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Metabolic hormones and ethnic aspects in obesity

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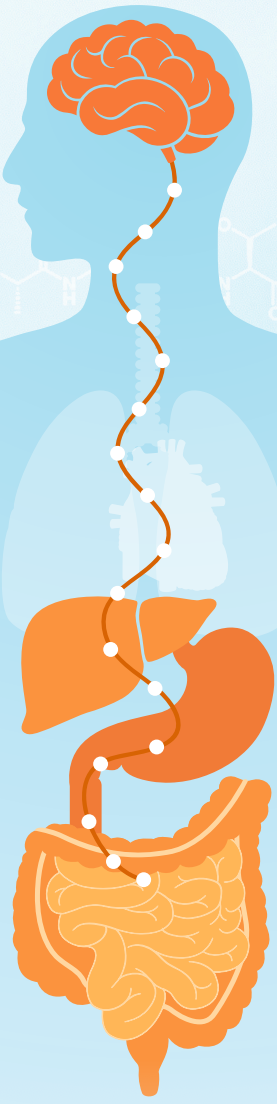
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METABOLIC HORMONES

AND ETHNIC ASPECTS IN OBESITY



CARLIJN ANNE-PAULINE HOEKX

Metabolic hormones and ethnic aspects in obesity

Carlijn Anne-Pauline Hoekx

Metabolic hormones and ethnic aspects in obesity
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Metabolic hormones and ethnic aspects in obesity

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE

GENERAL INTRODUCTION

1. Epidemiology of obesity

1.1. Definition of obesity

Obesity results from a positive energy balance (i.e., energy intake exceeds energy expenditure), and is defined by the World Health Organization (WHO) as a chronic complex disease defined by excessive fat deposits that can impair health (1). A person is considered to be living with overweight or obesity when body mass index (BMI), calculated by weight divided by height squared, is ≥ 25 kg/m^2 or ≥ 30 kg/m^2, respectively (1). Obesity is further categorized into either class I (BMI ≥ 30 and < 35 kg/m^2), class II (BMI ≥ 35 and < 40 kg/m^2), and class III (BMI ≥ 40 kg/m^2) (2). Classifications of obesity based on BMI are supplemented by an increased waist circumference (≥ 102 cm for males and ≥ 88 cm for females). The presence of comorbidities, such as cardiometabolic diseases, respiratory diseases, fertility issues, and psychological issues, is also included in the assessment (2).

1.2. Obesity throughout history

The first representation of obesity dates to at least 30,000 years Before Common Era (B.C.E.), with statuettes depicting women with obesity. These have been found throughout Europe, with the Venus of Willendorf among the most famous (3, 4). For years, obesity was viewed as a symbol of beauty, prosperity, and fertility, celebrated in the artworks of renowned painters like Rubens and Renoir (5, 6). Hippocrates noted the potential causes and detrimental consequences of obesity as early as the 5th century B.C.E. He linked obesity to infertility and attributed the sedentary lifestyle to excess fat mass in the tribe of Scythians. He recommended lifestyle modifications such as dietary adjustments and increased physical activity to maintain a healthy weight (7, 8). While people with obesity have been described throughout history, the incidence of obesity has always been rare (9). By the 20th century, particularly between 1960 and 1980, the obesity rate surged (10), as linked to economic growth, the growing availability of inexpensive nutrient-poor food, industrialization, urbanization, and mechanized transportation, resulting in reduced physical activity (9). This shift prompted the WHO in 1997 to declare an obesity pandemic (11).

1.3. Obesity is a worldwide problem

Since the declaration of obesity as a pandemic by the WHO, the prevalence of people living with obesity has kept on rising. In the Netherlands, 16% of adults aged 20 and above are currently living with obesity, which is triple the amount compared to the early

1980s, though it remains among the lowest in Europe (12). Worldwide, it is expected that 51% of the global population will be living with obesity by 2035 (13).

These increasing rates are alarming, as obesity impairs body health and is associated with the risk of developing various obesity-related diseases, like type 2 diabetes mellitus (T2DM), cardiovascular diseases, and even 13 types of cancer (1). This results in a reduced life expectancy of 3 years for people living with obesity and up to even 10 years for people with obesity class III (14). Combined, these factors have led to an economic impact of 2.4% of global gross domestic product in 2020, consisting of healthcare costs of treating obesity and its related diseases, as well as the effect of high BMI on economic productivity (13). This impact is expected to rise to 2.9% in 2035, which corresponds to 4.3 trillion dollars annually (13, 15).

1.4. Obesity stigma

Alongside the rising prevalence of obesity, in the twentieth century, the attitude towards people living with obesity shifted from a positive to a negative connotation (4). Currently, approximately 56-61% of people living with obesity have encountered weight stigma at some point in their lives, defined by discriminatory acts and ideologies towards individuals because of their weight and size (16, 17). The stigma often manifests in the belief that people who are living with obesity are responsible for their health solely due to laziness and overeating, suggesting a lack of motivation and willpower to lose weight (16, 17). Moreover, it coincides with beliefs that people with obesity are less intelligent and less capable of fulfilling leading positions at work (18). Encounters with the stigma result in diminished mental health, the development of unhealthy eating behavior, reduced physical activity, and increased stress, potentially exacerbating obesity development and the risk of comorbidities (16, 17, 19). Unfortunately, stigmatization not only occur in the general population. Two-thirds of people living with obesity report being stigmatized by their healthcare professionals (16, 20). Stigmatization can cause healthcare professionals to spend less time with individuals with obesity, reduce screening for underlying health conditions, and decrease their willingness to help them (20). Consequently, people with obesity may delay seeking help or avoid it altogether (16, 21). Addressing this issue requires training and education of healthcare professionals on the complex mechanisms of obesity and how to address weight-related concerns adequately (19).

2. Underlying causes and sustaining factors in obesity

According to the new Dutch guideline Overweight and obesity in adults, launched in 2023, the mechanisms driving obesity development and the factors that maintain it can be divided into seven categories: lifestyle, socioeconomic, psychological, medication

use, hormonal, hypothalamic, and genetic (i.e., monogenetic or syndromic) (22). Identifying the underlying cause(s) and sustaining factors in individuals living with obesity is an important first step for determining appropriate intervention options (2).

2.1. Lifestyle

Lifestyle factors play a significant role in the development of obesity. The intake of ultra-processed and excessive amounts of foods combined with a sedentary lifestyle can disrupt the energy balance within the body, leading to a positive energy balance. Consequently, excess nutrients (i.e., glucose and fatty acids) are stored as triglycerides in subcutaneous and visceral white adipose tissue (WAT) depots (23-25). Inadequate sleep is also linked to the development of obesity, as night shift workers are known to have an increased risk of developing obesity compared to day shift workers (26, 27). This may well be because changes in melatonin levels, as occurs in the case of night shifts, disrupt metabolic homeostasis and alter the regulation of hunger hormones, as will be further discussed in section 3.4. Next to disturbed sleep rhythmicity, shorter sleep duration and decreased sleep quality result in increased levels of the hunger hormone ghrelin and decreased levels of the satiety hormone leptin, leading to increased feelings of hunger, nutrient intake, and weight gain (28). In addition, individuals with sleep quality-impairing obstructive sleep apnea (OSA), characterized by recurrent episodes of upper airway collapse leading to apnea and hypopnea, are reported to gain weight (28). OSA-induced hypoxia and its associated complications, including inflammation and endothelial dysfunction, increase the risk of developing obesity and related complications in the long term, resulting in a vicious cycle (28).

2.2. Social economic position

Social economic position, determined by education, income, and occupation, has been linked to obesity (2, 29, 30). Financial instability, unemployment, and unfavorable work conditions contribute to psychological stress and limit access to healthy food options and safe exercise environments, thereby increasing the risk of obesity (30). In addition, children born in families with lower socioeconomic positions are more prone to develop obesity, often influenced by parental factors such as parental obesity and smoking during pregnancy (31, 32).

2.3. Psychological factors

Psychological factors play a significant role in the development of obesity, exhibiting a bidirectional relationship with weight gain. Childhood traumas, sexual abuse, depression, chronic stress, and eating disorders are all linked to the development of obesity (2, 33-35). Conversely, people living with obesity are more prone to developing depression, chronic stress, and eating disorders (2, 35). Depression development is

linked to hormonal changes, microbiota shifts, and inflammation, suggesting a shared biological pathway with obesity development (36). Chronic stress exacerbates obesity by increasing cortisol levels, leading to more appetite and abdominal obesity (34).

2.4. Medication use

Weight gain is a common side effect of various medications, including certain antihypertensives (e.g., beta-blockers), pain relievers, diabetes medication (e.g., insulin), antidepressants, anti-epileptics, and corticosteroids (37, 38). Various mechanisms, such as appetite stimulation, reduced energy expenditure, and disruption of the hypothalamic-pituitary axis, have all been linked to these medications and may facilitate weight gain (39).

2.5. Hormonal

In addition to the endocrine changes that occur in obesity, as discussed below, various common endocrine disorders are linked to the development of obesity. Hypothyroidism, polycystic ovary syndrome (PCOS), male hypogonadism, and menopause can all contribute to decreased resting energy expenditure, leading to weight gain (40-42). Rare forms of endocrine disorders associated with obesity development include Cushing's syndrome, insulinoma, and growth hormone deficiency (43).

2.6. Hypothalamic obesity

Hypothalamic obesity refers to abnormal weight gain resulting from the physical destruction of the hypothalamus, the center in which satiety and energy expenditure are regulated (44). Hypothalamic obesity is a rare underlying cause of obesity, and the most common causes include suprasellar tumors, cranial radiation, vasculitis, or head trauma (44). Weight gain typically occurs rapidly, within weeks to months, and is associated with massively increased appetite (i.e., hyperphagia) and reduced satiety. Additionally, affected individuals may experience disturbed sleep patterns and reduced energy expenditure, further contributing to obesity development (44).

2.7. Monogenetic/syndromic

On rare occasions, obesity is caused by mutations in either a single gene (monogenic obesity) or a part of a chromosome (syndromic obesity) (45, 46). Monogenic obesity typically involves genes involved in the melanocortin 4 receptor (MC4R)/pro-opiomelanocortin deficiency (POMC) pathway, resulting in disturbances in satiety (45). Key indicators of a genetic basis for obesity include severe obesity, early onset, hyperphagia, and a distinct familiar pattern (45). Other symptoms vary depending on the affected gene. Syndromal forms of obesity, such as 16p11.2 deletion syndrome and Bardet-Biedl syndrome, are additionally characterized by cognitive deficits and

behavioral alteration (45). While screening for genetic obesity is not routine, it is indicated for individuals exhibiting high clinical suspicion as more specific treatment options are being developed (2).

3. Physiology of adipose tissue and energy metabolism

Obesity is a complex chronic disease with intricate mechanisms affecting various physiological systems in the body. During the development of obesity, tissue function of different organs is negatively affected by metabolic disturbances due to a positive energy balance in addition to the impairment of the total body by its excess weight.

3.1. Physiology of white adipose tissue in healthy and obesity

WAT is the predominant type of adipose tissue in the human body. It is primarily located beneath the skin (subcutaneous adipose tissue; SAT) and around organs (visceral adipose tissue; VAT) (47). WAT consists of large adipocytes that contain a single lipid droplet. Its primary function is to store energy in the form of triglycerides during energy surplus, from which fatty acids can be released via intracellular lipolysis into the circulation for uptake and beta-oxidation by metabolically active organs such as skeletal muscles and heart during times of energy demand (25, 48).

In addition to storage, WAT is receptive to signals from other tissues, including several hormones (e.g., cortisol and insulin) and the central nervous system (via catecholamines). Furthermore, WAT also functions as an endocrine organ itself via the secretion of adipokines (49, 50). These adipokines can influence almost every other organ within our body in an autocrine, paracrine, and/or endocrine manner (50). Two well-known adipokines are leptin and adiponectin, which are involved in inducing satiety and regulating insulin sensitivity, as will be further discussed below.

During the development of obesity, a prolonged positive energy balance results in enhanced energy storage (contained in glucose and fatty acids) as triglycerides and subsequent expansion of lipid droplets in adipocytes, primarily in the SAT depot (50, 51). However, there is a limit to how much adipocytes within SAT can expand, constrained by the extracellular matrix and vascularization. Once these limits are reached, hypoxia and dysfunction of the tissue occur (50, 52). This dysfunction triggers stress in the adipocytes, promoting the secretion of inflammatory cytokines and the recruitment of immune cells, resulting in low-grade inflammation in the adipose tissue. Cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-alpha) impair adipocyte differentiation, reduce lipid accumulation, and increase intracellular lipolysis within adipocytes (50, 51, 53). Moreover, these cytokines can directly impair insulin sensitivity, resulting in insulin resistance within WAT. Due to the occurring insulin resistance, the

physiological inhibition of insulin on lipolysis is diminished, resulting in an increase in intracellular lipolysis in WAT. Consequently, the release of fatty acids into the circulation is enhanced, directing lipids towards VAT and other tissues such as skeletal muscle, liver, pancreas, and heart, a process known as ectopic fat deposition (53). Ectopic fat accumulation can impair the function of these metabolic organs, contributing to insulin resistance of these tissues and further enhancing insulin resistance and metabolic disturbances (50, 53).

3.2. Lipid metabolism and its implications for obesity

Triglyceride-derived fatty acids are an important energy source in our body. After a meal, triglycerides are broken down within the stomach and intestine into 2-monoacylglycerol and free fatty acids (FFA) by gastric lipase and pancreatic lipase and then absorbed by the epithelial cells of the gut (i.e., enterocytes) (54, 55). Here, 2-monoacylglycerol and fatty acids are re-esterified into triglycerides and packed with dietary cholesterol to form chylomicrons, which are then released into the lymphatic system and subsequently enter the circulation (54, 56). At the endothelial surface of the capillaries of metabolically active tissues, including the heart, skeletal muscles, and various adipose tissues, chylomicrons bind to lipoprotein lipase (LPL) that hydrolyses its triglycerides into glycerol and fatty acids. The liberated fatty acids are taken up by the parenchymal cells of the LPL-expressing tissues, and used for storage (WAT), or for beta-oxidation to produce ATP (skeletal muscle and heart) or heat (brown adipose tissue (BAT); see section 3.5.1. (57). This process results in the formation of partially delipidated and ApoE-enriched chylomicron remnants that hepatocytes recognize and take up via the LDL receptor (LDLR) and LDLR-related protein-1 (LRP1) (58).

The liver can also synthesize triglyceride-rich lipoproteins, called very low-density lipoproteins (VLDL), which are especially important in the fasted state. Just like chylomicrons, VLDL provides triglyceride-derived fatty acids to metabolically active tissues such as the heart, skeletal muscle, and BAT as fuel through the action of LPL (59). Like chylomicron remnants, resulting VLDL remnants are recognized and taken up via ApoE by the LDLR and LRP1 on hepatocytes. Alternatively, VLDL can be converted into low-density lipoproteins (LDL), the lipolytic end product of VLDL, which are primarily recognized and taken up by the liver through the interaction of their ApoB100 with the LDLR on hepatocytes (54).

ApoA1, the high-density lipoproteins (HDL) precursor, is synthesized and secreted by the intestine and liver. During LPL-mediated lipolysis, liberated phospholipids from chylomicrons and VLDL are acquired by circulating lipid-poor ApoA1. These newly formed pre-HDL particles can take up cholesterol from peripheral organs, forming

cholesterol-enriched pre-HDL. Finally, the enzyme lecithin cholesterol acyltransferase (LCAT) residing on HDL converts the acquired free cholesterol into cholesteryl esters, resulting in mature HDL. As such, HDL particles are involved in reverse cholesterol transport, i.e., translocating cholesterol from peripheral tissues to the liver for excretion into the feces primarily as bile acids (60).

Insulin plays a major role in triglyceride metabolism by increasing LPL activity, thereby enhancing triglyceride storage in adipose tissue. This process reduces the availability of FFA in the circulation that could be used by the liver to synthesize VLDL (61). Additionally, insulin inhibits the breakdown of intracellular triglycerides by inhibiting adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) (61, 62). The glucose-dependent insulinotropic polypeptide (GIP), produced by K-cells of the small intestine after a meal, also influences lipid metabolism by promoting postprandial lipid storage in adipose tissue by activating LPL and increasing blood flow through vasodilation (63). Conversely, catecholamines reduce lipid storage by stimulating intracellular lipolysis in adipose tissue, increasing circulating FFA and enhancing fatty acid-driven VLDL production by the liver (56, 64).

People living with obesity frequently develop dyslipidemia, defined as increased levels of circulating total cholesterol, LDL-cholesterol, and triglycerides in combination with decreased HDL-cholesterol (65). In obesity, dyslipidemia generally develops due to increased nutrient intake and adipose tissue insulin resistance, resulting in high circulating FFA levels both postprandially and during fasting (56, 66). The increased FFAs are taken up by the liver, where they are converted into triglycerides to drive the production of VLDL, ultimately resulting in increased LDL-cholesterol levels. Increased (V)LDL increases the exchange of VLDL-triglycerides with HDL-cholesteryl esters as mediated by the cholesteryl ester transfer protein (CETP), thereby decreasing HDL-cholesterol levels (60). Concomitantly, this leads to the accumulation of smaller dense LDL particles that are slowly catabolized and more easily oxidized when exposed to oxidative stressors, such as smoking and unhealthy diet (67, 68). The increase in LDL-cholesterol, probably in combination with increased oxidative stress, is causal to the formation of atherosclerosis, which can result in atherosclerotic cardiovascular diseases, including myocardial infarction and stroke (67, 69).

3.3. Regulation of glucose homeostasis and its disruption in obesity

While fatty acids are a prominent energy source for many tissues in the body like skeletal muscle and the heart, the brain relies primarily on glucose and cannot use fatty acids as an energy source (70). As the body's demand for glucose fluctuates throughout the day, various glucoregulatory hormones maintain stable circulating glucose levels.

Glucose is derived from three sources: via food, the breakdown of glycogen stored in the liver (glycogenolysis), and *de novo* generation by the liver (gluconeogenesis) (71). Glycogenolysis and gluconeogenesis are predominantly stimulated by glucagon, a hormone released from the alpha cells in the pancreas during fasting to ensure glucose availability in the body (71, 72).

After a meal, when glucose is abundant, glucose levels are lowered via different mechanisms. The hormones insulin and amylin, released by the pancreatic beta cells, and glucagon-like peptide 1 (GLP-1) and GIP, released by the intestinal L-cells and K-cells, respectively, all work to lower postprandial glucose levels.

Insulin lowers circulating glucose levels by binding insulin receptors on insulin-sensitive tissues, such as skeletal muscle and WAT, to increase glucose uptake. This is achieved by translocation and fusion of the glucose transporter GLUT4 to the cell membrane (73). Additionally, insulin inhibits glucagon secretion, thereby inhibiting glycogenolysis and gluconeogenesis (71, 72, 74, 75). Furthermore, as mentioned in section 3.2., insulin increases LPL activity and inhibits intracellular lipolysis, thereby enhancing triglyceride storage in adipocytes. Amylin complements the effects of insulin by suppressing glucagon secretion via efferent vagal signals, decreasing gastric emptying, and decreasing satiety by binding to its receptor in the area postrema in the hindbrain (71, 76).

The incretin hormones GLP-1 and GIP are responsible for the 'incretin effect', which refers to a 2-3 fold increase in insulin secretion when glucose is administered enterally compared to intravenously (77). GLP-1 and GIP both stimulate insulin release in a glucose-dependent manner. This means they only stimulate insulin when circulating glucose levels are above the normal range, preventing hypoglycemic episodes (72). GIP and GLP-1 are secreted within minutes after food ingestion (78), and rapidly degraded by dipeptidyl peptide IV (DPP4) (79). GLP-1 receptors (GLP-1R) and GIP receptors (GIPR) are expressed throughout the body in different tissues, especially the pancreas, heart, and brain (80). As mentioned in section 3.2., the interaction of GIP with GIPR on white adipocytes postprandially increases LPL-medicated uptake of fatty acids in adipocytes (81). GLP-1 also inhibits glucagon secretion, further contributing to glucose level reduction. Additionally, GLP-1 and GIP play roles in inducing satiety, as further described in section 3.4.2.

In addition to hyperinsulinemia, obesity-associated insulin resistance is characterized by basal hyperglucagonemia and reduced suppression of glucagon after a meal (82, 83). The higher levels of basal and post-prandial glucagon contribute to hyperglycemia

by increasing gluconeogenesis in the liver (83). The liver uses amino acids to increase gluconeogenesis, causing protein loss in skeletal muscles and thereby wasting muscle mass (83). This can contribute to the development of so-called 'sarcopenic obesity'.

The role of amylin in obesity and its connection to the development of insulin resistance is not yet fully elucidated. Until now, evidence points to no clear effect of dysfunction in the amylin system (both levels and receptor function) in obesity (84).

In obesity, the secretion of GLP-1 and GIP is often altered, resulting in higher levels of GIP and possibly lower levels of GLP-1 during fasting and postprandial; however, higher GLP-1 levels have also been described (85-87). The decreased GLP-1 levels together with reduced responsiveness of pancreatic islets to GIP result in a diminished incretin effect in people living with obesity. This leads, in the initial stages of obesity, to lower insulin release and higher glucose levels (88, 89). When the disease progresses, insulin levels increase due to the development of insulin resistance.

3.4. Hunger and satiety hormones and their role in energy balance

Various hunger and satiety hormones regulate the energy intake aspect of the energy balance. Among the most prominent are the previously discussed leptin, GLP-1, and GIP for satiety and ghrelin for hunger. However, there are several others, such as cholecystokinin (CKK), peptide YY (PYY), and growth differentiation factor 15 (GDF15) all inducing satiety.

3.4.1. Leptin

Leptin, the first discovered adipokine, is key in maintaining energy balance by regulating food intake and energy expenditure (49). It works by signaling through leptin receptors in the hypothalamus and brainstem, promoting satiety and increasing energy expenditure (90, 91). The importance of leptin is evident from patients who suffer from leptin deficiency, which leads to extreme hunger and severe obesity from a young age (92).

In common multifactorial obesity, leptin regulation also becomes disrupted. Circulating leptin levels are directly linked to the size of adipocytes, especially those located subcutaneously (93). Therefore, people living with obesity generally have higher circulating leptin levels. Leptin levels further fluctuate based on the nutritional status, decreasing during fasting and increasing during feeding (90, 93). However, despite the high leptin levels in people with obesity, they generally experience less satiety compared to individuals who are lean, suggesting that obesity is a state of leptin resistance (94). This may, at least in part, be a consequence of the development of hypothalamic inflammation in obesity (95).

3.4.2. Incretin hormones

While GLP-1 and GIP play an important role in glucose metabolism (see section 3.3), they also induce satiety by signaling via afferent neurons of the vagus nerve from the intestine to the GLP-1R and GIPR in the hypothalamus and hindbrain (88, 96, 97). Recent research has shown that GIP has an additive effect on GLP-1 in satiety induction, although the precise mechanisms are still largely unknown (98, 99).

People living with obesity often experience persistent increases in hunger and lower satiety compared to lean people, which can contribute to overconsumption and difficulty in reducing food intake (100, 101). Besides leptin resistance as described above, this disturbed function of other hunger and satiety hormones in people living with obesity contributes to this phenomenon. For example, the lower postprandial GLP-1 release as mentioned above can lead to a diminished satiety response but potentially also increase gut emptying (102). This results in overall less suppression of hunger and increased return after a meal (100, 102).

3.4.3. Ghrelin

Ghrelin is a key modulator of food intake, energy metabolism, gastric acid secretion, and motility (103, 104). When the body demands nutrients, ghrelin is secreted from the stomach and small intestine, preparing the body for a meal. Acting via vagal afferent neurons, ghrelin signals via the vagal nerve to its GH secretagogues receptor 1a (GHS-R1a) located in the hypothalamus, stimulating appetite (104). Beyond its role in appetite regulation, ghrelin influences glucose homeostasis by promoting glucose production in the liver by activating gluconeogenesis pathways. It also stimulates gastric secretion and motility (105). Furthermore, ghrelin plays a role in lipid metabolism by promoting lipid storage in adipocytes by upregulating several fat storage-related proteins, including fatty acid synthase (FAS), LPL, and perilipin (106, 107).

Although the exact underlying mechanism is not well known, it is reported that postprandial suppression of ghrelin in people living with obesity is often reduced, contributing to persistent feelings of hunger after a meal (108).

3.4.4. Growth Differentiation Factor 15

Growth Differentiation Factor 15 (GDF15) is a stress-regulated hormone secreted by various body organs, including the kidneys, placenta, prostate, and gastrointestinal tract. Recent reports suggest that it is also a potent appetite-suppressing hormone that signals via binding to the glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-like (GFRAL) located in the area postrema and solitary tract of the hindbrain (109-112). GDF15 is a stress-responsive hormone and in people living

with obesity, GDF15 levels are increased, especially in males (113, 114). However, the mechanism causing the increase in GDF15 during obesity and the consequences of obesity-induced GDF15 remains unclear (115).

3.5. Physiology of energy expenditure in health and obesity

Energy expenditure (EE) refers to the energy that an individual expends to maintain bodily functions, and consists of different components: resting energy expenditure (REE), food or diet-induced EE, adaptive thermogenesis, and activity-induced EE (116, 117). REE accounts for approximately two-thirds of the total EE (118). Many factors, like age, sex, body composition, genetics, and hormones influence REE (119). Environmental temperature can affect EE through adaptive thermogenesis; during colder temperatures, the body increases EE to maintain a stable core body temperature. Adaptive thermogenesis occurs in two forms: shivering thermogenesis, primarily caused by skeletal muscle contractions, and non-shivering thermogenesis, primarily driven by BAT but involving skeletal muscles (120-123).

As obesity results from an imbalance between energy intake and EE, people living with obesity might be expected to have decreased EE. However, REE is often increased compared to lean individuals (124). This is likely due to the increase in fat-free mass, which consists of highly active metabolic organs such as skeletal muscle, needed to support the excess body weight. When correcting for the increased fat-free mass, people living with obesity generally have similar REE compared to lean individuals (124).

3.5.1. Role of brown adipose tissue in thermogenesis and its detection in obesity

BAT contributes to adaptive thermogenesis by oxidizing glucose and triglyceride-derived fatty acids, thereby generating heat instead of adenosine triphosphate (ATP) (125). In adults, BAT is mainly located in the supraclavicular, cervical, and axial regions. BAT contains small multilocular brown adipocytes with mitochondria that contain uncoupling protein 1 (UCP1) (126). Long-chain fatty acids activate UCP1 and facilitates proton leak over the mitochondrial inner membrane, resulting in dissipation of energy as heat (121). Cold exposure is the main physiological activator of BAT, leading to the release of norepinephrine from the sympathetic nervous system, which binds to beta-adrenergic receptors on the cell membrane of brown adipocytes and triggers BAT thermogenesis (127). In addition, BAT has an autocrine, paracrine, and endocrine function, as it secretes several signaling molecules known as batokines that play a role in the homeostasis of different tissues, such as WAT, skeletal muscle, and liver (128, 129). While in mice the beta-adrenergic 3 receptor is the main receptor that stimulates BAT thermogenesis, the responsible beta-adrenergic receptor stimulating BAT thermogenesis in humans is less clear.

In humans, the uptake of glucose by BAT is used as a measure of BAT activity, often assessed via a [^{18}F]fluoro-D-deoxyglucose positron emission tomography/computed tomography ([^{18}F]FDG PET/CT) scan. This scan uses the tracer [^{18}F]FDG to quantify glucose uptake in BAT after an individualized cooling protocol. In people with obesity, the uptake of glucose by BAT often is lower or even absent (130). This is due to the visualization method of BAT. Both glucose and [^{18}F]FDG uptake by BAT are mediated via the GLUT4 and GLUT1 but GLUT4 is stimulated by insulin, so in case of reduced insulin sensitivity, less glucose, and [^{18}F]FDG are taken up by cells (131, 132). A large study using [^{18}F]FDG PET/CT scans showed that those people with obesity who had detectable BAT (i.e., with [^{18}F]DFG uptake above a certain threshold) have a healthier metabolic phenotype (lower VAT, insulin resistance, inflammation, odd to develop T2DM, dyslipidemia, and congestive heart failure) compared to those without detectable BAT, suggesting a contribution of BAT in whole-body metabolism (133-135).

3.5.2. Fibroblast growth factor 21

Fibroblast Growth Factor 21 (FGF21) is a stress-inducible hormone secreted by various organs. Following prolonged fasting (7-10 days), the liver mainly contributes to total FGF21 secretion (136). In hepatocytes, FGF21 secretion is controlled by the activation of peroxisome proliferator-activated receptor α (PPARα) which is, in turn, activated by non-esterified fatty acids released from adipocytes (137).

Other stressors that induce FGF21 release include cold exposure (FGF21 release by BAT, WAT, and liver) and exercise (FGF21 release by skeletal muscle). FGF21 is important in regulating lipid and glucose metabolism, enhancing insulin sensitivity, enhancing REE, and modulating mainly anti-inflammatory immune responses (138-140). FGF21 exerts its actions by binding to the isoforms of FGF 1,2,3, and 4 receptors, which forms a heterodimer with the co-receptor β-Klotho. Since β-Klotho is primarily expressed in the liver and in both white and brown adipose tissues, these are the main target tissues of FGF21. In adipose tissues, FGF21 enhances insulin sensitivity and glucose uptake. In the liver, FGF21 reduces lipogenesis and enhances fatty acid oxidation, lowering hepatic triglyceride content. However, during nutrient surplus, FGF21 suppresses lipolysis to lower triglyceride levels and prevent excessive hepatic lipid deposition (141, 142). Additionally, FGF21 reduces the expression of pro-inflammatory cytokines and inhibits inflammatory pathways in various tissues like the liver (138, 139).

Despite that FGF21 increases insulin sensitivity, reduces inflammation, and prevents excessive hepatic lipid depositions, circulating FGF21 levels are actually elevated in people living with obesity and positively correlate with BMI (143). This seeming discrepancy suggests that obesity is a state of FGF21 resistance, possibly with

downregulation of the FGF21 receptors. Indeed, different isoforms of the FGF receptor are downregulated in WAT in obese mice (144, 145). In obesity, higher FGF21 levels are positively associated with impaired glucose tolerance and lipid accumulation in the liver (145).

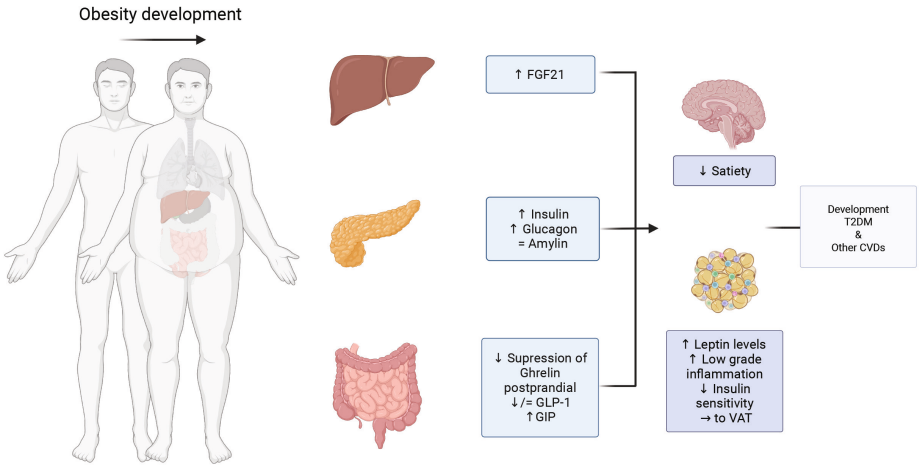


Figure 1. Dysregulation of various hormones during obesity development contributes to the development of obesity-related diseases. Obesity leads to dysregulation of hormones in the liver, pancreas, and gut, resulting in reduced satiety induction and alterations in white adipose tissue, all of which contribute to the development of obesity-related diseases. See sections 3.4 and 3.5.2. FGF21, fibroblast growth factor 21; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide 1; T2DM, type 2 diabetes mellitus; VAT, visceral adipose tissue.

4. Increased risk of South Asians to develop obesity and cardiometabolic diseases

4.1. Etiology of South Asians

Certain populations, such as the South Asian population, are more prone to develop obesity and obesity-related diseases as compared to e.g. Europids (146). South Asians (originally descended from Surinam, Bangladesh, India, Nepal, Pakistan, Afghanistan, Bhutan, and Sri Lanka) are predisposed to developing obesity and obesity-related diseases at a significantly younger age and lower BMI than other ethnic groups (146). The South Asian population constitutes approximately 25% of the world's population (147). In the Netherlands, there are about 240,000 people of South Asian descent (approximately 1.4% of the Dutch population), particularly concentrated in and around The Hague (148).

4.1.1. South Asians and migration towards the Netherlands

The large number of South Asians in the Netherlands is primarily due to the historical ties of the Netherlands with Surinam (148). In 1667, the Dutch defeated the British and occupied Surinam, establishing plantations for cotton, sugar, cocoa, and coffee. The labor-intensive work on these plantations was initially performed by enslaved Africans. However, after slavery was abolished in 1863 by the Emancipation Act, the Dutch recruited people from British India and the Dutch East Indies to replace the freed slaves as contract workers (149). Approximately 34,000 people migrated from Asia to Surinam. After the embellishment of contract work, supported by Mahatma Gandhi, about 25,000 people from South Asia remained in Surinam (150-152). Following Surinam's independence in 1975, around 40,000 individuals of South Asian descent migrated to the Netherlands (153). Therefore, many South Asians living in the Netherlands are of Surinam descent.

4.2. Underlying factors contributing to the high cardiometabolic disease risk in South Asians

An unfavorable metabolic phenotype in South Asians compared to Europids, consisting of central obesity, dyslipidemia, and insulin resistance, is an important factor that contributes to their high risk of developing cardiometabolic diseases, which will be discussed below.

4.2.1. Unfavorable metabolic phenotype of South Asians

South Asians have a higher body fat percentage, especially due to an increased abdominal visceral fat mass, and lower lean muscle mass than Europids (154, 155). These differences are already apparent in infancy, with South Asian neonates showing higher fat mass and lower fat free mass than Europid neonates (156). The higher fat mass in South Asians, especially in VAT and ectopic locations, may be explained by the adipose tissue overflow hypothesis (157). This hypothesis states that South Asians have less developed SAT than Europids, with a reduced capacity to store fatty acids. As a result, they utilize VAT for storage earlier than Europids (157). This theory is based on findings of higher VAT components in South Asian males and females, even though BMI and waist circumference differ slightly between South Asians and Europids (157). Another study showed that in healthy South Asians, adipocyte cell size was larger in subcutaneous fat biopsies compared to Europids with similar body fat content, and this was negatively correlated with insulin resistance and plasma adiponectin concentration (158).

Higher circulating insulin and glucose levels are already present in the cord blood of South Asian neonates compared to Europids neonates, suggesting that an unfavorable metabolic phenotype is already present from birth (159). This trend continues

throughout life, as young South Asians exhibit higher insulin responses to an oral glucose tolerance test (OGTT) than Europids (160). Increased insulin resistance in South Asians is likely partly explained by their increased VAT, ectopic fat, and lower muscle mass (161). As a result, South Asians are more prone to develop insulin resistance and T2DM at a younger age and lower BMI than Europids (162, 163). Moreover, an earlier decline in pancreatic beta cell function may also contribute to this increased risk (162).

Another pillar of the classical unfavorable metabolic phenotype in South Asians is dyslipidemia. This is characterized by high levels of triglycerides, low levels of HDL-C, and smaller, dysfunctional HDL particles despite having similar LDL-C levels (164). Dyslipidemia is a contributing factor to the increased risk of cardiovascular disease in the South Asian population (164). However, unfavorable fat distribution, dyslipidemia, and insulin resistance do not fully explain the increased risk of obesity-related diseases in South Asians. Other factors, such as lifestyle, inflammation, hormonal dysregulation, and energy expenditure changes, may also contribute (165). Additionally, there may be other yet unidentified factors that play a role.

4.2.2. Lifestyle of South Asians

As mentioned, unhealthy lifestyle factors can contribute to the development of obesity (2). Various unfavorable lifestyle factors are particularly prevalent in the South Asian population and may further aggravate their metabolic phenotype.

Food plays a main role in South Asian culture, especially during social gatherings. Offering abundant, flavorful, and nutrient-rich foods is seen as part of hospitality (166). In addition, the typical diet consists mainly of carbohydrates and saturated fats, with a low protein content, which is high in calories and can contribute to weight gain (154, 167).

Furthermore, South Asians tend to have lower levels of physical activity than other ethnic groups (166, 167). Different priorities, such as family and education, often take precedence, contributing to a more sedentary lifestyle (166). Moreover, the unfamiliarity with exercise, combined with cultural beliefs about appropriate clothing for exercise and concerns about the safety of women exercising alone, can discourage physical activity (166, 168).

Contributing to an unfavorable lifestyle is tobacco use in South Asians. Especially in males, the prevalence of smoking is much higher compared to the Europids (169). Alcohol use, on the other hand, is much lower in the South Asian population compared to Europids (170).

Finally, the quality and quantity of sleep among the South Asian population are lower compared to other ethnicities. Poor sleep quality is associated with greater VAT (171). Many South Asians sleep less than seven hours per night and have lower sleep quality, with a higher prevalence of obstructive sleep apnea. Poor sleep quality and quantity not only underlies obesity and increased VAT but also aggravates it (172).

4.2.3. Inflammation in South Asians

Inflammation has been increasingly acknowledged as a contributor to the development of obesity-related diseases (173). This is particularly evident in the South Asian population, who exhibit a more pro-inflammatory phenotype than Europids. C-reactive protein (CRP), an acute phase protein indicative of inflammation, is already elevated in the cord blood of South Asian neonates (174). These elevated CRP levels persist throughout life, with South Asians consistently showing higher CRP levels than Europids (175). Furthermore, South Asians with T2DM have a more activated interferon (IFN)-signaling pathway and higher B cell markers than Europids (176). These findings show the pro-inflammatory state of South Asians, which may contribute to their increased risk of obesity-related diseases (162). Indeed, IFN has been shown to accelerate insulin resistance in myocytes *in vitro* (177). Tackling the pro-inflammatory state of South Asians could potentially prevent or treat obesity-related diseases (178).

4.2.4. Hormone regulation in South Asians

South Asians exhibit higher leptin levels compared to Europids (179). This may well be due to their increased white adipocyte size. The differences in incretin levels between South Asians and Europids are not well researched; only two studies have investigated GLP-1 levels at fasting and after an OGTT in South Asians compared to Europids. These studies found that South Asians have higher postprandial GLP-1 levels (160, 180). Additionally, the regulation of other hunger and satiety hormones in South Asians, like GIP, ghrelin, and GDF15, remains inconclusive.

4.2.5. Resting Energy Expenditure in South Asians

So far, we have discussed the underlying mechanisms of the increased risk of obesity-related disease leading to an increased energy intake in South Asians. However, South Asians also exhibit lower REE, contributing to a positive energy balance (181, 182). Additionally, exercise-induced EE is also lower in South Asians (183, 184). This lower EE is attributed to a lower fat-free mass in South Asians than in Europids (181). Furthermore, South Asians also have a lower BAT volume than Europids, which may partly underlie their lower REE and adaptive thermogenesis, further contributing to their increased risk of developing obesity and obesity-related diseases (182).

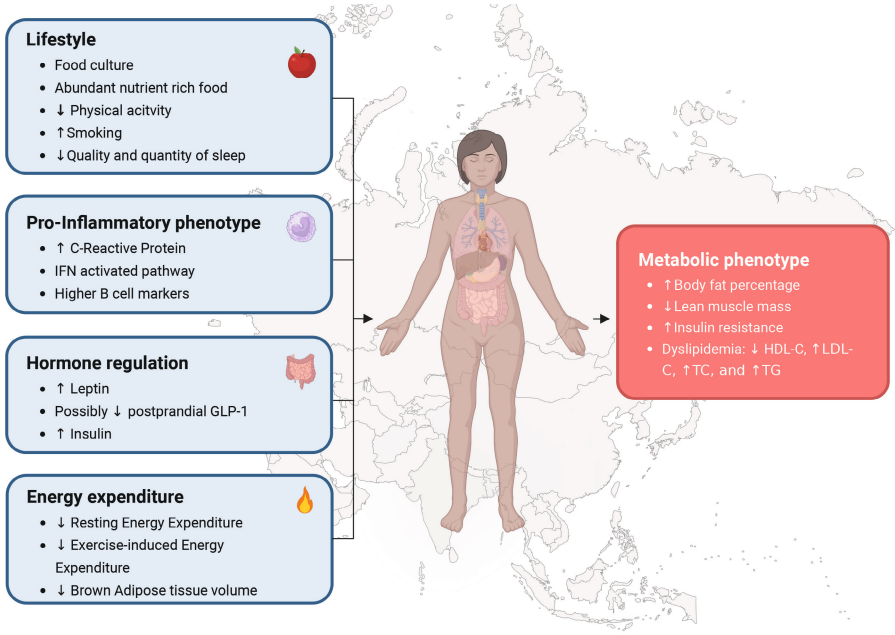


Figure 2. Various factors may contribute to the increased risks of South Asians developing obesity and obesity-related diseases including unhealthy lifestyle, pro-inflammatory phenotype, hormone dysregulation, and low energy expenditure (blue boxes), collectively contributing to an adverse metabolic phenotype (purple box). For more information, see the section 4.2. GLP-1, glucagon-like peptide 1; HDL-C, high-density lipoprotein cholesterol; IFN, interferon; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

5. Targets for prevention and treatment of obesity and related diseases

In the preceding sections, we have explored the physiology of and underlying mechanisms contributing to the development and consequences of obesity. This section will discuss possible strategies to counteract obesity and related diseases. Prevention and treatment options fall into two main categories: lifestyle interventions and pharmacological interventions. It is important to note that all individuals living with obesity are advised to adopt a combined lifestyle intervention, especially when additional pharmacological treatments are used (2).

5.1. Nonpharmacological intervention

5.1.1. Combined lifestyle intervention

Combined lifestyle intervention is the cornerstone of the treatment of obesity. Its goal is to help people adopt a healthy lifestyle. Combined lifestyle interventions encompass

advice on diet, exercise, sleep, and cognitive behavioral therapy, with an emphasis on behavioral change (2). People are advised to follow a healthy diet, minimize processed food, and engage in at least 150-200 minutes of exercise per week, including strength training twice a week, while reducing their sedentary lifestyle (2).

For the South Asian population, lifestyle interventions can be more difficult to adopt due to barriers caused by cultural influences (166). In addition, South Asians need to move more to achieve the same results as Europids. A previous study showed that 232 minutes of physical activity for South Asians is equivalent to 150 minutes for Europids in reaching the same cardio-metabolic risk benefits. This was measured using vertical axis accelerations with accelerometers and based on biochemical markers like glycemia variables, lipid measurements, and blood pressure (185). However, culturally tailored dietary and exercise interventions have shown promise in improving glycemic control in South Asians (186).

5.1.2. Cold

Although cold exposure is not currently included in the guidelines as a treatment for obesity, studies have shown that prolonged repetitive cold exposure has beneficial effects on fat mass and metabolic health (187, 188). More specifically, in patients with T2DM, repetitive cold exposure improved insulin sensitivity and even necessitated reducing daily insulin use in some patients after 10 days (187). The improvement of metabolic health by cold exposure may be mediated by increased thermogenesis in BAT and skeletal muscles, the secretion of hormones by BAT (batokines) and skeletal muscle (myokines), as well as decreased inflammation. However, more research and more long-term studies are necessary to confirm this (188). Since South Asians have lower BAT volume and skeletal muscle mass, cold exposure may be a useful therapy for increasing the amount of BAT and increasing skeletal muscle thermogenesis in this population. Although this must be further studied, a previous study showed that cold exposure of healthy South Asians could potentially influence the immune system towards a less pro-inflammatory phenotype by altering the expression of various immune genes (189).

The effects of cold exposure on energy metabolism depend on the time of day. Cold-induced thermogenesis (CIT) has been shown to be higher in the morning than in the evening, at least in males (190). These time-dependent effects of cold exposure in men are possibly due to the rhythmicity in BAT activity during the day (190). In mice, BAT has a higher uptake of TG-derived fatty acids at the onset of the dark period, which is the start of the active period in mice (191). However, the diurnal rhythm of BAT in humans and its secretory function are yet to be elucidated.

5.2. Pharmacotherapy

5.2.1. Pharmacological activation of BAT

Cold exposure can activate BAT but is not suitable or desirable for everyone (192). Therefore, pharmacological activation of BAT is a potentially interesting alternative. In addition, due to the lower BAT volume in South Asians compared to Europids, they may benefit more from pharmacological strategies.

The beta-adrenergic receptor on the cell membrane of brown adipocytes is a potential pharmacological target to activate BAT. ADRB3 is most prominent in the activation of BAT in mice and was assumed to be a prominent receptor for BAT activation in humans as well (193, 194). However, while single administration of the ADRB3 agonist mirabegron to healthy participants resulted in enhanced [^{18}F]DFG uptake by BAT and increased REE, it also resulted in increased heart rate and blood pressure (195), regulated by the ADRB1 and ADRB2 receptors (196, 197), suggesting overflow towards these receptor subtypes. The dose of mirabegron to activate BAT was 200 mg, whereas the advised dose for treating overactive bladder, for which mirabegron is registered, is around 50 mg (198). Thus, a supratherapeutic dose was used to show an increase in BAT activity (199). Therefore, it is possible that the effects of mirabegron on BAT and energy expenditure were also caused by overspill to the ADRB1 and/or ADRB2 receptors rather than directly activating the ADRB3. Indeed, the ADRB2 appeared the dominant beta-adrenergic receptor on human brown adipocytes *in vitro* (200). Whether human BAT can be activated via the ADRB2 *in vivo* remains to be seen.

