were <15% [24]. Precision or intra- and inter-assay CVs for ANGPTL8 were approximately 10%. Serum TG and free fatty acid (FFA) concentrations were determined with enzymatic kits in both the young, healthy, lean cohort (Roche Diagnostics, Woerden, the Netherlands and Wako Chemicals, Neuss, Germany, respectively) and the middle-aged overweight prediabetic cohort (ABX Pentra 400 autoanalyzer, HORIBA Medical, Montpellier, France). In the middle-aged overweight prediabetic cohort, plasma glucose was also measured with an enzymatic kit (ABX Pentra 400 autoanalyzer, HORIBA Medical, Montpellier, France) and plasma insulin via a commercially available radioimmunoassay kit (Human Insulin-specific Radioimmunoassay, Millipore Corporation, Burlington, MA, USA). Four young, healthy, lean subjects and 1 middle-aged subject with overweight and prediabetes were excluded from this study due to absent plasma samples.

Statistical Analysis

Statistical analyses were performed with SPSS Statistics version 25 for Windows (IBM, Armonk, NY, USA). Baseline characteristics were compared between cohorts and ethnicities with a two-tailed unpaired student's *t*-test, and with a two-way mixed-effect ANOVA in case temperature was an additional factor. A linear mixed-model analysis was performed with cohort, ethnicity, and temperature modelled as fixed effects, to investigate the effect of cold exposure on ANGPTLs both within and between cohorts and ethnicities. Temperature was additionally modelled as a random effect with intercepts and an unstructured covariance type. Correlations between changes in plasma ANGPTLs and serum lipids were performed using linear regression analysis and were assessed for interaction of ethnicity. Data are presented as mean ± SEM, unless stated otherwise. A p-value < 0.05 was considered statistically significant. No correction for multiple testing was applied.

RESULTS

Clinical Characteristics

Clinical characteristics of the healthy lean cohort as well as effects of short-term cooling on serum lipids and other metabolic parameters have been described elsewhere [21]. In brief, subjects were 24 ± 1 years of age, had a BMI of 21.9 ± 0.4 kg/m^2, and a body fat percentage of 21.4 ± 1.2% (**Table 1**). Short-term cooling increased both serum TG (+ 0.22 ± 0.06 mmol/L, $p < 0.01$) and FFA levels (+ 0.19 ± 0.05 mmol/L, $p < 0.001$; **Table 1**). Similar observations were made when taking into account South Asian and white Caucasian ethnicities separately (**Table S1**).

Clinical characteristics for the middle-aged men with overweight and prediabetes have also been described in detail elsewhere [22]. Compared with the young, healthy, lean men, middle-aged overweight prediabetic subjects were older (47 ± 2 vs. 24 ± 1 years, $p < 0.001$), had a higher BMI (30.6 ± 0.8 vs. 21.9 ± 0.4 kg/m^2, $p < 0.001$) and a higher body fat percentage (30.9 ± 0.9 vs. 21.4 ± 1.2%, $p < 0.001$) (**Table 1**). In addition, compared with the young, healthy, lean men, FFA levels were lower (0.54 ± 0.04 vs. 0.84 ± 0.08 mmol/L, $p < 0.01$) and TG levels higher (1.56 ± 0.14 vs. 0.87 ± 0.10 mmol/L, $p < 0.001$) in middle-aged overweight prediabetic subjects (**Table 1**). Short-term cooling increased serum TG (+ 0.18 ± 0.04 mmol/L, $p < 0.001$) but not FFA levels (+ 0.06 ± 0.04 mmol/L, $p = 0.11$) in middle-aged men with overweight and prediabetes. Similar observations were made when taking into account South Asian and white Caucasian ethnicities separately (**Table S1**).

Table 1. Clinical characteristics.

Clinical characteristics	Young healthy lean men (N = 20)	Middle-aged overweight prediabetic men (N = 19)
Age (years)	24 ± 1	47 ± 2 ***
Height (m)	1.79 ± 0.02	1.78 ± 0.01
Weight (kg)	70.6 ± 2.1	96.9 ± 2.9 ***
BMI (kg/m^2)	21.9 ± 0.4	30.6 ± 0.8 ***
Body fat percentage	21.4 ± 1.2	30.9 ± 0.9 ***
Thermoneutral TG (mmol/L)	0.87 ± 0.10	1.56 ± 0.14 ***
Cold-induced change TG (mmol/L)	+ 0.22 ± 0.06	+ 0.18 ± 0.04
Thermoneutral FFA (mmol/L)	0.84 ± 0.08	0.54 ± 0.04 **
Cold-induced change FFA (mmol/L)	+ 0.19 ± 0.05	+ 0.06 ± 0.04 $^{p=0.053}$

Data are mean ± SEM. ***$p < 0.001$, **$p < 0.01$ middle-aged overweight prediabetic men vs. young, healthy, lean men. BMI = body mass index, FFA = free fatty acids, TG = triglycerides. Four healthy, young, lean subjects and one middle-aged overweight subject were excluded from the original cohorts due to absent plasma samples.

Short-Term Cooling Increases Plasma ANGPTL3 and ANGPTL8 in Young, Healthy, Lean Men but not in Middle-Aged Men with Overweight and Prediabetes

In young, healthy, lean men, short-term cooling increased ANGPTL3 (142 ± 9 vs. 164 ± 11 ng/mL, +16%, $p < 0.05$; **Figure 1A**), ANGPTL4 (165 ± 19 vs. 190 ± 19 ng/mL, + 15%, $p < 0.05$; **Figure 1B**), and ANGPTL8 levels (1.8 ± 0.3 vs. 2.3 ± 0.3 µg/mL, + 28%, $p < 0.001$; **Figure 1C**).

In middle-aged men with overweight and prediabetes, short-term cooling also increased ANGPTL4 levels (193 ± 27 vs. 234 ± 31 ng/mL, + 15%, $p < 0.001$; **Figure 1E**). In contrary to the young, healthy, lean men, short-term cooling did not overtly increase ANGPTL3 (230 ± 9 vs. 251 ± 13 ng/mL, $p = 0.051$; **Figure 1D**) or ANGPTL8 levels (2.2 ± 0.5 vs. 2.3 ± 0.5 µg/mL, $p = 0.46$; **Figure 1F**). The effect of cold on plasma ANGPTL8 levels was significantly different between the young, healthy, lean men and the middle-aged men with overweight and prediabetes (+ 28% vs. + 3%, $p < 0.01$). Similar trends were observed for cold-induced ANGPTL4 and ANGPTL8 levels in both cohorts when taking into account South Asian and white Caucasian men separately. However, in case of ANGPTL3, plasma levels increased in white Caucasian but not South Asian middle-aged men with overweight and prediabetes (**Figure S1**).

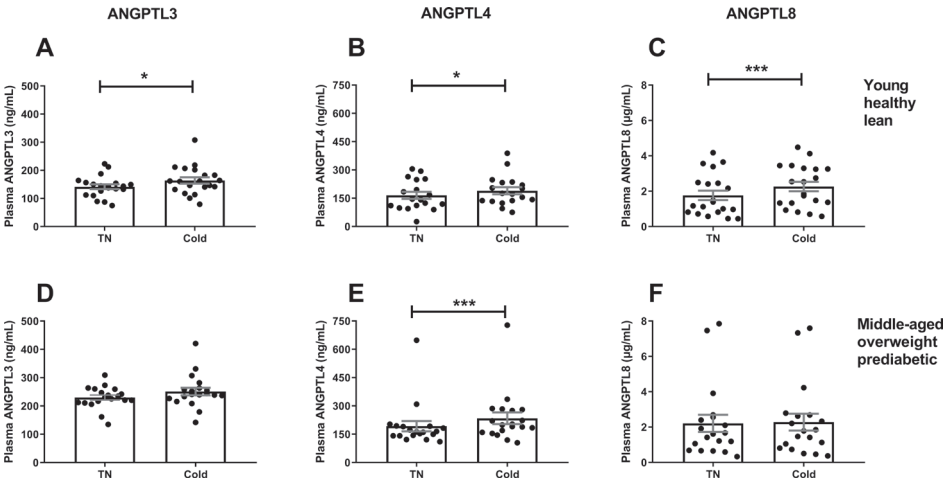

Figure 1. Effect of cold exposure on plasma ANGPTL3, ANGPTL4, and ANGPTL8 levels in young, healthy, lean men **(A–C)** and middle-aged men with overweight and prediabetes **(D–F)**. Data are mean ± SEM. ***$p < 0.001$, *$p < 0.05$ cold vs. thermoneutrality (TN).

The Change in Plasma ANGPTL4 Negatively Correlates with the Change in Triglycerides after Short-Term Cooling in Young, Healthy, Lean Men

To further investigate whether the change in ANGPTLs during short-term cooling was related to the increase in serum lipids, we performed correlation analyses between the changes in the ANGPTLs and TG and FFA levels in both study cohorts.

Data of both ethnicities were pooled, as ethnic origin did not show interaction with any of the correlation analyses. We did not observe a correlation between the cold-induced response in either one of the ANGPTLs and FFA levels in the young, healthy, lean men or middle-aged men with overweight and prediabetes. In the young, healthy, lean men, the cold-induced response in ANGPTL4 negatively correlated with the cold-induced response in TG ($R^2 = 0.39$, $p < 0.01$; **Figure 2B**), whereas no correlations were observed for ANGPTL3 (**Figure 2A**) or ANGPTL8 (**Figure 2C**) levels. In addition, no correlations between the cold-induced response in ANGPTL3, ANGPTL4, and ANGPTL8 and TG levels were observed in the middle-aged men with overweight and prediabetes (**Figure 2D–F**). Of note, body fat percentage did not correlate with cold-induced changes in levels of either of the ANGPTLs (**Figure S2**). Moreover, body fat percentage did not affect any of the correlation analyses between cold-induced changes in ANGPTLs and TG or FFA levels.

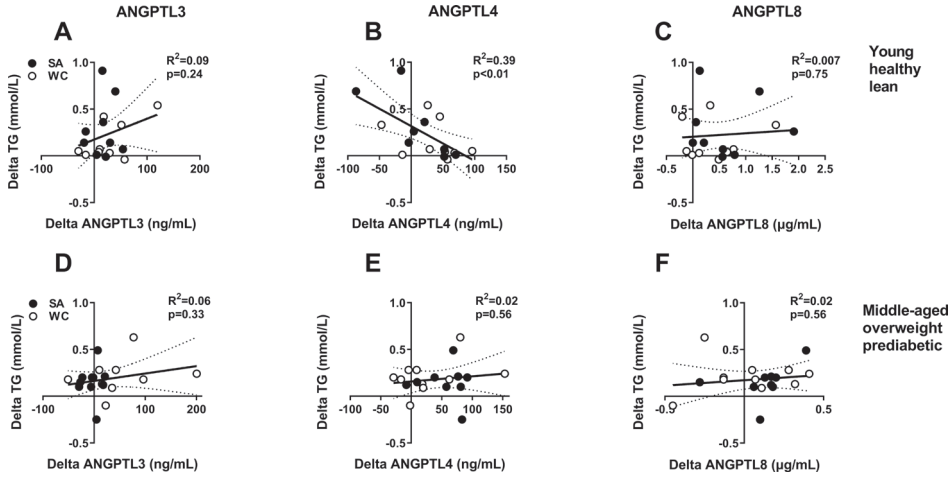

Figure 2. Correlation between cold-induced changes in serum triglyceride (TG) and plasma ANGPTL3, ANGPTL4, and ANGPTL8 levels in young, healthy, lean men **(A–C)** and middle-aged men with overweight and prediabetes **(D–F)**. Dotted lines represent 95% confidence interval. TG = triglycerides. Black circles are South Asians (SA), white circles are white Caucasians (WC).

Changes in ANGPTLs are not Overtly Correlated to [^{18}F]FDG Uptake by BAT or Plasma Glucose or Insulin Levels after Short-Term Cooling

Short-term cooling not only increases TRL-derived FA uptake, but also glucose uptake to stimulate thermogenesis by BAT, the latter likely to enhance de novo lipogenesis [26]. ANGPTLs have a well-established role in TRL-derived FA uptake by metabolic tissues, but their interplay with glucose metabolism is more controversial [27]. We therefore evaluated cold-induced changes in levels of ANGPTLs in relation to [^{18}F]FDG uptake by BAT on PET/CT scan in both young, healthy, lean men and middle-aged men with overweight and prediabetes. We additionally assessed cold-induced changes in ANGPTL levels in relation to delta glucose, delta insulin levels, as well as HOMA-IR under insulin resistant conditions (i.e., in the cohort of middle-aged men with oData of both ethnicities were pooled, as ethnic origin did not show interaction with any of the correlation analyses aside from ANGPTL8. We did not observe correlations between the cold-induced response in either one of the ANGPTLs and BAT volume, SUVmean, or BAT metabolic activity (BAT volume multiplied by SUVmean) in the young, healthy, lean men (**Figure S3**). In the middle-aged men with overweight and prediabetes, we only observed a negative correlation between the cold-induced change in ANGPTL4 levels and BAT volume, but no correlations between other ANGPTLs and BAT parameters (**Figure S4**). In addition, we did not observe correlations between the cold-induced response in either one of the ANGPTLs and delta glucose, delta insulin levels, or HOMA-IR in the middle-aged men with overweight and prediabetes (**Figure S5**).

DISCUSSION

ANGPTLs are inhibitors of LPL activity and function in modulating TRLs that traffic between tissues depending on specific situational energy demands. During cold exposure, LPL-mediated hydrolysis of TRL-TG by BAT is enhanced to meet the increased FA demand to facilitate thermogenesis [15]. Here, we confirmed that short-term cooling increases plasma ANGPTL4 levels in both young, healthy, lean men and middle-aged men with overweight and prediabetes. In addition, we now show that cooling increases plasma ANGPTL3 and ANGPTL8 levels, but only in young, healthy, lean men. We propose that the elevated circulating ANGPTL3 and ANGPTL8 levels during short-term cooling represent a compensatory response aimed at preventing an ANGPTL4-promoted excessive lipid accumulation in oxidative tissues in young, healthy, lean men.

First, we show that short-term cooling increased plasma ANGPTL4 levels in both young, healthy, lean men and middle-aged men with overweight and prediabetes. We previously obtained serum ANGPTL4 levels in this same young, healthy, lean cohort, and these findings are in line albeit measured with a different ELISA [20]. Plasma ANGPTL4 levels also increased after 48 hours of continuous mild cold exposure (16 °C) in young obese males [19]. In addition to cold exposure, both fasting and exercise increased circulating ANGPTL4 levels in humans [28–30]. Regulation of *Angptl4* expression is tissue-specific, as fasting upregulated *Angptl4* expression and impaired LPL activity in WAT in mice, thereby facilitating the uptake of TG-derived FA by tissues with an increased energy demand [28,31]. In line with this, in mice, cold exposure upregulated *Angptl4* expression and limited TRL-derived FA uptake in WAT, whereas *Angptl4* expression was downregulated and, as a consequence, TG-derived FA uptake was increased in BAT [19,31]. We previously hypothesized that during short-term cooling in humans, FAs derived from intracellular lipolysis bind to peroxisome proliferator-activated receptor-γ to stimulate ANGPTL4 expression in WAT, thereby increasing circulating ANGPTL4 levels [32]. This may subsequently limit TG-derived FA uptake by WAT and redirect TRLs towards active BAT for hydrolysis of TG [20]. In the current study, we also observed a negative correlation between the cold-induced changes in ANGPTL4 levels and TG levels in young, healthy, lean men. According to our hypothesis, a higher increase in plasma ANGPTL4 levels during short-term cooling possibly indicates more shuttling of TRLs away from WAT towards active BAT, which might be accompanied by enhanced TRL-TG hydrolysis by BAT that subsequently results in a less pronounced cold-induced increase in serum TG.

In addition to ANGPTL4, we now show that short-term cooling increased plasma ANGPTL3 levels in young, healthy, lean men. ANGPTL3 in humans is nearly exclusively expressed in the liver and inhibits LPL activity in metabolic tissues in an endocrine fashion [33,34]. In contrast to ANGPTL4, ANGPTL3 inhibits LPL activity and TG-derived

FA uptake by oxidative tissues after (re)feeding, thereby promoting lipid storage in WAT. This was demonstrated by a study showing that *Angptl3*$^{-/-}$ mice are unable to suppress LPL activity specifically in oxidative tissues in a fed state, thereby increasing very-low density-lipoprotein (VLDL)-TG-derived FA uptake by oxidative tissues (skeletal muscle, heart, and BAT) and reducing VLDL-TG-derived FA uptake by WAT. As a consequence, plasma TG levels were markedly lower in *Angptl3*$^{-/-}$ mice compared with wild-type mice [35]. In addition to TG levels, plasma FFA and glycerol levels were lower in *Angptl3*$^{-/-}$ mice, likely due to impaired inhibition of lipolysis [36]. Lowering circulating TG levels via ANGPTL3 inactivation by antisense oligonucleotides or monoclonal antibodies is a promising treatment strategy to target dyslipidemia and cardiovascular disease, as this reduced atherosclerosis progression in mice and significantly improved lipid profile in subjects with dyslipidemia in phase I trials [7,37]. However, the effect of pharmacologically targeting ANGPTL3 on risk factors for cardiovascular disease in specific metabolically challenged subjects remains to be elucidated in future studies.

We also show that short-term cooling increases plasma ANGPTL8 levels in young, healthy, lean men. This is in line with mouse studies showing that cold exposure enhances expression of *Angptl8* in liver, BAT, and WAT, although circulating ANGPTL8 levels were not reported in these studies [31,38]. The expression of ANGPTL8 is enriched in liver and present to a lesser extent in WAT and BAT and is highly upregulated in both tissues after (re)feeding [12,39]. Similar to ANGPTL3, ANGPTL8 likely inhibits LPL activity and TG-derived FA uptake by oxidative tissues after feeding to promote lipid storage in WAT. This was evident from a study showing that *Angptl8*$^{-/-}$ mice have increased LPL activity only in oxidative tissues (heart and skeletal muscle) and not in WAT upon (re)feeding [11]. Moreover, Wang et al. [13] showed that *Angptl8*$^{-/-}$ mice have impaired uptake of VLDL-TG-derived FA by WAT in a fed state.

ANGPTL3 and ANGPTL8 share sequence homology, form a protein–protein complex, and need each other's presence to sufficiently regulate circulating lipid levels [12]. Zhang et al. [40] proposed the idea of an ANGPTL3-4-8 axis that ensures adequate distribution of TRL-TG to energy-demanding tissues in different nutritional states. In this model, during fasting, ANGPTL4 negatively regulates LPL activity in WAT to redirect TRL-TG towards other energy requiring tissues for hydrolysis, whereas upon feeding, ANGPTL3 and ANGPTL8 negatively regulate LPL activity in oxidative tissues to make TG-derived FA available for storage by WAT. In this context, we hypothesize that the increase in plasma ANGPTL4 during short-term cooling indicates inhibition of LPL activity in WAT to shuttle TRLs towards active BAT for uptake of TG-derived FA, whereas ANGPTL3 and ANGPTL8 redirect TRLs towards WAT to prevent the accumulation of excess lipids in active BAT in young, healthy, lean men.

It is tempting to speculate about the underlying mechanisms that increase plasma ANGPTL3 and ANGPTL8 during short-term cooling in young, healthy, lean men. Both

ANGPTL3 and ANGPTL8 are regulated by the liver X receptor (LXR) [41–43]. Oxysterols are metabolites of cholesterol and are endogenous ligands of the LXR. As mouse studies have shown that cold exposure rapidly generates TRL-derived cholesterol-enriched remnants that are cleared by the liver [16], this might provide a source of cholesterol that enhances oxysterol formation and thereby stimulates LXR-induced expression of ANGPTL3 and ANGPTL8. Possibly, reduced formation of oxysterols as a consequence of decreased hepatic clearance of cholesterol-enriched remnants under insulin resistant conditions is involved in the absent response of ANGPTL3 and ANGPTL8 during short-term cooling in our cohort of middle-aged men with overweight and prediabetes [44]. Besides activation of the LXR, ANGPTL8 expression is upregulated in both hepatocytes and adipocytes by insulin [34,39,45]. As insulin release is stimulated during cold exposure [46], we speculate that insulin might contribute to the increased plasma ANGPTL8 levels during short-term cooling. Similar to other metabolic tissues, BAT becomes less sensitive to insulin during (pre)diabetes [47]. Therefore, we speculate that the absent cold-induced changes in ANGPTL3 and ANGPTL8 in middle-aged men with overweight and prediabetes might reflect an attempt of the body to overcome impaired glucose uptake by insulin-resistant BAT. It should be noted that we did not observe correlations between cold-induced changes in ANGPTL3 or ANGPTL8 and changes in glucose or insulin levels to support these hypotheses. However, circulating levels of insulin may not reflect local signaling function in metabolic tissues, including its effects on ANGPTL8 secretion. We also did not observe overt correlations between cold-induced changes in ANGPTLs and [^{18}F]FDG uptake by BAT measured with PET/CT scan. However, this only reflects the uptake of glucose by BAT. Taking into account the LPL-inhibitory function of ANGPTLs, it would be highly interesting to specifically investigate cold-induced changes in ANGPTLs in relation to the uptake of (TRL-derived) FA by BAT in future studies. Of note, we observed an increase in FFA levels upon short-term cooling in the young, healthy, lean cohort but not in the middle-aged overweight prediabetic cohort. Interestingly, an increased fat mass is associated with an impaired FFA release from subcutaneous WAT [48]. This likely reflects impaired lipolysis, which may be mediated in part via catecholamine-resistance during obesity (reviewed in [49]). We therefore propose that reduced activity of the sympathetic nervous system in our cohort of overweight and obese men contributed to their unchanged FFA levels upon short-term cooling.

A strong aspect of our study is that we evaluated the effects of short-term cooling on ANGPTLs in both a cohort of healthy and metabolically challenged men. However, this study also had its limitations. For example, its cross-sectional design, as it would be interesting to investigate whether differences in cold-induced ANGPTL levels arise during metabolic changes within individuals over time. In addition, the study designs of both cohorts, although comparable to a certain extent, are not identical with respect to timing and fasting duration (which may explain lower baseline FFA levels in the middle-

aged vs. young cohort). As the applied cooling protocols are also slightly different, we cannot exclude that variability in temperatures using individualized cooling protocols affected ANGPTL and lipid levels. Additionally, it is likely that a fasting state, maintained during the short-term cooling, partly contributed to an increase in circulating lipids and ANGPTL4, whereas this might have abolished the increase in ANGPTL3 and ANGPTL8. Importantly, we cannot exclude possible confounding in our analyses by a difference in age between both study cohorts, nor an effect of the placebo in the middle-aged overweight prediabetic cohort. Lastly, as the sample size of both studies is small, larger studies are warranted to confirm these observations.

Conclusion

In conclusion, we show that short-term cooling not only increases plasma ANGPTL4, but also plasma ANGPTL3 and ANGPTL8 levels in young, healthy, lean men. We propose that these ANGPTLs act in concert to facilitate TG partitioning between tissues in response to cold. While ANGPTL4 likely functions to shuttle TRLs away from WAT towards active thermogenic tissues during cold exposure, we suggest that ANGPTL3 and ANGPTL8 redirect TRLs away from thermogenic tissues to prevent excessive lipid accumulation. Whether these increases in circulating ANGPTLs reflect their ability to locally inhibit LPL-mediated TG hydrolysis and subsequent TG-derived FA uptake by metabolic tissues, remains to be elucidated.

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SUPPLEMENTAL APPENDIX

Supplementary tables

Table S1. Clinical characteristics per ethnicity.

Clinical characteristics	**Young healthy lean men**		**Middle-aged overweight prediabetic men**	
	SA (N=10)	**WC (N=10)**	**SA (N=9)**	**WC (N=10)**
Age (years)	24±1	25±1	46±3 ***	48±2 ***
Height (m)	1.75±0.02 ††	1.84±0.01	1.75±0.02 †	1.81±0.02
Weight (kg)	65.6±3.0†	75.6±2.2	93.5±4.2 ***	99.9±4.0 ***
BMI (kg/m^2)	21.4±0.7	22.3±0.4	30.4±1.1 ***	30.7±1.2 ***
Body fat percentage	23.4±1.6	19.4±1.5	31.6±1.4 **	30.1±1.0 ***
Thermoneutral TG (mmol/L)	0.91±0.18	0.82±0.08	1.58±0.26 *	1.54±0.16 **
Cold-induced change TG (mmol/L)	+0.29±0.11	+0.16±0.07	+0.15±0.06	+0.21±0.06
Thermoneutral FFA (mmol/L)	0.97±0.13	0.71±0.10	0.59±0.04 *	0.49±0.07
Cold-induced change FFA (mmol/L)	+0.04±0.06 ††	+0.34±0.05	+0.01±0.04	+0.11±0.06 **

Data are mean ± SEM. ***p<0.001, **p<0.01, *p<0.05 within same ethnicity between study cohorts. ††p<0.01, †p<0.05 between ethnicities within same study cohort. BMI=body mass index, FFA=free fatty acids, SA=South Asian, TG=triglycerides, WC=white Caucasian. Four healthy young lean subjects and 1 middle-aged overweight subject were excluded from the original cohorts due to absent plasma samples.

Supplementary figures

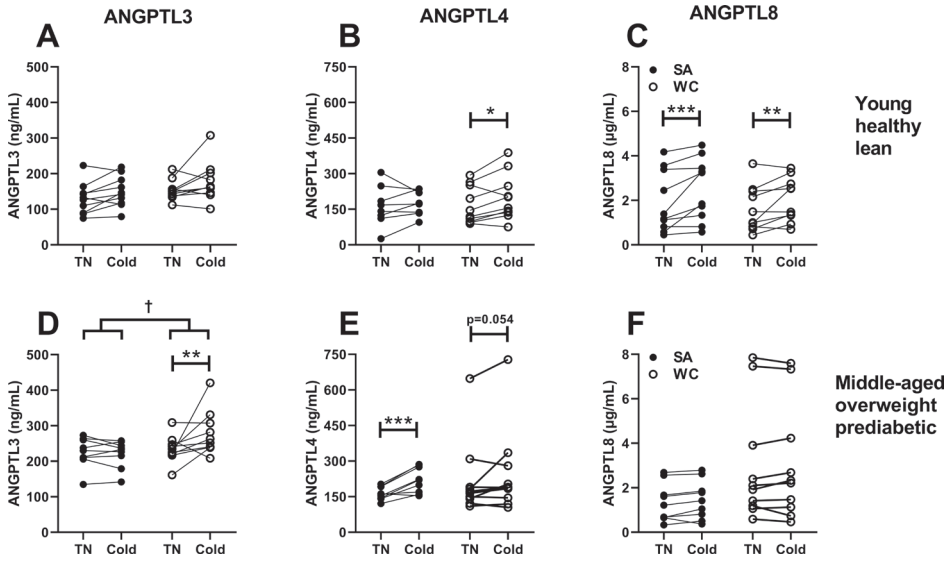

Figure S1. Effect of cold exposure on plasma ANGPTL3, ANGPTL4 and ANGPTL8 levels in young healthy lean South Asian (SA) and white Caucasian (WC) men **(A-C)** and SA and WC middle-aged men with overweight and prediabetes **(D-F)**. Data are mean. ***p<0.001, **p<0.01, *p<0.05 cold *vs* thermoneutrality (TN). †p<0.05 cold-induced delta SA *vs* WC. Black circles are SA, white circles are WC.

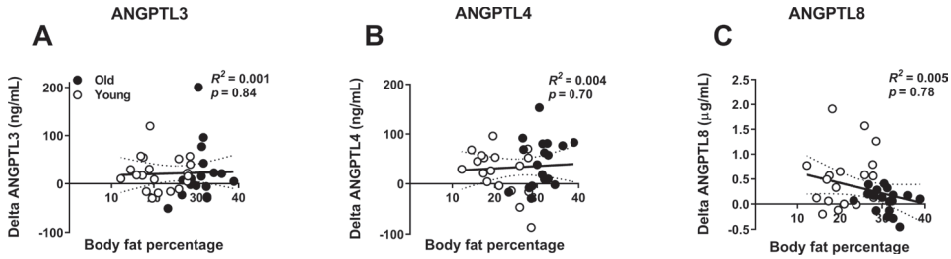

Figure S2. Correlation between body fat percentage and cold-induced changes in plasma ANGPTL3 (**A**), ANGPTL4 (**B**) and ANGPTL8 levels (**C**) in the young healthy lean and middle-aged overweight prediabetic cohorts combined. Dotted lines represent 95% confidence interval. Black circles are the middle-aged overweight prediabetic subjects (old) and white circles are the young healthy lean subjects (young).

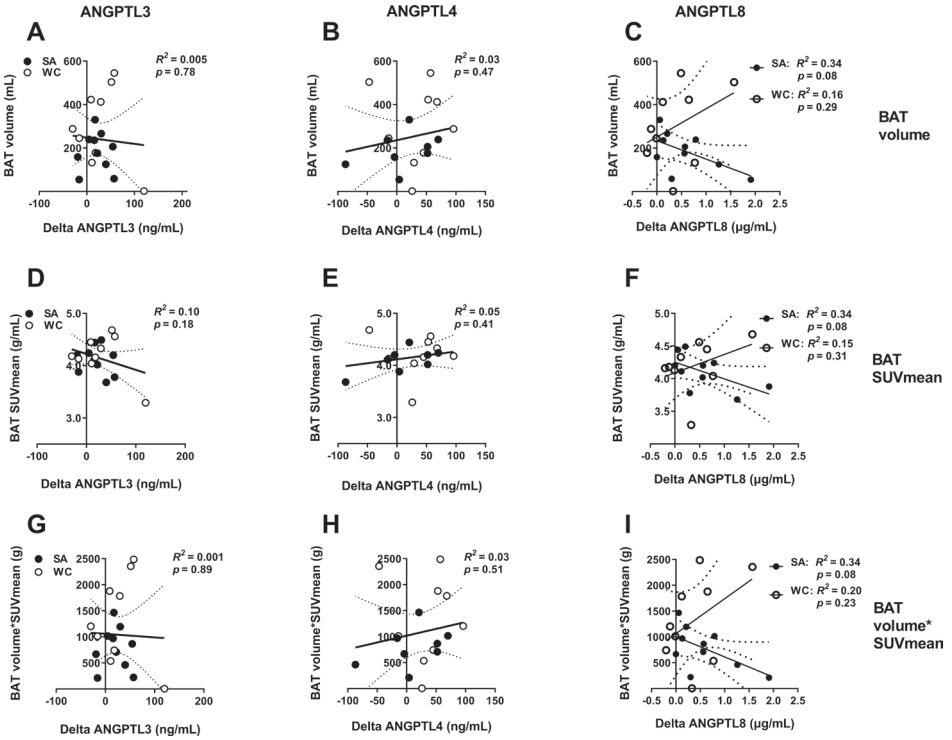

Figure S3. Correlation between cold-induced changes in plasma ANGPTL3, ANGPTL4 and ANGPTL8 levels and BAT volume (**A-C**), SUVmean (**D-F**) and metabolic activity, *i.e.* volume*SUVmean, (**G-I**) in young healthy lean men. Dotted lines represent 95% confidence interval. Black circles are South Asians (SA), white circles are white Caucasians (WC).

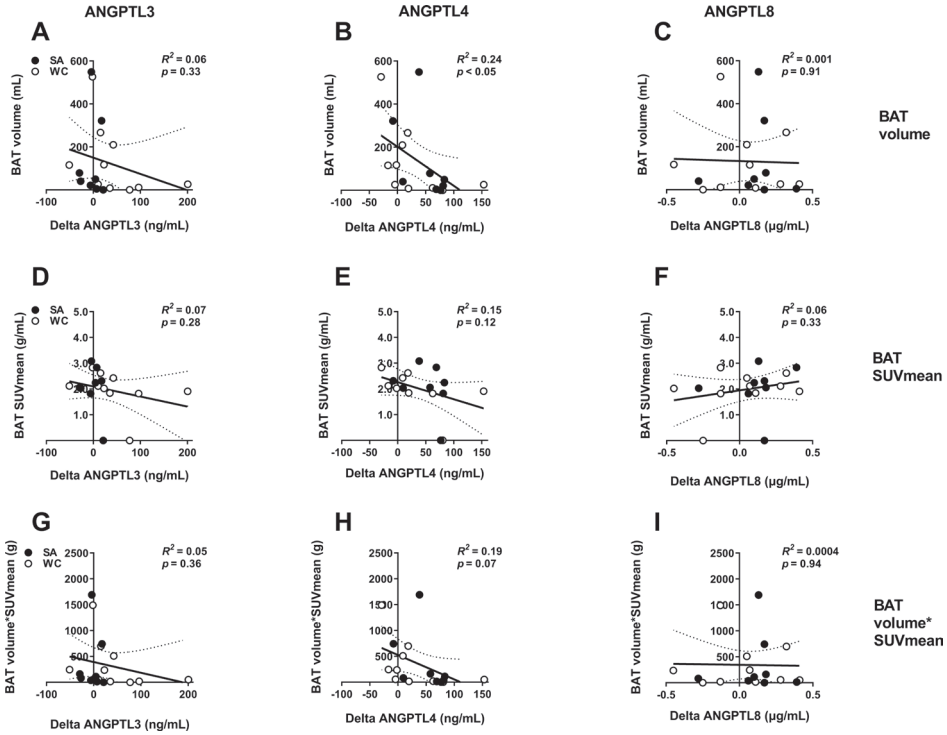

Figure S4. Correlation between cold-induced changes in plasma ANGPTL3, ANGPTL4 and ANGPTL8 levels and BAT volume (**A-C**), SUVmean (**D-F**) and metabolic activity, *i.e.* volume*SUVmean, (**G-I**) in middle-aged men with overweight and prediabetes. Dotted lines represent 95% confidence interval. Black circles are South Asians (SA), white circles are white Caucasians (WC).

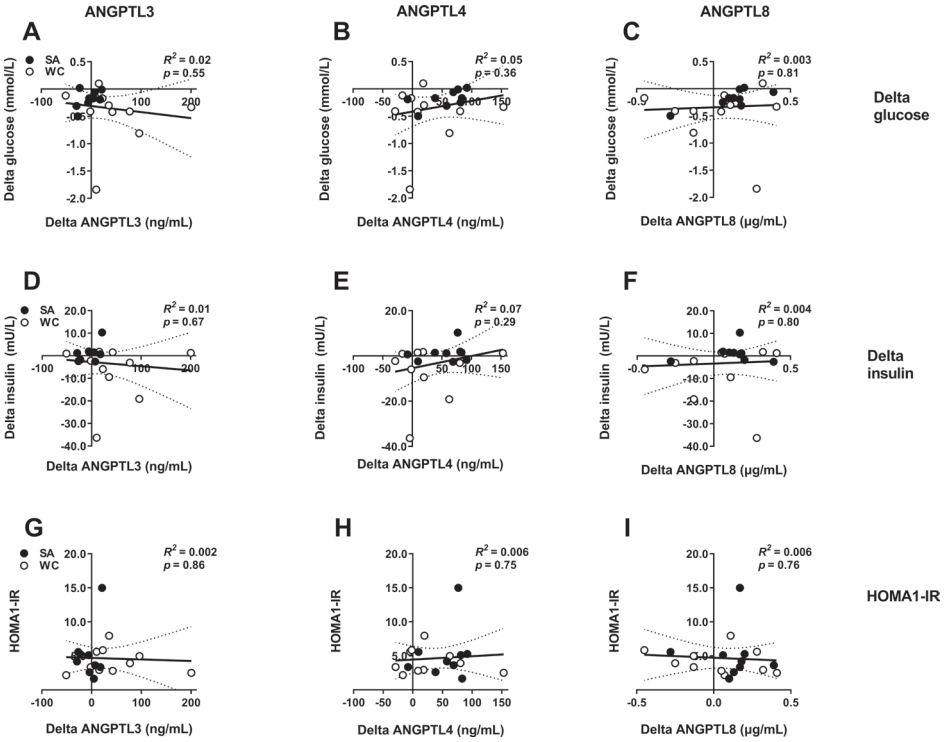

Figure S5. Correlation between cold-induced changes in plasma ANGPTL3, ANGPTL4 and ANGPTL8 levels and cold-induced changes in plasma glucose (**A-C**) and insulin (**D-F**) levels and HOMA1-IR (**G-H**) in middle-aged men with overweight and prediabetes. Dotted lines represent 95% confidence interval. Black circles are South Asians (SA), white circles are white Caucasians (WC).

3

Higher Plasma Sclerostin And Lower Wnt Signaling Gene Expression In White Adipose Tissue Of Prediabetic South Asian Men Compared With White Caucasian Men

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ABSTRACT

Background

South Asians generally have an unfavourable metabolic phenotype compared with white Caucasians, including central obesity and insulin resistance. The Wnt protein family interacts with insulin signaling, and impaired Wnt signaling is associated with adiposity and T2DM. We aimed to investigate Wnt signaling in relation to insulin signaling in South Asians compared with white Caucasians.

Methods

Ten Dutch South Asian men with prediabetes and overweight or obesity and ten matched Dutch white Caucasians were included. Blood samples were assayed for the Wnt inhibitor sclerostin. Subcutaneous white adipose tissue (WAT) and skeletal muscle biopsies were assayed for expression of genes involved in Wnt signaling and insulin signaling with qRT-PCR.

Results

Plasma sclerostin was markedly higher in South Asians compared with white Caucasians (+65 %, $p < 0.01$). Additionally, expression of multiple Wnt signaling genes and key insulin signaling genes were lower in WAT in South Asians compared with white Caucasians. Moreover, in WAT in both ethnicities, expression of Wnt signaling genes strongly positively correlated with expression of insulin signaling genes. In skeletal muscle, *WNT10B* expression in South Asians was lower, but expression of other Wnt signaling and insulin signaling genes did not differ between ethnicities. Wnt and insulin signaling gene expression also positively correlated in skeletal muscle, albeit less pronounced.

Conclusion

South Asian men with overweight or obesity and prediabetes have higher plasma sclerostin levels and lower Wnt signaling gene expression in WAT compared with white Caucasians. We interpret that reduced Wnt signaling could contribute to impaired insulin signaling in South Asians.

INTRODUCTION

The incidence of type 2 diabetes mellitus is increasing worldwide and its morbidity and mortality rates are accompanied by a high socioeconomical burden (1). In the South Asian population, originally deriving from the Indian subcontinent and comprising approximately one fifth of the world population, the prevalence of type 2 diabetes mellitus is particularly high (2, 3). This may at least partly be explained by a disadvantageous metabolic phenotype, including central obesity and insulin resistance (4-6). The underlying cause of this metabolic phenotype is not yet fully elucidated, but may be related to a difference in energy metabolism favouring energy storage.

The Wnt family is well-known for its function in embryonic development and oncogenesis. The canonical route of this signal transduction pathway comprises endogenous Wnt ligands that bind to Wnt coreceptors named Frizzleds (FZD) and low-density lipoprotein receptor-related proteins 5 (LRP5) and 6 (LRP6). Upon binding of Wnt ligands to these receptors, β-catenin is rescued from being degraded, facilitating its nuclear translocation and ultimately inducing transcription of Wnt target genes (7, 8).

Evidence has emerged that Wnt signaling also plays a role in metabolism, since an impairing mutation in the gene encoding for LRP6 was found in a family with hyperlipidaemia, type 2 diabetes mellitus and early coronary artery disease (9). Mutations in various Wnt genes have also been associated with an increased risk for insulin resistance and type 2 diabetes mellitus (10-12). Moreover, circulating levels of the Wnt inhibitor sclerostin were shown to be higher in individuals with prediabetes and type 2 diabetes mellitus compared with normoglycemic individuals (13). Additionally, the Wnt pathway is involved in adiposity, as mutations that probably impair Wnt signaling are associated with obesity and an increased waist circumference (14-16). In vitro studies have indeed shown that Wnt signaling suppresses adipogenesis and favors precursor cell commitment to other lineages, such as osteoblasts (17).

Altogether, we hypothesized that reduced Wnt signaling contributes to the increased risk to develop an impaired glucose homeostasis and high body fat percentage in South Asians. Therefore, we investigated expression levels of both Wnt and insulin signaling genes in subcutaneous white adipose tissue (WAT) and skeletal muscle as well as plasma sclerostin levels in South Asian and white Caucasian men with prediabetes and overweight or obesity.

MATERIAL AND METHODS

Participants

Ten Dutch South Asian males with prediabetes and overweight or obesity (BMI 25-35 kg/m^2, age 40-55 years) and ten age- and BMI-matched Dutch white Caucasian males were enrolled in this study. Participants were recruited via advertisements in local hospitals and universities. Ethnicity was defined as having four grandparents of South Asian or white Caucasian origin. To define South Asian ethnicity, we here adopted the geographical area of South Asia applied by the United Nations, with the exception of Iran (i.e. Afghanistan, Bangladesh, Bhutan, India, the Maldives, Nepal, Pakistan and Sri Lanka) (18). Participants underwent a medical screening including their medical history, a physical examination, blood chemistry tests and an OGTT to exclude individuals with undiagnosed type 2 diabetes mellitus according to 2014 ADA criteria. Prediabetes was defined as having either a fasting plasma glucose level between 5.6 and 6.9 mmol/L or a plasma glucose level between 7.8 and 11.1 mmol/L at 2 hours after the OGTT (19). Exclusion criteria included uncontrolled hypertension, hyper- or hypothyroidism, liver or kidney dysfunction, rigorous exercise, smoking and use of beta-blockers. Three South Asians were using antihypertensive medication before and during the study, two of whom used an ACE inhibitor and one of whom used an angiotensin II-receptor blocker. The Medical Ethical Committee of the Leiden University Medical Center (LUMC) and Maastricht University Medical Center (MUMC) approved the study protocol. The study was undertaken in accordance with the principles of the revised Declaration of Helsinki (2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO). All volunteers provided written informed consent prior to participation.

Study design

This study was part of a randomized clinical trial aiming to investigate the effects of L-arginine on brown adipose tissue metabolism (clinical trial registration number: NCT02291458). The study was conducted between November 2014 and October 2015 at the LUMC and MUMC. Participants were instructed to refrain from intense physical exercise for 48 hours prior to the experiment and consumed a standardized evening meal the day before the experiment. After a 10-hour overnight fast, body composition was determined by dual X-ray absorptiometry (Discovery A, Hologic, Marlborough, MA, USA) and a cannula was placed into the right antecubital vein for blood withdrawal. A fasting skeletal muscle biopsy from the musculus vastus lateralis and a subcutaneous WAT biopsy from the umbilical region were obtained under localized anaesthesia. Biopsies were frozen and stored at −80°C until further analyses.

Quantitative RT-PCR

Skeletal muscle and WAT biopsies were homogenized in 1 mL TriPure RNA Isolation reagent (Roche, Mannheim, Germany) and RNA was extracted according to the manufacturer's instructions. 1 µg RNA was reverse-transcribed using a Promega solution kit (Promega, Madison, WI, USA) for cDNA synthesis. Quantitative RT-PCR was performed with a CFX96 PCR device (Bio-Rad, Hercules, CA, USA) using SYBR green (Promega). Primer sequences of genes involved in Wnt and insulin signaling as well as housekeeping genes are listed in **Table S1**. mRNA expression was normalized to ribosomal protein S18 (RPS18) for WAT and LRP10 for skeletal muscle, and expressed as arbitrary units for South Asians compared with White Caucasians using the ΔΔCT method. Due to insufficient RNA yield, data on WAT of one South Asian and one white Caucasian participant were excluded, as well as data on skeletal muscle of one white Caucasian participant.

Laboratory measurements

Plasma sclerostin was measured with an electrochemiluminescence assay (MSD 96-Well MULTI-ARRAY Human Sclerostin Assay, Meso Scale Diagnostics, Rockville, MD, USA), as described previously (20). Plasma glucose, total cholesterol, NEFA and triglycerides were measured with an automated spectrophotometer (ABX Pentra 400 autoanalyzer, HORIBA, Kyoto, Japan) with enzymatic colorimetric kits. Plasma insulin was determined with commercially available radioimmunoassay kits (human insulin-specific radioimmunoassay, MilliporeSigma, Burlington, MA, USA). Plasma HbA1c was measured with ion exchange chromatography (Tosoh G8 HPLC analyzer, Sysmex, Kobe, Japan).