5.2.2. Metformin

Metformin is the first-line treatment for T2DM; it is also prescribed off-label for obesity due to its modest effects on satiety (2, 201). While the mechanism of metformin's satiety-inducing effect remained unknown for a long time, a recent study revealed that metformin stimulates satiety by increasing the levels of GDF15 (202). Preclinical studies showed that GDF15 induces satiety by binding to the glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-like (GFRAL) located in the area postrema and solitary tract of the hindbrain (109-112, 203, 204).

5.2.3. GLP-1 receptor agonists

A more effective pharmacological treatment for weight loss is incretin-based treatment using GLP-1 receptor agonists (205), mainly by reducing food intake through inducing satiety (206). This is thought to mainly occur via the interaction of GLP-1 receptor agonists with vagal afferent neurons, transmitting a signal to the hindbrain and inducing satiety (207). This is supported by the attenuated anorexic effect of GLP-1 receptor

agonism when the vagal afferents are denervated (208). However, the involvement of other appetite-regulating hormones, including GDF15, in the beneficial effects of GLP-1 receptor agonists on satiety cannot be ruled out. In addition to reducing food intake by inducing satiety, other factors such as delayed gastric emptying, influence on fat distribution, and possible involvement of BAT resulting in enhanced thermogenesis may contribute to GLP-1-induced weight loss (206).

OUTLINE OF THIS THESIS

Obesity is a complex chronic disease with many underlying mechanisms, as described in this **current chapter**. Numerous environmental and physical factors can disrupt energy balance, leading to changes like hormonal imbalances and inflammation. These disruptions ultimately contribute to the development and maintenance of obesity and its related diseases, including T2DM and other cardiometabolic diseases. Unfortunately, some populations, such as South Asians, are more at risk of developing obesity and related disorders compared to Europids. However, the underlying mechanisms are not yet fully elucidated. Novel and sustainable treatment options are necessary to combat the obesity epidemic. Promising approaches include cold exposure or pharmacological activation of BAT, as well as pharmacological options based on the mechanisms of incretin hormones and other satiety hormones.

All in all, in this thesis, we aim to i) unravel additional underlying causes of the disadvantageous metabolic profile of South Asians, and ii) comprehensively understand how different (non-)pharmacological interventions can modulate circulating levels of various hormones and regulate overall energy metabolism in humans with different comorbidities.

To answer the first objective, in **Chapter 2** we first investigated potential differences in circulating levels of inflammation-related proteins in Dutch South Asians compared to Dutch Europids with T2DM. These were measured using a large inflammation panel from Olink proteomics, with findings confirmed via ELISA. Next, in **Chapter 3** we compared circulating incretin hormones and glucagon, between young and lean Dutch South Asians and Dutch Europids before and during a mixed meal tolerance test (MMTT) in relation to glucose and insulin excursions. In **Chapter 4**, in the same cohort of young and lean Dutch South Asians and Dutch Europids, we focused on circulating PYY, ghrelin and leptin before and during the MMTT.

Cold exposure and pharmacological activation of BAT could be potential interventions for obesity. Therefore, for the second objective, in **Chapter 5** we investigated the effect of cold exposure on circulating FGF21 and GDF15 in Europids and examined whether cold-induced changes in FGF21 and GDF15 levels differ between morning and evening in males and females. Furthermore, in **Chapter 6**, we investigated whether pharmacological activation of the ADRB2 receptor using the specific agonist salbutamol increases glucose uptake by BAT of lean Europid males, as assessed by a dynamic [^{18}F] FDG PET/CT scan. We used a cross-over design to compare intravenous salbutamol without and with the oral ADRB1/2 blocker propranolol. Finally, in **Chapter 7**, we

examined whether GDF15 mediates the satiety-inducing effect of the GLP-1 receptor agonist liraglutide in Dutch Europid and Dutch South Asian patients with T2DM.

In the final chapter, **Chapter 8**, we discuss the most important findings of all studies, as well as their implications and future directives.

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CHAPTER 2

CIRCULATING FGF21 IS LOWER IN SOUTH ASIANS COMPARED TO EUROPIDS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

Objective

Inflammation contributes to the development of type 2 diabetes mellitus (T2DM). While South Asians are more prone to develop T2DM than Europids, the inflammatory phenotype of the South Asian population remains relatively unknown. Therefore, we aimed to investigate potential differences in circulating levels of inflammation-related proteins in South Asians compared to Europids with T2DM.

Method

In this secondary analysis of three randomized controlled trials, relative plasma levels of 73 inflammation-related proteins were measured using the Olink Target Inflammation panel and serum FGF21 concentration using an ELISA kit in Dutch South Asians (n=47) and Dutch Europids (n=49) with T2DM.

Results

Of the 73 inflammation-related proteins, the relative plasma levels of 6 proteins were higher (SCF, CASP-8, CCL28, IFN-gamma, ST1A1, CST5; q-value<0.05), while relative levels of 6 proteins were lower (FGF21, MMP-1, IL8, CCL4, CXCL6, MCP-1; q-value<0.05) in South Asians compared to Europids. Of these, the effect size of FGF21 was the largest, particularly in females. We validated this finding by assessing FGF21 concentration in serum. FGF21 concentration was indeed lower in South Asians compared to Europids with T2DM in both males (-42.2%; P<0.05) and females (-58.5%; P<0.001).

Conclusion

Relative plasma levels of 12 inflammation-related proteins differed between South Asians and Europids with T2DM, with a significantly pronounced reduction in FGF21. In addition, serum FGF21 concentration was significantly lower in South Asian males and females compared to Europids. Whether low FGF21 is an underlying cause or consequence of T2DM in South Asians remains to be determined.

INTRODUCTION

One in ten adults is living with diabetes, and this number is expected to rise to one in nine by 2040 (1, 2). Of those affected, ninety percent have type 2 diabetes mellitus (T2DM), with ethnic groups showing different susceptibility to developing T2DM and associated diseases (3). For example, South Asians originating from the Indian subcontinent develop T2DM at a significantly younger age and a lower body mass index (BMI) than other ethnic counterparts, and given that they present nearly a quarter of the world's population, understanding the underlying mechanism is of utmost importance (4, 5). The increased risk of the development of T2DM in South Asians is thought to be partially attributable to their metabolic phenotype, characterized by more central obesity, dyslipidemia, and more insulin resistance relative to Europids (6-8). However, these factors alone do not entirely explain the increased risk (9).

In recent years, research has shown that inflammation plays a prominent role in developing T2DM and its associated complications (10, 11). Inflammation results from stress on the adipose tissue, leading to the attraction of immune cells by releasing cytokines and chemokines that promote inflammation (12-14). Interestingly, previous studies suggest the presence of a more proinflammatory phenotype in the South Asian population. Our prior research revealed higher C-reactive protein (CRP) levels already in cord blood from South Asian neonates compared to Europid neonates (15). Additionally, South Asians living with T2DM display a more activated interferon (IFN)-signaling pathway than Europids living with T2DM (16).

Considering the pivotal role of inflammation in the development of T2DM and its associated comorbidities, identifying the inflammation-related protein signature could improve our understanding of South Asians' high T2DM risk. It may also provide valuable insight into the applicability of novel treatment modalities in this population. Therefore, in the current study, we aimed to investigate potential differences in circulating inflammation-related proteins in South Asians compared to Europid individuals living with T2DM.

METHODS

Participants and study design

Participants

This study is a secondary analysis of three previously performed randomized, double-blinded, placebo-controlled clinical trials.

The first two clinical trials were designed to investigate the effect of a 26-week liraglutide treatment on glycemic endpoints and ectopic fat deposition in participants with overweight, obesity, and T2DM (17, 18). In total, 50 patients of Dutch Europid (hereinafter: 'Europid') origin (study 1) (17) and 47 of Dutch South Asian (hereinafter: 'South Asian') origin (study 2) (18) were included. South Asian ethnicity was defined as having four grandparents who originally descended from Surinam, Bangladesh, India, Nepal, Pakistan, Afghanistan, Bhutan, or Sri Lanka. Inclusion criteria were males and females aged 18-69 years, BMI ≥ 25 kg/m^2, and Hemoglobin A_{1c} (HbA_{1c}) levels of 7.0-10.0% (53-86 mmol/mol) despite the use of metformin, sulfonylurea derivatives, and insulin. The main exclusion criteria were the use of other glucose-lowering therapy and renal, hepatic, or cardiovascular disease (i.e., presence of congestive heart failure New York Heart Association (NYHA) classification III-IV, uncontrolled hypertension (systolic blood pressure > 180 mmHg and/or diastolic blood pressure > 110 mmHg) or an acute coronary or cerebrovascular accident within 30 days prior to study inclusion); gastric bypass surgery; chronic pancreatitis or previous acute pancreatitis; pregnancy or lactation and MRI contra-indications. Both trials were performed between 2013 and 2018.

The third clinical trial was a randomized, double-blinded, placebo-controlled cross-over study designed to assess the effect of cold exposure and mirabegron on plasma lipids, energy expenditure, and brown adipose tissue fat fraction in 10 healthy lean South Asian males versus 10 age—and BMI-matched Europid males. Inclusion criteria were age 18-30 and healthy BMI between 18-25 kg/m^2. The study was performed between June 2017 and June 2018.

Study approval

All three trials were conducted according to the principles of the revised Declaration of Helsinki (19). Before inclusion, written informed consent was obtained from all participants. The local ethics committee approved all trials, which were conducted at Leiden University Medical Center and registered at clinicaltrial.gov (NCT01761318, NCT02660047, and NCT03012113).

Study designs

The designs of all trials have been extensively described elsewhere (17, 18, 20).

At baseline, all participants from the three studies arrived at the outpatient clinic after at least a 6-hour fast for those with T2DM and a 10-hour overnight fast for those without T2DM. Initial assessment in all trials included body composition and bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystat Ltd., Douglas, UK), followed by the collection of venous blood samples. In the trials with patients with T2DM, after the initial blood sample collection, individuals underwent an MRI and proton magnetic resonance spectroscopy (^{1}H-MRS) to measure subcutaneous and visceral adipose tissue and hepatic triglyceride content (HTGC).

Blood collection

Following the collection of venous blood samples, serum (BD Vacutainer® SST II Advanced tubes) and plasma (BD Vacutainer® EDTA tubes) were obtained by centrifugation in all studies and stored at -80°C until further analysis.

To measure relative levels of circulating inflammation-related proteins in patients with T2DM, the commercially available protein biomarker panel "Target 96 Inflammation" from Olink proteomics (Olink Bioscience, Uppsala, Sweden) was used. Olink Proteomics performed quality control; samples were excluded when their incubation and detection control deviated by more than ± 0.3 Normalized Protein Expression (NPX) from the plate median (21). This resulted in the exclusion of 1 sample. 73 of the 96 (i.e., 76%) proteins were detected in at least 75% of the plasma samples included in the analysis.

Plasma levels of total cholesterol, high-density lipoprotein-cholesterol (HDL-C), triglycerides, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured on a Modular P800 analyzer (Roche Diagnostic, Mannheim, Germany) for the patients with T2DM. Low-density lipoprotein-cholesterol (LDL-C) was calculated according to the Friedewald formula (22). HbA_{1c} was initially measured with boronate-affinity high-performance liquid chromatography (Primus Ultra; Siemens Healthcare Diagnostics, Breda, the Netherlands) due to logistical reasons and later with ion-exchange high-performance liquid chromatography (Tosoh G8, Sysmex Nederland B.V., Etten-Leur, the Netherlands). To ensure accurate and consistent results, HbA_{1c} levels obtained from the boronate affinity method were corrected based on the correlation coefficient obtained from validation samples measured on both analyzers. Plasma CRP concentrations were measured on a Roche Modular analyzer (Roche Diagnostics). Serum fibroblast growth factor 21 (FGF21) concentrations in samples from all three

trials were measured using the human FGF21 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

MRI

The patients with T2DM underwent an MRI in the supine position at baseline, using a 3.0 Tesla MRI scanner (Ingenia, Philips Healthcare, Best, The Netherlands) to assess visceral, abdominal adipose tissue volumes and HTGC, as extensively described previously (18).

Statistical analysis

Data are expressed as mean ± standard deviation. The normality of data was confirmed using the Shapiro-Wilk test, visual histograms, and Q-Q plots. Baseline characteristics for the patients with T2DM were compared between ethnicities and sexes using a Chi-square test for binary values (i.e., use of diabetic medication) and an independent t-test for normally distributed data. Not normally distributed data were log10 transformed (i.e., subcutaneous adipose tissue, visceral adipose tissue, visceral/subcutaneous adipose tissue ratio, total cholesterol, triglycerides, and CRP). Non-parametric tests were performed on data not normally distributed after log 10 transformation (i.e., age, diabetes duration, body fat percentage, HTGC, HbA_{1c}, LDL-C, AST, ALT, and metformin dose).

Olink data were analyzed using the Mann-Whitney U test to determine the difference in relative plasma levels of inflammation-related proteins between ethnicities. All proteomic analyses were corrected for multiple testing using Benjamin-Hochberg's false discovery rate (FDR). FDR corrected p-value (i.e., q-value) was set at < 0.05. Volcano plots were constructed by calculating the fold change (FC) between South Asians and Europids on a log2 scale. From here on, all data was split for both sexes.

To study the difference between serum FGF21 concentrations between ethnicities, an independent t-test was performed with log10 transformed data. To determine whether the observed differences in FGF21 levels between ethnic groups were attributable to baseline phenotypical characteristics of the two cohorts, we conducted sensitivity analyses (**Supplemental Table 2**). In these analyses, we examined FGF21 level differences between ethnicities while adjusting separately for age, BMI, waist circumference, VAT, HTGC, TC, LDL-C, AST, ALT, metformin dose, and T2DM duration. The adjustments did not alter the results, indicating that the observed differences in FGF21 levels were not explained by these variables. Furthermore, nonparametric Spearman-rank correlations (rho) were applied to examine the association between relative plasma FGF21 levels from the Olink database and relative plasma IFN-gamma

levels, HTCG, and serum triglycerides and between serum FGF21 concentrations with serum CRP, HTGC, and serum triglycerides, as the data were not normally distributed.

The statistical analysis of the baseline characteristics of the cohort without T2DM has been extensively described elsewhere (23). To compare serum FGF21 concentrations between ethnicities, an independent t-test was performed with log10 transformed data of baseline values to attain a normal distribution. All statistical analyses of the Olink database were performed using RStudio (version 4.3.2, 2023), and other statistical analyses were performed using Statistical Package for the Social Sciences v.29.0.1.0. Armonk, NY: IBM Corp.). All graphs were created with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Significance for the analysis of the difference in relative plasma levels of inflammation-related proteins between ethnicities was set at $q < 0.05$ and for all other analyses at $P < 0.05$.

RESULTS

Baseline characteristics

At baseline, significant differences were seen in the characteristics between the South Asians and Europids with T2DM, as described previously (**Supplemental Table 1**) (17, 18). In short, South Asians exhibited a lower age ($P = 0.012$), weight ($P < 0.001$), length ($P < 0.001$), BMI ($P = 0.002$), waist circumference ($P < 0.001$), waist-to-hip ratio ($P < 0.001$), visceral adipose tissue volume ($P = 0.006$), HTGC ($P < 0.001$), total cholesterol ($P = 0.003$), LDL-C ($P = 0.003$), AST ($P < 0.001$) and dose of metformin ($P = 0.037$) compared to Europids. On the other hand, South Asians had a longer duration of diabetes ($P < 0.001$) and higher levels of ALT ($P < 0.001$) than their Europid counterparts. When splitting the groups per sex, the differences in body composition and clinical parameters between South Asians and Europids persisted (**Supplemental Table 1**).

Relative plasma levels of inflammation-related proteins differ between South Asians versus Europids with T2DM.

Relative plasma levels of six inflammation-related proteins were higher in South Asians compared to Europids with T2DM (i.e., stem cell factor (SCF), caspase-8 (CASP-8), C-C motif chemokine ligand 28 (CCL28), interferon-gamma (IFN-gamma), sulfotransferase 1A1 (ST1A1), cystatin D (CST5); fold change > 0.27, $q < 0.05$; **Fig. 1A**). Also, relative plasma levels of six inflammation-related proteins were lower in South Asians compared to Europids (i.e., FGF21, human fibroblast collagenase (MMP-1), interferon-8 (IL-8), C-C motif chemokine ligand 4 (CCL4), C-X-C motif chemokine ligand 6 (CXCL6), monocyte chemoattractant protein-1 (MCP-1); fold change < -0.24, $q < 0.05$; **Fig. 1A**). When splitting the data per sex, the effects appeared to be specific for females, since no significant

differences in relative plasma levels were found in South Asian versus Europid males (**Fig. 1B)**. In contrast, in South Asian versus Europid females, relative plasma levels of two inflammation-related proteins were higher (i.e., ST1A1, IFN-gamma; fold change > 0.74, q < 0.04) while relative plasma levels of six proteins were lower (i.e., FGF21, MCP-1, MMP-1, IL-8, vascular endothelial growth factor A (VEGFA), CXCL6; fold change < -0.28, q < 0.04; **Fig. 1C**).

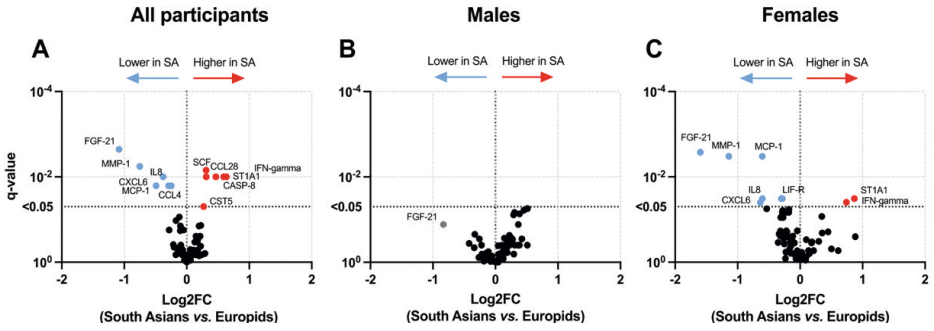


Figure 1. Comparison of plasma levels of inflammation-related proteins in South Asian compared to Europid males and females with type 2 diabetes mellitus.
Volcano plot showing the comparison of relative plasma levels of 73 inflammation-related proteins in all South Asians (SA) (n=47) compared to all Europids (EU) (n=48) with type 2 diabetes mellitus **(A)**. Additionally, comparisons are shown for South Asian (n=19) versus Europid (n=27) males with type 2 diabetes mellitus **(B)** and for South Asian (n=28) versus Europid (n=20) females with type 2 diabetes mellitus **(C)**. The x-axes show the log2 fold change (log2FC) between South Asians and Europids; the y-axes show the q-value on a log scale. P-values were obtained from a Wilcox-U test and then corrected using Benjamin-Hochberg's false discovery rate to yield q-values. Red circles represent significantly higher relative protein levels in South Asians, and blue circles represent significantly lower relative protein levels in South Asians, compared to Europids (q < 0.05). The value of one Europid male was excluded due to failure of the Quality Control of Olink, one Europid male was excluded as insufficient plasma was available to perform protein analysis, and data of 23 proteins were omitted from the 96-inflammation panel because their levels were below the detection limit in more than 75% of the samples.

Circulating FGF21 concentrations are significantly lower in South Asians versus Europids with T2DM.

Of note, of the 73 inflammation-related proteins measured, the relative plasma level of FGF21 differed most between South Asians and Europids with T2DM. The relative plasma level of FGF21 was lower in South Asians compared to Europids with T2DM, both in males and females combined (fold change -1.08; q = 0.002, **Fig 1A**) and in females (fold change -1.60; q = 0.003; **Fig 1C, Supplementary Fig. 1B**). No significant difference in relative plasma level of FGF21 was observed between South Asian males compared to Europid males with T2DM (fold change -0.82; q = 0.120; **Fig 1B, Supplementary Fig. 1A**).

To validate this finding, we next quantified FGF21 concentrations in the serum of the same cohort of South Asians and Europids with T2DM by ELISA. A strong correlation was found between relative plasma levels of FGF21 and serum FGF21 concentration (rho > 0.860; P < 0.001) (data not shown). Similarly to the relative plasma levels, serum FGF21 concentrations were lower in South Asians compared to Europids with T2DM (215 ± 136 pg/ml *vs.* 420 ± 337 pg/ml; -48.8%; P < 0.001; **Fig. 2A**). In addition, we now also observed lower serum FGF21 concentrations in South Asian compared to Europid males (182 ± 100 pg/mL *vs.* 315 ± 249 pg/mL; -42.2%; P = 0.020; **Fig. 2B**) and in females (238 ± 154 pg/mL *vs.* 574 ± 393 pg/mL; -58.5%; P < 0.001; **Fig. 2C**). Due to significant differences in baseline characteristics, we repeated the analysis with adjustments for potential confounders. However, this did not affect the results (**Supplemental Table 2**).

When measuring FGF21 concentrations in the serum of males without T2DM, serum FGF21 levels were generally lower compared to the males with T2DM, and without differences between South Asians and Europids (83 ± 58 pg/mL *vs.* 96 ± 76 pg/mL; -13.1%; P = 0.675; **Supplementary Fig. 2**).

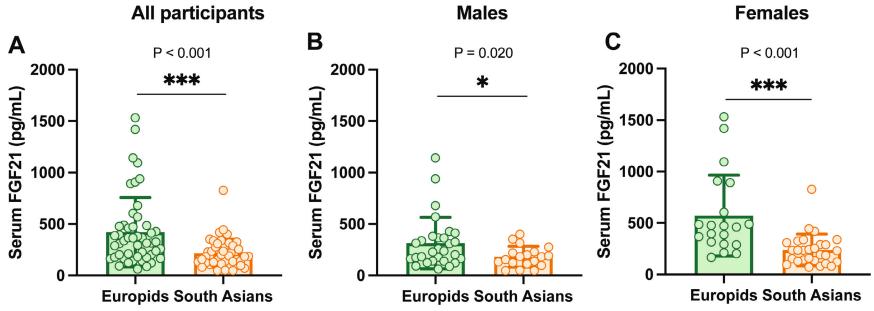


Figure 2. Comparison of serum fibroblast growth factor 21 concentration in South Asian and Europid males and females with type 2 diabetes mellitus.
Box plots showing serum concentration of fibroblast growth factor 21 (FGF21) in all South Asians (n=47, orange circles) compared to all Europids (n=49, green circles) with type 2 diabetes mellitus **(A).** In addition, FGF21 concentrations are shown for South Asian (n=19, orange circles) compared to Europid (n=29, green circles) males **(B)** and South Asian (n=28, orange circles) compared to Europid (n=20, green circles) females **(C)**. Circles represent individual values, boxes represent means, and deviations represent standard deviations.

Circulating FGF21 does not correlate with inflammation markers in both sexes.

Since we previously showed that South Asians have a more activated IFN-signaling pathway compared to Europids with T2DM (16) and FGF21 is known for its anti-inflammatory properties in the context of T2DM (24), we next assessed whether relative FGF21 levels and circulating FGF21 concentration were related to inflammation markers. First, we performed correlations with IFN-gamma levels. However, we did not find a significant correlation between relative plasma levels of FGF21 and IFN-gamma in South Asian males (rho = 0.088, P = 0.721) and Europid males (rho = -0.318, P = 0.106) (**Supplementary Fig. 3A**), or in South Asian females (rho = -0.171, P = 0.385) and Europid females (rho = 0.268, P = 0.254) (**Supplementary Fig. 3C**). In addition, we did not find a significant correlation between serum FGF21 concentrations and plasma CRP levels in South Asian males (rho = 0.125, P = 0.632) and Europid males (rho = 0.141, P = 0.466) (**Supplementary Fig. 3A**) or in South Asian females (rho = 0.034, P = 0.866) and Europid females (rho = -0.056, P = 0.819) (**Supplementary Fig. 3B**).

FGF21 relative levels and concentration positively correlate with serum triglycerides and hepatic triglyceride content in South Asians with T2DM

FGF21 is mainly synthesized by the liver and is known to play a role in lipid metabolism. (25) Therefore, we next assessed whether relative plasma FGF21 levels and serum FGF21 concentrations were related to HTGC and serum triglyceride levels. We found that relative plasma FGF21 levels tended to be positively associated with HTGC in South Asian males (rho = 0.486, P = 0.035; **Supplementary Fig. 4A**) and were significantly positively related to HTGC in South Asian females (rho = 0.460, P = 0.014; **Supplementary Fig. 4C**). However, relative plasma FGF21 levels did not correlate with HTCG in both Europid males (rho = 0.155, P = 0.458; **Supplementary Fig. 4A**) and females (rho = 0.368, P = 0.110; **Supplementary Fig 4B**). Of note, relative plasma FGF21 levels were positively correlated with serum triglycerides in South Asian males (rho = 0.639, P = 0.003; **Supplementary Fig. 4B**) and females (rho = 0.528, P = 0.004; **Supplementary Fig. 4D**). Relative plasma FGF21 levels did tend to positively relate to serum triglycerides in Europid males (rho = 0.358, P = 0.067, **Supplementary Fig. 4B**). However, no such correlation was found in Europid females (rho = 0.224, P = 0.342, **Supplementary Fig. 4D**). Comparable results were found for serum FGF21 concentrations with both serum triglycerides and HTCG levels (**Supplementary Fig. 5**).

DISCUSSION

In this study, we compared the relative plasma levels of 73 inflammation-related proteins between South Asians and Europids with T2DM. Relative plasma levels of six inflammation-related proteins were higher, and relative plasma levels of six proteins were lower in South Asians compared to Europids with T2DM. FGF21 was the most distinctive of all inflammation-related proteins measured and was lower in South Asians compared to Europid females with T2DM. We could validate this finding by measuring circulating serum FGF21 concentrations and observed lower concentrations in both male and female South Asians compared to Europids with T2DM. However, serum FGF21 concentrations did not differ in healthy South Asian versus Europid males, suggesting that the difference in FGF21 levels between ethnicities develops later in life. Furthermore, we found a tendency towards a positive correlation between relative plasma FGF21 levels, serum FGF21 concentration, and both hepatic triglycerides and circulating triglycerides in especially South Asians with T2DM.

Among the six inflammation-related proteins with higher relative levels in South Asians (i.e., SCF, CASP-8, CCL28, IFN-gamma, ST1A1, and CST5), all proteins are described in pro-inflammatory pathways (26-31). Additionally, our findings align with our previous research showing higher mRNA levels of B-cell markers and interferon signaling genes, indicating a more activated IFN signaling pathway at the gene level in the blood of South Asians compared to Europids with T2DM (16). In this study, we replicate these findings at the protein level, showing a higher relative protein level of IFN-gamma and ST1A1, a central IFN-gamma intracellular mediator, in South Asians compared to Europids. Given the potential role of IFN-gamma in inducing insulin resistance in metabolic tissues, these findings may at least in part underly the increased insulin resistance among South Asians compared to Europids (30).

Significant differences in relative levels of inflammation-related proteins between South Asians and Europids were only found in females. However, serum FGF21 concentrations were significantly different in both males and females. Given that we assessed 73 inflammatory-related proteins simultaneously, we adjusted our statical analysis to account for multiple comparisons by controlling for the false discovery rate. Consequently, it is possible that, if we had measured solely FGF21 protein in plasma, we might also have a significant difference in FGF21 among males. Furthermore, the substantial difference in relative protein levels between South Asians and Europids in females only also suggests a potential influence of sex hormones on inflammation-related protein levels. The role of sex hormones on inflammation is a known factor (32, 33). Females exhibit higher inflammatory markers (C-reactive protein, tumor necrosis

factor-alpha, and interleukin 6) compared to males during their reproductive years and variations in inflammatory markers throughout the menstrual cycle (34, 35). Furthermore, pro-inflammatory markers are typically increased in postmenopausal females (36). In addition, sex hormones contribute to differences in body composition, particularly in fat distribution between males and females (37). Unfortunately, we have not measured sex hormones in this population nor have information on the menstrual cycles, use of hormonal anticonception, or menopausal status in this population. However, in this study, both South Asian and Europid females with T2DM had significantly higher body fat percentages compared to the males. Higher fat percentage is positively associated with more inflammatory markers (38), which could contribute to the significant differences in relative protein levels between females but not males of both ethnicities.

Serum FGF21 concentrations were lower both in male and female South Asians compared to Europids with T2DM. FGF21 is an essential mediator of lipid metabolism by regulating lipolysis in white adipose tissue and increasing substrate utilization by increasing fatty acid oxidation in the liver (39, 40). It is mainly released from the liver and adipose tissue upon different metabolic stressors on the body, such as fasting, cold exposure, and overfeeding (41). In addition, FGF21 has anti-inflammatory properties (42). People with obesity have a higher concentration of FGF21 than healthy people (43), likely in response to the increased metabolic stress on the body. Despite this, in Europids, 48 weeks of treatment with the FGF21 analog Pegbelfermin improved signs of metabolic dysfunction-associated steatohepatitis (MASH) (44). The increased FGF21 concentration in people living with obesity could indicate a compensatory mechanism for the possible reduction of FGF21 sensitivity in obesity. In our study, serum FGF21 concentration was significantly lower in South Asians compared to Europids with T2DM. This could indicate that South Asians may not adequately compensate for this reduced sensitivity by increasing the FGF21 levels, potentially contributing to their increased risk of developing obesity-associated complications, including T2DM. Furthermore, exogenous FGF21 treatment could hold promise as a potential preventative and therapeutical option for obesity-associated complications in South Asians. Alternatively, since South Asians are known to exhibit higher inflammation compared to Europids, lower FGF21 concentration observed in South Asians could be a compensatory response to the increased inflammation in this population (16, 45). However, we did not find a significant negative correlation between the circulating FGF21 concentrations and pro-inflammatory markers CRP and IFN-gamma. Given the complex mechanisms regulating FGF21, this does not rule out the possibility of a compensatory mechanism to increased inflammation. We did not observe significant differences in serum FGF21 levels between young South Asians and Europids without T2DM, suggesting that the

differences observed in individuals with T2DM may contribute to the development of metabolic diseases. However, potential (epi-)genetic factors influencing the South Asian phenotype later in life cannot be ruled out. Therefore, further research is necessary to identify the potential underlying factors. Measuring circulating FGF21 levels in larger groups of lean individuals without metabolic disease or in individuals with obesity and pre-diabetes could provide more insight into its potential relationship with the development of T2DM.

South Asians are known to develop T2DM at a lower BMI and younger age compared to Europids. This pattern is consistent with our study population as BMI and waist circumference were lower in the South Asians compared to Europids, while their diabetes duration was more prolonged. A recent study analyzed a panel of inflammation-related proteins among people living with obesity, with and without metabolic syndrome. They found a significant upregulation of FGF21 among those with both obesity and metabolic syndrome compared to people living with obesity without metabolic syndrome (46). Given that South Asians in our study had lower total cholesterol and hepatic triglyceride content than Europids, we cannot exclude that they were less metabolically compromised than Europids, potentially explaining their lower FGF21 concentration.

On the other hand, despite the lower HTGC in South Asians, the hepatic stress marker ALT was significantly elevated compared to Europids. This suggests that despite the lower HTGC, South Asians may still have elevated (metabolic) stress on the liver. Additionally, South Asians had a significantly longer duration of T2DM and consequently (beneficial) treatment, which might have impacted HTGC. Therefore, while the lower HTGC observed in the South Asian population could explain the lower FGF21 concentration compared to Europids, elevated liver stress indicated by higher ALT levels could suggest that other factors may contribute to the lower FGF21 concentration observed.

One of the strengths of our study is the extensive analysis of inflammation-related proteins in a large sample of males and females of two ethnicities, allowing us to identify differences between ethnicities objectively. In addition, our population's almost equal distribution of sexes will enable us to explore potential sex differences. However, our study is not without limitations. Due to differences in the metabolic phenotype in South Asians compared to Europids, matching both ethnic groups remains difficult. In addition, we have only blood samples to provide information about inflammation. Ideally, we would have measured inflammation proteins in metabolically active tissues, like the liver and white adipose tissue. This would allow us to localize the assessment of the sources of inflammation.

In conclusion, we showed that of the 73 measurable inflammation-related proteins, the relative plasma levels of 12 proteins were significantly different in South Asians compared to Europids with T2DM, with FGF21 being the most prominent concerning effect size. This observation was further supported by the finding of lower serum FGF21 concentration in South Asians compared to Europids with T2DM. The difference in FGF21 between these ethnicities warrants further tissue-specific studies, given its potential as a metabolic target.

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Supplemental Table 1. Baseline characteristics

	Europids		South Asians		Europids	South Asians
	Males (n=29)	Females (n=20)	Males (n=19)	Females (n=28)	Combined (n=49)	Combined (n=47)
Demographics						
Age, years	60.8 ± 6.3	57.7 ± 6.5	56.0 ± 9.9	54.1 ± 10.4	59.6 ± 6.5	54.9 ± 10.1$^{\#}$
Diabetes duration, years	12.6 ± 7.6	8.9 ± 4.5	18.3 ± 11.1	17.6 ± 9.3**	11.1 ± 6.7	17.9 ± 10.0$^{\#\#\#}$
Clinical parameters						
Body weight, kg	97.1 ± 13.1	95.2 ± 14.2	85.4 ± 11.1**	75.9 ± 10.8***	96.3 ± 13.4	79.7 ± 11.8$^{\#\#\#}$
Body length, cm	178.7 ± 5.2	165.6 ± 7.1	172.9 ± 7.0**	159.0 ± 4.1***	173.3 ± 8.8	164.6 ± 8.7$^{\#\#\#}$
BMI, kg/m^2	30.3 ± 3.0	34.6 ± 3.6	28.6 ± 3.9	30.0 ± 4.0***	32.1 ± 3.9	29.5 ± 4.0$^{\#\#\#}$
Waist circumference, cm	108.8 ± 8.3	111.9 ± 10.3	102.5 ± 7.9*	100.0 ± 10.4***	110.1 ± 9.2	101.0 ± 9.5$^{\#\#\#}$
Hip circumference, cm	104.0 ± 6.1	112.0 ± 6.8	100.8 ± 7.0	106.4 ± 8.1*	107.2 ± 7.5	104.1 ± 8.1
Waist-to-hip ratio	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1**	1.0 ± 0.1	1.0 ± 0.1$^{\#\#\#}$
Body fat percentage, %	29.3 ± 3.7	46.4 ± 5.0	27.7 ± 4.2	43.1 ± 5.4**	36.4 ± 9.5	37.1 ± 9.1
Subcutaneous adipose tissue, cm^2	276 ± 91	442 ± 97	283 ± 107	347 ± 126**	344 ±124	321 ± 121
Visceral adipose tissue, cm^2	211 ± 64	197 ± 89	169 ± 49	165 ± 61	206 ± 75	166 ± 56$^{\#\#}$
Visceral/subcutaneous adipose tissue ratio	0.8 ± 0.3	0.5 ± 0.2	0.7 ± 0.3	0.5 ± 0.3	0.7 ± 0.3	0.6 ± 0.3
Hepatic Triglyceride Content, %	14.6 ± 8.7	23.2 ± 10.2	8.7 ± 6.4*	10.1 ± 10.9***	18.3 ± 10.2	9.5 ± 9.3$^{\#\#\#}$
HbA_{1c}, mmol/mol	67.4 ± 10.5	63.1 ± 10.8	68.7 ± 11.9	67.2 ± 11.0	65.6 ± 10.7	67.8 ± 11.3
Total cholesterol, mmol/L	4.6 ± 1.0	5.1 ± 1.0	4.0 ± 1.0*	4.4 ± 0.9**	4.8 ± 1.0	4.2 ± 0.9$^{\#\#}$
HDL-C, mmol/L	1.2 ± 0.3	1.3 ± 0.3	1.1 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.3
LDL-C, mmol/L	1.2 ± 0.3	2.8 ± 0.9	1.9 ± 0.8*	2.3 ± 0.8*	2.6 ± 0.9	2.1 ± 0.8$^{\#\#}$
Triglyceride, mmol/L	2.1 ± 1.5	2.2 ± 0.8	2.0 ± 2.0	1.7 ± 1.0*	2.1 ± 1.3	1.8 ± 1.4
AST, IU/L	28.7 ± 8.6	40.1 ± 22.7	26.7 ± 12.6	20.4 ± 5.9***	33.4 ± 16.7	22.9 ± 9.6$^{\#\#\#}$
ALT, IU/L	13.3 ± 5.5	14.6 ± 6.3	29.4 ± 21.3***	20.9 ± 9.1**	13.9 ± 5.8	24.3 ± 15.6$^{\#\#\#}$
CRP, mg/L	2.3 ± 1.8	3.1 ± 2.1	2.2 ± 2.0	4.9 ± 4.6	2.6 ± 1.9	3.8 ± 4.0

Supplemental Table 1. Baseline characteristics (continued)

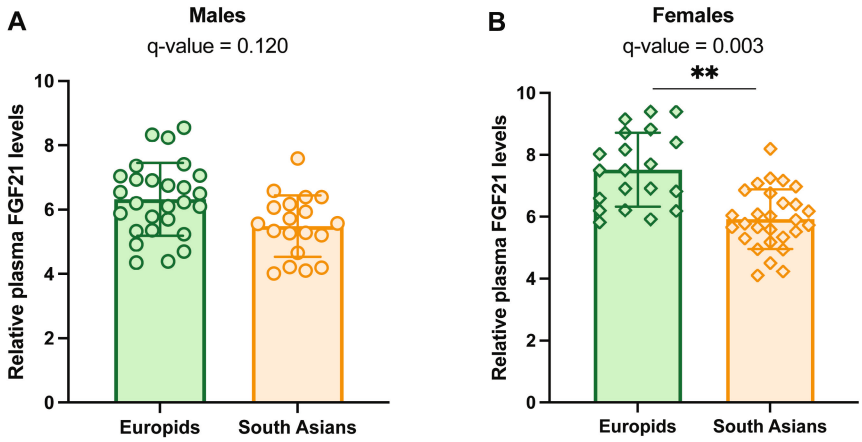
	Europids		South Asians		Europids	South Asians
	Males (n=29)	**Females (n=20)**	**Males (n=19)**	**Females (n=28)**	**Combined (n=49)**	**Combined (n=47)**
Diabetes medication						
Metformin use, n, %	*n*=29, 100%	*n*=20, 100%	*n*=18, 95%	*n*=27, 96%	*n*=49, 100%	*n*=45, 96%
Metformin, mg/day	2012 ± 627	2038 ± 609	1808 ± 694	1693 ± 622	2022 ± 613	1739 ± 646$^{\#}$
Sulfonylurea, n, %	*n*=6, 21%	*n*=8, 40%	*n*=2, 11%	*n*=6, 21%	*n*=14, 29%	*n*=8, 17%
Insulin use, n, %	*n*=19, 66%	*n*=13, 65%	*n*=14, 74%	*n*=22, 79%	*n*=32, 65%	*n*=36, 77%

Data adapted from the original data of South Asian (1) and Europid (2) individuals with type 2 diabetes mellitus. Data are presented as mean ± standard deviation. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; HbA_{1c}, hemoglobin A1c; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; Asterisk signs (*) indicate significant differences between ethnicities within a specific sex group *P < 0.05, **P < 0.01, ***P < 0.001. Hash sign ($^{\#}$) indicates significant differences between ethnicities when both sex groups are combined $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001.

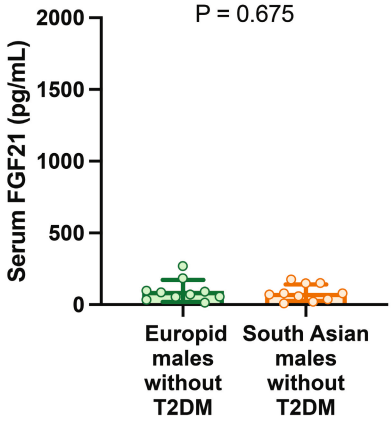
Supplemental Table 2. Differences in serum FGF21 concentrations between South Asians and Europids corrected for baseline values

	Males	Females	Combined
Adjusting for:			
Age	P = 0.075	P < 0.001	P < 0.001
BMI	P = 0.020	P < 0.001	P < 0.001
Waist circumference	P = 0.098	P = 0.002	P = 0.004
Visceral adipose tissue	P = 0.131	P < 0.001	P = 0.002
Hepatic Triglyceride Content	P = 0.072	P = 0.015	P = 0.013
Total cholesterol	P = 0.041	P < 0.001	P = 0.001
LDL-C	P = 0.006	P < 0.001	P < 0.001
AST	P = 0.027	P = 0.049	P = 0.009
Dose of metformin	P = 0.052	P < 0.001	P < 0.001
Duration T2DM	P = 0.013	P = 0.002	P < 0.001
ALT	P = 0.084	P < 0.001	P < 0.001

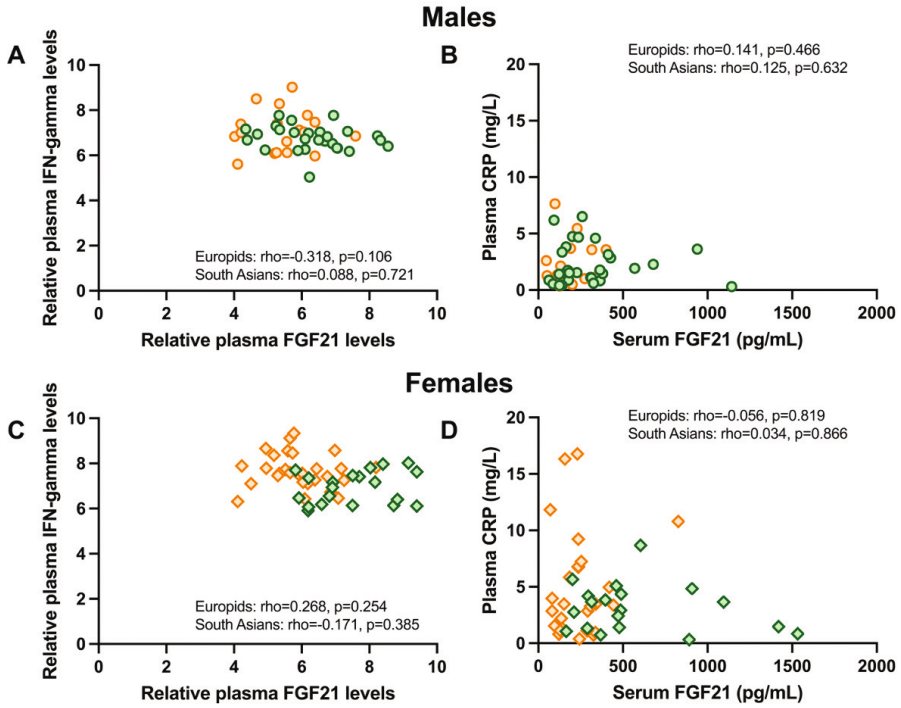
P values of the differences in serum FGF21 concentrations between South Asian males, females and combined compared to Europids corrected for the baseline characteristics that were significantly different between the two ethnicities. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; LDL-C, low-density lipoprotein-cholesterol; T2DM, type 2 diabetes mellitus.



Supplementary Figure 1. Comparison of relative plasma levels of fibroblast growth factor-21 in South Asian and Europid males and females with type 2 diabetes mellitus.
Box plots showing relative plasma levels of fibroblast growth factor 21 (FGF21) in South Asian (n=19; orange circles) versus Europid (n=27; green circles) males with type 2 diabetes mellitus (**A**) and in South Asian (n=28; orange diamonds) versus Europid (n=20; green diamonds) females with type 2 diabetes mellitus (**B**). The value of one Europid male was excluded due to failure of the Quality Control of Olink; one Europid male was excluded as insufficient plasma was available to perform the protein analysis. Circles and diamonds represent individual values; boxes represent means, and deviations represent standard deviations.

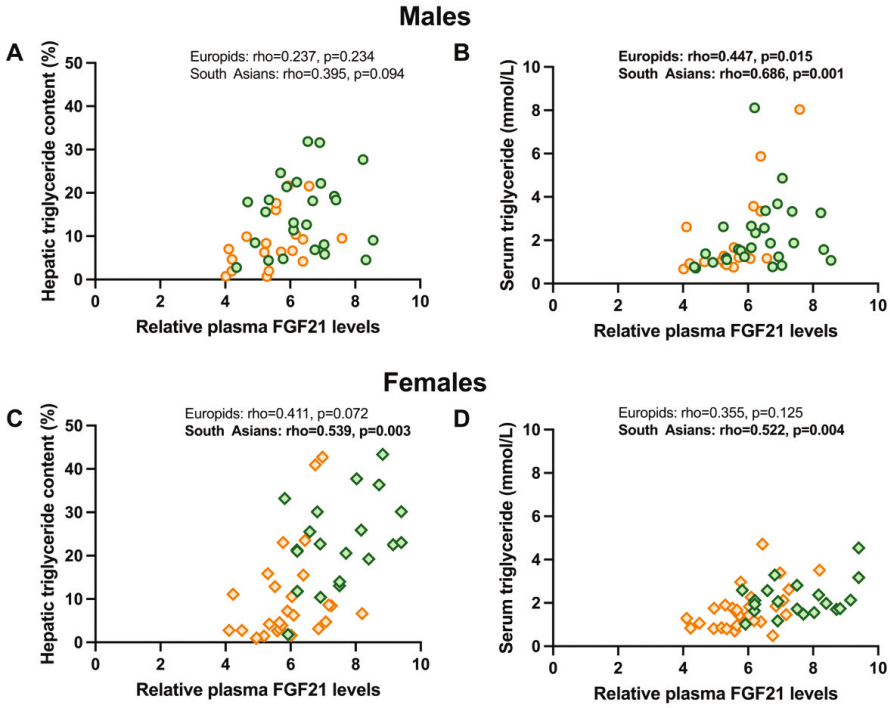


Supplementary Figure 2. Comparison of fibroblast growth factor-21 serum concentration in South Asians and Europid males without type 2 diabetes mellitus.
Box plots showing serum concentration of fibroblast growth factor 21 (FGF21) in South Asian males (n=10, orange circles) compared to Europid males (n=10, green circles) without T2DM. Circles represent individual values, boxes represent means, and deviations represent standard deviations.



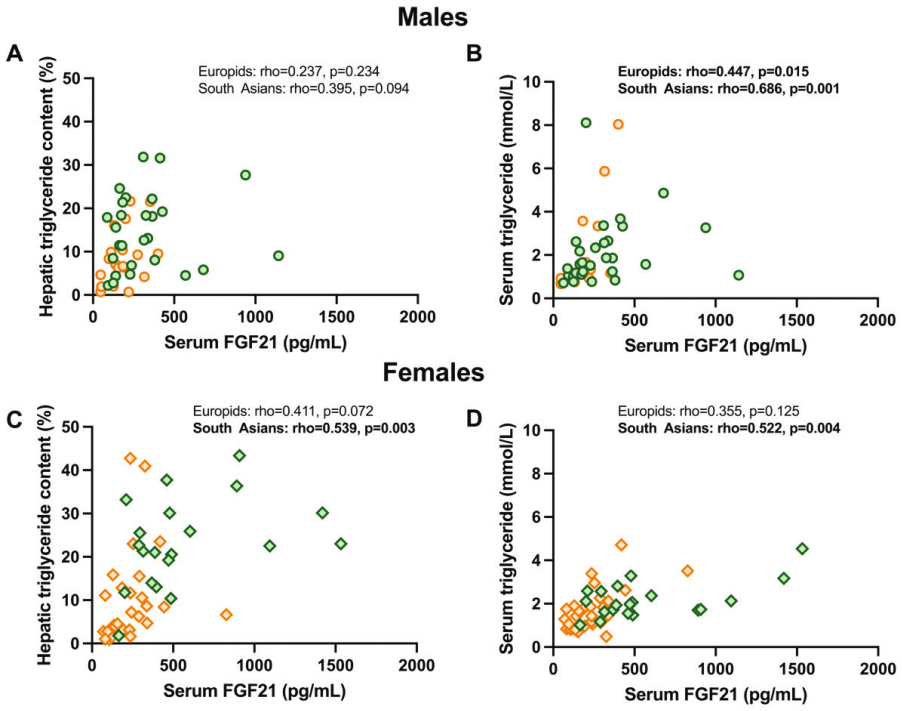
Supplementary Figure 3. Correlations between relative plasma levels of fibroblast growth factor 21 and interferon-gamma and serum fibroblast growth factor 21 concentrations and plasma C-reactive protein concentrations in South Asian compared to Europid males and females with type 2 diabetes mellitus.

Spearman correlations, in both South Asians and Europids, between relative plasma levels of fibroblast growth factor 21 (FGF21) and interferon-gamma (IFN-gamma) in South Asian (n=19, orange circles) and Europid (n=27, green circles) males with type 2 diabetes mellitus **(A)** and in South Asian (n=28, orange diamonds) and Europid (n=20, green diamonds) females with type 2 diabetes mellitus **(C)**. Spearman correlation of serum FGF21 concentration and plasma concentration of C-reactive protein (CRP) in South Asian (n=17, orange circles) and Europid (n=29, green circles) males with type 2 diabetes mellitus **(B)** and in South Asian (n=27, orange diamonds) and Europid (n=19, green diamonds) females with type 2 diabetes mellitus **(D)**. The value of one Europid male was excluded due to failure of the Quality Control of Olink; one Europid male was excluded as insufficient plasma was available to perform the protein analysis. Two South Asian males had plasma CRP levels below the detection limit, one South Asian female had insufficient plasma material for CRP analysis, and one Europid female had serum CRP levels out of range and therefore excluded.



Supplementary Figure 4. Correlations between relative plasma fibroblast growth factor 21 levels, hepatic triglyceride content, and serum triglyceride concentration in South Asian compared to Europid males and females with T2DM.

Spearman correlations, in both South Asians and Europids, between relative plasma Fibroblast Growth Factor 21 (FGF21) levels and hepatic triglyceride content (HTGC) in South Asian (n=19, orange circles) and Europid (n=25, green circles) males with type 2 diabetes mellitus **(A)** and in South Asian (n=28, orange diamonds) and Europid (n=20, green diamonds) females with type 2 diabetes mellitus **(C)**. Spearman correlation of relative plasma FGF21 levels and serum triglyceride concentration in South Asian (n=19, orange circles) and Europid (n=27, green circles) males with type 2 diabetes mellitus **(B)** and in South Asian (n=28, orange diamonds) and Europid (n=20, green diamonds) females with type 2 diabetes mellitus **(D)**. The hepatic triglyceride content was missing for two Europid males due to technically unsuccessful ^{1}H-MRS of the liver. The value of one Europid male was excluded due to failure of the Quality Control of Olink; one Europid male was excluded as insufficient plasma was available to perform protein analysis.



Supplementary Figure 5. Correlations between serum fibroblast growth factor 21 concentration, hepatic triglyceride content, and serum triglyceride concentration in South Asian compared to Europid males and females with T2DM.

Spearman correlations, in both South Asians and Europids, between serum Fibroblast Growth Factor 21 (FGF21) concentration and hepatic triglyceride content (HTGC) in South Asian (n=19, orange circles) and Europid (n=27, green circles) males with type 2 diabetes mellitus **(A)** and in South Asian (n=28, orange diamonds) and Europid (n=20, green diamonds) females with type 2 diabetes mellitus **(C)**. Spearman correlation of serum FGF21 concentration and serum triglyceride concentration in South Asian (n=19, orange circles) and Europid (n=29, green circles) males with type 2 diabetes mellitus **(B)** and in South Asian (n=28, orange diamonds) and Europid (n=20, green diamonds) females with type 2 diabetes mellitus **(D)**. The hepatic triglyceride content was missing for two Europid males due to technically unsuccessful ^{1}H-MRS of the liver.

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CHAPTER 3

GLP-1, GIP, AND GLUCAGON EXCURSIONS DURING A MIXED MEAL TOLERANCE TEST IN YOUNG AND LEAN SOUTH ASIANS VERSUS EUROPIDS

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Submitted

ABSTRACT

Objectives

South Asians exhibit an unfavorable metabolic phenotype characterized by visceral obesity, insulin resistance and dyslipidemia. Since various hormones play a critical role in regulating energy metabolism, we aimed to study the meal-induced excursion of incretin hormones and glucagon in South Asians and Europids.

Method

49 young, lean South Asian (n=24), and Europid (n=25) males and females underwent an extended (up to 240 min) mixed meal tolerance test (MMTT). At seven time points circulating incretins (active and total GLP-1 and GIP), glucagon, and parameters related to glucose and lipid metabolism were measured.

Results

While a single peak (t=30 min) in circulating glucose levels was observed in Europids, a biphasic peak (t=30 and t=90 min) was found in South Asian males and females. In addition, South Asian males exhibited an increased insulin response, with elevated levels at the corresponding glucose peaks. On the other hand, South Asian females demonstrated a drop in circulating glucagon at t=90 min, and double peaks of total and active GLP-1 and GIP (t=30 and t=120 min). Postprandial lipid excursions did not differ between ethnicities.

Conclusion

South Asians respond to an MMTT with a biphasic peak in glucose levels, without differences in postprandial lipid excursions. Potentially as a consequence, female South Asians demonstrated a second peak of active GLP-1 and GIP and a drop in glucagon. Interestingly, an increased insulin response was only observed in South Asian males. We speculate that these effects result from biphasic gastric emptying in South Asians.

INTRODUCTION

Obesity is defined by the World Health Organization as abnormal or excessive fat accumulation that presents a health risk (1). Currently, 16 percent of adults are living with obesity worldwide (1). This number has more than doubled since 1980 and is expected to continue to rise (1, 2). Obesity not only impacts health directly but also increases the risk of various obesity-related diseases, such as type 2 diabetes mellitus (T2DM), cardiovascular diseases, and various forms of cancer (3, 4). Therefore, understanding the underlying mechanisms of obesity development is important to effectively prevent and treat obesity.

Certain ethnic populations, such as the South Asian population, are more prone to develop obesity and obesity-related diseases. For instance, South Asians have a fourfold increased risk of developing T2DM compared to other ethnicities (i.e., Whites, Chinese Americans, African Americans, and Hispanics) (5). In addition, they develop obesity-related diseases at a younger age and a lower BMI than Europids (6, 7). The underlying mechanisms for their increased risk to develop T2DM are only partly known. South Asians exhibit an unfavorable metabolic phenotype characterized by higher fat mass, especially in visceral and ectopic areas, lower muscle mass, and dyslipidemia (8). They typically also show insulin resistance, which is already observable at birth (9-12). Furthermore, they have lower resting energy expenditure, and brown adipose tissue volume, which may further contribute to increased fat storage (13).

While various metabolic hormones released by the gastrointestinal tract play a role in regulating energy balance (e.g., satiety and energy expenditure) and insulin sensitivity, their potentially different regulation in South Asians versus Europids has not been studied in detail. Incretin hormones, such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), secreted by the intestinal L-cells and K-cells, respectively, are important mediators in lowering postprandial glucose levels. These hormones bind to their respective receptors (GLP-1R and GIPR) on the beta cells in the pancreas (14-16), and promote the release of insulin in a glucose-dependent manner (17). Both GLP-1 and GIP also induce satiety in the hypothalamus and hindbrain (15, 17, 18). Glucagon is released from the alpha cells of the pancreas in response to a drop in circulating glucose levels. It regulates glucose homeostasis by stimulating glycogenolysis and gluconeogenesis, with the aim to increase plasma glucose levels. Furthermore, it has been shown to contribute to energy balance by increasing energy expenditure (19). A novel therapeutical intervention for the treatment of obesity and T2DM is based on the potential of GLP-1, GIP, and glucagon to concomitantly induce satiety and increase energy expenditure (20). Understanding the release of these

hormones in response to a meal in South Asians versus Europids could provide valuable insights into underlying mechanisms that contribute to the higher cardiometabolic disease risk in South Asians. Moreover, it could offer insight in the potential benefit of interventions based on these hormones in South Asians. The mixed meal tolerance test (MMTT) is a low-invasive and well-established method, used to assess pancreatic beta cell function and the release of incretin and glucagon hormones, with the variety of nutrients in the used liquid meal providing a more accurate reflection of postprandial metabolic processes compared to the common used oral glucose tolerance test (21-23).

Therefore, in the current study, we aimed to investigate the effect of an extended MMTT (up to 240 min) on the excursion of GLP-1, GIP, and glucagon and parameters related to glucose and lipid metabolism (i.e., glucose, insulin, FFA, triglycerides, and total cholesterol) in young and lean South Asians and Europids.

METHODS

Study Design

This study uses samples and data obtained from the CAMI project (Elucidating the high cardiovascular disease risk in South Asians: focus on monocyte phenotype and incretin hormones), an observational study conducted at the Leiden University Medical Center (LUMC) between June and October 2023. The study was approved by the Medical Ethics Committee of the LUMC and undertaken in accordance with the principles of the revised Declaration of Helsinki (24). Written informed consent was obtained from all participants prior to inclusion. The clinical trial is registered at ClinicalTrial.gov (no. NCT05829018). The primary objective of the CAMI study was to compare immune cell composition between lean adolescent Dutch South Asians (hereinafter: 'South Asians') and BMI- and age-matched Dutch Europids (hereinafter: 'Europids'). In this manuscript, we report on one of the secondary objectives.

Participants

A total of forty-nine lean and healthy participants were included in the study, namely South Asian males (n=12) and females (n=12), and Europid males (n=13) and females (n=12). Additional inclusion criteria were a body mass index (BMI) of 18-25 kg/m^2 and age of 18-30 years. We included an additional Europid male (resulting in 13 instead of 12 Europid males) as we encountered a technical problem during the collection of the samples for the primary endpoint of this study from one Europid male.

Participants were recruited especially through social media advertisements and by recalling participants from previous studies. Eligibility to participate in the study was

tested primarily during a telephonic screening that consisted of questions about their heritage, body weight, height, and medical history. South Asian ethnicity was defined as having all four grandparents from Surinam, Bangladesh, India, Nepal, Pakistan, Afghanistan, Bhutan, or Sri Lanka. Europid ethnicity was defined by having 4 grandparents originally descent from Europe. Exclusion criteria were the presence of an (auto-)immune disease, genetic lipid-associated disorders, chronic renal or hepatic disease, use of medication known to influence glucose and/or lipid metabolism, abuse of alcohol or other substances, smoking, vigorous exercise (more than 3 times per week), and milk or soy allergy.

Procedure

Participants were asked to withhold from vigorous exercise 48 hours preceding the study days and not to drink alcohol or caffeinated drinks 24 hours preceding the study days. In addition, they were instructed to eat a standardized meal (prepared supermarket meal including pasta or noodles, ranging from 450–600 kcal, or similar meal prepared by themselves) in the evening before the experiment and not to eat or drink anything other than water until the completion of the study day.

Questionnaires and anthropometric measurements

After an overnight fast, participants arrived at the LUMC at 08:00 am, where they underwent questionnaires about their medical history and current health. Thereafter, body weight and body composition were assessed using bioelectrical impedance analysis (BIA) (InBody720, InBody CO., Ltd., CA, USA). In addition, height, waist, and hip circumference were obtained using a measuring lint. BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Mixed meal tolerance test

A catheter was inserted in the antecubital vein for venous blood sampling, whereafter a screening sample was obtained using Vacutainer SST II Advance Gel and EDTA tubes, to determine inclusion into the study. Measurements included full blood count, glucose, insulin, kidney function, liver function, and lipid metabolism. If the participants were eligible for inclusion they proceeded with the study. Next, a baseline sample prior to ingestion of the mixed meal test was obtained using Vacutainer SST II Advance Gel tubes, a BD™ P800 collection tube, and an EDTA tube. At approximately 9.00 am, within 5 minutes, the participants ingested a standardized liquid meal (200 mL, 300 kcal, 36.8 g carbohydrates, 12.0 g protein, and 11.6 g fat; Nutridrink strawberry flavor, Nutricia). Blood samples were collected at 7 time points in total (-10 or baseline, 30, 60, 90, 120, 180, and 240 minutes). The BD™ P800 collection tubes and EDTA tubes were directly stored on ice after obtaining blood, and blood obtained using the Vacutainer SST II

Advance Gel tubes was clotted for at least 30 minutes at room temperature. Thereafter, the samples were centrifuged to obtain plasma or serum, respectively, and stored at -80°C until batch-wise analyses. Plasma levels of total and active GLP-1, total and active GIP, and glucagon were measured from blood collected in the BD™ P800 collection tubes using a U-Plex Assay Platform (Meso-Scale Diagnostics, Gaithersburg, MD, USA). Commercially available kits were used for the measurements of serum free fatty acids (FFA) (Wako chemicals, Neuss, Germany), serum triglycerides and serum total cholesterol (Roche Diagnostics, Woerden, the Netherlands), plasma glucose (Instruchemie, Delfzijl, the Netherlands), and serum insulin (Chrystal Chem, Elk Grove Village, IL, USA).

Statistical analysis

Data are expressed as mean ± standard deviation. The normality of data was assessed using the Shapiro-Wilk test, visual histograms, and Q-Q plots. For the baseline characteristics, waist-hip ratio (WHR) was calculated as waist circumference divided by hip circumference. Lean mass was calculated by subtracting the fat mass, obtained by BIA, from the total body weight. Body fat percentage was calculated by dividing fat mass by body weight and multiplying by 100. HOMA-IR was calculated by multiplying fasting insulin levels (mU/L) with fasting glucose levels (mmol/L) and dividing this by 22.5.

Baseline characteristics of the participants were compared between ethnicities within the same sex using an independent t-test for normally distributed data (age, weight, length, BMI, hip circumference, fat mass, lean mass, and fasting glucose). Not normally distributed data were log10 transformed to yield normal distribution (i.e., total cholesterol) and analyzed using an independent t-test. Non-parametric tests were performed on data that were not normally distributed even after log 10 transformation (i.e., waist circumference, waist-hip ratio, body fat percentage, fasting insulin, HOMA-IR, and triglycerides).

For the comparison of the excursion of GLP-1, GIP, and parameters for glucose and lipid metabolism in response to a mixed meal test, we calculated the total area under the curve ($tAUC_{0\text{-}240}$, $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$) with the trapezoid rule (25). To determine the incremental AUC ($iAUC_{0\text{-}240}$, $iAUC_{0\text{-}60,}$ and $iAUC_{60\text{-}240}$), defined as the AUC corrected for baseline, we subtracted the area below the baseline value from the $tAUC_{0\text{-}240}$. To compare the tAUC and iAUC between the two ethnicities, the non-parametric Mann-Whitney U test was used, as not all the data was normally distributed. In addition, we used a two-way repeated measures ANOVA with the within-subject factor 'time' and the between-subject factor 'ethnicity' for the comparison of the response of various hormones throughout a mixed meal test between ethnicities. We compared the means of each time point

between ethnicities of the general linear model using the estimated marginal means comparison corrected with the Bonferroni methods to correct for multiple tests.

All statistical analyses were performed using SPSS v.29.0.1.0. Armonk, NY: IBM Corp. All graphs were created with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

All groups were comparable with respect to age. Both South Asian males and females were significantly shorter than their Europid counterparts (males: P = 0.015; females P = 0.003; **Table 1**). In males, there was no difference in body weight between ethnicities, which resulted in a higher BMI in South Asians (P = 0.004). On the other hand, South Asian females had a lower body weight compared to Europid females (P = 0.020), which combined with the shorter stature resulted in a similar BMI for the two ethnicities. The South Asian females had a lower lean mass compared to the Europid females ($P < 0.001$). Furthermore, the South Asian males had a higher fat mass (P = 0.002), and both South Asian males and females had a higher body fat percentage compared to the Europids (males: $P < 0.001$; females: P = 0.020). Fasting glucose, insulin and, as a consequence, HOMA-IR did not differ between ethnicities in both males and females. Lastly, the South Asian males had a higher serum total cholesterol compared to Europid males (P = 0.027).

South Asians exhibit biphasic glucose excursions during an MMTT

Following the mixed meal, South Asian and Europid males and females showed a single peak in circulating plasma glucose levels after 30 minutes (**Fig. 1**). Interestingly, South Asian males and females exhibited an additional glucose peak at 90 minutes, with glucose levels being significantly higher compared to both Europid males and females (P = 0.026 and P = 0.044, respectively) which pursued until 120 min in South Asian females (P = 0.028). Although these changes did not result in significant differences over time between the ethnicities (males: $P_{Interaction}$ = 0.368; females: $P_{Interaction}$ = 0.156; **Fig. 1**, **Suppl. Tables 1 and 2**), this biphasic response led to a significantly elevated $tAUC_{0\text{-}240}$ of the glucose excursion in South Asian compared to Europid females (P = 0.043, **Suppl. Table 2 and Fig. 1**). After evaluating both peaks individually and calculating $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of the glucose response, we observed no difference in $tAUC_{0\text{-}60}$, while $tAUC_{60\text{-}240}$ was significantly higher in South Asian compared to Europid females (P = 0.020; **Suppl. Fig. 1**. Furthermore, the $iAUC_{0\text{-}240}$ for the glucose excursion did not differ between ethnicities in either sex (P = 0.949 and P = 0.114, **Suppl. Tables 1 and 2** and **Fig. 1**).

Table 1. Baseline characteristics

	Males		Females	
	Europids (*n*=13)	**South Asians (*n*=12)**	**Europids (*n*=12)**	**South Asians (*n*=12)**
Age, years	21.7 ± 2.9	23.3 ± 3.2	23.1 ± 2.1	23.3 ± 3.3
Body length, m	1.86 ± 0.07	1.79 ± 0.06*	1.74 ± 0.08	1.63 ± 0.06**
Body weight, kg	73.6 ± 6.0	74.9 ± 7.2	68.1 ± 9.1	60.3 ± 5.7*
BMI, kg/m^2	21.3 ± 1.5	23.3 ± 1.5**	22.5 ± 1.2	22.6 ± 1.8
Waist circumference, cm	69.9 ± 12.9	75.7 ± 5.9	69.8 ± 4.6	68.9 ± 3.8
Hip circumference, cm	93.3 ± 3.1	94.1 ± 5.0	89.8 ± 6.4	87.9 ± 5.9
Waist to hip ratio	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Lean mass, kg	66.4 ± 5.3	61.4 ± 8.2	51.5 ± 6.6	41.2 ± 3.6***
Fat mass, kg	7.3 ± 2.0	13.5 ± 5.5**	16.6 ± 6.1	19.1 ± 4.6
Fat percentage, %	9.8 ± 2.3	18.0 ± 7.2***	24.1 ± 6.5	31.5 ± 5.9*
Fasting glucose, mmol/L	4.9 ± 0.5	4.9 ± 0.3	4.8 ± 0.3	4.8 ± 0.3
Fasting insulin, mU/L	4.0 ± 2.0	4.4 ± 1.8	4.4 ± 1.6	4.4 ± 3.3
HOMA-IR	0.8 ± 0.5	0.8 ± 0.5	0.9 ± 0.4	0.9 ± 0.7
Serum Total Cholesterol, mmol/L	3.1 ± 0.4	3.6 ± 0.5*	3.4 ± 0.5	3.7 ± 0.9
Serum Triglycerides, mmol/L	0.6 ± 0.2	0.8 ± 0.7	0.5 ± 0.2	0.6 ± 0.3

Asterisk signs (*) indicate significant differences between ethnicities within a specific sex. *P < 0.05, **P < 0.01, ***P < 0.001. BMI, Body Mass Index; HOMA-IR, Homeostatic Model Assessment for Insulin Resistance.

In South Asian males, serum insulin levels were higher compared to Europid males at 30 and 90 minutes (**Fig. 2**). This resulted in significantly different levels of circulating serum insulin over time (P = 0.046), a higher $tAUC_{0\text{-}240}$ (P = 0.044), and a higher $iAUC_{0\text{-}240}$ (P = 0.016) in South Asian compared to Europid males (**Suppl. Table 1** and **Fig. 2**) but not females (**Suppl. Table 2** and **Fig. 2**). For males and females, both the $tAUC_{0\text{-}60}$ and the $tAUC_{60\text{-}240}$ for the insulin excursion was not significantly different between South Asians and Europids (**Suppl. Fig. 2**).

South Asian females have lower circulating plasma glucagon levels at 90 min after MMTT compared to Europid females

In males, we did not find any difference in excursion of circulating plasma glucagon levels between South Asians and Europids over time ($P_{Interaction}$ = 0.409). Correspondingly, $tAUC_{0\text{-}240}$ and $iAUC_{0\text{-}240}$ did not differ between ethnicities (P = 0.242 and P = 0.671, respectively; **Suppl. Table 1** and **Fig. 3**). In females, however, South Asians exhibited lower circulating plasma glucagon levels at 30 and 90 minutes (P = 0.040 and P =

0.013, respectively; **Fig. 3**). This resulted into significant differences in circulating plasma glucagon levels over time during the MMTT between South Asian and Europid females ($P_{Interaction}$ = 0.045, **Suppl. Table 2** and **Fig. 3**). The $tAUC_{0-240}$ tended to be lower (P = 0.095) in South Asian compared to Europid females (**Suppl. Table 2** and **Fig. 3**). We did not find a significant different $tAUC_{0-60}$ or $tAUC_{60-240}$ for the glucagon excursion; **Suppl. Fig. 3**). Furthermore, $iAUC_{0-240}$ did not differ between ethnicities in females.

South Asian males exhibit lower circulating plasma total GLP-1 levels while females exhibit higher active GLP-1 during an MMTT compared to Europids

Next, we assessed the excursions of plasma incretins during the MMTT. In males, circulating plasma levels of both total GLP-1 and active GLP-1 were not significantly different at different time points and over time between ethnicities (total GLP-1: $P_{Interaction}$ = 0.774, active GLP-1: $P_{Interaction}$= 0.655; **Fig. 4 and 5**). However, in South Asian males, the $tAUC_{0-240}$ of circulating total GLP-1, but not active GLP-1, was significantly lower compared to Europids (total GLP-1: P = 0.030; active GLP-1: P = 0.487; **Fig 4 and 5** and **Suppl. Table 1**). For excursions of total GLP-1, $tAUC_{0-60}$ tended to be lower and $tAUC_{60-240}$ was significantly lower in South Asian males compared to Europids (P = 0.098 and P = 0.026, respectively, **Suppl. Fig. 4**). The $iAUC_{0-240}$ for circulating total and active GLP-1 excursions were not significantly different in males in either ethnicity (total GLP-1: P = 0.936; active GLP-1: P = 0.487; **Fig 4 and 5** and **Suppl. Table 1**).

In females, South Asians had higher levels of circulating plasma total and active GLP-1 at time point 120 minutes during the MMTT (P = 0.016 and P = 0.002, respectively; **Fig. 4 and 5**). Circulating plasma active GLP-1 was also higher at 240 minutes in South Asian females compared to Europid females (P = 0.003; **Fig 5**). However, this did not result in a different excursion of circulating plasma total and active GLP-1 over time between ethnicities (total GLP-1: $P_{Interaction}$ = 0.394, active GLP-1: $P_{Interaction}$= 0.387; **Fig. 4 and 5** and **Suppl. Table 2**) or $tAUC_{0-240}$ (total GLP-1: P = 0.148; active GLP-1: P = 0.058) and $iAUC_{0-240}$ (total GLP-1: P = 0.219; active GLP-1: P = 0.129) (**Suppl. Table 2**, **Fig. 4 and 5**). Interestingly, the $tAUC_{60-240}$ for active GLP-1 was significantly higher in South Asian compared to Europid females (P = 0.015; **Suppl. Fig. 4**).

South Asian females exhibit higher circulating plasma total and active GIP levels during an MMTT compared to Europid females

In males, circulating plasma total and active GIP levels did not differ over time between both ethnicities (total GIP: $P_{Interaction}$ = 0.715, active GIP: $P_{Interaction}$= 0.729; **Suppl. Table 1** and **Fig. 6**). As a result, $tAUC_{0-240}$ did not differ between ethnicities (P = 0.538 and P = 0.319, respectively), also not for the $tAUC_{0-60}$ and the $tAUC_{60-240}$ (**Suppl. Fig 5**). However,

the $iAUC_{0\text{-}240}$ was significantly higher in South Asian compared to Europid males (total GIP: P = 0.040; active GIP: P = 0.033).

In females, circulating plasma total and active GIP levels were higher in South Asians at 120, 180, and 240 minutes during the MMTT (**Suppl. Table 2** and **Fig. 6 and 7**). This resulted in a tendency towards higher circulating plasma total GIP and active GIP levels over time ($P_{Interaction}$ = 0.069 and $P_{Interaction}$ = 0.052, respectively; **Suppl. Table 2**, **Fig. 6 and 7**). The $tAUC_{0\text{-}240}$ and $iAUC_{0\text{-}240}$ of both circulating plasma total and active GIP were not significantly different in females between South Asians and Europids. However, similarly to active GLP-1 in South Asian females, plasma active GIP increased during the second part of the MMTT with a significantly higher $tAUC_{60\text{-}240}$ of plasma active GIP (P = 0.041; **Suppl. Fig. 5**).

Postprandial lipid excursions do not differ between South Asian and Europid males and females

Finally, we assessed lipid levels (serum FFA, triglycerides, and total cholesterol) during the MMTT. In males, circulating serum FFA levels did not differ between time points or over time between South Asians and Europids (**Suppl. Table 1** and **Suppl. Fig. 6**). In females, circulating serum FFA levels remained suppressed longer over time in South Asians compared to Europids, which resulted in lower circulating serum FFA levels at 180 minutes (P = 0.038) and over time ($P_{Interaction}$ = 0.022; **Suppl. Table 2** and **Suppl. Fig. 6**). While the $tAUC_{0\text{-}240}$ of circulating serum FFA levels did not differ (P = 0.557; **Suppl. Tables 1 and 2**), the iAUC was lower in South Asians compared to Europids (P = 0.029). The responses of circulating serum triglyceride levels were similar in both South Asian males and females compared to their Europid counterparts (**Suppl. Tables 1 and 2** and **Suppl. Fig. 6**).

In males, circulating serum total cholesterol levels were higher in South Asians compared to Europids during all time points (all P < 0.05). Since no difference in excursion in total cholesterol was observed over time in both ethnicities, this resulted in no significant difference over time ($P_{Interaction}$ = 0.306), but in a significantly higher $tAUC_{0\text{-}240}$ (P = 0.007) in South Asian males compared to Europid males, with no significant different $iAUC_{0\text{-}240}$ (P = 0.059) (**Suppl. Table 1** and **Suppl. Fig. 6**). In females, circulating serum cholesterol levels remained similar between South Asians and Europids at the various time points and over time (**Suppl. Table 2** and **Suppl. Fig. 6**).

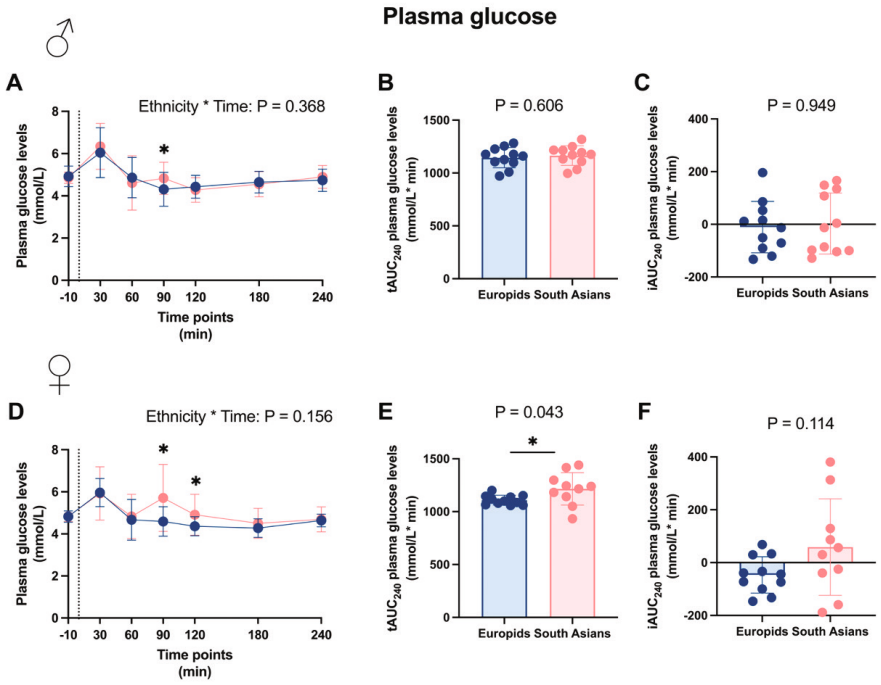


Figure 1. Plasma glucose levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing plasma glucose levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=11) compared to Europid (n=11) males **(A)**. Box plots showing total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing plasma glucose levels during an MMTT in South Asian females (n=10) compared to Europid females (n=11) **(D)** and box plots showing $tAUC_{0\text{-}240}$ **now** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D** and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). We were unable to retrieve a blood sample of one South Asian male at two time points, and from one Europid male, two South Asian females, and one Europid female at one time point.

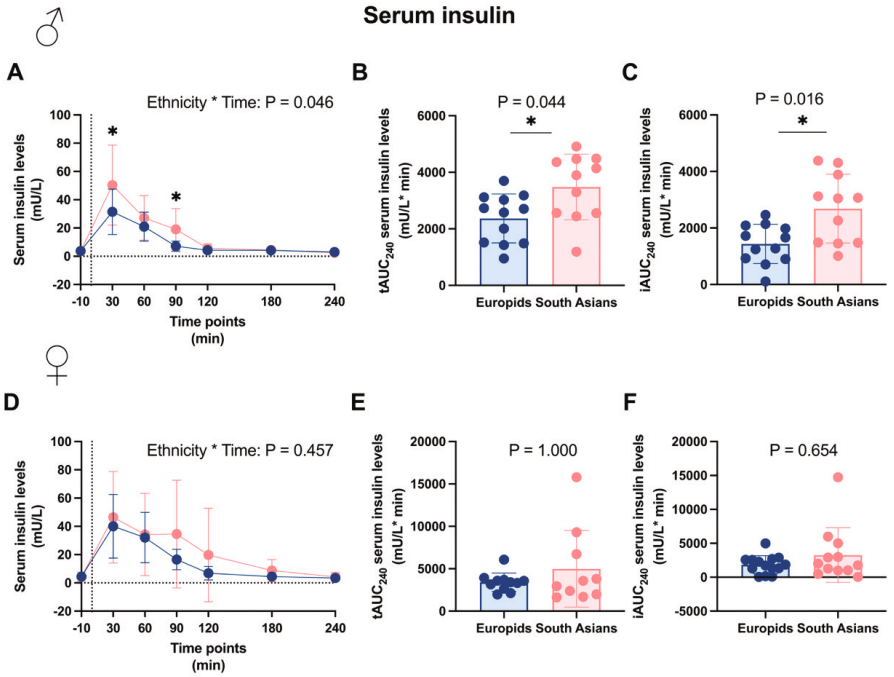


Figure 2. Serum insulin levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing serum insulin levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=11) compared to Europid (n=12) males **(A)**. Box plots showing the total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing serum insulin levels during an MMTT in South Asian females (n=10) compared to Europid females (n=11) **(D)** and box plots showing the $tAUC_{0\text{-}240}$ **(E)** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D**, and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). We were unable to retrieve a blood sample of one South Asian female at two time points, and from one South Asian male, Europid male, one South Asian female, and one Europid female at one time point.

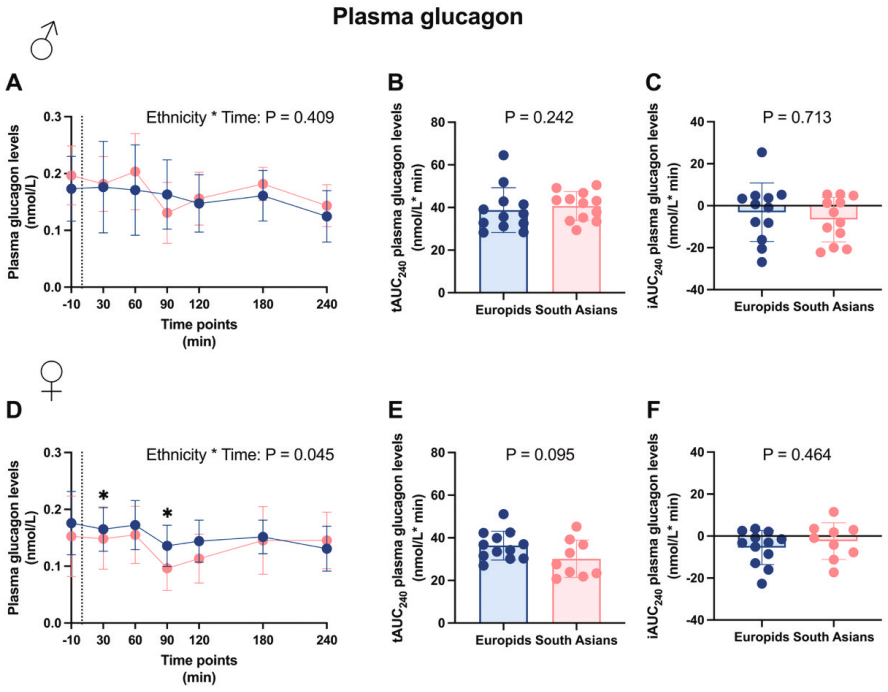


Figure 3. Plasma glucagon levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing plasma glucagon levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=12) compared to Europid (n=12) males **(A)**. Box plots showing the total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing plasma glucagon levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(D)** and box plots showing the $tAUC_{0\text{-}240}$ **(E)** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D**, and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). Due to a technical error, one sample of one Europid male is missing and we were unable to retrieve a blood sample and of three South Asian females at one time point.

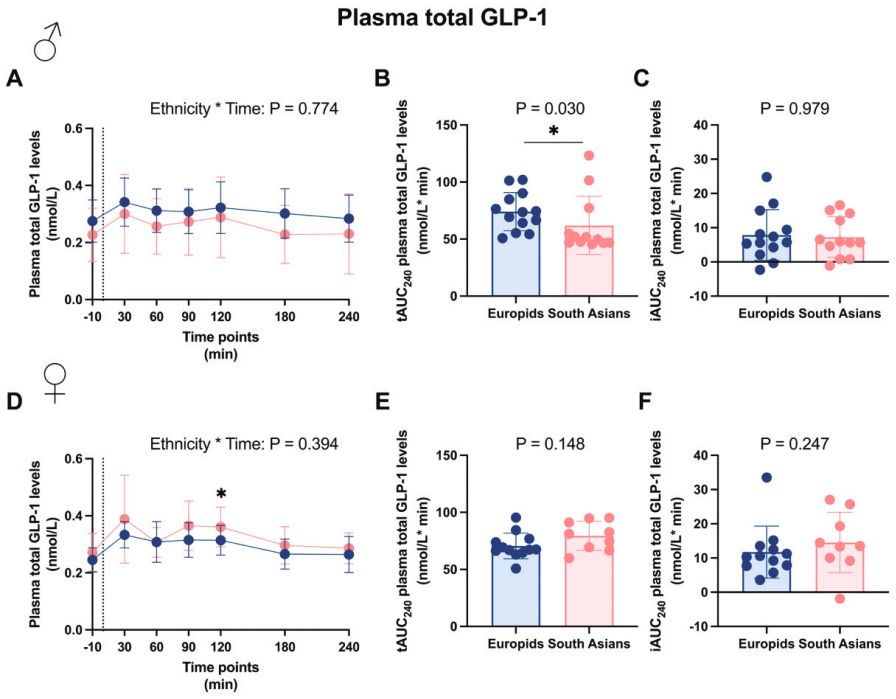


Figure 4. Plasma total glucagon-like peptide-1 levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing plasma total glucagon-like peptide-1 (GLP-1) levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=12) compared to Europid (n=13) males **(A)**. Box plots showing the total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing the plasma GLP-1 levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(D)** and box plots showing the $tAUC_{0\text{-}240}$ **(E)** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D**, and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). We were unable to retrieve a blood sample of three South Asian females at one time point.

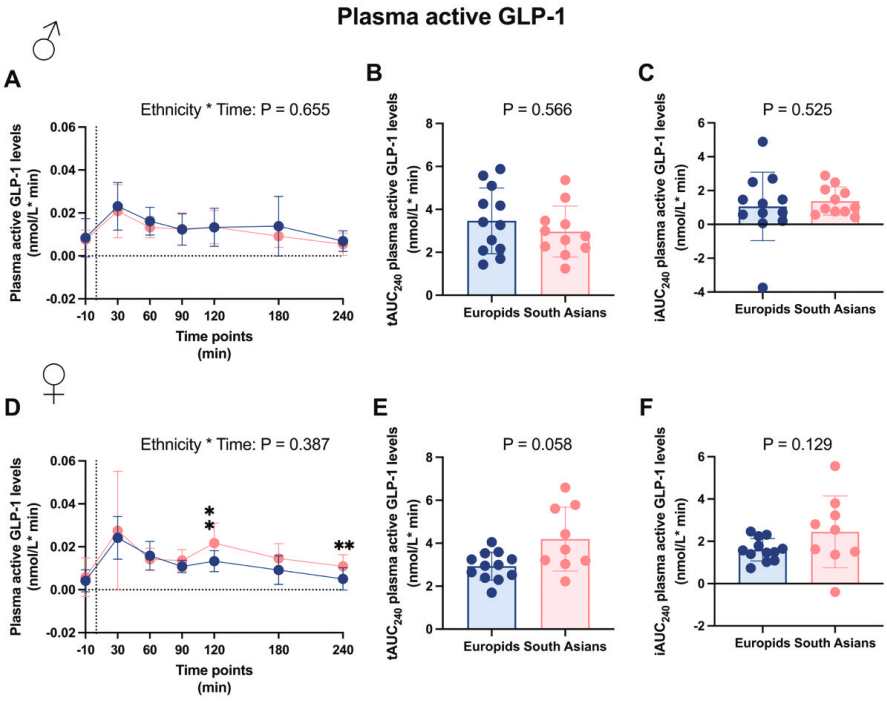


Figure 5. Plasma active glucagon-like peptide-1 levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing plasma active glucagon-like peptide-1 (GLP-1) levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=12) compared to Europid (n=13) males **(A)**. Box plots showing the total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing the plasma active GLP-1 levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(D)** and box plots showing the $tAUC_{0\text{-}240}$ **(E)** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D**, and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). We were unable to retrieve a blood sample of three South Asian females at one time point.

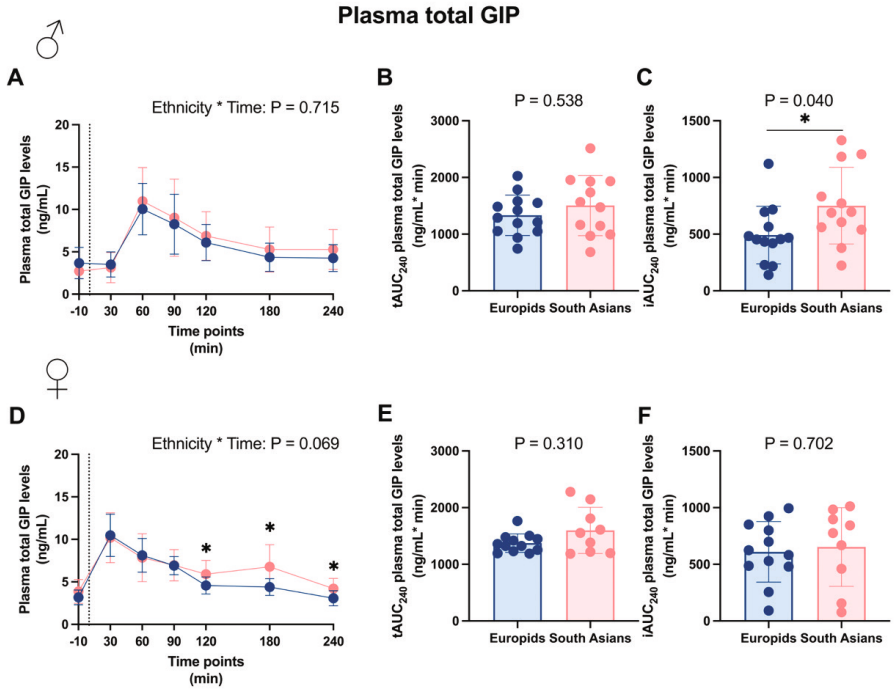


Figure 6. Plasma total glucose-dependent insulinotropic polypeptide levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing plasma total glucose-dependent insulinotropic polypeptide (GIP) levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=12) compared to Europid (n=13) males **(A)**. Box plots showing the total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing the plasma GIP levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(D)** and box plots showing the $tAUC_{0\text{-}240}$ **(E)** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D**, and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). We were unable to retrieve a blood sample of three South Asian females at one time point.

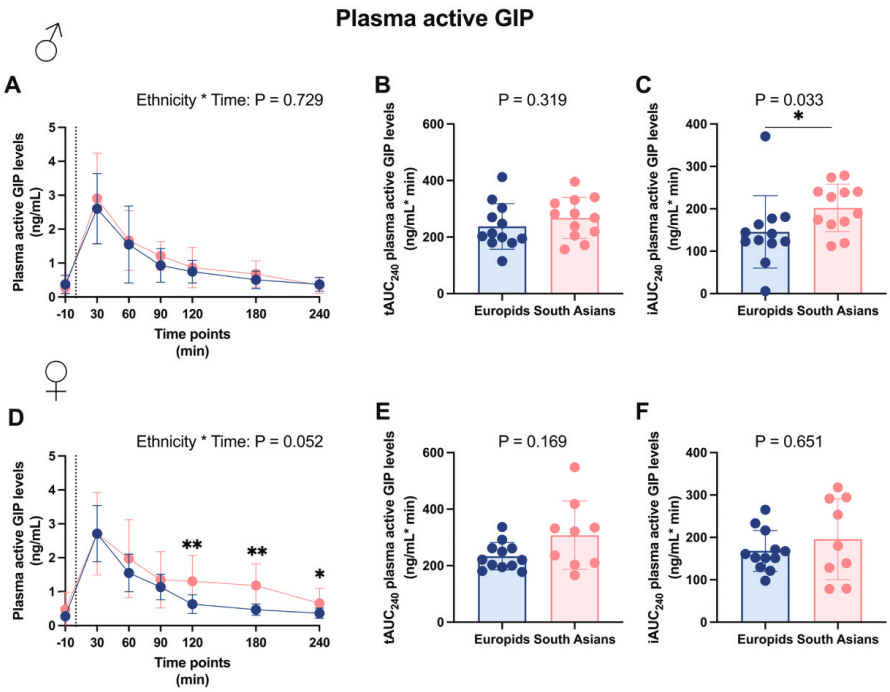


Figure 7. Plasma active glucose-dependent insulinotropic polypeptide levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing plasma active glucose-dependent insulinotropic polypeptide (GIP) levels before and during a mixed meal tolerance test (MMTT) in South Asian (n=12) compared to Europid (n=12) males **(A)**. Box plots showing the total area under the curve ($tAUC_{0\text{-}240}$) **(B)** and incremental area under the curve ($iAUC_{0\text{-}240}$) **(C)** in South Asian and Europid males. Similarly, line graphs showing the plasma active GIP levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(D)** and box plots showing the $tAUC_{0\text{-}240}$ **(E)** and $iAUC_{0\text{-}240}$ **(F)** for South Asian and Europid females **(F)**. Circles represent means in **A** and **D**, and individuals' values in **B, C, E,** and **F**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Dotted lines represent the time of the ingestion of the liquid meal (t=0). We were unable to retrieve a blood sample of three South Asian females at one time point and of one Europid male one time point is missing due to a technical failure.

DISCUSSION

In this study, we aimed to compare the excursions of GLP-1, GIP, and glucagon as well as the response of markers related to glucose and lipid metabolism between young and lean South Asians and Europids in response to an extended MMTT. We observed several differences between the two ethnicities: South Asian males and females both exhibited a biphasic rather than monophasic glucose excursion. South Asian males exhibited an increased insulin response, with elevated levels at the corresponding glucose peaks. South Asian females demonstrated higher active GLP-1 and active GIP excursion of in the second phase of the MMTT, and a tendency towards lower $tAUC_{0\text{-}60}$ of circulating glucagon compared to Europid females.