Statistical analysis

Statistical analysis were performed with PASW Statistics 23.0 for Windows (IBM, Armonk, NY, USA). Baseline characteristics, plasma concentrations and gene expression levels were compared between South Asians and white Caucasians with two-sided independent sample t tests. Linear regression analysis was used to perform correlations between Wnt and metabolic parameters. In case of interaction between ethnicity and the correlated parameters, linear regression analysis was performed for South Asians and white Caucasians separately by including ethnicity as a covariate. No adjustments (for e.g. BMI or age) were made, as participants were matched for these parameters. Data are presented as mean ± SEM, unless states otherwise. A p-value <0.05 was considered statistically significant.

RESULTS

Clinical characteristics

Participant characteristics are summarized in **Table 1**. In this study, all South Asian subjects were Surinamese-Hindustani with grandparents originating from the Indian subcontinent. All white Caucasians were native Dutch. South Asians and white Caucasians were comparable with respect to age and BMI, as they were matched for these criteria. Plasma glucose, insulin, HbA1c and lipid levels as well as body fat percentage did not statistically significantly differ between South Asians and white Caucasians in this study.

Table 1. Participant characteristics.

	white Caucasians (n =10)	**South Asians (n =10)**
Age (yr)	48 ± 6	47 ± 7
Body weight (kg)	99.9 ± 12.5	93.0 ± 12.1
BMI (kg/m^2)	30.7 ± 3.9	30.1 ± 3.3
Fat mass (%)	30.1 ± 3.1	31.2 ± 4.1
Bone mineral content (kg)	3.0 ± 0.4	2.8 ± 0.2
Total bone mineral density (g/cm^2)	1.26 ± 0.07	1.25 ± 0.1
Glucose (mmol/L)	5.7 ± 0.7	5.6 ± 0.5
Insulin (mU/L)	16.8 ± 6.7	20.3 ± 15.0
HbA1c (mmol/mol)	36.0 ± 2.7	38.3 ± 3.3
HbA1c (%)	5.4 ± 0.3	5.7 ± 0.3
HOMA1-IR	4.2 ± 1.9	4.9 ± 3.8
Triglycerides (mmol/L)	1.5 ± 0.5	1.6 ± 0.7
Total cholesterol (mmol/L)	5.6 ± 0.9	5.5 ± 0.8

Data are presented as mean ± SD

Plasma sclerostin levels are higher in South Asians, while bone mass is not affected

Firstly, we determined plasma sclerostin, a circulating inhibitor of the Wnt signaling pathway (**Fig. 1**). Sclerostin levels were markedly higher in South Asians compared with white Caucasians (81 ± 8 vs 49 ± 6 pg/mL, p <0.01, respectively). This was not accompanied by a difference in bone mass between South Asians and white Caucasians, as indicated by a similar total bone mineral density (1.25 ± 0.03 vs 1.26 ± 0.02 g/cm^2, p =0.75, respectively). Plasma sclerostin levels negatively correlated with plasma glucose (R^2 =-0.20; p <0.05, **Fig. S1A**). We did not observe correlations between plasma sclerostin levels and plasma insulin (**Fig. S1B**), HbA1c (**Fig. S1C**), HOMA1-IR (**Fig. S1D**) or fat mass (data not shown).

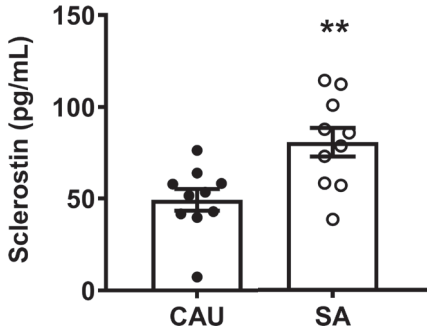

Fig 1. Plasma sclerostin levels in white Caucasian (CAU) and South Asian (SA) men. Data are expressed as mean. Error bars show SEM. ** p <0.01 South Asians versus white Caucasians.

Wnt signaling gene expression is lower in white adipose tissue of South Asians

To study whether Wnt signaling is altered in metabolic tissues, we investigated expression of Wnt family genes in WAT and skeletal muscle. In addition, we investigated insulin signaling gene expression in both tissues.

In WAT, expression of FZD1 (-34 %, p <0.05) as well as LRP5 (-44 %, p <0.05) and LRP6 (-30 %, p <0.05) were lower in South Asians compared with white Caucasians (**Fig. 2A**). Furthermore, gene expression of the intracellular signal transducer GSK3B (-33 %, p <0.05) and the transcription factor TCF7L2 (-45 %, p <0.05) were lower in South Asians (**Fig. 2A**). Multiple genes involved in insulin signaling (INSR -31 %, p <0.05; TBC1D4 -29 %, p <0.05; SLC2A4 -38 %, p <0.05 and GYS1 -46 %, p <0.05) were also expressed to a lower extent in WAT of South Asians compared with white Caucasians (**Fig. 2B**). In skeletal muscle, besides a marked lowering of WNT10B expression (-73 %, p <0.001), genes involved in Wnt signaling did not differ between South Asians and white Caucasians (**Fig. 2C**). Genes involved in insulin signaling were also not differentially expressed in skeletal muscle between South Asians and white Caucasians (**Fig. 2D**).

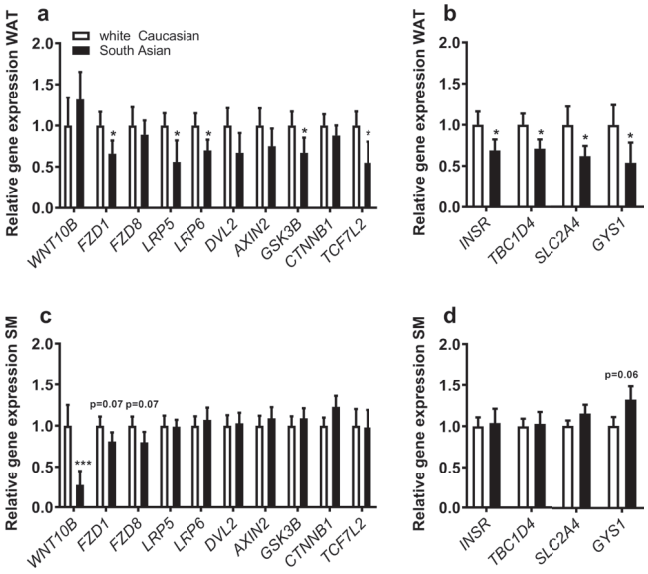

Fig 2. Gene expression of key players in Wnt and insulin signaling in (**A,B**) white adipose tissue (WAT) and (**C,D**) skeletal muscle (SM) in white Caucasian and South Asian men. Data are expressed as mean. Error bars show SEM. Black bars are South Asians and white bars are white Caucasians. * p <0.05 South Asians versus white Caucasians, *** p <0.001 South Asians versus white Caucasians.

Wnt and insulin signaling gene expressions strongly and positively correlate in white adipose tissue

To investigate whether the lower Wnt signaling gene expression in WAT of South Asians could be related to impaired insulin signaling, we performed correlation analysis between expression of Wnt genes and insulin signaling genes.

We observed a strong positive correlation between expression of multiple Wnt family genes and key insulin signaling genes in WAT. Both LRP5 and LRP6 expression strongly correlated with INSR (LRP5: R^2 =0.77; p <0.0001, LRP6: R^2 =0.47; p <0.001, **Fig. 3A,B**), TBC1D4 (LRP5: R^2 =0.67; p <0.0001, LRP6: R^2 =0.61; p <0.001, **Fig. 3C,D**), SLC2A4 (LRP5: R^2 =0.59; p <0.001, LRP6: R^2 =0.62; p <0.0001, **Fig. 3E,F**) and GYS1 (LRP5: R^2 =0.85; p <0.0001, LRP6: R^2 =0.74; p <0.0001, **Fig. 3G,H**) expression. In addition, we observed positive correlations between expression of FZD1, FZD8, DVL2, TCF7L2, AXIN2 and GSK3B and insulin signaling genes (data not shown). For skeletal muscle, we also observed positive correlations between expression of LRP6 and TBC1D4 (R^2 =0.39; p <0.01, **Fig. S2D**), SLC2A4 (R^2 =0.43; p <0.01, **Fig. S2F**) and GYS1 (R^2 =0.31; p <0.05, **Fig. S2H**). Additionally, expression of LRP5 positively correlated with TBC1D4 (R^2 =0.68; p <0.01, **Fig. S2C**) and SLC2A4 (R^2 =0.68; p <0.01, **Fig. S2E**) in skeletal muscle only in South Asians. Furthermore, expression of DVL2, CTNNB1, TCF7L2, AXIN2 and GSK3B also positively correlated with insulin signaling genes in skeletal muscle (data not shown).

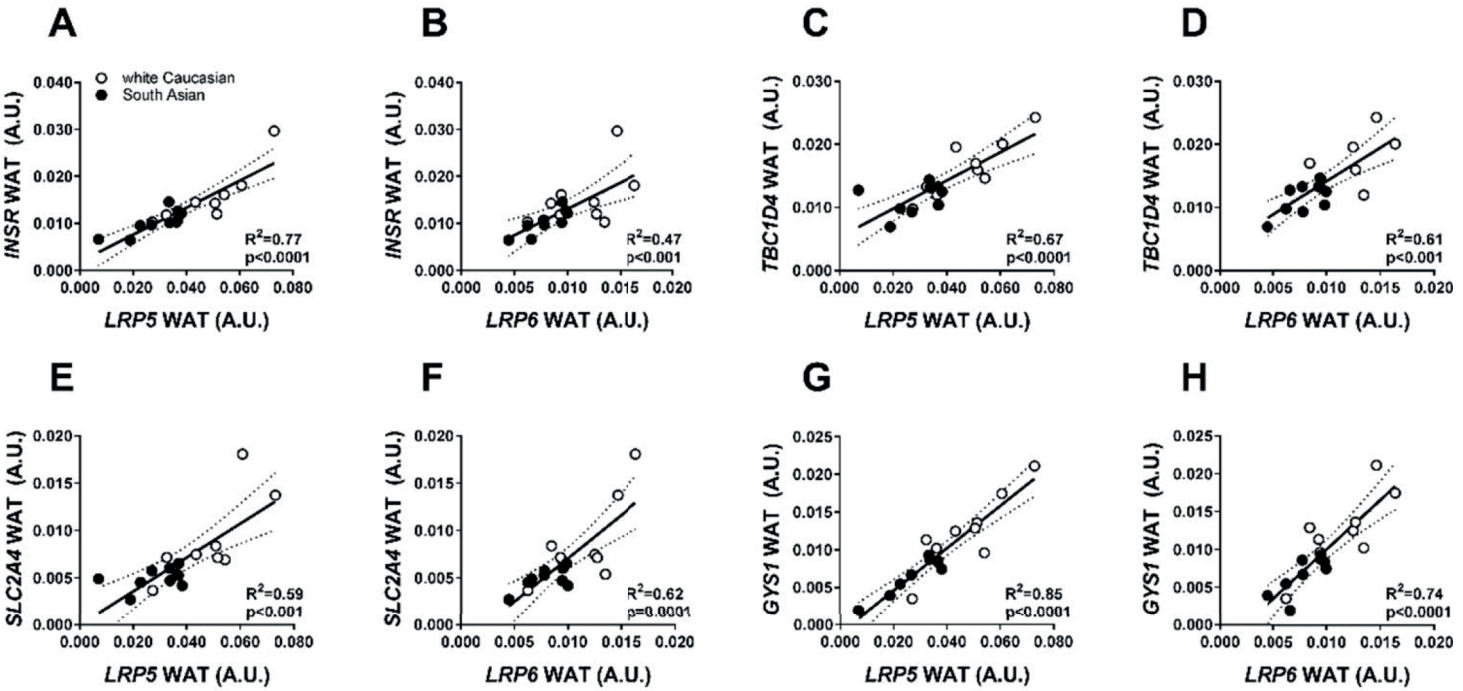

Fig. 3. Correlations between expression of Wnt signaling coreceptors low-density lipoprotein receptor-related proteins 5 (LRP5) and 6 (LRP6) and insulin signaling genes (A, B) insulin signaling receptor (INSR), (C, D) TBC1 domain family member 4 (TBC1D4), (E, F) solute carrier family 2 member 4 (SLC2A4), and (G, H) glycogen synthase 1 (GYS1) in white adipose tissue (WAT) in South Asian and white Caucasian men. Correlations are shown for both groups combined, black circles are South Asians and white circles are white Caucasians. Dotted lines represent 95% confidence interval.

DISCUSSION

Impaired Wnt signaling is associated with obesity and type 2 diabetes mellitus. As South Asians generally have a high body fat percentage and are at risk for the development of type 2 diabetes mellitus, we hypothesized that Wnt signaling is reduced in South Asian men compared with white Caucasian men. We showed that plasma sclerostin was higher in South Asians without evident effect on its primary target, bone. Additionally, expression of various Wnt family genes in WAT was lower in South Asians than in white Caucasians and this was accompanied by a lower expression of insulin signaling genes. Moreover, there was a strong positive correlation between expression of Wnt family genes and insulin signaling genes in WAT. These observations suggest that Wnt and insulin signaling in WAT are associated and that lower Wnt signaling might, at least in part, affect insulin signaling in South Asians.

Sclerostin is a glycoprotein produced predominantly by mature osteocytes within the mineralized bone matrix, that negatively regulates bone formation via inhibiting canonical Wnt signaling (21, 22). We observed higher sclerostin levels in South Asians compared with white Caucasians. Whether these higher sclerostin levels are due to increased production by osteocytes or decreased renal or hepatic elimination, is unknown. Of note, as sclerostin is suggested to be involved in arterial stiffness and vascular calcifications (23, 24) we critically looked at the plasma sclerostin values of the 3 South Asian participants with well-controlled hypertension, but these were well within the range of the study population. Interestingly, previous studies showed that circulating sclerostin levels are higher in individuals with prediabetes and diabetes compared with normoglycemic individuals, and that sclerostin levels positively correlate with HbA1c and HOMA-IR (13, 25-29), albeit we could not reproduce this in our study. We observed a negative correlation between plasma sclerostin and glucose levels, which is against our hypothesis that sclerostin negatively affects glucose homeostasis. However, this fairly weak correlation does not necessarily reflect the effect of altered insulin signaling by sclerostin-induced Wnt inhibition. Moreover, our sample size is limited and previous studies show inconsistent results in different study populations (13, 27-30). The mechanism by which sclerostin may affect insulin signaling and glucose homeostasis remains to be established, but likely involves Wnt signaling.

To further investigate Wnt signaling in metabolic tissues, we obtained WAT and skeletal muscle biopsies. Firstly, we consistently observed lower expression of genes involved in Wnt signaling (FZD1, LRP5, LRP6, GSKS3B and TCF7L2) in WAT of South Asians compared with white Caucasians. Notably, inhibition of canonical Wnt signaling by sclerostin favors differentiation of adipocytes over osteoblastogenesis (31, 32). In line with this, in vivo evidence showed that mice with overexpression of sclerostin indeed have reduced WAT expression of genes involved in Wnt signaling and increased expres-

sion of genes involved in adipocyte differentiation, together with adipocyte hypertrophy, fat mass accumulation and an impaired glucose homeostasis, whereas the opposite was observed for Sost-/- mice and mice administered a sclerostin-neutralizing antibody (31). Based on these observations, we speculate that impaired Wnt signaling contributes to the central adiposity that is commonly observed in South Asians. Whether these alterations in the Wnt pathway are congenital or possibly induced by increased sclerostin levels later in life, remains to be explored. Interestingly, Karczewska-Kupczewska et al. (33) also previously showed in an euglycemic clamp study that expression of various Wnt signaling genes in WAT was lower in nondiabetic individuals with low versus high insulin sensitivity. Indeed, in our study expression of key insulin genes (INSR, TBC1D4, SLC2A4 and GYS1) in WAT was also lower in South Asians compared with white Caucasians, suggesting that Wnt and insulin signaling in WAT are associated and that lower Wnt signaling might partially reduce insulin signaling in South Asians.

Aside from a markedly lower WNT10B expression, we did not observe overt differences in skeletal muscle with respect to Wnt and insulin signaling gene expression between South Asians and white Caucasians. It is not unlikely that the canonical Wnt pathway is more involved in adipogenic insulin signaling, as adipocytes and osteoblasts share a direct common cellular progenitor and the involvement of Wnt signaling in adipogenesis is well established (34). Possibly, Wnt signaling in skeletal muscle is influenced more by other Wnt ligands than sclerostin. Alternatively, Wnt signaling in skeletal muscle might be more regulated by other pathways, including the non-canonical pathway. However, Wnt signaling is likely to affect insulin signaling and sensitivity in skeletal muscle to some extent, as human carriers of an LRP6 loss-of-function mutation have impaired skeletal muscle insulin sensitivity and insulin signaling (11). In addition, Kim. et al (31) showed that Sost-/- mice had increased skeletal muscle insulin sensitivity compared with control mice, despite unaltered Wnt signaling gene expression in this tissue. In concordance, serum sclerostin levels positively correlated with skeletal muscle and adipose tissue insulin resistance in a hyperinsulinemic-euglycemic clamp study in human subjects with either normoglycemia or prediabetes (13). Alternative mechanisms that may contribute to peripheral insulin resistance in South Asians are a pro-inflammatory status, including downregulation of genes involved in anti-inflammatory type 1 IFN signaling in WAT and skeletal muscle (35), lower adiponectin levels (36, 37), as well as a lower skeletal muscle oxidative capacity (38) and cardiorespiratory fitness compared with white Caucasians (39).

Although we cannot draw conclusions regarding causality in the current study set-up, we observed strong correlations between expression of various Wnt family genes, including Wnt coreceptors LRP5 and LRP6, and insulin signaling gene expression in WAT, and to a lesser extent in skeletal muscle. This is in line with evidence showing that Wnt and insulin signaling are intertwined in WAT, as Palsgaard et al. (40) have shown that

knocking down LRP5 in murine preadipocytes resulted in reduced phosphorylation of insulin signaling proteins. Whether this is a result of direct interaction between LRP5 and the insulin receptor or due to a mutual downstream docking protein is yet to be unraveled. However, when knocking down LRP5 in human stromovascular cells, Loh et al. (16) did not observe differences in insulin receptor expression or insulin signaling pathway activity.

To the best of our knowledge, this is the first study investigating Wnt signaling and its link with glucose homeostasis in South Asians. A strong point of this study is our ability to investigate Wnt signaling on a tissue-specific metabolic level. Unfortunately, no waist and hip circumference measurements were obtained, therefore we cannot explore Wnt signaling in relation to body fat distribution in this cohort. The fact that we did not observe correlations between plasma sclerostin levels and HbA1c and HOMA1-IR may be due to our relatively small sample size, as such correlations have repeatedly been shown in larger cohorts. Although we did not observe evident differences in socioeconomical factors between South Asians and white Caucasians, we cannot exclude that socioeconomical status contributed to metabolic differences between ethnicities in this study. It is important to note that the results in these Surinamese-Hindustani cannot be directly extrapolated to other Asian subgroups, as metabolic characteristics differ between Asian populations (41).

To conclude, we show that circulating sclerostin levels are higher and expression of Wnt signaling genes is lower in WAT of South Asian men with prediabetes and overweight or obesity compared with white Caucasian men. Therefore, we speculate that Wnt signaling in adipose tissue might be reduced in South Asians, which could contribute to their susceptibility to develop a disadvantageous metabolic phenotype, including insulin resistance. Future research investigating the interaction between Wnt and insulin signaling is warranted to reveal whether Wnt targeting therapy, such as sclerostin antibodies, can improve insulin sensitivity in South Asians.

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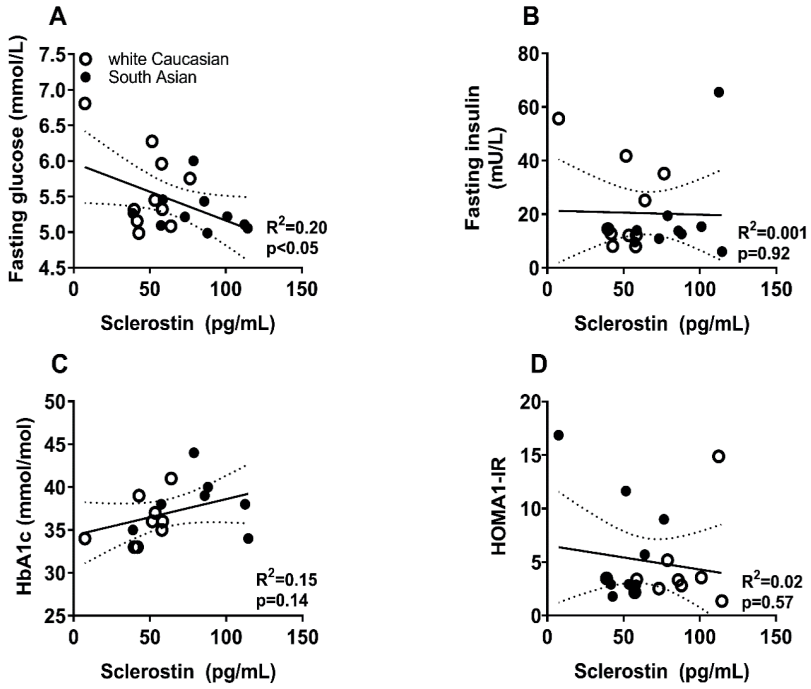
SUPPLEMENTAL APPENDIX

Supplementary Table 1. Primer sequences for quantitative reverse transcription polymerase chain reaction on white adipose tissue and skeletal muscle

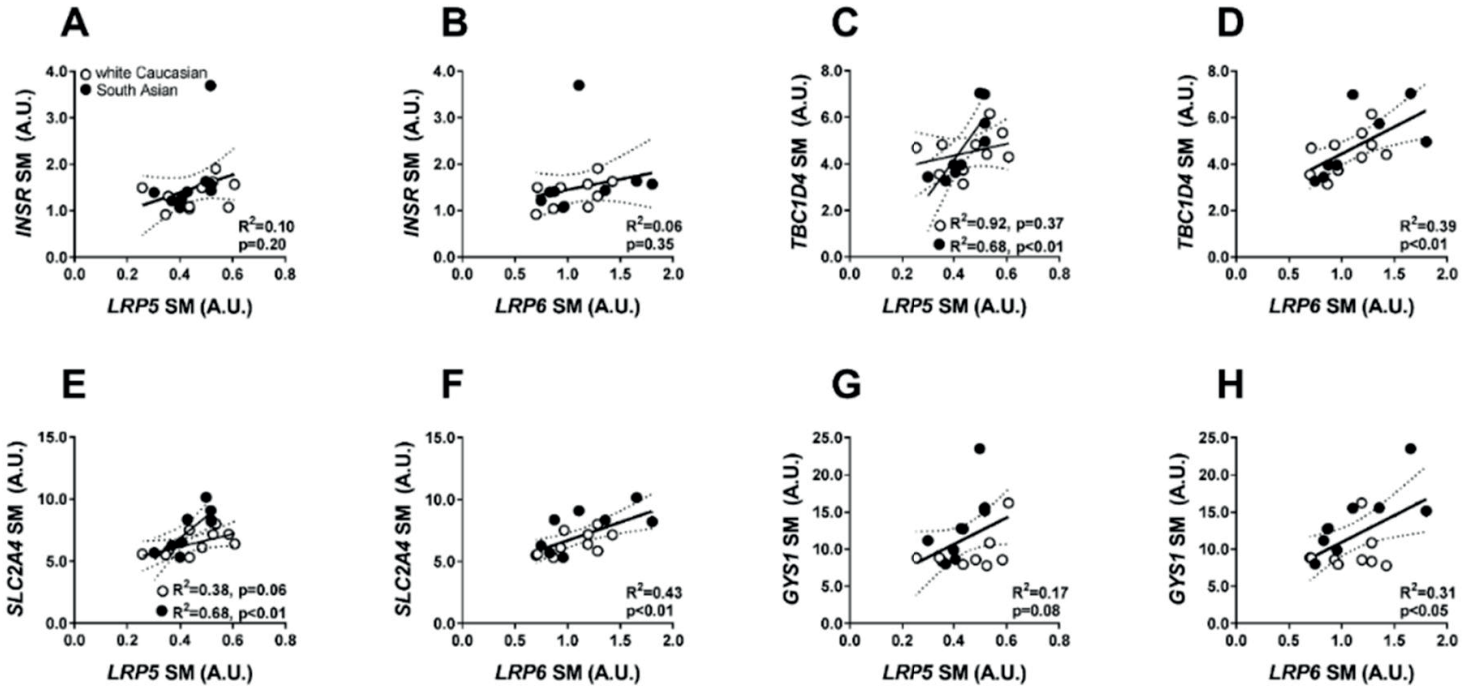
	Forward primer sequence	Reverse primer sequence
AXIN2	CCACACCCTTCTCCAATCC	TGCCAGTTTCTTTGGCTCTT
CTNNB1	TTGGATATCGCCAGGATGAT	CATGGGGTCCATACCCAAG
DVL2	TATTTCACTCTCCCCCGAAA	GGAAGGTGCCAGTCAGAGC
FZD1	CGGCAAGACCCTCAACTC	CCTTGTTTGCTGTTGGTGAG
FZD8	CGCCACGCGTTAATTTCT	ATCTCGGGTTCTGGAAACG
GSK3B	TCTTCAACTTCACCACTCAAGAA	CCGAGCATGAGGAGGAATAA
GYS1	TGGGGCTACACACCGGCTGA	GCGGAACCGCCGGTCAAGAA
INSR	GGGCAACGGCTCTTGGACGG	CGGCCCATCTGGCTGCCTCTT
LRP5	Commercially available via QIAGEN	Commercially available via QIAGEN
LRP6	Commercially available via QIAGEN	Commercially available via QIAGEN
LRP10	CAGACTGTCACCATCAGGTTC	GAGAGGGGAGCGTAGGGTTA
RPS18	AGGATCCATTGGAGGGCAAGT	TCCAACTACGAGCTTTTTAACTGCA
SLC2A4	GGCTGGAGTCCTGCTTCTGCAC	GCTGGTACATTTGAATCTGCAGCGA
TBC1D4	GCTACCTCTACATCATCCAGAATCTC	CCAGAAACATCGGCCCA
TCF7L2	TGAACACAGCGAATGTTTCC	CTGTTGATCAAGGCCAAAGC
WNT10B	CCCAGGACACATGGGAAT	TCCAAGAAATCCCGAGAGAA

CTNNB1, catenin beta 1; DVL2, dishevelled segment polarity protein 2; FZD, Frizzleds; GSK3B, glycogen synthase kinase 3 beta; GYS1, glycogen synthase 1; INSR, insulin signaling receptor; LRP, low-density lipoprotein receptor-related protein; RPS18, ribosomal protein S18; SLC2A4, solute carrier family 2 member 4; TBC1D4, TBC1 domain family member 4; TCF7L2, transcription factor 7 like 2; WNT10B, wingless-type MMTV integration site family member 10B.

Supplementary figures



Supplementary Fig. 1. Correlations between plasma sclerostin levels and (A) fasting plasma glucose, (B) insulin, (C) glycosylated hemoglobin (HbA1c), and (D) homeostasis model assessment 1 of insulin resistance (HOMA1-IR) in South Asian and white Caucasian men. Black circles are South Asians and white circles are white Caucasians. Dotted lines represent 95% confidence interval.



Supplementary Fig. 2. Correlations between expression of Wnt signaling coreceptors low-density lipoprotein receptor-related proteins 5 (LRP5) and 6 (LRP6) and insulin signaling genes (A, B) insulin signaling receptor (INSR), (C, D) TBC1 domain family member 4 (TBC1D4), (E, F) solute carrier family 2 member 4 (SLC2A4), and (G, H) glycogen synthase 1 (GYS1) in skeletal muscle (SM) in South Asian and white Caucasian men. Correlations are shown for both groups combined and per ethnicity in case of interaction. Black circles are South Asians and white circles are white Caucasians. Dotted lines represent 95% confidence interval.

4

LDL aggregation susceptibility is higher in healthy South Asian compared with white Caucasian men

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ABSTRACT

Background

South Asians are more prone to develop atherosclerotic cardiovascular disease (ASCVD) compared with white Caucasians, which is not fully explained by classical risk factors. We recently reported that the presence of aggregation-prone LDL in the circulation is associated with increased ASCVD mortality.

Objective

We hypothesized that LDL of South Asians is more prone to aggregate, which may be explained by differences in their LDL lipid composition.

Methods

In this cross-sectional hypothesis-generating study, LDL was isolated from plasma of healthy South Asians (n=12) and age- and BMI-matched white Caucasians (n=12), and its aggregation susceptibility and lipid composition were analyzed.

Results

LDL from South Asians was markedly more prone to aggregate compared with white Caucasians. Among all measured lipids, sphingomyelin 24:0 and triacylglycerol 56:8 showed the highest positive correlation with LDL aggregation. In addition, LDL from South Asians was enriched in arachidonic acid containing phosphatidylcholine 38:4 and had less phosphatidylcholines and cholesteryl esters containing monounsaturated fatty acids. Interestingly, body fat percentage which was higher in South Asians (+26%), positively correlated with LDL aggregation and highly positively correlated with triacylglycerol 56:8, sphingomyelin 24:0 and total sphingomyelin.

Conclusions

LDL aggregation susceptibility is higher in healthy young South Asians compared with white Caucasians. This may be partly explained by the higher body fat percentage of South Asians, leading to sphingomyelin-enrichment of LDL. We anticipate that the presence of sphingomyelin-rich, aggregation-prone LDL particles in young South Asians may increase LDL accumulation in the arterial wall and thereby contribute to their increased risk of developing ASCVD later in life.

INTRODUCTION

Atherosclerotic cardiovascular disease (ASCVD) is the primary cause of death worldwide and puts a major burden on global health care.[1] People originating from the South Asian subcontinent (India, Nepal, Bangladesh, Bhutan, Pakistan and Sri Lanka), who comprise one fourth of the world population, are particularly prone to develop ASCVD compared with other ethnic groups.[2] Moreover, South Asians suffer from higher ASCVD morbidity and mortality rates and experience their first myocardial infarction on average ten years prior to western white Caucasians.[3] Factors contributing to this ASCVD risk that are highly present in the South Asian population include smoking, a low level of physical activity and a diet enriched with carbohydrates and saturated fats. In addition to these lifestyle factors, South Asians have a high body fat percentage and are susceptible to develop obesity, insulin resistance, hypertension and dyslipidemia. However, the high ASCVD risk of South Asians cannot be solely explained by a high prevalence of classical risk factors in this population.[4-6]

Even though a high plasma LDL-cholesterol level is a risk factor for ASCVD,[7] measurement of LDL-cholesterol levels only does not capture the qualitative properties of LDL particles affecting the progression of atherosclerosis.[8] Current theories of atherogenesis emphasize retention of LDL by the subendothelial proteoglycans in the arterial intima as an initial step.[9] The retained LDL is susceptible to modification by oxidation, glycation, and proteolytic and lipolytic enzymes,[10] which can induce aggregation of the modified LDL particles.[11] An enzyme that induces formation of extremely large LDL aggregates is sphingomyelinase.[12-15] LDL aggregation enhances its binding to arterial proteoglycans[11] and thereby can increase extracellular lipid accumulation, thereby further aggravating atherosclerosis development.[16] In addition, macrophages and other inflammatory cells can take up aggregated LDL particles, leading to foam cell formation.[17] Over decades, these processes result in formation of atherosclerotic lesions containing aggregated LDL-derived particles.[13, 18-20] We have recently developed a test to measure the susceptibility of LDL particles isolated from plasma to aggregate, and observed substantial inter-individual variation in LDL aggregation.[21] Importantly, LDL aggregation susceptibility was found to predict future cardiovascular deaths independently of conventional risk factors for ASCVD, such as LDL-cholesterol levels.[21]

Differences in the aggregation susceptibility of LDL particles depend on their surface lipid composition. We observed that LDL particles having a high proportion of sphingomyelins (SMs) and ceramides are prone to aggregate, whereas LDL particles having a relatively high content of phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs) are more resistant to aggregation.[21] These data are in accordance with studies showing that plasma SM levels are higher in patients having coronary artery disease than in controls[22] and that plasma ceramides predict future cardiovascular deaths.[23,48] Of

LDL core lipids, a high proportion of several triacylglycerol (TAG) species and cholesteryl esters (CEs) containing monounsaturated fatty acids was associated with decreased LDL aggregation.[21]

In this study, we hypothesized that, compared with age- and BMI-matched white Caucasians, the susceptibility of LDL to aggregate is higher in healthy South Asian individuals, which could partly contribute to their increased risk to develop ASCVD later in life. In addition, since we have previously shown that LDL aggregation is associated with specific characteristics of the LDL lipidome, we hypothesized that such a difference in LDL aggregation would be mirrored by differences in LDL lipid composition between South Asians and white Caucasians. Finally, as South Asians have relatively more body fat than BMI-matched white Caucasians, and obesity is associated with higher levels of sphingolipids, including both SM and ceramide,[24] we assessed whether body composition is related to both the aggregation susceptibility and lipidome of LDL.

MATERIAL AND METHODS

Participants

Twelve healthy Dutch South Asian and twelve Dutch white Caucasian men were matched for age (18-32 years) and BMI (18-27 kg/m^2) and were included in this study. South Asian subjects were eligible in case of being born and raised in the Netherlands and having 4 grandparents from South Asian descent. Major exclusion criteria included smoking, recent weight-loss, a significant chronic disease and/or a renal, hepatic or endocrine disease. None of the participants used any medication. The study was performed in accordance with the principles of the revised declaration of Helsinki and approved by the medical ethical committee of the Leiden University Medical Center in the Netherland.[25] All study participants provided written informed consent prior to the study.

Study design

This study was conducted as part of a clinical trial that investigated the effects of the glucagon-like peptide 1 receptor agonist exenatide on brown adipose tissue (BAT) activity and energy metabolism (Janssen & Nahon et al, in preparation, trial register number clinicaltrials.gov NCT03002675). That study was conducted between September 2016 and February 2018 at the Leiden University Medical Center, the Netherlands. In the present study, we analyzed only samples collected at the baseline. Participants had been instructed to refrain from physical exercise 48 hours prior to the study day. After a 10-hour overnight fast, body composition was determined by bio-impedance analysis (BIA; Bodystat 1500, Bodystat, Douglas, Isle of Man, UK) and blood samples were drawn.

Serum and plasma measurements

Commercially available enzymatic kits were used to measure concentrations of triglycerides, total cholesterol, HDL-cholesterol (all Roche Diagnostics, Woerden, the Netherlands) and insulin (Meso Scale Diagnostics LLC, Rockville, MD, USA) in serum, and glucose (Instruchemie, Delfzijl, the Netherlands) in plasma. LDL-cholesterol was calculated by the Friedewald equation.[26]

LDL isolation

LDL (d = 1.019 to 1.063 g/ml) was isolated from 300 µl plasma samples by D2O-based sequential ultracentrifugation,[27] and 300 µl of LDL was collected. The concentration of LDL is expressed as protein concentration, which was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA)

Production of human recombinant acid sphingomyelinase

The human recombinant acid sphingomyelinase protein was produced at the University of Helsinki, Finland. The cDNA was ordered from GenScript (Piscataway, USA) as pUC57 plasmid and subcloned to pEFIRES-P vector with an EF1a promoter[28] or to another proprietary mammalian expression vector with a CAG-promoter. Both plasmid vectors yielded essentially similar protein expression in CHO-S cells.

For production of the protein, Chinese hamster ovary (CHO) cells were transfected with the expression construct via lipofection (Fugene 6; Promega, Madison, WI) and selected with puromycin (Corning, Manassas, VA). During selection, cells were grown in F12 (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mmol/l Ultraglutamine (Lonza, Verviers, Belgium), 100 µl/ml streptomycin, 100 IU/ml penicillin (Corning, Mediatech Inc, Manassas, VA) and 10% FBS (Gibco, LifeTechnologies, Paisley, UK). For large-scale expression, cells were adapted to CD OptiCHO medium (Gibco, LifeTechnologies, Paisley, UK) supplemented with 2 mmol/l Ultraglutamine and grown in suspension in an orbital shaker. Cell culture supernatants were clarified by filtration through 0.22 µm membranes (Steritop, Millipore, Darmstadt, Germany) and the solution was pumped through a Protino column (Macherey-Nagel, Duren, Germany). The protein was eluted with imidazole, dialyzed against 140 mM NaCl and finally concentrated with Amicon Ultra concentrator (30 kDa MWCO, Millipore Ireland Ltd, Tullagreen, Ireland).

LDL aggregation susceptibility measurement

The measurement of LDL aggregation susceptibility was performed essentially as described before.[21] Briefly, isolated LDL particles were diluted to 200 µg/ml in 20 mM MES, pH 5.5, containing 150 mM NaCl and 50 µM ZnCl2. The size of the LDL particles was measured (0 h) using dynamic light scattering (Wyatt DynaPro Plate Reader II; Wyatt Technology, CA). Sphingomyelinase was added to the wells and the wells were coated

with paraffin. Particle aggregation was followed by measuring their size approximately every 15 minutes for 6 hours. Aggregation data was collected with Dynamics V7 software (Wyatt Technology, CA).

Lipid mass spectrometry analyses

For mass spectrometry (MS), total lipids of the blood plasma LDL isolates were extracted into chloroform according to Folch et al.[29] Before MS analysis aliquots of the lipid extracts were dissolved in chloroform/methanol (1:2 v/v) and spiked with the quantitative internal standard mixture designed for human plasma lipids (SPLASH® LIPIDOMIX® Mass Spec Standard No 330707; Avanti Polar Lipids, Inc., AL). This mixture contained separate deuterium labelled standard compounds with exact concentration for each of the LDL lipid classes, which thus were quantified against their own standards having similar efficiency of detection as the natural lipid species in LDL. Just before the analysis NH_4OH was added to sample aliquots (to give 1% solution by vol) to support ionization and prevent sodium adduct formation. The sample solutions were infused via a syringe pump into the electrospray ionization (ESI) source of a triple quadrupole mass spectrometer (Agilent 6490 Triple Quad LC/MS; Agilent Technologies, Inc., Santa Clara, CA) at a flow rate of 10 µl/min. The MS+ scan was used to detect TAG species as (M+NH4)+ ions[30], whereas MS/MS precursor ion scans were used to detect PC, LPC and SM species (precursors of m/z 184) and CE species (precursors of m/z 369). The ESI-MS/MS instrument was set to a source temperature of 250°C and collision energies of 10-30 eV (optimized for each lipid class) were used. Nitrogen was used as the collision, nebulizing (20 psi) as well as drying gas (11 µl/min). Data analysis of the mass spectra were performed by using MassHunter Workstation qualitative analysis software (Agilent Technologies, Inc.) and the individual lipid species were quantified using the internal standards and Lipid Mass Spectrum Analysis software.[31] The concentrations generated by LIMSA were converted to molar percent data. In addition, the acyl chain assemblies in such TAG and PC species that clearly separated the South Asian and white Caucasian samples were studied by recording their acyl chain specific MS/MS fragments.[32] For TAG species, positive ion mode neutral loss scans of different acyl chains were detected. For PC species, their formate adducts served as mother ions for negative ion mode precursor scans of the acyl fragments.

Statistical analyses

Raw data of LDL aggregate size was analyzed with GraphPad Prism (version 8.0.1, GraphPad Software, La Jolla, CA). Due to limitations of dynamic light scattering sensitivity for large particles, the maximum aggregate size was limited to 3000 nm and the minimum size to 14 nm. LDL aggregation curves were fitted with nonlinear regression curve fit

([Agonist] vs. response – Variable slope [4 parameters]) and aggregate size at the 2-h time point was interpolated.

The results are presented as mean ± SD and the statistical significance between groups was determined by unpaired Student´s t-test or by Mann-Whitney test. These

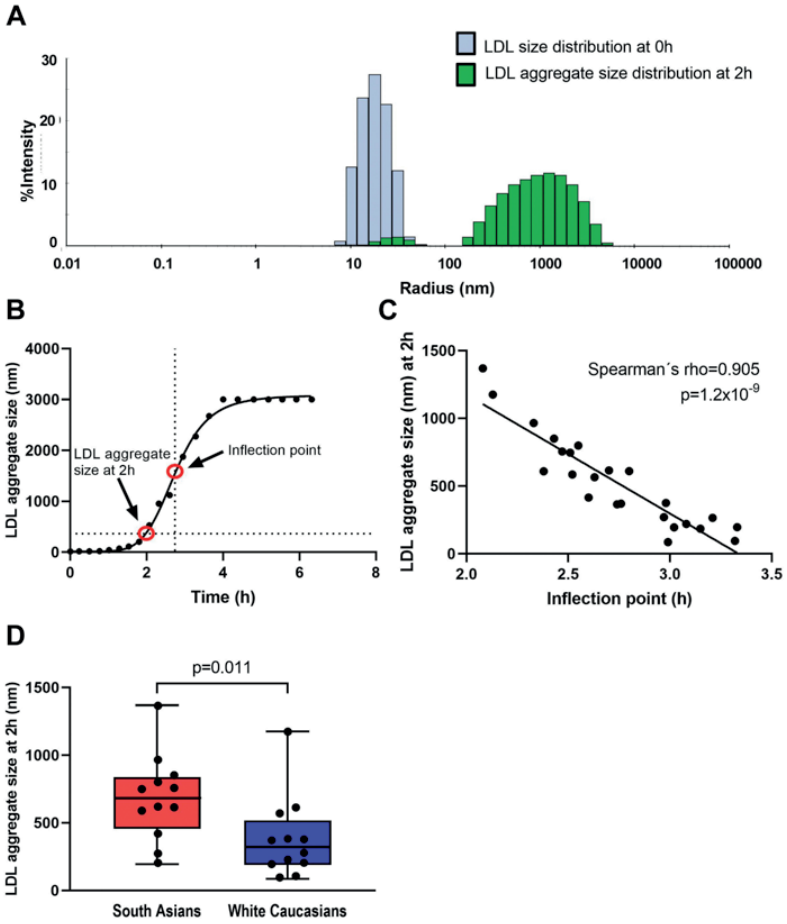

Figure 1. (A) The size distribution of both native LDL and LDL treated with human recombinant sphingomyelinase for 2 h was determined by dynamic light scattering. (B) LDL aggregation curve (LDL aggregate size (nm) vs. time (h)) after inducing aggregation with human recombinant sphingomyelinase. LDL aggregate size at time point 2 h (365 nm) is marked in the curve as well as the calculated inflection point (2.74 h). (C) Correlation of LDL aggregate size (nm) at 2 h and the calculated inflection point (h) (Spearman's rho=0.905, p=1.2x10-9). (D). LDL particles were isolated from plasma of South Asian men (n=12) and white Caucasian men (n=12), treated with sphingomyelinase, and aggregate size was measured using dynamic light scattering. Aggregate size at the 2-h time point was calculated from aggregation curves. The box plot diagram shows the median and the upper and lower quartiles of the aggregate size at 2 h in both ethnicities, the whiskers presenting the lowest and the highest values. Statistical significances of the differences between the ethnicities were studied by using Mann-Whitney U test. LDL, low-density lipoprotein. .

tests and two-tailed Spearman correlation coefficient analysis were performed using IBM SPSS Software (version 25.0, North Castle, NY). P-values < 0.05 were considered to be significant. Correlation analyses were performed for the total study population as well as per ethnicity. In addition, the PC species profiles of the LDL isolates were subjected to principal component analysis using Sirius, PRS, Bergen, Norway (version 8.5). False discovery rate (FDR) was controlled by using two-stage step-up method of Benjamini, Krieger and Yekutieli using GraphPad Prism.

RESULTS

Baseline characteristics

Twelve healthy Dutch men of South Asian descent and twelve age- and BMI-matched white Caucasian men participated in this study. **Table 1** shows the clinical characteristics of the participants. Compared with white Caucasians, South Asians had a higher body fat percentage (18.9 ± 3.2 vs. 14.5 ± 4.7%, p=0.015, unpaired Student´s t-test) at a similar BMI (24.7 ± 2.7 vs. 23.9 ± 2.4 kg/m^2, p=0.47). There were no significant differences between ethnicities in plasma glucose and serum insulin, triglycerides, HDL-cholesterol or LDL-cholesterol, while serum total cholesterol was higher in South Asians than in white Caucasians (4.8 ± 0.8 vs. 4.2 ± 0.5 mmol/l, p=0.032).

Table 1. Clinical characteristics of study participants.

Clinical characteristics	South Asians (n=12)	White Caucasians (n=12)	p-value
Age (years)	27.5 ± 3.2	25.6 ± 3.2	
Body mass index (kg/m^2)	24.7 ± 2.7	23.9 ± 2.4	
Body fat (%)	18.9 ± 3.2	14.5 ± 4.7	0.015
Systolic blood pressure (mmHg)	119 ± 6	124 ± 9	
Diastolic blood pressure (mmHg)	75 ± 9	82 ± 10	
Triglycerides (mg/dL)	73 ± 36	73 ± 25	
Total cholesterol (mg/dL)	186 ± 31	162 ± 19	0.032
HDL-cholesterol (mg/dL)	46 ± 12	44 ± 9	
LDL-cholesterol (mg/dL)	126 ± 35	104 ± 15	
Insulin (pg/ml)	137 ± 133	136 ± 89	
Glucose (mmol/l)	4.8 ± 0.3	4.6 ± 0.2	

Data are presented as mean ± SD. Statistical differences between the ethnicities were determined using the unpaired Student´s t-test, and the p-value is reported in the case of a statistically significant difference between the South Asians and white Caucasians. LDL, low-density lipoprotein; HDL, high-density lipoprotein.

LDL from South Asians is more prone to aggregate than LDL from white Caucasians

To determine if LDL of South Asians is more prone to aggregate than LDL of white Caucasians, we isolated LDL from the plasma samples and measured LDL aggregation susceptibility. Treatment of LDL with sphingomyelinase induced rapid formation of large aggregates (**Figure 1A**). In accordance with our earlier report,[21] LDL aggregate size at 2 hours correlated tightly and significantly with the calculated inflection point of the curve describing the aggregate size as a function of time (Spearman's rho = -0.905, $P=1.2 \times 10^{-9}$) (**Figures 1B** and **C**). There were no significant differences in the size of LDL particles in the beginning of the incubation or in the end of the incubation. However, LDL from South Asians aggregated more rapidly than LDL from white Caucasians, as indicated by a larger LDL aggregate size at 2 h (620 ± 320 nm vs 350 ± 290 nm, p=0.011; **Figure 1D**).

LDL lipid composition correlates with LDL aggregation and differs between the ethnic groups

LDL particles have an amphiphilic surface monolayer containing phospholipids and unesterified cholesterol and a hydrophobic core containing CEs and TAGs. A single copy of apoB-100 surrounds the particle. Since LDL lipid composition has been shown to influence the aggregation susceptibility of LDL particles,[21] we next analyzed the LDL lipidome in both ethnicities (n=24). Within this pooled data, SM 23:0, SM 24:0 and TAG 56:8 were associated with aggregation-prone LDL, while TAG 54:1, TAG 52:2 and CE 16:0 and CE18:1 were associated with aggregation-resistant LDL (**Figure 2A**). When the relative amounts of these lipids in LDL particles were compared between the 2 ethnic groups, South Asians had significantly more SM 24:0 and TAG 56:8, 2 lipids associated with aggregation-prone LDL, and less CE 18:1 and TAG 52:2, two lipids that were associated with aggregation-resistant LDL (**Figure 2B–D**). In addition, we determined associations between LDL lipid composition and LDL aggregation susceptibility in South Asians and white Caucasians separately (**Supplemental Figure 1**). Although in South Asians only LPC 20:3 correlated with LDL aggregation, in white Caucasians several lipid species correlated with LDL aggregation, including lipids that positively (TAG 56:8 and SM 24:0) and negatively (TAG 54:1) correlated with LDL aggregation when ethnicities were combined.

In addition to these lipids that significantly associated with LDL aggregation, there were also other differences between the LDL lipidomes of South Asians and white Caucasians (**Figure 3** and **Supplementary Table 1**). LDL from white Caucasians had a higher proportion of 2 highly unsaturated PC species; 36:5 and 38:5, but lower proportion of PC38:4 (**Figure 3A**). The PC species profiles, trait of the LDL surface, were also studied by multivariate principal component analysis (**Figure 3B**), and also in this analysis, the South Asians were found to have relative enrichment of PC 38:4 and 36:2, which species

contained arachidonic acid (20:4) and its precursor linoleic acid (18:2), respectively (as evidenced by acyl chain specific MS/MS precursor scans). In addition, several monounsaturated PC species were present with higher proportions in white Caucasians (**Figure 3A and 3B**). Of all core lipids, monounsaturated CEs (16:1 and 18:1) were higher in white Caucasians, while CE 18:2, the most common lipid in LDL, was higher in South Asians (Figure 3C). Of note, the elevated TAG 56:8 of South Asians was comprised of two main molecular species 16:0/18:2/22:6 and 16:0/20:4/20:4 (as evidenced by studying the acyl chain specific MS/MS neutral losses from the TAG molecule). Of these, the amount of 16:0/18.2/22:6 was similar in different samples but the amount of the arachidonic acid containing varied from sample to sample and was on average higher in the South Asian samples.

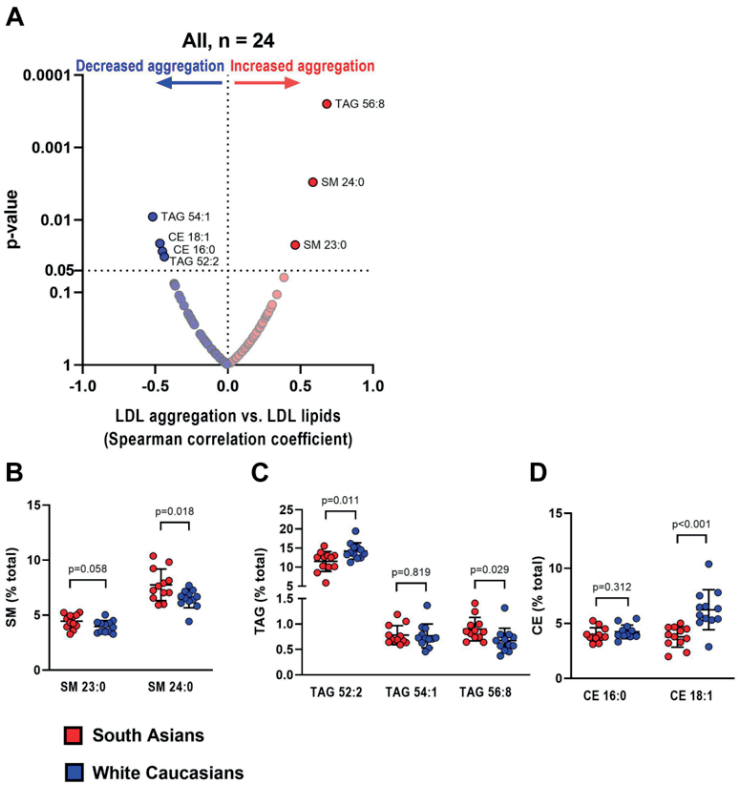

Figure 2. (A) Volcano plot showing the Spearman´s correlation coefficients of LDL aggregate size at 2 h (LDL aggregation susceptibility) vs. LDL lipids (n=24). Only those lipids with significant p-values ($p<0.05$) are annotated within the figure. Positive correlations are indicated with red circles and negative correlations with blue circles. (B-D) Scatter plot diagrams (mean ± SD) showing the proportions of the (B) surface lipid species (SM, sphingomyelin), (C) core triacyclglycerols (TAGs) and (D) core cholesteryl esters (CE), which significantly correlate with aggregate size at 2 h, in South Asians and white Caucasians. Statistical significances of the differences between the groups were determined using the unpaired Student's t-test. The differences remain significant after FDR correction for all lipids with p-value < 0.05. .

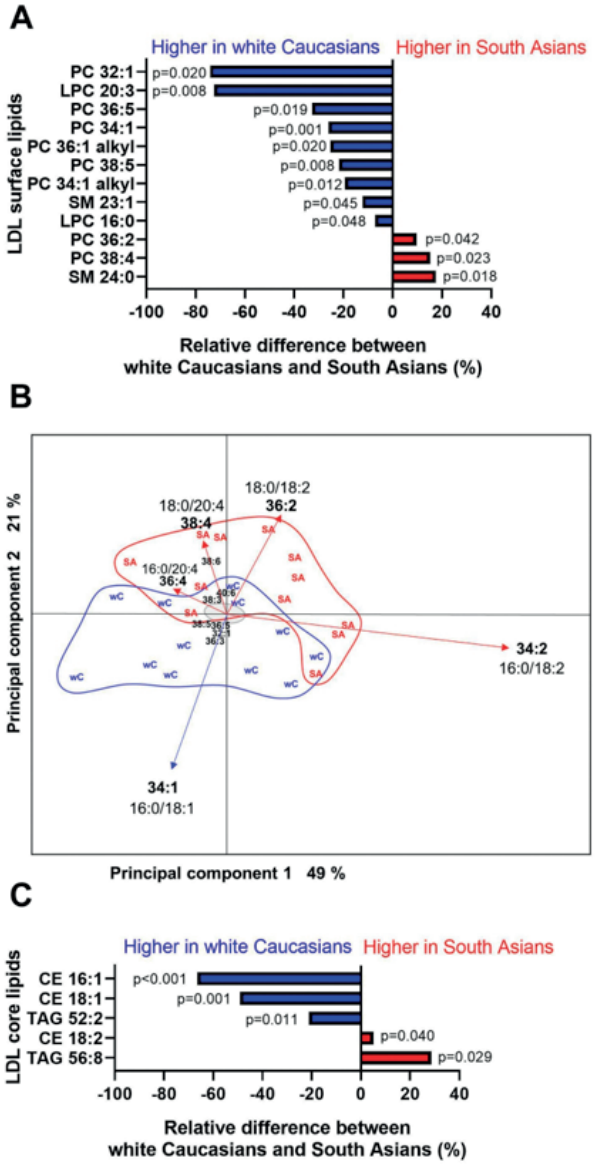

Figure 3. (A) Relative differences between South Asians and white Caucasians (((Average South Asians – Average white Caucasians) / Average all) x 100) in their LDL surface lipids. (B) Phosphatidylcholine (PC) species profile differences between the ethnicities were further demonstrated by PCA using untransformed data (the grey area at the origin of the PCA biplot represents the location of several species having little separation power in the analysis). (C) Relative differences between South Asians and white Caucasians (((Average South Asians – Average white Caucasians) / Average all) x 100) in their LDL core lipids. Statistical differences between the groups were determined using the unpaired Student's t-test. The difference in LPC 16:0, PC 36:2, SM 23:1, SM 24:0, TAG 52:2 and TAG 56:8 did not remain significant after FDR correction. CE, cholesteryl ester; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PCA, principal component analysis; SM, sphingomyelin; TAG; triacylglycerol.

Body fat percentage positively correlates with LDL aggregation susceptibility

Since obesity may modulate ASCVD risk by inducing alterations in the plasma lipidome, we next examined whether anthropometric measurements correlated with LDL lipid components and LDL aggregation. While no correlation with BMI was observed (data not shown), LDL aggregate size at 2 h significantly and positively correlated with body fat percentage (Spearman's rho=0.486, p=0.016) (**Figure 4A**). Interestingly, a higher body fat percentage was associated with a higher proportion of total SM and lower proportion of total PC in the surface of LDL particles (**Figure 4B**), which were previously shown to be characteristics of aggregation-prone LDL.[21] High body fat percentage was also associated with a high relative content of CE 18:2 and TAG 56:8 and low proportion of monounsaturated CE species 16:1 and 18:1 in the core of LDL particles. Notably, many of these LDL components were associated in a similar manner with LDL aggregation (**Figure 2**). Moreover, some of these specific lipid species that correlated positively with both LDL aggregation and body fat percentage were present to a higher extent in LDL of South Asians (SM 24:0 and TAG 58:6), and vice versa, CE 18:1 with low levels in South Asians correlated negatively with both LDL aggregation and body fat percentage (**Figure 2** vs. **Figure 4B**). Strikingly, the LDL lipid surface proportion of many PCs (PC 38:5, PC 34:1 and PC 32:1) correlated negatively with body fat percentage and were lower in South Asians than in white Caucasians. When the groups were evaluated separately, higher total SM and lower total PC content statistically significantly correlated with a higher body fat percentage only in South Asians but not in white Caucasians (**Supplemental Figure 2**).

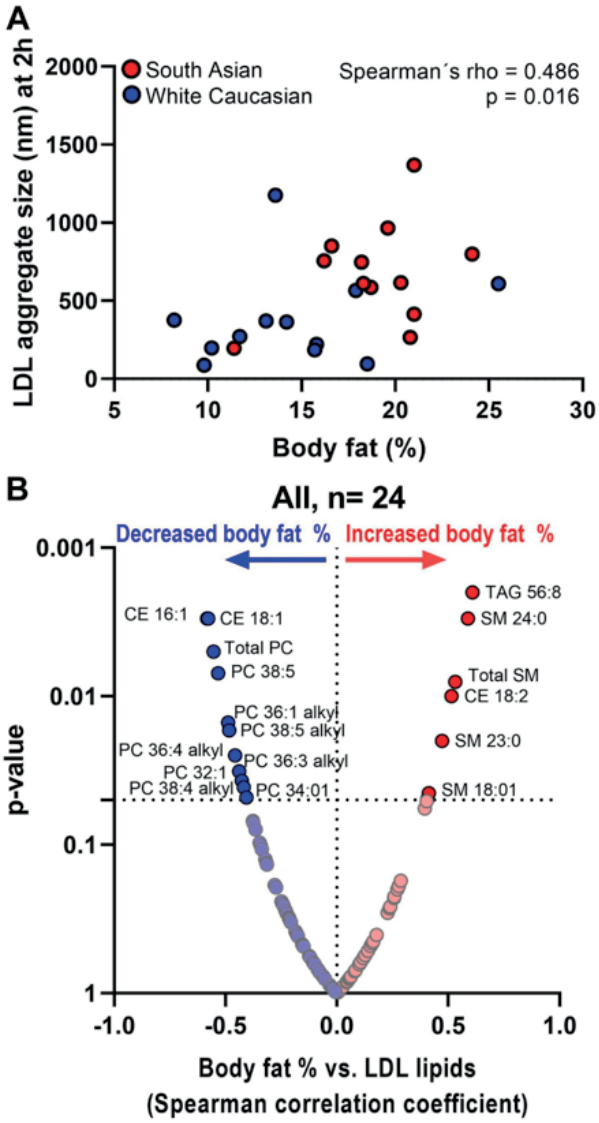

Figure 4. (A) Correlation between LDL aggregation susceptibility and body fat percentage (r=0.486, p=0.016). (B) Volcano plot showing the Spearman correlation coefficients of body fat percentage vs. LDL lipids (n=24). Only those lipids with significant p-values (p<0.05) are identified in the figure. Positive correlations are indicated with red circles and negative correlations with blue circles. CE, cholesteryl ester; LDL, low-density lipoprotein; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; SM, sphingomyelin; TAG; triacylglycerol.

DISCUSSION

In this study we show that South Asians, who have higher ASCVD morbidity and mortality than white Caucasians,[4-6, 33] have LDL that is more prone to aggregate than LDL from white Caucasians. Recently, we showed that increased LDL aggregation susceptibility is a marker of increased ASCVD risk independently of conventional risk factors.[21] As aggregation of modified LDL is one of the key steps in atherogenesis by promoting LDL retention,[9, 11, 16] foam cell formation,[35-37] inflammation[20] and plaque destabilization,[21] the presence of aggregation-prone LDL, besides being a marker, can also be a maker in several crucial steps in atherogenesis. Therefore, the unbeneficial LDL quality among South Asians could partly explain their increased risk for ASCVD compared with white Caucasians.

The susceptibility of LDL to aggregation is influenced by the lipid composition of the surface monolayer of LDL, which, in turn, influences the conformation of apo-B100.[21] Such conformational changes mediate formation of LDL aggregates.[15, 21] In addition to the surface lipids, also the core lipid composition has been identified to influence the conformation of apoB-100 within LDL.[37, 38] We previously showed that a high proportion of SM within the surface of circulating LDL is associated with aggregation-prone LDL, whereas a high proportion of PC is associated with aggregation-resistant LDL. Moreover, causality of the differences in the lipid composition in LDL aggregation was previously shown by modifying the proportions of SM and PC *in vitro* or *in vivo* in mice.[21] In the present study, we observed that the proportion SM 23:0 and SM 24:0 were higher in aggregation-prone LDL, which is thus in accordance with our previous results. In addition, of the core lipids, the proportion of highly unsaturated TAG 56:8 was higher in aggregation-prone LDL, whereas the proportion of TAGs 52:2 and 54:1 and CEs 18:1 and 16:0 were higher in aggregation-resistant LDLs. In accordance, TAGs and CEs harbouring saturated and monosaturated fatty acids in LDL, were also previously shown to associate with lower aggregation susceptibility of LDL particles.[21]

Of the individual lipid species that correlated with LDL aggregation susceptibility, TAG 56:8 is comprised largely of two main molecular species (16:0/18:2/22:6 and 16:0/20:4/20:4) of which particularly the arachidonic acid (20:4)-containing TAG species was higher in South Asians. Furthermore, the LDL surface PC profile of the South Asians was characterized by high proportion of PC 38:4 and PC 36:2, which also contained arachidonic acid or its precursor linoleic acid (18:2), respectively. In line with these findings, plasma levels of arachidonic acid have been reported to be higher in South Asians than in white Caucasians.[39] Arachidonic acid can be converted intracellularly into either pro-inflammatory or pro-resolving lipid mediators, while docosahexaenoic acid (22:6) is converted into pro-resolving lipid mediators. The balance between these pro-inflammatory and pro-resolving lipid mediators controls the inflammatory state of an

atherosclerotic plaque.[40] We propose that LDL enriched in arachidonic acid-containing TAGs and PCs species contributes to increased LDL aggregation in South Asians.

We observed for the first time that body fat percentage (mean 16.7%, range 8.2%-25.5%) correlated positively with aggregation susceptibility of LDL particles. Thus, in addition to having aggregation-prone LDL particles, South Asians had a higher body fat percentage than white Caucasians (18.9 ± 3.2 vs. 14.5 ± 4.7%) at a similar BMI (24.7 ± 2.7 vs. 23.9 ± 2.4 kg/m^2). This latter finding is in accordance with previous reports.[41, 42] Of note, BMI is a relatively poor predictor of body fat,[45] and indeed, we did not observe any association between BMI and LDL aggregation in either this study or in our previous study cohorts.[21] When investigating the LDL lipidome in relation to adiposity, we observed a positive correlation between body fat percentage and total SM content of LDL particles only in South Asians (Spearman's rho=0.678, p=0.014). SMs are bioactive lipids that are modulated by adiposity, as obesity-induced inflammation has been suggested to increase SM biosynthesis, and SMs have the potential to increase metabolic dysfunction and ASCVD risk.[22, 23, 44] In line with this, loss of visceral fat was recently associated with a decrease in plasma SM levels and reduced inflammation in athletes with a healthy body weight.[45] Furthermore, similar changes in circulating SM levels and inflammation status were observed during a 7-year follow-up period of individuals who lost body weight, whereas opposite effects were observed in individuals who gained weight.[45] Indeed, in addition to a higher body fat percentage in South Asians compared with white Caucasians, the surface of LDL particles of South Asians was enriched in SM 24:0. Collectively, these data support a link between being overweight and increased ASCVD risk,[46,47] and we suggest that adiposity may modulate the lipidome of LDL particles to affect their aggregation susceptibility.

Limitations of the study

The sample size in this study is relatively small and we investigated LDL aggregation susceptibility in blood samples only from healthy subjects in a cross-sectional study. Therefore, these results should be verified in larger study cohorts, also including patients with ASCVD. In addition, it would be highly interesting to investigate in a prospective study setting whether the susceptibility of LDL particles to aggregate indeed contributes to the development of ASCVD particularly in South Asians. Dietary information of the participants in this study is limited and the relative effects of diet vs. body composition on LDL aggregation susceptibility remain to be examined in future studies. In addition, the LDL aggregation assay requires isolation of LDL particles, which may challenge the clinical use and the use in of the assay in large cohorts. In the present study we found differences in the lipid composition of LDL particles between South Asian and white Caucasian participants. However, ethnicity may also influence the apolipoproteins other

than apoB-100 carried in LDL particles. Such differences and their potential effect on LDL aggregation remain to be studied.

Conclusions

This study provides evidence that LDL aggregation susceptibility is higher in young lean South Asians compared with BMI-matched white Caucasians. Mechanistically, this may be explained by the higher body fat percentage of South Asians, leading to SM enrichment of the LDL particle surface. We anticipate that the presence of SM- and arachidonic acid-rich, aggregation-prone LDL particles in young South Asians may increase LDL accumulation in the arterial wall and thereby contribute to their increased risk of developing ASCVD later in life.

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Authors' contributions: KÖ and PCNR designed and supervised the study together with MRB. LJ recruited the study participants and collected the samples, participant data, and analyzed baseline characteristics together with KJN. MR, LÄ, and FTS performed the experiments and analyzed the data together with HR, RK, and KÖ. OR produced the human recombinant sphingomyelinase. MR prepared the figures and tables and wrote the first draft of the manuscript to which LJ, KÖ, and PCNR provided critical comments and edits. All authors commented on the manuscript and have read and approved the final manuscript.

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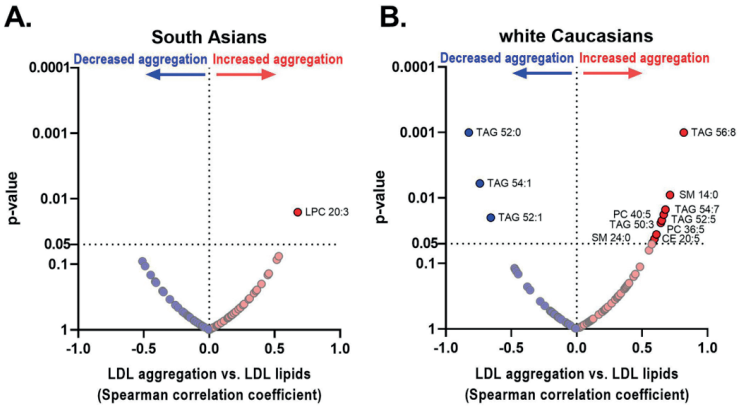
SUPPLEMENTAL APPENDIX

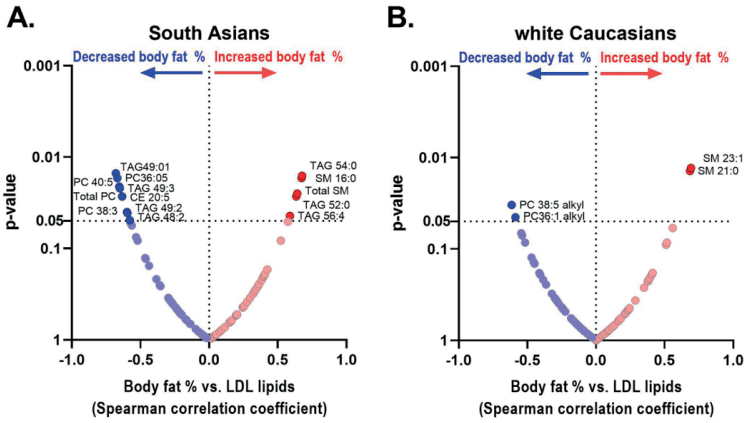
Supplementary Table 1. LDL lipids that differ significantly between white Caucasians and South Asians

LDL Surface Lipids	White Caucasians (mean % of lipid class)	South Asians (mean % of lipid class)	*P*-value
LPC 16:0	50.20 ± 3.85	46.75 ± 4.23	.048
LPC 20:3	0.80 ± 0.41	0.38 ± 0.27	.008
PC 32:1	0.61 ± 0.40	0.28 ± 0.18	.020
PC 34:1	11.47 ± 1.74	8.83 ± 1.58	.001
PC 34:1 alkyl	0.57 ± 0.10	0.47 ± 0.08	.012
PC 36:1 alkyl	0.26 ± 0.04	0.20 ± 0.07	.020
PC 36:2	14.02 ± 1.46	15.44 ± 1.75	.042
PC 36:5	1.21 ± 0.36	0.84 ± 0.29	.019
PC 38:4	5.94 ± 0.79	6.92 ± 1.14	.023
PC 38:5	2.95 ± 0.36	2.37 ± 0.57	.008
SM 23:1	3.40 ± 0.39	3.00 ± 0.51	.045
SM 24:0	6.50 ± 0.83	7.75 ± 1.44	.018
LDL core lipids			
CE 16:1	1.7 ± 0.6	0.9 ± 0.6	<.001
CE 18:1	6.2 ± 1.8	3.9 ± 1.5	.001
CE 18:2	66.5 ± 4.7	69.9 ± 3.5	.040
TAG 52:2	14.2 ± 2.1	11.5 ± 2.4	.011
TAG 56:8	0.67 ± 0.24	0.90 ± 0.26	.029

CE, cholesteryl ester; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; TAG; triacylglycerol. LDL lipids are expressed as mean percentages per lipid class (mean ± SD, n = 12 per group). Statistical differences between the groups were determined using the unpaired Student's t-test. The difference in LPC 16:0, PC 36:2, SM 23:1, and SM 24:0 did not remain significant after FDR correction.



Supplementary Figure 1. Volcano plot showing the Spearman correlation coefficients of LDL aggregate size at 2 h (LDL aggregation susceptibility) vs LDL lipids in (A) South Asians (n = 12) and (B) white Caucasians (n = 12). Only those lipids with significant P-values (P < .05) are annotated within the figure. Positive correlations are indicated with red circles, and negative correlations with blue circles. CE, cholesteryl ester; LPC lysophosphatidylcholine; PC phosphatidylcholine; SM, sphingomyelin; TAG; triacylglycerol.



Supplementary Figure 2. Volcano plot showing the Spearman correlation coefficients of body fat percentages vs LDL lipids in (A) South Asians (n = 12) and (B) white Caucasians (n = 12). Only those lipids with significant P-values (P < .05) are annotated within the figure. Positive correlations are indicated with red circles, and negative correlations with blue circles. CE, cholesteryl ester; LPC, lysophosphatidylcholine; PC phosphatidylcholine; SM, sphingomyelin; TAG; triacylglycerol.

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

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ABSTRACT

Aims

To compare the effects of cold exposure and the β3-adrenergic receptor agonist mirabegron on plasma lipids, energy expenditure and brown adipose tissue (BAT) activity in South Asians versus Europids.

Material and Methods

Ten lean Dutch South Asian (age 18–30 years; body mass index (BMI) 18–25 kg/m^2) and 10 age- and BMI-matched Europid men participated in a randomized, double-blinded, cross-over study consisting of three interventions; short-term (~ 2 hours) cold exposure, mirabegron (200 mg one dose p.o.) and placebo. Before and after each intervention, we performed lipidomic analysis in serum, assessed resting energy expenditure (REE) and skin temperature, and measured BAT fat fraction by magnetic resonance imaging.

Results

In both ethnicities, cold exposure increased the levels of several serum lipid species, whereas mirabegron only increased free fatty acids. Cold exposure increased lipid oxidation in both ethnicities, whereas mirabegron increased lipid oxidation in Europids only. Cold exposure and mirabegron enhanced supraclavicular skin temperature in both ethnicities. Cold exposure decreased BAT fat fraction in both ethnicities. After the combination of data from both ethnicities, mirabegron decreased BAT fat fraction compared with placebo.

Conclusions

In South Asians and Europids, cold exposure and mirabegron induced beneficial metabolic effects. When combining both ethnicities, cold exposure and mirabegron increased REE and lipid oxidation, coinciding with a higher supraclavicular skin temperature and lower BAT fat fraction.

INTRODUCTION

Obesity and associated diseases, including type 2 diabetes and cardiovascular diseases, are a major public health problem worldwide[1]. Certain ethnic subgroups, such as the South Asian, are particularly vulnerable to develop cardiometabolic disease. This is likely due, at least in part, to their disadvantageous metabolic profile, consisting of susceptibility to develop abdominal obesity, dyslipidaemia and insulin resistance [2-4]. The underlying mechanisms that explain this susceptibility are not fully understood but may involve differences in skeletal muscle metabolism, size of metabolic organs and regulation of adipocytokines in South Asians [5-8]. As a result, treatment options to improve the metabolic profile of the South Asian population are limited and unfocused, and specific strategies are needed.

Activation of brown adipose tissue (BAT) is an interesting therapeutic strategy to improve energy metabolism. BAT takes up triglyceride (TG)-derived fatty acids (FA) [9] and glucose from the systemic blood supply for combustion into heat, thereby increasing energy expenditure and improving lipid and glucose metabolism [10,11]. BAT is strongly innervated by the sympathetic nervous system. Cold exposure, resulting in sympathetic nervous system activation, is a potent physiological activator of BAT. Upon sympathetic nervous system activation, noradrenalin released from sympathetic nerve endings [12] acts on β-adrenergic receptors (β-AR) of brown adipocytes to promote thermogenesis [13-15]. Simultaneously, BAT releases endocannabinoids, which are believed to inhibit noradrenalin signalling to prevent excessive activation of BAT [16,17]. Circulating endocannabinoid levels are elevated in obesity [18-20] and, interestingly, South Asians have higher basal circulating endocannabinoid levels compared with Europids [21]. This might, at least partly, explain the reduced ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake by BAT and lower resting energy expenditure (REE) observed in South Asians compared to Europids during cold exposure [22]. In addition, the cold-induced increase in free fatty acid (FFA) levels, which generally results from lipolysis induced by sympathetic stimulation of white adipose tissue, is lower in South Asians compared to Europids [22]. Taken together, these data suggest that South Asians have a lower sympathetic outflow upon cold exposure compared to Europids.

Repetitive cold exposure is an effective strategy to enhance BAT metabolism, as cold acclimation increases BAT volume and even reduces fat mass in healthy lean men [23]. However, as a treatment or even lifestyle 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 β-ARs, agonists of such receptors might be a potent way to activate BAT. Indeed, preclinical studies have shown that treatment with the selective β3-AR agonist CL316,243 strongly stimulates BAT activity, prevents fat accumulation, improves dys-

lipidemia and insulin sensitivity, and attenuates the development of atherosclerosis [24]. Likewise, in humans, the β3-AR agonist mirabegron increased ^{18}F-FDG uptake by BAT as well as REE in healthy young men [25,26].

The aim of the current study was to assess the effects of cold exposure and the β3-AR agonist mirabegron on serum lipids, energy expenditure and BAT fat fraction and compare these in healthy lean South Asian versus Europid men.

MATERIAL AND METHODS

For more details of methods, see the supporting information.

Participants

Ten healthy, young (aged 18–30 years), lean body mass index ((BMI) 18–25 kg/m^2) Dutch South Asian men and 10 age- and BMI-matched Europid men were included in the study. The study (clinical trial registration number: NCT03012113) 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 [27]. Written informed consent was obtained from all volunteers prior to participation.

Study design

Participants were enrolled in a randomized, double-blinded, placebo-controlled cross-over study conducted between June 2017 and June 2018. The study consisted of three different interventions. During the first study visit, participants were exposed to an individualized water-cooling protocol to activate BAT as previously described [22]. Estimated supraclavicular BAT volume and fat fraction were assessed with chemical-shift encoded MRI. Only if BAT could be detected on MRI after cold exposure, were participants then randomized to receive first either 200 mg mirabegron (Betmiga®, Astellas BV, the Netherlands) or placebo in one oral dose. An overview of the study design is depicted in **Fig. S6**. Before each study day, subjects were fasted for 10 hours overnight and remained fasted until the end of the experiment.

Study visit 1: Cold exposure

During the first visit, a medical screening was performed to assess if participants met the inclusion criteria. In case of eligibility, body composition was measured by bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystat, UK). Precooling (thermoneutral conditions), a fasted blood sample was collected, and REE, lipid and glucose oxidation were measured via indirect calorimetry (Oxycon Pro, CareFusion, Germany) and cardiovascular parameters (including heart rate and blood pressure) were assessed with

Finapres Nova (Finapres Medical Systems BV, Netherlands). Thereafter, a precooling MRI scan (3T MRI, Philips Ingenia, Philips Healthcare, Best, the Netherlands) was performed to assess supraclavicular BAT fat fraction, transverse relaxation time (T2*) and estimated BAT volume using a three-dimensional six-point chemical-shift encoded gradient echo sequence, as described previously[28]. Next, 18 wireless iButtons were placed to monitor skin temperature (iButton®, Maxim Integrated Products, CA, USA), and an individualized water cooling protocol was applied to activate BAT, as described previously [22]. After maximal non-shivering thermogenesis was reached, cold exposure continued for 60 more minutes. Thereafter, after ~ 2 hours, a blood sample was obtained and cold-induced REE, lipid and glucose oxidation were measured again. Lastly, a second MRI scan was performed to assess changes in supraclavicular BAT after cold exposure.

Study visits 2 and 3: mirabegron and placebo treatment
During these study days, all measurements were performed under thermoneutral conditions. After 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 minutes), two hours (t=120 minutes), and three hours (t=180 minutes) after administration, REE, lipid and glucose oxidation were assessed again. At 3,5 hours (t=210 minutes), when reaching the maximum plasma concentration of mirabegron (*i.e.* Tmax ~ 3–4 hours), another blood sample was drawn and an MRI scan was performed to assess changes in supraclavicular BAT. Between study visit 1 and 2 there was a minimum wash-out period of 1 week, and between study visit 2 and 3 it was 2 weeks.

Analyses

Serum measurements
Commercially available enzymatic kits were used to measure serum concentrations of TG and total cholesterol (Roche Diagnostics, the Netherlands), HDL-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). LDL-cholesterol (LDL-C) was calculated using the Friedewald equation [29].

Serum lipidomic analysis by high performance liquid chromatography-mass spectrometry
Serum lipidomic analysis was performed essentially as described previously [30,31]. The dataset was processed using an in-house developed metabolomics pipeline, written in the R programming language (*http://www.r-project.org*).

Skin temperature

Eighteen wireless iButton temperature sensors were placed as adapted from 14 prescribed ISO-defined positions [32] (forehead, left chest, right abdomen, right thigh, right shinbone, right foot, back of the neck, right scapula, left lower back, left upper leg, right deltoideus, right forearm, right fingertip, left supraclavicular) and four additional positions (left hand, left lower leg, left elbow, and right armpit) [33]. Data were analysed using Temperatus software [34]. Armpit temperature was estimated and used as a proxy of core body temperature [35]. Supraclavicular skin temperature was estimated from an iButton placed above the left clavicula. Distal skin temperature was calculated as the average temperature of the left hand and right foot [36]. Proximal skin temperature was defined as the average of the iButtons on the chest, abdomen, scapula, and lower back [37].

Indirect calorimetry

VO_2 and carbon dioxide production were determined every minute. Mean VO_2 and VCO_2 obtained by indirect calorimetry were entered into Weir's abbreviated equation (see below) to estimate energy expenditure, and REE was calculated as VCO_2/VO_2:

$$REE\ (Kcal/min) = 3.941 \times VO_2(l/min) + 1.106 \times VCO_2(l/min)$$

Additionally, nutrient oxidation rates (i.e. carbohydrate and fat oxidation) were determined using Frayn equations [38].

MRI analysis

An in-house water-fat separation algorithm was used to reconstruct fat fraction maps, combined with a region-growing scheme to mitigate main field inhomogeneity effects [39-42]. Regions of interest encompassing the known location of the left supraclavicular BAT depot [43] were drawn manually by one observer (**Fig. S7)**. Registration was performed using the image registration software Elastix [44,45]. The average fat fraction, T2* and estimated BAT volume of the supraclavicular adipose depot were computed for pre- and postcooling, postmirabegron and post-placebo scans. Only voxels with a fat fraction between 50-100% were included for data analysis. One participant was excluded from all MRI analyses because of a failure to reconstruct the scan caused by excessive movement.

Statistical analysis

Data were analysed using IBM SPSS Statistics for Windows version 22.0 (SPSS Inc, Chicago, IL, USA). Figures were created by GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA). Paired t-tests were used to study the effect of cold exposure, mirabegron and placebo treatments on serum lipids and skin temperature. Furthermore, paired t-

tests were used to study the effect of cold on REE and nutrient oxidation, and two-way repeated measures ANOVA was applied to study the effect of placebo *vs.* mirabegron on REE and nutrient oxidation. To study differences between interventions (cold exposure versus mirabegron versus placebo) in BAT MRI outcomes and the deltas (value after minus before intervention) of serum lipids and skin temperature, we performed one-way ANOVA with Bonferroni adjustments for posthoc comparisons. Moreover, to study changes in REE and nutrient oxidation over time and to assess differences between mirabegron and placebo treatments herein, we performed a two-way repeated measures ANOVA with the variables 'time' (0, 1, 2 and 3 hours) and 'treatment' (mirabegron or placebo) as within-subject factors. For the lipidomics data, mixed model analyses were used. P-values were adjusted for false rate of discovery (FDR) using the Benjamini-Hochberg procedure. All main analyses are presented per ethnicity (Europids *vs.* South Asians), as well as combined for both ethnicities since we did not observe interaction between ethnicity, treatment and metabolic outcome parameters. A *P*-value <0.05 was considered statistically significant.

RESULTS

Participant characteristics

Participant characteristics are summarized in **Table 1.** Europid and South Asian participants were equal with respect to age (24.4±1.0 *vs.* 22.9±0.7 years) and BMI (22.7±0.6 *vs.* 22.3±0.3 kg/m^2). South Asians were however shorter (1.77±0.1 *vs.* 1.86±0.02 m, p<0.01), had a higher body fat percentage (16.7±1.2 *vs.* 12.9±0.8%, p<0.05) and lower fat free mass (59.5±1.9 *vs.* 67.6±1.3 kg, p<0.01) in comparison to Europids. Basal fasting glucose, insulin, and lipid levels were comparable between ethnicities, except for LDL-C levels that tended to be higher in South Asians compared to Europids (4.3±0.4 *vs.* 3.1±0.4 mmol/L, p=0.051).

Table 1. Participant characteristics

	Europids (*n*=10)	**South Asians (*n*=10)**
Age (years)	22.9 (2.2)	24.4 (3.1)
Height (m)	1.86 (0.06)	1.77 (0.05) **
Weight (kg)	77.7 (5.9)	71.5 (7.6)
Body mass index (kg/m^2)	22.3 (1.1)	22.7 (1.8)
Waist circumference (cm)	82.1 (5.6)	78.2 (5.2)
Hip circumference (cm)	86.7 (4.7)	86.1 (5.4)
Fat mass (%)	12.9 (2.5)	16.7 (3.7) *

Table 1. Participant characteristics (continued)

	Europids (*n*=10)	**South Asians (*n*=10)**
Fat body mass (kg)	10.1 (2.5)	11.9 (3.2)
Fat free mass (kg)	67.6 (4.2)	59.5 (6.3) **
Glucose (mmol/L)	4.5 (0.4)	4.6 (0.3)
Insulin (pg/mL)	126 (59.1)	203 (182.8)
Free fatty acids (mmol/L)	0.43 (0..2)	0.48 (0.1)
Triglycerides (mmol/L)	0.79 (0.5)	0.87 (0.7)
Total cholesterol (mmol/L)	4.8 (1.5)	6.0 (1.3)
HDL-cholesterol (mmol/L)	1.4 (0.2)	1.2 (0.3)
LDL-cholesterol (mmol/L)	3.1 (1.3)	4.3 (1.3)

Values are presented as mean (standard deviation). Unpaired t-tests were used for comparison between South Asians vs. Europids. * $p<0.05$ and ** $p<0.01$. .

Mirabegron increases serum FFA and insulin levels

Because active BAT takes up lipids and glucose from the circulation, we first compared the effect of cold exposure and mirabegron on these serum variables in Europids and South Asians.

Two hours of cold exposure increased total cholesterol (TC) in Europids only (+16%, $p<0.05$; **Fig. 1A**). This was accompanied by an increase in HDL-cholesterol (HDL-C) (+9%, $p<0.05$) in Europids, an observation that also reached significance in South Asians (+11%, $p<0.01$; **Table S1**). TG levels were not changed upon cold exposure (**Fig. 1B**), while FFA levels were increased, but only in Europids (+61%, $p<0.001$; **Fig. 1C**). Glucose, LDL-C (**Table S1**) and insulin (**Fig. 1D**) levels were not affected by cold exposure in either ethnicity. There was no significant interaction between ethnicities and, therefore, we performed combined analyses of data from both ethnicities. Pooling of ethnicities showed that cold exposure significantly increased TC (**Fig. S1A**), TG (**Fig. S1B**) and FFA (**Fig. S1C**).

One dose of mirabegron did not affect TC (**Fig. 1A**), TG (**Fig. 1B**), LDL-C or HDL-C (**Table S1**) in Europids or South Asians, nor when both groups were combined in a single analysis (**Fig. S1** and **Table S1**). Mirabegron increased FFA levels in Europids (+214%, $p<0.001$) and South Asians (+155%, $p<0.001$) (**Fig. 1C**). In addition, mirabegron similarly increased insulin levels in Europids (+23%, $p<0.05$) and South Asians (+38%, $p<0.01$) (**Fig. 1D**), without affecting glucose levels (**Table S1**).

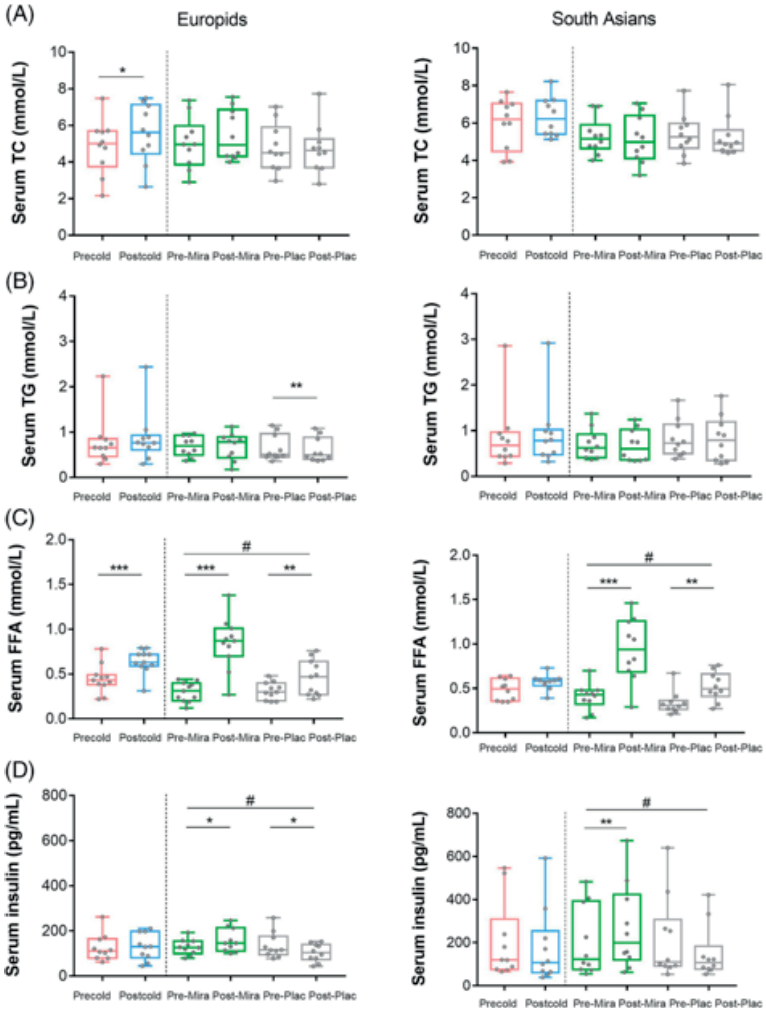

Figure 1. Effect of cold exposure, mirabegron and placebo on serum lipids and insulin in Europids and South Asians

Serum was collected pre-cold and post-cold, mirabegron (mira) or placebo (plac) in Europids (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 means ± 95% CI. Paired t-tests were used to assess the effect of the different treatments on serum parameters * $p<0.05$, ** $p<0.01$, *** $p<0.001$ before vs. after intervention. One-way ANOVA was performed to study the delta's in time (after treatment minus before) between treatments. # $p<0.05$ delta time between treatments.