Firstly, we found that both South Asian males and females exhibited a biphasic glucose response following the MMTT, which was not observed in Europids. In healthy individuals, the circulating glucose levels in response to a meal or an oral glucose tolerance test often follow either a single peak or a biphasic curve, both of which are part of a normal physiological response with the biphasic curve often resulting in a lower $tAUC_{0\text{-}240}$ than with a monophasic curve (26, 27). Of note, a more pronounced biphasic glucose curve also seemed to be found in healthy South Asians males following an MMTT in a recent study in healthy lean South Asian and Europid males (28), supporting an ethnic variation in glucose excursion following an MMTT. We speculate that delayed gastric emptying rates in South Asians compared to Europids at least in part contributed to the biphasic glucose peak. Interestingly, variations of gastric emptying rates between other ethnicities (i.e., Mexican Americans and American Indians compared to Caucasians) have been described in (29). The consequences of the observed biphasic glucose curve for healthy South Asians and the possible development of T2DM later in life remain unknown and warrant further research. However, considering the well-established link between elevated glucose levels and cardiometabolic diseases, the enhanced glucose $tAUC_{0\text{-}240}$ that accompanied the biphasic glucose excursion in South Asians might already contribute to an enhanced T2DM risk.

Postprandial increases in glucose levels are an important stimulus for the release of the incretin hormones GLP-1 and GIP by intestinal L-cells and K-cells, respectively. Indeed, this is consistent with the biphasic peaks in total and active GLP-1 and GIP we found in South Asian females. More specifically, for GLP-1, the increase in both total and active GLP-1 levels in South Asian females from 90 minutes onwards coincided with the increased $tAUC_{60\text{-}240}$ of plasma glucose observed in South Asian females compared to Europids. This pattern was also found for active GIP, having higher $tAUC_{60\text{-}240}$ in South Asians compared to Europid females. The excursions of postprandial incretin hormones

have only scarcely been studied in healthy lean South Asians compared to Europids. Our finding of higher postprandial GLP-1 levels in South Asian females is in line with a previous study executed in lean Dutch South Asians compared to Dutch Europid males, in which a higher GLP-1 peak was found in the South Asian men following an oral glucose tolerance test (30). However, in that study, only a single increased peak of GLP-1 was found in South Asians, which may have been due to the fact that an oral glucose load rather than a mixed meal was used. Remarkably, in our study, plasma total GLP-1 excursion was lower in the South Asian compared to Europid males. This could have been explained by the fact that baseline levels of total GLP-1 levels seemed to be lower, which persisted throughout the MMTT. An important question is how this sex difference in GLP-1 excursions can be explained between ethnicities. Potentially, South Asian males are more sensitive to the effects of GLP-1 compared to females and therefore require lower postprandial GLP-1 levels, which warrants further investigation. Regarding the mechanism, the lower total GLP-1 excursion throughout the MMTT in South Asian males could indicate a reduced release of GLP-1 by the enterocytes in response to the mixed meal. To the best of our knowledge, DPP4 activity has not been investigated in South Asians yet. Importantly, GLP-1 promotes the glucose-stimulated release of insulin by pancreatic beta cells, thereby lowering circulating glucose levels postprandially (31). Even though we observed a lower $tAUC_{0\text{-}240}$ of plasma total GLP-1 levels in South Asian males compared to Europids, their insulin response was higher. This could indicate that the diminished exposure to postprandial circulating plasma GLP-1 levels at least did not affect serum insulin excursion. However, GIP also plays an important role in the release of insulin postprandially. Of note, although in males, total GIP levels were equal between South Asians and Europids, the $iAUC_{240}$ was higher for both total and active GIP, supporting a steeper increase in GIP release during the MMTT. Indeed, for total GIP, this was especially evident between 30 and 60 minutes during the MMTT.

The biphasic peak in circulating glucose levels accompanied by the differences in excursions of GLP-1 (South Asian females) and GIP (South Asian males) could have contributed to the elevated insulin response in South Asians compared to Europids. The higher $tAUC_{0\text{-}240}$ of plasma glucose, along with the higher peak of serum insulin, which was especially evident in South Asian males, could indicate that, even in our young and lean cohort, South Asian males are already exhibiting decreased insulin sensitivity. However, this was not reflected in a difference in HOMA-IR index. Our data are in line with a recent study in which a mixed meal tolerance test was performed in young and lean South Asian and Europid males both at baseline and after 5-7% weight gain following 4-6 weeks of overfeeding. Although in that study the HOMA-IR index and fasting and postprandial glucose levels did not differ between South Asian and

Europid males, postprandial insulin levels were 62% higher in South Asians at baseline compared to their Europid counterparts (28).

Insulin decreases glucagon levels to help maintain glucose homeostasis. However, despite the higher serum insulin excursion in South Asian males, we did not observe a significant difference in plasma glucagon levels compared to Europid males. In females, however, plasma glucagon excursion was significantly different during the MMTT in South Asians compared to Europids, with a trend toward a lower total $AUC_{0\text{-}240}$, primarily driven by lower plasma glucagon levels at 30 and 90 minutes. The reduced plasma glucagon levels in South Asian females do not appear to be driven by higher insulin levels, as we did not observe significantly higher serum insulin levels in this group. However, the lower glucagon levels at 30 and 90 minutes coincided with the biphasic glucose curve observed in our cohort. The increased glucose levels in South Asian females could be contributing to lower glucagon levels by acting directly on the pancreatic alpha cells (32) or through other hormones, such as GLP-1 (33), which was significantly higher after 120 minutes in South Asian females compared to Europid females. Given that glucagon increases energy expenditure (34), the lower plasma glucagon levels in South Asian females could contribute to the lower energy expenditure known in the South Asian population (13). However, to our knowledge, differences in (postprandial) glucagon levels between South Asians and Europids and their relationship to variations in resting energy expenditure have not been previously reported.

Various incretin-based pharmacological interventions are emerging for the treatment of obesity and T2DM, which improve insulin sensitivity and reduce nutrient intake by inducing satiety (35). If the lower $tAUC_{0\text{-}240}$ of total GLP-1 levels, driven by lower baseline levels, observed in South Asian males in our study indeed impairs satiety, then these interventions could be especially beneficial for the South Asian population, especially the males. These treatments might be initiated earlier, given the already existing differences observed in young and lean South Asians compared to Europids in the current study. A previous study from our group showed similar beneficial effects in South Asians and Europids for improving the glycemic index and improving body composition in the treatment of T2DM with GLP-1 receptor agonist liraglutide (36, 37). Interestingly, a recent meta-analysis showed that GLP-1 receptor agonists have more benefit in cardiovascular outcomes in Asians compared to whites (38). Although the applicability for South Asian males remains to be determined, the fact whether South Asians benefit more from and potentially earlier treatment with incretin-based pharmacological intervention is an interesting topic for future studies.

A strength of this study is that we were able to measure a wide variety of hunger and satiety hormones during the MMTT up to 240 minutes after food ingestion in both males and females. However, this study is not without its limitations. Despite having a young and lean population with a healthy BMI, we already noticed metabolic differences. Matching South Asians and Europids remains a challenge due to differences in fat mass, fat percentage, and possibly insulin sensitivity already observed. Therefore, finding additional markers to match these ethnicities, could overcome these discrepancies. Furthermore, similar to the standard MMTT procedure, all participants ingested the same amount of liquid meal. However, due to variations in body composition between sexes and ethnicities, the caloric content of the liquid meal may represent a different proportion of energy intake relative to their basal metabolic rate.

In conclusion, South Asians respond differently to an MMTT compared to Europids, with a noticeable biphasic peak in glucose levels, and, potentially as a consequence, higher active GLP-1 and active GIP levels towards the end of the MMTT in South Asian compared to Europid females. Our findings suggest that various metabolic hormones are differentially regulated in South Asians compared to Europids, although the precise contribution to their disadvantageous metabolic phenotype remains to be determined.

Acknowledgments

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SUPPLEMENTAL DATA

Supplemental Table 1. Overview of the area under the curve of the excursions of glucose, insulin, hormones, and lipids during a mixed meal tolerance test in South Asian compared to Europid males

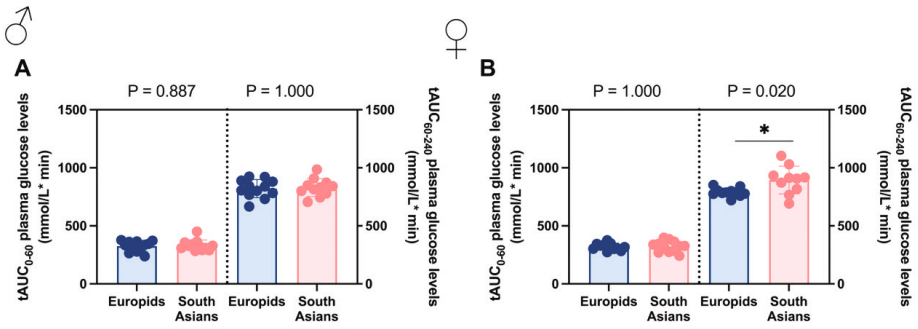
	Europids		South Asians				
	Males		Males		P values		
	$tAUC_{0\text{-}240}$	$iAUC_{0\text{-}240}$	$tAUC_{0\text{-}240}$	$iAUC_{0\text{-}240}$	$tAUC_{0\text{-}240}$	$iAUC_{0\text{-}240}$	$P_{Interaction}$
Glycemic parameters							
Plasma Glucose (mmol/L * min)	1145±99^{c}	1107±94^{c}	1165±93^{c}	1126±94^{c}	0.606^{w}	0.949^{w}	0.368^{w}
Serum Insulin (mU/L * min)	2369±865^{b}	2338±855^{b}	3483±1161^{c}	3457±115^{c}	0.044^{x}	0.016^{x}	0.046^{x}
Plasma Glucagon (nmol/L * min)	38.8±10.5^{b}	37.4±10.4^{b}	40.7±6.7^{b}	39.1±6.5^{b}	0.242^{y}	0.713^{y}	0.409^{y}
Incretin hormones							
Plasma Total GLP-1 (nmol/L * min)	74.1±16.7^{a}	71.9±16.2^{a}	62.0±25.5^{b}	60.2±24.7^{b}	0.030^{z}	0.979^{z}	0.774^{z}
Plasma Active GLP-1 (nmol/L * min)	3.5±1.5^{b}	3.4±1.5^{b}	3.0±1.2^{c}	2.9±1.2^{c}	0.566^{x}	0.525^{x}	0.655^{x}
Plasma Total GIP (ng/mL * min)	1333±357^{a}	1308±349^{a}	1505±532^{b}	1483±522^{b}	0.538^{z}	0.040^{z}	0.715^{z}
Plasma Active GIP (ng/mL * min)	238±81^{c}	234±80^{c}	268±72^{c}	266±71^{c}	0.319^{w}	0.033^{w}	0.660^{w}
Lipids							
Serum FFA (mmol/L * min)	63.2±19.0^{b}	59.3±17.6^{b}	73.7±25.5^{c}	69.7±24.0^{c}	0.316^{x}	0.651^{x}	0.921^{x}
Serum TG (mmol/L * min)	132.3±53.4^{b}	128.3±52.1^{b}	114.8±29.4^{c}	110.9±28.6^{c}	0.413^{x}	0.169^{x}	0.416^{x}
Serum TC (mmol/L * min)	708±104^{b}	683±101^{b}	847±114^{c}	819±110^{c}	0.007^{x}	0.059^{x}	0.306^{x}

Table showing the mean and standard deviation of the total area under the curve ($tAUC_{0\text{-}240}$) and incremental area under the curve ($iAUC_{0\text{-}240}$) of the excursions of glucose, insulin, hormones, and lipids during a mixed meal tolerance test for both South Asian and Europid males. P-values of the comparisons between the two ethnicities were obtained via the non-parametric Man-Whitney U test and the p-values of the interactions were analyzed via a repeated measurement ANOVA. FFA, free fatty acids; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; TC, total cholesterol; TG, triglycerides. Letters indicate n values of each ethnicity, *a*n=13; *b*n=12; *c*n=11; *d*n=10, and *w*n=22; *x*n=23; *y*n=24, *z*n=25.

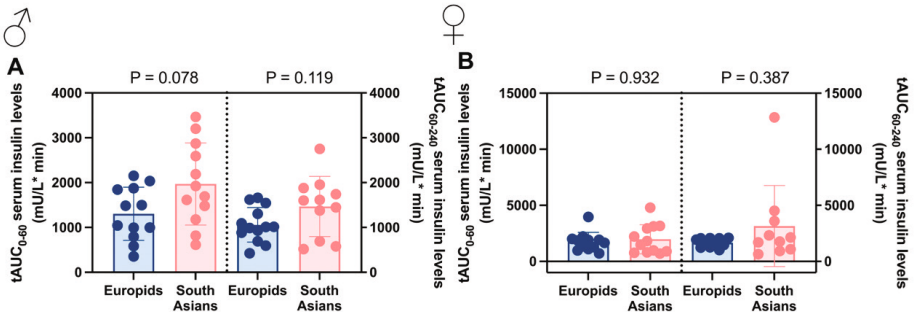
Supplemental Table 2. Overview of the area under the curve of the excursion of glucose, insulin, hormones, and lipids during a mixed meal tolerance test in South Asian compared to Europid females

	Europids		South Asians				
	Females		Females		P values		
	$tAUC_{0-240}$	$iAUC_{0-240}$	$tAUC_{0-240}$	$iAUC_{0-240}$	$tAUC_{0-240}$	$iAUC_{0-240}$	$P_{Interaction}$
Glycemic parameters							
Plasma Glucose (mmol/L * min)	1110±46[c]	1071±45[c]	1216±154[d]	1178±154[d]	0.043[v]	0.114[v]	0.156[v]
Serum Insulin (mU/L * min)	3409±1089[c]	3373±1086[c]	4988±4519[d]	4951±4506[d]	1.000[v]	0.654[v]	0.457[v]
Plasma Glucagon (nmol/L * min)	36.3±6.8[b]	35.0±6.4[b]	30.2±8.7[e]	29.1±8.3[e]	0.095[v]	0.464[v]	0.045[v]
Incretin hormones and glucagon							
Plasma Total GLP-1 (nmol/L * min)	70.5±11.3[b]	68.6±11.0[b]	79.5±12.8[e]	77.3±12.4[e]	0.148[v]	0.247[v]	0.394[v]
Plasma Active GLP-1 (nmol/L * min)	2.9±0.7[b]	2.9±0.6[b]	4.2±1.5[e]	4.1±1.5[e]	0.058[v]	0.129[v]	0.387[v]
Plasma Total GIP (ng/mL * min)	1374±164[b]	1351±164[b]	1598±408[e]	1571±402[e]	0.310[v]	0.702[v]	0.069[v]
Plasma Active GIP (ng/mL * min)	233±48[b]	231±48[b]	308±121[e]	304±118[e]	0.169[v]	0.651[v]	0.052[v]
Lipids							
Serum FFA (mmol/L * min)	94.6±25.1[c]	90.5±24.5[c]	84.2±30.0[d]	78.6±28.6[d]	0.349[v]	0.029[v]	0.022[v]
Serum TG (mmol/L * min)	137±45[c]	133±44[c]	170±72[d]	165±70[d]	0.314[v]	0.132[v]	0.538[v]
Serum TC (mmol/L * min)	805±129[c]	778±125[c]	850±195[d]	821±188[d]	0.654[v]	0.197[v]	0.454[v]

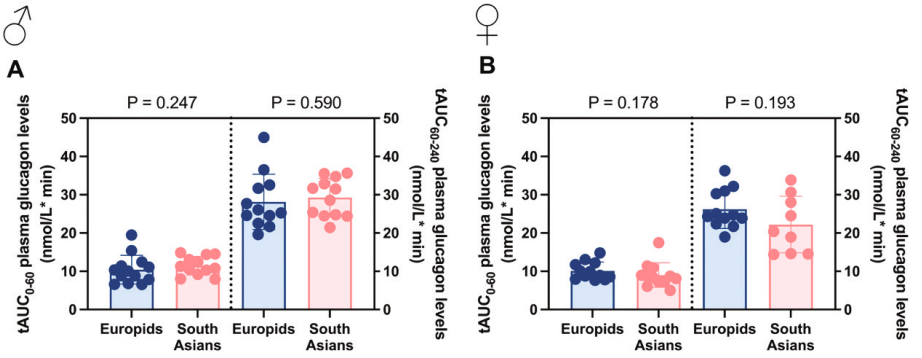
Table showing the mean and standard deviation of the total area under the curve ($tAUC_{0-240}$) and incremental area under the curve ($iAUC_{0-240}$) of the excursions of glucose, insulin, hormones, and lipid during a mixed meal tolerance test for both South Asian and Europid females. P-values of the comparisons between the two ethnicities were obtained via the non-parametric Man-Whitney U test and the p-values of the interaction were analyzed via a repeated measurement ANOVA. FFA, free fatty acids; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; TC, total cholesterol; TG, triglycerides. Letters indicate n values of each ethnicity, *b*n=12; *c*n=11; *d*n=10, *e*n=9, and *v*=21



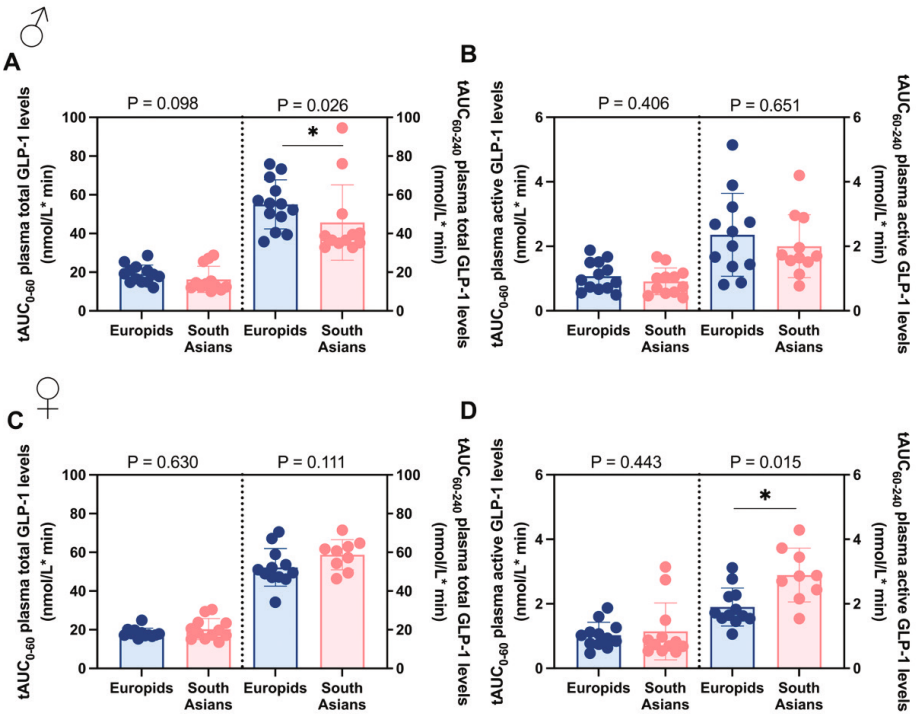
Supplemental Figure 1. Total areas under the curve of the glucose excursion within two periods during the mixed meal tolerance test in South Asian and Europid males and females
Box plots showing the total areas under the curve within two periods ($tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$) of the glucose excursion during the mixed meal tolerance test in South Asian (n=11) compared to Europid (n=12) males (**A)** and in South Asian (n=10) compared to Europid (n=11) females (**B**). Circles represent individuals' values and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. We were unable to retrieve a blood sample of one South Asian male at two time points, and of one Europid male, two South Asian females, and one Europid female at one time point.



Supplemental Figure 2. Total areas under the curve of the insulin excursion within two periods during the mixed meal tolerance test in South Asian and Europid males and females
Box plots showing the total areas under the curve within two periods ($tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$) of the insulin excursion in South Asian (n=11) compared to Europid (n=12) males (**A)** and in South Asian (n=10) compared to Europid (n=11) females (**B**). Circles represent individuals' values and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. We were unable to retrieve a blood sample of one South Asian female at two time points, and from one South Asian male, Europid male, one South Asian female, and one Europid female at one time point.

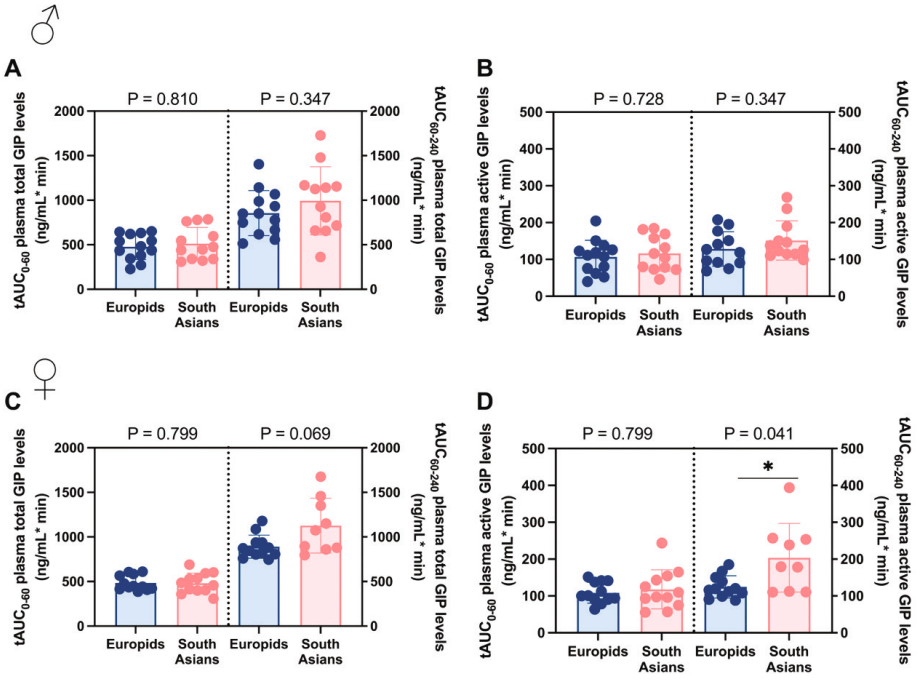


Supplemental Figure 3. Total areas under the curve of the glucagon excursion within two periods during the mixed meal tolerance test in South Asian and Europid males and females
Box plots showing the total areas under the curve within two periods ($tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$) of the glucagon excursion during the mixed meal tolerance test in South Asian (n=12) compared to Europid (n=12) males (**A)** and in South Asian (n=9) compared to Europid (n=12) females (**B**). Circles represent individuals' values and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. Due to a technical error, one sample of one Europid male is missing and we were unable to retrieve a blood sample of three South Asian females at one time point.



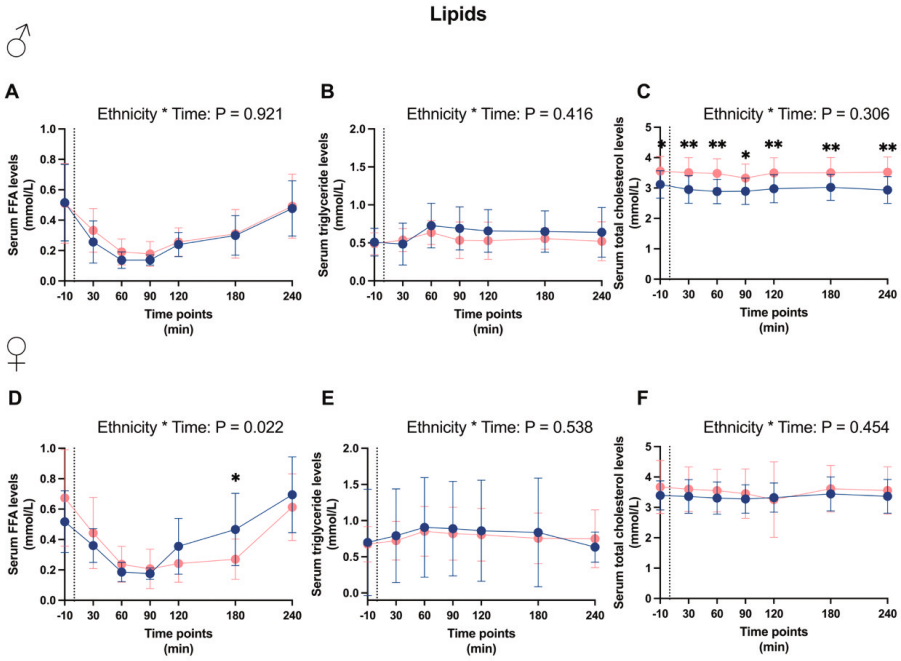
Supplemental Figure 4. Total areas under the curve of the total and active glucagon-like peptide-1 excursions within two periods during the mixed meal tolerance test in South Asian and Europid males and females

Box plots showing the total areas under the curve within two periods ($tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$) of the total glucagon-like peptide-1 (GLP-1) excursion in South Asian (n=12) compared to Europid (n=13) males (**A**) and box plots showing the $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of active GLP-1 excursion in South Asian (n=11) and Europid (n=12) males (**B**). Box plots showing the $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of the GLP-1 excursions in South Asian (n=9) compared to Europid (n=12) females (**C**) and box plots showing the $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of active GLP-1 excursion in South Asian (n=9) and Europid (n=12) females (**D**). Circles represent individuals' values and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. We were unable to retrieve a blood sample of one South Asian female at two time points, and from one South Asian male, Europid male, one South Asian female, and one Europid female at one time point.



Supplemental Figure 5. Total areas under the curve of total and active glucose-dependent insulinotropic polypeptide excursions within two periods during the mixed meal tolerance test in South Asian and Europid males and females

Box plots showing the total areas under the curve within two periods ($tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$) of the total glucose-dependent insulinotropic polypeptide (GIP) excursions during the mixed meal tolerance test (MMTT) in South Asian (n=12) compared to Europid (n=13) males (**A**) and box plots showing the $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of active GIP excursions in South Asian (n=12) and Europid males (n=12) (**B**). Box plots showing $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of total GIP excursion during the MMTT in South Asian (n=9) compared to Europid (n=12) females (**C**) and box plots showing $tAUC_{0\text{-}60}$ and $tAUC_{60\text{-}240}$ of active GIP excursions in South Asian (n=9) and Europid (n=12) females (**D**). Circles represent individuals' values and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. We were unable to retrieve a blood sample of three South Asian females at one time point and of one Europid male one time point missing due to a technical failure.



Supplemental Figure 6. Free fatty acids, triglycerides, and total cholesterol excursions before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graphs showing in South Asian (n=12) compared to Europid (n=13) males the free fatty acid (FFA) (**A)**, triglyceride **(B)**, and total cholesterol **(C)** excursions before and during a mixed meal tolerance test (MMTT). Similarly, showing in South Asian (n=12) compared to Europid (n=12) females line graphs showing the FFA, triglyceride, and total cholesterol excursions during an MMTT. Circles represent means and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. The dotted line is the time of the ingestion of the liquid meal. We were unable to retrieve a blood sample of one South Asian female at two time points, and from one South Asian male, Europid male, one South Asian female, and one Europid female at one time point.



CHAPTER 4

YOUNG AND LEAN SOUTH ASIANS HAVE HIGHER LEPTIN AND LOWER GHRELIN LEVELS BEFORE AND DURING A MIXED MEAL TOLERANCE TEST COMPARED TO EUROPIDS

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Submitted

ABSTRACT

Objectives

In South Asians, an unfavorable metabolic phenotype characterized by central obesity, insulin resistance, and dyslipidemia is more common than in Europids. Since various hunger and satiety hormones play a crucial role in managing energy balance, we aimed to investigate excursions of peptide YY (PYY), ghrelin, and leptin in response to a mixed meal tolerance test (MMTT) in young and lean South Asians and Europids.

Method

PYY, ghrelin, and leptin were measured in plasma obtained during an extended MMTT (up to 240 min) in young and lean South Asian (n=24) and Europid (n=25) males and females.

Results

At baseline and throughout the MMTT, no differences in PYY levels were observed between South Asian and Europid males and females. South Asian males had lower ghrelin levels before and throughout the MMTT compared to Europid males. Both South Asian males and females exhibited higher leptin levels at baseline and throughout the MMTT, resulting in a higher total area under the curve ($tAUC_{0\text{-}240}$) of leptin in South Asians compared to Europids. In addition, baseline leptin levels strongly positively correlated with fat mass and fat percentage in South Asian males and females, but generally not in Europids.

Conclusion

South Asian males and females exhibit higher leptin, while only South Asian males showed lower ghrelin levels at baseline and throughout an MMTT compared to Europids. The potential contribution of high leptin levels to the disadvantageous metabolic phenotype in South Asians remains to be determined.

INTRODUCTION

In 2022, 1 in 8 people were living with obesity (1, 2), a disease resulting from a long-term positive energy balance leading to excess storage of nutrients in adipose tissue (3). Obesity is associated with impaired health and an increased risk of developing various diseases including type 2 diabetes mellitus (T2DM), cardiovascular diseases, and certain types of cancers (4). In most individuals, obesity results from a combination of genetic predisposition, psychosocial factors, and exposure to obesogenic environments (4). Moreover, individuals from certain ethnic backgrounds, such as those of South Asian descent, have an increased risk of developing obesity and obesity-related diseases (5).

In South Asia, and particularly in Pakistan and India, the prevalence of obesity markedly increased since the last decades and is expected to increase further in the coming years (2, 6, 7). Moreover, South Asians have a metabolic phenotype that makes them more susceptible to health impairments arising from obesity, such as insulin resistance and cardiovascular disease (8). This increased risk for obesity-related diseases is not only seen in people currently living in South Asia but is even more pronounced among people of South Asian descent residing in Western countries (9). The disadvantageous metabolic phenotype of South Asians is characterized by a low lean mass, high fat mass, insulin resistance, and dyslipidemia (10). In addition, we (11) and others (12) reported a lower resting energy expenditure in South Asians, which predisposes them to a positive energy balance. This phenotype is hypothesized to be partly an adaptation to repeated periods of severe famine in the 19th and 20th centuries (13). Further contributing to this positive energy balance could be the previously found higher dietary energy intake in South Asians compared to Europids (14). Although numerous other factors contributing to this phenotype have been studied in recent years, many underlying factors remain unknown. For example, it is still unknown whether impaired satiety signaling could play a role in increasing their susceptibility to maintaining a positive energy balance.

Obesity is often associated with disruption of various hunger and satiety hormones, such as ghrelin, leptin, and peptide YY (PYY) (15-19). The hunger hormone ghrelin is released from the stomach and small intestine to prepare the body for a meal. Ghrelin stimulates appetite directly via receptors located in the hypothalamus and indirectly via ghrelin receptors on the vagal nerve (20, 21). In addition to regulating hunger, ghrelin promotes gluconeogenesis in the liver, stimulates gastric secretion and motility, and promotes lipid storage in adipocytes (22). On the other hand, the satiety hormone leptin, which is produced by adipocytes, signals through leptin receptors in the hypothalamus and brainstem, where it promotes satiety and increases energy expenditure (23, 24). Similarly, the satiety-inducing hormone PYY is released postprandially from the distal gut and binds

to its receptors (i.e., Y1 and Y2 receptors) in the area postrema and dorsal vagal complex located in the hindbrain, where it induces satiety and increases energy expenditure (19).

Understanding baseline levels and postprandial excursions of these hormones in South Asians versus Europids could provide valuable insights into underlying mechanisms that contribute to energy balance and the unfavorable metabolic phenotype of South Asians. Therefore, we aimed to investigate baseline levels and excursions of ghrelin, leptin, and PYY in young and lean South Asians compared to Europids during an extended (up to 240 min) mixed meal tolerance test (MMTT).

METHODS

Study Design

This study used samples obtained from the CAMI study (Elucidating the high cardiovascular disease risk in South Asians: focus on monocyte phenotype and incretin hormones), an observational study conducted at the Leiden University Medical Center (LUMC) between June and October 2023. The study was approved by the Medical Ethics Committee of the LUMC and undertaken in accordance with the principles of the revised Declaration of Helsinki (25). Written informed consent was obtained from all participants prior to inclusion. The clinical trial is registered at ClinicalTrial.gov (no. NCT05829018). The primary objective of the CAMI study was to compare immune cell composition between lean adolescent Dutch South Asians (hereinafter: 'South Asians') and BMI- and age-matched Dutch Europids (hereinafter: 'Europids'). In this manuscript, we report on one of the secondary objectives.

Participants

A total of forty-nine lean and healthy participants were included in the study, namely South Asian males (n=12) and females (n=12), and Europid males (n=13) and females (n=12). Additional inclusion criteria were a body mass index (BMI) of 18.0-25.0 kg/m^2 and age of 18-30 years. We included an additional (13th) Europid male as we encountered a technical problem during the collection of the samples for the primary endpoint of this study from one Europid male.

Participants were recruited especially through social media advertisements and by recalling participants from previous studies. Eligibility to participate in the study was tested primarily during a telephonic screening that consisted of questions about their heritage, body weight, height, and medical history. South Asian ethnicity was defined as having all four grandparents from Surinam, Bangladesh, India, Nepal, Pakistan, Afghanistan, Bhutan, or Sri Lanka. Europid ethnicity was defined by having

four grandparents originating from Europe. Exclusion criteria were the presence of an (auto-)immune disease, genetic lipid-associated disorders, chronic renal or hepatic disease, use of medication known to influence glucose and/or lipid metabolism, abuse of alcohol or other substances, smoking, vigorous exercise (more than 3 times per week), and milk or soy allergy.

Screening procedure

Participants refrained from vigorous exercise for 48 hours and from alcohol or caffeinated beverages for 24 hours prior to the study day. They consumed a standardized meal the evening before, which consisted of either a prepared supermarket meal with comparable ratio of carbohydrates and lipids (ranging from 450–600 kcal) or a similar meal at home and drank only water. After an overnight fast, participants arrived at the LUMC at 08:00 am, where they completed questionnaires about their medical history and current health. Subsequently, body weight and body composition were measured using bioelectrical impedance analysis (BIA) (InBody720, InBody CO., Ltd., CA, USA). In addition, height, waist, and hip circumference were measured with a measuring tape. BMI was calculated as weight in kilograms divided by height in meters squared (kg/m^2). To complete the screening procedure, a blood sample was collected by inserting a catheter into the antecubital vein for venous blood sampling. This was done using Vacutainer SST II Advance Gel and EDTA tubes to measure full blood count, glucose, insulin, and parameters related to kidney function, liver function, and lipid metabolism. If participants met the inclusion criteria based on the questionnaires, body composition measurements, and screening blood sample data, the MMTT was started directly after.

Mixed meal tolerance test

Upon inclusion, a baseline sample was taken using Vacutainer SST II Advance Gel tubes, a BD™ P800 collection tube, and an EDTA tube. Around 9.00 am, within 5 minutes, participants consumed a standardized liquid meal (200 mL, 300 kcal, 36.8 g carbohydrates, 12.0 g protein, and 11.6 g fat; Nutridrink strawberry flavor, Nutricia, The Netherlands). Blood samples were drawn at 7 time points (0 or baseline, 30, 60, 90, 120, 180, and 240 minutes). Blood collected in the BD™ P800 collection tubes and EDTA tubes was immediately stored on ice, while the Vacutainer SST II Advance Gel tube was clotted for at least 30 minutes at room temperature. The samples were then centrifuged to separate the plasma from the serum and were subsequently stored at -80°C until batch-wise analyses. Plasma levels of ghrelin, leptin, and PYY were measured from blood samples collected in the BD™ P800 collection tubes using a U-Plex Assay Platform (Meso-Scale Diagnostics, Gaithersburg, MD, USA). Commercial kits were used to measure serum total cholesterol (Roche Diagnostics, Woerden, The Netherlands), plasma glucose (Instruchemie, Delfzijl, The Netherlands), and serum insulin (Crystal Chem, Elk Grove Village, IL, USA).

Statistical analysis

Data are presented as means ± standard deviation. The normality of data was evaluated using the Shapiro-Wilk test, along with visual histograms, and Q-Q plots. For the baseline characteristics, waist-hip ratio (WHR) was calculated as waist circumference divided by hip circumference. Lean mass was calculated by subtracting the fat mass from the total body weight, both measured by BIA. Body fat percentage was calculated by dividing fat mass by body weight and multiplying by 100. HOMA-IR was calculated by multiplying fasting insulin levels (mU/L) with fasting glucose levels (mmol/L) and dividing by 22.5. Baseline characteristics were compared between ethnicities within the same sex using an independent t-test for normally distributed data (age, weight, length, BMI, hip circumference, fat mass, lean mass, and fasting glucose). Data that were not normally distributed data were log10 transformed to achieve normality (i.e., total cholesterol) and then analyzed using an independent t-test. For data that remained not normally distributed after log10 transformation (i.e., waist circumference, waist-hip ratio, body fat percentage, fasting insulin, HOMA-IR, and total triglycerides), a non-parametric test was used.

To compare the excursion of the hunger and satiety hormones during an MMTT, the total area under the curve (tAUC) was calculated using the trapezoid rule (26). To determine the incremental AUC (iAUC), which represents the area under the curve starting from the baseline, the area below the baseline value was subtracted from the tAUC. The Mann-Whitney U test, a non-parametric test, was used to compare tAUC and iAUC between the two ethnicities, as not all data followed a normal distribution. In addition, a two-way repeated measures ANOVA was applied, with 'time' as the within-subject factor and 'ethnicity' as the between-subject factor, to compare the hormone excursion during an MMTT between ethnicities. The general linear model's estimated marginal means comparison, corrected using the Bonferroni method to account for multiple testing, was used to compare the means at each time point between ethnicities. The leptin/ghrelin ratio was calculated by dividing baseline leptin levels (ng/mL) by baseline plasma ghrelin levels (ng/mL) and comparing the results between ethnicities by sex with the non-parametric Mann-Whitney U test, as the data were not normally distributed. Correlations between variables were assessed using Spearman's correlations due to the non-normal distribution of some data.

All statistical analyses were performed using SPSS v.29.0.1.0. Armonk, NY: IBM Corp. All graphs were created with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). The threshold for significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

As described elsewhere (27), South Asian males were shorter than Europid males (1.79 ± 0.06 m *vs.* 1.86 ± 0.07 m; P = 0.015), while body weight was similar (74.9 ± 7.2 kg *vs.* 73.6 ± 6.0 kg), resulting in a higher BMI among South Asian compared to Europid males (23.3 ± 1.5 kg/m^2 *vs.* 21.3 ± 1.5 kg/m^2; P = 0.004). In addition, South Asian males had more fat mass (13.5 ± 5.5 kg *vs.* 7.3 ± 2.0 kg; P = 0.002) and a higher body fat percentage (18.0 ± 7.2% *vs.* 9.8 ± 2.3%; P < 0.001) than Europid males. Furthermore, South Asian males had higher serum total cholesterol levels than Europid males (3.6 ± 0.5 mmol/L *vs.* 3.1 ± 0.4 mmol/L; P = 0.027).

Similarly, South Asian females were shorter than Europid females (1.63 ± 0.06 m *vs.* 1.74 ± 0.08 m; P = 0.003), and in addition had a lower body weight (60.3 ± 5.7 kg *vs.* 68.1 ± 9.1 kg; P = 0.020), resulting in a similar BMI compared to Europid females (22.6 ± 1.8 kg/m^2 *vs.* 22.5 ± 1.2 kg/m^2). South Asian females had a lower lean mass than Europid females (41.2 ± 3.6 kg *vs.* 51.5 ± 6.6 kg; P < 0.001).

No significant differences were observed in fasting glucose, insulin, or HOMA-IR between South Asian versus Europid males and between South Asian and Europid females.

South Asian males and females have similar plasma peptide YY levels at baseline and throughout an MMTT compared to Europid males and females

Generally, plasma levels of the satiety-inducing hormone PYY remained largely stable during the MMTT in males and females. In male South Asians *vs.* Europids, plasma PYY levels did not differ at baseline (1.08 ± 0.47 ng/mL *vs.* 1.43 ± 0.57 ng/mL; P = 0.109) or at other time points during the MMTT (**Fig. 1A**). In addition, plasma PYY did not change over time between ethnicities ($P_{Interaction}$ = 0.708, **Suppl. Table 1**) with no difference in tAUC (P = 0.168; **Fig. 1B**).

In female South Asians *vs.* Europids, plasma PYY levels also did not differ at baseline (1.51 ± 0.75 ng/mL *vs.* 1.15 ± 0.31 ng/mL; P = 0.233) or at other time points during the MMTT except at 240 minutes, being higher in South Asians compared to Europids (**Fig. 1C**). However, this did not result in a difference over time ($P_{Interaction}$ = 0.490; **Suppl. Table 2**) or a difference in tAUC (P = 0.310) between both ethnicities (**Fig. 1D**).

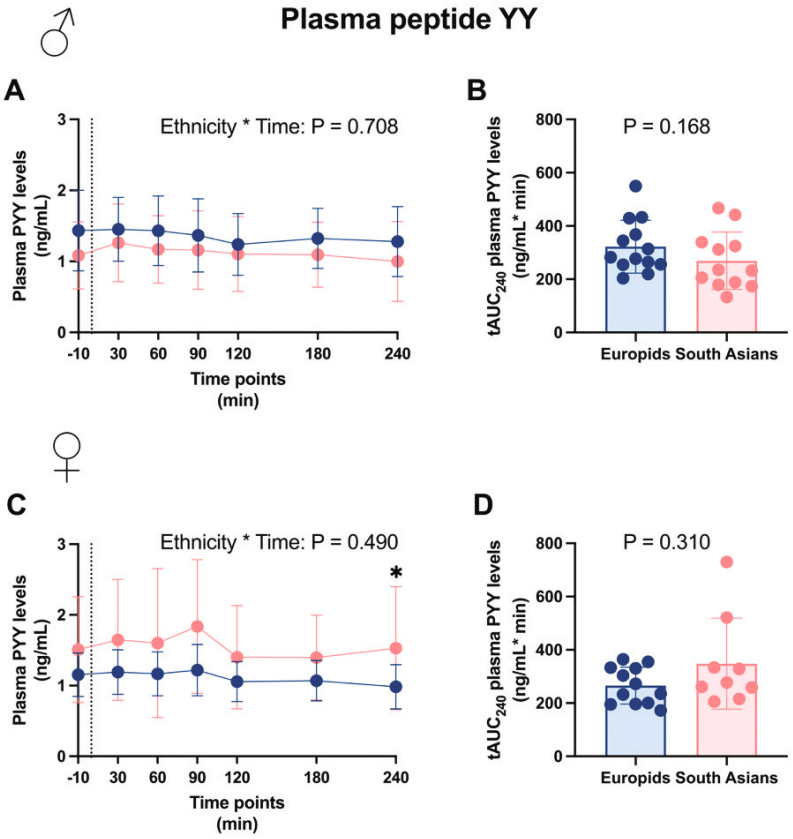


Figure 1. Plasma peptide YY levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graph showing plasma peptide YY (PYY) levels before and during a mixed meal tolerance test (MMTT) in South Asian males (n=12) compared to Europid males (n=13) **(A)**. Box plots showing the total area under the curve (tAUC) **(B)** in South Asian and Europid males. Similarly, a line graph showing the plasma PYY levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(C)** and box plots showing the tAUC **(D)** for South Asian and Europid females. Circles represent means in **A** and **C**, and individuals' values in **B** and **D** and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. The dotted lines in **A** and **C** indicate the time of the ingestion of the liquid meal. We were unable to retrieve a blood sample of three South Asian females at one time point.

South Asian males have lower plasma ghrelin levels at baseline and throughout an MMTT compared to Europid males

Generally, plasma levels of the hunger hormone ghrelin first decreased during the MMTT, and from 120 min on increased. In male South Asians *vs.* Europids, plasma ghrelin levels were lower at baseline (2.25 ± 1.42 ng/mL *vs.* 3.74 ± 2.08 ng/mL; P = 0.049). In addition, during the MMTT plasma ghrelin levels remained lower at 30, 60, 90, 120, and 240 minutes (all P < 0.05, **Fig. 2A**), resulting in a tendency towards a lower tAUC in South Asian compared with Europid males (P = 0.060; **Fig. 2B**). No significant difference in the excursion of plasma ghrelin levels was observed over time between ethnicities ($P_{Interaction}$ = 0.484; **Suppl. Table 1**).

In females, baseline plasma ghrelin levels did not differ between South Asians and Europids (2.88 ± 1.58 ng/mL *vs.* 3.47 ± 1.95 ng/mL; P = 0.651) nor during the MMTT (**Fig. 2C**). As in males, excursion of plasma ghrelin levels did not differ between ethnicities ($P_{Interaction}$ = 0.295; **Suppl. Table 2**), and tAUC was not different between ethnicities (**Fig. 2D**).

South Asian males and females have higher plasma leptin levels at baseline and throughout an MMTT compared to Europid males and females

Plasma levels of the satiety-inducing hormone leptin initially decreased, and from 120 min on increased. In South Asian males, plasma leptin levels were higher at baseline (82.9 ± 53.5 ng/mL *vs.* 21.7 ± 21.5 ng/mL; P < 0.001) and during all other time points during the MMTT (all P < 0.01) compared to Europid males (**Fig. 3A**). In addition, excursion of plasma leptin levels differed between ethnicities ($P_{Interaction}$ = 0.015; **Suppl. Table 1**) and South Asians had a higher tAUC (P < 0.001; **Fig. 3B**) compared to Europids. Compared to males, leptin levels were markedly higher in females of both ethnicities, which is in line with their higher fat mass. In South Asian females, circulating plasma leptin levels were significantly higher at baseline (410 ± 236 ng/mL *vs.* 183 ± 129 ng/mL; P = 0.030) and at 180 minutes during the MMTT (P = 0.043) (**Fig. 3C**) compared to Europid females. Again, excursion of plasma leptin levels differed between ethnicities ($P_{Interaction}$ = 0.047; **Suppl. Table 2**) and South Asians had a higher tAUC (P = 0.041) compared to Europids (**Fig. 3D**).