Mirabegron does not change the serum lipidome

To obtain a more comprehensive understanding of changes in the lipid profile induced by cold exposure and mirabegron, we next performed a semi-targeted high performance liquid chromatography-mass spectrometry-based analysis of the lipidome in serum.

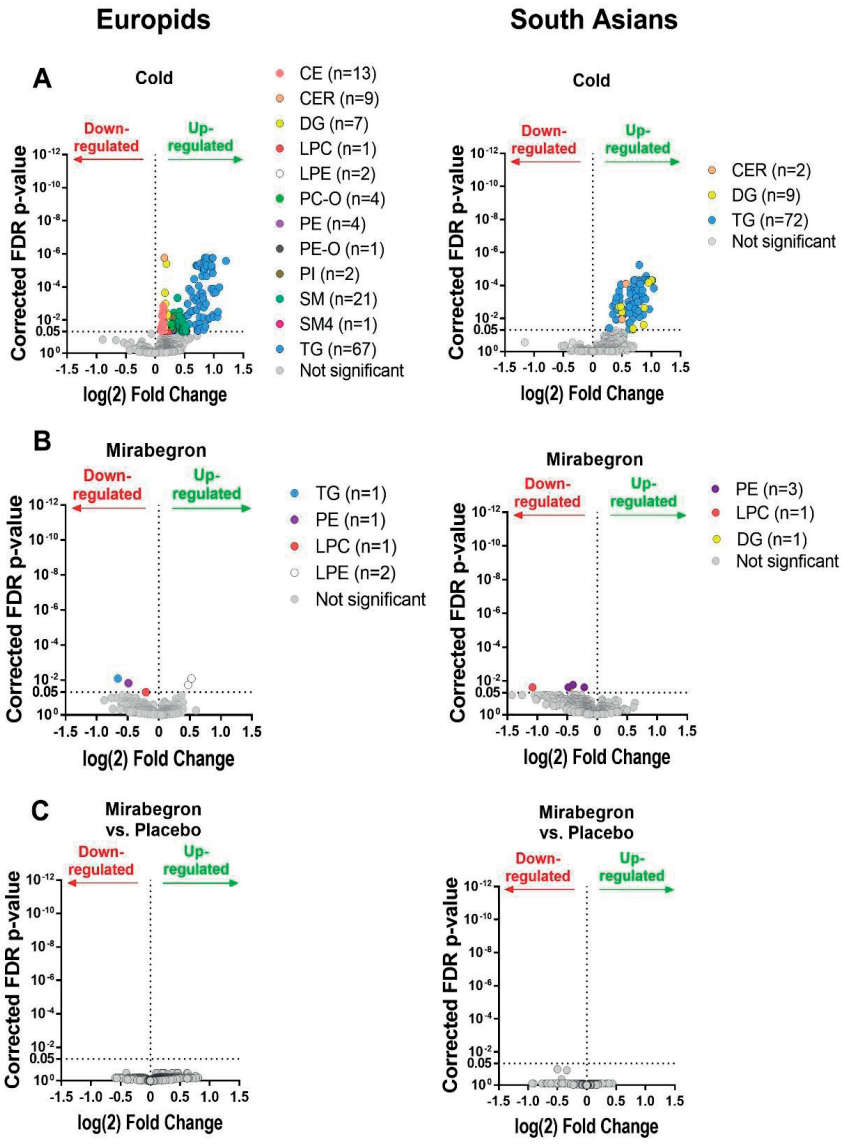

Figure 2. Effect of cold exposure, mirabegron and placebo on serum lipidome in Europids and South Asians
Volcano plots showing lipidomics data in response to cold exposure (A), mirabegron (B) or the difference between mirabegron and placebo (C) in Europids (n=10) and South Asians (n=10). Fold change represents the change of these lipids in comparison to the baseline (log (2)) (x-axis). P-value was corrected by the false rate of discovery (FDR). The horizontal dash line shows the level of significance (FDR corrected p<0.05). CE: Cholesteryl ester; CER: Ceramide; DG: diglyceride; LPC: Lysophosphatidylcholine; PC-O: phosphatidylcholine etherphospholipid; (L)PE: (Lyso)phosphatidylethanolamine; PE-O: phosphatidylethanolamine etherphospholipid; PI: Phosphatidylinositol; PS: Phosphatidylserine; SM: sphingomyelin; SM4: sulfatide; TG: triglyceride. q-value represents p-value after FDR corrections.

Cold exposure increased 132 and 83 out of ~1000 annotated lipid species in Europids and South Asians, respectively (**Fig. 2A**). Of these increased lipid species, 67 (51%) and 72 (87%) were long-chain TG in Europids and South Asians, respectively. These changes were accompanied by increases in diglycerides in both ethnicities. Cold exposure also increased 21 sphingomyelins, 13 cholesteryl esters, 9 ceramides and 4 phosphatidylethanolamines in Europids, whereas in South Asians only 2 ceramides were increased. Although this suggests an ethnicity-specific response to the cooling protocol, there were no statistically significant differences between Europids and South Asians upon cold exposure for any of the lipid species.

Interestingly, none of these cold-induced changes in the lipidome were observed in Europids or South Asians after treatment with mirabegron (**Fig. 2B**) or placebo (not shown). In fact, mirabegron downregulated 3 lipid species in Europids and 4 in South Asians. In addition, there was no statistically significant difference between mirabegron and placebo treatment for any of the lipid species in either ethnicity (**Fig 2C**), or when data of the individuals from both ethnicities were combined into a single analysis (**Fig. S2C**).

Mirabegron increases lipid oxidation

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

Precooling REE was lower in South Asians compared with Europids (1347±46 *vs*. 1563±66 kcal/day, $p<0.05$; **Fig. 3A**), while lipid oxidation and carbohydrate oxidation were comparable. Of note, the ethnic differences in REE were no longer present after correction for lean body mass (data not shown). Cold exposure increased REE in both Europids (+20%; $p<0.01$) and South Asians (+29%; $p<0.05$) (**Fig. 3A**). In addition, cold exposure increased lipid oxidation in Europids (+114%, $p<0.01$) and South Asians (+97%; $p<0.05$) (**Fig. 3B**), whereas carbohydrate oxidation remained unchanged in both ethnicities (**Fig. 3C**). The increases in REE and lipid oxidation upon cold exposure were still observed when both groups were analysed together (**Fig. S3A** and **3B**).

Mirabegron treatment did not increase REE over time when compared to placebo in Europids or South Asians (**Fig. 3A).** However, mirabegron did promote lipid oxidation in Europids when compared to placebo (p for time*treatment=0.035, **Fig. 3B**), whereas this was not the case in South Asians (p for time*treatment=0.270, **Fig. 3B**). Mirabegron did not affect carbohydrate oxidation (**Fig. 3C**).

Because two-way ANOVA with repeated measurements did not reveal interaction between ethnicities in any of the tests (all $p>0.05$), we performed combined analyses. These analyses showed that mirabegron significantly increased REE compared with placebo treatment, specifically in the second hour after treatment (**Fig. S3A**). This was

because of an increase in lipid oxidation (p for time*treatment <0.001, **Fig. S3B**), while carbohydrate oxidation was slightly decreased after 2 hours of treatment compared with baseline (p<0.05, **Fig. S3C**).

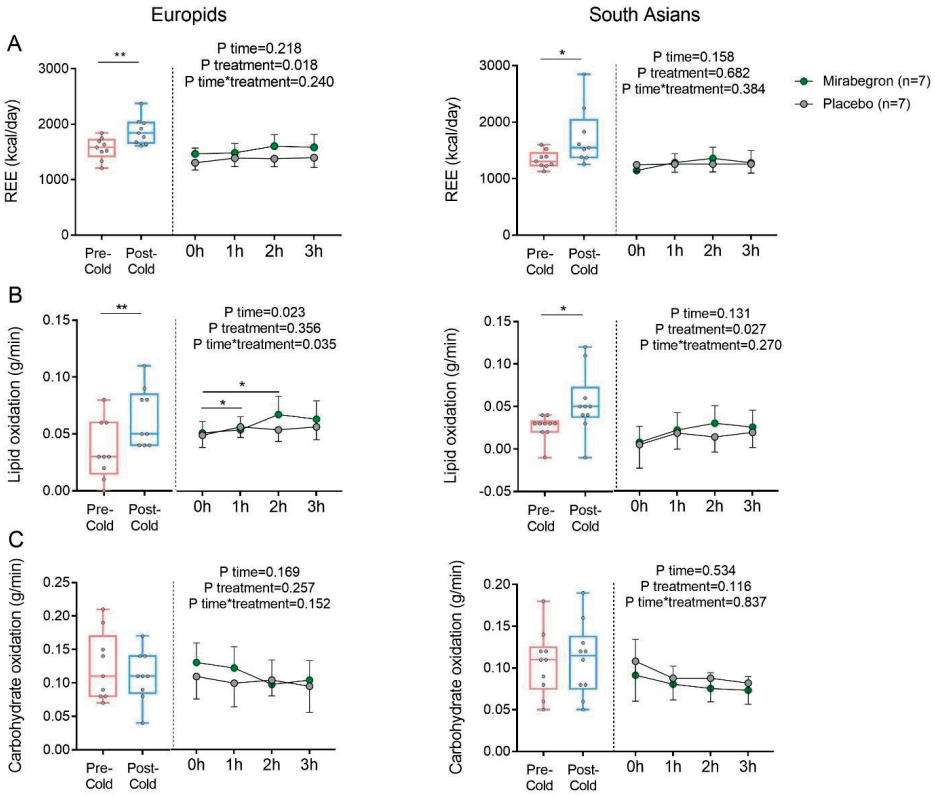

Figure 3. Effect of cold exposure and mirabegron in comparison with placebo on resting energy expenditure and nutrient oxidation in Europids and South Asians. Precold (red boxes) and postcold (blue boxes) REE and nutrient oxidation in Europids (n=10) and South Asians (n=10). Paired t-tests were performed to study the effect of cold exposure. The effect of mirabegron or placebo on REE (A), lipid oxidation (B) and carbohydrate oxidation (C) were studied by a repeated measures two-way ANOVA with 'time' (0, 1, 2 and 3 h) and 'treatment' (mirabegron or placebo) as within-subject factors. The analyses were performed per ethnicity. P for time, P for treatment and P for time*treatment were obtained from the two-way ANOVA. Data are presented as mean ± 95% CI. * p<0.05 and ** p<0.01.

Mirabegron increases supraclavicular skin temperature

The main function of BAT is heat production. Because supraclavicular skin temperature positively associates with ^{18}F-FDG uptake by BAT in young healthy lean men [46], we compared the effects of cold exposure and mirabegron on skin and core temperature in Europids and South Asians.

Cold exposure increased armpit skin temperature (as a proxy of core temperature) in Europids (+1.0°C, p<0.01) and South Asians (+0.8°C, p<0.05) (**Fig. 4A**). Likewise, supraclavicular skin temperature was increased in Europids (+1.6°C, p<0.001) and South Asians (+1.7°C, p<0.001) (**Fig. 4B**). Furthermore, as expected, cooling decreased proximal skin temperature in Europids (-3.2°C, p<0.001) and South Asians (-4.9°C, p<0.001; **Fig 4C**) as well as distal skin temperature (-2.4°C, p<0.01 and -3.1°C, p<0.01, respectively) (**Fig. 4D**).

Mirabegron also increased armpit skin temperature in Europids (+0.6°C, p<0.05) and South Asians (+0.3°C, p<0.01) (**Fig. 4A**). Furthermore, mirabegron increased supraclavicular skin temperature in both Europids (+0.4°C, p<0.05) and South Asians (+0.7°C, p<0.01). (**Fig. 4B**). In contrast to cold exposure, mirabegron increased proximal skin temperature in Europids (+1.2°C, p<0.001) and South Asians (+1.4°C, p<0.001) (**Fig. 4C**), without affecting distal skin temperature (**Fig. 4D)**. Of these measures, only the increase in supraclavicular skin temperature after mirabegron treatment in Europids was higher when compared to placebo (p<0.05, **Fig. 4B**). Combining individuals of both ethnicities to perform a single analysis resulted in comparable results (**Fig. S4)**.

Mirabegron reduces supraclavicular BAT fat fraction without affecting T2* or estimated BAT volume

As BAT combusts intracellular lipids [24], studying changes in fat fraction of the supraclavicular fat depot by MRI has been used as a read-out for BAT activity. Therefore, we next compared the effect of cold exposure, mirabegron and placebo on BAT fat fraction, T2* and estimated BAT volume in Europids and South Asians. Hereby, T2* is defined as the effective transverse relaxation time which is influenced by both perfusion of oxygen-rich blood and the removal of deoxygenated blood in the tissue [47]. When BAT becomes activated, oxygen consumption increases due to enhanced metabolic activity and at the same time, perfusion increases to keep up with this demand. Deoxygenated blood causes a local distortion of the magnetic field resulting in signal loss, and thereby a shorter T2*. However, increased perfusion leads to a longer T2* due to the presence of more blood, and therefore more oxyhemoglobin. Thus, oxygen consumption leads to a decrease in T2*, whereas increased blood perfusion leads to an opposite effect [27]. Cold exposure lowered BAT fat fraction, both in Europids (-3.2%, p<0.001, **Fig. 5A)** and South Asians (-1.5%, p<0.05). Cold did not affect fat fraction in the dorsocervical and deltoid subcutaneous adipose tissues, as well as in deltoid skeletal muscle (data not shown). There was no difference in BAT fat fraction after mirabegron versus placebo treatment in Europids or South Asians. Also, as compared to placebo, mirabegron dit not affect fat fraction in the dorsocervical and deltoid subcutaneous adipose tissues, as well as in deltoid skeletal muscle (data not shown). Furthermore, while there was no effect of any of the treatments on BAT T2*, cold exposure lowered estimated BAT volume in Europids only, probably as a result of lowered fat fraction (**Fig. 5B** and **5C)**. When both

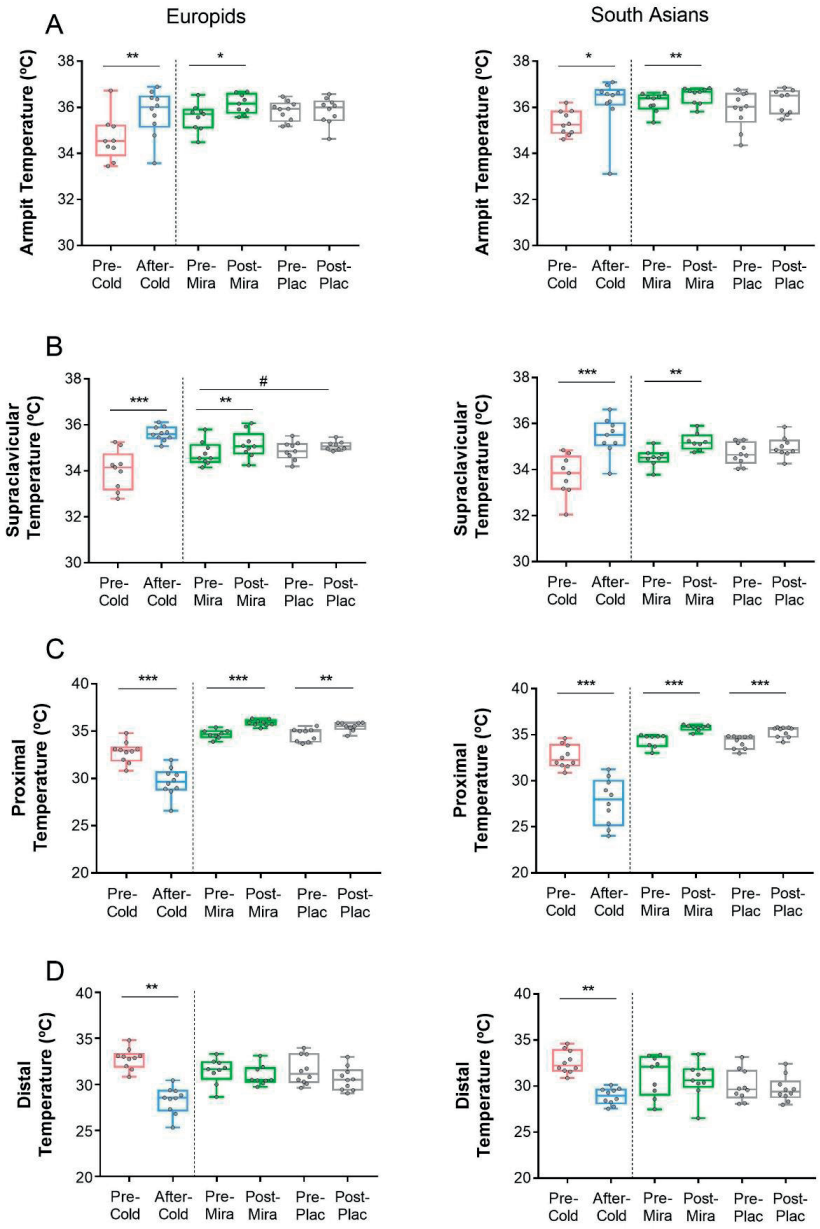

Figure 4. Effect of cold exposure, mirabegron and placebo on skin temperature in Europids and South Asians. Skin temperature was measured pre- and postcold, mirabegron (mira) and placebo (plac) in Europids (n=10) and South Asians (n=10). We directly measured armpit (A) and supraclavicular (B) skin temperatures, whereas proximal (C) and distal (D) skin temperatures were calculated following equations described in the supporting information. Data are presented as mean ± 95% CI. Paired t-tests were used to evaluate the effect of the interventions. * p<0.05, ** p<0.01, *** p<0.001 before vs. after intervention. # p<0.05 differences between the delta between treatments.

ethnicities were combined in a single analysis, cold exposure still lowered BAT fat fraction (-2.3%, p<0.001, **Fig S5A**) as well as the estimated BAT volume (-1.5%, p<0.05, **Fig S5C**). Of note, the average BAT fat fraction was lower after mirabegron versus placebo treatment (-1.4%, p<0.01, **Fig S5A**). Furthermore, BAT T2* still remained unaltered after all treatments (**Fig. S5B**).

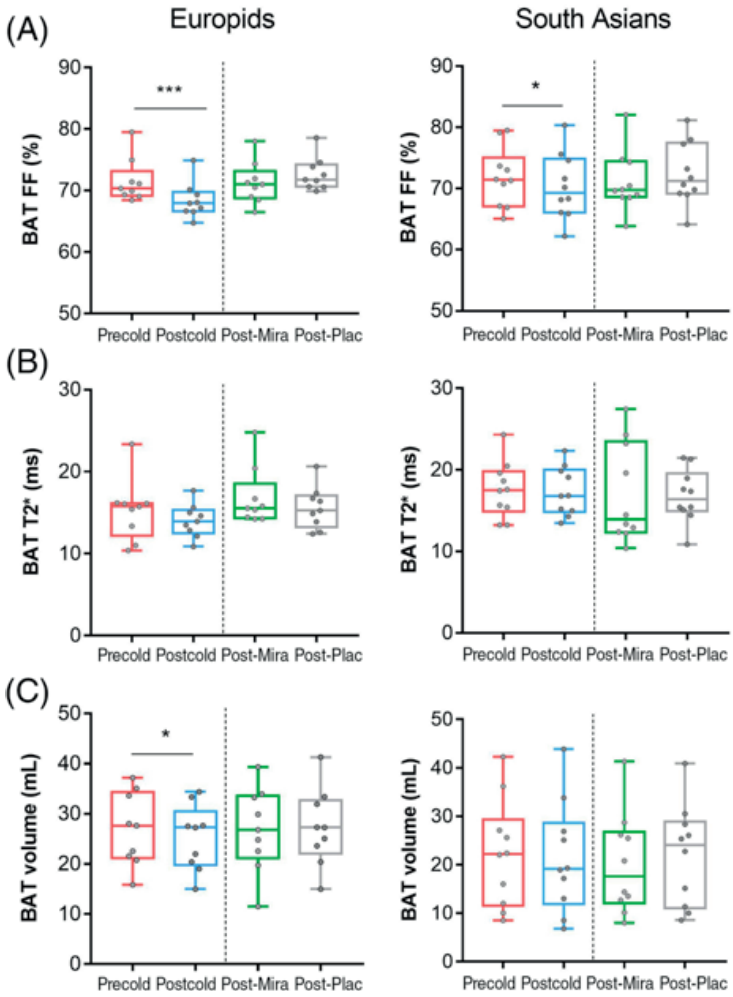

Figure 5. Effect of cold exposure, mirabegron and placebo on brown adipose tissue (BAT) fat fraction (FF), T2* and estimated volume in Europids and South Asians. MRI was used to determine BAT FF (A), T2* (B) and estimated volume (C) in Europids (n=10) and South Asians (n=9). Red boxes represent BAT-related outcomes before cold exposure and blue boxes represent BAT-related outcomes after cold exposure, green boxes after mirabegron (post-mira) and grey boxes after placebo (post-plac) treatment. All analyses were performed per ethnicity. One-way ANOVA was performed to study differences in BAT variables between treatments. ** p<0.01 and *** p<0.001 between treatments. One South Asian was excluded from analyses because of movement in the MRI.

Mirabegron increases heart rate

Although mirabegron is a comparatively specific β3-AR agonist, it does cross-react with β1-AR and β2-AR. Since subtypes of β-adrenergic receptors are abundantly present on heart and blood vessels, we investigated the effects of mirabegron on heart rate and blood pressure. Cold exposure decreased heart rate in white Caucasians (-2 beats/minute, $p<0.01$) and tended to decrease heart rate in South Asians (-1 beats/minute, $p=0.10$) (**Suppl. Table 2**). In addition, cooling increased systolic (+9%, $p<0.05$) and diastolic (+22%, $p<0.05$) blood pressure in South Asians only (**Suppl. Table 2**). Mirabegron increased heart rate both 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 significantly changed.

DISCUSSION

Targeting BAT by cold exposure or adrenergic receptor agonism is considered as a treatment strategy to combat cardiometabolic disease, which is more prevalent in South Asians compared with Europids. In the current study, we investigated the effect of targeting BAT by cold exposure and the β3-AR agonist mirabegron on the serum lipidome, REE, lipid oxidation, skin temperature parameters and BAT fat fraction, T2* and estimated BAT volume in healthy lean South Asians versus Europids. We found that the response to cold and mirabegron on these parameters was largely comparable between both ethnicities. We report that, in all subjects combined, both cold exposure and mirabegron increase serum FFA levels, lipid oxidation and supraclavicular skin temperature, while they decrease BAT fat fraction as compared to placebo. Cold exposure, but not mirabegron treatment, induced changes in the serum lipidome including appearance of long-chain triglycerides and diglycerides. This study supports the notion that both cold exposure and mirabegron may induce beneficial metabolic effects in Europid and South Asian subjects.

Because we included both South Asians and Europids in the current study, this gave us the opportunity to investigate whether the response to cold exposure and mirabegron would be different between ethnicities. We had reason to hypothesize this, because South Asians have a lower FFA response upon cold exposure [22] and higher circulating endocannabinoid levels compared with Europids [21], suggesting they have lower cold-induced sympathetic outflow to BAT. Because mirabegron is believed to activate BAT directly via β-ARs, we thus expected a more pronounced effect of mirabegron on REE and BAT fat fraction in South Asians compared with cold by circumventing sympathetic activation. Here, we confirmed a lower FFA response in South Asians upon cold exposure. However, counteracting our hypothesis, mirabegron enhanced lipid oxidation

compared with placebo only significantly in Europids, and responses of other metabolic parameters to mirabegron were comparable between Europids and South Asians. It could still be possible that the extent to which noradrenalin is released from sympathetic nerve endings is lower in South Asians, contributing to a lower sympathetic stimulation of BAT. Alternatively, β3 independent pathways may contribute to cold-induced activation of BAT and these may differ between ethnicities. Clearly, future studies are needed to clarify whether there is a true difference in sympathetic output upon cooling between South Asians and Europids. We also aimed to compare the effects of mirabegron with cold exposure on several metabolic variables, and 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 exposure and mirabegron was larger than after placebo in both groups. A possible explanation for the more pronounced increase in FFA levels after mirabegron compared to cold exposure may be a higher relative effect of mirabegron on liberating FFA from white adipose tissue compared with stimulating FFA uptake or combustion (*e.g.* by BAT). To further investigate specific changes in the lipidome, we also performed lipidomic analysis. We observed in both ethnicities that cold exposure, but not mirabegron, increased levels of long-chain TG, as well as a set of diglycerides. This is suggestive of increased hepatic production of (VLDL-)TG, probably due to globally enhanced sympathetic outflow as induced by cold exposure, coupled to increased peripheral lipolysis (*e.g.* by BAT). Indeed, we previously showed that a comparable duration and mode of cooling increased serum concentration of large VLDL-TG particles accompanied by increased mean size of VLDL particles, further supporting enhanced hepatic VLDL production [48]. The fact that the changes in lipidome mainly point towards increased hepatic VLDL production, probably induced by global sympathetic activation following cold exposure, may well explain the lack of effect of mirabegron on the lipidome.

It would be interesting to study the effect of mirabegron in combination with a treatment that further stimulates FFA combustion (*e.g.* by inducing 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 will ultimately be compensated by increased energy expenditure.

In addition, in contrast to cold exposure, we observed that a single dose of mirabegron increased serum insulin levels without affecting glucose levels. This is in line with data of Cypess et al. [26], who also showed increased insulin levels upon administration of the same dose of mirabegron in healthy lean volunteers. While this may be a very early sign of insulin resistance, the mirabegron-induced increase in FFA may also stimulate the pancreas to release insulin [49], which has been reported essential for efficient energy replenishment of activated BAT, at least in mice [50]. Alternatively, mirabegron may induce

insulin release through acting on the β3-AR on the pancreas. Stimulation of β3-AR on blood vessels in the pancreas might induce local vasodilatation resulting in increased blood flow [51], 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 [52]. In addition, insulin increases glucose uptake by tissues due to increased translocation of GLUT4 to the cell membrane. 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 [50]. In contrast, two recent studies have shown that long-term treatment (4-12 weeks) with mirabegron improves insulin sensitivity in healthy slightly overweight and obese subjects, possibly due to enhanced adiponectin levels and/or improved β cell function [53,54]. An interesting result of the current study was that, in contrast to cold exposure, mirabegron did not affect resting energy expenditure in Europids nor in South Asians. A small increase in fat oxidation was observed in Europids only. Interestingly, this increase was found after 2 hours, while the T_{max} of mirabegron is 3-4 hours. We can only speculate about the underlying cause. Possibly, the effect on fat oxidation occurs rather acute resulting in a quick peak, at least in the Europids. When both ethnicities were combined in a single analysis, mirabegron did increase resting energy expenditure. In a previous study of Cypess et al [26], a similar dose of mirabegron did induce a significant increase in resting metabolic rate (+203 ± 40 kcal/day). Our data support the notion that mirabegron is less efficient in activating BAT as compared to cold exposure. Possibly, cold activates BAT via other mechanisms besides β adrenergic signaling, such as via FFA release.

We also observed an increase in supraclavicular skin temperature upon mirabegron treatment, which may reflect local heat production possibly as a consequence of BAT activation [46]. Alternatively, this may be due to a direct effect of mirabegron on skin blood flow. Supporting increased BAT activation, we found a reduced BAT fat fraction upon mirabegron treatment in the combined group analysis. As was expected, we did not find an increase in the estimated BAT volume after acute cold exposure and mirabegron treatment. Such an increase may have been foreseen, if participants were acclimated to cold conditions or treated with mirabegron for a longer period, resulting in the recruitment of beige/brown adipocytes [55]. Instead, we found a reduction in the estimated BAT volume after cold exposure due to the exclusion of MRI voxels, for which the fat fraction fell below the segmentation threshold, as is more extensively described in our previous work [27]. On the contrary, the estimated BAT volume after mirabegron treatment remained unaltered, which is most likely due to the smaller effect compared to cold exposure. Cypess et al. [26] previously reported a massive increase in uptake of the glucose label ^{18}F-FDG by BAT as measured via positron emission tomography-computed tomography (PET-CT) scan after the same dose of mirabegron as used in the current study. Besides resulting from more active BAT, this increased ^{18}F-FDG uptake might also result from

vasodilation within BAT due to binding of mirabegron on β3-AR on the endothelium of arteries or due to stimulation of other adrenergic receptors on blood vessels within BAT [56,57]. It would be of interest to further investigate the extent by which mirabegron activates BAT, also because of the lack of increase in REE as mentioned above. Future studies should probably investigate BAT activity also with other imaging modalities and tracers, such as ^{11}C-acetate to investigate the oxidative capacity of the tissue. 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, amongst others, prolonged fasting. A limitation of the current study is that we measured BAT fat fraction at only one time point after cold exposure, mirabegron and placebo treatment. Because activated BAT also takes up lipids from the blood to restore intracellular lipid stores, we cannot exclude that this interfered with measurement of fat fraction as a proxy of BAT activity. This may thus result in an underestimation of the effect size of cold exposure and mirabegron on combustion of intracellular TG by BAT. For future studies, it would be preferable to combine fat fraction measurement by MRI with tracers that measure lipid uptake by PET-CT scan [58]. Furthermore, because we only found significant effects on REE and fat fraction after combining both ethnicities, the study may have been underpowered for these variables. 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 Europids have a comparable beneficial metabolic response to mirabegron and cold exposure. More specifically, both mirabegron and cold exposure increased FFA, lipid oxidation, and supraclavicular skin temperature, while they decreased supraclavicular BAT fat fraction. Only cold exposure induced changes in the lipidome indicative of changes in VLDL-TG production and lipolysis. Future studies should aim at unravelling the relative effect of both treatments on BAT activity by using alternative tracers such as those that assess glucose and lipid uptake, or oxidative capacity.

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SUPPLEMENTAL APPENDIX

Participants

Ten healthy, young (aged 18–30 years), lean (BMI 18–25 kg/m^2) Dutch South Asian men and ten age- and BMI-matched Europid 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 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 [1]. Written informed consent was obtained from all volunteers prior to participation. Trial register clinicaltrials.gov number NCT03012113 (registration date: January 6, 2017).

Study design

Participants were enrolled in a randomized, double-blinded, placebo-controlled crossover study consisting of three different interventions that were each performed on a different study day. The study was performed at the Leiden University Medical Center (Leiden, the Netherlands). During the first study visit, participants were exposed to an individualized water-cooling protocol to activate BAT as previously described [2]. Supraclavicular BAT volume and fat fraction were assessed with chemical-shift encoded MRI. As BAT combusts intracellular lipids [3], the intracellular fat fraction of BAT is expected to decrease upon activation. Therefore, the quantification of the fat fraction 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 mirabegron and placebo treatment. Thereafter, participants were randomised to receive first either 200 mg mirabegron (Betmiga®, Astellas BV, the Netherlands) or placebo in one oral dose. After cold exposure, there was a wash-out period of at least one week, and the wash-out period between the mirabegron and placebo treatment was at least two weeks. An overview of the study design is depicted in **Fig. S6**. Before every study day, 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. The primary outcome measure of the study was BAT fat fraction. The secondary outcome measures were changes in REE, skin temperature and plasma lipidome. Based on a previous study assessing the effect of a cold intervention on BAT activity using MRI [4], we expected a decrease in TG content of 4% in BAT (SD = 1.7%). We expected the same effect for mirabegron and therefore, we considered a decrease in TG content of BAT by mirabegron of 4% as therapeutically relevant as this is as effective as cold exposure. Thus, with an SD of 1.7, $\alpha = 0.05$, $\beta = 20\%$ we needed 10 subjects per group.

Randomisation and treatment allocation

Mirabegron tablets were repacked into capsules by the LUMC pharmacy to resemble the placebo. All capsules were consecutively numbered for each subject according to the randomisation schedule. Researchers received the capsules in pre-packed idential looking bottles from the pharmacy. Thus, both participants and researches were blinded to treatment allocation. Randomisation was performed by the LUMC pharmacy. The random allocation sequence was generated by means of excel. Stratification was done on ethnicity and subjects were randomized in blocks of 4. In short, 2 groups were made of 12 numbers (number 1 up to 12 for South Asians and 13 up to 24 for Europids) and within each group of 12 numbers, 50% started with mirabegron and 50% with placebo. Allocation of randomization numbers was done in the order of inclusion.

Study visit 1: Cold exposure

During the first visit, a medical screening consisting of a medical questionnaire, measurement of height, weight and blood pressure, and a blood draw were 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 bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystat, UK). Pre-cooling (thermoneutral conditions), a fasted blood sample was collected, and resting energy expenditure (REE), lipid and glucose oxidation were measured via indirect calorimetry (Oxycon Pro, CareFusion, Germany) and cardiovascular parameters (including heart rate and blood pressure) were assessed with Finapres Nova (Finapres Medical Systems BV, Netherlands). Thereafter, a pre-cooling MRI scan (3T MRI, Philips Ingenia, Philips Healthcare, Best, the Netherlands) was performed to assess supraclavicular BAT fat fraction, transverse relaxation time (T2*) and volume using a three-dimensional six-point chemical-shift encoded gradient echo sequence as described before [5]. 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 ΔTE=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 retrospective signal averages. Next, 18 wireless iButtons were placed to monitor skin temperature (iButton®, Maxim Integrated Products, CA, USA), and an individualized water cooling protocol was applied to activate BAT (as described previously [2]). 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 by 5°C every 10 minutes, until the participants started to shiver or until the minimum water temperature of 9°C was reached. Shivering was reported by the participants and confirmed visually by researchers. At that point, the water temperature was increased by 3°C to ensure maximal non-shivering thermogenesis. This cold exposure continued for 60 more minutes. Thereafter, after approximately 2 hours, a blood sample was obtained and cold-induced REE, lipid and glucose oxidation were measured again. Lastly, a second MRI scan was performed to assess changes in supraclavicular BAT after cold exposure.

Study visit 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 (again via BIA), 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 min), two hours (t=120 min), and three hours (t=180 min) after administration, REE, lipid and glucose oxidation were assessed again. At three and a half hours (t=210 min), when reaching the maximum plasma concentration of mirabegron (*i.e.* Tmax ~3–4 h), another blood sample was drawn and an MRI scan was performed to assess changes in supraclavicular BAT.

All participants were followed up up to 1 week after the last study visit.

Analyses

Serum measurements

Commercially available enzymatic kits were used to measure serum concentrations of triglycerides and total cholesterol (Roche Diagnostics, the Netherlands), HDL-cholesterol (HDL-C) (Roche Diagnostics), free fatty acids (Wako Chemicals, Germany) and glucose (Instruchemie, the Netherlands). Insulin concentrations were measured using ELISA

(Crystal Chem Inc., IL, USA). LDL-cholesterol (LDL-C) was calculated using the Friedewald equation [6]. Data were analysed using SoftMaxPro 5.4.1 software.

Serum lipidomic analysis

Serum lipidomic analysis was performed essentially as described previously [7,8]. In short, lipids were extracted from 20 µL of serum and four HPLC-MS runs were performed on a reversed phase and a normal phase column and ions were detected by a Q Exactive Plus (Thermo Fisher Scientific, MA, USA) mass spectrometer in both the positive and negative ionisation mode yielding four raw data files per sample. The dataset was processed using an in-house developed metabolomics pipeline written in the R programming language (http://www.r-project.org) that identified and annotated features (peaks), performed isotope correction, and normalized data to the intensities of the added internal standards.

Skin temperature

18 wireless iButton temperature sensors were placed and adapted from 14 prescribed ISO-defined positions [9] (forehead, left chest, right abdomen, right thigh, right shinbone, right feet, back of the neck, right scapula, left lower back, left upper leg, right deltoideus, right forearm, right fingertip, left supraclavicular) and 4 additional positions (left hand, left lower leg, left elbow, and right armpit) [10]. Data were analysed using Temperatus® software [11]. Armpit temperature was estimated and used as a proxy of core body temperature [12]. Supraclavicular skin temperature was estimated from an iButton placed above the left clavicula. Distal skin temperature was calculated as the average temperature of the left hand and right foot [13]. Proximal skin temperature was defined as the average of the iButtons on the chest, abdomen, scapula, and lower back [14].

Indirect calorimetry

Indirect calorimetry was performed in time-frames of 30 minutes. Participants were instructed to lie still and were not allowed to talk. VO_2 and carbon dioxide production were determined every minute. REE was calculated and substrate utilization was assessed by calculation of lipid and glucose oxidation after correction for protein oxidation, as described previously [15]. Both the thermoneutral and cold-induced measurement represent the average of the last twenty minutes.

MRI analysis

An in-house water-fat separation algorithm based on the known frequencies of the multi-peak fat spectrum and assuming mono-exponential effective transverse relaxation time (T2*) was used to reconstruct fat fraction maps, combined with a region-growing scheme to mitigate main field inhomogeneity effects [16-19]. Regions of interest

encompassing the known location of the left supraclavicular BAT depot [20] were drawn manually by one observer, exclusively on pre-cooling scans as described before (Abreu-Vieira 2020) (**Fig. S7)**. Registration was performed using the image registration software Elastix [21,22]. 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 10×10×10 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). The calculated deformation field was subsequently used to map the pre-cooling region of interest to the coordinate space of the post-cooling images. Data analysis was performed within a 50-100% fat fraction interval. This segmentation range was applied for both the pre-cooling and post-cooling supraclavicular adipose tissue volumes. In this work, we set the lower FF threshold to 50%, which was based on our previously published work [5], wherein we analysed the effects of FF segmentation thresholds on MRI BAT-related outcomes and has been used by others in the field [23,24]. Post-mirabegron and post-placebo scans were analysed independently. The average fat fraction, T2* and total volume of the supraclavicular BAT depot were computed for pre-and post-cooling, post-mirabegron and post-placebo scans. One participant was excluded from all MRI analyses because of failure to reconstruct the scan due to excessive movement.

Statistical analysis

Data were analysed using IBM SPSS Statistics for Windows version 22.0 (SPSS Inc, Chicago, IL, USA). Figures were created by GraphPad Prism version 7.00 (GraphPad Software, La Jolla, CA, USA). Paired t-tests were used to study the effect of cold exposure, mirabegron and placebo treatments on serum lipids and skin temperature. Furthermore, paired t-tests were used to study the effect of cold on REE and nutrient oxidation and two-way repeated measures ANOVA was applied to study the effect of placebo *vs.* mirabegron on REE and nutrient oxidation. In addition, to study differences between interventions (cold exposure *vs.* mirabegron *vs.* placebo) in BAT MRI outcomes and the deltas (value after *minus* before intervention) of serum lipids and skin temperature, we performed one-way repeated measures ANOVA. Moreover, to study changes in REE and nutrient oxidation over time and to assess differences between mirabegron and placebo treatments herein, we performed a two-way repeated measures ANOVA with the variables 'time' (0, 1, 2 and 3 hours) and 'treatment' (mirabegron or placebo) as within-subject factors. For the lipidomics data, mixed model analyses with ethnicity, intervention and study visit as fixed effects and subject specific deviances from the mean as random effects were used to assess the effects of mirabegron and cold exposure. In case the mixed model failed to converge, an ordinary linear model with only the fixed effects was used. P-values were adjusted for false rate of discovery (FDR) using the Benjamini-Hochberg procedure. All

main analyses are presented per ethnicity (Europids *vs.* South Asians). However, as we did not observe interaction between ethnicity, treatment and metabolic outcome parameters in any statistical test (all p>0.05), we also show all analyses combined for both ethnicities to increase the statistical power (**Fig. S1-S5**).A p-value<0.05 was considered statistically significant. Data are shown as mean and 95% CI, unless stated otherwise.

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Table S1. Effect of cold exposure, mirabegron and placebo on serum glucose, HDL-cholesterol and LDL-cholesterol in Europids, South Asians and both groups combined.

Europids (n=10)						
	Pre-cold	Post-cold	Pre-Mira	Post-Mira	Pre-Placebo	Post-Placebo
Glucose (mmol/L)	4.5 (0.4)	4.4 (0.4)	4.5 (0.2)	4.5 (0.2)	4.4 (0.2)	4.4 (0.4)
HDL-C (mmol/L)	1.4 (0.2)	1.5 (0.3)*	1.4 (0.3)	1.4 (0.3)	1.4 (02)	1.4 (0.3)
LDL-C (mmol/L)	3.1 (1.3)	3.6 (1.4)	3.3 (1.3)	3.7 (1.2)	3.1 (1.2)	3.1 (1.2)
South Asians (n=10)						
	Pre-cold	Post-cold	Pre-Mira	Post-Mira	Pre-Placebo	Post-Placebo
Glucose (mmol/L)	4.6 (0.3)	4.5 (0.4)	4.6 (0.3)	4.7 (0.4)	4.6 (0.5)	4.5 (0.3)
HDL-C (mmol/L)	1.2 (03)	1.4 (0.4)**	1.2 (0.2)	1.2 (0.3)	1.2 (0.3)	1.3 (0.2)
LDL-C (mmol/L)	4.3 (1.3)	4.5 (1.0)	3.8 (1.0)	3.6 (1.3)	3.8 (1.0)	3.6 (0.1.0)
All (n=20)						
	Pre-cold	Post-cold	Pre-Mira	Post-Mira	Pre-Placebo	Post-Placebo
Glucose (mmol/L)	4.5 (0.3)	4.4 (0.3)	4.6 (0.2)	4.6 (0.3)	4.5 (0.4)	4.5 (0.3)
HDL-C (mmol/L)	1.3 (0.3)	1.4 (0.3)***	1.3 (0.3)	1.3 (0.3)	1.3 (0.3)	1.4 (0.3)
LDL-C (mmol/L)	3.7 (1.4)	4.1(1.3)	3.5 (1.1)	3.7 (1.2)	3.5 (1.1)	3.3 (1.1)

HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; Mira: mirabegron. Data are presented as mean and Standard Deviation. P-values were obtained from paired t-tests. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ after vs before treatment.

Table S2. Cardiovascular parameters.

	Europids (n=10)			South Asians (n=10)		
	Cooling	Mirabegron	Placebo	Cooling	Mirabegron	Placebo
Heart rate (bpm)	-2 **	+7 ***	0	-1	+10 **	+2
Diastolic BP (mmHg)	+13	+6	+4	+13 *	+1	+4
Systolic BP (mmHg)	+16	+4	+7 *	+13 *	+9	+6

Values are presented as delta (post- minus pre-intervention). Two-tailed paired (within ethnicities) was used for comparison before and after intervention. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ difference compared to baseline measurement. BP, blood pressure

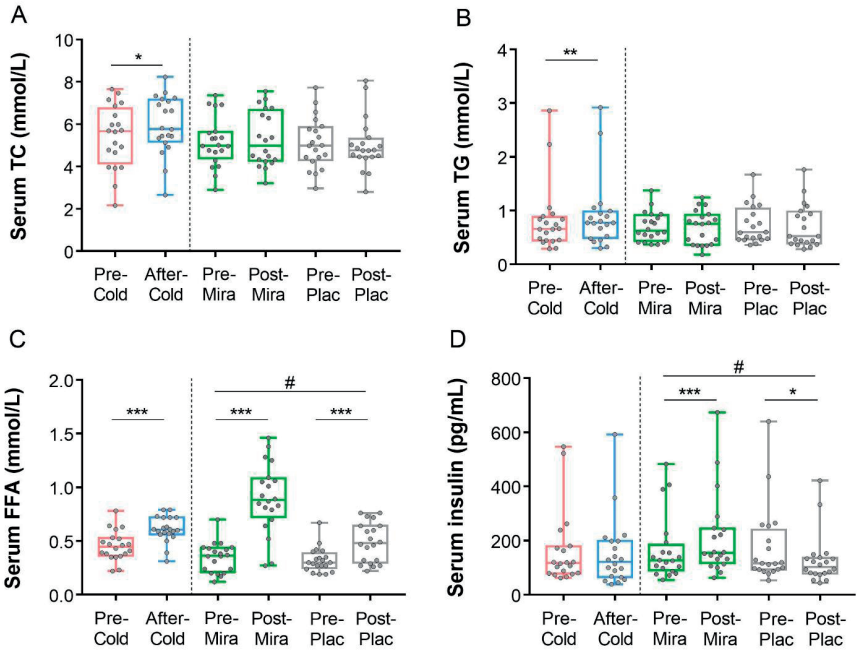

Figure S1. Effect of cold exposure, mirabegron and placebo on serum lipids and insulin in all subjects combined. Serum was collected pre- and post-cooling, mirabegron (mira) and placebo (plac) in all participants (n=20), and assayed for total cholesterol (TC) (A), triglycerides (TG) (B), free fatty acids (FFA) (C), and insulin (D). Data are presented as mean ± 95% CI. Paired t-tests were used to evaluate the effect of the interventions. * p<0.05, ** p<0.01, *** p<0.001 before vs. after intervention. One-way ANOVA was performed to study the delta's in time (after treatment minus before) between treatments. # p<0.05 delta time between treatments.

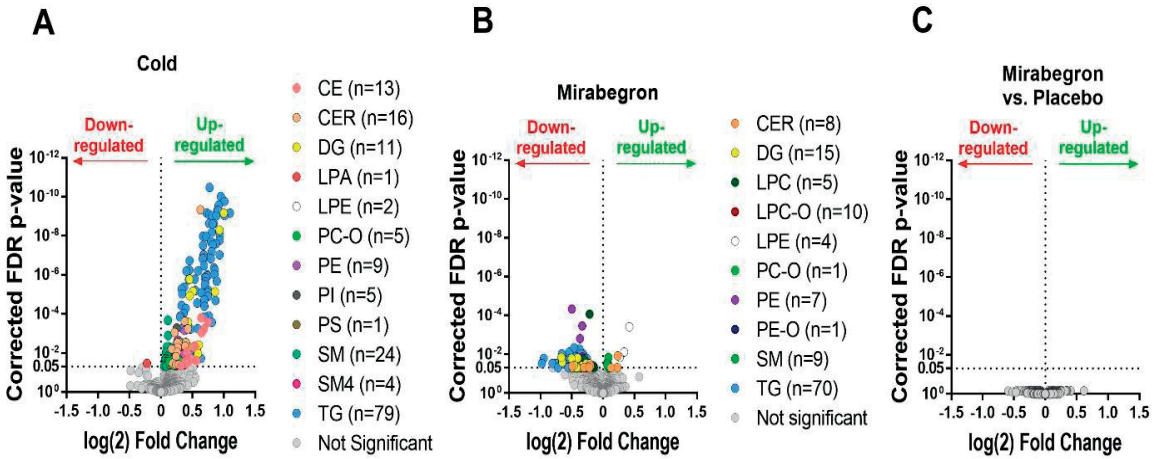

Figure S2. Effect of cold exposure, mirabegron and placebo on serum lipidome in all subjects combined. Volcano plots showing lipidomics data in response to cold exposure (A), mirabegron (B) or the difference between mirabegron and placebo (C) in all subjects combined. Fold change represents the change of these lipids in comparison to the baseline (log (2)) (x-axis). P-value was corrected by the false rate of discovery (FDR). The horizontal dash line shows the level of significance (FDR corrected $p<0.05$). CE: Cholesteryl ester; CER: Ceramide; DG: diglyceride; LPA: Lysophosphatidic acid; LPC: Lysophosphatidylcholine; (L)PC-O: (Lyso)phosphatidylcholine etherphospholipid; (L)PE: (Lyso)phosphatidylethanolamine; PE-O: phosphatidylethanolamine etherphospholipid; PI: Phosphatidylinositol; PS: Phosphatidylserine; SM: sphingomyelin; SM4: sulfatide; TG: triglyceride. q-value represents p-value after FDR corrections.

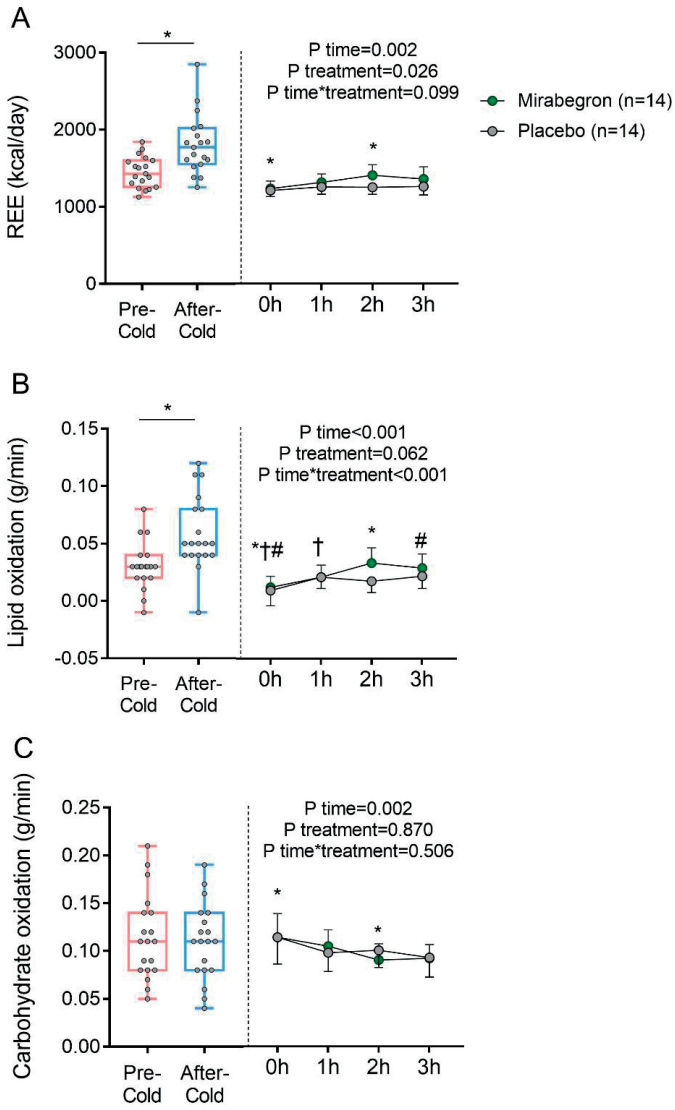

Figure S3. Effect of cold exposure and mirabegron in comparison to placebo on resting energy expenditure and nutrient oxidation in all subject combined**.** Pre-cold (red boxes) and post-cold (blue boxes) resting energy expenditure (REE) and nutrient oxidation in all subjects combined (n=20). Paired t-tests were performed to study the effect of cold exposure with The effects of mirabegron or placebo on REE (A), lipid oxidation (B) and carbohydrate oxidation (C) were studied by a repeated measures two-way ANOVA with 'time' (0, 1, 2 and 3 h) and 'treatment' (mirabegron or placebo) as within-subject factors. These analyses were performed with both ethnicities combined. P for time, P for treatment and P for time*treatment were obtained from the two-way ANOVA. Data are presented as mean ± 95% CI. *P<0.05.

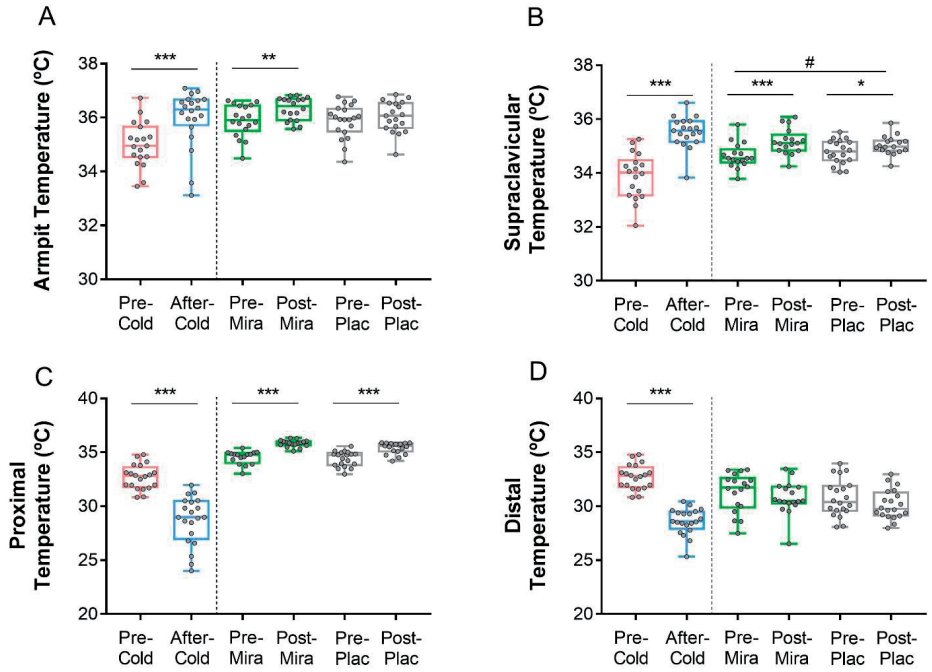

Figure S4. Effect of cold exposure, mirabegron and placebo on skin temperature in all subjects combined. Skin temperature was measured pre- and post-cold, mirabegron (mira) and placebo (plac) in all subjects combined (n=20). We directly measured armpit (A) and supraclavicular (B) skin temperatures, whereas proximal (C) and distal (D) skin temperatures were calculated following equations described in the ESM. Data are presented as mean ± 95% CI. Paired t-tests were used to evaluate the effect of the interventions. * p<0.05, ** p<0.01, *** p<0.001 before vs. after intervention. # p<0.05 differences between the delta between treatments.

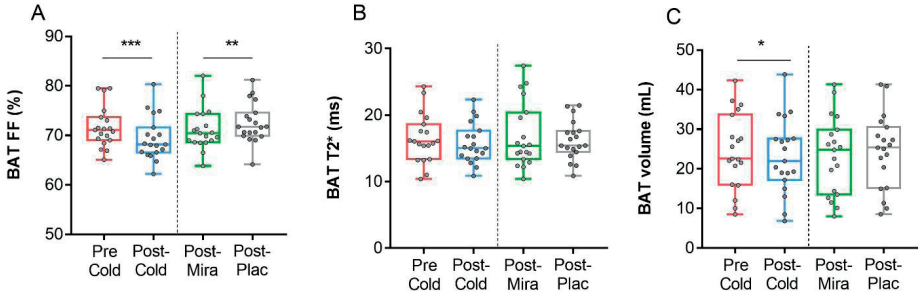

Figure S5. Effect of cold exposure, mirabegron and placebo on brown adipose tissue (BAT) fat fraction (FF), T2* and volume in all subjects combined. MRI was used to determine BAT FF (A), T2* (B) and volume (C). Red boxes represent BAT-related outcomes before cold exposure and blue boxes represent BAT-related outcomes after cold exposure, green boxes after mirabegron (mira) and grey boxes after placebo (plac) treatment. All analyses were performed with all available subject data (n=19). One-way ANOVA was performed to study differences in BAT parameters between treatments. ** p<0.01; *** p<0.001 between treatments. One South Asian was excluded from analyses due to movement in the MRI.

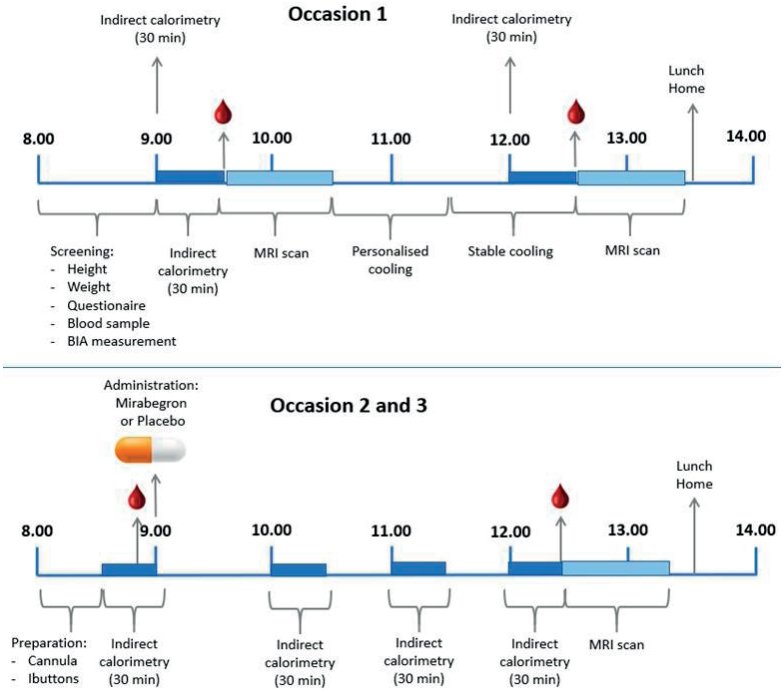

Figure S6. Overview of the study design**.** See text for explanation.

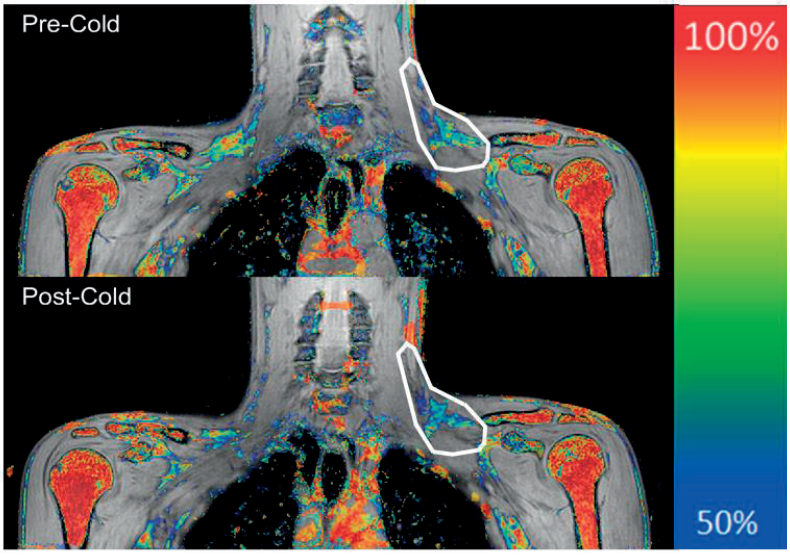

Figure S7. Region of interest drawn on the MRI scan**.** Example of supraclavicular region of interest drawn on MRI scan before and after cold exposure.

6

Twelve weeks of exenatide treatment increases [^{18}F]fluorodeoxyglucose uptake by brown adipose tissue without affecting oxidative resting energy expenditure in nondiabetic males

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ABSTRACT

Aims/hypothesis

Brown adipose tissue (BAT) improves energy metabolism by combusting glucose and lipids into heat. Agonism of the glucagon-like peptide-1 receptor (GLP-1R) within the central nervous system activates BAT in mice. Moreover, in patients with type 2 diabetes, GLP-1R agonism lowers body weight and improves glucose and lipid levels, possibly involving BAT activation. Interestingly, people from South Asian descent are prone to develop cardiometabolic disease. We studied the effect of GLP-1R agonism on BAT in humans, specifically in South Asians and Europids without obesity or type 2 diabetes.

Methods

Twelve Dutch South Asian and 12 age- and BMI-matched Europid nondiabetic men received 12 weeks extended-release exenatide (Bydureon) in this single-arm prospective study. Before and after treatment, BAT was visualized by a cold-induced [^{18}F]FDG-PET/CT scan and a thermoneutral MRI scan, and resting energy expenditure (REE), substrate oxidation, body composition and fasting plasma glucose and serum lipids were determined. Appetite was rated using a visual analogue scale.

Results

Since the effect of exenatide on metabolic parameters did not evidently differ between ethnicities, data of all participants were pooled. Exenatide decreased body weight (-1.5 ± 0.4 kg, $p < 0.01$), without affecting REE or substrate oxidation, and transiently decreased appetite ratings during the first weeks. Exenatide also lowered triglycerides (-15%, $p < 0.05$) and total cholesterol (-5%, $p < 0.05$), and tended to lower glucose levels. Notably, exenatide increased BAT metabolic volume ($+28\%$, $p < 0.05$) and mean standardized uptake value ($+11\%$, $p < 0.05$) ([^{18}F]FDG-PET/CT), without affecting supraclavicular adipose tissue fat fraction (MRI).

Conclusions/interpretation

We show for the first time that GLP-1R agonism increases [^{18}F]FDG uptake by BAT in South Asian and Europid men without obesity or type 2 diabetes.

Trial registry: Clinicaltrials.gov NCT03002675

INTRODUCTION

Obesity has a major impact on healthcare costs, by contributing to dysregulated glucose metabolism and dyslipidaemia which may eventually culminate in type 2 diabetes and cardiovascular disease [1]. People from South Asian descent are especially prone to develop these unfavorable metabolic traits, with also higher morbidity and mortality rates compared with other ethnicities [2]. In presence of cardiometabolic disease during obesity, pharmacotherapy may be considered as an adjunct to lifestyle therapy to further enhance weight loss [3,4]. Glucagon-like peptide-1 receptor (GLP-1R) agonists have proven efficacy in the treatment of both obesity and type 2 diabetes by lowering body weight and improving glucose regulation [3–5].

Glucagon-like peptide-1 (GLP-1) is produced by the intestine upon food intake. GLP-1 subsequently lowers blood glucose levels via stimulating pancreatic insulin secretion and lowering glucagon secretion, an effect that can be mimicked by GLP-1R agonism [6]. In addition to improving postprandial glycaemia, GLP-1R agonists are well-known to induce weight loss. This weight-lowering effect of GLP-1R agonists occurs at least in part by lowering food intake via a combination of reducing appetite, increasing satiety and delaying gastric emptying [6]. Furthermore, GLP-1R agonists are associated with a modest improvement of lipid profile [7] and, albeit not consistently, an increase in resting energy expenditure (REE) in patients with type 2 diabetes [8,9]. Interestingly, preclinical evidence indicates that energy-combusting brown adipose tissue (BAT) contributes to the various beneficial metabolic effects of GLP-1R agonists [8,10,11]. More specifically, central GLP-1R agonism in mice was shown to increase plasma triglyceride-derived fatty acid and glucose uptake by BAT and to shift substrate utilization towards lipid oxidation [10,12].

In this study, we hypothesized that chronic GLP-1R agonism activates BAT in humans, thereby contributing to weight loss and improved plasma glucose and lipid levels. As a proof of concept, we investigated the effect of 12 weeks extended-release exenatide on BAT measured by [^{18}F]fluorodeoxyglucose positron emission tomography/computed tomography ([^{18}F]FDG-PET/CT) and magnetic resonance imaging (MRI) scans in South Asian and Europid men without obesity or diabetes. In addition, we evaluated the effect of exenatide on body weight and composition, energy metabolism and plasma glucose and lipid levels.

METHODS

See the supplemental material for an extensive description of all analyses.

2.1. Power calculation and participants

We regarded an increase in BAT activity assessed by [^{18}F]FDG-PET/ CT of 13% as clinically relevant, and with an SD of 13, α of 0.05 and β of 80% this resulted in 12 subjects per arm. Therefore, twelve healthy nondiabetic Dutch South Asian and 12 Dutch Europid men were included in this study. South Asians and Europids were matched for age (20–36 years) and BMI (18–27 kg/m^2). Exclusion criteria were smoking, recent participation in a weight loss or exercise program, any significant chronic disease or renal, hepatic or endocrine disease, use of medication known to influence glucose or lipid metabolism or BAT activity (e.g. beta blockers), participation in another study including a pharmaceutical drug and any contra-indications to undergo an MRI scan.

2.2. Study approval

This study was performed in accordance with the principles of the revised declaration of Helsinki [13] and approved by the medical ethical committee of the Leiden University Medical Center (LUMC). All participants provided written informed consent prior to participation.

2.3. Study design

This single-arm prospective study was conducted between September 2016 and February 2018 at the LUMC. Participants received extended-release exenatide (Bydureon, AstraZeneca B.V., The Hague, the Netherlands) 2 mg s.c. once weekly during 12 weeks. Side-effects and general wellbeing were monitored weekly. Changes in dietary habits and physical activity were discouraged. Appetite ratings were monitored every 4 weeks with a visual analogue scale (VAS), which was filled in during the day in between meals. A study day was conducted before and after exenatide treatment (**Supplemental Fig. 1**). The post-exenatide study day was performed one week after the last injection. Participants were instructed to refrain from physical exercise 48 h prior to these study days and to consume a standardized meal the evening prior to the study days. After a 10-hour overnight fast, body composition was determined by bio-impedance analysis (Bodystat 1500, Bodystat, Douglas, Isle of Man, UK) and an intravenous cannula was placed in the antecubital vein. An MRI scan (3 T MRI, Philips Ingenia, Philips Healthcare, Best, the Netherlands) was then performed at room temperature to assess the fat fraction and volume of the supraclavicular adipose tissue depot. Afterwards, wireless iButton temperature loggers were attached to 14 ISO-defined positions to measure skin temperature [14], and participants took place in a semi supine position on a bed between two water-

perfused blankets (Blanketrol® III, Cincinnati Sub-Zero Products, Inc., Cincinnati, Ohio, USA) set at a temperature of 32 °C (considered thermoneutrality) for a period of 45 min. During the final 30 min, REE was measured by indirect calorimetry (JAEGER™ Vyntus™ CPX, Carefusion, Hochberg, Germany), followed by a blood draw. Next, a personalized cooling protocol was applied as described previously [15]. Briefly, the water temperature was gradually decreased to a minimum of 9 °C during 1 h, followed by a gradual increase of 2–3 °C, which happened earlier in case of shivering. Shivering was reported by the participants and visually assessed by the researchers. Subsequently, cold-induced REE was measured, whereafter 74 MBq [^{18}F]FDG was administered intravenously and followed by a PET/CT scan after 1 h of incubation (Horizon with TrueV option, Siemens Healthcare, Knoxville, USA). The cooling protocol continued until start of the PET/CT. One South Asian participant was excluded from all [^{18}F]FDG-PET/CT analyses due to excessive movement during a scan.

2.4. Serum and plasma measurements

Commercially available enzymatic kits were used to measure serum concentrations of triglycerides, total cholesterol and HDL-cholesterol (all Roche Diagnostics, Woerden, the Netherlands), free fatty acids (Wako chemicals, Nuess, Germany) and insulin (Meso Scale Diagnostics LLC, Rockville, MD, USA), and plasma glucose (Instruchemie, Delfzijl, the Netherlands). LDL-cholesterol was calculated by the Friedewald equation [16].

2.5. Statistical analysis

Statistical analyses were performed with SPSS Statistics (version 20.0, IBM Corporation, Armonk, NY, USA) and GraphPad Prism (version 8.0.1.244, GraphPad Software, La Jolla, CA, USA). Baseline characteristics were compared between ethnicities with a two-tailed unpaired Student's t-test. Comparisons between variables measured at a similar temperature were performed with a two-factor mixed design ANOVA, as they included two factors: exenatide treatment (within-subjects) and ethnicity (between subjects). Comparisons between variables measured during both thermoneutrality and cold were analysed with linear mixed models, which included temperature, exenatide treatment and ethnicity as fixed factors, and temperature and exenatide treatment as random effects. For the random effects, i.e. random slopes and intercepts, the model used an unstructured covariance matrix. P-values are shown for main effects and interactions as well as for post hoc tests. Correlation analyses were performed using linear regression analysis and assessed for interaction of ethnicity. A p-value < 0.05 was considered statistically significant. Data are presented as mean ± SEM.

RESULTS

3.1 Participant characteristics and compliance

One participant dropped out of the study prior to the first study day and was replaced by a newly recruited participant. Twenty-four participants completed the study. Clinical characteristics are shown in **Table 1**. Blood pressure and heart rate as well as fasting total cholesterol, triglyceride and glucose levels were within a healthy range. When comparing baseline characteristics between ethnicities, South Asians were shorter than Europids (1.78 ± 0.02 *vs* 1.85 ± 0.02 m, $p < 0.01$). Age and BMI were comparable between South Asians and Europids, as they were matched for these parameters. Total cholesterol, triglyceride and glucose levels were also comparable between ethnicities.

The weekly s.c. injections with exenatide were generally well tolerated. The most frequently reported side-effects were mild and transient, and were of gastro-intestinal (e.g. nausea, vomiting) and dermatological origin (e.g. subcutaneous nodule). No serious adverse events occurred.

Table 1. Participant characteristics

	All participants (N=24)	Europids (N=12)	South Asians (N=12)
Age (yr)	26.5±0.7	25.6±0.9	27.5±0.9
Height (m)	1.82±0.01	1.85±0.02	1.78±0.02 ††
Weight (kg)	79.3±2.1	81.6±2.6	77.0±3.3
BMI (kg/m^2)	23.9±0.5	23.8±0.7	24.1±0.8
Serum total cholesterol (mmol/L)	4.6±0.2	4.5±0.2	4.8±0.2
Serum triglycerides (mmol/L)	0.98±0.07	0.96±0.10	1.00±0.11
Plasma glucose (mmol/L)	5.0±0.1	5.0±0.1	4.9±0.2
Systolic blood pressure (mmHg)	121±2	125±4	118±2
Diastolic blood pressure (mmHg)	76±2	76±3	75±2
Heart rate (bpm)	62±2	66±4	58±3 ˜

Fasted serum lipid and plasma glucose levels are shown. Data were analysed by an unpaired students t-test and presented as mean ± SEM. ˜$p < 0.1$, ††$p < 0.01$, South Asians *vs* Europids.

3.2. Exenatide lowers body weight without affecting resting energy expenditure

We firstly assessed the effect of exenatide on body weight and composition in our cohort of non-obese men (**Supplemental Table 1**). Exenatide lowered body weight in the total cohort (−1.5 ± 0.4 kg, $p < 0.01$), mainly due to a reduction in lean mass (−1.1 ± 0.4 kg, $p < 0.01$) rather than fat mass, without affecting the waist-to-hip ratio. We then evaluated whether ethnicity modifies the effect of exenatide on these parameters. Here, we

observed a trend towards interaction between the effects of ethnicity and exenatide on fat mass ($p = 0.059$), reflecting a decreased fat mass after exenatide only in South Asians (-1.0 ± 0.4 kg, $p < 0.05$) but not in Europids (0.2 ± 0.4, $p = 0.68$, **Supplemental Table 1**). Body fat percentage was higher in South Asians compared with Europids at baseline (18.9 ± 0.9 *vs* $14.5 \pm 1.4\%$, $p < 0.01$), which remained present after exenatide treatment (18.1 ± 0.8 *vs* $14.9 \pm 1.2\%$, $p < 0.05$, **Supplemental Table 1**). Of note, we observed a negative correlation between baseline body fat percentage and the exenatide-induced delta fat percentage (data not shown).

Hereafter, we investigated whether an altered energy metabolism could underlie these weight-lowering effects of exenatide. Aside from a trend towards a lower glucose oxidation, exenatide did not evidently affect the REE (nor when corrected for lean mass), respiratory quotient (RQ) or substrate oxidation in the total study cohort (**Fig. 1**) or when studying ethnicities separately (**Supplemental Fig. 2**). As expected, both before and after exenatide, cold exposure increased REE (+7%, $p < 0.01$ and + 11%, $p < 0.001$) and lowered the RQ (−5%, $p < 0.001$ and −3%, $p < 0.05$), reflected by an increased lipid oxidation (+35%, $p < 0.001$ and +28%, $p < 0.001$) and decreased glucose oxidation (−28%, $p < 0.01$ and −14%, $p = 0.12$) in the total study cohort (**Fig. 1**), which was not significantly different before and after exenatide.

We next investigated the role of a lowered appetite in the weightlowering effect of exenatide (**Fig. 2**). In the total study cohort exenatide reduced the hunger sensation (−27%, $p < 0.05$) and desire to eat (−25%, $p < 0.05$) after 4 weeks, which was transient. Furthermore, exenatide did not overtly affect the sensation of fullness or satiety. Ethnicity did not modify the effect of exenatide on these appetite ratings (**Supplemental Fig. 3**).

3.3. Exenatide lowers serum lipid levels and tends to lower plasma glucose levels

We next evaluated the effect of exenatide on glucose and lipid levels (**Fig. 3**). In the total study cohort, the main treatment effect showed that exenatide lowered triglycerides (−15%, $p < 0.05$), without affecting free fatty acids. In addition, exenatide lowered total cholesterol (−5%, $p < 0.05$), which was attributable to a trend towards a lowering of LDLcholesterol (−5%, $p = 0.10$) probably in addition to lowering of VLDL/ remnant-cholesterol, rather than HDL-cholesterol. Lastly, exenatide tended to lower plasma glucose (4.6 ± 0.1 *vs* 4.7 ± 0.0 mmol/L, $p = 0.09$) without affecting serum insulin levels. Ethnicity did not modify these effects of exenatide on lipid and glucose levels (**Supplemental Fig. 4**). However, when comparing ethnicities at baseline (**Supplemental Fig. 4**), total cholesterol tended to be higher in South Asians compared with Europids (4.8 ± 0.2 *vs* 4.2 ± 0.1 mmol/L, $p = 0.05$), explained by a trend towards higher LDL-cholesterol in South Asians compared with Europids (3.3 ± 0.3 *vs* 2.7 ± 0.1 mmol/L, $p = 0.07$). Furthermore,

plasma glucose tended to be higher in South Asians compared with Europids at baseline (4.8 ± 0.1 *vs* 4.6 ± 0.1 mmol/L, p = 0.08).

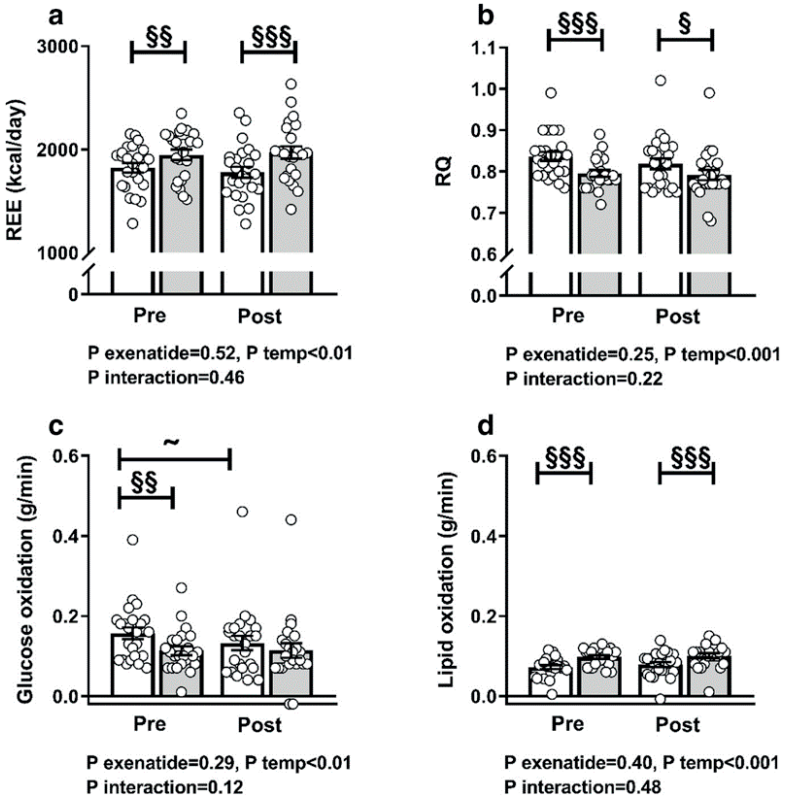

Fig. 1. Exenatide does not affect energy metabolism.
The effect of exenatide on thermoneutral and cold-induced resting energy expenditure (REE) (a), respiratory quotient (RQ) (b), glucose oxidation (c) and lipid oxidation (d) in the total study cohort (N = 24). Pre = before exenatide, post = after exenatide. Data were analysed by linear mixed models and are presented as mean ± SEM. White bars are thermoneutral, grey bars are during short term cooling. p-Values for the main effect of exenatide treatment (exenatide) and temperature (temp) and their interaction (exenatide*temp) are shown below the figures. ˜p < 0.1 post-hoc p-value post vs pre exenatide. §p < 0.05, §§p < 0.05, §§§p < 0.001 post-hoc p-values cold vs thermoneutrality.

3.4. Exenatide decreases the systolic blood pressure and increases the heart rate

As GLP-1R agonists are known to affect the cardiovascular system [17], we evaluated the effect of exenatide on blood pressure and heart rate in our study (**Supplemental Table 2**). In the total study cohort, exenatide lowered the systolic blood pressure (–4 ± 1 mm Hg, p < 0.01) without affecting the diastolic blood pressure, and increased the heart rate (+6 ± 1 bpm, p < 0.001). When assessing whether ethnicity modifies the effect

of exenatide on these outcome parameters, we observed that the increase in heart rate was more pronounced in South Asians compared with Europids (+8 ± 1 *vs* +3 ± 1 bpm, $p < 0.05$; **Supplemental Table 2**).

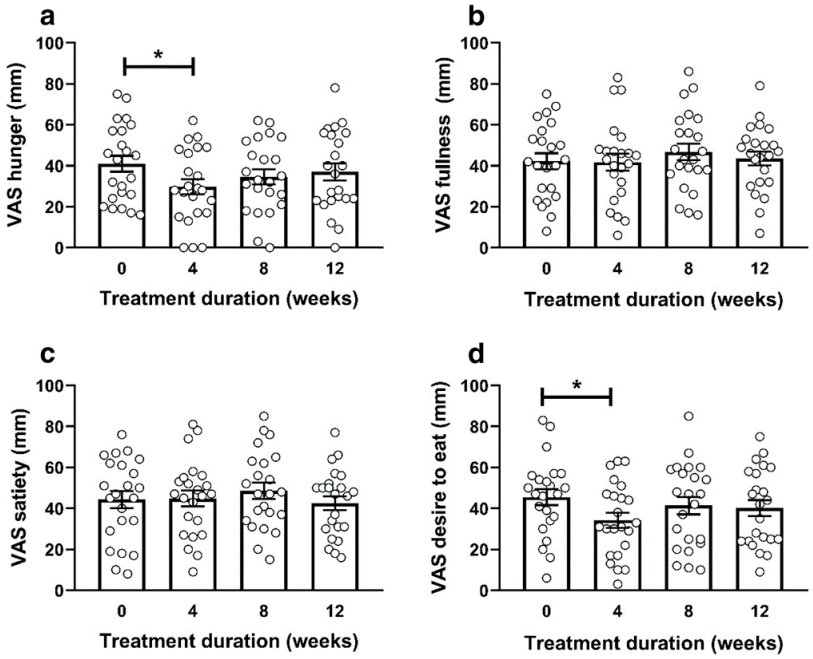

Fig. 2. Exenatide lowers the sensation of hunger and desire to eat during the first weeks of treatment. The effect of exenatide on subjective ratings for hunger (a), fullness (b), satiety (c) and desire to eat (d) measured every 4 weeks in the total study cohort (N = 23). One of the 12 South Asian participants was excluded due to incomplete questionnaires. Higher values (mm) indicate higher ratings. Data were analysed by a two factor mixed design ANOVA and are presented as mean ± SEM. *$p < 0.05$ effect of exenatide.

3.5. Exenatide enhances [^{18}F]FDG uptake by brown adipose tissue

We next studied the effect of exenatide on BAT [^{18}F]FDG uptake (**Fig. 4**). Notably, in the total study cohort exenatide increased the metabolic volume (+28%, $p < 0.05$) and mean standardized uptake value (SUVmean) (+11%, $p < 0.05$) of classical BAT regions, i.e. cervical and supraclavicular depots. Similar results were observed when additionally including the upper mediastinal, axillary and paravertebral BAT depots (**Supplemental Table 3**). Of note, the effect of exenatide on BAT parameters could not be explained by seasonal variation or by changes in body weight or composition (data not shown). The mean water temperature to which participants were exposed during the personalized cooling protocol was also comparable after exenatide (data not shown). Ethnicity did not interact with the effect of exenatide on BAT parameters, and BAT parameters

were comparable between South Asians and Europids at baseline (**Supplemental Table 3**). Moreover, exenatide did not affect the [^{18}F]FDG uptake of the subcutaneous or visceral white adipose tissue depots (**Supplemental Table 4**). Exenatide did increase

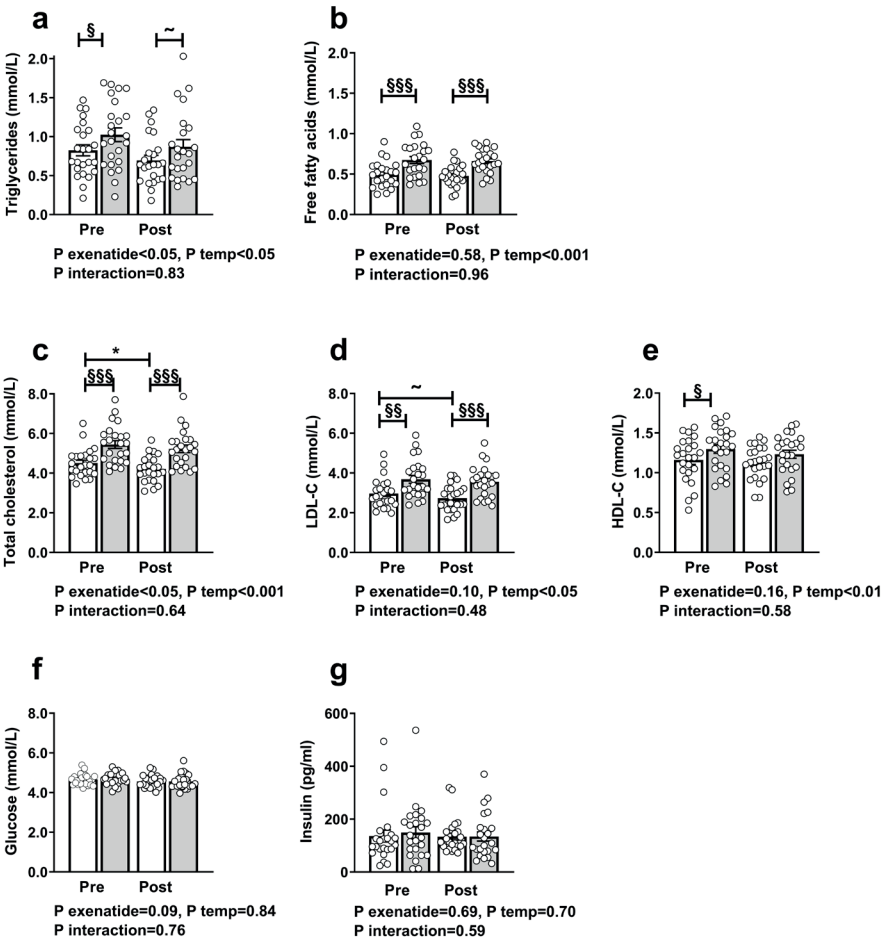

Fig. 3. Exenatide lowers total triglyceride and cholesterol levels, and tends to lower plasma glucose levels. The effect of exenatide on thermoneutral and cold-induced fasted serum triglycerides (a), free fatty acids (b), total cholesterol (c), LDL-cholesterol (LDL-C) (d), HDL-cholesterol (HDL-C) (e), plasma glucose (f) and serum insulin (g) levels in the total study cohort (N = 24). Pre = before exenatide, post = after exenatide. Data were analysed by linear mixed models and are presented as mean ± SEM. White bars are thermoneutral, grey bars are during short term cooling. P-values for the main effect of exenatide treatment (exenatide) and temperature (temp) and their interaction (exenatide*temp) are shown below the figures. ˜p < 0.1, *p < 0.05 post-hoc p-values post vs pre exenatide. ˜p < 0.1, §p < 0.05, §§p < 0.01, §§§p < 0.001 post-hoc p-values cold *vs* thermoneutrality

the SUVmean of the pectoralis major muscle (+16%, $p < 0.05$) and psoas major muscle (+27% g/mL, $p < 0.001$) and decreased the SUVmean of the trapezius muscle (−15%, $p < 0.05$) (**Supplemental Table 4**). Ethnicity affected the exenatide-induced decrease in SUVmean of the trapezius muscle ($p < 0.05$ for interaction), reflecting a significant decrease in SUVmean of the trapezius muscle only in South Asians (−24%, $p < 0.01$) but not in Europids (−4%, $p = 0.72$) (**Supplemental Table 4**).

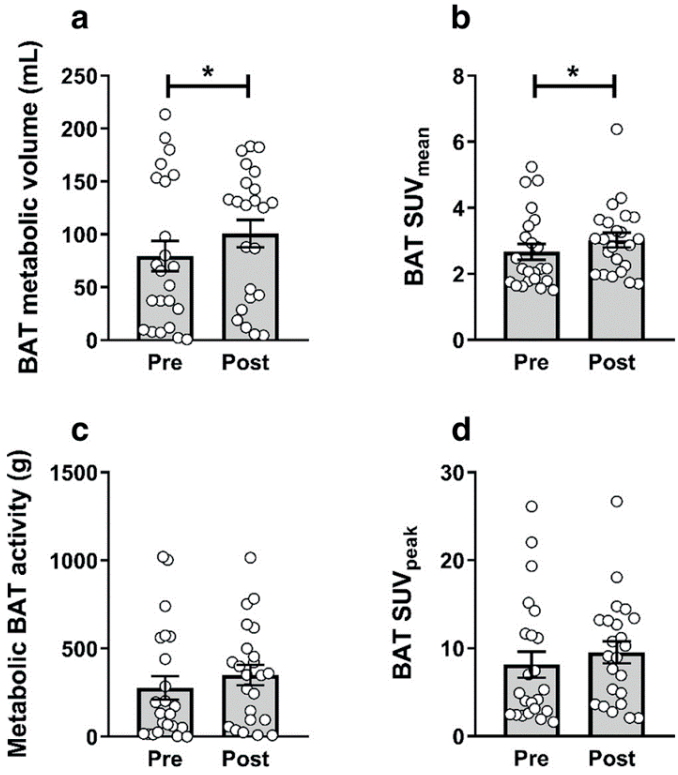

Fig. 4. Exenatide increases brown adipose tissue metabolic volume and SUVmean.
The effect of exenatide on metabolic volume (a), mean standardized uptake value (SUVmean) (b), metabolic activity (c) and peak standardized uptake value (SUVpeak) (d) of classical brown adipose tissue (BAT) depots in the total study cohort (N = 23). One of the 12 South Asian participants was excluded due to movement during a scan. Pre = before exenatide, post = after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean ± SEM. *$p < 0.05$ post vs pre exenatide.

3.6. Exenatide does not affect supraclavicular adipose tissue fat fraction measured with MRI

Lastly, as BAT activation lowers the fat fraction of classical BAT depots by combusting intracellular triglycerides [18], we evaluated the effect of exenatide on supraclavicular

adipose tissue by MRI. Both the fat fraction (post 0.745 ± 0.008 *vs* pre 0.745 ± 0.009, p = 0.96) and volume (post 30.2 ± 2.9 *vs* pre 31.0 ± 2.8 mL, p = 0.22) of this adipose tissue depot remained unaltered after exenatide in the total study cohort (**Fig. 5**) or in either ethnicity (**Supplemental Table 5**). Interestingly, albeit exenatide did not affect supraclavicular adipose tissue mean fat fraction or volume, Δfat fraction negatively correlated with ΔSUVmean (**Fig. 6**) and ΔSUVpeak (data not shown) on [^{18}F]FDG-PET/CT scan. Ethnicity did not affect these correlation analyses.