South Asian males and females have higher leptin/ghrelin ratio before and throughout the MMTT compared to Europid males and females

Given the reciprocal function of leptin (satiety-inducing) and ghrelin (hunger-inducing), the leptin/ghrelin ratio is a measure for hunger suppression (28, 29). The ghrelin/leptin ratio was overall higher at baseline and during the MMTT in South Asian *vs.* Europid males (**Fig. 4A**) and females (**Fig. 4B**).

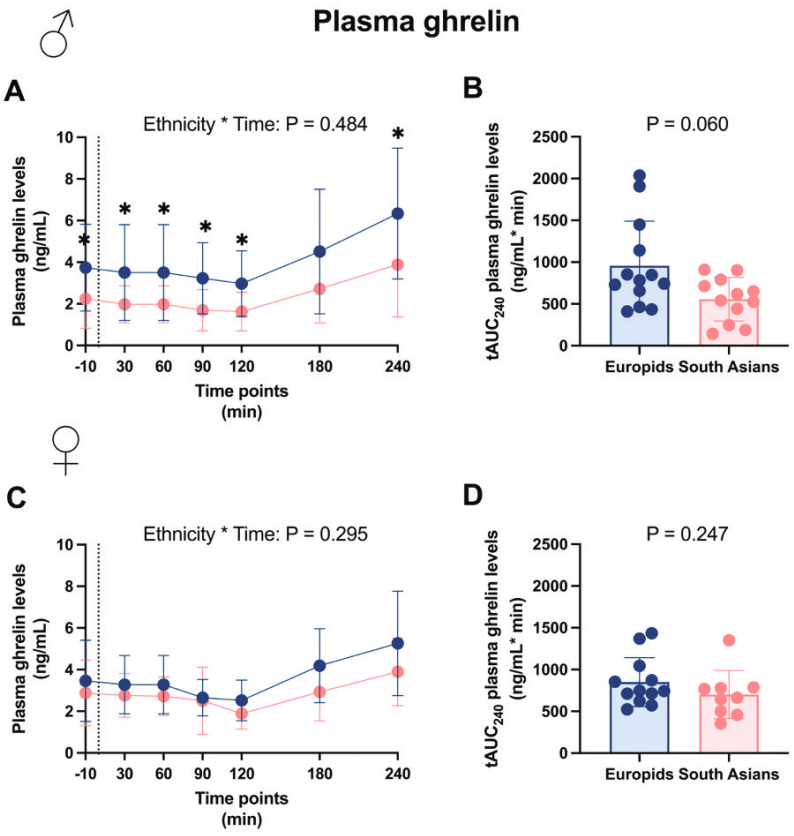


Figure 2. Plasma ghrelin levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graph showing plasma ghrelin levels before and during a mixed meal tolerance test (MMTT) in South Asian males (n=12) compared to Europid males (n=13) **(A)**. Box plots showing the total area under the curve (tAUC) **(B)** in South Asian and Europid males. Similarly, a line graph showing the plasma ghrelin levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(C)** and box plots showing the tAUC **(D)** for South Asian and Europid females Circles represent means in **A** and **C**, and individuals' values in **B** and **D**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. The dotted lines in **A** and **C** indicate the time of the ingestion of the liquid meal. We were unable to retrieve a blood sample of three South Asian females at one time point.

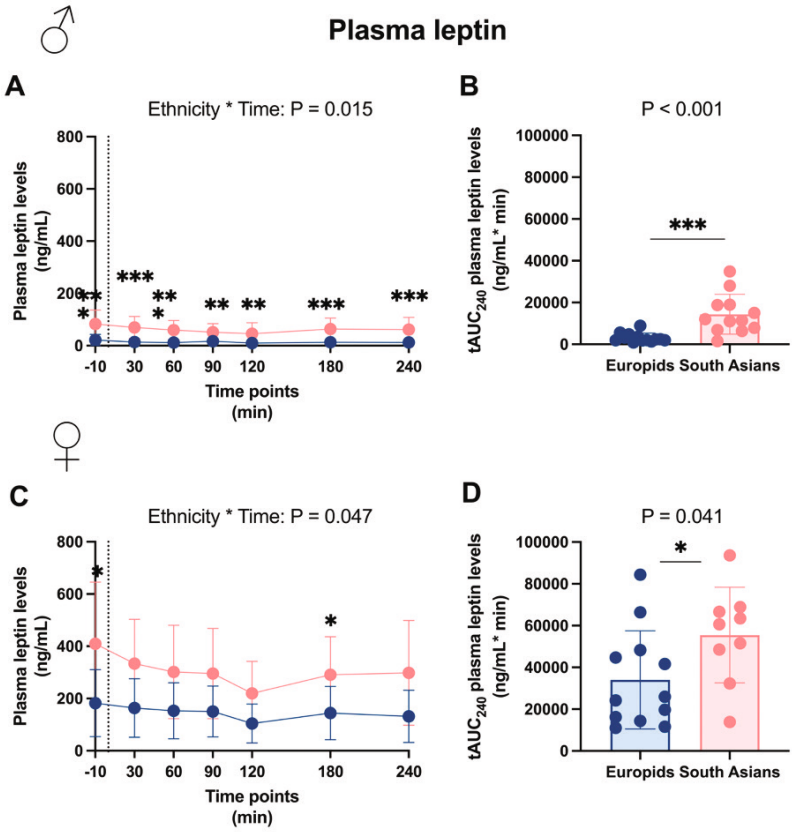


Figure 3. Plasma leptin levels before and during a mixed meal tolerance test in South Asian and Europid males and females

Line graph showing plasma leptin levels before and during a mixed meal tolerance test (MMTT) in South Asian males (n=12) compared to Europid males (n=13) **(A)**. Box plots showing the total area under the curve (tAUC) **(B)** in South Asian and Europid males. Similarly, a line graph showing the plasma leptin levels during an MMTT in South Asian females (n=9) compared to Europid females (n=12) **(C)** and box plots showing the tAUC **(D)** for South Asian and to Europid females. Circles represent means in **A** and **C**, and individuals' values in **B** and **D**, and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. The dotted lines in **A** and **C** indicate the time of the ingestion of the liquid meal. We were unable to retrieve a blood sample of three South Asian females at one time point.

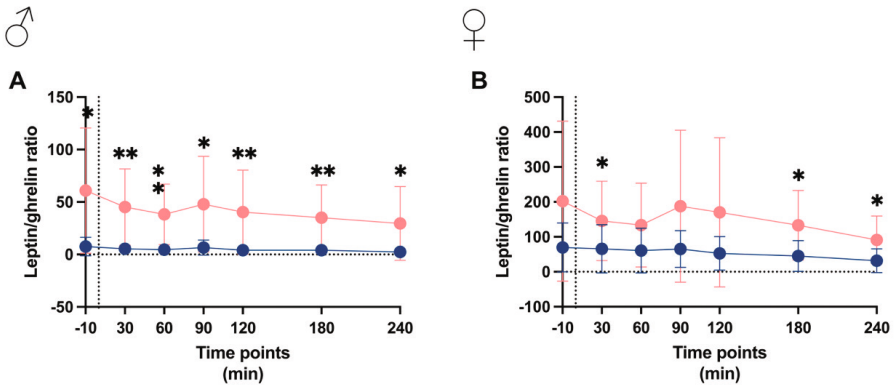


Figure 4. Plasma leptin/ghrelin ratio before and during a mixed meal tolerance test in South Asian compared to Europid males and females

Line graphs showing the plasma leptin/ghrelin ratio at baseline in South Asian males (n=12) compared to Europid males (n=13) **(A)** and in South Asian females (n=12) and Europid females (n=12) **(B)**. Circles represent means and deviations are the standard deviations. Blue circles, lines, and boxes represent Europids, and pink circles, lines, and boxes represent South Asians. The dotted lines indicate the time of the ingestion of the liquid meal.

Since previous studies showed a relationship between a higher leptin/ghrelin ratio and increased BMI, as well as reduced insulin sensitivity (29, 30), we correlated the baseline leptin/ghrelin ratio with both BMI and HOMA-IR in both ethnicities. In South Asian males, no correlation was observed between leptin/ghrelin ratio and BMI (rho = 0.266, P = 0.404; **Suppl. Fig. 2A**) or HOMA-IR (rho = 0.455, P = 0.138; **Suppl. Fig. 2B**). In Europid males, we did find a positive correlation between plasma leptin/ghrelin ratio and BMI (rho = 0.676, P = 0.011; **Suppl. Fig. 2A**) but not with HOMA-IR (rho = 0.214, P = 0.482; **Suppl. Fig. 2B**). In South Asian females, no correlations were found between leptin/ghrelin ratio and BMI (rho = 0.252, P = 0.430; **Suppl. Fig. 2C**) and HOMA-IR (rho = 0.210, P = 0.513; **Suppl. Fig. 2D**). Similarly, in Europid females no correlations were found between leptin/ghrelin ratio and BMI (rho = 0.210, P = 0.513; **Suppl. Fig. 2C**) and HOMA-IR (rho = 0.252, P = 0.430; **Suppl. Fig. 2D**).

Baseline plasma leptin levels positively correlate with fat mass and fat percentage in South Asian males and females

Given that fat percentage is higher in South Asians, which is not reflected by a higher BMI at least for females, we reasoned that plasma leptin levels may be a better marker for adiposity. Therefore, we next assessed whether baseline plasma leptin levels were related to fat mass and fat percentage in both South Asian and Europid males and females.

Baseline plasma leptin levels strongly positively correlated with fat mass in South Asian males (rho = 0.825, P < 0.001; **Fig. 5A**), and South Asian females (rho = 0.587, P = 0.045; **Fig. 5C**). A similar correlation was observed in Europid males (rho = 0.587, P = 0.035; **Fig. 5A**) but not Europid females (rho = 0.420, P = 0.175; **Fig. 5C**). Likewise, baseline plasma leptin levels were strongly positively correlated with fat percentage in South Asian males (rho = 0.909, P < 0.001; **Fig. 5B**) and South Asian females (rho = 0.608, P = 0.036; **Fig. 5D**). No such correlation was observed in Europid males (rho = 0.489, P = 0.090; **Fig. 5B**) and Europid females (rho = 0.266, P = 0.404; **Fig. 5D**).

In contrast, baseline plasma ghrelin and plasma PYY levels did not correlate with fat mass or fat percentage in South Asian or Europid males and females (data not shown).

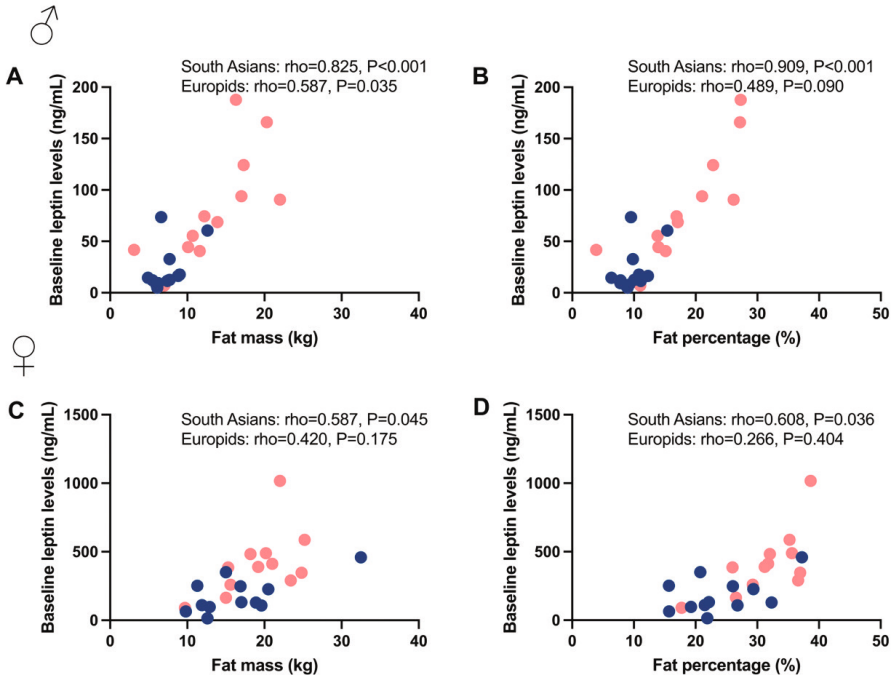


Figure 5. Correlations between baseline circulating plasma leptin levels and fat mass and fat percentage in South Asian compared to Europid males and females

Spearman correlation plots between baseline plasma leptin levels and fat mass in South Asian males (n=12, pink circles) and Europid males (n=13, blue circles) **(A)** and in South Asian females (n=12, pink circles) and Europid females (n=12, blue circles) **(C)**. Spearman collections of baseline plasma leptin levels and fat percentage in South Asian males (n=12, pink circles) and Europid males (n=13, blue circles) **(B)** and South Asian females (n=12, pink circles) and Europid females (n=12, blue circles) **(D)**.

DISCUSSION

In this study, we aimed to investigate the baseline levels of the hunger and satiety hormones ghrelin, leptin, and PYY, as well as their response to an extended MMTT, in young and lean South Asians compared to Europids. We observed several differences: Discussion

In this study, we aimed to investigate the baseline levels of the hunger and satiety hormones ghrelin, leptin, and PYY, as well as their response to an extended MMTT, in young and lean South Asians compared to Europids. We observed several differences: lower plasma ghrelin levels at baseline and throughout the MMTT in South Asian males, and higher plasma leptin levels at baseline and throughout the MMTT in both South Asian males and females. This resulted in a significantly higher leptin/ghrelin ratio especially in South Asian compared to Europid males. Furthermore, baseline plasma leptin levels were strongly positively correlated with both fat mass and fat percentage in South Asian males and females. The potential contribution of high leptin levels to the disadvantageous metabolic phenotype in South Asians remains to be determined.

With respect to the satiety hormone PYY, we did not observe differences between the two ethnicities at baseline and during the MMTT. While PYY has been generally described to peak 1-2 hours after a meal in healthy individuals (19), we did not observe a peak of PYY in our participants. However, this is in line with a previous study where PYY was measured in healthy individuals in an extended MMTT with multiple meals during different time points. This study showed no major change in circulating PYY levels after the first meal up to 180 minutes, after which another meal was provided (31). Theoretically, we may have missed a potential change of plasma PYY after the meal, as our study stopped after 240 minutes. A more prolonged study using various meals could give more information on PYY excursion in South Asians compared to Europids.

In contrast, plasma levels of the hunger hormone ghrelin were consistently lower in South Asian compared to Europid males at baseline and during the MMTT. Likewise, a previous study showed a lower fasting acylated ghrelin concentration in healthy South Asian *vs.* Europid men (32). Notably, they measured only the acylated ghrelin levels, while our study assessed total ghrelin, which includes both acylated and des-acyl ghrelin. Des-acyl ghrelin has both independent and opposing functions to acylated ghrelin (33, 34). Based on the disadvantageous metabolic phenotype of South Asians, lower plasma ghrelin levels would seem counterintuitive. However, both acylated and des-acyl ghrelin excursions have been found to be decreased in people at risk for T2DM after an oral glucose tolerance test (35). The underlying mechanisms behind the

observed lower ghrelin levels in this population, as well as the consequences, remain unclear and require further research.

The largest difference observed between South Asians and Europids throughout the MMTT in the current study was in circulating leptin levels. At baseline and throughout the MMTT circulating leptin levels were higher in both South Asian males and females compared to Europids, resulting in higher $tAUC_{0\text{-}240}$ in South Asians. Higher leptin levels in females compared to males within the same ethnicity and in South Asians compared with other ethnicities, including Europids, are in line with previous studies (32, 36, 37). Since leptin is secreted by adipocytes, adipocyte size and mass influence leptin secretion. Indeed, South Asians are known to have larger adipocytes compared to Europids (38). Since adipocyte size has been shown to be positively associated with leptin secretion (36), this could at least in part explain the higher circulating leptin levels observed in South Asians. Unfortunately, we did not measure adipocyte size in our cohort to study this directly. However, we did show that fat mass and fat percentage strongly positively correlated with leptin levels in both South Asian males and females and, to a lesser extent, in Europid males, suggesting that this may largely explain the higher leptin levels. In fact, the correlation between plasma leptin levels and fat mass and fat mass percentage was particularly strong in South Asian men, with a rho of 0.91 for fat mass percentage. Since BMI does not accurately reflect body composition in South Asians, these data suggest that leptin may form a more reliable measure for body fat in South Asians, especially in men. Interestingly, leptin levels were particularly high in South Asian females. Although this could at least in part be explained by their higher fat mass, potentially also a more leptin resistant state could be present in females, further contributing to higher leptin levels.

As ghrelin and leptin have opposing effects with respect to hunger and satiety, we reasoned the leptin/ghrelin ratio would provide insight into the balance between hunger and satiety signals. We observed a higher plasma leptin/ghrelin ratio throughout the MMTT in both South Asian compared to Europid males and females. This elevated ratio in males was mainly driven by a higher baseline ratio in South Asians. For females, the differences in plasma leptin/ghrelin ratio seemed more pronounced in the postprandial state, with higher ratios at 30, 180, and 240 min during the MMTT. Higher leptin/ghrelin ratios, whether in fasting conditions or postprandially, have been observed in people living with obesity (28). Interestingly, a higher leptin/ghrelin ratio is also associated with lower resting metabolic rate and insulin resistance (28, 29, 39), characteristics also known in the South Asian population (11, 40). This suggests that a higher leptin/ghrelin ratio in South Asians could potentially reflect a more compromised metabolic status, particularly in males. However, we did not find a significant correlation between the

baseline leptin/ghrelin ratio and BMI or HOMA-IR. The elevated plasma leptin/ghrelin ratio in South Asians could potentially influence the outcome of some intervention strategies. Previous research showed that individuals living with obesity with a higher baseline leptin/ghrelin ratio lose less body weight and fat mass after 12 weeks on a low-calorie diet (39). Whether the observed difference in the leptin/ghrelin ratio exacerbates as South Asians become more metabolically compromised, and how it affects the efficacy of caloric restriction in this population, or upon anti-obesity medication, remains to be seen.

A strength of this study is our ability to measure various hormones during an extended MMTT, lasting up to 240 minutes postprandially. The inclusion of a young and lean cohort of South Asian males and females allows us to gain insight into this population before significant metabolic derangements occur, as well as to observe differences between the sexes. However, this study does have its limitations. We used a 200 mL liquid meal, and while it offers a more comprehensive nutrient composition than an OGTT, the relatively small volume may have resulted in some hormonal differences being overlooked. Furthermore, while we extended the MMTT duration to 240 minutes, there remains the possibility that we missed observing changes in excursions of hormones that peak later than 240 minutes.

In conclusion, in South Asians *vs*. Europids, plasma ghrelin levels are lower and leptin levels are higher at baseline and during an MMTT, resulting in a higher leptin/ghrelin ratio particularly in South Asian men. Although the exact impact of these differentially regulated satiety hormones on the adverse metabolic profile of South Asians warrants further investigation.

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SUPPLEMENTAL DATA

Supplemental Table 1. Overview of the total and incremental areas under the curve in South Asian compared to Europid males

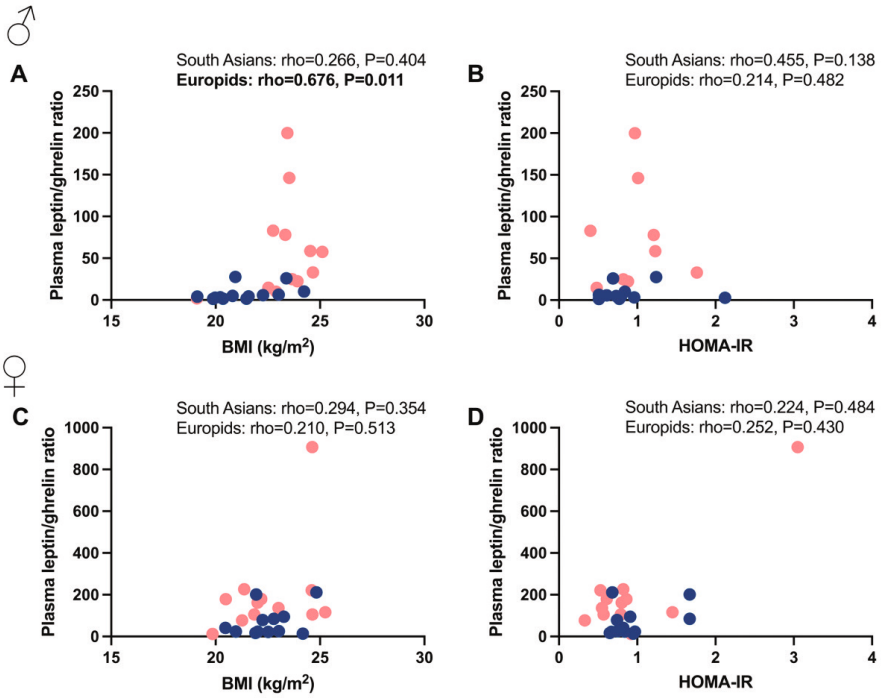
	Europid males	South Asian males	P values	
	$tAUC_{0-240}$	$tAUC_{0-240}$	$tAUC_{0-240}$	$P_{Interaction}$
Hunger & satiety hormones				
Plasma PYY (ng/mL * min)	323±99[a]	269±108[b]	0.168[c]	0.708[c]
Plasma Ghrelin (ng/mL *min)	958±532[a]	556±261[b]	0.060[c]	0.484[c]
Plasma Leptin (ng/mL * min)	3168±2173[a]	14404±9543[b]	<0.001[c]	0.015[c]

Means and standard deviations of the total area under the curve (tAUC) of the PYY, ghrelin, and leptin excursions for both South Asian and Europid males. P-values for the comparison between the two ethnicities were obtained via the non-parametric Man-Whitney U test and the p-value of the interaction was analyzed via repeated measurement ANOVA. PYY, peptide YY. Letters indicate n values of each ethnicity, *a*n=13; *b*n=12; and *c*n=25.

Supplemental Table 2. Overview of the total and incremental areas under the curve in South Asian compared to Europid females

	Europid females	South Asian females	P values	
	$tAUC_{0-240}$	$tAUC_{0-240}$	$tAUC_{0-240}$	$P_{Interaction}$
Hunger & satiety hormones				
Plasma PYY (ng/mL * min)	266±69[a]	348±171[b]	0.310[c]	0.490[c]
Plasma Ghrelin (ng/mL * min)	851±29[a]	701±288[b]	0.247[c]	0.295[c]
Plasma Leptin (ng/mL * min)	34046±23463[a]	55491±22860[b]	0.041[c]	0.047[c]

Means and standard deviations of the total area under the curve (tAUC) for both South Asian and Europid females with the p-value of the comparison between the two ethnicities via the non-parametric Man-Whitney U test and the p-value of the interaction analyzed via a repeated measurement ANOVA. PYY, peptide YY. Letters indicate n values of each ethnicity, *a*n=12; *b*n=9; and *c*n=21.



Supplemental Figure 1. Correlations between baseline circulating plasma leptin/ghrelin ratio and body mass index and HOMA-IR in South Asian compared to Europid males and females
Spearman correlation plots between baseline plasma leptin/ghrelin ratio and body mass index (BMI) in South Asian males (n=12, pink circles) and Europid males (n=13, blue circles) **(A)** and in South Asian females (n=12, pink circles) and Europid females (n=12, blue circles) **(C)**. Spearman collections of baseline plasma leptin/ghrelin ratio and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) in South Asian males (n=12, pink circles) and Europid males (n=13, blue circles) **(B)** and South Asian females (n=12, pink circles) and Europid females (n=12, blue circles) **(D)**.



CHAPTER 5

COLD EXPOSURE INCREASES CIRCULATING FIBROBLAST GROWTH FACTOR 21 IN THE EVENING IN MALES AND FEMALES

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ABSTRACT

Objectives

Cold exposure is linked to cardiometabolic benefits. Cold activates brown adipose tissue (BAT), increases energy expenditure, and induces secretion of the hormones fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15). The cold-induced increase in energy expenditure exhibits a diurnal rhythm in men. Therefore, we aimed to investigate the effect of cold exposure on serum FGF21 and GDF15 levels in humans and whether cold-induced changes in FGF21 and GDF15 levels differ between morning and evening in males and females.

Method

In this randomized cross-over study, serum FGF21 and GDF15 levels were measured in healthy lean males (n=12) and females (n=12) before, during, and after 90 minutes of stable cold exposure in the morning (7:45 am) and evening (7:45 pm) with a one-day washout period in between.

Results

Cold exposure increased FGF21 levels in the evening compared to the morning both in males (+61% *vs.* -13%; $P < 0.001$) and in females (+58% *vs.* +8%; $P < 0.001$). In contrast, cold exposure did not significantly modify serum GDF15 levels, and no diurnal variation was found. Changes in FGF21 and GDF15 levels did not correlate with changes in cold-induced energy expenditure in the morning and evening.

Conclusion

Cold exposure increased serum FGF21 levels in the evening, but not in the morning, in both males and females. GDF15 levels were not affected by cold exposure. Thus, this study suggests that the timing of cold exposure may influence cold-induced changes in FGF21 levels but not GDF15 levels and seems to be independent of changes in energy expenditure.

INTRODUCTION

Over the past decade, the popularity of ice baths and cold showers has been on the rise (1). Interestingly, the beneficial effects of cold exposure have already been described in the Edwin Smith Surgical Papyrus, the oldest known medical document (2, 3). Recent studies have shown the potential benefits of cold exposure, including its ability to reduce anxiety and improve insulin sensitivity in patients with type 2 diabetes (T2D) (2, 4, 5). The exact mechanism(s) underlying the metabolic improvement associated with cold exposure remain unknown.

A possible mechanism by which cold exposure improves metabolic health may be activating brown adipose tissue (BAT). Cold exposure stimulates the release of norepinephrine that binds to beta-adrenergic receptors located on BAT, of which the beta-2-adrenergic receptor seems to be the dominant receptor in humans (6, 7). Binding of norepinephrine triggers the oxidation of fatty acids (FA) and glucose to generate heat, a process facilitated by uncoupling protein 1 (UCP1) (8). Given its capacity to burn energy, activation of BAT holds promise as a potential tool for improving cardiometabolic diseases (9). We previously showed that metabolic BAT activity follows a diurnal rhythm in mice, with the highest uptake of triglyceride (TG)-derived FA by BAT at the onset of the active period, independent of environmental temperature (10). In humans, previous research has shown that glucose uptake by BAT might be higher in the morning (11). In addition, we recently showed that cold-induced thermogenesis, assessed by cold-induced energy expenditure and supraclavicular skin temperature, was higher in the morning than in the evening in humans (12). This difference was observed in males but not in females, suggesting a potential influence of a diurnal rhythm on BAT, which may be sex-dependent. Indeed, sex differences in thermoregulation and the release of various metabolic hormones following a stimulus have previously been reported and could potentially influence BAT activation and metabolic outcomes between males and females (13, 14).

It is known that BAT can release regulatory factors known as batokines with autocrine, paracrine, and endocrine actions that could enhance energy metabolism (15). Preclinical experiments have identified two batokines that have a prominent effect on energy balance: fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15) (16, 17). FGF21, when secreted by activated brown adipocytes, promotes thermogenesis through both UCP1-dependent and UCP1-independent mechanisms (18, 19). Additionally, FGF21 is secreted by the liver in a process dependent on the action of peroxisome proliferator-activated receptor α (PPARα) during fasting conditions (20). An increase in FGF21 levels leads to higher hepatic lipid oxidation, ketogenesis, and

gluconeogenesis (20). Similarly, GDF15 is secreted by brown adipocytes upon activation and targets macrophages to downregulate proinflammatory signals (21). GDF15 also induces satiety by acting on the glial cell line-derived neurotrophic factor (GDNF) family receptor alpha-like (GFRAL) located in the area postrema and nucleus solitary tract of the hindbrain (22-24).

Previous studies, conducted at room temperature, have shown a diurnal variation in FGF21 and GDF15 levels in humans, with the peak early in the morning and nadir in the afternoon (25, 26). Both FGF21 and GDF15 play important roles in metabolic health and therefore are potential tools in the treatment of metabolic diseases such as obesity and atherosclerosis (27-29). Understanding the diurnal influence on cold-stimulated FGF21 and GDF15 levels and potential sex differences may provide valuable insight into the most effective timing for BAT activation in both males and females.

Therefore, in the current study we aimed to investigate 1) the effect of cold exposure on serum FGF21 and GDF15 levels and 2) whether cold-induced changes in FGF21 and GDF15 levels differ between morning and evening in both males and females.

METHODS

Participants and study design

Participants

This study is a secondary analysis of a previously performed randomized cross-over study conducted at the Leiden University Medical Center (12). The study aimed to assess whether cold-induced thermogenesis varies between the morning and the evening in healthy lean males and females (12). A total of 24 participants, 12 males, and 12 females, were included. To be eligible for inclusion, participants had to be aged 18-31 years, with a body mass index (BMI) ranging from 18 to 26 kg/m^2. Participants were excluded if they had any active endocrine, renal, or hepatic disease, were taking medication that could affect glucose and/or lipid metabolism or BAT activity, were smokers, substance abusers, were pregnant, had recent weight changes, or had a disturbed day-night rhythm. The study was performed between December 2019 and December 2020.

Study approval

The study was undertaken in accordance with the principles of the Declaration of Helsinki (30) and approved by the local ethics committee of the Leiden University Medical Center, Leiden, the Netherlands. All participants provided written informed

consent before participation. The trial is registered at ClinicalTrials.gov (registration no. NCT04406922).

Study design

An extensive description of the design of the study has been published elsewhere (12). In short, each participant underwent a personal cooling protocol twice, once in the morning (7:45 am) and once in the evening (7:45 pm), with a one-day interval between the two study days. At the start of the study day, height, and waist- and hip circumference were measured. This was followed by the measurement of body weight and body composition using bioelectrical impedance analysis (digital balance; E1200, August Sauter GmBH, Albstadt, Germany and InBody720, InBody Co., Ltd., CA, USA). Afterward, an intravenous cannula was inserted into the antecubital vein to obtain blood samples during the study day. Finally, the participants lay down between two blankets filled with water (Blanketroll III, Cincinnati Sub-Zero Products, Inc, Cincinnati, Ohio, USA) at a thermoneutral temperature of 32°C for 45 minutes. Thereafter, the temperature was gradually decreased until either the participant began shivering or the minimal temperature of 9°C was reached. The temperature was then increased by 2-3°C and maintained at this level for 90 minutes. During the personalized cooling protocol energy expenditure was measured twice using indirect calorimetry (Vyntus CPX, Carefusion, Hochberg, Germany). In addition, blood pressure and heart rate were measured at different times during the personalized cooling protocol (t = 45, 65, and 180 minutes) using a cuff connected to a digital blood pressure device (Welch Allyn, Skaneateles, New York, USA).

Randomization

Participants were randomized to decide in which order the participant underwent the two study days, either morning-evening or evening-morning. The unequal distribution between the two randomization groups (with 10 and 14 participants, respectively) resulted from the randomization of newly recruited participants who replaced those unable to participate due to COVID-19-related restrictions.

Blood collection

For this study we used blood samples collected at three time points: after 45 minutes of thermoneutral conditions, 15 minutes after starting the cooling-down period, and after 90 minutes of stable cold exposure. Fasted venous blood samples were collected using Vacutainer SST II Advanced tubes, and serum was obtained after centrifugation and stored at -80°C until analysis. Serum FGF21 levels were measured using an immunoassay of the U-PLEX Human FGF21 assay platform (Meso Scale Discovery, Rockville, Maryland, USA). Serum GDF15 levels were measured with a custom-built

Luminex Screening assay (R&D Systems, Minneapolis, Minnesota, USA) was used in combination with the Bio-Plex Multiplex system (Bio-Rad, Hercules, California, USA). Serum cortisol levels were measured with the commercially available enzymatic kit (Roch Diagnostics, Woerden, the Netherlands).

Supraclavicular skin temperature measurement

The supraclavicular skin temperature was measured at 1-minute intervals using wireless iButton temperature loggers (31) and through infrared thermographic (IRT) images (FLIR T450sc, FLIR systems Inc., Wilsonville, OR, USA) focused on the upper thorax/neck region at the start of the study visit and after cold exposure. Analysis of the data recorded by the iButton temperature loggers was analyzed using Temperatus software (http://profith.ugr.es/temperatus) (31), while the IRT images were processed via an open-source IRT toolbox software. Further details of the methods were described previously (12).

Energy expenditure

A metabolic cart equipped with a ventilated hood system (Vyntus CPX, Carefusion, Hochberg, Germany) was used to collect data on total carbon dioxide production and oxygen consumption. At each study visit, energy expenditure (EE) was measured twice using indirect calorimetry: first at the end of the thermoneutral period when a stable cold temperature was reached and second during the last 30 minutes of stable cold exposure. The methods were described in detail previously (12).

Statistical analysis

Data are expressed as means ± standard error of the mean. Data normality was confirmed using the Shapiro-Wilk test, visual histograms, and Q-Q plots. All statistical analyses were done separately for males and females. For this clinical study, the statistical analysis of the baseline characteristics of this cohort has been extensively described elsewhere (12). To study the influence of circadian rhythm on cold-induced changes in serum GDF15 and FGF21 levels, a general linear model with repeated measures was used, with 2 within-subject factors. First, the timing during the day (morning *vs*. evening), and second, the cooling phase (thermoneutral, cooling down, and end of cooling). As the serum FGF21 levels, GDF15 levels, and cortisol levels were not normally distributed at all time points, we log10-transformed all serum FGF21 and GDF15 outcomes at the different time points. To compare the change of serum FGF21 and GDF15 levels between the morning and the evening, a delta was created (Δ_{cold}; values at the end of cooling phase *minus* values at thermoneutral period and $\Delta_{cooling}$; cooling-down *minus* thermoneutral) and two-tailed paired Student's t-tests were used. Moreover, to compare the serum FGF21 levels at thermoneutral conditions between

morning and evening as well as the serum GDF15 levels at thermoneutral conditions between morning and evening, paired Student's t-tests were used. To study the association between the change in energy expenditure with the change in serum FGF21 and GDF15 levels, deltas (Δ_{cold} and $\Delta_{cooling\ down}$) were created for all outcomes. Thereafter, nonparametric Spearman-rank correlations (rho) were applied to the raw data as the data were not normally distributed. All statistical analyses were performed using the Statistical Package for the Social Sciences v.25.0 (IBM Corp. Released 2018. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.), whereas all graphs were created with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

As previously reported (12), there were no significant differences in age, weight, and BMI between the males and females included in this study. However, the females were shorter with a higher body surface area (BSA), waist circumference, waist-to-hip ratio, fat mass, and fat percentage compared to males. Cold-induced thermogenesis, as measured by cold-induced energy expenditure and supraclavicular skin temperature, was higher in the morning compared to the evening for males only. In females, there was no diurnal variation in cold-induced thermogenesis, though they displayed an extended time to shivering and had a lower shivering temperature in the morning compared to the evening (12).

Cold exposure in the evening, but not in the morning, increases serum FGF21 in both sexes

At thermoneutral conditions, there were no significant differences in serum FGF21 levels in the morning compared to the evening in both males (332 ± 72 pg/mL *vs.* 288 ± 69 pg/mL; P = 0.088, **Fig. 1A**) and females (285 ± 50 pg/mL *vs.* 242 ± 39 pg/mL; P = 0.403, **Fig. 1D**). Cold exposure significantly increased serum FGF21 levels in the evening compared to the morning in both males ($P_{Interaction} < 0.001$; morning: -13 ± 10% *vs.* evening: +61 ± 21%; P < 0.001, **Fig. 1B** and **C**) and in females ($P_{Interaction} = 0.001$; morning: +8 ± 10% *vs.* evening: +58 ± 13%; P < 0.001, **Fig. 1E** and **F**).

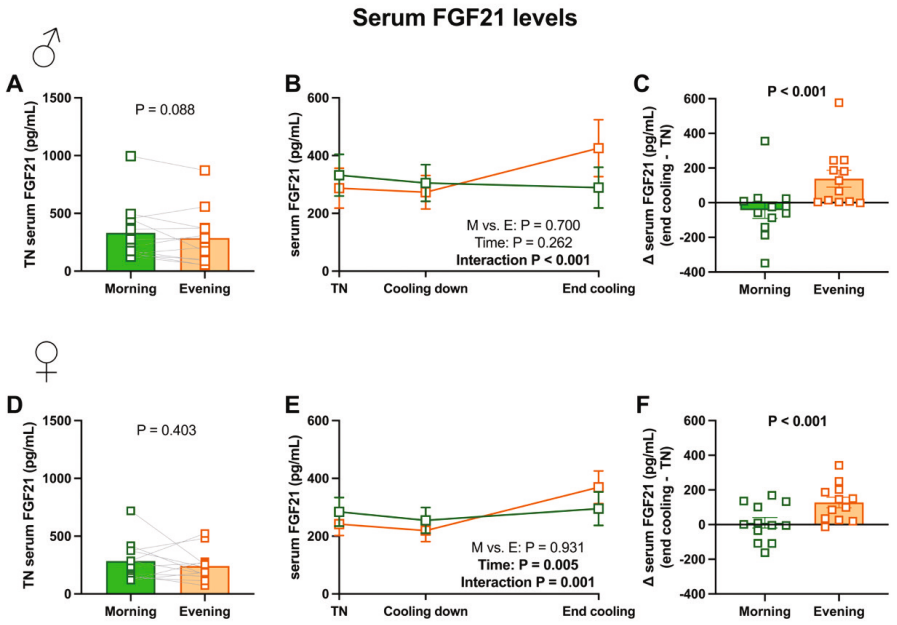


Figure 1. Changes in serum FGF21 levels after cold exposure in the morning compared to the evening in both males and females

Box plots showing serum FGF21 levels at thermoneutral (TN) conditions in the morning and the evening in males (n=12) **(A)** and females (n=12) **(D)**, and the cold-induced changes of serum FGF21 levels (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(C)** and females (n=12) **(F)**. Squares represent individual values, boxes represent means, and deviations represent the standard error of the mean. Linear model showing the changes of serum FGF21 levels after cold exposure in the morning and the evening in males (n=12) **(B)** and females (n=12) **(F)**. The changes were assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). However, for two participants, serum FGF21 levels in the cooling down phase in the evening could not be measured (n=10). Squares represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.

Neither cold exposure in the morning nor in the evening affects serum GDF15 levels in both sexes

At thermoneutrality, we observed similar GDF15 levels in the morning and in the evening in males (297 ± 41 pg/mL *vs.* 295 ± 32 pg/mL; P = 0.674, **Fig. 2A**) and females (315 ± 65 pg/mL *vs.* 289 ± 40 pg/mL; P = 0.865, **Fig. 2D**). Additionally, cold exposure did not modify serum GDF15 levels (P_{time} = 0.126, **Fig. 2B**). Further analyses showed that serum GDF15 levels before and after cold exposure were similar in the morning compared to the evening ($P_{Interaction}$ = 0.257; morning: +31 ± 18% *vs.* evening: +4 ± 7%; P = 0.293 **Fig. 2B** and **C**). Similarly, in females, cold exposure did not modify serum GDF15 levels (P_{time} = 0.929, **Fig. 2E**) and we did not detect significant differences in the GDF15 responses

upon cold exposure in the morning and the evening ($P_{Interaction}$ = 0.402; morning: +3 ± 15% *vs.* evening: +8 ± 6%; P = 0.395, **Fig. 2E** and **F**).

We also performed sensitivity analyses comparing $\Delta_{cooling\ down}$ of serum FGF21 and GDF15 levels during cooling compared to the thermoneutral phase in the morning and the evening. However, we observed no effect of cold exposure and therefore, no significant differences between morning and evening (data not shown).

In addition, we correlated circulating FGF21 levels with serum GDF15 levels, however we did not find a significant correlation (data not shown).

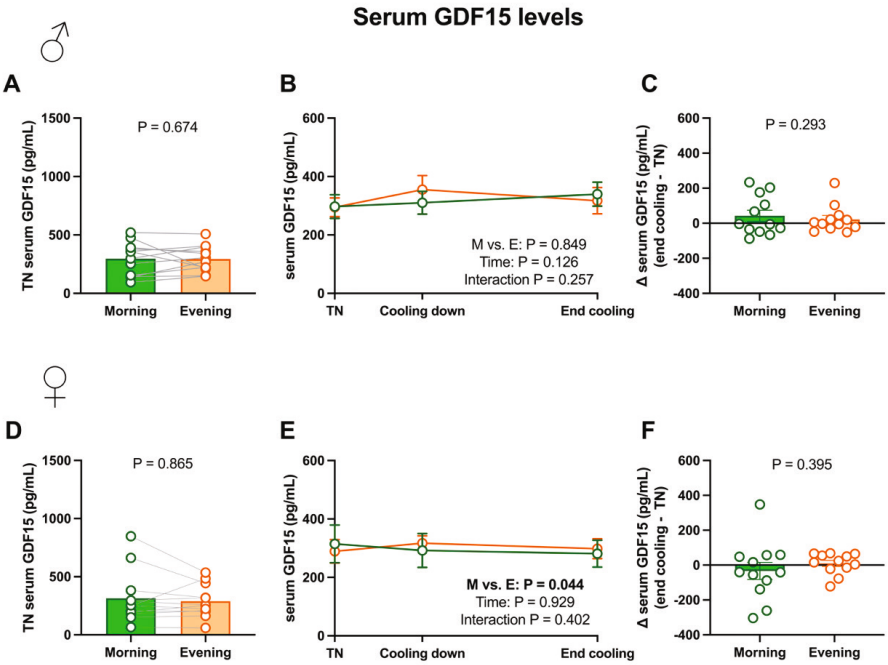


Figure 2. Changes in serum GDF15 levels after cold exposure in the morning compared to the evening in both males and females

Box plots showing serum GDF15 levels at thermoneutral (TN) conditions in the morning and the evening in males (n=12) **(A)** and females (n=12) **(D)**, and the cold-induced changes of serum GDF15 levels (end of cooling minus thermoneutral) in the morning and the evening in males (n=12) **(C)** and females (n=12) **(F)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean. Linear model showing the changes of serum GDF15 levels after cold exposure in the morning and the evening in males (n=12) **(B)** and females (n=12) **(E)**. The changes were assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). However, for two participants serum GDF15 levels in the cooling-down phase in the evening could not be measured (n=10). Dots represent means, and deviations represent the standard error of the mean. Green circles, lines, and boxes represent the morning, and orange circles, lines, and boxes represent the evening.

Serum cortisol increases after cold exposure in the evening in males only

Since cold exposure induces a stress response, we also compared the effects of cold exposure on circulating cortisol levels between morning and evening. First, as expected and in accordance with its diurnal rhythm, at thermoneutrality serum cortisol levels were higher in the morning compared to the evening in both males (318 ± 20 nmol/L *vs.* 114 ± 25 nmol/L; P < 0.001, **Supplemental Fig. 1A**) and females (526 ± 80 nmol/L *vs.* 270 ± 45 nmol/L; P = 0.002, **Supplemental Fig. 1C**). Additionally, serum cortisol only increased after cold in the morning in males compared to the evening (+20 ± 12% *vs.* -28 ± 8%; P = 0.008, **Supplemental Fig. 1B**). However, in females, circulating cortisol did not change significantly in the morning nor in the evening (-16 ± 11% *vs.* -5 ± 21%; P = 0.953, **Supplemental Fig. 1D**).

In addition, we correlated circulating FGF21 levels with serum cortisol, however, we did not find a significant correlation (data not shown).

The change of cold-induced serum FGF21 levels in the evening is correlated with the change of supraclavicular skin temperature but not with energy expenditure, in only females

We next assessed whether the effects of cold exposure on circulating FGF21 and GDF15 levels were associated with the cold-induced effects on supraclavicular skin temperature and energy expenditure. We found a significant and positive correlation between the change of serum FGF21 levels and the change of supraclavicular skin temperature in the evening in females (rho = 0.836, P < 0.001, **Supplemental Fig. 2B**), but not in males (P = 0.247, **Supplemental Fig. 2A**). We did not find a significant correlation in the morning for any sex (all P ≥ 0.142, **Supplemental Table 1**). Furthermore, the change in GDF15 levels did not correlate with the change in supraclavicular skin temperature at any time point (**Supplemental Table 1**). A lack of significance was also observed in the correlation between the change of serum FGF21 levels and the percental change in cold-induced energy expenditure in the morning and the evening in males (rho = -0.455, P = 0.160 and rho = 0.264, P = 0.433, respectively, **Supplemental Fig. 3A** and **B**) or females (rho = -0.300, P = 0.370 and rho = -0.082, P = 0.811, **Supplemental Fig. 3C** and **D**). Similarly, the change of cold-induced GDF15 levels was not correlated with the cold-induced changes of energy expenditure in the morning and the evening in males (rho = 0.591, P = 0.056 and rho = -0.255, P = 0.450, **Supplemental Fig. 4A** and **B**) or females (rho = -0.145, P = 0.670 and rho = 0.082, P = 0.811, **Supplemental Fig. 4C** and **D**). Finally, we assessed the effect of cold exposure on blood pressure and heart rate and found that cold exposure increased systolic (SBP) and diastolic blood pressure (DBP) similarly in the morning and evening in males (SBP: $P_{Interaction}$ = 0.499; morning: + 20 ± 2% *vs.* evening: +17 ± 2%; P = 0.337; **Supplemental Fig. 5A and B;** DBP:

$P_{Interaction}$ = 0.123; morning: +25 ± 4% *vs.* evening: +20 ± 3%; P = 0.150; **Supplemental Fig. 6A and B**) and females (SBP: $P_{Interaction}$ = 0.275; morning: +11 ± 1% *vs.* evening: +9 ± 2%; P = 0.428; **Supplemental Fig. 5C and D**; $P_{Interaction}$= 0.218; DBP: morning: +17 ± 2% *vs.* 13 ± 2%; P = 0.258; **Supplemental Fig. 6C and D**), without affecting heart rate (males: $P_{Interaction}$=0.426; morning: -9 ± 4% *vs.* evening: -7 ± 4%**;** P = 0.652; **Supplemental Fig. 7A and B**; females: $P_{Interaction}$ = 0.157; morning: +0 ± 4% *vs.* evening: -4 ± 5%; P = 0.289; **Supplemental Fig. 7C and D**).

The change in cold-induced serum FGF21 levels is negatively correlated with the change in serum triglycerides

Here, we investigated whether the changes in FGF21 and GDF15 levels are correlated with changes in circulating lipids. We observed that the change of serum FGF21 levels was negatively and significantly related to the change of serum free fatty acids exclusively in the morning in males (rho = -0.874, P < 0.001, **Supplementary Table 1**). However, this correlation was neither observed in the morning for females (rho = -0.085, P = 0.794) nor in the evening for both sexes (**Supplementary Table 1)**. On the other hand, we found a significant and negative correlation between the change of serum FGF21 levels and the change in circulating triglycerides, but only in the evening for females (rho = -0.580, P = 0.048, **Supplementary Table 1**). No significant correlations were observed in the other analyses (**Supplementary Table 1**). Lastly, the change in serum GDF15 did not correlate with any of the circulating lipids (**Supplementary Table 1**).

DISCUSSION

In this study, we observed that cold exposure increased serum FGF21 levels in the evening but not in the morning in both males and females. However, serum GDF15 levels were not affected by cold exposure, and no diurnal variation was found. Furthermore, no correlations were seen between the changes in serum FGF21 and GDF15 and changes in cold-induced energy expenditure in both sexes. Our findings thus suggest that the timing of cold exposure influences cold-induced changes in FGF21 levels.

Although circulating FGF21 has been reported to exhibit a diurnal rhythm, peaking early in the morning and reaching a nadir in the afternoon (26), we did not find higher thermoneutral FGF21 levels in the morning compared to the evening. This seeming discrepancy may be explained by the fact that the early peak of FGF21 already occurred prior to our FGF21 measurements during the study days in the morning (26). Moreover, the response of this rhythm to cold stimulation has remained unclear so far (26). Our study indicates that FGF21 levels only increase after cold exposure in the evening for

both males and females. The increase of FGF21 in the evening only could be attributed to the natural diurnal effect of FGF21, as the nadir is in the afternoon which increases towards the peak early in the morning. However, previous research showed that the biggest increase in FGF21 levels starts around midnight, which was much later than the completion of our study days (26). In addition, previous research showed that the natural increase of FGF21 levels was about 20% from nadir to peak (26). We found an increase in the evening of 61% in males and 58% in females after 90 minutes of cold exposure, supporting that the increase in FGF21 levels is likely not only attributed to the diurnal variation of FGF21. Nonetheless, this response is still modest compared to the approximately tenfold higher circulating FGF21 levels seen in patients with sepsis compared to healthy individuals (32).

Fasting is also known to influence FGF21 levels. This is a consequence of enhanced FFA release from white adipose tissue following fasting, resulting in activation of hepatic PPARα and FGF21 release (20). Of note, cold exposure seems to have similar effects on circulating FGF21 levels as fasting (33). A previous study where participants fasted for up to 72 hours resulted in increased FGF21 levels specifically in the evening, similar to our findings where cold exposure only increased serum FGF21 levels in the evening (34). To exclude the effect of fasting on serum FGF21 levels, participants fasted for the same time period before the study day in the morning and evening. In addition, the increase of FG21 in the evening only might have resulted from a stress response of the body to cold exposure. However, we did not find a significant increase in serum cortisol levels after cold exposure, and no significant correlation was found between the change in serum FGF21 levels and serum cortisol levels. This implies that the increase of serum FGF21 levels in response to cold may not be attributed to a higher stress level induced by cold exposure.

Interestingly, we found a positive correlation between the cold-induced change in supraclavicular skin temperature and serum FGF21 levels in the evening, specifically in females. The change in skin temperature may indicate alterations in either BAT activity or blood flow in the supraclavicular area (28). This suggests that FGF21 might play a more prominent role in maintaining normothermia or influencing BAT activity in the evening compared to the morning. The prominent role of FGF21 in cold-induced thermogenesis is supported by a previous study showing that individuals with lower FGF21 secretion shivered more intensely compared to individuals with higher FGF21 secretion and that a higher increase in FGF21 independently predicted a greater increase in cold-induced thermogenesis (35, 36). However, this correlation was not evident during the evening in males, hinting at a potential influence of female sex hormones on cold response. These sex-specific response differences align with our

previous findings, emphasizing the need for further studies to understand the role of sex hormones in cold-induced responses (12).

We previously showed that cold-induced energy expenditure was higher in the morning compared to the evening (12). Since cold increases energy expenditure via the activation of BAT, and FGF21 has a role in increasing cold-induced thermogenesis, we hypothesized that the FGF21 would also increase more in the morning compared to the evening. However, since we also did not find a correlation between cold-induced energy expenditure and cold-induced FGF21 levels, it suggests that the cold-induced increase in FGF21 levels might occur independently of changes in energy expenditure. This increase in FGF21 levels could potentially be a consequence of enhanced release by BAT and the liver following sympathetic stimulation.

In addition, we found a negative correlation between the cold-induced change in serum FGF21 levels and the change in FFA levels in the morning in males only, with the lowering of serum FGF21 levels with an increase in serum FFA. The decrease of FGF21 could have led to a decreased hepatic fat oxidation and thereby an accumulation of FFA in the circulation in the morning in males only.

In females, we found a negative correlation between the cold-induced change in serum FGF21 levels and triglyceride levels in the evening only. This supports the hypothesis of an increased BAT activity in the evening in females only, as indicated by a positive correlation between the cold-induced change in supraclavicular skin temperature and serum FGF21 levels in the evening in females. Increased BAT activity leads to increased skin temperature and the utilization of circulating triglyceride-derived FA, resulting in lower circulating triglycerides (10). The lack of correlations of change in serum FGF21 levels and the changes in skin temperature and triglycerides in males could indicate potential differences in BAT activity or metabolic responses to cold exposure between sexes (13, 14).

Interestingly, and in contrast to our expectation, cold exposure did not significantly increase circulating GDF15 levels, neither in the morning nor in the evening. These findings are in line with results in mice that were subjected to prolonged cold exposure (4°C) for up to 21 days. While prolonged cold exposure increased GDF15 gene expression, particularly in BAT and WAT, there was no corresponding rise in plasma GDF15 levels (21). While it is known that GDF15 is released by BAT in mice, hence it is called a batokine, GDF15 release in humans is more complex. Human GDF15 is expressed by many different tissues specifically after different stressors. GDF15 is expressed particularly in the gastrointestinal system, kidneys, placenta, and prostate,

suggesting that BAT may not be the primary contributor to GDF15 levels and could explain the lack of observed effects in our study (37). Additionally, it is possible that rapid breakdown and/or clearance of GDF15, with its short half-life of 3 hours, contributed to the lack of effect (38). We cannot exclude that other cellular stressors besides cold could have influenced our study as well (39). However, since all participants were healthy young individuals who were not subjected to any other (metabolic) stressors known to influence GDF15 levels this is unlikely (40). Furthermore, the circadian rhythm observed in GDF15 could have influenced our results as well. GDF15 is shown to peak at midnight and show a nadir at noon (25). As our study days were conducted at 7:45 am and 7:45 pm, the serum levels of GDF15 could have been in the decreasing phase during the morning and on the rise at 7:45 pm. Consequently, it could therefore be that we could not find a significant difference in cold-induced responsiveness of serum GDF15 levels between morning and evening. Additionally, previous research observed a considerable variation in the natural diurnal rhythm of GDF15 levels among individuals, with not all participants displaying diurnal fluctuations (25). Therefore, we may have missed the variation in GDF15 levels and/or that cold-induced GDF15 levels are not influenced by the timing of the day in our study.

A strength of this study was the use of a personalized cooling protocol, which accounted for individual differences in shivering time and temperature. Females, for example, had a longer time to reach shivering and a lower shivering temperature in the morning compared to the evening (12). Applying the same temperature to all individuals may have led to a failure to elicit cold-induced changes due to insufficient cold exposure. However, this study is not without limitations. It was not specifically powered to compare males and females, as the focus was on within-subject comparisons. Ideally, we would have incorporated a control group consisting of both males and females who had undergone measurements of FGF21 and GDF15 levels under thermoneutral conditions in both morning and evening. Then we could have accounted for the individual natural diurnal rhythm of FGF21 and GDF15. Additionally, the duration of stable cold exposure was relatively short (90 minutes) compared to previous research that utilized longer cold exposure periods to observe effects on FGF21 and GDF15 (21, 41). While mice have a more pronounced and faster release of FGF21 upon cold exposure compared to humans, studies in humans have shown a significant increase in circulating FGF21 after fasting for 7-10 days and after prolonged exposure to mild cold for 10 days in men (19, 42, 43). Therefore, it is possible that longer cold exposures may reveal effects that were not detected in our study.

In conclusion, we observed that 90 minutes of cold exposure specifically increases serum FGF21 levels in the evening, regardless of sex, while serum GDF15 levels are not affected. Further research is needed to explore the underlying mechanisms.

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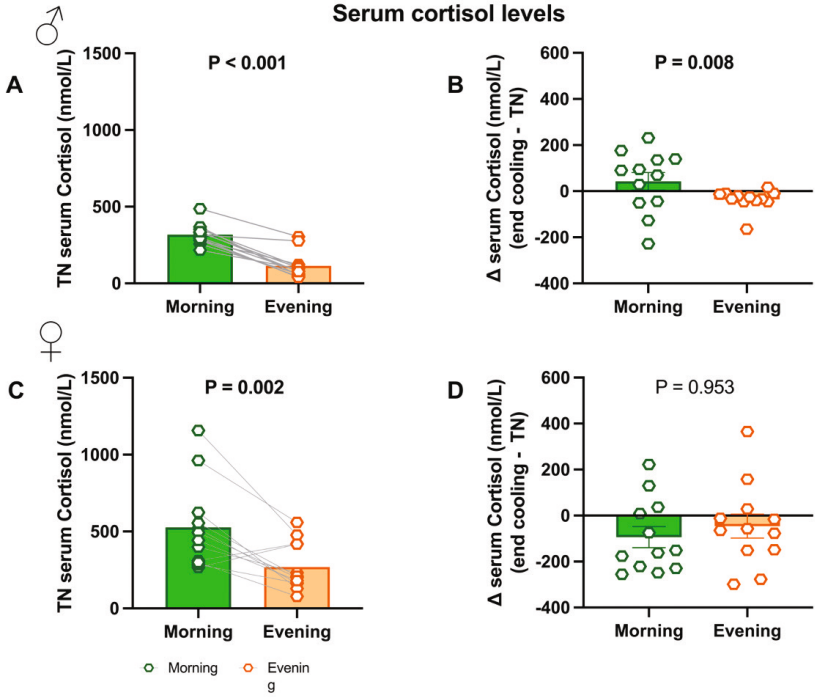
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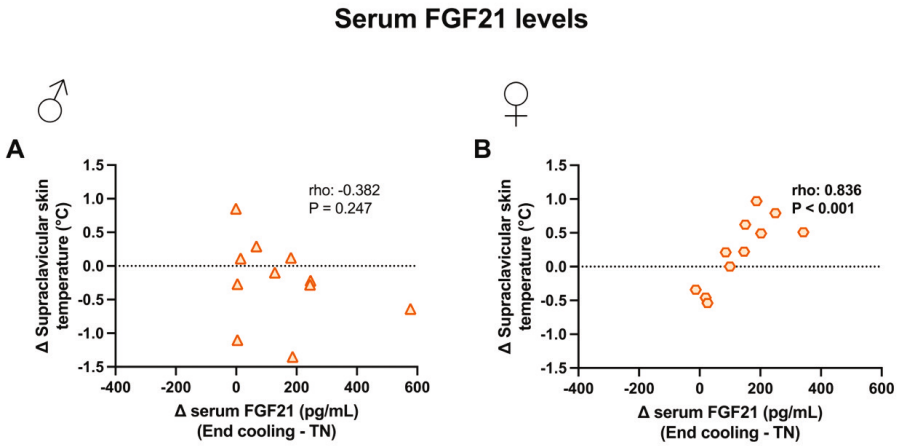
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SUPPLEMENTAL DATA



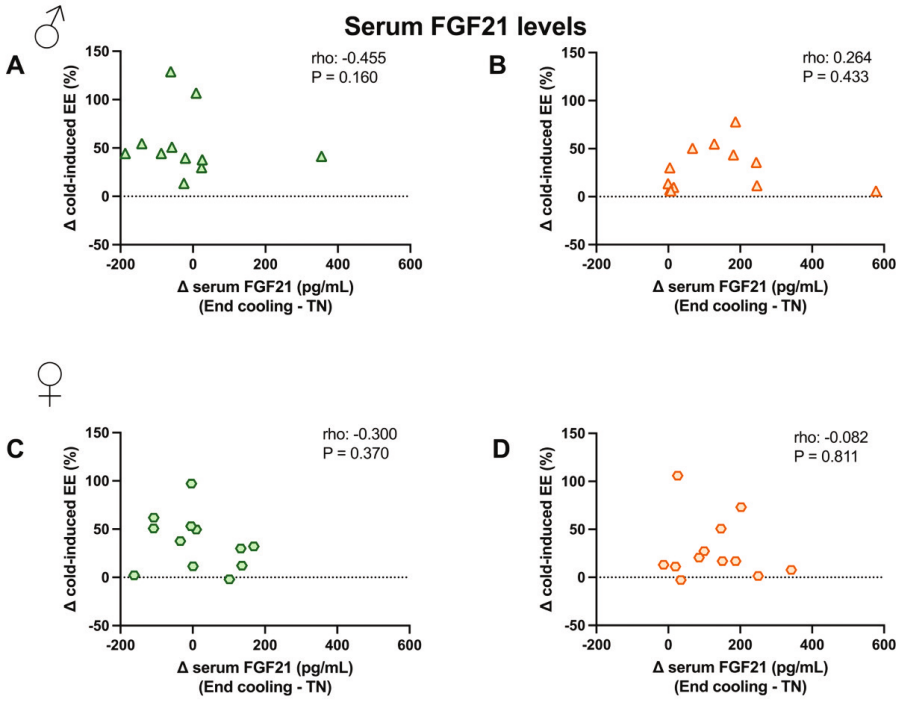
Supplemental Figure 1. Changes in serum cortisol levels after cold exposure in the morning compared to the evening in both males and females

Box plots showing serum cortisol levels at thermoneutral (TN) conditions in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**, and the cold-induced changes of serum cortisol levels (end of cooling minus thermoneutral) in the morning and the evening in males (n=12) **(B)** and females (n=12) **(D)**. Hexagons represent individual values, boxes represent means, and deviations represent the standard error of the mean.



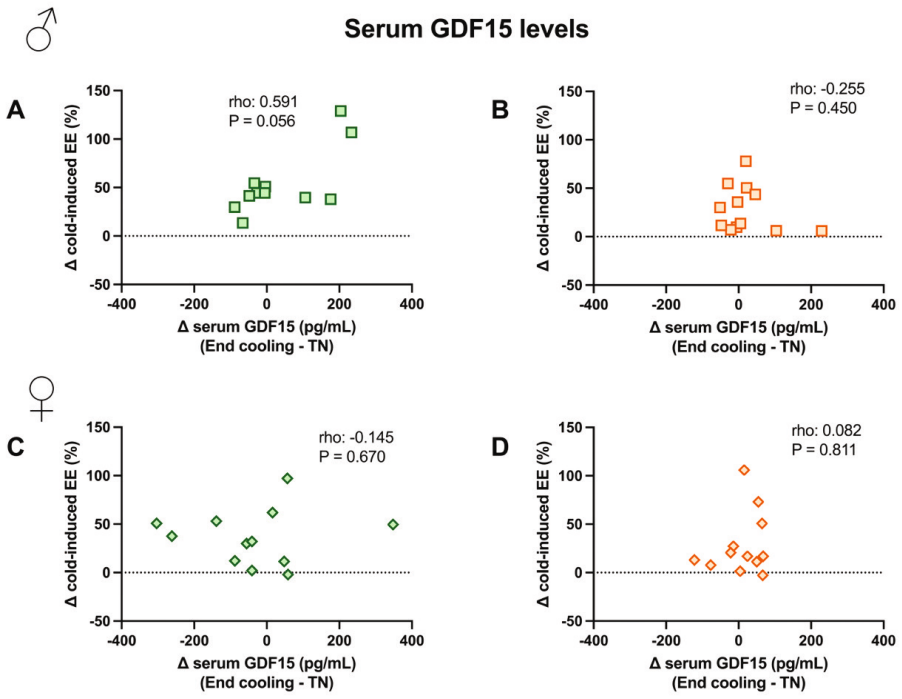
Supplemental Figure 2. Correlations between the change of serum FGF21 levels and the change of supraclavicular skin temperature after cold exposure in the evening

Spearman's correlations between the cold-induced change of serum FGF21 levels and change of supraclavicular skin temperature (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males (n=11; tiangles) **(A)** and females (n=11; hexagons) **(B)**, in the evening. Due to a problem with the data acquisition, data on the supraclavicular skin temperature of 1 male and 1 female were excluded. Symbols represent individual values.



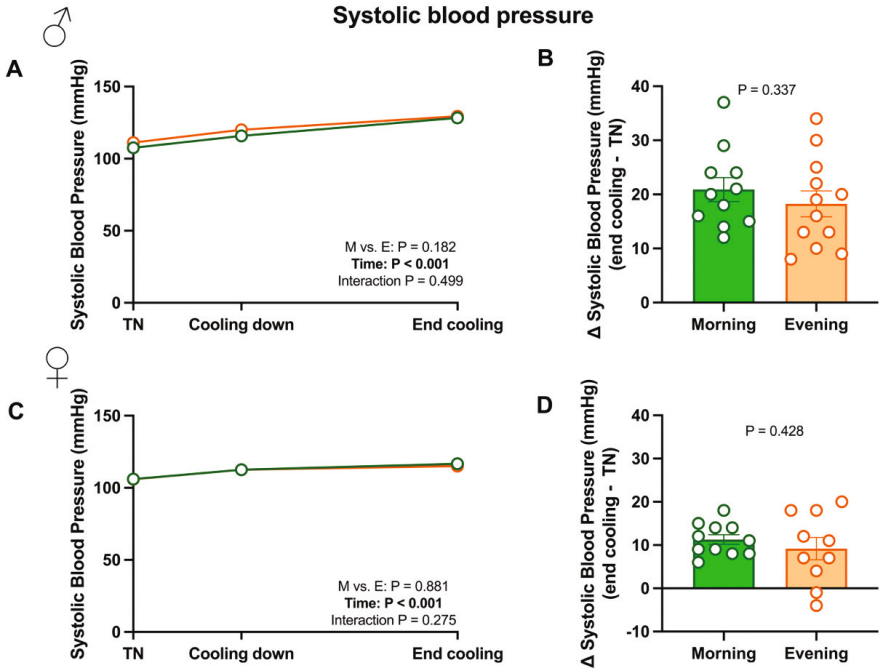
Supplemental Figure 3. Correlations between the change of serum FGF21 levels and the change of energy expenditure after cold exposure in the morning and evening

Spearman's correlation plots between the cold-induced change of serum FGF21 levels and the percentual change in energy expenditure (EE) (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males (triangles) **(A** and **B)** and females (hexagons) **(C** and **D)**, in the morning (n=11; green triangles and hexagons) **(A** and **C)** and evening (n=11; orange triangles and hexagons) **(B** and **D)**. The gas exchange measurement failed for 1 male and 1 female due to technical issues. Symbols represent individual values.



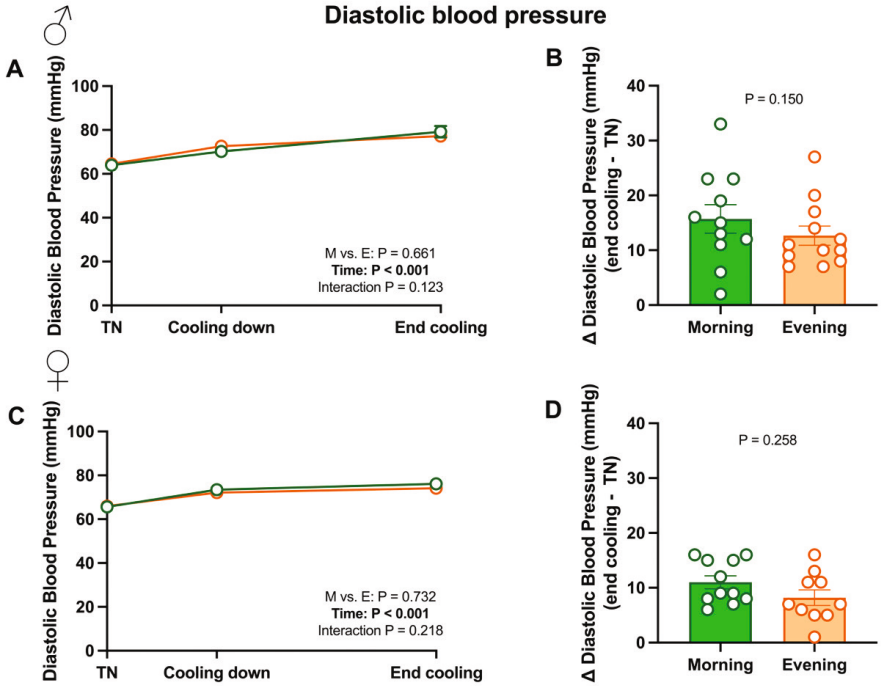
Supplemental Figure 4. Correlations between the change of serum GDF15 levels and the change of energy expenditure after cold exposure in the morning and evening

Spearman's correlation plot between the cold-induced change of serum GDF15 levels and the percentual change in energy expenditure (EE) (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males (squares) **(A** and **B)** and females (diamonds) **(C** and **D)**, in the morning (n=11; green squares and diamonds) **(A** and **C)** and evening (n=11; orange squares and diamonds) **(B** and **D)**. The gas exchange measurement failed for 1 male and 1 female due to technical issues. Symbols represent individual values.



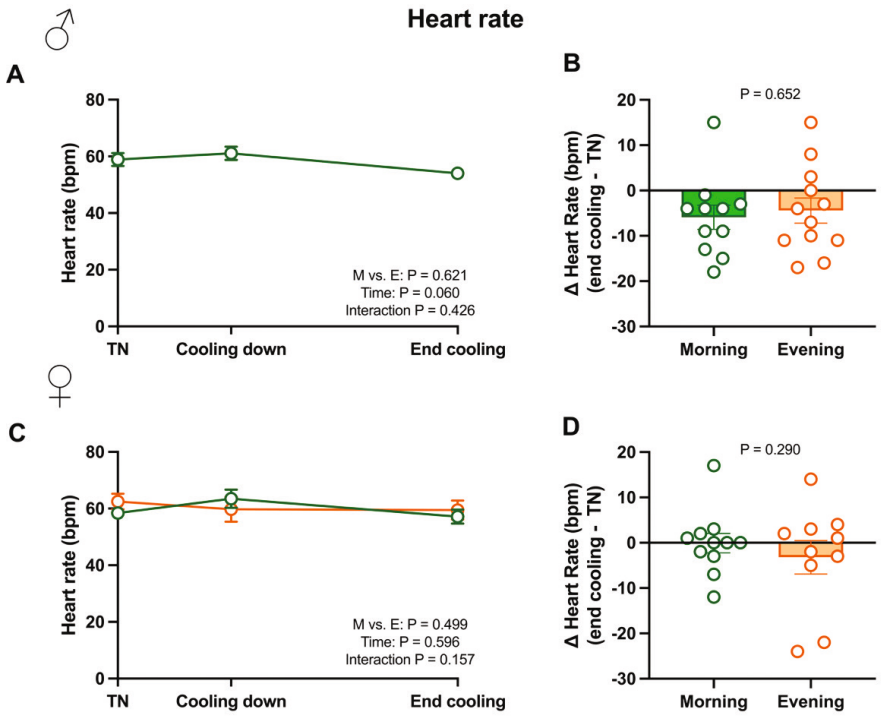
Supplemental Figure 5. Changes in serum systolic blood pressure after cold exposure in the morning compared to the evening in both males and females

Linear model showing the changes of systolic blood pressure after cold exposure in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**. Blood pressure was assessed at three time points (i.e., thermoneutral, cooling down, and end of cooling). Box plots showing the cold-induced changes of systolic blood pressure (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(C)** and females (n=12) **(D)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean. However, for one male and one female participant, systolic blood pressure could not be measured in the morning at the end of cooling (n=11). In the evening, systolic blood pressure could not be measured for four female participants during cooling (n=8), and for two female participants at the end of cooling (n=10). Dots represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.



Supplemental Figure 6. Changes in serum diastolic blood pressure after cold exposure in the morning compared to the evening in both males and females

Linear model showing the changes of diastolic blood pressure after cold exposure in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**. Blood pressure was assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). Box plots showing the cold-induced changes of diastolic blood pressure (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(B)** and females (n=12) **(D)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean. However, for one male and one female participant, diastolic blood pressure could not be measured in the morning at the end of cooling (n=11). In the evening, diastolic blood pressure could not be measured for four female participants during cooling (n=8), and for two female participants at the end of cooling (n=10). Dots represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.



Supplemental Figure 7. Changes in serum heart rate after cold exposure in the morning compared to the evening in both males and females

Linear model showing the changes in heart rate after cold exposure in the morning and the evening in males (n=12) **(A)** and females (n=12) **(C)**. Heart rate was assessed during three time points (i.e., thermoneutral, cooling down, and end of cooling). Box plots showing the cold-induced changes of heart rate (end of cooling minus thermoneutral) during the morning and the evening in males (n=12) **(B)** and females (n=12) **(D)**. Dots represent individual values, boxes represent means, and deviations represent the standard error of the mean.). However, for one male and one female participant, heart rate could not be measured in the morning at the end of cooling (n=11). In the evening, heart rate could not be measured for four female participants during cooling (n=8), and for two female participants at the end of cooling (n=10). Dots represent means, and deviations represent the standard error of the mean. Green boxes, lines, and boxes represent the morning values, and orange boxes, lines, and boxes represent the evening values.

Supplemental Table 1. Correlations between the change of serum FGF21 and GDF15 levels and the change of supraclavicular skin temperature and serum lipids after cold exposure in males and females, in the morning and evening

	Males		**Females**	
	Morning	**Evening**	**Morning**	**Evening**
Supraclavicular skin temperature				
		Δ Serum FGF21 levels (pg/mL)		
	Rho, P	Rho, P	Rho, P	Rho, P
Δ Supraclavicular skin temperature (°C)	-0.473; 0.142	-0.382; 0.247	-0.264; 0.433	0.836; 0.001**
		Δ Serum GDF15 levels (pg/mL)		
Δ Supraclavicular skin temperature (°C)	0.427; 0.190	0.109; 0.750	0.027; 0.937	0.227; 0.502
Lipids				
		Δ Serum FGF21 levels (pg/mL)		
Δ Serum Free fatty acids (mmol/mL)	-0.874; <0.001***	-0.085; 0.794	-0.273; 0.391	0.084; 0.795
Δ Serum Triglycerides (mmol/mL)	-0.294; 0.354	-0.014; 0.966	-0.245; 0.443	-0.580; 0.048*
		Δ Serum GDF15 levels (pg/mL)		
Δ Serum Free fatty Acids (mmol/mL)	-0.168; 0.602	0.269; 0.400	-0.147; 0.649	-0.263; 0.409
Δ Serum Triglycerides (mmol/mL)	0.559; 0.059	0.266; 0.403	-0.329; 0.297	0.119; 0.731

Spearman's correlations between the cold-induced change of serum FGF21 and GDF15 levels and change of supraclavicular skin temperature and serum lipids (Δ_{cold}: end of cooling minus thermoneutral (TN)) in males and females, in the morning and evening. *P < 0.05, **P < 0.01, ***P < 0.001,



CHAPTER 6

STIMULATION OF THE BETA-2-ADRENERGIC RECEPTOR WITH SALBUTAMOL ACTIVATES HUMAN BROWN ADIPOSE TISSUE

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SUMMARY

While brown adipose tissue (BAT) is activated by the beta-3-adrenergic receptor (ADRB3) in rodents, in human brown adipocytes the ADRB2 is dominantly present and responsible for noradrenergic activation. Therefore, we performed a randomized double-blinded crossover trial in young lean men to compare the effects of single intravenous bolus of the ADRB2 agonist salbutamol without and with the ADRB1/2 antagonist propranolol on glucose uptake by BAT, assessed by dynamic 2-[^{18}F]fluoro-2-deoxy-D-glucose PET-CT scan (i.e., primary outcome). Salbutamol, compared to salbutamol with propranolol, increases glucose uptake by BAT, without affecting the glucose uptake by skeletal muscle and white adipose tissue. The salbutamol-induced glucose uptake by BAT positively associates with the increase in energy expenditure. Notably, participants with high salbutamol-induced glucose uptake by BAT have lower body fat mass, waist-hip ratio and serum LDL-cholesterol concentration. In conclusion, specific ADRB2 agonism activates human BAT, which warrants investigation of ADRB2 activation in long-term studies (EudraCT: 2020-004059-34).

INTRODUCTION

Over the last decades, brown adipose tissue (BAT) has become an attractive target to stimulate energy dissipation to improve cardiometabolic health (1). BAT is a highly vascularized thermogenic organ mainly located in the deep neck region, along large blood vessels and in the supraclavicular area, and combusts triglyceride-derived fatty acids and glucose into heat (2-4). Naturally, the most potent activator of BAT is cold exposure, which increases sympathetic outflow towards beta-adrenergic receptors on BAT (5, 6).

In rodents, the beta-3-adrenergic receptor (ADRB3) is the main adrenergic receptor found on brown adipocytes and activation of the ADRB3 has been shown to effectively activate BAT and improve cardiometabolic outcomes in mice (7-9). In humans, however, the involvement of ADRB3 in BAT activation is less clear (10-16). The ADRB3 agonist mirabegron increases the uptake of the glucose analogue 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) by BAT, increases whole body lipolysis and increases resting energy expenditure. Nevertheless, this only occurs after administration of a supratherapeutic dose of 200 mg,(10-12) which highly exceeds the therapeutic dose of 50 mg to treat hyperactive bladder in the clinic. In addition, at 200 mg cardiovascular side effects occur, such as an increase in heart rate and systolic blood pressure, raising the possibility that mirabegron cross-reacts with other beta-adrenergic receptors such as the ADRB1 and ADRB2 that are also present on the cardiovascular system and as such contribute to the increase in energy expenditure (17).

Indeed, we recently showed that the therapeutic dose of 50 mg mirabegron is ineffective to increase oxidative metabolism in BAT, and that the ADRB2 is in fact the dominant adrenergic receptor expressed in human BAT biopsies and brown adipocytes, while the expression of ADRB3 is negligible (12). Accordingly, evidence supporting the hypothesis that ADRB2 is responsible for stimulating thermogenesis in human BAT stems from our *in vitro* experiments in human brown adipocytes, where we demonstrated that: 1) stimulation with mirabegron did not increase oxygen consumption; 2) stimulation with the ADRB2 agonist formoterol increased oxygen consumption, which was inhibited when pre-exposed with a selective ADRB2 antagonist; and 3) knock-down of ADRB2, but not ADRB1 or ADRB3, reduced norepinephrine-stimulated oxygen consumption (12). Therefore, the aim of this study was to investigate the role of ADRB2 in activation of human BAT *in vivo*. To this end, as a proof of concept, we evaluated the acute effect of the specific ADRB2 agonist salbutamol on glucose uptake by BAT without and with the ADRB1/2 antagonist propranolol in healthy lean men.

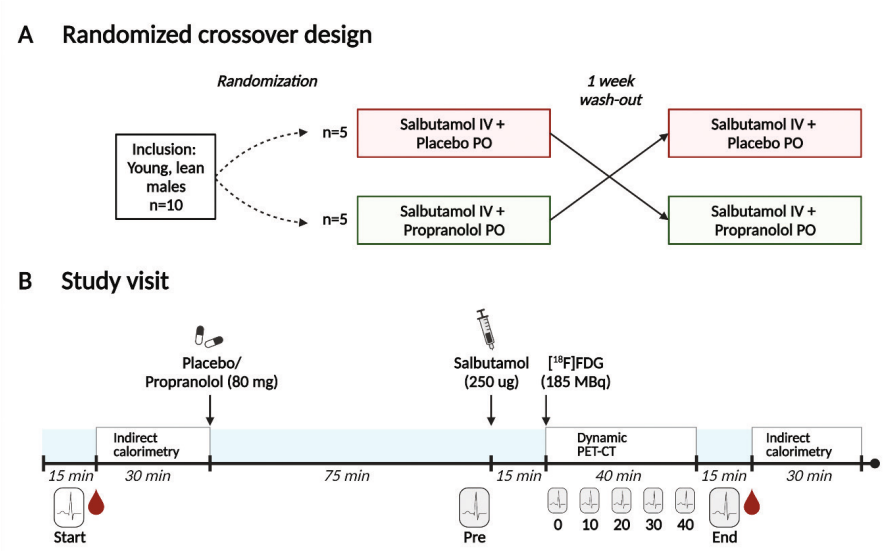


Figure 1. Study design and time line of study procedures.
A) This study had a randomized, double-blinded, crossover design.
B) Both study visits started with the measurement of blood pressure and heart rate (indicated by the ECG icon). Thereafter, the first blood sample (indicated by blood drop icon) was drawn, followed by an indirect calorimetry measurement for 30 min. Then, participants received either placebo or propranolol (80 mg, in two capsules; per oral; PO), depending on the study visit. After 75 min, blood pressure and heart rate were measured again and a single bolus of salbutamol (250 µg, intravenous; IV) was injected over a continuous time course of 5 min. 15 min after initiation of the injection, a low dose computed tomography (CT) scan was performed, directly followed by injection of 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG; 185 MBq) and a dynamic positron emission tomography (PET) acquisition, during which heart rate was monitored. After termination of the scan, blood pressure and heart rate were measured, the final blood sample was drawn, and indirect calorimetry was performed for 30 min.

RESULTS

Salbutamol increases heart rate and tends to increase energy expenditure

In total, 10 young (age: 24.4 ± 4.3 years) and lean (body mass index: 23.1 ± 2.3 kg/m^2) males were included in this study and participated in two experimental study visits (see **Figure 1A and B**). A single intravenous bolus of salbutamol (250 µg) acutely increased heart rate (+16.9 ± 10.5 bpm, P = 0.001), but not when combined with propranolol (-2.8 ± 8.9 bpm, P = 0.35; interaction between treatments P < 0.001; **Figure 2A**). This initial effect of salbutamol injection on heart rate gradually faded (**Figure 2B**), resulting in a non-significant difference between treatments at the end of the study visit (**Figure 2C**). Salbutamol did not affect systolic blood pressure (+2.4 ± 8.0 mmHg; P = 0.38) or diastolic blood pressure (+3.2 ± 9.7 mmHg, P = 0.35) (i.e., end of treatment visit *versus* before treatment). Salbutamol combined with propranolol tended to decrease systolic

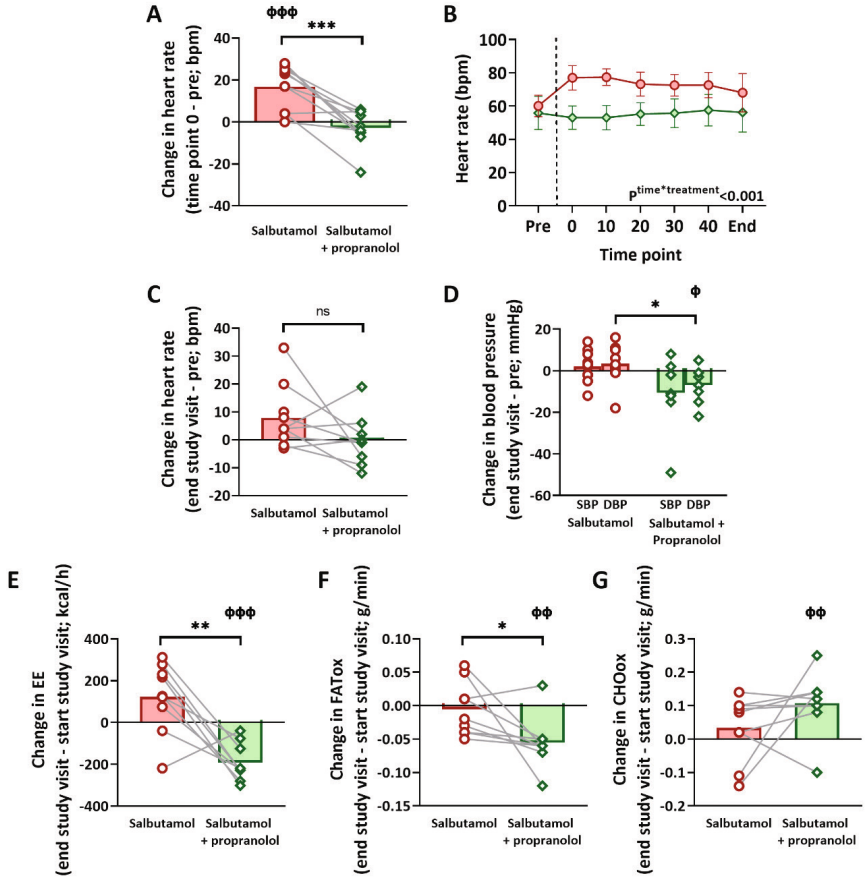


Figure 2. The effect of salbutamol *vs*. salbutamol with propranolol on heart rate, blood pressure, energy expenditure and nutrient oxidation rates.

The direct change in heart rate (n=10) (A) and change over the study day of heart rate (n=9) (C), systolic blood pressure (SBP) (n=10) and diastolic blood pressure (DBP) (n=9) (D), energy expenditure (EE) (n=9) (E), fatty acid oxidation (FATox) (n=9) (F), and carbohydrate oxidation (CHOox) (n=9) (G) after salbutamol (red bars with circles) *vs*. salbutamol with propranolol (green bars with diamonds). For one participant, EE measurement failed due to technical issues. In one participant a measurement of heart rate at the end of the study after salbutamol with propranolol is missing. General linear models with repeated measures and pairwise comparisons were used to test the effect of treatment and to compare the treatment regimens. Bars represent means, circles/diamonds represent individual values, and grey lines represent paired data. Before *vs*. after treatment: ΦP≤0.05, ΦΦP≤0.01, ΦΦΦP≤0.001. Salbutamol *vs*. salbutamol with propranolol: *P≤0.05, **P≤0.01, ***P≤0.001.

B) The effect of salbutamol (n=10; red circles) *vs*. salbutamol with propranolol (n=9; green diamonds) on heart rate over time. Vertical dash-line represents the moment of the administration of salbutamol. General linear model with repeated measures was used to test for an interaction between treatment regime and the effect of treatment over time. Bars represent means, error bars represent standard deviation.

blood pressure (-10.7 ± 16.5 mmHg, P = 0.09) and decreased diastolic blood pressure (-7.0 ± 8.0 mmHg, P = 0.03) (i.e., end of treatment visit *versus* before treatment) (**Figure 2D**). It should be noted that blood pressure could not be assessed in between these measurements and initial effects of salbutamol on systolic and diastolic blood pressure may have been missed.

Next, we assessed the effect of salbutamol on energy expenditure and substrate utilization. For one male, the gas exchange measurement failed due to technical issues, leaving a total of 9 males for these analyses. Salbutamol tended to increase energy expenditure (+7.2%, +122 ± 168 kcal/day, P = 0.06), whereas salbutamol with propranolol decreased energy expenditure (-9.4%, -192 ± 91 kcal/day, P < 0.001), leading to a significantly different change in energy expenditure between the two treatment regimens (P = 0.005; **Figure 2E**). Of note, the salbutamol-induced percentual change in energy expenditure did not correlate with the increase in heart rate, both parameters defined as the change between "end study visit" *minus* "start study visit" (Spearman's rho: -0.59, P = 0.10; **Supplementary Figure 1**). Fat oxidation did not change after salbutamol (-0.004 ± 0.04 g/min, P = 0.77), but decreased after salbutamol with propranolol (-0.06 ± 0.04 g/min, P = 0.003; change between treatments P = 0.03; **Figure 2F**). Carbohydrate oxidation did not change after salbutamol (+0.03 ± 0.10 g/min, P = 0.34), but increased after salbutamol with propranolol (+0.12 ± 0.09 g/min, P = 0.009; change between treatments: P = 0.19; **Figure 2G**).

Salbutamol increases net glucose uptake by brown adipose tissue, and this positively correlates to the change in energy expenditure

We next assessed the effect of the ADRB2 agonist salbutamol on net glucose uptake by supraclavicular BAT, as calculated from the [^{18}F]FDG influx rate. Salbutamol, compared to salbutamol with propranolol, increased the net glucose uptake by BAT (salbutamol *vs.* salbutamol with propranolol: 67.1 ± 87.0 nmol/g/min *vs.* 16.2 ± 5.2 nmol/g/min, P = 0.03; **Figure 3A-C**). In contrast, after salbutamol, glucose uptake by skeletal muscle (9.5 ± 3.0 nmol/g/min *vs.* 12.6 ± 2.4 nmol/g/min, P = 0.06) and scWAT (21.4 ± 3.7 nmol/g/min *vs.* 24.5 ± 3.4 nmol/g/min, P = 0.06; **Figure 3A**) tended to be lower compared to after salbutamol with propranolol. Interestingly, we observed a physiologically plausible outlier with very high glucose uptake values by BAT (285.1 nmol/g/min, see identification number 10 in **Figure 4A** and **Supplementary Figure 2**). After sensitivity analyses excluding the values of this participant, differences in glucose uptake by BAT after salbutamol *vs*. salbutamol with propranolol remained significant (salbutamol *vs.* salbutamol with propranolol: 36.7 ± 31.2 nmol/g/min *vs.* 14.9 ± 3.2 nmol/g/min, P = 0.05; data not shown). BAT volumes were not calculated as these are markedly influenced by various factors (e.g., diet, intracellular triglyceride stores, and thresholds for standard uptake value) (18, 19).

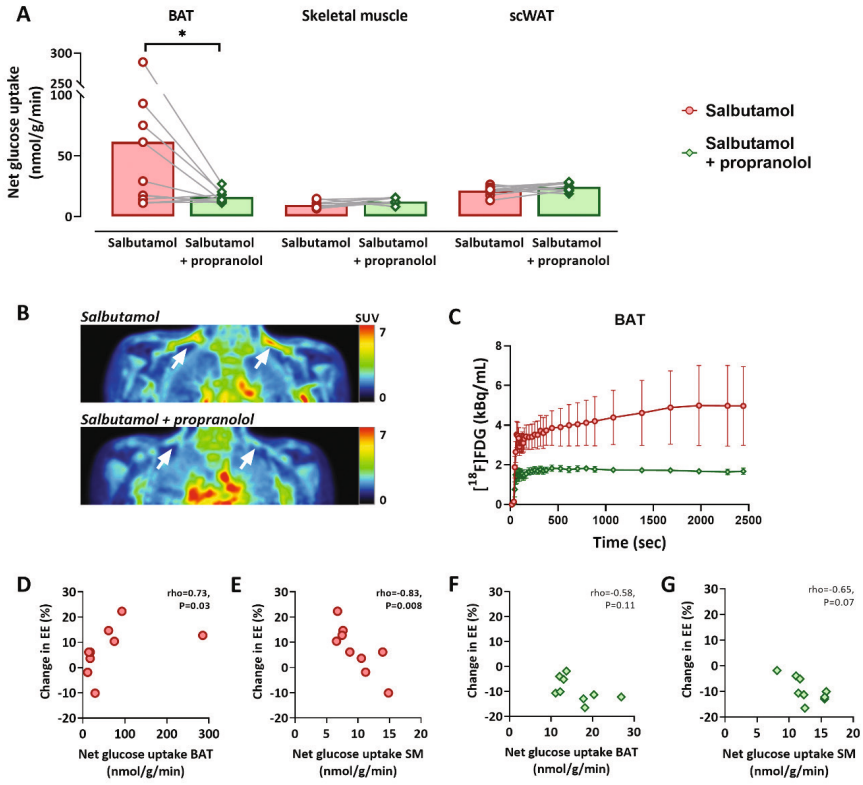


Figure 3. The effect of salbutamol *vs*. salbutamol with propranolol on glucose uptake by brown adipose tissue, skeletal muscle, and subcutaneous white adipose tissue, and the association with the change in energy expenditure.

A) The glucose uptake by human brown adipose tissue (BAT), skeletal muscle (i.e., average of m. pectoralis, m. trapezius, m. deltoideus, and m. sternocleidomastoideus), and subcutaneous white adipose tissue (scWAT) after salbutamol (n=10) *vs.* salbutamol with propranolol (n=10). A paired Student's t-test, or nonparametric equivalent, was used to compare the two treatment regimes. Bars represent means, dots/diamonds represent individual values, and grey lines represent paired data. *P≤0.05.

B) Positron emission tomography images of the supraclavicular area illustrating the [^{18}F] fluorodeoxyglucose [^{18}F]FDG uptake, expressed by body-weighted standardized uptake values (SUV), in response to salbutamol (top) and salbutamol with propranolol (bottom). The same representative participant is presented for both images. White arrows show supraclavicular BAT depots.

C) Time-activity curve showing the concentration of [^{18}F]FDG in BAT depots. Left and right, and all participants (n=10) are averaged. Data represent mean with standard error of the mean.

D+E) Correlation plots between the change in energy expenditure (EE) (%) and the glucose uptake by human BAT after salbutamol (D) and skeletal muscle (SM) after salbutamol (E) (n=9). For one participant, EE measurement failed due to technical issues.