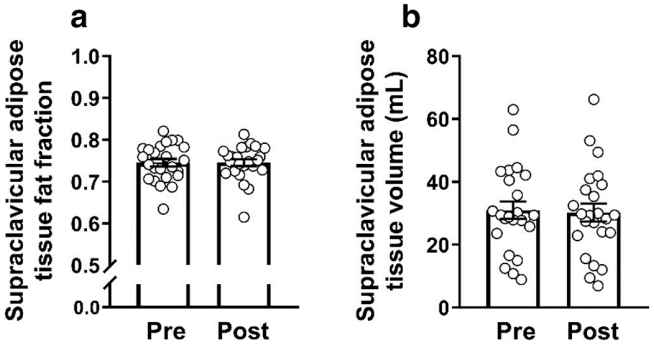

Fig. 5. Exenatide does not affect the supraclavicular adipose tissue depot fat fraction or volume.
The effect of exenatide on the supraclavicular adipose tissue depot fat fraction (a) and volume (b) in the total study cohort (N = 24). Fat fraction thresholds were set at 0.5–1.0. Pre = before exenatide, post = after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean ± SEM.

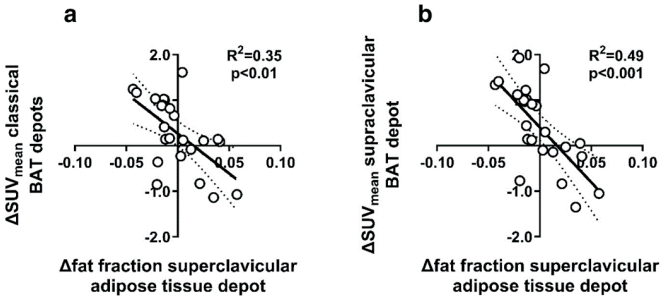

Fig. 6. Exenatide-induced changes in supraclavicular adipose tissue fat fraction (MRI) and SUVmean ([18F] FDG-PET/CT) negatively correlate.
Correlation analyses between the Δfat fraction (MRI) and ΔSUVmean ([18F]FDG-PET/CT) upon exenatide treatment of classical BAT depots (a) and only the unilateral left supraclavicular BAT depot (b). MRI fat fraction thresholds were set at 0.5–1.0. Data were analysed with linear regression analysis and assessed for interaction of ethnicity, and are presented as mean ± SEM. Dotted lines represent 95% CI.

DISCUSSION

GLP-1R agonists have multiple favorable metabolic effects additional to improving glycaemia, including weight loss and lowering lipid levels. Since preclinical studies have shown a role for central agonism of the GLP-1R in activating energy-combusting BAT, we aimed to investigate in a proof-of-principle study the effect of GLP-1R agonism on BAT metabolism in non-obese nondiabetic men. We included both South Asian and Europid participants, as we have previously shown that South Asians have an unfavorable energy metabolism compared with Europids [15]. Here, we show that exenatide lowered body weight and serum lipids already in healthy young men, without affecting REE or substrate utilization. Intriguingly, exenatide increased [^{18}F]FDG uptake by BAT, suggesting more metabolically active BAT. The metabolic effects of exenatide were largely comparable between ethnicities. Our findings support a role for the GLP-1R in BAT activation in South Asian and Europid men.

Exenatide lowered body weight in this cohort of lean to mildly overweight men. This reduction in body weight was mainly attributable to a loss of lean mass in both ethnicities, with an additional loss of fat mass in South Asian participants, without significantly affecting overall body fat percentage. The reduction in body weight in this study is in line with a meta-analysis investigating randomized controlled trials conducted in normoglycemic severe overweight or obese participants, showing a body weight reduction of 2.5–5.1 kg after 12–24 weeks of treatment with short-acting exenatide [19]. There were however only two trials included in this meta-analysis that reported body fat measurements, and these showed no significant change in overall fat mass [20] or percentage [21] compared with placebo. In concordance with previous studies [15,22], we observed a higher body fat percentage in South Asians compared with Europids. Since exercise may preserve lean mass during weight loss [23], we cannot exclude that reduced physical activity to any extent during the 12-week exenatide treatment period might have contributed to the loss of lean mass observed in our cohort of healthy young men, albeit we encouraged participants not to alter their lifestyle. We observed a negative correlation between baseline fat percentage and the delta fat percentage upon exenatide treatment, which is in line with evidence showing that higher baseline adiposity is associated with a lower relative contribution of lean mass to weight loss [24]. We therefore propose that the higher baseline body fat percentage in South Asians compared with Europids underlies their loss of fat mass after exenatide treatment.

Food intake is another major determinant of energy balance. Activation of the GLP-1R has an anorexigenic effect that contributes to weight loss [25,26]. Here, exenatide treatment did not affect overall appetite as measured by VAS. However, the sensation of hunger and desire to eat were lower during the first 4 weeks of treatment. This fits with recent studies showing that GLP-1R agonists cause changes in central nervous system

activation to food cues resulting in less reward to food [26]. These effects are temporarily, which might explain the plateau that is often reached with respect to weight loss. A study using short-term liraglutide suggests that this might be due to a decrease in fasting leptin levels, resulting in increased reward thereby counteracting the beneficial effects of liraglutide [27]. Albeit that in our study these appetite changes were transient and restored within a few weeks, a temporarily reduced food intake presumably contributed to the weight loss of some participants. Interestingly, however, preclinical evidence showed that weight loss was less pronounced in mice that were pair-fed to mice treated with centrally administrated exendin-4 [10]. Moreover, in mice treated chronically with peripherally administered exendin-4, weight loss continued even after food intake was restored within a few weeks after initiation of treatment [28]. These murine studies thus suggest that the weight-lowering effect of GLP-1R agonism goes beyond merely reducing food intake.

To investigate whether GLP-1R agonism induces weight loss in part by stimulating energy combustion, we evaluated the effect of exenatide on REE and substrate utilization. In our study, exenatide did not affect REE in the total study cohort or in either ethnicity. So far, only two clinical trials have reported an effect of exenatide on REE; one study investigating short-term treatment with liraglutide in patients with type 2 diabetes [9], and one study investigating long-term treatment with either liraglutide or exenatide in patients with type 2 diabetes [8]. However, our results are in agreement with most studies investigating the effect of prolonged (5–16 weeks) treatment with a GLP-1R agonist on energy metabolism, which did not observe differences in REE or any of the other components of total energy expenditure (i.e. the thermic effects of feeding and physical activity) [21,29–31]. We cannot exclude that a possible increase in REE by exenatide is masked in our study by a lowering in REE that generally accompanies weight loss [32]. Exenatide did also not affect substrate utilization in our study. This observation is in concordance with Beiroa et al. [8], who showed an unchanged substrate utilization in patients with type 2 diabetes after one year of treatment with either exenatide or liraglutide on top of metformin. On the contrary, another study showed that 4 weeks liraglutide in nondiabetic obese subjects shifted substrate oxidation from glucose towards lipids [31]. This is in line with preclinical studies showing that centrally administered GLP-1 [12] or exendin-4 [10] increased lipid oxidation in diet-induced obese mice. Possibly, the use of different variants of exenatide (long-acting vs short-acting) and the time after administration affects measures of energy balance. We can also not exclude that a shift in substrate utilization might be too subtle to detect via indirect calorimetry, especially in our study involving non-obese normoglycemic humans.

Exenatide tended to lower plasma glucose levels in our study, which may be mediated by enhanced insulin secretion due to GLP-1R agonism in pancreatic beta cells, and possibly increased peripheral insulin sensitivity [5]. Intriguingly, exenatide reduced

serum triglycerides and total cholesterol, and tended to lower LDL-cholesterol already in these normolipidemic participants. We can only speculate about the underlying mechanisms contributing to the lipid-lowering effects of exenatide. Firstly, impaired secretion of triglyceride-rich lipoproteins into the circulation (i.e. VLDL from the liver and chylomicrons from the small intestines) following GLP-1R agonism may have lowered lipid levels. In line with this, one to four weeks peripherally administered exendin-4 lowered circulating VLDL-triglyceride levels in mice, accompanied by reduced hepatic VLDL particle production. This resulted, together with decreased hepatic lipogenesis, even in reversal of high-fat diet-induced hepatic steatosis [33,34]. Likewise, an acute infusion with exenatide reduced postprandial triglyceride excursions and intestinal lipoprotein production in both healthy [35] and insulin-resistant humans [36]. On the other hand, we hypothesize that exenatide may increase lipids and glucose clearance by peripheral metabolic tissues, as we have previously shown that central administration of exendin-4 increased lipid and glucose uptake by skeletal muscle and BAT in mice [10].

To further investigate the contribution of peripheral metabolic tissues to the beneficial effects of exenatide, especially that of energycombusting BAT, we performed a cold-induced [^{18}F]FDG-PET/CT scan. This is the current gold standard to assess BAT metabolic volume and activity and involves quantifying glucose uptake by several BAT depots [37]. Here, we show for the first time that GLP-1R agonism increased BAT volume and SUVmean in humans. This is fully compatible with our previous observation that exendin-4 enhances glucose uptake by BAT in both lean and diet-induced obese mice [10], although the contribution of BAT to whole-body metabolism in rodents is more pronounced compared to humans [38]. Exenatide did not increase [^{18}F]FDG uptake by either subcutaneous or visceral white adipose tissue in the current study, suggesting that GLP-1R agonism in humans might be more involved in promoting substrate utilization by classical BAT depots rather than browning of white adipose tissue. Glucose uptake as measured by [^{18}F]FDG PET/CT scan is influenced by insulin sensitivity of the tissue [39]. Therefore, this method may underestimate measures of BAT metabolism in older subjects and/or subjects with type 2 diabetes, circumstances in which BAT becomes more insulin resistant. In our study, it could be argued that exenatide treatment increases whole-body insulin sensitivity, which would enhance glucose uptake by BAT. However, we did not observe a consistent increase in [^{18}F]FDG uptake by skeletal muscles after exenatide, supporting that the enhanced glucose uptake by BAT truly represents expansion of BAT volume. In addition, to exclude an acute effect of exenatide on BAT metabolism, the postexenatide study day was performed one week after the last injection. It would be interesting to assess whether other GLP-1R agonists, for instance liraglutide 3.0 mg that is currently approved for the treatment of obesity [40], also enhances glucose uptake specifically by BAT using [^{18}F]FDG-PET/CT. Importantly, centrally administering either exendin4 [10] or liraglutide [8] was shown to enhance sympathetic

outflow to BAT in mice. We therefore propose that increased sympathetic output may mediate the enhanced BAT volume and [^{18}F]FDG uptake during GLP-1R agonism in our human study. The increased heart rate we observed after exenatide in this study is in line with previous research [41] and may also reflect this increased sympathetic outflow.

Profound BAT activation, e.g. via applying a potent sympathetic stimulus by cooling humans until shivering, has been shown to burn intracellular lipids and thereby decrease the fat fraction of classical BAT depots [18]. However, the fat fraction and volume of the supraclavicular adipose tissue depot as measured by MRI remained unchanged after exenatide. As lipid uptake and utilization by activated BAT are strictly regulated, we cannot exclude that MRI might be unable to quantify a net increase in intracellular lipid combustion after exenatide. Although MRI is used less often to assess BAT volume compared with the [^{18}F]FDG PET/scan, a recent study showed that these two methods correlate well ($R^2 = 0.52$) in healthy adult subjects [42]. Interestingly, despite an unchanged overall supraclavicular adipose tissue fat fraction after exenatide, the Δfat fraction on MRI negatively correlated with the Δ[^{18}F]FDG uptake on PET/CT. This suggests that BAT metabolism of some participants was more sensitive to GLP-1R agonism ('responders') than that of others ('non-responders'), with a lower fat fraction being associated with more [^{18}F]FDG uptake and vice versa. Since GLP-1R agonism in mice increased the uptake of triglyceride-derived fatty acids by BAT much more robustly compared with deoxyglucose [10], it would be highly interesting to investigate whether GLP-1R agonism in humans also increases BAT fatty acid uptake and oxidative metabolism, using [^{18}F]fluorothiaheptadecanoic acid and [^{11}C]acetate tracers by PET/CT, respectively [43]. However, in this respect it should be noted that BAT in rodents has a larger contribution to resting energy expenditure as compared to BAT in humans. The precise contribution of activated human BAT to resting energy expenditure remains unclear so far but estimations based on static and dynamic [^{18}F]FDG PET/CT scans vary between 115 and 256 kcal/day [44,45]. Furthermore, we can only speculate about the contribution of increased BAT activity to the metabolic improvements observed after exenatide in this human study.

This study is not without limitations. As this study was designed without a placebo arm, we cannot exclude that intra-individual variations in energy metabolism that may occur over time have affected our results. Reassuringly, including seasonality as a covariate did not influence the statistical analyses. Furthermore, despite increased [^{18}F]FDG uptake by BAT, with our experimental set-up no effect of exenatide on resting energy expenditure was found. The absence of effect may be inherent to indirect calorimetry that we used to estimate energy expenditure, which only measures oxygen-dependent energy metabolism, and/or relate to the error of the measurement of indirect calorimetry. The increase in resting energy expenditure in humans that is expected from enhanced BAT activity is in fact modest (115 to 256 kcal/day), which may be below

the threshold of a detectable increase in energy expenditure. Notably, we previously showed by pair-feeding experiments in mice that exendin-4 reduces body fat mass despite equal food intake. Although these data by definition imply that exendin-4 increases energy expenditure in mice, increased energy expenditure was not apparent from indirect calorimetry [10]. Future studies investigating the effects of long-term BAT activation on resting energy expenditure and its metabolic consequences are therefore highly warranted, probably with more accurate and/or more advanced techniques. For future studies, we propose to include continuous measurements by means of a room calorimeter system, and employ techniques to assess the contribution of non-oxidative energy expenditure. In addition, as mentioned above, to further explore the effect of GLP-1R agonism on BAT metabolism and energy expenditure, we propose to conduct a future study with the GLP-1R agonist liraglutide 3.0 mg, that is especially potent with respect to inducing weight loss [46].

In summary, we show that prolonged GLP-1R agonism activates BAT and improves the metabolic phenotype, including serum lipid profile, already in non-obese normoglycemic young men, with largely similar effects observed in South Asian and Europid individuals. Further research investigating the effect of GLP-1R agonism on thermogenesis and substrate utilization by BAT, and the contribution of BAT to an improved cardiometabolic phenotype upon GLP-1R agonism in patients with type 2 diabetes is warranted.

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SUPPLEMENTAL APPENDIX

METHODS

Indirect calorimetry analysis

Carbon dioxide production (VCO_2) and oxygen consumption (VO_2) were determined per minute. VCO_2 and VO_2 were used to calculate the resting energy expenditure, respiratory quotient (RQ) and glucose and lipid oxidation as described previously [1]. Formulas were adjusted for the estimated male urinary nitrogen excretion rate. Formulas for substrate oxidation rates were as follows:

Glucose oxidation (g/min) = (4.57 VCO_2) - (3.23 * VO_2) - 0.0274456*
*Lipid oxidation (g/min) = (1.69 * VO_2) - (1.69 * VCO_2) - 0.02142868*

[^{18}F]fluorodeoxyglucose positron emission tomography/computed tomography data acquisition and analysis

The scanning protocol started with a low dose CT-scan (30 mAeff, 120kV), followed by a PET scan (ten bed positions, each lasting 5.5 minutes, from the top of the head until the iliac crest) in accordance with European Nuclear Medicine guidelines [2]. Positron emission tomography (PET) and computed tomography (CT) thresholding were applied according to BARCIST 1.0 criteria, comprising an individualized lower standardized uptake value (SUV) threshold and a Hounsfield unit range (-190;-10) [3]. In addition to assessing [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) uptake in classical BAT depots (i.e. cervical and supraclavicular regions), [^{18}F]FDG uptake was evaluated in the upper mediastinal, axillary and paravertebral adipose tissue depots ('total body BAT'). Lastly, [^{18}F]FDG uptake was investigated in reference tissues (liver, cerebellum and descending aorta) as well as various muscles; m. sternocleidomastoideus, m. longus colli, m. trapezius, m. deltoideus, m. pectoralis major, m. psoas major and m. gluteus maximus.

Skin temperature analysis

Supraclavicular skin temperature was measured on the right supraclavicular fossa. Skin temperature at the right axilla was used as a proxy for core body temperature. Proximal skin temperature was calculated by the formula of Schellen et al. [4], as the average of the skin temperature at the scapula, lumbar zone, chest area and abdomen. Distal skin temperature was determined as the average temperature of the right hand and left foot.

Magnetic resonance imaging data acquisition and analysis

Participants were placed in a head-first supine position in the MRI scanner, with their head in a 16-channel head and neck coil, while a 16-channel anterior array was placed on their torso. A three-dimensional six-point chemical-shift encoded gradient-echo acquisition was applied with the following parameters: repetition time TR=16.5 ms, first echo time TE=1.79 ms, echo time separation ΔTE=1.98 ms, flip angle=3°, field-of-view of 480 mm × 300 mm × 90 mm (Right-Left, Foot-Head, Anterior-Posterior), 1.1 mm isotropic resolution, 4 retrospectively averaged signal averages.

An in-house water-fat separation algorithm based on the estimation of main magnetic field inhomogeneity was used for magnetic resonance imaging (MRI) characterization of the supraclavicular adipose tissue depot. The algorithm comprises a-priori knowledge regarding the multi-peak fat spectrum and assumes mono-exponential effective transverse relaxation time T2*. Initially, a low-resolution reconstruction was performed by using an estimate for the main magnetic field inhomogeneity. Subsequently, a region growing scheme was used to extrapolate the solution from correctly reconstructed parts in order to acquire the reconstructed water and fat images at high resolution [5-8]. Fat fraction maps were reconstructed according to formula [1], where x, y and z denote

$$Signal\ fat\ fraction(x,y,z) = \frac{Fat(x,y,z)}{Water(x,y,z)+Fat(x,y,z)} \quad [1]$$

the coordinates of a voxel in the directions: Right-Left, Foot-Head, Anterior-Posterior, respectively. Lipid content of the supraclavicular adipose depot was calculated as the product of the average fat fraction and volume.

Regions of interest encompassing the established location of the unilateral left supraclavicular adipose tissue depot were manually drawn on the baseline scans by A.S.M. and K.F.M.B. Registration was performed using the open-source image registration toolbox Elastix [9, 10]. The first echoes of the pre- and post-intervention image stacks were co-registered by first aligning them in an affine manner. Afterwards, deformable registration was performed with a three-dimensional B-spline transform with a 10×10×10 mm3 grid. To calculate the deformation field, we used adaptive stochastic gradient descent with two resolutions for optimization and Mattes mutual information as the similarity measure. The parameter file that was used for performing the registration can be downloaded from http://elastix.bigr.nl/wiki/index.php/Par0048. Finally, the calculated deformation field from the registration was used to transform the baseline regions of interest to the post-intervention image coordinates for the assessment of estimated BAT volume and fat fraction. A fat fraction threshold range of 0.5–1.0 was used for data analysis.

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Tables

Supplementary Table 1 Effect of exenatide on body weight and composition.

	All participants (N=24)		Europids (N=12)		South Asians (N=12)		
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	p-value for interaction
SBP (mmHg)	120±2	116±2 **	123±2	119±3 *	116±2 †	113±2 *†	0.879
DBP (mmHg)	78±2	76±1	81±3	78±2	75±2 †	75±1	0.319
Heart rate (bpm)	61±2	67±2 ***	67±2	70±3 *	55±3 ††	64±2 $^{***\sim}$	0.010

Pre=before exenatide, post=after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean±SEM. P-value for interaction is exenatide treatment*ethnicity. ~p<0.1, *p<0.05, **p<0.01 post *vs* pre exenatide. †p<0.05, ††p<0.01 South Asians *vs* Europids.

Supplementary Table 2 Effect of exenatide on blood pressure and heart rate.

	All participants (N=24)		Europids (N=12)		South Asians (N=12)		
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	p-value for interaction
SBP (mmHg)	120±2	116±2 **	123±2	119±3 *	116±2 †	113±2 *†	0.879
DBP (mmHg)	78±2	76±1	81±3	78±2	75±2 †	75±1	0.319
Heart rate (bpm)	61±2	67±2 ***	67±2	70±3 *	55±3 ††	64±2 $^{***\sim}$	0.010

Pre=before exenatide, post=after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean±SEM. P-value for interaction is exenatide treatment*ethnicity. *p<0.05, **p<0.01, ***p<0.001 post vs pre exenatide. ~p<0.1, †p<0.05, ††p<0.01 South Asians vs Europids. DBP=diastolic blood pressure, SBP=systolic blood pressure.

Supplementary Table 3 Effect of exenatide on [18F]FDG uptake by brown adipose tissue

	All participants (N=23)		Europids (N=12)		South Asians (N=11)		
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	p-value for interaction
Classical BAT depots							
BAT metabolic volume (mL)	79±14	101±13 *	63±18	84±19	97±22	119±16	0.933
SUV_{mean}	2.7±0.2	3.0±0.2 *	2.3±0.2	2.7±0.2	3.0±0.4	3.4±0.4	0.862
SUV_{peak}	8.1±1.5	9.5±1.2	6.0±1.3	7.6±1.5	10.4±2.7	11.6±1.9	0.865
Metabolic BAT activity (g)	277±67	350±58	186±63	268±76	375±117	439±84	0.842
Total body BAT depots							
BAT metabolic volume (mL)	123±21	154±19 *	100±27	132±30	147±32	178±24	0.974
SUV_{mean}	2.5±0.2	2.8±0.2 ˜	2.2±0.2	2.5±0.2	2.8±0.3	3.1±0.3	0.895
SUV_{peak}	8.2±1.5	9.5±1.2	6.1±1.2	7.6±1.5	10.4±2.7	11.6±1.9	0.888
Metabolic BAT activity (g)	382±87	486±78 ˜	270±86	390±107	503±151	591±111	0.788

Classical brown adipose tissue (BAT) is defined as the bilateral cervical and supraclavicular BAT depots. Total body BAT is defined as the bilateral cervical, supraclavicular, upper mediastinal, axillary and paravertebral BAT depots. One of the 12 South Asian participants was excluded due to movement of the participant during a scan. Pre=before exenatide, post=after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean±SEM. P-value for interaction is exenatide treatment*ethnicity. ˜p<0.1, *p<0.05 post vs pre exenatide.

Supplementary Table 4 Effect of exenatide on [18F]FDG uptake by reference tisues, skeletal muscle and white adipose tissue

SUVmean	All participants (N=23)		Europids (N=12)		South Asians (N=11)		p-value for interaction
	Pre	Post	Pre	Post	Pre	Post	
Reference tissues							
Liver	2.12±0.07	2.08±0.06	2.06±0.07	1.99±0.05	2.20±0.12	2.18±0.11	0.663
Cerebellum	7.47±0.34	7.59±0.28	7.36±0.37	7.34±0.35	7.60±0.60	7.86±0.45	0.583
Aorta descendens	1.39±0.06	1.34±0.04	1.35±0.07	1.23±0.04	1.44±0.08	1.45±0.06 †	0.249
Skeletal muscle							
M. Sternocleidomastoideus	1.03±0.08	1.02±0.10	0.93±0.10	1.00±0.14	1.13±0.12	1.06±0.12	0.555
M. Longus colli	1.55±0.18	1.78±0.31	1.16±0.21	1.58±0.25	1.94±0.25	1.99±0.58	0.527
M. Trapezius	0.55±0.03	0.47±0.03 *	0.52±0.05	0.50±0.03	0.58±0.05	0.44±0.04 **	0.040
M. Deltoideus	0.56±0.04	0.54±0.04	0.58±0.08	0.57±0.08	0.54±0.03	0.50±0.03	0.557
M. Pectoralis major	0.51±0.03	0.59±0.03 *	0.50±0.04	0.63±0.05 *	0.52±0.06	0.55±0.04	0.141
M. Psoas major	0.78±0.07	0.99±0.07 ***	0.84±0.11	1.11±0.11 **	0.72±0.07	0.86±0.07 ˜	0.207
M. Gluteus maximus	0.45±0.02	0.46±0.02	0.44±0.03	0.46±0.03	0.45±0.02	0.46±0.04	0.926
White adipose tissue							
Subcutaneous, abdominal	0.29±0.03	0.30±0.03	0.30±0.03	0.30±0.06	0.27±0.05	0.30±0.04	0.778
Subcutaneous, dorsocervical	0.47±0.03	0.46±0.03	0.48±0.04	0.52±0.04	0.46±0.04	0.40±0.04 †	0.847
Visceral, paracolic	0.71±0.08	0.74±0.08	0.71±0.11	0.75±0.11	0.72±0.12	0.73±0.11	0.106

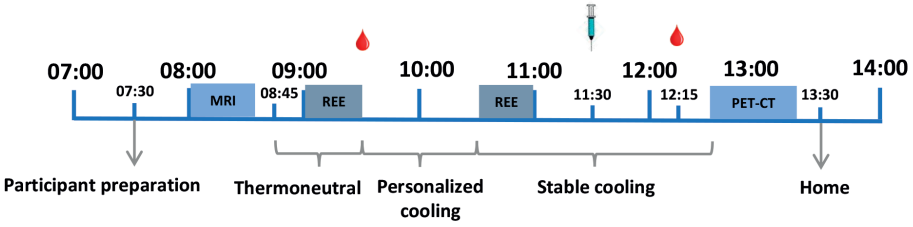
One of the 12 South Asian participants was excluded from all [18F]FDG-PET/CT analyses due to movement of the participant. Pre=before exenatide, post=after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean±SEM. P-value for interaction is exenatide treatment*ethnicity. ˜p<0.1, *p<0.05, **p<0.01, ***p<0.001 post vs pre exenatide. ˜p<0.1, †p<0.05 South Asians vs Europids.

Supplementary Table 5: Effect of exenatide on the supraclavicular adipose tissue fat fraction and volume per ethnicity

	Europids (N=12)		South Asians (N=12)		p-value for interaction
	Pre	**Post**	**Pre**	**Post**	
Supraclavicular adipose tissue fat fraction	0.751±0.011	0.748±0.009	0.739±0.015	0.743±0.015	0.519
Supraclavicular adipose tissue volume (mL)	29.7±3.1	29.1±3.3	32.3±4.7	31.4±4.9	0.827

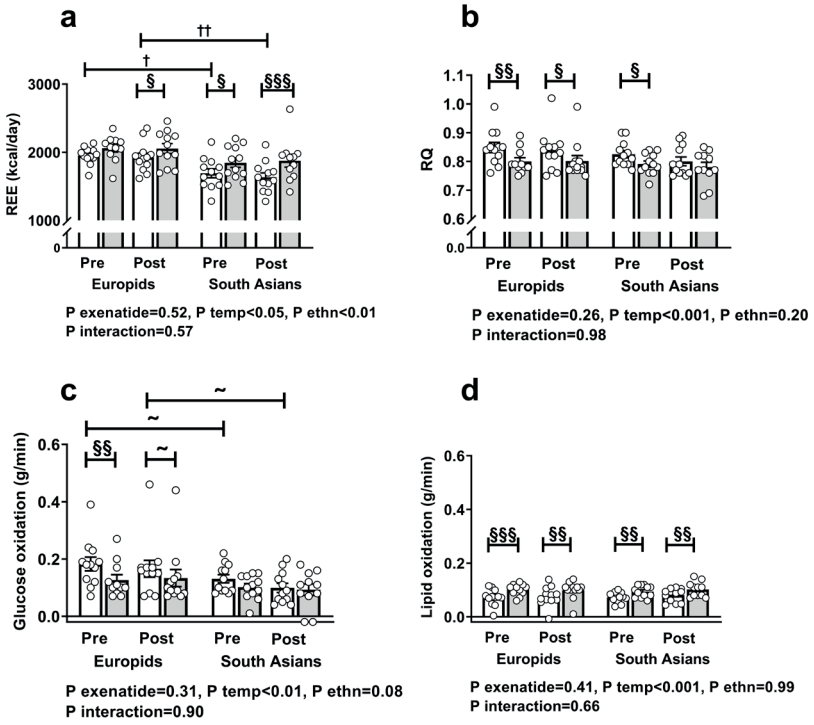
Fat fraction thresholds were set at 0.5-1.0. Pre=before exenatide, post=after exenatide. Data were analysed by a two factor mixed design ANOVA and are presented as mean±SEM. P-value for interaction is exenatide treatment*ethnicity.

Figures



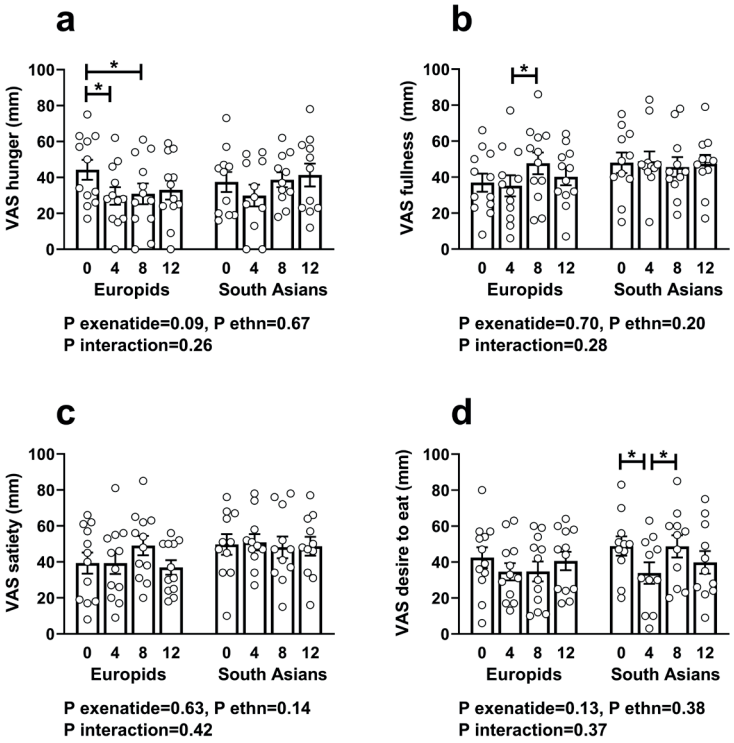
- Body impedance analysis
- Insert cannula
- 08:45-09:00 Attach iButtons
- 09:00-09:30: Indirect calorimetry
- 09:30: Blood draw
- 10:30-11:00: Indirect calorimetry
- 11:30: [^{18}F]FDG injection
- 12:15: Blood draw

Supplementary Fig. 1 Overview identical study day before and after the exenatide treatment period



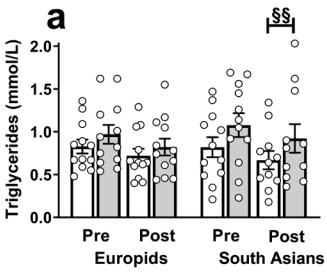
Supplementary Fig. 2 Exenatide does not affect energy metabolism in either ethnicity

The effect of exenatide on thermoneutral and cold-induced resting energy expenditure (REE) (**a**), respiratory quotient (RQ) (**b**), glucose oxidation (**c**) and lipid oxidation (**d**) per ethnicity (N=12 South Asians and N=12 Europids). Pre=before exenatide, post=after exenatide. White bars are thermoneutral, grey bars are during short term cooling. Data were analysed by linear mixed models and are presented as mean±SEM.. P-values for the main effect of exenatide treatment (exenatide), temperature (temp) and ethnicity (ethn) and their interaction (exenatide*temp*ethn) are shown below the figures. §p<0.05, §§p<0.01, §§§p<0.001 post-hoc p-values cold *vs* thermoneutrality. ˜p<0.1, †p<0.05, ††p<0.01 post-hoc p-values South Asians *vs* Europids.

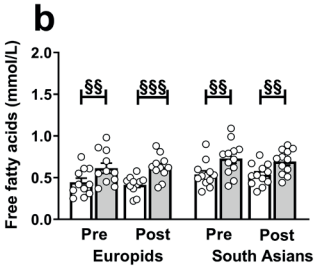


Supplementary Fig. 3 Exenatide reduces the feeling of hunger in Europids and desire to eat in South Asians during the first weeks of treatment

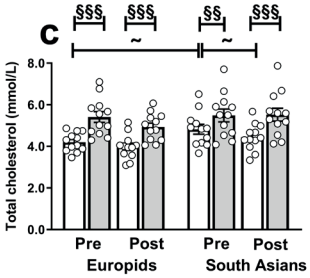
The effect of exenatide on subjective ratings for hunger (**a**), fullness (**b**), satiety (**c**) and desire to eat (**d**) measured every 4 weeks per ethnicity (N=11 South Asians and N=12 Europids). One of the 12 South Asian participants was excluded due to incomplete questionnaires. Higher values (mm) indicate higher ratings. Data were analysed by a two factor mixed design ANOVA and presented as mean±SEM. P-values for the main effect of exenatide treatment (exenatide), and ethnicity (ethn) and their interaction (exenatide*ethn) are shown below the figures. *p<0.05 post-hoc p-value effect of exenatide.



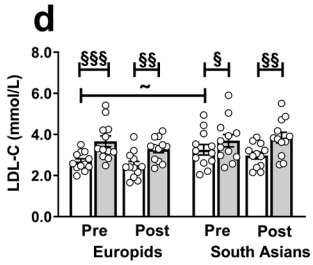
P exenatide=0.33, P temp=0.24, P ethn=0.71
P interaction=0.90



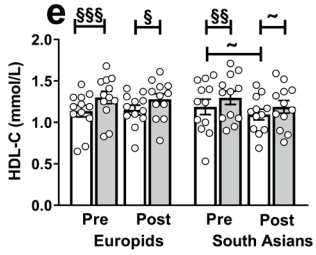
P exenatide=0.59, P temp<0.001, P ethn=0.12
P interaction=0.38



P exenatide<0.05, P temp<0.001, P ethn=0.11
P interaction=0.05



P exenatide=0.13, P temp<0.001, P ethn=0.13
P interaction=0.05



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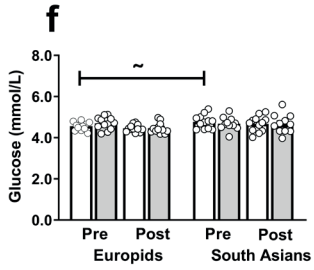
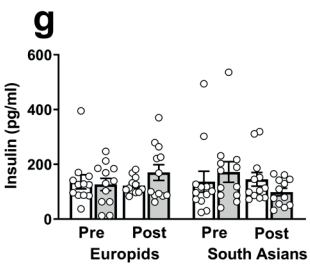
P interaction=0.77

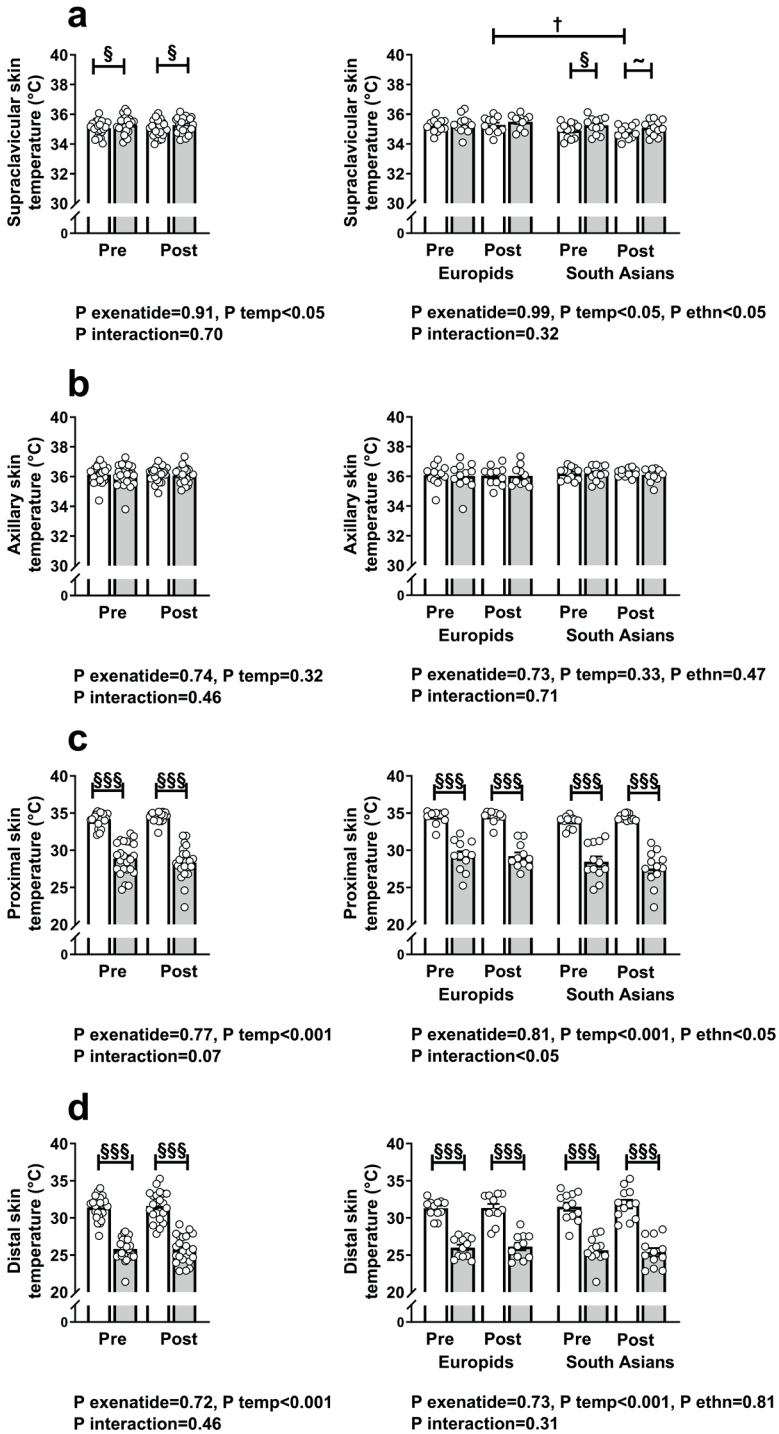


P exenatide=0.09, P temp=0.84, P ethn=0.10
P interaction=0.16



P exenatide=0.69, P temp=0.70, P ethn=0.98
P interaction<0.01

Supplementary Fig 4. Exenatide does not significantly affect lipid, glucose or insulin levels in either ethnicity The effect of exenatide on thermoneutral and cold-induced fasted serum triglycerides (**a**), free fatty acids (**b**), total cholesterol (**c**), LDL-cholesterol (LDL-C) (**d**)**,** HDL-cholesterol (HDL-C) (**e**), plasma glucose (**f**) and serum insulin (**g**) per ethnicity (N=12 South Asians and N=12 Europids). Pre=before exenatide, post=after exenatide. White bars are thermoneutral, grey bars are during short term cooling. Data were analysed by linear mixed models and are presented as mean±SEM. P-values for the main effect of exenatide treatment (exenatide), temperature (temp) and ethnicity (ethn) and their interaction (exenatide*temp*ethn) are shown below the figures. ˜p<0.1, §p<0.05, §§p<0.01, §§§p<0.001 post-hoc p-values cold *vs* thermoneutrality. ˜p<0.1 post-hoc p-values South Asians *vs* Europids.


a
Supraclavicular skin temperature (°C)
40
38
36
34
32
30
0
§
§
Pre
Post
P exenatide=0.91, P temp<0.05
P interaction=0.70
†
§
~
Pre
Post
Pre
Post
Europids
South Asians
P exenatide=0.99, P temp<0.05, P ethn<0.05
P interaction=0.32
b
Axillary skin temperature (°C)
P exenatide=0.74, P temp=0.32
P interaction=0.46
P exenatide=0.73, P temp=0.33, P ethn=0.47
P interaction=0.71
c
Proximal skin temperature (°C)
35
25
20
§§§
P exenatide=0.77, P temp<0.001
P interaction=0.07
P exenatide=0.81, P temp<0.001, P ethn<0.05
P interaction<0.05
d
Distal skin temperature (°C)
P exenatide=0.72, P temp<0.001
P interaction=0.46
P exenatide=0.73, P temp<0.001, P ethn=0.81
P interaction=0.31

Supplementary Fig 5. Exenatide does not affect the supraclavicular, axillary, proximal or distal skin temperature in the total study population or in either ethnicity

The effect of exenatide on thermoneutral and cold-induced supraclavicular skin temperature (a), axillary skin temperature (b), proximal skin temperature (c) and distal skin temperature (d) in the total study cohort and per ethnicity (N=12 South Asians and N=12 Europids). Pre=before exenatide, post=after exenatide. White bars are thermoneutral, grey bars are during short term cooling. Data were analysed by linear mixed models and are presented as mean±SEM. For ethnicities combined, p-values for the main effect of exenatide treatment (treat) and temperature (temp) and their interaction (exenatide*temp) are shown below the figures. When taking ethnicity into account as a factor, p-values for the main effect of exenatide treatment (treat), temperature (temp) and ethnicity (ethn) and their interaction (exenatide*temp*ethn) are shown below the figures. ˜p<0.1, §p<0.05, §§§p<0.001 post-hoc p-values cold vs thermoneutrality. †p<0.05 post-hoc p-value South Asians vs

7 General discussion and future perspectives

Cardiovascular disease (CVD) has been the global leading cause of death since many decades, and is, together with diabetes, responsible for almost one third of all deaths worldwide. The morbidity and mortality rates of these cardiometabolic diseases are particularly high in people from South Asian descent, especially when adopting a 'Western' lifestyle after urbanization or migration to Western countries. In this thesis we aimed to unravel underlying mechanisms contributing to CVD and type 2 diabetes (T2D) in South Asians. We therefore investigated pathophysiological aspects of these cardiometabolic diseases in both healthy and metabolically compromised South Asians compared with white Caucasians. To this end, we assessed whether Wnt signaling, a pathway that has recently been implicated in insulin resistance and T2D, is altered in metabolic tissues in South Asians. Next, we assessed whether LDL of South Asians is more prone to aggregate *in vitro*, since this was recently shown to robustly predict future cardiovascular events in patients with CVD. After identifying novel mechanisms that could contribute to cardiometabolic disease in South Asians, we focused on novel treatment strategies to treat cardiometabolic disease. We specifically investigated brown adipose tissue (BAT), which has been a topic of interest during the last decade as a potential target to alleviate, or possibly even prevent, cardiometabolic disease. BAT takes up lipids and glucose from the circulation for combustion into heat, thereby increasing energy expenditure. We therefore performed two clinical trials in which we investigated the potential of the sympathomimetic agent mirabegron and the glucagon-like peptide-1 (GLP-1) receptor agonist exenatide to activate BAT and thereby improve energy and nutrient metabolism, and compared this between South Asians and white Caucasians. In these studies we included both ^{18}F-fluorodeoxyglucose ([^{18}F]FDG) positron emission tomography computed tomography (PET/CT) scan and magnetic resonance imaging (MRI) to visualize BAT. To gain further insight into the physiological function of BAT, we investigated the effect of cold exposure on several angiopoietin-like proteins (ANGPTLs), which are involved in lipid trafficking between metabolic tissues, in South Asians and white Caucasians. Novel pathophysiological aspects of T2D and CVD in South Asians have emerged from this thesis, as well as insights into the effects of novel BAT-activating treatment strategies on cardiometabolic parameters. In this chapter we will address these observations, together with ongoing challenges and future prospects in BAT research.