F+G) Correlation plots between the change in EE and the glucose uptake by human BAT after salbutamol with propranolol (F) and SM after salbutamol with propranolol (G) (n=9).

After salbutamol, glucose uptake by BAT was positively associated with the percentage change in energy expenditure (Spearman's rho = 0.73, P = 0.03; **Figure 3D**, whereas the glucose uptake by skeletal muscle was negatively associated (Spearman's rho = -0.83, P = 0.008; **Figure 3E**). After salbutamol with propranolol, no significant correlation was found between the glucose uptake by BAT and energy expenditure (Spearman's rho = -0.58, P = 0.11; **Figure 3F**), and a tendency between the change in glucose uptake by skeletal muscle and energy expenditure (Spearman's rho = -0.65, P = 0.07; **Figure 3G**). In addition, we observed a tendency between a delta of EE (i.e., the change of EE after 45 minutes of salbutamol injection *minus* the change of EE after salbutamol with propranolol) with the delta of net glucose uptake by BAT (i.e., the value of BAT after 45 minutes of salbutamol injection *minus* the value of BAT after salbutamol with propranolol) (Spearman's rho = 0.70, P = 0.05; **Supplementary Figure 3A**) and a negative correlation with the delta of net glucose uptake by skeletal muscle (SM) (i.e., the value of SM after 45 minutes of salbutamol injection *minus* the value of SM after salbutamol with propranolol (Spearman's rho = -0.73, P = 0.025 **Supplementary Figure 3B**). Furthermore, during both treatment regimes, no significant correlations were found between the change in glucose uptake by scWAT and energy expenditure (salbutamol: Spearman's rho = -0.16, P = 0.68; salbutamol with propranolol: Spearman's rho = -0.32, P = 0.41; not shown).

High responders to salbutamol-induced glucose uptake by BAT have a more beneficial metabolic phenotype than low responders

A marked variability was observed between participants in the salbutamol-induced glucose uptake by BAT. Specifically, as is evident from **Figure 4A** and **Supplementary Figure 2**, five participants showed a high glucose uptake by BAT (ranging from 29.2 to 285.1 nmol/g/min; 'responders'), whereas the other five participants showed low glucose uptake (ranging from 11.1 to 17.6 nmol/g/min; 'non-responders'; P = 0.008). Compared to non-responders, responders had a lower body fat mass (11.8 ± 1.3 % *vs.* 16.9 ± 2.3 %, P = 0.008; **Figure 4B**), lower WHR (0.8 ± 0.02 *vs.* 0.9 ± 0.1, P = 0.03; **Figure 4C**), and lower serum concentrations of total cholesterol (3.0 ± 0.7 mmol/L *vs.* 4.5 ± 0.5 mmol/L, P = 0.008; **Figure 4D**) and LDL-cholesterol (1.6 ± 0.4 mmol/L *vs.* 2.7 ± 0.5 mmol/L, P = 0.02; **Figure 4D**). No significant differences were observed in glucose, insulin or C-peptide levels between non-responders and responders (all P ≥ 0.1, see **Supplementary Table 1**). Moreover, responders tended to have a higher heart rate at the start of the study visit (77.8 ± 10.0 bpm *vs.* 66.5 ± 8.3 bmp, P = 0.06), without differences in baseline energy expenditure (1887 ± 384 kcal/day *vs.* 2056 ± 68 kcal/day, P = 1.0). There was no significant difference between the groups in salbutamol-induced change in energy expenditure (+10.0 ± 12.1 % *vs.* +3.5 ± 3.8 %, P = 0.35) or heart rate (+12.2 ± 13.2 bpm *vs.* +21.6 ± 4.4 bpm, P = 0.55). A full overview of baseline characteristics between the two phenotypes can be found in **Supplementary Table 1**.

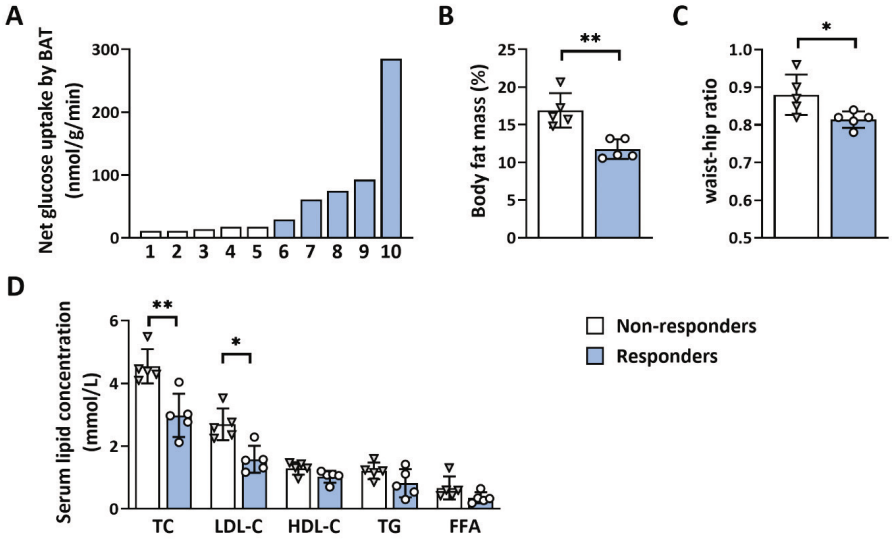


Figure 4. Differences in body composition and baseline serum lipid concentrations between non-responders and responders in terms of salbutamol-induced glucose uptake by brown adipose tissue.

A) Waterfall plot showing the distribution in net glucose uptake by supraclavicular brown adipose tissue (BAT; left and right averaged). Each bar represents the individual value of a participant.

B+C+D) Differences in body fat mass percentage (B), waist-hip ratio (C), and baseline serum concentrations of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), and free fatty acids (FFA) (D) between participants who showed a high salbutamol-induced net glucose uptake by brown adipose tissue ('responders', blue bars with circles; n=5) *vs.* participants who showed low salbutamol-induced net glucose uptake by brown adipose tissue ('non-responders', white bars with triangles; n=5). Values illustrated in the figures were measured at baseline during the placebo-visit.

A paired Student's t-test, or nonparametric equivalent, was used to compare the two groups. Bars represent means, error bars represent standard deviation. *P≤0.05, **P≤0.01.

Salbutamol does not affect serum lipid or glucose concentrations

Lastly, we aimed to assess the acute effects of salbutamol on measures of lipoprotein and glucose metabolism. Salbutamol did not affect serum concentrations of triglycerides (Start *vs.* end study day: 1.0 ± 0.4 mmol/L *vs.* 1.0 ± 0.4 mmol/L, P = 0.78), FFA (0.5 ± 0.3 mmol/L *vs.* 0.4 ± 0.1 mmol/L, P = 0.21), total cholesterol (3.8 ± 1.0 mmol/L *vs.* 3.8 ± 0.9 mmol/L, P = 0.74), HDL-cholesterol (1.2 ± 0.2 mmol/L *vs.* 1.2 ± 0.2 mmol/L, P = 0.70), or LDL-cholesterol (2.1 ± 0.7 mmol/L *vs.* 2.2 ± 0.7 mmol/L, P = 0.78; **Figure 5**). Salbutamol with propranolol only decreased serum FFA levels (0.6 ± 0.3 mmol/L *vs.* 0.2 ± 0.1 mmol/L, P < 0.001; **Figure 5B**). Moreover, salbutamol did not affect serum concentrations of glucose (5.5 ± 0.2 mmol/L *vs.* 5.6 ± 0.6 mmol/L, P = 0.45), insulin (12.7 ± 4.3 μU/mL *vs.* 14.0 ± 7.3 μU/mL, P = 0.75), or C-peptide (1.6 ± 0.4 ng/mL *vs.* 1.8 ± 0.6 ng/mL, P = 0.08),

whereas salbutamol with propranolol decreased serum concentrations of glucose (5.5 ± 0.4 mmol/L *vs.* 5.2 ± 0.5 mmol/L, P = 0.002), insulin (12.6 ± 7.1 µU/mL *vs.* 4.4 ± 2.5 µU/mL, P<0.001), and C-peptide (1.5 ± 0.6 ng/mL *vs.* 1.0 ± 0.4 ng/mL, P = 0.001; **Figure 5B**). Importantly, the glucose uptake by BAT after salbutamol with propranolol was not associated with the decrease in glucose (Spearman's rho = 0.28, P = 0.43) nor insulin (Spearman's rho = 0.45, P = 0.19; not shown).

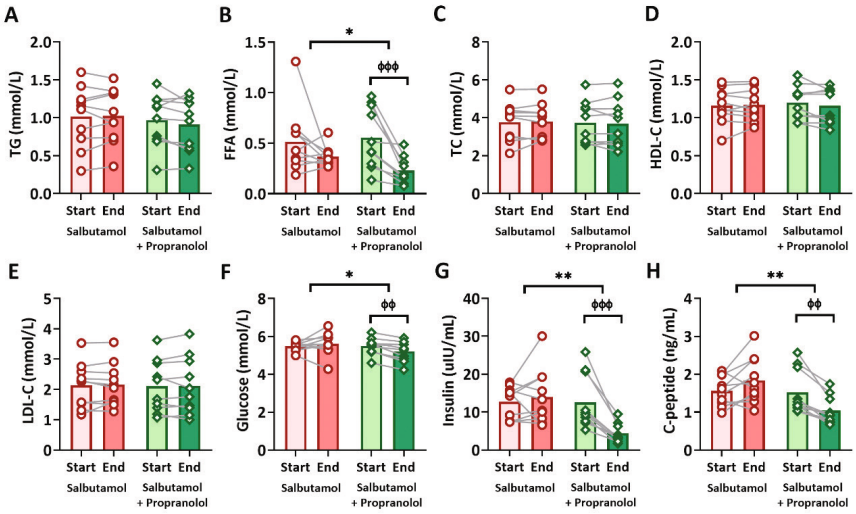


Figure 5. The effect of salbutamol *vs*. salbutamol with propranolol on serum concentrations of lipid and glucose metabolism.
The effect of salbutamol (red bars with circles; n=10) *vs*. salbutamol with propranolol (green bars with diamonds; n=10) on serum concentrations of triglycerides (TG; A), free fatty acids (FFA; B), total cholesterol (TC; C), high density lipoprotein cholesterol (HDL-C; D), low density lipoprotein cholesterol (LDL-C, E), glucose (F), insulin (G), and C-peptide (H). General linear models with repeated measures and pairwise comparisons were used to test the effect of treatment and to compare the treatment regimens. Bars represent means, dots/diamonds represent individual values, and grey lines represent the paired nature of the data. Start *vs*. end: ΦΦP≤0.01, ΦΦΦP≤0.001. Salbutamol *vs*. salbutamol with propranolol: *P≤0.05, **P≤0.01.

DISCUSSION

In this proof-of-concept study, we show that pharmacological stimulation of the ADRB2 acutely increases glucose uptake by human BAT in vivo. Specifically, we demonstrate that a single intravenous administration of the specific ADRB2 agonist salbutamol increased glucose uptake by BAT, which could be largely prevented when blocking the ADRB1/2 with propranolol. The salbutamol-induced uptake of glucose by BAT, but not skeletal muscle, was positively associated with whole-body energy expenditure.

Notably, participants with high salbutamol-induced glucose uptake by BAT had a more favorable metabolic phenotype (among other lower WHR and LDL-cholesterol levels) compared to participants with low glucose uptake by BAT. Together, our data underline the relevance of the ADRB2 for sympathetically induced glucose uptake by human BAT.

By revealing that stimulation of the ADRB2 increases glucose uptake by human BAT, we now provide in vivo evidence for our recent findings that the ADRB2 is the adrenergic receptor that activates human brown adipocytes in vitro (12). Moreover, since blocking the ADRB1/2 effectively inhibited the salbutamol-induced glucose uptake by BAT in our study, the ADRB3 apparently was not involved. Over the last decades, many research groups have focused on mimicking the sympathetic activation of BAT by targeting the ADRB3 (10, 13, 14, 20). Direct targeting the ADRB3 using CL 316,243, one of the most commonly used ADRB3 agonists, effectively activates BAT and improves cardiometabolic outcomes in mice (7, 9). However, pharmacological targeting of the ADRB3 by the use of mirabegron led to inconsistent results in humans (10-16). In fact, mirabegron only activates human BAT oxidative metabolism at maximal allowable dose (i.e., 200 mg), which also induces cardiovascular side effects, (11, 12, 21) suggesting cross-activation with other beta-adrenergic receptors. Indeed, the human heart mainly expresses ADRB1 (80%) and ADRB2 (20%), with negligible expression of ADRB3 (22). In human BAT, in principle all three receptors are present (12, 23-26). In immortalized brown adipocytes originating from just a single donor, stimulation of the ADRB1, but not ADRB2 or ADRB3, increased uncoupling protein 1 (UCP1) expression and lipolysis (26). In contrast, we demonstrated using both immortalized and in vitro differentiated primary brown adipocytes from multiple donors from independent labs that ADRB2 agonism increases respiration, and knockdown of the ADRB2, but not ADRB1 and ADRB3, hampers norepinephrine-induced respiration (12). Taken together, although the involvement of the ADRB1 cannot be excluded, the ADRB2 seems to play the most important role in sympathetic activation of human BAT in vivo.

We show that ADRB2 stimulation with salbutamol tended to increase whole-body energy expenditure, and the change in energy expenditure correlated positively with the net glucose uptake by BAT. This is in line with previous studies that also demonstrated that intravenous administration of salbutamol with and without atenolol (i.e., ADRB1 blocker) increased energy expenditure, although in those studies the tissues involved had not been explored (27-29). Besides BAT, skeletal muscles are also considered responsible for the increase in thermogenesis during cold exposure, and deeper and more centrally located skeletal muscles have been shown to contribute to the cold-induced glucose turnover (30). However, we here show that the change in energy expenditure after salbutamol in fact negatively correlates with the net glucose uptake by skeletal muscle.

Moreover, comparable to previous findings that acute ADRB2 stimulation does not affect insulin-stimulated glucose uptake by skeletal muscle, we did not find an increase in glucose uptake by skeletal muscle after acute salbutamol administration. Interestingly, long term (four weeks) daily inhalation of terbutaline (i.e., an ADRB2 agonist) increases insulin-stimulated whole-body glucose disposal, without changes in GLUT4 in skeletal muscle or abdominal WAT (31). Although it has been shown that this may in part be due to muscle hypertrophy (31), we propose a potential role for BAT through increased glucose uptake coinciding with increased energy dissipation.

Previous studies demonstrated that continuous or repetitive intravenous administration of salbutamol with and without atenolol increases lipolysis and fat oxidation together with an increase in energy expenditure (27-29). In the present study, we did not observe a change in circulating lipids or in fat oxidation after a single bolus of salbutamol. Importantly, we performed indirect calorimetry and blood sampling approx. 70 min after salbutamol administration. Hence, we may have missed an acute effect of salbutamol on lipolysis and fat oxidation. Indeed, salbutamol with atenolol (i.e., resulting in specific ADRB2 stimulation), increases fat oxidation only after 45 min of continuous intravenous administration (27). Furthermore, we used a single intravenous bolus of salbutamol, which may have not been sufficiently potent to induce a lipolytic effect in white adipose tissue. Interestingly, it has been reported previously that the salbutamol-induced increase in energy expenditure is independent of circulating fatty acid levels, as substrate utilization switches from fat to carbohydrate when peripheral lipolysis is blocked with acipimox (i.e., a niacin derivative that inhibits adipose triglyceride lipase) (28). It is conceivable that the single bolus of salbutamol only transiently increases lipolysis, circulating fatty acid levels, and fat oxidation, and eventually increases energy expenditure due to an increase in both fat and glucose oxidation.

The finding that a single bolus of salbutamol activates human BAT and whole-body energy metabolism, is a promising lead for the development of BAT targeted drugs to treat obesity-related cardiometabolic disorders. Nevertheless, although salbutamol is approved in the clinic for the treatment of respiratory diseases, it is unlikely that conventional tissue-unspecific ADRB2 agonism will be applied to treat cardiometabolic pathophysiology due to common cardiovascular side effects (i.e., increase in heart rate, blood pressure and tremors). Moreover, pharmacological stimulation of the ADRB2 using salbutamol does not fully mimic the sympathetic cold-induced activation of BAT just yet, as during cold exposure heart rate decreases (12), possibly explained by cold-induced local norepinephrine release affecting tissue specific beta- as well as alpha-receptors. Nevertheless, it is conceivable that BAT-targeted drug delivery systems may be developed. Such delivery systems have already been developed to target the liver,

taking advantage of the exclusive expression of the asialoglycoprotein receptor on hepatocytes, and hepatocyte-targeted modulation of PCSK9 by Inclirisan has recently been approved by the FDA to reduce cardiovascular disease risk (32). Of note, a recent study indeed showed the feasibility of targeting adipose tissue using an adipose homing peptide (33).

Interestingly, we found notable individual variability in the glucose uptake by BAT after salbutamol administration, with responders having a lower body fat mass percentage and lower WHR as well as lower total cholesterol and LDL-cholesterol levels compared to non-responders. This seems consistent with previous studies showing that [^{18}F]FDG uptake by cold exposed BAT (34, 35) or thermoneutral BAT (36, 37) is associated with a lower body fat mass percentage and/or less central adiposity. As an explanation, a higher body fat mass may influence the detectability of BAT using [^{18}F]FDG PET acquisitions, as human BAT is scattered between white adipocytes and the proportion between classical BAT, beige/brite and white adipose tissue varies substantially between individuals (38, 39). Also, relatively high circulating triglyceride levels at baseline may interfere with glucose uptake by BAT. Under stimulated conditions, BAT increases oxidation of intracellular triglyceride-derived fatty acids, followed by replenishment of the intracellular lipid stores via the uptake of glucose and triglyceride-derives fatty acids from the circulation (40). A higher triglyceride-derived fatty acid flux towards the BAT will thus reduce its glucose uptake (41). However, in the current study baseline triglyceride levels were not significantly different. Alternatively, variations in responsiveness of the ADRB2, due to gene polymorphisms, receptor sensitivity, and/or receptor density could explain differences in salbutamol-induced glucose uptake by BAT and disparities in metabolic phenotype. Various polymorphisms in the ADRB2 gene have been identified, of which the two most commonly studied gene polymorphisms have a frequency of approx. 35 to 50% in Europe (42). Indeed variations in polymorphisms of the ADRB2 gene can result in different thermogenic responses upon ADRB2 stimulation (29). In addition, loss-of-function mutations of the ADRB2 gene are related to higher body fat and circulating lipid variability (43-49), and low ADRB2 sensitivity and low ADRB2 density are linked to higher circulating triglycerides and LDL-cholesterol, respectively (50, 51). With respect to the current study, the latter would suggest that the responsiveness of the ADRB2 influences both the metabolic phenotype as the salbutamol-induced glucose uptake by BAT, which may be an important factor to consider when targeting BAT to improve cardiometabolic health.

Conclusions

In conclusion, we provide evidence that stimulation of the ADRB2 using salbutamol acutely increases the rate of glucose uptake by human BAT *in vivo*, which is suppressed

after blocking the ADRB1/2, suggesting that this effect is not mediated by cross-reaction of salbutamol with the ADRB3. As such, these findings provide *in vivo* evidence for our recent findings that the ADRB2 is responsible for adrenergic stimulation of human brown adipocytes (12). Identification of ADRB2 as the most important receptor involved in the sympathetic activation of human BAT thermogenesis allows to maximize the therapeutic potential of targeting BAT to combat cardiometabolic diseases.

Limitations of the study

This study is not without limitations. While we used [^{18}F]FDG as tracer for BAT activation, triglyceride-derived fatty acids are probably much more important as fuel for BAT oxidative metabolism (40). Nonetheless, a PET-compatible triglyceride tracer has not been described as yet. Alternative tracers include 14(R,S)-[^{18}F]fluoro-6-thia-heptadecanoic acid ([^{18}F]FTHA) or [^{11}C]acetate, which trace circulating fatty acid uptake by BAT and oxidative metabolism and perfusion of BAT, respectively. Using these tracers it has been reported that BAT glucose uptake can be uncoupled from BAT oxidative metabolism, as shown in individuals with obesity or type 2 diabetes whom have maintained BAT oxidative metabolism and fatty acid uptake, despite reduced glucose uptake (52). Hence, future work should examine how the salbutamol-induced changes in BAT glucose uptake reflect changes in BAT oxidative metabolism. Furthermore, in the current study we compared salbutamol treatment with salbutamol combined with propranolol. The addition of a third, vehicle-treated control condition would have been ideal. However, the [^{18}F]FDG PET-CT scans that are required to assess BAT activity in a reliable way are subjected to a high radiation burden (i.e., 4.2 mSv per scan). As the total annual radiation burden is not allowed to exceed 10 mSv in the Netherlands, we were unfortunately restricted to a maximum of two [^{18}F]FDG PET-CT scans per person (53). One of our main research objectives was to eliminate the possibility that the stimulatory effect of salbutamol on glucose uptake by BAT is mediated via the ADRB3. Hence, we reasoned that we would gain most scientific knowledge by studying the effects of salbutamol without and with the ADRB1/2 antagonist propranolol. Of note, a previous study showed similar rates of glucose uptake by BAT when participants were exposed to room temperature as we found in our study when participants were treated with salbutamol and propranolol, suggesting that the stimulatory effect of salbutamol was indeed effectively inhibited by propranolol to values that would have been observed under non-stimulated conditions (54). Another general limitation is that with [^{18}F]FDG PET-CT analysis reliable measures of BAT volume cannot be obtained, as accumulation of [^{18}F]FDG in BAT and therefore the volume of BAT that meets the arbitrary threshold used to define BAT is dependent upon: (i) amount of tracer administered; (ii) time between tracer administration and image acquisition; (iii) whether the tissue activity is normalized to body weight or lean body mass; (iv) and the type of PET scan used (each

manufacturer and/or model from same manufacturer will have different sensitivities). A final limitation is that we could not measure the dynamic responses of blood pressure, energy expenditure, and nutrient oxidation rates during the PET image acquisition as we did for heart rate. Finally, only young, lean males were included in this study. As we already observe baseline differences between responders and non-responders considering body composition and circulating lipid levels, future studies should focus on metabolically compromised individuals and additionally include women.

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STAR METHODS

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Dr. Patrick C.N. Rensen (p.c.n.rensen@lumc.nl).

Materials availability

This study did not generate new unique reagents

Data and code availability

- All data reported in this paper will be shared by the lead contact upon reasonable request
- This paper does not report original code
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study design

We performed a single center randomized double-blinded crossover trial to assess whether ADRB2 agonism activates human brown adipose tissue (BAT). The intervention consisted of a single intravenous bolus of salbutamol (250 µg) in combination with orally administered propranolol (80 mg) or placebo, in random order after a seven-day wash-out period (**Figure 1A**). On both study days all participants underwent a dynamic [^{18}F]FDG Positron Emission Tomography and (low dose) Computed Tomography (PET-CT) scan. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and undertaken in accordance with the principles of the revised Declaration of Helsinki (see the three versions of the study protocol in **Data S1**, **Data S2** and **Data S3**). Written informed consent was obtained from all participants prior to participation. The clinical trial is registered at the Netherlands Trial Register (NTR; NL9345), and at the European Union Drug Regulating Authorities Clinical Trials (EudraCT; 2020-004059-34).

Participants

Participants were recruited via emails, flyers and website advertisements. In total, 10 healthy white Caucasian men were enrolled in this study, aged 19 to 35 years and with a body mass index between 19.2 and 26.5 kg/m^2. Inclusion criteria were: white Caucasian

males, age between 18 and 35 years old and BMI ≥ 18 and ≤ 25 kg/m^2. Exclusion criteria were the presence of any endocrine, cardiac, renal, of hepatic disease, a first-degree family member with sudden cardiac death, the use of medication known to influence glucose and/or lipid metabolism, the use of beta-adrenergic receptor agonists (e.g., for asthma), any contra-indications for the use of salbutamol or propranolol, abuse of alcohol or other substances, smoking, participation in an intensive weight-loss program or vigorous exercise program during the last year before the start of the study, and/or clinically relevant abnormalities in clinical chemistry or electrocardiogram. Eligibility for inclusion was assessed during a screening that consisted of anthropometry, electrocardiography, a questionnaire on medical history, and an overnight 10 h fasted blood sample.

METHOD DETAILS

Randomization

After inclusion, participants (n=10) were randomized to determine whether they would receive salbutamol in combination with placebo on the first study day (n=5), or salbutamol in combination with propranolol on the first study day (n=5). Randomization was executed by the LUMC department of Clinical Pharmacology and Toxicology.

Procedures

After inclusion, participants were asked to adhere to several lifestyle rules prior to the study visits: no vigorous exercise 48 hours preceding the study days and no alcohol or drinks with caffeine 24 hours preceding the study visits. In addition, they were instructed to eat a standardized meal (prepared supermarket meal including pasta or noodles, ranging from 450-600 kcal) in the evening prior to the study visits, and not to eat or drink anything (with an exception for water) afterwards until completion of the study visits.

Anthropometric measurements

After arrival (9:00 AM), body weight (digital balance; E1200, August Sauter GmBH, Albstadt, Germany), height, and waist and hip circumference were obtained. Waist-hip-ratio (WHR) was calculated as: 'waist circumference'/'hip circumference'. Body composition (fat mass and fat percentage) was estimated using bioelectrical impedance analysis (InBody720, InBody CO., Ltd., CA, USA). In addition, heart rate and blood pressure were measured using a cuff connected to a digital blood pressure device (Model). In total, heart rate and blood pressure were measured at three time points (at the start of the study day, before administration of salbutamol and at the end of the study day). In addition, heart rate was measured at 5 time points (t=0, 10, 20, 30, 40

min) during the PET scan using a 3-lead ECG connected to a bedside patient monitor (Intellivue MP5, Philips Healthcare, Best, the Netherlands).

Indirect calorimetry

At the start and at the end of the study day, resting energy expenditure and substrate utilization were measured for 30 min with a metabolic cart (Vyntus™ CPX, Carefusion, Hochberg, Germany) equipped with a ventilated hood system that measures total carbon dioxide production (VCO_2) and oxygen consumption (VO_2) every 10 sec. Before each measurement, volume and gas calibrations were performed. The Weir formula was used to estimate energy expenditure (ignoring urinary nitrogen excretion): energy expenditure (kcal/day) = (3.941*VO_2 (L/min)) + (1.106*VCO_2 (L/min))*1440. The first 5 min of gas exchange data of every new recording was discarded, whereafter the most stable 5 min were selected for further analyses, as previously described (55).

Administration of medication

Directly after the first indirect calorimetry measurement, participants received either placebo or 80 mg propranolol *per os*, divided over two capsules each, followed by 75 min of rest to reach peak plasma concentrations of propranolol. The dose, timing and mode of administration are conform the European Association of Nuclear medicine guidelines for tumor imaging **(56, 57).** Afterwards, participants were placed in supine position within the PET-CT scanner where salbutamol (250 µg) was administered via a single intravenous bolus (10 mL) into the antecubital vein over a time course of 5 min. The administration of salbutamol intravenously was selected instead of the more commonly used administration via inhalation as high variability in inhalation techniques could not guarantee similar salbutamol exposure in all participants **(58).** In addition, even with an adequate inhalation technique, only a small portion of the administered dose after inhalation will reach the blood **(59).** Currently, the approved dosage of intravenous salbutamol for symptomatic treatment of a severe asthmatic attack is 250 µg **(60).** As salbutamol administered intravenously reaches a maximum concentration within seconds after administration, the medication was injected fifteen minutes prior to the injection of [^{18}F] FDG tracer **(58).**

[^{18}F]FDG PET-CT scan

Fifteen minutes after initiation of salbutamol administration, a low dose (30 mA, effective dose 0.7 mSv) CT scan of the cervicothoracic area centered on the supraclavicular region was performed. This was directly followed by the administration of a single bolus of [^{18}F]FDG tracer in a dosage of 185 MBq using an injection pump, after which the line was flushed with saline and the dynamic list-mode PET acquisition was started. The list-mode data were reconstructed into 32 time frames (1 x 30 sec, 12 x 10 sec, 8 x 30

sec, 6 x 90 sec and 5 x 300 sec). The time radioactivity curves for [^{18}F]FDG of metabolic tissues were analyzed using the Patlak linearization method (61), with the plasma input function taken from the aortic arch (62). The slope of the linear phase of the Patlak plot denotes the net influx rate (influx constant, Ki, in min^{-1}), which is the accumulated [^{18}F]FDG relative to the amount of [^{18}F]FDG that has been available in plasma. Ki was then multiplied by circulating glucose levels at the time of the PET image acquisition, and divided by the lumped constant, to calculate the net glucose uptake. Finally, this value was divided by tissue density and multiplied with 1,000 to obtain the net glucose uptake in the preferred unit: nmol·g^{-1}·min^{-1}. For adipose tissue, the lumped constant of 1.14 and tissue density 0.925 g/mL was used, for skeletal muscle, the lumped constant of 1.16 and tissue density 1.06 g/mL was used (63, 64). In summary, the following formula was used to estimate the net glucose uptake by metabolic tissues after intervention:

$$Net\ glucose\ uptake\ (\mathrm{nmol/g/min}) = ((Ki * circulating\ glucose)/(Lumped\ constant))/(Tissue\ density * 1000)$$

PET-CT image data were analyzed using PMOD software (PMOD technologies LLC, Zürich, Switzerland). Regions of interest (ROIs) were drawn independently by two researchers (M.E.S, C.A.H) on the aortic arch for the plasma input function, four skeletal muscles (e.g., m. sternocleidomastoid, m. trapezius, m. pectoralis major, m. deltoideus; all left and right), posterior cervical subcutaneous white adipose tissue (scWAT) and supraclavicular BAT (left and right). For skeletal muscles and supraclavicular BAT, values from left and right side were averaged.

Blood samples

At the start of the study visit, a catheter was inserted in the antecubital vein, for venous blood sampling and for administration of salbutamol and [^{18}F]FDG tracer. At two time points 10 h fasted blood samples were collected: at the start and at the end of the study visit. Blood was collected in Vacutainer® SST™ II Advance tubes. After a clotting time of at least 30 min, samples were centrifuged to obtain serum, which was aliquoted and stored at -80°C until batch-wise analyses. Commercially available enzymatic kits were used to measure serum concentrations of free fatty acids (FFA; Wako chemicals, Nuess, Germany), triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C; all Roche Diagnostics, Woerden, the Netherlands), glucose (Instruchemie, Delfzijl, the Netherlands), insulin and C-peptide (both Meso Scale Diagnostics, Rockville, Maryland, USA). Low-density lipoprotein cholesterol (LDL-C) was estimated using the Friedewald equation (65).

QUANTIFICATION AND STATICIAL ANALYSIS

Sample size

Our power calculation was based on previous studies performed by Blondin et al.(12) and Orava et al.(54) Based on these studies, we considered a difference in net glucose uptake rate by BAT of +13 nmol/g/min after salbutamol administration as clinically relevant. From this, we calculated that a sample size of 10 participants would provide a power of 80% to show a clinical relevant effect with a standard deviation of 10.

Statistical analysis

Statistical analyses were performed with SPSS® Statistics (version 25, IBM® Corporation, Armonk, NY, USA). Normal distribution of the data was tested using the Shapiro-Wilk test, visual histograms, and Q-Q plots. To assess the effect of treatment, and to compare the changes after treatment between the treatment regiments, general linear models with repeated measures and pairwise comparisons were used with two within-subject factors: treatment (salbutamol *vs.* salbutamol with propranolol) and timepoint (e.g., before and after treatment). Not normally distributed data were log10 transformed (e.g., energy expenditure, serum FFA, and serum insulin levels). To compare the glucose uptake by BAT and skeletal muscles between treatments, non-parametric Wilcoxon Signed Rank tests were used. To compare the glucose uptake by scWAT between treatments two-tailed paired Student's t-tests was used. Associations between parameters were tested using Pearson correlations (r) or nonparametric Spearman-rank correlations (rho). Baseline characteristics were compared between non-responders and responders using Mann-Whitney U tests. Absolute changes in heart rate, blood pressure, expenditure, fat oxidation, carbohydrate oxidation, and serum markers were calculated as: 'end study visit'-'start study visit'. Changes in energy expenditure were calculated using the following formula: ('end study visit'-'start study visit')/('start study visit')*100%. The homeostasis model assessment-estimated insulin resistance (HOMA-IR) levels were calculated as: 'fasting glucose in mmol/L'*'fasting insulin in μU/mL'/22.5. A P-value of $P \leq 0.05$ was considered statistically significant. All data are presented as mean ± standard deviation. **Figure 1** was created with BioRender.com. All other figures were prepared with Prism 9 for Windows (version 9.0.1, 2021, GraphPad Software, LLC, San Diego, California, USA).

ADDITIONAL RESOURCES

Netherlands Trial Register Number (NTR; NL9345) and the European Union Drug Regulating Authorities Clinical Trials Number (EudraCT; 2020-004059-34)

(https://www.clinicaltrialsregister.eu/ctr-search/search?query=2020-004059-34).

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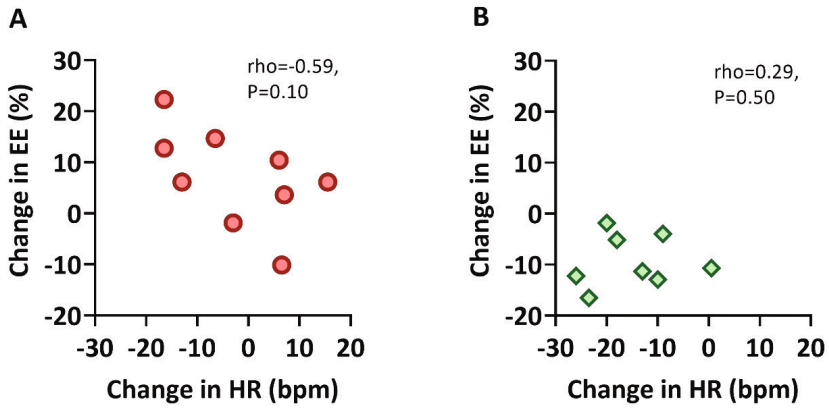
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SUPPLEMENTAL DATA

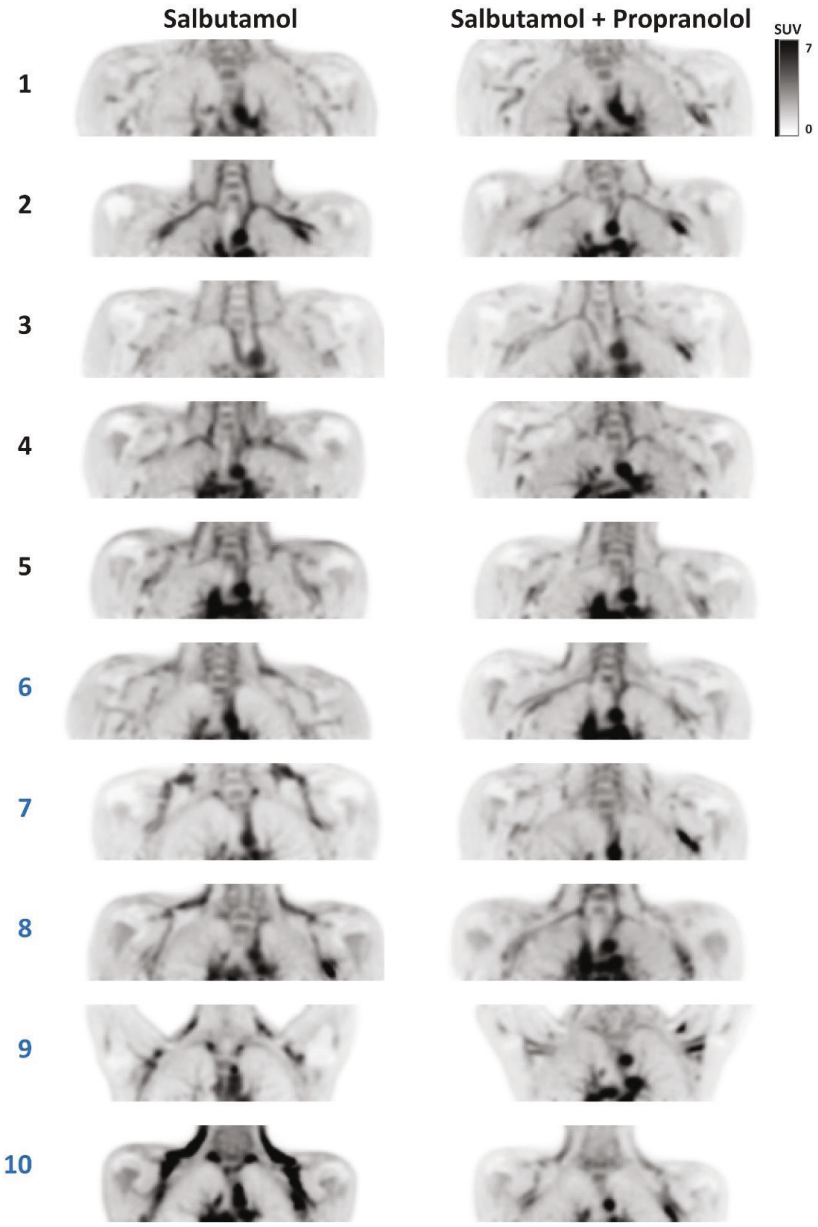
Supplementary Table 1. Differences in clinical characteristics, serum measurements, and the effect of salbutamol on metabolic parameters between non-responders and responders.

	Non-responders (n=5)	Responders (n=5)
Glucose uptake by BAT after salbutamol, nmol/g/min	14.4 ± 3.1	108.7 ± 101.3 **
Glucose uptake by BAT after salbutamol with propranolol, nmol/g/min	13.7 ± 2.8	18.4 ± 5.6
Clinical characteristics		
Age, years	25.6 ± 5.5	23.2 ± 2.7
Weight, kg	83.6 ± 8.3	76.1 ± 12.6
Body mass index	24.4 ± 1.2	21.9 ± 2.4 #
Fat mass, %	16.9 ± 2.3	11.8 ± 1.3 **
Waist circumference, cm	84.9 ± 6.3	74.3 ± 7.3 *
Hip circumference, cm	96.7 ± 7.5	91.0 ± 8.2
Waist-hip ratio	0.9 ± 0.1	0.8 ± 0.02 *
Systolic blood pressure (start), mmHg	125.3 ± 10.7	128.9 ± 8.5
Diastolic blood pressure (start), mmHg	79.9 ± 7.1	72.4 ± 2.9 #
Heart rate (start), bpm	66.5 ± 8.3	77.8 ± 10.0 #
Heart rate (pre), bpm	57.8 ± 3.7	62.4 ± 8.2
Heart rate (end), bpm	63.6 ± 8.3	72.4 ± 13.5
Baseline energy expenditure, kcal/h	2056 ± 68	1887 ± 384
Serum measurements		
Triglycerides, mmol/L	1.2 ± 0.3	0.8 ± 0.4
Free fatty acids, mmol/L	0.7 ± 0.4	0.4 ± 0.2 #
Total cholesterol, mmol/L	4.5 ± 0.5	3.0 ± 0.7 **
HDL-cholesterol, mmol/L	1.3 ± 0.2	1.0 ± 0.2 #
LDL-cholesterol, mmol/L	2.7 ± 0.5	1.6 ± 0.4 *
Glucose, mmol/L	5.5 ± 0.1	5.4 ± 0.3
Insulin, µU/mL	13.2 ± 5.3	12.3 ± 3.6
C-peptide, ng/mL	1.5 ± 0.5	1.6 ± 0.3
HOMA-IR	3.2 ± 1.3	2.9 ± 0.8
Effect salbutamol		
Glucose uptake by skeletal muscle, nmol/g/min	10.5 ± 2.3	8.6 ± 3.5
Glucose uptake by scWAT, nmol/g/min	22.2 ± 3.4	20.6 ± 4.3
Change in energy expenditure, %	+3.5 ± 3.8	+10.0 ± 12.1
Change in heart rate, bpm	+21.6 ± 4.4	+12.2 ± 13.2

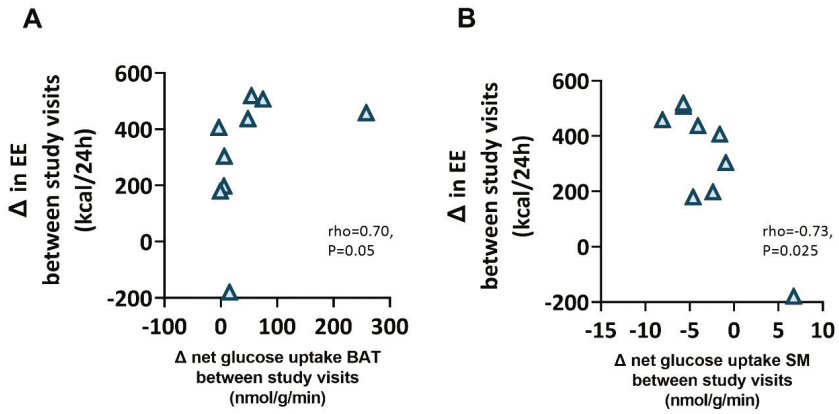
Values are presented as mean ± standard deviation. Values are measured at baseline during the salbutamol + placebo-visit. Significance levels are obtained from independent-Samples Mann-Whitney U tests, comparing values from participants that showed a high salbutamol-induced glucose uptake by brown adipose tissue (BAT) ('responders') *vs.* participants that showed low salbutamol-induced glucose uptake by BAT ('non-responders').
#P<0.1, *P<0.05, **P<0.01. HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; LDL, low-density lipoprotein; scWAT, subcutaneous white adipose tissue. Related to **Figure 4**.



Supplementary Figure 1. Correlations plots between the change in energy expenditure (EE) and the change in heart rate (HR) after salbutamol (A) and salbutamol with propranolol (B). The change in heart rate and EE was calculated as "end study visit" minus "start study visit" after salbutamol or salbutamol with propranolol. EE measurement failed due to technical issues for one participant (i.e., missing value, panel A; n=9 red circles). Additionally, the heart rate measurement at the end of the study after salbutamol with propranolol was missing for a second participant (i.e., missing value, panel B; n=8 green diamonds). Related to **Figure 2**.



Supplementary Figure 2. Tissue 2-[18F]fluoro-2-deoxy-D-glucoseuptake in response to salbutamol and salbutamol with propranolol.
Positron emission tomography (PET) images after salbutamol are shown on the left and PET images after salbutamol with propranolol are shown on the right. Numbers of the scans coincide with the numbers in the waterfall plot of **Figure 4A** and are ordered from lowest (non-responders to salbutamol, black numbers (n=5)) to highest (responders to salbutamol, blue numbers, (n=5)) glucose uptake by brown adipose tissue (BAT). SUV, body-weighted standardized uptake value.



Supplementary Figure 3. Correlation plot between the delta in energy expenditure (EE) and the delta in net glucose uptake by brown adipose tissue (BAT; A) or skeletal muscle (SM; B). All deltas (Δ) were calculated as the change of EE or glucose uptake by brown adipose tissue (BAT) or skeletal muscle (SM) after salbutamol injection minus the change of EE or glucose uptake by BAT after salbutamol injection with propranolol. EE measurement failed due to technical issues for one participant (n=9). Related to **Figure 3**.



CHAPTER 7

GROWTH DIFFERENTIATION FACTOR 15 IS NOT MODIFIED AFTER WEIGHT LOSS INDUCED BY LIRAGLUTIDE IN SOUTH ASIANS AND EUROPIDS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

Objectives

Glucagon-like peptide-1 receptor (GLP-1R) agonists induce weight loss in patients with type 2 diabetes mellitus (T2DM), but the underlying mechanism is unclear. Recently, the mechanism by which metformin induces weight loss could be explained by an increase in the growth differentiation factor 15 (GDF15), which suppresses appetite. Therefore, we aimed to investigate whether the GLP-1R agonist liraglutide modifies plasma GDF15 levels in patients with T2DM.

Methods

GDF15 levels were measured in plasma samples obtained from Dutch Europids and Dutch South Asians with T2DM before and after 26 weeks of treatment with daily liraglutide (n=44) or placebo (n=50) added to standard care.

Results

At baseline, circulating GDF15 levels did not differ between South Asians and Europids with T2DM. Treatment with liraglutide, compared to placebo, decreased body weight, but did not modify plasma GDF15 levels in all patients, or when data were split by ethnicity. Also, the change in plasma GDF15 levels after treatment with liraglutide did not correlate with changes in body weight or HbA_{1c} levels. In addition, the dose of metformin used did not correlate with baseline plasma GDF15 levels.

Conclusion

Compared to placebo, liraglutide treatment for 26 weeks does not modify plasma GDF15 levels in Dutch Europid or South Asian patients with T2DM. Thus, the weight loss induced by liraglutide is likely explained by other mechanisms beyond the GDF15 pathway.

INTRODUCTION

The number of people living with type 2 diabetes mellitus (T2DM) has rapidly increased globally in recent years and is expected to continue to rise (1). Obesity is a major risk factor for the development of T2DM (2). South Asian ethnicity is another well-known risk factor, as South Asians have a significantly higher risk of developing T2DM at a younger age and a lower body mass index (BMI) than other ethnic groups, including Europids (3). The underlying mechanism for this increased risk of developing T2DM in South Asians is not entirely known; however, it is likely multifactorial. Their disadvantageous body composition, consisting of a higher fat mass percentage with more visceral adipose tissue and a lower muscle mass than Europids, is a significant contributing factor to a higher insulin resistance state (4-6). Hormonal cues may also play a role in the increased risk of developing T2DM in South Asians. Differences in appetite-regulating hormones such as glucagon-like peptide-1, leptin, and ghrelin have previously been described in South Asians compared to other ethnicities (7-9). These hormonal variations could further contribute to the increased risk of South Asians to develop T2DM.