1. NOVEL PATHOPHYSIOLOGICAL ASPECTS OF CARDIOMETABOLIC DISEASE IN SOUTH ASIANS

Insulin resistance and type 2 diabetes in South Asians

T2D is characterized by hyperglycemia, which has various detrimental effects on the vasculature, amongst others via glycemic modification of proteins, promoting oxidative stress and impairment of mitochondrial function, thereby inducing inflammation and endothelial dysfunction. This ultimately results in damage of the microvasculature presenting as retinopathy, nephropathy and neuropathy, and macrovasculature resulting in atherosclerotic CVD that presents as myocardial infarction and/or stroke (1). These processes are further aggravated by the dyslipidemia often present in diabetes, especially by high levels of free fatty acids, and underlying insulin resistance. Insulin resistance is characterized by the loss of intracellular signaling in response to insulin by metabolic tissues, which is fundamental to the development of T2D. Insulin resistance leads to a compensatory increase in pancreatic insulin release. This hyperinsulinemia eventually exhausts β-cell function, resulting in an insulin production deficit and the subsequent need for *e.g.* exogenous insulin. South Asians, who are at particular high risk to develop T2D and suffer from high rates of organ damage and mortality due to T2D, show insulin resistance already at a young age when signs of cardiometabolic disease have not yet developed. As described in **chapter 1**, many factors can contribute to insulin resistance, with an important and causal role for adiposity and ectopic lipid deposition. It is well established that South Asians have a higher body fat percentage compared with other ethnicities, including visceral white adipose tissue (WAT). This phenotype is already present in young generally healthy subjects, an observation which we confirmed in **chapters 5** and **6**. Considering the significant contribution of adiposity to the development and aggravation of insulin resistance, we continue to believe that a relatively large amount of – especially visceral and ectopic – body fat in South Asians contributes to their high risk of insulin resistance and T2D. Further research investigating additional mechanisms involved in impaired insulin signaling in metabolic tissues in South Asians is warranted.

Impaired Wnt and insulin signaling in WAT in South Asians

We therefore assessed insulin signaling in WAT and skeletal muscle in South Asians compared with white Caucasians, and investigated its relation with Wnt signaling. The Wnt protein family is involved in at least one canonical and two noncanonical signaling transduction pathways, and is mainly known for its role in carcinogenesis and embryonic development via affecting various essential cellular processes including regulation of cell fate, proliferation and migration. In addition, there is ample evidence that defects in Wnt signaling promote cardiometabolic disease. Firstly, Wnt signaling suppresses adipogenesis and favors development of precursor cells into other lineages,

in particular osteoblasts (2), and mutations in multiple Wnt genes are associated with the amount as well as the distribution of adipose tissue (3-5). Secondly, impaired Wnt signaling in metabolic tissues is proposed to negatively affect glucose homeostasis, since mutations in various Wnt genes are associated with more insulin resistance and occurrence of T2D (6-9). Moreover, evidence points towards a possible role for reduced Wnt signaling in promoting CVD, since an impairing mutation in the gene encoding for LRP6, a key receptor involved in Wnt signaling, was identified in a family with T2D but also with hyperlipidemia and early onset atherosclerotic CVD (6). Hence, impaired Wnt signaling may contribute to obesity, disadvantageous fat distribution, impaired glucose regulation and atherosclerotic CVD, all of which South Asians are at increased risk for. In **chapter 3** we showed that expression of key genes involved in Wnt signaling, in addition to insulin signaling, are indeed lower in WAT of South Asians compared with white Caucasians. Moreover, expression levels of genes in Wnt signaling and insulin signaling were strongly positively correlated, suggesting that Wnt and insulin signaling pathways are intertwined. This is in line with preclinical evidence showing that knocking down LRP5, another key receptor involved in Wnt signaling, reduces phosphorylation of insulin signaling proteins in murine preadipocytes (10).

We wondered which factors could be involved in this altered Wnt signaling in WAT in South Asians. Interestingly, Wnt signaling can be impaired by sclerostin, a protein mainly produced by osteocytes. Sclerostin negatively regulates bone formation by directing precursor cell differentiation away from the osteocyte lineage, and is increased in subjects with (pre)diabetes compared with normoglycemic subjects (11). Of note, sclerostin is suggested to be involved in vascular calcifications, and sclerostin levels are increased even further in individuals with T2D in case of significant carotid atherosclerosis (12). Interestingly, in **chapter 3** we observed higher plasma sclerostin levels in South Asians compared with white Caucasians. Whether these higher sclerostin levels in South Asians are due to increased production by osteocytes or decreased renal or hepatic elimination is currently unknown. We propose that higher sclerostin levels in South Asians could impair Wnt signaling in WAT, thereby negatively affecting local insulin signaling. In addition, since inhibition of Wnt signaling by sclerostin was shown to increase expression of genes involved in adipocyte differentiation, increase adipocyte hypertrophy and result in accumulation of fat mass in mice (13), we propose that reduced Wnt signaling by sclerostin in WAT in South Asians contributes to their higher amount of abdominal fat mass compared with white Caucasians. Notably, our study included biopsies derived from the subcutaneous adipose tissue depot, and whether these observations also hold true for the visceral adipose tissue depot is not yet known. Whether these changes in Wnt signaling are congenital or induced by higher sclerostin levels later in life, also remains to be investigated. Importantly, whether reduced gene expression truly reflects impaired Wnt and insulin signaling in WAT of South Asians remains to be elucidated in

future *in vitro* studies, including the possible causal role of sclerostin herein. Recently, the sclerostin-neutralizing antibody romosozumab (Evinity) was approved by the FDA to increase bone mineral density and reduce fracture risk in postmenopausal women with osteoporosis, based on the osteoanabolic function of the Wnt signaling pathway (14-16). This development is particularly interesting from a metabolic point of view, since sclerostin deficiency in mice, induced either via a sclerostin-neutralizing antibody or by using a *Sost* knockout model, reduces body fat accumulation and improves glucose homeostasis (13). Therefore, such a sclerostin-neutralizing antibody may yield potential to improve obesity and diabetes by stimulating Wnt signaling in metabolic tissues, which could be of specific interest to South Asians.

Impaired mitochondrial function in South Asians

In **chapter 3**, we did not observe consistent differences in expression levels of genes involved in Wnt or insulin signaling in skeletal muscle between overweight prediabetic South Asians and white Caucasians. These comparable gene expression levels suggests that insulin signaling is not evidently hampered in skeletal muscle under insulin resistant conditions in South Asians. Since gene expression does not necessarily reflect signaling activity, these results should however be interpreted with caution. A previous study indeed observed lower levels of phosphorylated proteins involved in insulin signaling in skeletal muscle of nondiabetic South Asians compared with white Caucasians (17). An additional factor involved in local insulin signaling may be the oxidative capacity of mitochondria. More specifically, an impaired mitochondrial oxidative capacity is associated with reduced whole-body insulin sensitivity, possibly via lipid accumulation in skeletal muscle leading to disruption of insulin signaling and thereby promoting insulin resistance (18). South Asians were indeed shown to have more skeletal muscle lipid deposition compared with white Caucasians (19-21). Cardiorespiratory fitness is a proxy for the oxidative capacity of the local skeletal muscle as well as the total body, and is positively correlated with insulin sensitivity (22-24). Interestingly, cardiorespiratory fitness levels are consistently lower in South Asians compared with white Caucasians (17, 25-27). To date, three studies compared skeletal muscle oxidative capacity between South Asians and white Caucasians. In two of these studies, skeletal muscle oxidative capacity was comparable between South Asians and white Caucasians (17, 28). A third and in-depth study from our group however investigated mitochondrial respiratory function by measuring *ex vivo* skeletal muscle oxygen consumption, and did observe multiple lower respiration states in South Asians compared with white Caucasians (29). Increasing skeletal muscle mitochondrial capacity may be achieved by performing physical activity, which also accompanies improved hyperglycemia in subjects with T2D (30). It would be highly interesting to investigate whether promoting physical activity could enhance skeletal muscle oxidative capacity and thereby improve whole-body insulin sensitiv-

ity, especially in South Asians since this population generally exercises little. Another potentially interesting treatment strategy to improve skeletal muscle oxidative capacity in South Asians is administering the naturally occurring polyphenolic compound resveratrol. Resveratrol increases skeletal muscle mitochondrial function in subjects with T2D (31), as well as in their first-degree relatives who are at increased risk to develop diabetes (32). In neither of these studies did this however coincide with changes in local or whole-body insulin sensitivity. Another potentially interesting polyphenol that could positively affect mitochondrial function is quercetin. Quercetin stimulates skeletal muscle mitochondrial biogenesis in mice (33), an observation which could however not be reproduced in a human trial (34). Furthermore, animal studies showed various anti-diabetic properties of quercetin, including an improved insulin resistance and lowering of glucose levels, amongst others via antioxidant and anti-inflammatory functions (35). Notably, quercetin also stimulated browning of WAT in mice, which could partly underly its beneficial triglyceride-lowering effects (36). These data demonstrate the relevance of further investigating the effect of physical activity and dietary compounds on mitochondrial functionality and whole-body energy metabolism, which could be especially relevant to target T2D in South Asians.

Altered endocrine function of WAT in South Asians

In addition to functioning as an energy reservoir, WAT also actively secretes a variety of lipids, adipokines and cytokines that act in concert to regulate whole-body energy metabolism and systemic inflammation (37). Alterations in this endocrine function of WAT could contribute to the high rate of T2D in South Asians. For example, adiponectin levels (an insulin-sensitizing, anti-atherogenic and anti-inflammatory adipokine) were repeatedly shown to be lower in South Asians compared with white Caucasians (38-41). In contrary, leptin levels (an insulin-sensitizing adipokine) are higher in South Asians compared with white Caucasians (41-45). Higher leptin levels could however be due to relatively more leptin-secreting adipose tissue mass, or reflect a leptin-resistant state, similar to obesogenic conditions (46). Furthermore, higher circulating levels of CRP (47, 48), the pro-inflammatory cytokines interleukin-6 (IL-6) (42, 49) and tumor necrosis factor-α (TNF-α) (42) suggest a more pro-inflammatory state in South Asians compared with white Caucasians. These observations are corroborated by an altered expression of inflammatory genes, especially those involved in interferon signaling, in subcutaneous WAT and skeletal muscle in both healthy young (38) and older overweight prediabetic (50) South Asians compared with white Caucasians. Altogether, an altered secretory function of WAT might induce a pro-inflammatory state, thereby impairing local insulin signaling and promoting whole-body insulin resistance in South Asians. The non-classical mechanisms contributing to T2D in South Asians described in these paragraphs are summarized in **Figure 1**.

Cardiovascular disease in South Asians

A cardiovascular event, *i.e.* myocardial infarction or stroke, results from complete vascular occlusion due to progressive atherosclerosis. During the atherosclerotic process, damaged and activated endothelium recruits inflammatory cells, whereafter monocytes move into the subendothelial intima of the vessel wall and transform into macrophages. Simultaneously, LDL particles deposited within the intima become oxidized and aggregate into larger particles, and are engulfed by the macrophages which then become lipid-loaded foam cells. Meanwhile, a series of pro-inflammatory cytokines, including IL-6, TNF-α and interferon-γ, are secreted from the atherosclerotic plaque, which further promote transmigration of inflammatory cells and foam cell formation. Smooth muscle cells that reside within the intima proliferate to co-generate a plaque, with migration of additional smooth muscle cells towards the intima leading to plaque expansion. A fibrous cap, consisting of muscle cells and collagen, is then formed around the plaque lipid core. Matrix metalloproteinases (MMPs) produced by macrophages induce fibrous cap thinning, which can ultimately lead to plaque rupture. This exposes the underlying subendothelial space to the blood, leading to platelet activation and adhesion and further vascular occlusion. Ultimately, upon significant occlusion of the artery, an ischemic event occurs (51). Notably, South Asians are very prone to develop atherosclerosis and have higher rates of cardiovascular events compared with white Caucasians. This increased risk of CVD highlights the need for research identifying underlying mechanisms and risk factors that could serve as treatment targets against CVD in the vulnerable South Asian population.

High LDL-cholesterol levels are such an important and causal risk factor for atherosclerotic CVD, since LDL particles promote plaque formation by transporting cholesterol towards the vessel wall (51, 52). LDL-cholesterol levels are generally higher in South Asians compared with whites, already prior to development of clinically relevant CVD (53). Aside from investigating total LDL-cholesterol levels, further assessing LDL functionality and characterizing LDL atherogenic characteristics could aid in identifying subjects at risk for CVD. In line with this, the aggregation capacity of LDL particles, which is a key feature within the etiology of atherosclerosis, was recently shown to predict future cardiovascular deaths in a cohort of CVD patients, independently of classical CVD risk factors (54). Since higher LDL-levels only partially explain the high risk of CVD in South Asians (55), in **chapter 4** we investigated whether aggregation susceptibility of LDL particles differs between healthy young South Asian and white Caucasian subjects without established CVD. We indeed observed that LDL particles of South Asians are more prone to aggregate compared with those of white Caucasians. The surface of LDL particles of South Asians were also enriched in specific lipids, including the bioactive sphingomyelins SM 23:0 and 24:0. Moreover, LDL sphingomyelins positively correlated with LDL aggregation, which is in line with evidence showing that plasma sphingomy-

elin levels are positively associated with atherosclerotic CVD risk (56). Now the question remains; why are LDL particles of South Asians enriched in these specific sphingomyelins and therefore more prone to aggregate? Interestingly, preclinical evidence showed that plasma sphingomyelin levels are increased during obesity, which possibly contributes to inflammation (57). In **chapter 4** we indeed observed a positive correlation between body fat percentage and LDL sphingolipids. We therefore propose that adiposity in South Asians could affect their LDL lipidome, thereby promoting LDL aggregation susceptibility which increases the risk of atherosclerotic CVD. As we only performed a small cross-sectional study in a healthy cohort, future larger studies that follow LDL-aggregation susceptibility over time and also include patients with established CVD are needed to investigate the true contribution of increased LDL aggregation to the high CVD burden in South Asians. It would also be highly interesting to investigate whether beneficially modulating the LDL lipidome, for example by a healthy diet in combination with moderate intense exercise resulting in a loss of fat mass, also reduces LDL aggregation susceptibility in South Asians.

Besides LDL aggregation, LDL oxidation is also believed to trigger atherogenesis, and oxidation of LDL particles can be prevented by HDL (58). This raises the, currently unanswered, question whether an impaired HDL functionality could also contribute to the increased LDL aggregation that we observed in South Asians in **chapter 4**. Intriguingly, we indeed previously showed that the anti-oxidative capacity of HDL is lower in middle-aged South Asians compared with white Caucasians, a feature which was gained throughout ageing as this was not yet present in neonates or adolescents (59). HDL is a multifunctional lipoprotein that is also believed to exert anti-atherogenic effects via reducing inflammation. More specifically, HDL prevents expression of cytokine-induced adhesion molecules on endothelial cells and monocytes, including vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1), thereby reducing monocyte recruitment and vessel wall infiltration (60, 61). Interestingly, we indeed previously observed that TNF-α-induced VCAM-1 expression was lower in South Asian neonates compared with white Caucasians, an observation lost in young and middle-aged subjects (59). In addition, we observed higher levels of the adhesion molecule E-selectin, a marker for endothelial activation, in cord blood of South Asian neonates compared with white Caucasians (62). These data suggest that the possibly impaired anti-inflammatory function of HDL observed upon birth in South Asians restores throughout life, but it might then at a young age have already contributed to the basis for a pro-atherogenic endothelial environment. Endothelial activation is characterized by impaired endothelium-dependent vasodilatation, which is mediated by the vasodilatory agent nitric oxide (NO) (60, 61), a feature present in patients with CVD (63, 64). Also in South Asians there is evidence supporting reduced endothelium-dependent vasodilatation compared with white Caucasians (65, 66). Since

NO is produced by the endothelium upon interaction with HDL, dysfunctional HDL in South Asians might impair NO production and subsequent vasodilatation, and thereby contribute to a pro-atherogenic environment (reviewed in (67)). Another major function of HDL is the reverse transport of cholesterol; *i.e.* the efflux of cholesterol from foam cells residing within the vessel wall back to the liver. To date only one study assessed the cholesterol efflux capacity of HDL between South Asians and white Caucasians, but observed no differences herein (59). Altogether, dysfunctional HDL in South Asians may promote oxidative modification of LDL particles and induce endothelial dysfunction, thereby possibly contributing to the increased risk of atherosclerotic CVD in this population. The non-classical mechanisms contributing to CVD in South Asians, partly revealed by the studies described in this thesis and summarized in this paragraph, are depicted in **Figure 1**.

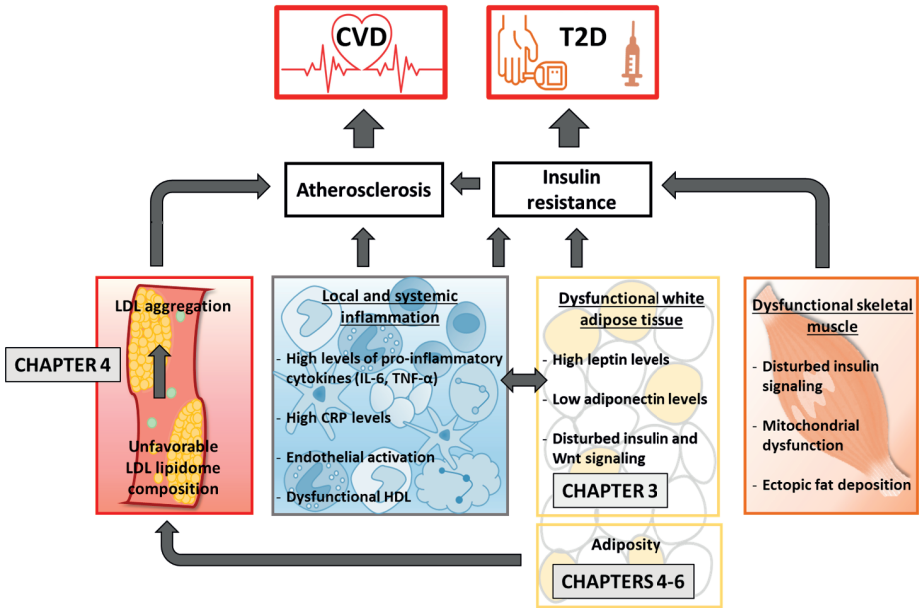


Figure 1. Non-classical mechanisms contributing to type 2 diabetes (T2D) and cardiovascular disease (CVD) in South Asians

2. PHARMACOLOGICAL ACTIVATION OF BAT TO COMBAT CARDIOMETABOLIC DISEASE IN SOUTH ASIANS

Cold exposure stimulates thermogenesis by BAT in order to maintain core body temperature. To generate heat, upon stimulation BAT increases combustion of its intracel-

lular lipids, thereby enhancing energy expenditure and increasing uptake of fatty acids and glucose from the circulation to restock its fuel. Targeting BAT is therefore regarded a potential promising treatment strategy to reduce adiposity, dyslipidemia and insulin resistance. However, as cold exposure is often uncomfortable and probably difficult to comply to on the long term, many researchers focus on identifying pharmacological agents that stimulate BAT activity or enhance 'browning' of WAT. Pharmacological agents that stimulate BAT thermogenesis can be crudely divided into two main categories; those directly activating receptors on the brown adipocyte, and those that enhance the sympathetic outflow towards BAT. In parallel to studies investigating pharmacological agents to stimulate BAT thermogenesis, the complex modulations of tissue-specific and whole-body energy metabolism and nutrient utilization induced by cold exposure are still being unraveled step by step. Such studies are however essential to grasp the complex physiology of BAT and may reveal insights important for the clinical utility of novel treatment strategies aiming to stimulate BAT thermogenesis and thereby improve cardiometabolic health.

Lipid trafficking between metabolic tissues during BAT activation

As described in **chapter 1**, stimulated BAT mainly takes up fatty acids from the circulation via lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride-rich lipoproteins (TRLs) (68). LPL, which is expressed by the brown adipocytes and exposed to the blood by binding to endothelial cells, is also involved in the uptake of TRL-derived fatty acids by other metabolic tissues, including WAT, skeletal muscle and the heart. As energy demands can quickly change (*e.g.* in a fasting *vs* fed state, during exercise *vs* rest and under cold *vs* warm conditions), the tissue-specific clearance of fatty acids is regulated by a variety of proteins, including the LPL-inhibitory ANGPTLs (69). During the past years it has become clear that there are various ANGPTLs, aside from the well-investigated ANGPTL4, that act in concert to inhibit tissue-specific LPL activity, including the novel members ANGPTL3 and ANGPTL8. On the one hand, overexpression of either of these ANGPTLs in mice markedly inhibits LPL activity and subsequently impairs hydrolysis of circulating TRLs, thereby resulting in hypertriglyceridemia (70-75). On the other hand, deficiency of either of these ANGPTLs in mice results in lower triglyceride levels (70-75). Moreover, loss-of-function mutations in either of these ANGPTLs are associated with lower triglyceride levels in humans (76-79) and inactivating variants of ANGPTL4 are even associated with reduced cardiovascular risk (77). A better understanding of the effect of cold exposure on the various lipid species could reveal further insight into the key role of this well-orchestrated family of ANGPTLs in lipid trafficking during BAT activation.

As a general rule, during periods of an increased energy demand (*e.g.* fasting, exercise and cold) LPL is mainly active in oxidative tissues to take up fatty acids as fuel. In contrary, during periods of energy overload, LPL is mainly active in WAT to take up

fatty acids for storage in the form of triglycerides. During increased energy demands, ANGPTL4 inhibits LPL activity specifically in WAT, thereby shuttling TRLs away towards the energy-demanding oxidative tissues for fatty acid uptake. This was elegantly demonstrated by cold exposing mice. In WAT, cold exposure increases *Angptl4* expression and reduces triglyceride-derived fatty acid uptake, whereas in BAT *Angptl4* expression decreases and triglyceride-derived fatty acid uptake increases (80, 81). ANGPTL3 and ANGPTL8 function together in a protein-protein complex (73) and seem to function opposite from ANGPTL4 in modulating tissue-specific lipid uptake. During periods of nutrient excess (*i.e.* under fed conditions), ANGPTL3 and ANGPTL8 inhibit LPL activity and triglyceride-derived fatty acid uptake by oxidative tissues (BAT, skeletal muscle and heart), thereby promoting lipid storage in WAT (82). Aside from two mouse studies curiously showing increased expression of *Angptl8* in liver, BAT and WAT upon cold exposure (81, 83), no other studies so far have reported on the effect of cold exposure on either ANGPTL3 or ANGPTL8.

To this end, in **chapter 2** we evaluated the effect of cold exposure on human plasma ANGPTL3 and ANGPTL8 levels, in addition to ANGPTL4. We previously showed that cold exposure increases circulating ANGPTL4 levels (84) in the same cohort of healthy lean men that we included in our study described in **chapter 2**, an observation that we now validated. ANGPTL4 is mainly expressed in WAT and produced in response to an increase in intracellular fatty acids. We therefore propose that in our study, cold-induced lipolysis in WAT increases the release of intracellular triglyceride-derived free fatty acids, resulting in upregulated ANGPTL4 expression. Increased ANGPTL4 subsequently limits the LPL-dependent triglyceride-derived fatty acid uptake by WAT as a negative feedback mechanism, thereby shuttling TRLs towards heat-producing BAT for uptake of fatty acids (85). In our study in **chapter 2** we observed an increase not only in plasma ANGTPL4 levels, but also in ANGPTL3 and ANGPTL8 levels. Since ANGPTL3 and ANGPTL8 were previously established to inhibit LPL activity and subsequent TRL-derived fatty acid uptake in oxidative tissues upon feeding, we propose that this also occurs during cold exposure. Via this way, the increase in ANGPTL3 and ANGPTL8 may counteract a potentially toxic fatty acid overload in oxidative tissues induced by the increased ANGPTL4 levels . Notably, this increase in ANGPTL3 and ANGPTL8 levels only occurred in young healthy lean subjects and not in older overweight prediabetic subjects. This might be due to impaired glucose uptake by insulin resistant BAT in the older subjects, as it was previously demonstrated that BAT indeed is very sensitive to become insulin resistant (86). We suggest that the unchanged ANGPTL3 and ANGPTL8 levels in older overweight prediabetic subjects during cold exposure might reflect an attempt to overcome a reduced glucose uptake by insulin resistant BAT, via promoting TRL-derived fatty acid uptake by BAT. It remains to be studied whether these cold-induced changes in circulating levels of the various ANGPTLs also correspond with their tissue-specific LPL-inhibitory function and

tissue-specific lipid uptake, although this is challenging since collecting BAT biopsies in humans is difficult for ethical and practical reasons.

Considering the overt effects of overexpression, deficiency or genetic defects in ANGPTLs on lipid levels, pharmacologically neutralizing the LPL-inhibitory action of either of the ANGPTLs has been investigated as a potential lipid-lowering therapy. So far, monoclonal antibodies against both ANGPTL4 (77) and ANGPTL8 (70, 87) resulted in an improved lipid profile in preclinical studies. Moreover, a monoclonal antibody (Evinacumab) and antisense oligonucleotide targeting ANGPTL3 successfully improved the lipid profile in subjects with dyslipidemia in multiple phase I trials (76, 88, 89) and even reduced atherosclerosis in mice (76, 88). A successful translation may be more difficult for the ANGPTL4 neutralizing monoclonal antibody, as preclinical studies unfortunately showed lipotoxic effects of this antibody upon a high-fat diet. More specifically, feeding a high-fat diet to mice or primates receiving a neutralizing ANGPTL4 monoclonal antibody resulted in pathological accumulation of lipids in the lymphatic system (77, 90). Moreover, mice genetically deficient for ANGPTL4 eventually developed a lethal phenotype (90, 91). A decade ago, Lichtenstein et al. (91) already showed the disastrous phenotype in these mice, characterized by peritonitis, intestinal fibrosis and cachexia, which was preceded by a tremendous acute phase-response upon the intake of saturated fats. Before ANGPTL4-neutralizing pharmacological agents can make a clinical translation, it needs to be investigated whether ANGPTL4 also protects against the inflammatory effects of saturated fats in humans, and tissue-specific downregulation of ANGPTL4 expression may be necessary to prevent such a dramatic immune response. Promising, no abdominal lymphatic disorders were observed in a limited amount of subjects with an inactivating *ANGPTL4* mutation (77).

In our study in **chapter 2** we did not observe overt and consistent differences between South Asians and white Caucasians in cold-induced changes in ANGPTL3, ANGPTL4 or ANGPTL8 levels, aside from an absent increase in ANGPTL3 in the middle-aged overweight prediabetic South Asians. We did observe a lower cold-induced increase in free fatty acids, which derive from WAT, in South Asians compared with white Caucasians in our studies in both **chapters 2** and **5**. Since cold exposure induces WAT lipolysis via stimulation of the sympathetic nervous system, we hypothesize that South Asians have a lower sympathetic tone compared with white Caucasians upon cold exposure. Increasing WAT lipolysis to promote the liberation of free fatty acids for uptake for combustion by oxidative tissues, such as BAT and skeletal muscle, may therefore be particularly useful to enhance lipid utilization and energy expenditure in South Asians. Combining this therapeutic strategy with neutralization of ANGPTLs that inhibit LPL activity mainly in oxidative tissues, thereby promoting lipid clearance by energy-combusting thermogenic tissues such as BAT, could improve lipid levels particularly efficiently. Based

on the currently available data and its translational success, ANGPTL3 seems the most promising candidate to date.

Activation of BAT through sympathomimetics and improving sympathetic outflow

Cold exposure stimulates the release of noradrenalin from sympathetic nerve endings, which binds to beta-adrenergic receptors (β-ARs) on brown adipocytes and activates an intracellular signaling cascade leading to thermogenesis. In addition to the classical ARs α_1, α_2, β_1 and β_2, in the 1980s the presence of another AR in humans was shown; the β_3-AR (92). Whereas the β_1-AR and β_2-AR are mainly present on the cardiovascular system, the β_3-AR mainly increased thermogenesis and reduced fat mass, at least in rodents. Much effort was therefore put into the development of β_3-AR agonists to mimic these beneficial metabolic effects in humans, unfortunately but without much success (93). Since it however became apparent a decade ago that adult humans still have sufficient amounts of BAT that can be stimulated via cold exposure, pharmacologically targeting the β_3-AR receptor to improve cardiometabolic health regained interest. A previous study from our department showed that β_3-AR agonism in mice increases TRL-derived fatty acid uptake by BAT with subsequently increased clearance of cholesterol-enriched remnants by the liver, resulting in lower circulating triglyceride and cholesterol levels and even attenuated progression of atherosclerosis (94). In addition, in that same year a key paper from Cypess et al. (95) showed that the β_3-AR agonist mirabegron indeed increases resting energy expenditure and [^{18}F]FDG uptake by BAT in healthy young men, at that time suggesting that β_3-AR agonism activates human BAT.

In **chapter 5**, we aimed to more thoroughly investigate the metabolic effects of mirabegron in humans, by assessing its effects on BAT activity measured by MRI and changes in specific lipid species, and to compare this between South Asians and white Caucasians. We firstly confirmed an increase in resting energy expenditure and lipid oxidation after administration of mirabegron. We then assessed the effect of mirabegron on BAT activity by measuring changes in BAT fat fraction, *i.e.* its amount of fat versus fat+water. It has repeatedly been shown that acute cold exposure lowers the fat fraction of the supraclavicular adipose tissue depot (96-100), likely due to intracellular lipid combustion preceding replenishment of BAT lipid storage pools. In our study, mirabegron indeed appeared to lower BAT fat fraction as measured via MRI and increased the supraclavicular skin temperature, suggestive of more lipid-combusting heat-producing BAT. We also hypothesized that in South Asians the effect of mirabegron on BAT activity would be more pronounced compared with that of cold exposure, by circumventing the sympathetic nervous system. We proposed this based on our previous data showing a lower cold-induced increase in plasma free fatty acids in South Asians (101), suggestive of less sympathetically-driven WAT lipolysis which could represent a lower sympathetic

outflow, an observation that we corroborated in our study in **chapter 5**. The effects of mirabegron and cold exposure on BAT parameters were however largely comparable between ethnicities, underlining the need for further research investigating whether sympathetic tone is truly lower in South Asians.

Although we (**chapter 5**) and others (95, 102, 103) have shown that a single dose of 200 mg mirabegron increases both energy expenditure, plasma free fatty acids and [^{18}F]FDG uptake in healthy subjects, this was hardly the case for a dose of 50 mg (102). Important to note is that 50 mg per day is the dose approved by the FDA and EMA to treat patients with overactive bladder disease. Furthermore, the supratherapeutic dose of 200 mg mirabegron increases heart rate and systolic blood pressure, of which we confirmed the former in our study described in **chapter 5**, whereas these effects are minimal with the dose of 50 mg (104, 105). This points towards off-target binding of mirabegron to the β_1-AR, which is expressed in amongst others the heart. A collaborative study in which our group participated recently investigated these dose-dependent and β-AR-specific effects of mirabegron on energy metabolism within a single study set-up (106). This study showed that mirabegron only increased whole-body lipolysis and fatty acid oxidation at the 200 mg dose. When further investigating receptor-specific actions of mirabegron *in vitro*, we observed that the stimulating effects of noradrenalin and mirabegron on intracellular lipolysis of human brown adipocytes were completely abolished by a specific β_2-AR antagonist. In line with this, knocking down the β_2-AR, but not the β_1-AR or β_3-AR, significantly suppressed cellular respiration in human brown adipocytes. In fact, intracellular lipolysis and *UCP1* expression were potently increased by the specific β_2-AR agonist formoterol. These novel data highlight an essential role for β_2-AR signaling rather than β_3-AR signaling in human BAT. It would therefore be highly relevant to investigate the potential of a truly β_2-AR-specific agonist on human BAT functionality, in the search for a specific BAT-activating pharmacological agent without unwanted cardiovascular side-effects. Strikingly, a recent study successfully demonstrated the capacity of a sympathicomimetic drug that signals via the peripheral β_2-AR, as its central effects were blocked, to enhance lipolysis and heat generation by BAT in mice, protecting them against diet-induced obesity (107). It would be very interesting to assess whether such a compound can make a successful clinical translation and improve cardiometabolic health by stimulating BAT thermogenesis without unwanted cardiac side-effects in humans. In addition, as the β_2-AR is expressed in various immune cells and noradrenergic signaling via the β_2-AR affects the inflammatory response (108), agonism for this receptor may have additional favorable effects on energy metabolism via affecting the immune system.

Activity of the sympathetic nervous system could also be modified by targeting the endocannabinoid system. The endocannabinoid system is comprised of lipid-derived ligands that bind to cannabinoid receptors types 1 (CB1R) and 2 (CB2R), which

are present in both the brain and peripheral tissues. The endocannabinoid system is linked to maintaining energy balance, via affecting amongst others appetite as well as energy expenditure, and endocannabinoid levels are increased in obesity (109, 110). Interestingly, endocannabinoids are proposed to negatively regulate BAT activity by dampening noradrenalin signaling (111). Since we previously observed higher circulating endocannabinoid levels in South Asians compared with white Caucasians (112), we speculate that an upregulated endocannabinoid system in South Asians might impair their sympathetic activity and thereby lower their BAT activity. In line with this, pharmacologically antagonizing the CB1R with the compound rimonabant increases glucose uptake and lipolysis in a human brown adipocyte cell line, and [^{18}F]FDG uptake as well as TG-derived free fatty acids by BAT in mice (113, 114). Moreover, clinical trials showed that rimonabant effectively reduces body weight and improves lipid profile in subjects with overweight or obesity, and improves glycemic control in subjects with T2D (115). Due to serious psychiatric side-effects, rimonabant was however withdrawn from the European market and has never been approved by the FDA (116). These serious psychiatric side-effects are based on the central effects of rimonabant due to crossing the blood-brain-barrier. Blocking of the CB1R specifically in peripheral organs, such as adipose tissue, may overcome these side-effects (114) and in my opinion still yields potential to improve whole-body energy metabolism. As we observed increased endocannabinoid levels in South Asians, targeting the endocannabinoid system to improve cardiometabolic health could be of specific interest for South Asians and remains an interesting topic of future studies.

Activation of BAT through mimics of incretin hormones

Whereas the aforementioned sympathomimetics and CB1R antagonists are currently not in use in clinical practice to treat obesity or its related cardiometabolic diseases, incretin-based therapies have been successful in improving glucose regulation, lowering body weight and reducing plasma lipid levels for a number of years. Moreover, a meta-analysis, including 4 major trials with a total of over 30,000 patients, showed an overall reduction in CVD mortality of 13% in subjects with T2D using GLP-1 receptor agonists (117). GLP-1 receptor agonists mimic the insulinotropic effects of incretin hormones. Upon food intake, the incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 are produced by K-cells and L-cells of the intestine, respectively, and stimulate postprandial insulin release from pancreatic β-cells to prevent hyperglycemia. Incretin hormones lose their insulinotropic effects in T2D, which can be partly restored upon administration of synthetic GLP-1 (118). Therefore, agonists of the GLP-1 receptor and inhibitors of the GLP-1-degrading enzyme dipeptidyl peptidase-4 (DPP-IV) have been successfully developed and used as co-treatment with other antidiabetics to improve hyperglycemia in T2D. In contrary to the anabolic effects of insulin,

incretin-based therapies have the benefit of not promoting weight gain, with DPP-IV inhibitors being considered weight-neutral (119) and GLP-1 receptor agonists even lowering body weight (120, 121). This is especially useful for the large population of subjects with T2D who mostly have either overweight or obesity, and may even partly counteract the weight-increasing effect of exogenous insulin in case of insulin co-treatment. The weight-reducing effect of GLP-1 receptor-targeted therapy occurs at least in part by lowering food intake via a combination of reducing appetite, increasing satiety and delaying gastric emptying (122). Reduced food intake however only partially explains the body weight loss observed with these drugs. This was demonstrated by an abolished weight gain by DPP-IV inhibitors in mice on a high-fed diet without affecting food intake (123). In line with this, central administration of a GLP-1 receptor agonist reduced body weight in lean mice and diet-induced obese mice to a larger extent compared with mice that were pair-fed to these mice receiving the GLP-1 receptor agonist (124, 125). Furthermore, GLP-1 receptor agonists lower CVD risk in clinical practice by improving glucose regulation, lowering body weight and reducing plasma lipid levels, and have additional beneficial effects on blood pressure, endothelial function, inflammation and cardiac function under ischemic conditions (126). These data further support the involvement of a broad range of underlying mechanisms involving various organ systems in the beneficial cardiometabolic effects of GLP-1 receptor-targeted therapy.

In line with these extensive favorable effects of incretin therapy, preclinical evidence showed that DPP-IV inhibitors increase BAT activity (123, 127) and promote browning of WAT (127). Moreover, we previously showed that the DPP-IV inhibitor sitagliptin increases [^{18}F]FDG uptake by WAT in overweight prediabetic subjects, which may suggest browning of WAT (128). Furthermore, we (124) and others (125) have previously shown in preclinical studies that central GLP-1 receptor agonism with liraglutide or exendin-4 increases sympathetic outflow towards BAT and lipid and glucose clearance by BAT. This made us hypothesize that BAT contributes to the elaborate beneficial cardiometabolic effects observed with GLP-1 receptor-targeted therapy in humans, which we investigated in a proof-of-principle study in **chapter 6**. Here, we treated subjects without obesity or diabetes for 12 weeks with Bydureon, a long-acting formula of exenatide. Notably, we observed an increase in the metabolic volume of BAT and standardized uptake value measured by cold-induced [^{18}F]FDG-PET/CT scan, indicating more glucose uptake by BAT. We therefore propose that more active BAT contributes to the negative energy balance induced by GLP-1 receptor agonists. It should however be noted that in our study, the increased uptake of [^{18}F]FDG by BAT did not coincide with higher resting energy expenditure or substrate oxidation rates. This might be due to the method of indirect calorimetry that we used, which only calculates oxygen-dependent energy metabolism, and/or possibly relate to the measurement error of this method. We can also not exclude that an increase in energy expenditure after exenatide is too subtle to measure with this

method, as the contribution of BAT to energy expenditure in humans is modest, with an estimated 100-250 kcal/day during maximal BAT activation in healthy adult males (129, 130).

In our study in **chapter 6** we also aimed to investigate whether the effect of GLP-1 receptor agonism on BAT was different between South Asians and white Caucasians. We hypothesized that GLP-1 receptor agonism yields more cardiometabolic benefit by stimulating BAT thermogenesis in South Asians, since we previously showed that healthy young South Asians have less BAT and a lower resting energy expenditure compared with white Caucasians (101). It should however be noted that the few other studies investigating this topic did not confirm less BAT in either healthy young (131) or overweight prediabetic older South Asian subjects (29). Furthermore, since South Asians are generally more insulin resistant compared with white Caucasians, the insulin-sensitizing effects of GLP-1 receptor agonism, also on BAT, may be larger in South Asians. There is indeed evidence pointing towards a more pronounced HbA1c-lowering effect of DPP-IV inhibitors and GLP-1 receptor agonists in Asians (132, 133). Most of these studies however concern East Asians (subjects from *e.g.* Japan, South Korea and China), in whom the primary underlying mechanism of T2D is an insulin secretion defect, making extrapolation to the primarily insulin resistant South Asians not possible. In our study in **chapter 6** we generally observed comparable effects of GLP-1 receptor agonism on parameters for BAT activity and other cardiometabolic outcomes between ethnicities. As we only included healthy young subjects in this study, we cannot exclude that the beneficial effect of GLP-1 receptor signaling on metabolism perhaps becomes more pronounced in South Asians with deteriorating glycemic control during ageing and/or increased adiposity.

Interestingly, the beneficial effects of GLP-1 receptor agonists are highly heterogenous, with approximately one quarter of subjects being non-responders (134). Therefore, there is much interest in combining GLP-1 receptor-targeted therapy with targeting other related receptors to possibly further improve its cardiometabolic effects. In particular, combining GLP-1 receptor agonism with GIP receptor agonism by using a dual agonist targeting both receptors, resulted in a dose-dependent substantially higher body weight loss compared with GLP-1 receptor agonism alone, as well as an improved glucose regulation and lowering in lipid levels in patients with T2D (135). However, in contrast to these clinical beneficial metabolic effects of combining GIP with GLP-1 receptor agonism, systemic disruption of GIP action in mice and monkeys also improves metabolism by decreasing high-fat diet-induced weight gain and increasing fat oxidation and energy expenditure (136-138). Due to these controversial varying effects of GIP signaling on energy metabolism, both GIP receptor agonists and antagonists are currently being investigated as treatment strategies to improve body weight and nutrient metabolism. Interestingly, a recent study pointed towards a role for BAT in the

effects of GIP signaling on whole-body metabolism. This study showed that during cold exposure, rectal temperature, *i.e.* as a proxy for core temperature, dropped less in mice with systemic loss of GIP receptor signaling compared with wild-type mice. In addition, oxygen consumption after a β-adrenergic stimulus was higher in these mice compared with wild-type mice (139). Moreover, this lesser drop in rectal temperature remained preserved when the GIP receptor was specifically knocked out in BAT. However, neither at room temperature nor under chronic cold conditions did read outs evidently show increased BAT thermogenesis or an improved whole-body metabolism. Additional dedicated experiments investigating the effects of GIP signaling and its interaction with central GLP-1 signaling on BAT functionality and whole-body metabolism are necessary to further understand the underlying mechanisms of the beneficial effects of prolonged GIP/GLP-1 receptor co-agonism in humans, and to distinguish its central from peripheral effects. The possibility of GIP(/GLP-1) receptor (co-)agonism to further improve weight regulation and whole-body energy metabolism during profound BAT activation, *e.g.* during cold exposure or β-AR agonism, may also yield potential and remains to be explored. Possibly, targeting incretin receptor signaling in a tissue-specific manner will optimize its beneficial effects on body weight and energy metabolism.

Activation of BAT by altering the gut microbiome

As described above, the GLP-1 receptor can be directly targeted via GLP-1 receptor agonism, and endogenous GLP-1 levels can be increased by preventing its DPP-IV-mediated breakdown. In addition, endogenous GLP-1 production is increased via administering short-chain fatty acids (140). Short-chain fatty acids, of which acetate, propionate and butyrate are the most common, are formed after fermentation of indigestible dietary fibers by gut bacteria. There has been much interest to alter whole-body energy metabolism by modulating the gut microbiome, and short-chain fatty acids have been proposed to play a role in these effects of the microbiome on energy and nutrient metabolism (141). Notably, a study from our group showed that oral administration of butyrate prevented diet-induced obesity, hyperinsulinemia, hyperlipidemia and hepatic steatosis in mice. This was largely attributed to a lowering of food intake, and additionally to increased BAT activity via enhanced sympathetic outflow, for which an intact gut-brain axis proved to be essential (142). This study elegantly showed that targeting the gut microbiome by dietary supplementation of short-chain fatty acids could be a promising treatment strategy to improve metabolic health. In addition, recent research showed that butyrate treatment of mice fed a high-fat diet indeed reduces plasma glucose and lowers the glucose excursion during a glucose tolerance test (Li Z. et al, unpublished). To date, only one small study investigated the role of butyrate in human metabolism. In this study, 4 weeks oral butyrate administration was beneficial for glucose regulation, however only in lean subjects and not in subjects with characteristics of the metabolic syndrome,

without affecting [^{18}F]FDG uptake by BAT in any of these groups (143). Validation in larger metabolically challenged study populations with different short-chain fatty acid dosages and treatment duration is warranted, as well as visualization of BAT nutrient combustion and thermogenic capacity via other methods as described below in this chapter. Alternatively, and probably more feasible, since butyrate is the fermentation product of dietary fiber, adapting the daily food intake towards increasing fiber intake will beneficially change cardiometabolic health. Indeed, a diet rich in dietary fiber promoted butyrate production in the gut, increased GLP-1 release and alleviated T2D in humans (144). The value of dietary fiber for the population at large to reduce T2D, CVD and even all-cause mortality is underscored by a recent series of systematic reviews and meta-analyses (145).

These findings may be of specific relevance for South Asians, as their diet is traditionally relative poor in fiber. One study compared the gut microbiome composition between one-year old South Asian and white Caucasian infants in Canada via analyzing stool samples, and showed amongst others lesser numbers of anaerobic Gram-negative bacteria from the *Clostridium* group, belonging to the cluster of *Firmicutes*, which are involved in the endogenous production of butyrate (146). It would be interesting to investigate how the gut microbiome composition develops throughout life in South Asians compared with white Caucasians. Moreover, it would be relevant to investigate whether a fiber-rich diet or administration of butyrate improves energy metabolism, possibly by stimulating nutrient utilization by activated BAT, specifically in South Asians.

Activation of BAT by targeting the inflammatory response

Another interesting compound that could prove to be beneficial for cardiometabolic health is salsalate, an anti-inflammatory drug from the class of salicylates. Salicylates have already long been used in clinical practice for their anti-inflammatory properties, and have also gained interest for the treatment of T2D during the past years. More specifically, salsalate lowers glucose and triglyceride levels in subjects both with and without T2D (147), and in mice even reduces non-alcoholic fatty liver disease (NAFLD) (148). Underlying mechanisms may include activation of AMP-activated protein kinase (AMPK) and inhibition of mechanistic target of rapamycin (mTOR), thereby lowering inflammation and lipid accumulation and promoting insulin availability. Furthermore, salicylates decrease the activity of nuclear factor kappa beta (NF-κβ), a transcription factor involved in the pro-inflammatory response. Salicylates also increase levels of adiponectin (147), an anti-inflammatory adipokine that was repeatedly shown to be lower in South Asians (38-41). A previous study from our department showed that in mice fed a high-fat diet, salsalate not only lowers weight accumulation, especially fat mass, and glucose and lipid levels, but also activates BAT (149). These data indicate that, at least in part, BAT activation is one of the underlying mechanisms involved in the beneficial

effects of salsalate on cardiometabolic health. Since South Asians generally also show a more pro-inflammatory phenotype, it would be especially interesting to investigate the effect of salsalate on BAT activity and cardiometabolic health in South Asians.

3. CHALLENGES IN METHODS TO QUANTIFY BAT ACTIVITY

[^{18}F]FDG-PET/CT SCAN TO ESTIMATE GLUCOSE UPTAKE BY BAT

The first studies showing the potential of cold exposure to increase BAT 'activity' used the imaging modality of [^{18}F]FDG-PET/CT (150-152), which we also applied in our studies in **chapters 2** and **6**. [^{18}F]FDG-PET/CT is mainly used in clinical practice in the field of oncology to diagnose, stage and assess therapy response of malignant tumors (153). This imaging modality measures tissue accumulation of FDG, a glucose analogue that is resistant to intracellular degradation, labeled with the radioactive isotope ^{18}F. [^{18}F]FDG is taken up by tissues via cellular membrane transporters similar to glucose, but in contrast to glucose it only enters the first step of glycolysis and then remains trapped within the cell as FDG-6-phosphate, whereafter [^{18}F]FDG-avid tissues can be visualized by means of PET scanning.

Although it is an elegant and readily available strategy to assess glucose uptake by tissues, the use of [^{18}F]FDG-PET/CT to assess BAT activity however has some important limitations. Firstly, measuring [^{18}F]FDG-uptake is a proxy for glucose uptake. During thermogenesis, BAT however takes up large quantities of triglyceride-derived fatty acids from the circulation to replenish its depleted lipid pools, whereas it takes up glucose – likely used mainly for the novo lipogenesis (154) – to a much lesser extent (155). Secondly, BAT is a highly insulin-sensitive organ, as shown by a 5-fold increase in [^{18}F] FDG uptake by BAT upon insulin administration in healthy subjects (156), rendering its glucose uptake dependent on an individual's insulin sensitivity. *Vice versa*, [^{18}F]FDG uptake by BAT reduces after fasting, *e.g.* when insulin levels are low (157). In line with this, [^{18}F]FDG uptake by BAT is impaired in subjects with T2D and subjects with increasing age (86). Interestingly, this latter study showed that in contrary to [^{18}F]FDG, the uptake of free fatty acids, traced using via [^{18}F]fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA), and BAT oxidative metabolism, traced by [^{11}C]acetate, were not reduced in these groups (86), indicating that BAT lipid metabolism and oxidative capacity are not impaired under insulin resistant conditions. These observations confirm the necessity of developing additional tracers to enable the quantification of lipid uptake by BAT and its thermogenic potential. As most circulating free fatty acids bind to albumin and are subsequently taken up by the liver, the distribution pattern of [^{18}F]FTHA also shows very high hepatic uptake, so far preventing its successful implementation to measure lipid uptake by BAT.

Since mouse studies from our department consistently show that BAT mainly takes up fatty acids derived from TRLs by LPL-mediated hydrolysis of triglycerides (68), which is in line with the crucial involvement of LPL in BAT activity (158), assessing the uptake of radiolabeled fatty acids derived from triglycerides residing within TRL(-like) particles will likely reflect lipid uptake by BAT more accurately. Developing such a tracer may be difficult, but if successful it likely represents a conceptual advance over the currently much used [^{18}F]FDG and more experimental [^{18}F]FTHA tracers to demonstrate and quantify metabolic BAT activity. It would also be of specific relevance to use such a tracer when evaluating the effect of compounds on BAT activity that also affect insulin sensitivity, for example the GLP-1 receptor agonist that we used in our study in **chapter 6**, albeit the uptake of TRL-derived fatty acids has been shown to be sensitive to the effects of insulin to a certain extent (159).

Lastly, an important general limitation of PET/CT is its accompanying radiation burden. There is a strictly regulated maximum allowed radiation burden for healthy volunteers and patients participating in clinical trials to protect against radiation-induced carcinogenesis. This maximum radiation dose is based on the estimated lifetime attributable cancer risk due to exposure to radioactivity, and is dependent on a combination of age, sex and the expected benefit gained by the study subject (160). For example, the maximum allowed radiation burden is lower with declining age, female sex, and when a study aims to gain knowledge not resulting in clinical health benefit for the individual per se *vs* when aiming to directly reduce serious illness. Due to this maximum radiation burden only a limited number of PET/CT scans can be performed in each study subject, thereby prohibiting the evaluation of longitudinal changes in BAT, which would be required either to assess physiological changes with time or effects of treatment. Strict legislation to protect study subjects against the potential carcinogenic effects of radiation is important from an ethical point of view, and therefore justified in my opinion. Nevertheless, this makes it problematic to perform studies aimed at gaining insight into underlying mechanisms of BAT activation that are important, essential even, for future progression in this field of research. In this respect, it is reassuring that BAT activation in mice results in an approximately 10-fold higher specific uptake of triglyceride-derived fatty acids than glucose when injected concomitantly (161). This can be partly explained by the fact that the brain requires a lot of glucose but not fatty acids from the circulation for its energy supply. If this also holds true for human BAT, the use of a triglyceride-derived fatty acid tracer may largely reduce radiation burden compared to [^{18}F]FDG and permit repeated PET/CT scans.

From a practical point of view, improvements can also be made in the methods applied for data acquisition and reconstruction with PET/CT, and subsequent analyses to calculate BAT volume and activity. Recommendations regarding many aspects of data generation, analyses and description, aiming to standardize [^{18}F]FDG-PET/CT imaging in

human studies, have been captured in a report by a panel of experts (162). We recognize this importance and have adopted these criteria in our study involving [^{18}F]FDG-PET/CT described in **chapter 6**. For example, variation in the thresholding applied to the PET signal intensity (standardized uptake value; SUV) and CT tissue radiodensity (Hounsfield Unit; HU) strongly affects the quantification of BAT-related outcomes. To tackle this issue, personalizing the SUV threshold to an individual's amount of lean body mass, instead of using a predefined value that assumes a similar ratio between fat and lean mass in all subjects, overcomes the issue of differences in tracer distribution between fat and lean tissues. Additionally, standardizing the HU threshold aids in preventing underestimation of BAT volume and activity by including all [^{18}F]FDG-avid BAT depots, and in preventing overestimation of these parameters by excluding [^{18}F]FDG uptake by non-BAT areas. To conclude, analyzing tracer uptake by BAT is challenging at least, and research investigating the role of BAT in cardiometabolic disease is in desperate need of additional imaging modalities that are preferably without radioactivity.

MRI TO MEASURE BAT LIPID CONTENT

MRI is a particularly promising radiation-free imaging modality able to assess the lipid content of adipose tissue, which we have applied for the classical supraclavicular BAT region in our studies in **chapters 5** and **6**. MRI estimates fat and water content based on radiofrequency pulses, and is able to distinguish BAT from WAT based on its lower fat fraction. However, since human BAT depots are heterogenous in terms of the types of adipocytes they contain (brown, beige and white), the delineation and separation of BAT and WAT depots is complex and these methods are currently still being optimized. Since BAT combusts intracellular lipids during non-shivering thermogenesis, activation of BAT at least acutely results in a lowering of its fat fraction. This has repeatedly been shown for the classical supraclavicular adipose tissue depot upon cold exposure (96-100), an observation that we confirmed in our study in **chapter 5**.

A major advantage of MRI compared with [^{18}F]FDG-PET/CT is that it can also distinguish BAT from WAT based on its lower fat fraction already at room temperature, while both BAT and WAT hardly take up [^{18}F]FDG at such temperatures (96, 99, 163-166). Furthermore, the fat fraction of the supraclavicular adipose tissue depot under thermoneutral as well as cold conditions negatively correlates with [^{18}F]FDG uptake on cold-induced PET/CT (165). This shows that subjects with the highest [^{18}F]FDG uptake by BAT have the lowest fat fraction, and *vice versa*, suggesting that MRI at thermoneutrality is able to identify subjects with the most 'active' BAT in terms of lipid combustion and uptake of glucose from the circulation. This should of course still be solidly confirmed in additional larger future studies, but could possibly lead to bypassing the need for an extra unpleasant cold exposure procedure when investigating BAT activity. In our study in **chapter 6** we did not observe an effect of prolonged treatment with the GLP-1 recep-

tor agonist exenatide on the fat fraction of the supraclavicular adipose tissue depot, whereas we did observe a significant increase in [^{18}F]FDG uptake by BAT measured by PET/CT. Nevertheless, in this study we also observed a negative correlation between the delta in fat fraction and [^{18}F]FDG uptake upon exenatide treatment, suggesting that subjects with the largest decrease in fat fraction in BAT also had the highest glucose uptake from the circulation by BAT. Perhaps the effect of exenatide on the fat fraction of the supraclavicular adipose tissue depot is too subtle to allow detection with MRI, as the decline in fat fraction generally measured after cold exposure, which is the most potent stimulus of BAT thermogenesis, is only in the order of a few percent (100). As we did not include a cold-induced MRI, we can also not exclude that the maximum potential of BAT, *i.e.* during the extra cold stimulus, might have been increased after exenatide treatment.

Importantly, the fat fraction of BAT is a net balance of the dynamic process of intracellular lipid combustion and replenishment of its lipid droplets by fatty acid and glucose uptake and lipogenesis. Therefore, true lipid combustion as a marker for thermogenic BAT activity may be difficult to capture in a static water-fat MRI scan. Other variants of MRI yielding potential to quantify BAT activity are currently widely investigated (reviewed in (167)). For example, blood-oxygen-level-dependent (BOLD) imaging is mainly used in functional MRI of the brain and is based on changes in blood flow. During metabolic activity, tissues extract oxygen from the blood, thereby decreasing oxygenated hemoglobin and increasing deoxygenated hemoglobin levels. Hereafter, blood flow towards such tissues is quickly restored, increasing oxygenated hemoglobin levels again and eliminating deoxyhemoglobin. As deoxyhemoglobin is accompanied by a lower MRI signal in terms of T2-star (T2*) relaxation time, dynamic changes in perfusion during BAT activation may firstly lower T2*, and upon restoration of blood flow towards BAT increase T2*. One small exploratory study in humans assessed the dynamic effect of cold exposure on BAT characteristics with T2*-weighted BOLD MRI, and indeed showed signal fluctuations coinciding with temperature adjustments and [^{18}F]FDG uptake measured by PET/CT (168). Such methods investigating dynamic changes in BAT activity seem promising and could provide further insight into BAT metabolism.

MEASUREMENTS OF SKIN TEMPERATURE

Since heat production reflects true BAT activity, and there is a desire to eliminate the radiation burden of methods quantifying BAT, the use of skin temperature rather than imaging as an indirect marker for BAT thermogenesis has also gained much interest in BAT research. Skin temperature can be measured either via infrared thermography (IRT), which generates images of temperature distribution by a camera a few meters in front of a subject that detects infrared electromagnetic radiation, or via iButtons, which are wireless data loggers that can easily be attached directly to the skin. Both these methods are also much cheaper and more practical to implement in a study set-up

compared with advanced imaging techniques. Such methods have shown that upon cold exposure, temperature in the supraclavicular area, *i.e.* overlying an important BAT depot, increased or remained stable, whilst it lowered in other body surface areas. We have also demonstrated this in our studies performed in **chapters 5** and **6** by using iButtons, in line with many others who have used iButtons or IRT (169-173). Moreover, this cold-induced change in supraclavicular skin temperature positively correlates with [^{18}F]FDG uptake on PET/CT (169, 170, 173). A disadvantage of using wireless data loggers as a proxy for supraclavicular BAT heat production is the subcutaneous WAT depot overlying this BAT area. This was indeed demonstrated by a study showing a negative correlation between subcutaneous fat thickness and supraclavicular skin temperature measured with IRT (174). This means that in obese subjects, with a relatively thick layer of subcutaneous WAT, BAT thermogenesis may be underestimated. This makes it difficult to compare temperature measurements between subjects with different body phenotypes. Another issue arises with skeletal muscle tissue and vasculature surrounding BAT, which also produces heat and could therefore bias the heat truly produced by BAT. In our study in **chapter 5**, we observed an increase in supraclavicular skin temperature after mirabegron, which coincides with an apparently lower fat fraction, suggestive of more active lipid-combusting BAT. Neither in **chapter 5** or **6**, did we observe differences in supraclavicular skin temperature between South Asians and white Caucasians. Altogether, 'simply' measuring skin temperature may be a practical and cheap alternative to estimate BAT thermogenesis compared with CT- and MR-based imaging methods. Nevertheless, it remains an indirect measure of BAT thermogenesis and it is difficult, if not impossible, to compare between subjects with a different body composition. This renders it questionable whether such techniques are of high value and have the potency to eliminate the use for imaging modalities in quantification of BAT activity.

4. CONCLUDING REMARKS

T2D and CVD are particularly common and progress rapidly in people from South Asian descent, which is likely explained for an important part by the presence of risk factors shared by these cardiometabolic diseases; especially (visceral) adiposity, glucose intolerance and dyslipidemia. In this thesis we firstly focused on further unraveling underlying mechanisms contributing to T2D and CVD in South Asians. We demonstrated that Wnt and insulin signaling are correlated and are likely impaired in WAT of South Asians, thereby potentially contributing to a disrupted glucose homeostasis. This could be due, at least in part, to higher circulating levels of the Wnt-inhibitor sclerostin in South Asians, and targeting sclerostin may therefore yield potential to improve glucose homeostasis in this ethnic group. We also showed that LDL is more prone to aggregate in South Asians,

which is probably related to alterations in LDL surface lipids and could contribute to the high risk of atherosclerotic CVD in this population. Modulating the LDL lipidome, for example by a healthy diet, could be a potential treatment strategy to reduce LDL aggregation capacity and thereby lower atherosclerotic CVD in South Asians.

Increasing energy expenditure and nutrient utilization by stimulating BAT thermogenesis has emerged as a potential treatment strategy to tackle obesity, T2D and CVD. In this thesis we confirmed that β-AR agonism improves cardiometabolic parameters in humans, and our data points towards more active BAT. Since recent evidence showed that it is the β_2-AR rather than the β_3-AR that is responsible for BAT activity in humans (106), and crosstalk with the β_1-AR results in unwanted cardiovascular (side-)effects, future studies specifically stimulating the β_2-AR in humans are essential. Incretin mimetics also yield potential to promote cardiometabolic health via stimulating BAT activity in humans. In this thesis we confirmed that GLP-1 receptor agonism improves cardiometabolic characteristics, and now also show that it increases [^{18}F]FDG uptake by BAT in humans. Combining agonism for receptors of the incretin hormones GIP and GLP-1, *i.e.* dual GIP/GLP-1 receptor agonism, is an even more promising treatment strategy, as this was shown to improve glucose regulation and weight loss to a larger extent than GLP-1 receptor agonism alone (135). Parallel to research focused on strategies to enhance BAT activity, studies aimed at improving the currently available imaging modalities to quantify BAT activity are essential to assess true BAT thermogenic capacity and nutrient clearance. Development of a tracer including radiolabeled fatty acids derived from triglycerides incorporated within TRL(-like) particles is difficult for many reasons, but may yield the most clinical potential.

To conclude, in my opinion unraveling the role of BAT in health and disease and assessing how to modulate BAT functionality to improve, or even prevent, cardiometabolic disease, remains a relevant topic for future research, and may be especially useful for populations prone to develop cardiometabolic disease such as South Asians. Whether a successful BAT-targeting treatment strategy can be developed with sufficient clinical potential to improve cardiometabolic health and without significant side-effects, remains to be elucidated in the forthcoming years. Such a strategy may then serve as a welcome add-on treatment for those subjects at high risk, *e.g.* South Asians, as a healthy lifestyle remains the foundation of improving cardiometabolic health.

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8

Summary

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SUMMARY

Cardiometabolic diseases such as type 2 diabetes (T2D) and cardiovascular diseases (CVD) put a major burden on the health care system, with CVD accounting for most annual deaths globally. People from South Asian descent are especially at risk to develop T2D and CVD. This may, at least in part, be due to their unfavorable body fat distribution with relatively large amounts of abdominal white adipose tissue (WAT) and ectopic fat deposition, contributing to insulin resistance and negatively affecting whole-body energy metabolism. Reducing the rates of obesity and its complications can be accomplished by shifting towards a negative energy balance. This can be achieved by lowering food intake, which is however challenging to maintain and on the long term most often results in weight regain. Parallel to lowering food intake, increasing energy expenditure can help to further induce and maintain weight loss. To this end, enhancing the thermogenic capacity of energy-combusting brown adipose tissue (BAT) is a promising novel treatment strategy. This could be especially useful in South Asians whom were previously shown to exhibit a lower BAT volume. In this thesis, we firstly investigated mechanisms underlying the unfavorable metabolic phenotype of South Asians. Secondly, we studied the potential of pharmacological agents to promote BAT activity and improve cardiometabolic health, in South Asian compared with white Caucasian men.

Chapter 1 serves as a general introduction into the South Asian heritage and their establishment in the Dutch society today. Here we describe the metabolic characteristics of South Asians and elaborate on pathophysiological aspects contributing to their high risk of T2D and CVD. We then continue to elaborate on the role of BAT in energy metabolism, and illustrate treatment strategies, either by implementing lifestyle changes or the use of pharmacological agents, that may improve cardiometabolic health by promoting BAT activity.

BAT generates heat by combusting fatty acids, which are stored within intracellular lipid droplets. To replenish these lipid pools, fatty acids are taken up from the circulation by lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride-rich lipoproteins (TRLs). LPL activity can be inhibited by a family of angiopoietin-like proteins (ANGPTLs). More specifically, it has been shown that cold exposure modulates ANGPTL4 expression and thus LPL activity in a tissue-specific manner. ANGPTL4 acts in concert with its family members ANGPTL3 and ANGPTL8 to orchestrate lipid distribution between different metabolic tissues under alternating energy demands. In **chapter 2**, we investigated the effect of cold exposure on plasma levels of ANGPTL3 and ANGPTL8, in addition to ANGPTL4, in South Asian and white Caucasian men. We confirmed that cold exposure increases ANGPTL4 levels both in young healthy lean men and middle-aged men with overweight and prediabetes. We now also show that cold exposure increases ANGPTL3 and ANGPTL8 levels, but only in young healthy lean men. We previously proposed that

during cold exposure, ANGPTL4 may function to redirect TRLs away from WAT towards active BAT and skeletal muscle for uptake of TRL-derived fatty acids to facilitate thermogenesis. We here suggest that as a counter response, the increase in ANGPTL3 and ANGPTL8 may reduce the interaction of TRLs with thermogenic tissues to prevent excessive lipid combustion and/or accumulation in these tissues. This response could be absent in middle-aged men with overweight and prediabetes in an attempt to overcome reduced glucose uptake by BAT resulting from insulin resistance.

The Wnt signaling pathway is involved in embryonic development and oncogenesis. Interestingly, impaired Wnt signaling is also associated with adiposity and T2D in humans, and *in vitro* studies have indeed confirmed that Wnt signaling interacts with insulin signaling. Since South Asians are prone to develop obesity and insulin resistance, in **chapter 3** we next investigated whether Wnt signaling is impaired in subcutaneous WAT and skeletal muscle biopsies in overweight prediabetic South Asian men compared with white Caucasian men. We showed markedly higher plasma levels of the Wnt-inhibitor sclerostin in South Asians compared with white Caucasians. In addition, expression of various genes involved in Wnt and insulin signaling was lower in WAT in South Asians, and expression of genes involved in Wnt and insulin signaling were strongly positively correlated. In skeletal muscle, only *WNT10B* expression was lower in South Asians compared with white Caucasians, and the positive correlation between Wnt and insulin signaling gene expression was less pronounced. We conclude that Wnt signaling may be lower in WAT of South Asians compared with white Caucasians, thereby possibly contributing to impaired insulin signaling and development of T2D in South Asians.

The excessive risk of CVD in South Asians cannot be fully explained by classical risk factors such as increased LDL-cholesterol levels. Interestingly, it was recently shown that the susceptibility of circulating LDL particles to aggregate *in vitro* is related to their lipid composition, and that the presence of such aggregation-prone LDL particles is associated with mortality from cardiovascular events in patients with CVD. In **chapter 4** we therefore assessed the aggregation susceptibility and lipid composition of LDL particles isolated from plasma of healthy South Asians compared with white Caucasians. LDL particles from South Asians were indeed considerably more prone to aggregate. In addition, body fat percentage, which was higher in South Asians, correlated positively with LDL aggregation. Furthermore, when investigating the LDL lipidome, we observed that body fat percentage positively correlated with LDL-sphingomyelins, especially sphingomyelin 24:0 which was also higher in South Asians. We conclude that LDL aggregation susceptibility is higher in South Asians compared with white Caucasians, which may be explained in part by their higher body fat percentage leading to enrichment of LDL particles with sphingomyelins. Presence of such aggregation-prone circulating LDL particles could contribute to the higher CVD risk in South Asians later in life.

We next switched our focus to studying the effect of two different pharmacological compounds on BAT metabolism in both South Asian and white Caucasian subjects. Firstly, in **chapter 5** we performed a randomized placebo-controlled trial in which we compared the effects of cold exposure and one dose of 200 mg of the beta-3-adrenergic receptor (β_3-AR) agonist mirabegron on BAT and markers of energy metabolism in healthy lean South Asian and white Caucasian men. In both ethnicities, cold exposure increased the skin temperature in the supraclavicular area, as a proxy for BAT thermogenesis, lowered the fat fraction of the supraclavicular adipose tissue depot, increased free fatty acid levels, and increased resting energy expenditure due to enhanced lipid oxidation. Mirabegron also increased the supraclavicular skin temperature in both ethnicities, and lowered the fat fraction of the supraclavicular adipose tissue depot after pooling data from all subjects. Furthermore, mirabegron increased free fatty acid levels in both ethnicities, and resting energy expenditure when pooling data from all subjects, due to an increase in lipid oxidation which was only significant in white Caucasians. Recently, it has been shown that the beneficial metabolic effects of mirabegron on BAT are mediated via β_2-AR signaling rather than β_3-AR signaling. Investigating the potential of a selective β_2-AR agonist to enhance BAT activity would therefore be very interesting, also in the South Asian population.

In addition to directly stimulating β-ARs on brown adipocytes, mimicking incretin hormones is a promising treatment strategy to indirectly stimulate BAT thermogenesis. More specifically, central agonism of the glucagon-like peptide-1 (GLP-1) receptor agonist activates BAT in mice, and GLP-1 receptor agonists lower body weight and improve glucose and lipid levels in patients with T2D. To investigate whether GLP-1 receptor agonism also activates BAT in humans, in **chapter 6** we determined the effect of 12 weeks administration of the GLP-1 receptor agonist exenatide on BAT and markers for energy metabolism in nondiabetic men, and compared this between South Asians and white Caucasians. Exenatide lowered body weight, without affecting energy expenditure or substrate oxidation rates, and lowered serum triglycerides and total cholesterol, as well as plasma glucose. Notably, exenatide increased the metabolic volume and standardized uptake value of BAT measured with [^{18}F]FDG-PET/CT scan, whereas the fat fraction of the supraclavicular adipose tissue depot measured with MRI remained unaltered. The overall effect of exenatide on these metabolic parameters was comparable between ethnicities. These results show that also in humans GLP-1 receptor agonism has multiple beneficial metabolic effects in addition to improving glucose levels, and that this may at least be partly mediated by enhanced glucose disposal by BAT.

Lastly, in **chapter 7** we placed the results of the studies performed in this thesis into the perspective of the available evidence described in the scientific literature. We described novel pathophysiological aspects contributing to T2D and CVD, with a focus on the South Asian population. We then shifted our focus towards BAT and firstly

described mechanisms involved in its lipid uptake. Thereafter, we expanded on the potential of pharmacological compounds that could improve cardiometabolic health in part by stimulating BAT thermogenesis. Finally, we briefly highlighted methodological challenges in the quantification of BAT functionality, and discussed methods that are currently being developed in an attempt to overcome these issues in future BAT research. We concluded that continuing to unravel underlying mechanisms contributing to the high risk of T2D and CVD in South Asians remains important in the search for novel treatment options to tackle these cardiometabolic diseases. Whether successfully stimulating BAT thermogenesis can be of significant aid in the treatment of cardiometabolic disease on the long term, remains to be elucidated in the forthcoming years.

In summary, the studies described in this thesis provide novel insight into the effect of cold exposure on the lipid-trafficking regulating ANGPTLs, underlying mechanisms contributing to cardiometabolic disease in South Asians in terms of Wnt signaling in metabolic tissues and the aggregation susceptibility as well as the lipidome composition of LDL, and the effect of the two promising pharmacological treatment strategies of β-AR agonism and GLP-1 receptor agonism on BAT activity and whole-body energy metabolism. As such, our studies contribute to further unraveling risk factors for cardiometabolic diseases and the development of novel therapeutic handles in strategies to combat these diseases, especially in the vulnerable South Asian population.

NEDERLANDSE SAMENVATTING

De behandeling van cardiometabole ziekten zoals type 2 diabetes (suikerziekte) en hart- en vaatziekten vormt wereldwijd een zware belasting voor zorgstelsels. Hart- en vaatziekten zijn wereldwijd verantwoordelijk voor de meeste sterfgevallen. Vergeleken met mensen van West-Europese afkomst hebben mensen van Zuid-Aziatische afkomst een hoog risico om type 2 diabetes en hart- en vaatziekten te ontwikkelen. Dit wordt voor een deel veroorzaakt doordat zij een ongunstige lichaamssamenstelling hebben, met relatief veel buikvet en vetopslag in organen die normaal gesproken geen vet opslaan, zoals bijvoorbeeld de lever. Deze overmatige hoeveelheden (wit) vetweefsel dragen bij aan de ontwikkeling van insulineongevoeligheid en hebben een negatieve invloed op de stofwisseling in het hele lichaam. Het verminderen van ernstig overgewicht (obesitas) en de daarmee gepaard gaande cardiometabole ziekten, zoals type 2 diabetes en hart- en vaatziekten, kan gerealiseerd worden door het bewerkstelligen van een zogenaamde negatieve energiebalans, waarbij het lichaam meer calorieën verbrandt dan opneemt. Het verminderen van voedselinname kan hier voor een belangrijk deel aan bijdragen. In de praktijk is dit echter lastig vol te houden en op de lange termijn leidt diëten meestal weer tot gewichtstoename: het jojo-effect. Naast het verminderen van de voedselinname kan ook het verhogen van het energieverbruik bijdragen aan gewichtsverlies. Een veelbelovende nieuwe aanpak om het energieverbruik te verhogen, is het stimuleren van de vet- en suikerverbranding door bruin vetweefsel. Dit kan met name interessant zijn voor de Zuid-Aziatische bevolking. Hierom hebben wij in dit proefschrift mechanismen onderzocht die aan de verminderde cardiometabole gezondheid van Zuid-Aziaten ten grondslag liggen. Daarna hebben wij de effectiviteit onderzocht van geneesmiddelen om de activiteit van bruin vetweefsel te verhogen en de cardiometabole gezondheid te verbeteren, waarbij deze effecten vergeleken werden tussen Zuid-Aziaten en West-Europeanen.

Hoofdstuk 1 van dit proefschrift vormt een algemene introductie in het migratiepatroon van Zuid-Aziaten (specifiek Hindostanen) vanuit India via Suriname naar Nederland. Hier beschrijf ik de metabole kenmerken van deze specifieke groep Zuid-Aziaten en licht ik onderliggende mechanismen toe die zouden kunnen bijdragen aan hun hogere risico op type 2 diabetes en hart- en vaatziekten vergeleken met West-Europeanen. Hierna beschrijf ik de rol van bruin vetweefsel in de energiebalans in het lichaam. Als laatste laat ik voorbeelden zien van leefstijlveranderingen en geneesmiddelen die de cardiometabole gezondheid kunnen verbeteren door de activiteit van bruin vetweefsel te stimuleren.

Bruin vetweefsel produceert warmte door het verbranden van vetzuren die opgeslagen liggen in vetdruppels in zijn vetcellen. Om deze vetdruppels weer van nieuwe vetzuren te voorzien, worden vetzuren opgenomen vanuit het bloed. Hiervoor verant-

woordelijk is het enzym lipoproteïnelipase (LPL), dat vetzuren vrijmaakt uit vetrijke deeltjes die in de bloedbaan circuleren; de zogenaamde triglyceridenrijke lipoproteïnen. De activiteit van LPL kan geremd worden door een familie van eiwitten die angiopoietin-like proteins (ANGPTLs) worden genoemd. Interessant is dat onderzoekers hebben aangetoond dat koudeblootstelling de expressie van ANGPTL4 kan beïnvloeden, wat leidt tot een weefselspecifieke modulatie van LPL. Later werd ontdekt dat ANGPTL4 samenwerkt met ANGPTL3 en ANGPTL8 om onder verschillende omstandigheden (zoals tijdens vasten en bewegen) vetten te verdelen tussen de organen die vet verbranden dan wel opslaan. In **hoofdstuk 2** onderzochten wij hierom het effect van koudeblootstelling op de bloedconcentraties van ANGPTL3 en ANGPTL8, naast ANGPTL4, in Zuid-Aziaten en West-Europeanen. Wij bevestigden dat koudeblootstelling de concentratie van ANGPTL4 in het bloed verhoogt in zowel jonge gezonde mannen als oudere mannen met overgewicht en prediabetes, een voorstadium van suikerziekte. Wij toonden nu ook aan dat koudeblootstelling de concentraties van ANGPTL3 en ANGPTL8 in het bloed verhoogt, maar alleen in jonge gezonde mannen. Eerder rapporteerden wij al dat ANGPTL4 er tijdens koudeblootstelling voor kan zorgen dat triglyceridenrijke lipoproteïnen hun vetzuren niet afstaan aan wit vetweefsel maar aan bruin vetweefsel en skeletspieren, zodat deze kunnen worden verbrand voor het produceren van warmte. Op basis van onze nieuwe onderzoeksgegevens stellen wij nu voor dat als een tegenrespons, de verhoging in ANGPTL3 en ANGPTL8 de interactie van triglyceridenrijke lipoproteïnen met bruin vetweefsel en skeletspier kan verminderen, om een overmatig verbruik door en/of ophoping van vet in deze weefsels te voorkomen. Mogelijk is deze tegenrespons afwezig in oudere mannen met overgewicht en prediabetes omdat hun bruin vetweefsel minder gevoelig is voor de insulineafhankelijke opname van suiker, en daardoor meer afhankelijk is van de opname van vetten uit het bloed.

De Wnt signaalroute is betrokken bij de embryonale ontwikkeling en tumorvorming. Daarnaast is verminderde Wnt signaaltransductie geassocieerd met overgewicht en type 2 diabetes, en hebben in vitro studies bevestigd dat Wnt signaaltransductie en insuline signaaltransductie met elkaar communiceren. Aangezien Zuid-Aziaten in de regel meer lichaamsvet hebben en minder insulinegevoelig zijn dan West-Europeanen, hebben wij in **hoofdstuk 3** onderzocht of de Wnt signaaltransductie verminderd is in biopten van onderhuids wit vetweefsel en skeletspieren van Zuid-Aziatische mannen met overgewicht en prediabetes in vergelijking met West-Europese mannen. Wij vonden in het bloed opvallend hogere niveaus van de Wnt-remmer sclerostin in Zuid-Aziaten in vergelijking met West-Europeanen. Daarnaast was de expressie van diverse genen die betrokken zijn bij Wnt signaaltransductie en insuline signaaltransductie lager in wit vetweefsel van Zuid-Aziaten, en was er in wit vetweefsel sprake van een sterke samenhang tussen expressie van genen betrokken bij Wnt signaaltransductie en insuline signaaltransductie. In de skeletspier was alleen de expressie van het Wnt signaaltransductiegen

WNT10B lager in Zuid-Aziaten in vergelijking met West-Europeanen, en was de samenhang tussen expressie van genen betrokken bij Wnt signaaltransductie en insuline signaaltransductie minder uitgesproken. Wij concludeerden dat Wnt signaaltransductie verlaagd kan zijn in wit vetweefsel in Zuid-Aziaten in vergelijking met West-Europeanen. Dit kan mogelijk bijdragen aan verminderde insuline signaaltransductie en daardoor de ontwikkeling van type 2 diabetes in deze bevolkingsgroep.

Het sterk verhoogde risico op hart- en vaatziekten van Zuid-Aziaten kan niet geheel worden verklaard door de aanwezigheid van klassieke risicofactoren zoals een hoog LDL-cholesterol ('slecht cholesterol') niveau in het bloed. Recentelijk is aangetoond dat de gevoeligheid van LDL deeltjes om samen te klonteren *in vitro* samenhangt met hun vetsamenstelling. Daarnaast is de aanwezigheid van zulke LDL deeltjes die geneigd zijn om samen te klonteren geassocieerd met sterfte in patiënten met hart- en vaatziekten. In **hoofdstuk 4** hebben wij hierom LDL deeltjes geïsoleerd uit het bloed van gezonde Zuid-Aziatische en West-Europese mannen en hebben wij hun gevoeligheid tot samenklonteren en vetsamenstelling vergeleken. LDL deeltjes van Zuid-Aziaten waren inderdaad aanzienlijk meer geneigd om samen te klonteren. De neiging van LDL deeltjes om samen te klonteren hing daarnaast samen met het lichaamsvetpercentage, wat hoger is in Zuid-Aziaten. Het lichaamsvetpercentage hing vooral samen met de sphingomyelinen in de LDL deeltjes, in het bijzonder met sphingomyeline 24:0 welke hoger was in Zuid-Aziaten in vergelijking met West-Europeanen. Wij concludeerden dat LDL deeltjes meer geneigd zijn om samen te klonteren in Zuid-Aziaten dan in West-Europeanen. Dit kan mogelijk voor een deel verklaard worden door het hogere lichaamsvetpercentage in Zuid-Aziaten, wat leidt tot verrijking van LDL deeltjes met sphingomyelinen. De aanwezigheid van zulke LDL deeltjes die makkelijk samenklonteren kan bijdragen aan het hogere risico op hart- en vaatziekten in Zuid-Aziaten in hun latere leven door het proces van slagaderverkalking te versnellen.

Hierna verlegden wij onze focus naar het effect van twee verschillende geneesmiddelen op de verbranding door bruin vetweefsel in zowel Zuid-Aziaten als West-Europeanen. Als eerste voerden wij hierom in **hoofdstuk 5** een gerandomiseerde placebo-gecontroleerde studie uit waarin wij de effecten onderzochten van koudeblootstelling en een eenmalige dosis van 200 mg mirabegron, dat aan ontvangers genaamd beta-adrenerge receptoren bindt die zich onder andere op bruine vetcellen bevinden, op maten voor de activiteit van bruin vetweefsel en de stofwisseling in gezonde slanke Zuid-Aziatische en West-Europese mannen. Koudeblootstelling verhoogde de temperatuur boven het sleutelbeen, een indirecte maat voor de warmteproductie door bruin vetweefsel, en verlaagde de hoeveelheid vet op die plek. Daarnaast verhoogde koudeblootstelling de bloedconcentratie van vrije vetzuren, en verhoogde het de ruststofwisseling door toename van de vetverbranding. Interessant was dat mirabegron ook de huidtemperatuur boven het sleutelbeen verhoogde, en ook de hoeveelheid vet

op die plek verlaagde nadat we de onderzoeksgegevens van alle studiedeelnemers samenvoegden. Daarnaast verhoogde mirabegron ook de vrije vetzuurniveaus in het bloed en de stofwisseling in rust na het samenvoegen van onderzoeksgegevens van alle studiedeelnemers. Recentelijk is het aangetoond dat de gunstige metabole effecten van mirabegron met name plaatsvinden door signaaltransductie via de beta-2-adrenerge receptor. Het is daarom interessant om de potentie van een selectieve beta-2-adrenerge receptoragonist om bruin vetweefsel te activeren te onderzoeken, vooral in mensen van Zuid-Aziatische afkomst.

Naast het stimuleren van beta-adrenerge receptoren op bruine vetcellen, is het nabootsen van de effecten van bepaalde darmhormonen die 'incretines' worden genoemd, een veelbelovende strategie om indirect via de hersenen de verbranding door bruin vetweefsel te stimuleren. Eerder hebben we namelijk aangetoond dat binding van het darmhormoon glucagon-like peptide-1 (GLP-1) aan een receptor in de hersenen het bruine vetweefsel in muizen activeert. Daarnaast is vastgesteld dat middelen die aan de GLP-1 receptor binden, zogenaamde 'GLP-1 receptor agonisten', in patiënten met type 2 diabetes zorgen voor gewichtsverlies en het verbeteren van de suiker- en vetniveaus in het bloed. In **hoofdstuk 6** onderzochten wij daarom het effect van 12 weken toediening van de GLP-1 receptoragonist exenatide op bruin vetweefsel en de stofwisseling in gezonde jonge Zuid-Aziatische en West-Europese mannen. Exenatide verlaagde het lichaamsgewicht, zonder significante veranderingen in de stofwisseling, en verlaagde de niveaus van vetten (triglyceriden en totaal cholesterol) en suiker in het bloed. Opvallend was dat exenatide de hoeveelheid suikeropname door bruin vetweefsel verhoogde, zoals gemeten met een zogenaamde [^{18}F]FDG PET-CT scan. Daarentegen bleef het vetpercentage van het vetdepot boven het sleutelbeen, gemeten met MRI, onveranderd. Het effect van exenatide op deze metabole uitkomsten was grotendeels vergelijkbaar tussen Zuid-Aziaten en West-Europeanen. Deze resultaten tonen aan dat GLP-1 receptoragonisme ook in mensen gunstige effecten heeft op het lichaamsgewicht en de vetten en suikers in het bloed, waaraan een toegenomen suikeropname en -verbranding door bruin vetweefsel mogelijk gedeeltelijk bijdraagt.

Als laatste hebben wij in **hoofdstuk 7** de resultaten van de uitgevoerde studies die in dit proefschrift beschreven zijn in het perspectief van de beschikbare wetenschappelijke literatuur geplaatst. Wij onderzochten onderliggende mechanismen die bijdragen aan de ontwikkeling en verergering van type 2 diabetes en hart- en vaatziekten, met name in mensen van Zuid-Aziatische (specifiek Hindostaanse) afkomst. Daarna richtten wij ons op de rol van bruin vetweefsel in deze ziektebeelden en beschreven wij mechanismen die betrokken zijn bij de opname van vet door bruin vetweefsel voor verbranding tot warmte. Hierna weidden wij uit over de mogelijkheid om met geneesmiddelen de cardiometabole gezondheid te verbeteren, mogelijk gedeeltelijk via het stimuleren van de verbranding door bruin vetweefsel. Als laatste beschreven wij beknopt de uitdagin-

gen in het vaststellen van de activiteit van bruin vetweefsel met de huidige beschikbare methoden, en bediscussieerden wij methoden die momenteel ontwikkeld worden om de verbranding door bruin vetweefsel nog beter in kaart te kunnen brengen. Wij concludeerden dat het verder blijven onderzoeken van onderliggende mechanismen die bijdragen aan het hoge risico op type 2 diabetes en hart- en vaatziekten, vooral in Zuid-Aziaten, belangrijk blijft in de zoektocht naar nieuwe behandelstrategieën in de aanpak van deze ziekten. Of het succesvol stimuleren van de verbranding door bruin vetweefsel kan bijdragen aan de behandeling van cardiometabole ziekten op de lange termijn, zal onderzoek in de komende jaren moeten gaan uitwijzen.

Samenvattend hebben de studies beschreven in dit proefschrift inzicht gegeven in 1) het effect van koudeblootstelling op de angiopoietin-like proteins die betrokken zijn bij de verdeling van vetzuren uit het bloed over de diverse organen in het lichaam, 2) onderliggende mechanismen die bijdragen aan cardiometabole ziekten in Hindostanen, zoals de Wnt signaaltransductie in metabole weefsels en de vetsamenstelling van LDL en de neiging van LDL deeltjes om samen te klonteren, en 3) het effect van de twee veelbelovende farmacologische methoden beta-adrenerg receptoragonisme en GLP-1 receptoragonisme op de activiteit van bruin vetweefsel en de vet- en suikerstofwisseling. Als zodanig hebben onze studies bijgedragen aan een beter begrip van risicofactoren en de ontwikkeling van mogelijk nieuwe therapeutische handvatten die gebruikt kunnen worden om cardiometabole ziekten tegen te gaan, vooral in de kwetsbare Zuid-Aziatische bevolkingsgroep.

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*shared authorship

CURRICULUM VITAE

Laura Gerarda Maria Janssen werd geboren op 25 juni 1991 te Brunssum, Limburg, waar zij opgroeide met haar ouders en zusje Linda. Zij behaalde haar VWO diploma in 2009 aan het Romboutscollege in Brunssum.

Hierna startte zij in 2009 met de bachelor Geneeskunde aan de Universiteit Leiden. Tijdens haar middelbare schooltijd en studie Geneeskunde had zij diverse bijbanen in de zorg. Van juni 2011 tot en met december 2012 verrichtte zij een oriëntatie op een wetenschappelijke stage op de afdeling Endocrinologie naar oorzaken van het vroegtijdig optreden van diabetes mellitus en hart- en vaatziekten bij Hindostaanse mannen, onder begeleiding van dr. Leontine Bakker en dr. Ingrid Jazet. In maart 2013 startte zij met twee aaneensluitende wetenschapsstages gedurende 9 maanden op de afdeling Endocrinologie in samenwerking met de afdeling Klinische Oncologie. Hierbij onderzocht zij onder supervisie van dr. Stefanie de Groot, dr. Judith Kroep en prof. dr. Hanno Pijl enerzijds de voorspellende en prognostische waarde van groeifactoren bij het mammacarcinoom, en anderzijds de effecten van voeding op nutriënt sensoren in humane lymfocyten. In 2014 startte zij met haar coschappen en in december 2015 behaalde zij haar masterdiploma Geneeskunde.

In januari 2016 startte zij met haar promotietraject gefinancierd door een beurs van AstraZeneca op basis van een onderzoeker-geïnitieerde projectaanvraag op de afdeling Endocrinologie onder begeleiding van dr. Mariëtte Boon en prof. dr. Patrick Rensen, waarvan de resultaten in dit proefschrift beschreven zijn. Gedurende dit promotietraject presenteerde zij haar data op verscheidene (inter)nationale congressen en won zij in 2018 een travel grant voor de presentatie van een *late breaking* abstract tijdens het *International Congress of Endocrinology* in Kaapstad. Ook won zij tijdens de *Dutch Endocrine Meeting* in Noordwijkerhout in 2019 de prijs voor het beste klinische abstract.

Vanaf november 2019 werkte zij gedurende 7 maanden als arts-assistent niet in opleiding (ANIOS) op de afdeling Cardiologie in het Alrijne ziekenhuis te Leiderdorp. Sinds juni 2020 is zij werkzaam als ANIOS op de afdeling Interne Geneeskunde, ook in het Alrijne ziekenhuis.

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