Metformin is the first-line pharmacological treatment for patients with new-onset T2DM. It acts by reducing hepatic gluconeogenesis, enhancing peripheral insulin sensitivity, and increasing the secretion of the gut hormone GLP-1, ultimately leading to reduced plasma glucose levels. Besides improving glucose regulation, metformin also reduces body weight (10, 11). Recent translational studies from two independent research groups have demonstrated that metformin increases circulating growth differentiation factor 15 (GDF15) levels to suppress appetite, thereby inducing weight loss (12, 13). GDF15 is a stress-induced cytokine and a member of the transforming growth factor-beta superfamily (14). It induces satiety by binding to the glial cell line-derived neurotrophic factor (GDNF) family Receptor alpha-like (GFRAL) located in the area postrema and solitary tract of the hindbrain. GFRAL subsequently interacts with the tyrosine kinase co-receptor RET which induces phosphorylation of the signaling molecules AKT, ERK1/2, and phospholipase C, thereby inducing anorexia (15-20). In addition, GDF15 induces satiety by signalling through satiety-inducing cholecystokinin neurons located in the hindbrain (19, 21). Clinical trials involving individuals with overweight and obesity who were treated with a long-acting GDF15 receptor analogue showed a reduction in food intake and body weight, supporting the weight-reducing effect of GDF15 (22). Beyond appetite suppression, GDF15 also contributes to weight loss by enhancing energy expenditure, at least in mice (19, 23). Accordingly, metformin also increases energy expenditure in preclinical studies, which was mechanistically at least in part through enhancing activation of energy-combusting brown adipose tissue (24, 25).

Despite its effectiveness, metformin monotherapy is insufficient in some patients with T2DM to maintain glucose regulation (26). Therefore, additional therapeutic strategies have been developed to improve glycaemic parameters, reducing the risk of T2DM-associated complications. One of these therapies is GLP-1 receptor (GLP-1R) agonism, which mimics the effects of the incretin hormone GLP-1 to stimulate glucose-dependent insulin secretion and reduce glucagon secretion, both contributing to its glucose-lowering effects (27, 28). In addition, GLP-1R agonism has been shown to induce weight loss in patients with T2DM to a greater extent than metformin (29, 30), and its weight loss effect is partly attributed to its ability to promote satiety (31).

Unlike metformin, the underlying mechanism of appetite suppression due to GLP-1R agonism is not entirely known. GLP-1 receptors are expressed throughout the hindbrain, including the area postrema and nucleus solitary tract where GLP-1 receptor agonism induces appetite suppression. Of note, these regions also harbor GFRAL receptors, which mediate the effects of GDF15 in inducing satiety and reducing food intake, as mentioned above (32, 33). This spatial overlap in receptor expression raises the question of whether GLP-1R agonism may modulate the GFRAL/GDF15 signaling pathways and thereby contribute to its appetite-suppressing and weight-reducing effects.

Understanding the mechanism involved in appetite suppression following GLP-1R agonism is of great significance, considering its increasing popularity as a treatment for both diabetes and obesity. Furthermore, with approximately 20% of the world's population of South Asian descent and considering their markedly increased risk to develop obesity and type 2 diabetes compared to subjects of Europid descent, studying potential differences in the GFRAL/GDF15 system in South Asians compared to Europids as a potential underlying mechanism is relevant as well.

Therefore, in the current study, we aimed to study 1) whether circulating GDF15 levels differ between Dutch South Asian and Dutch Europid patients with T2DM; 2) whether the GLP-1R agonist liraglutide modifies plasma levels of GDF15 in both ethnicities and 3) whether changes in GDF15 levels are related to the reduction in body weight after liraglutide treatment in both ethnicities.

METHODS

Participants and study design

Participants

This study is a secondary analysis of two previously performed double-blind, placebo-controlled, randomized clinical trials that were both designed to study the effect of treatment with liraglutide for 26 weeks on glucose regulation and cardiovascular endpoints in patients with overweight and obesity and T2DM (34, 35) and performed at the Leiden University Medical Center (LUMC). In total, 50 patients of Dutch Europid (hereinafter: 'Europid') origin (study 1) (34) and 47 participants of Dutch South Asian (hereinafter: 'South Asian') origin (study 2) (35) were included. South Asian ethnicity is defined as having four grandparents who originally descended from either Surinam, Bangladesh, India, Nepal, Pakistan, Afghanistan, Bhutan, or Sri Lanka. Inclusion criteria were males and females aged 18-69 years, BMI ≥ 25 kg/m^2, and HbA_{1c} levels of 7.0-10.0% (53-86 mmol/mol) despite the use of metformin and/or sulfonylurea derivatives and/or insulin. General exclusion criteria were the use of other glucose-lowering medication than mentioned above, a history of renal or hepatic disease, surgery, pancreatitis, pregnancy or lactation, and the presence of any contra-indication for magnetic resonance imaging (MRI). Both trials were performed between 2013 and 2018.

Study approval

Both studies were performed by the principles of the Declaration of Helsinki (36) and approved by the local ethics committee of the Leiden University Medical Center, Leiden, the Netherlands. All participants provided written informed consent before participation. The trials were registered at ClinicalTrials.gov (registration no. NCT01761318 and NCT02660047).

Treatment regimen

After inclusion, all participants were randomized to receive daily treatment with liraglutide (Victoza®, Novo Nordisk A/S, Bagsværd, Denmark) or placebo (provided by Novo Nordisk A/S, Bagsværd, Denmark) with 1:1 stratification for sex and insulin use. At baseline, the dose of liraglutide was 0.6 mg per day (administered subcutaneously), which was titrated in two steps in three weeks to the maximum amount of 1.8 mg once daily. The dose was reduced, if necessary, in case of adverse events. During the study, participants were contacted weekly by telephone to assess adverse events and to discuss glucose management. During the entire 26 weeks, regular treatment options for optimal glycemic control, and regulation of cholesterol levels and blood pressure were given according to current clinical guidelines. Two Europid participants discontinued

treatment. One of these participants was in the liraglutide group and discontinued treatment due to repeated hypoglycemic events and was later diagnosed with type 1 diabetes mellitus. The other participant was in the placebo group and could not continue treatment because he was in detention. There were no serious adverse events related to study drug use, and adverse events reported were mild gastrointestinal problems (i.e., nausea and vomiting).

Study design

Extensive descriptions of the design of both trials have been published elsewhere (34, 35). In short, at baseline and after 26 weeks of treatment, all included participants arrived at the outpatient clinic after at least a 6-hour fast. First, body weight, body composition, and lean body mass were assessed by bioelectrical impedance analysis (BIA; scale Bodystat 1500, Bodystat Ltd., Douglas, UK). Then, venous blood samples were collected, and the participants underwent an MRI and proton magnetic resonance spectroscopy (^{1}H-MRS) to measure subcutaneous, visceral, epicardial, and paracardial adipose tissue volume.

Blood collection

After the collection of venous blood samples, serum and plasma were obtained by centrifugation and stored in the freezer at -80°C until analysis. Serum levels of total cholesterol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured on a Modular P800 analyzer (Roche Diagnostic, Mannheim, Germany). Due to logistical reasons, HbA_{1c} was initially measured with boronate-affinity high-performance liquid chromatography (Primus Ultra; Siemens Healthcare Diagnostics, Breda, the Netherlands) and later with ion-exchange high-performance liquid chromatography (Tosoh G8, Sysmex Nederland B.V., Etten-Leur, the Netherlands). To ensure accurate and consistent results, HbA_{1c} levels obtained from the boronate affinity method were corrected based on the correlation coefficient obtained from validation samples measured on both analyzers. Plasma GDF15 levels were measured with Human Magnetic bead-based multiplex for the Luminex platform (LXSAHM; R&D systems, Minneapolis, Minnesota, USA) according to the manufacturer's protocol.

MRI

At baseline and after 26 weeks of treatment with liraglutide or placebo, participants underwent an MRI in the supine position, using a 3.0 Tesla MRI scanner (Ingenia, Philips Healthcare, Best, the Netherlands) to assess epicardial and paracardial adipose tissue as well as visceral and abdominal adipose tissue volumes, as extensively described previously (35).

Statistical Analyses

Data are expressed as means ± standard deviation. Data normality was confirmed using the Shapiro-Wilk test, visual histograms, and Q-Q plots. Baseline characteristics were compared between the treatment groups and ethnicities using the Chi-square test for binary values (i.e., sex and use of diabetes medication), independent t-test, and Mann-Whitney U for normality distributed data. Not normally distributed data were log10 transformed (e.g., baseline creatine, baseline subcutaneous adipose tissue, visceral adipose tissue, subcutaneous/visceral adipose tissue ratio, paracardial adipose tissue, total cholesterol, and LDL-C). Non-parametric tests were performed on data that were not normally distributed after log10 transformation (e.g., diabetes duration, baseline fat percentage, baseline $HbA_{1c,}$ and metformin dose at baseline). To study the difference between circulating GDF15 levels between ethnicities, an independent t-test was performed with log10 transformed data of baseline GDF15 levels to follow a normal distribution. A delta (Δ; 26-week treatment *minus* baseline value) was created for every outcome. To study the effect of liraglutide on plasma GDF15 levels, body weight, and $HbA_{1c,}$ an analysis of covariance (ANCOVA) was performed, adjusting for baseline values. Moreover, to examine the association of baseline and Δ plasma GDF15 levels with Δ body weight, Δ HbA_{1c}, metformin dose, Δ glucose, Δ total cholesterol, Δ adipose tissue deposition, and Δ kidney function, nonparametric Spearman-rank correlations (rho) were applied. All statistical analyses were performed using the Statistical Package for the Social Sciences v.25.0 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.), whereas all graphs were created with GraphPad Prism software version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA). Significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

At baseline, no significant differences in age, BMI, sex distribution, or other cardiometabolic parameters were observed between participants receiving liraglutide or placebo in both Europid and South Asian individuals with T2DM, as described previously for both trials (34, 35) (**Table 1**). However, waist circumference ($P = 0.029$), paracardial ($P = 0.013$), and pericardial ($P = 0.039$) adipose tissue volumes were higher in participants from the liraglutide group than those from the placebo group at baseline when both ethnicities were combined (35). When comparing the baseline characteristics between Europid and South Asian individuals, age ($P = 0.013$), body weight ($P < 0.001$), body length ($P < 0.001$), BMI ($P = 0.001$), waist circumference ($P < 0.001$), waist to hip ratio ($P = 0.001$), visceral ($P = 0.007$), paracardial ($P < 0.001$), and pericardial adipose tissue volumes ($P < 0.001$), total cholesterol ($P = 0.003$), LDL-C ($P = 0.022$) and metformin dose ($P = 0.031$) were significantly higher in Europids compared to South Asians. Conversely, South Asians had a longer duration of T2DM compared to the Europids (**Table 1**).

Table 1. Baseline characteristics

	Europids		South Asians		Combined	
	Placebo (*n*=25)	**Liraglutide (*n*=22)**	**Placebo (*n*=25)**	**Liraglutide (*n*=22)**	**Placebo (*n*=50)**	**Liraglutide (*n*=44)**
Demographics						
Females, n, %	*n*=11, 44%	*n*=9, 41%	*n*=14, 56%	*n*=14, 64%	*n*=25, 50%	*n*=23, 52%
Age, years	58.9 ± 6.7	59.9 ± 6.4	54.6 ± 9.4	55.2 ± 11.1	56.7 ± 8.4	57.5 ± 9.2
Diabetes duration, years	10.5 ± 6.9	10.4 ± 5.0	17.0 ± 9.8$^{\#}$	18.8 ± 10.3$^{\#\#}$	13.8 ± 9.0	14.6 ± 9.0
Clinical parameters						
Body weight, kg	93.8 ± 12.9	98.6 ± 14.1	77.8 ± 12.4$^{\#\#\#}$	81.9 ± 11.0$^{\#\#\#}$	85.8 ± 14.9	90.3 ± 15.1
Body length, cm	172.4 ± 9.7	173.4 ± 7.8	164.8 ± 9.4$^{\#\#}$	164.3 ± 8.1$^{\#\#\#}$	168.6 ± 10.2	168.9 ± 9.1
BMI, kg/m2	31.5 ± 3.5	32.8 ± 4.3	28.6 ± 4.0$^{\#\#}$	30.4 ± 3.8	30.1 ± 4.0	31.6 ± 4.2
Waist circumference, cm	108.4 ± 8.1	111.9 ± 9.6	98.4 ± 10.1$^{\#\#\#}$	104.1 ± 7.8* $^{\#\#}$	103.4 ± 10.4	108.0 ± 9.5*
Hip circumference, cm	106.2 ± 6.7	108.5 ± 8.5	104.0 ± 9.0	104.3 ± 7.1	105.1 ± 7.9	106.4 ± 8.0
Waist to hip ratio	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1$^{\#\#}$	1.0 ± 0.1*	1.0 ± 0.1	1.0 ± 0.1
Body fat percentage, %	36.7 ± 9.0	36.7 ± 10.1	36.9 ± 9.8	37.2 ± 8.4	36.8 ± 9.3	37.0 ± 9.2
Subcutaneous adipose tissue, cm2	330 ± 109	367 ± 143	326 ± 141	316 ± 97	328 ± 125	341 ± 123
Visceral adipose tissue, cm2	200 ± 62	211 ± 88	149 ± 49$^{\#\#}$	187 ± 57*	174 ± 61	199 ± 74
Visceral/subcutaneous adipose tissue ratio	0.7 ± 0.3	0.7 ± 0.4	0.5 ± 0.3	0.7 ± 0.3	0.6 ± 0.3	0.7 ± 0.3
Epicardial adipose tissue, cm2	9.6 ± 4.1	8.9 ± 4.4	9.1 ± 2.7	10.4 ± 3.2	9.3 ± 3.4	9.6 ± 3.9
Paracardial adipose tissue, cm2	20.6 ± 10.0	25.8 ± 11.2	9.0 ± 4.5$^{\#\#\#}$	12.3 ± 4.4* $^{\#\#\#}$	14.4 ± 9.5	19.1 ± 10.8*
Pericardial adipose tissue, cm2	30.2 ± 12.3	34.7 ± 13.7	18.2 ± 5.6$^{\#\#\#}$	22.7 ± 6.5* $^{\#\#}$	23.9 ± 11.1	28.7 ± 12.2*
HbA1c, mmol/mol	64.6 ± 10.3	66.5 ± 11.7	70.5 ± 12.1	64.8 ± 9.7	67.5 ± 11.5	65.7 ± 10.7
Total cholesterol, mmol/L	4.8 ± 1.0	4.9 ± 1.0	4.5 ± 1.1	4.0 ± 0.7$^{\#\#}$	4.6 ± 1.1	4.4 ± 1.0
HDL-C, mmol/L	1.3 ± 0.4	1.2 ± 0.3	1.2 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.2 ± 0.3
LDL-C, mmol/L	2.5 ± 0.9	2.6 ± 0.9	2.2 ± 1.0	2.0 ± 0.7$^{\#\#}$	2.4 ± 1.0	2.4 ± 0.9

Table 1. Baseline characteristics (continued)

	Europids		South Asians		Combined	
	Placebo (*n*=25)	**Liraglutide (*n*=22)**	**Placebo (*n*=25)**	**Liraglutide (*n*=22)**	**Placebo (*n*=50)**	**Liraglutide (*n*=44)**
Diabetes medication						
Metformin use, n, %	*n*=25, 100%	*n*=22, 100%	*n*=23, 92%	*n*=22, 100%	*n*=48, 96%	*n*=44, 100 %
Metformin, mg/day	1982 ± 553	2093 ± 700	1728 ± 643	1750 ± 665	1860 ± 605	1922 ± 697
Sulfonylurea, n, %	*n*=8, 32%	*n*=6, 27%	*n*=5, 20%	*n*=3, 14%	*n*=13, 26%	*n*=9, 21%
Insulin use, n, %	*n*=16, 64%	*n*=14, 64%	*n*=19, 76%	*n*=17, 77%	*n*=35, 70%	*n*=31, 71%

Adapted from the original data from treatment in Europids (34). and South Asians (35). Two Europids were not included in the analyses since they discontinued treatment. BMI, body mass index; HbA_{1c}, hemoglobin A1c; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; Asterisk signs (*) indicate significant differences between treatments within a specific ethnicity, and hash signs (#) indicate significant differences between ethnicities within a specific treatment group. *P < 0.05, #P < 0.05, ##P < 0.01, ###P < 0.001. Data are presented as mean ± standard deviation.

Liraglutide reduces body weight but not HbA_{1c} levels

As previously published, 26 weeks of liraglutide treatment, as compared to placebo, decreased body weight in both Europids (-4.3 ± 3.8 kg *vs.* +0.1 ± 2.5 kg*;* P < 0.001) (34) and South Asians (-3.9 ± 3.6 kg *vs.* -0.6 ± 2.2 kg; P < 0.001) (35). The reduction in body weight induced by liraglutide was not different between ethnicities (P = 0.287). In addition, as previously published, 26 weeks of liraglutide treatment, as compared to placebo, did not decrease HbA_{1c} in both Europids (-11.6 ± 11.1 mmol/mol *vs.* -7.7 ± 9.4 mmol/mol; P = 0.265) (34) and South Asians (-8.5 ± 11.2 mmol/mol *vs.* -6.8 ± 9.3 mmol/mol; P = 0.156) (35).

Liraglutide does not modify plasma GDF15 levels

Next, we assessed plasma GDF15 levels at baseline and after 26 weeks of liraglutide treatment and ethnic differences herein. At baseline, plasma GDF15 levels were similar between the liraglutide and placebo groups in both Europids (1495 ± 838 pg/mL *vs.* 1702 ± 1056 pg/mL; P = 0.651) and South Asians (1834 ± 790 pg/mL *vs.* 1814 ± 825 pg/mL; P = 0.774). We combined the baseline GDF15 levels from the participants assigned to the liraglutide and placebo groups to increase the sample size. We observed that baseline levels of GDF15 were not statistically different in South Asians compared to Europids (1823 ± 800 pg/mL *vs.* 1605 ± 956 pg/mL; P = 0.077, **Fig. 1**). Additionally, we performed sensitivity analyses by splitting the data by sex but did not observe significant differences (data not shown).

Twenty-six weeks of treatment with liraglutide, as compared to placebo, did not affect the change (i.e*.,* 26 weeks *minus* baseline) in plasma GDF15 levels in either Europids (-124 ± 962 pg/mL *vs.* -55 ± 911 pg/mL; P = 0.403, **Fig. 2A**), South Asians (-163 ± 853 pg/mL *vs.* -74 ± 732 pg/mL; P = 0.695, **Fig. 2B**). To increase the statistical power, we combined the data from both ethnicities. However, we still did not find significant differences in plasma GDF15 levels between the placebo and liraglutide groups (-144 ± 899 pg/mL *vs.* -64 ± 818 pg/mL *vs.* liraglutide; P = 0.386, **Fig. 2C**). Additionally, we repeated all analyses separately for men and women, and for men and women with different ethnicities and the lack of effect also persisted (**Figs. 3A** and **B**).

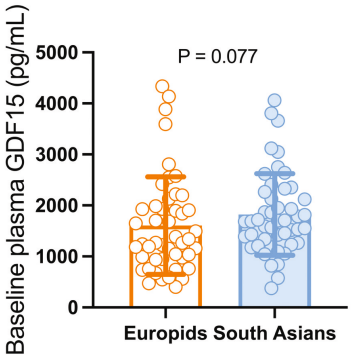


Figure 1. Comparison of plasma GDF15 levels in Europids and South Asians at baseline.
Box plots showing plasma GDF15 levels in Europids (n=47; orange box with circles) compared to South Asians (n=47; blue box with circles) at baseline combined for the placebo and liraglutide treatment groups. Dots represent individual values, boxes represent means, and deviations represent standard deviation (SD).

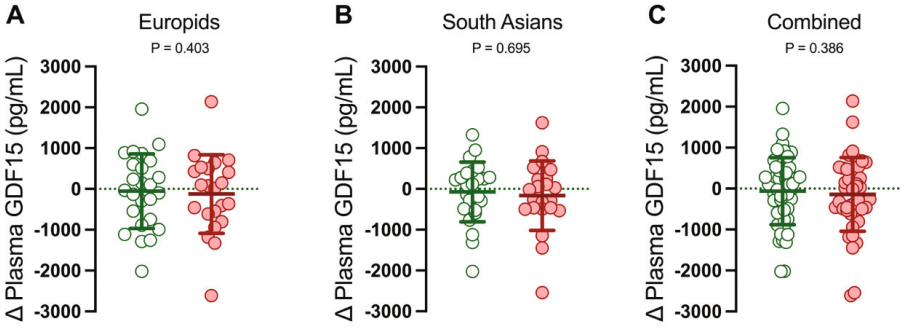


Figure 2. Changes in plasma GDF15 levels in Europids, South Asians, and both ethnicities combined after 26 weeks of placebo or liraglutide treatment.
Scatter plots showing changes in plasma GDF15 (26-week treatment minus baseline values) of (**A**) Europids, (**B**) South Asians, and (**C**) both ethnicities combined after treatment with placebo (n=50; green box and circles) or liraglutide (n=44; red box and circles). Dots represent individual values, horizontal lines represent means, and deviations represent standard deviation (SD).

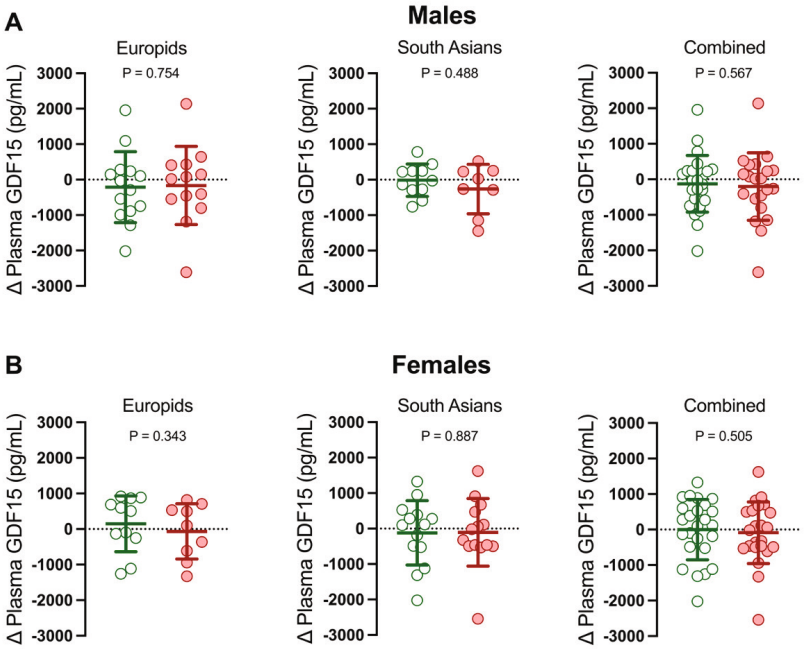


Figure 3. Changes in plasma GDF15 levels in male and female Europids, South Asians, and both ethnicities combined after 26 weeks of placebo or liraglutide treatment

Scatter plots showing changes in plasma GDF15 levels (26-week treatment minus baseline values) of **(A)** Europid males (placebo: n=14; green circles; liraglutide: n=13; red circles), South Asian males placebo: n=11; green circles; liraglutide: n=8; red circles) and both ethnicities combined after placebo (n=25; green circles) or liraglutide (n=21; red circles) and **(B)** Europid females (placebo: n=11; green circles; liraglutide: n=9; red circles), South Asian females (placebo: n=14; green circles; liraglutide: n=14; red circles) of both ethnicities combined after placebo (n=25; green circles) or liraglutide (n=23; red circles). Dots represent individual values, horizontal lines represent means, and deviations represent standard deviation (SD)

Changes in plasma GDF15 levels do not relate to liraglutide-induced decreases in body weight

We next assessed whether the changes in body weight were related to changes in plasma GDF15 levels. Since we did not find any statistical interaction of ethnicity or sex on the changes in plasma GDF15 levels (P ≥ 0.5, data not shown), we pooled all data together to enhance statistical power for the subsequent analyses. After 26 weeks of treatment with liraglutide, as compared to placebo, changes in plasma GDF15 levels were not related to changes in body weight (rho = -0.182; P = 0.236 *vs.* rho = 0.071; P = 0.623, **Fig. 4A**) or changes in HbA_{1c} levels (rho = -0.189; P = 0.220 *vs.* rho = 0.094; P = 0.516 Fig. **4B**). Additionally, we observed that changes in plasma GDF15 levels were not related to changes in blood parameters related to glucose and lipid metabolism (e.g., glucose or total cholesterol) and subcutaneous, visceral, epicardial, paracardial adipose tissue volumes or markers for kidney function (**Figs. 5**-**7**).

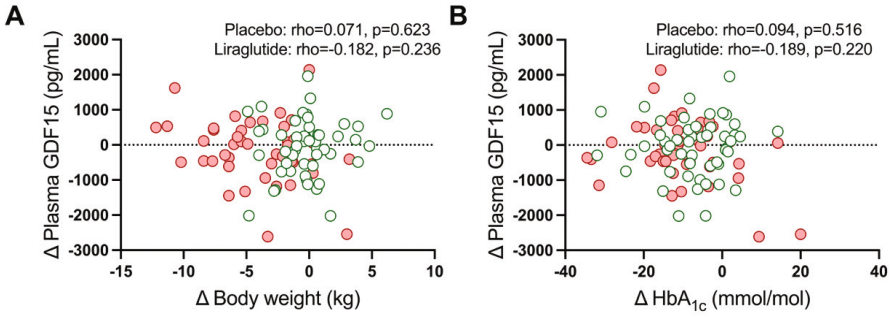


Figure 4. Correlations between changes in body weight and HbA_{1c} levels and changes in plasma GDF15 levels after 26 weeks of treatment with placebo or liraglutide.
Spearman correlation, in both Europids and South Asians, combined, between the change of body weight and the change in plasma GDF15 levels **(A)** and change in plasma HbA_{1c} levels and change in plasma GDF15 levels after treatment with placebo (n=50; green circles) or liraglutide (n=44; red circles) **(B)**. Dots represent individual values.

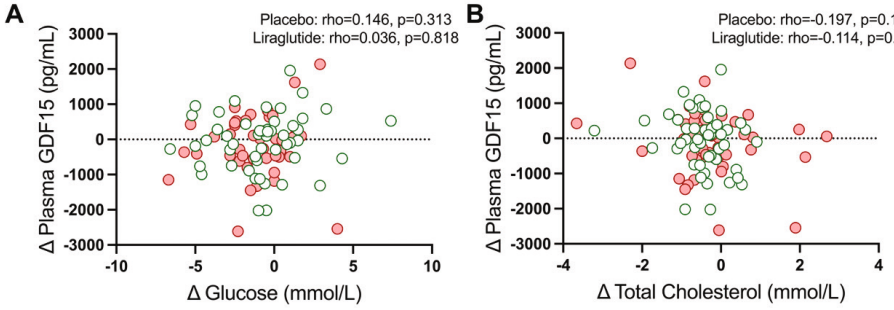


Figure 5. Correlations between changes in metabolic parameters and changes in plasma GDF15 levels after 26 weeks of treatment with placebo or liraglutide
Spearman's correlation plots between changes in fasting plasma glucose and changes in plasma GDF15 levels **(A)** and changes between total cholesterol levels and changes in plasma GDF15 levels **(B)** in both ethnicities combined after treatment with placebo (n=50; green circles) or liraglutide (n=44; red circles). For one participant in the placebo treatment group, the fasting glucose at the end of the study was missing (n=43). Dots represent individual values.

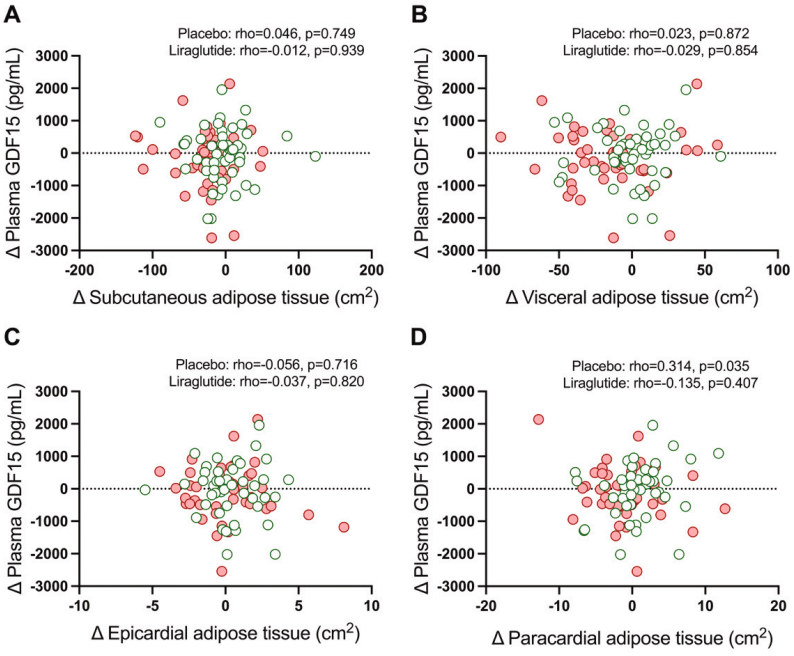


Figure 6. Correlations between changes in subcutaneous adipose tissue, visceral adipose tissue, epicardial adipose tissue, and paracardial adipose tissue volumes with changes in plasma GDF15 levels after 26 weeks of treatment with placebo or liraglutide

Spearman's correlation plots between changes in subcutaneous adipose tissue **(A)**, visceral adipose tissue **(B)**, epicardial adipose tissue **(C),** or paracardial adipose tissue volumes **(D)** with the change of plasma GDF15 levels in both ethnicities combined after treatment with placebo (n=50; green circles) or liraglutide (n=44; red circles). For six participants in the placebo treatment group, the epicardial adipose tissue (n=44) and for five participants in the placebo group the paracardial adipose tissue (n=45) was not reported. For four participants in the liraglutide treatment group, both epicardial adipose tissue and paracardial adipose tissue volumes could not be reported (n=40) at either the start of the study, at the end of the study, or both. This was due to either unsuccessful ^{1}H-MRS of the heart due to low signal-to-noise ratio incorrect peak frequency, or due to missing data (35, 37). Dots represent individual values.

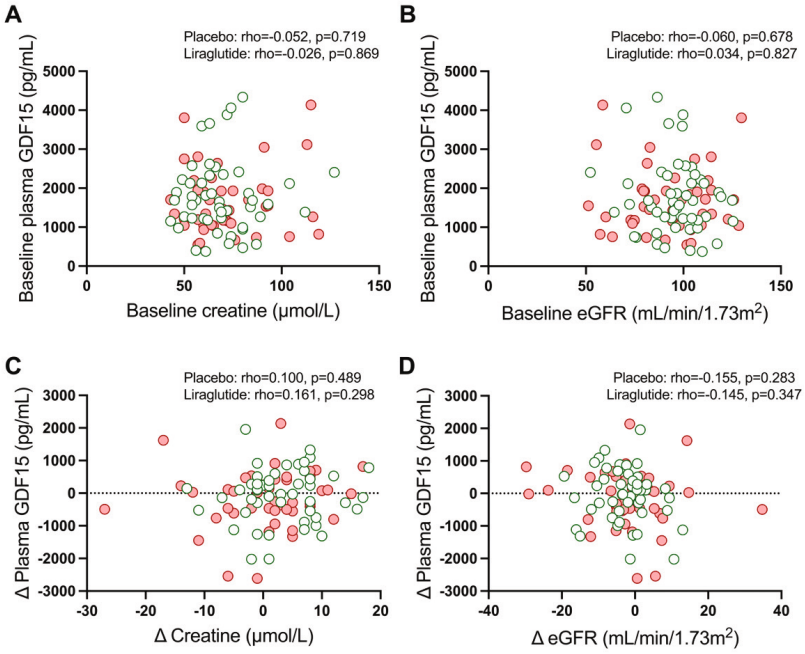


Figure 7. Correlations between markers for kidney function with plasma GDF15 levels at baseline and after 26 weeks of treatment with placebo or liraglutide
Spearman's correlation plots between baseline serum creatinine levels **(A)** or estimated Glomerular Filtration Rate (eGFR) calculated based on serum creatine levels **(B)** with baseline plasma GDF15 levels in both ethnicities combined. Spearman's correlations between changes in plasma creatine levels **(C)** or changes in eGFR **(D)** with changes in plasma GDF15 levels in both ethnicities combined after placebo (n=50; green circle) or liraglutide (n=44; red circles). Dots represent individual values.

Plasma GDF15 levels do not correlate with metformin doses

As previously published, metformin increases plasma GDF15 levels to suppress appetite, thereby inducing weight reduction (12, 13). Here we found that the metformin dose was not related to plasma GDF15 levels, either at baseline (liraglutide: rho = 0.077; P = 0.617; placebo: rho = 0.071; P = 0.629, **Fig. 8A**) or after 26 weeks of treatment (liraglutide: rho = -0.047; P = 0.762; placebo: rho = -0.004; P = 0.978, **Fig. 8B**).

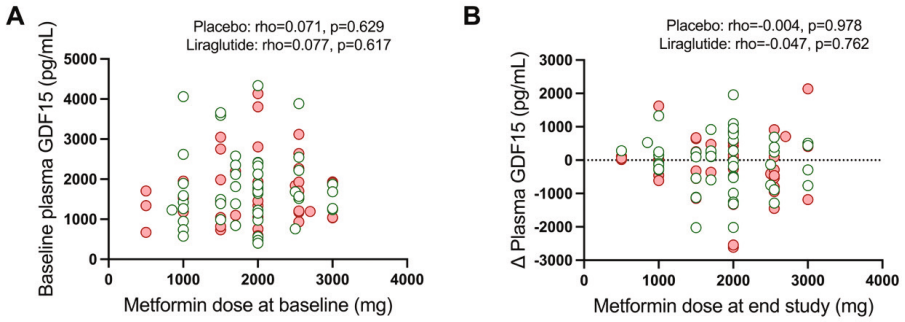


Figure 8. Correlation between metformin dose with change of plasma GDF15
Spearman correlations between the metformin dose at baseline with baseline GDF15 levels **(A)** and between metformin dose at the end of the study with the change in plasma GDF15 levels **(B)** in both ethnicities combined after placebo (n=48; green circles) or liraglutide (n=44; red circles). Two participants in the placebo intervention arm did not use metformin at baseline (placebo; n=48), however, one participant started metformin during the trial (placebo; n=49). Dots represent individual values.

DISCUSSION

The current study showed that plasma GDF15 levels were similar in South Asians compared to Europids with T2DM. Furthermore, 26 weeks of daily treatment with the GLP-1R agonist liraglutide, compared to placebo, did not modify plasma GDF15 levels in both ethnicities. Additionally, we showed that the change in GDF15 levels did not correlate with the decreases in body weight and changes in HbA_{1c} as induced by liraglutide nor with the use of metformin.

In our study, we observed that plasma GDF15 levels at baseline were similar between South Asians compared to Europids. While GDF15 levels have not been previously compared between South Asians and Europids, some studies have investigated GDF15 gene expression and circulating levels between different ethnic groups. For example, one study (38) found that mRNA expression of GDF15 in prostate tissue was lower in African American males compared to American Caucasians who underwent a prostatic biopsy. Another study (39) found that serum GDF15 levels were higher in black people with chronic kidney disease in South Africa compared to the white racial group with chronic kidney disease. Our study specifically focused on differences in plasma GDF15 levels between Europids and South Asians, a population known for their unfavorable metabolic phenotype and high risk of developing cardiometabolic complications (4-6, 40). GDF15 is a well-known biomarker for metabolic diseases, with

higher GDF15 levels corresponding to metabolic diseases such as atherosclerosis, cardiomyopathies, obesity, insulin resistance, and chronic kidney diseases (41-43). Considering the disadvantageous metabolic phenotype of the South Asian population in addition to the previously found differences in the regulation of appetite-regulating hormones, we hypothesized that GDF15 levels would be different in the South Asian population compared to the Europids. However, we did not find a significant difference in plasma GDF15 levels between South Asians and Europids. A possible explanation for this could be the longer duration of diabetes in South Asians. Metformin is the first-line pharmacological intervention for patients with T2DM (44). Since the South Asian participants in this study had longer T2DM duration than the Europid participants, it is possible that they have used metformin for a longer time than Europids, influencing GDF15 levels in this study. Additionally, GDF15 could already have reached a plateau and did not further increase after liraglutide treatment.

Our finding that 26 weeks of liraglutide treatment did not affect plasma GDF15 levels in either ethnicity suggests that the liraglutide-induced weight reduction is independent of the GDF15/GFRAL system. This vision is in line with a recent preclinical study showing that the absence of GDF15 or GFRAL signaling did not affect the ability of liraglutide to reduce food intake in mice (33). There, it was found that while many GFRAL neurons in the area postrema of the mouse and human hindbrain also contained various amounts of GLP-1R mRNA, the majority of the GLP-1R neurons did not express GFRAL mRNA. When mice were injected with liraglutide subcutaneously, food intake decreased independent of whether the mice lacked GFRAL or GDF15. In addition, when mice that lacked the GLP1-R on a whole-body level were injected subcutaneously with GDF15, their food intake was reduced to the same extent as in mice that did not lack the GLP1R. Of note, when mice were injected with the combination of GDF15 and liraglutide, they had a higher reduction of food intake and weight loss than injection of either treatment alone, indicating an independent effect of liraglutide on the GDF15/GFRAL system with possible synergetic potential. Furthermore, our data align with a recent human study that appeared during the preparation of our manuscript (45). In that study, GDF15 levels in male and female patients with obesity (n=20) did not change upon treatment with liraglutide for 5 weeks. Since a previous study involving metformin (12) showed that plasma GDF15 levels continued to rise after a more extended period of metformin treatment (26, 52, and 78 weeks), 5 weeks of treatment with liraglutide may have been too short to modify plasma GDF15 levels. However, in the current study, in which participants were treated for 26 weeks, plasma GDF15 levels also remained unaffected, demonstrating that an extended treatment period of liraglutide treatment is unable to modify plasma GDF15 levels as well. In addition, we did not find a correlation between the change in GDF15 levels upon liraglutide treatment and the change in

weight loss. Altogether, our study supports that the beneficial effects induced by the GLP-1R agonism liraglutide do not involve the GDF15/GFRAL system.

Taken together, we postulate that the weight-reducing effect of liraglutide is likely mediated by other systems. GLP-1 interacts with vagal afferent neurons to transmit gut signals to the hindbrain where it induces satiety, possibly serving as the primary mechanism for satiety induction by GLP-1 receptor agonism (46). This is supported by the attenuated anorexic effect of GLP-1 agonism when the vagal afferents are denervated (47). In addition, other factors such as delayed gastric emptying, influencing fat distribution, and the possible involvement of brown adipose tissue resulting in enhanced thermogenesis may contribute to liraglutide-induced weight loss as well (48-50). We previously found that treating healthy men with the GLP-1R agonist exenatide for 12 weeks enhanced glucose uptake by brown adipose tissue, pointing to enhanced brown adipose tissue volume (48). Although our current study points towards an effect of liraglutide on weight loss independent of GDF15, the concept of GDF15 as a potential treatment strategy for people living with obesity remains intriguing. A recent study showed that a long-acting GDF15 analogue was successful in reducing food intake in rodents and in humans living with overweight and obesity (22).

Although metformin has previously been shown to increase plasma GDF15 levels, in the current study, we did not find a correlation between the dose of metformin and plasma GDF15 levels, not at baseline nor the end of the intervention period. Although multiple research groups previously described that metformin decreases food intake via increasing plasma GDF15 levels, GDF15 levels were measured up to only 18 months of treatment (12, 13). The participants in our study had been living with diabetes ranging from 11-19 years. To our knowledge, no long-term studies have studied GDF15 levels during prolonged metformin use. Therefore, the GDF15 levels may have reached a plateau or even decreased after long-term treatment, which could explain why we did not find a correlation between metformin dose and plasma GDF15 levels. In addition, the majority of patients included in the study had T2DM for over 10 years and used other pharmacological therapies, such as insulin and/or sulfonylurea derivates, in addition to metformin. Thus, the presence of polypharmacy resulting from prolonged diabetes duration may also have influenced plasma GDF15 levels in our study. In addition, diabetes-related complications, such as atherosclerosis development, as well as the presence of obesity itself may have influenced GDF15 levels.

The strengths of our study are the large sample size of 94 participants, with about 50% females. Secondly, we also included patients of South Asian descent, an ethnic population with a high cardiometabolic disease risk to assess whether ethnic differences

exist in plasma GDF15 levels. Moreover, this would give us the opportunity to assess whether liraglutide-induced effects on GDF15 levels differed between ethnicities. However, this study is not without limitations. We only measured plasma GDF15 levels at two time points: baseline and after 26 weeks of intervention. Therefore, any changes in plasma GDF15 levels within that period could have been missed. Plasma GDF15 is known to be influenced by many factors, such as pharmacological agents (51, 52). Almost half of our population received multiple pharmaceutical agents during the intervention period, which could have influenced plasma GDF15 values as well.

In conclusion, this study showed that plasma GDF15 levels were similar in Dutch South Asians and Dutch Europids. Additionally, we observed that 26 weeks of liraglutide treatment does not modify plasma GDF15 levels in both ethnicities. Therefore, we conclude that the GDF15/GFRAL system likely does not play a role in the weight loss induced by liraglutide.

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CHAPTER 8

GENERAL DISCUSSION AND FUTURE PERSPECTIVES



GENERAL DISCUSSION

Obesity is a chronic disease characterized by excessive fat deposits caused by a positive balance between energy intake and energy expenditure (1). The prevalence of obesity and its related diseases is rising, especially in certain ethnic populations (2, 3). In this thesis, we aimed to i) unravel the underlying causes of the disadvantageous metabolic profile of South Asians, and ii) comprehensively understand how different (non-) pharmacological interventions can modulate various circulating hormones and regulate overall energy metabolism in humans with different comorbidities. In this chapter, we discuss new insights based on experimental studies addressing these objectives, their implications for the general population and the South Asian population specifically in clinical practice, and future challenges.

Unraveling influential factors leading to increased risk of obesity and related diseases in the South Asian population

South Asians are prone to develop obesity and obesity-related diseases, including type 2 diabetes mellitus (T2DM), at a significantly younger age and lower body mass index (BMI) than other ethnic groups (4). As described in **Chapter 1**, their increased risk of developing T2DM is significantly influenced by their unfavorable metabolic phenotype, characterized by increased visceral fat mass, dyslipidemia, and reduced insulin sensitivity (5). Apart from this phenotype, several other factors likely contribute to their increased risk of developing T2DM, including lifestyle factors, inflammation, and hormonal dysregulation (5). In this thesis, inflammatory factors (**Chapter 2**) and various appetite-regulating hormones (**Chapter 3** and **Chapter 4**) were studied in South Asians compared to Europids.

Inflammation as a potential catalyzer of the South Asian metabolic phenotype

Inflammation plays a crucial role in the development of T2DM and its associated complications, often triggered by stress on the adipose tissue due to a positive energy balance. This stress leads to the attraction of different immune cell types, including monocytes, and the release of cytokines and chemokines that promote inflammation, exacerbating insulin resistance both locally in white adipose tissue (WAT) and systemically (6). While a more pro-inflammatory phenotype is already found in healthy South Asians, the precise differences in various immune cell types that coincide with the South Asian phenotype are still unknown (7, 8). This is important when developing specific treatment options for South Asians.

In **Chapter 2**, we demonstrated that the relative plasma levels of six inflammation-related proteins were higher in South Asians with T2DM compared to Europids, and

six proteins were lower among a large panel of inflammation-related proteins. Among them, fibroblast growth factor 21 (FGF21) was significantly lower in South Asians than Europids in both males and females (**Figure 1**). FGF21 is a pleiotropic hormone that affects glucose and lipid metabolism. It enhances insulin sensitivity in brown adipose tissue (BAT) and WAT and suppresses lipolysis in WAT (9). Furthermore, FGF21 has anti-inflammatory effects. These anti-inflammatory properties include targeting macrophages to shift from a pro-inflammatory to a pro-repair phenotype. This shift occurs through the paracrine effect of hepatocytes on Kupffer cells and in adipocytes via the secretion of adiponectin (10). The subsequent question arising from this study is whether the lower FGF21 levels in South Asians are a cause or a consequence of the already present metabolic complications in this cohort. To get more insight into this question, we also measured FGF21 levels in a small cohort of healthy South Asians since they had not developed metabolic complications as yet. Of note, in this cohort of males, FGF21 levels were comparable between South Asians and Europids, which may indicate that FGF21 regulation becomes disturbed later in life. However, since we only measured baseline FGF21 levels, we cannot exclude that stress-induced FGF21 release (e.g. following cold exposure) is disturbed in this population prior to the development of metabolic diseases. This may be of relevance since it implies that certain metabolic pathways may already be targeted in an early stage to prevent the development of metabolic diseases in this population (see below).

Next to measuring inflammation-related proteins, to better understand the increased pro-inflammatory phenotype observed in South Asians, it is crucial to focus on identifying specific immune cell types that may be involved. This can improve understanding of their vulnerable metabolic phenotype and give direction to possible intervention options. We previously showed that South Asians with T2DM had higher gene expression of B cell markers in blood than Europids (8). The role of B cells in developing insulin resistance has been investigated in mice, showing that a high-fed diet induces the accumulation of B cells in VAT after four weeks. Mice lacking mature B cells had body weight and VAT adipocyte size similar to those of mice with mature B cells, although with lower fasting glucose and insulin levels and improved glucose tolerance and insulin sensitivity (11). The accumulation of B cells in VAT may also contribute to the development of insulin resistance in humans with obesity, since B cells are known to stimulate the production of interferon-gamma (IFN-gamma) and tumor necrosis factor-alpha (TNF-alpha) from T-cells. These factors, in turn, are known to induce insulin resistance in other tissues than VAT, including skeletal muscle (12). Therefore, as increased B cells contribute to the development of insulin resistance, and South Asians with T2DM have higher expression of B cell markers in blood, this could have contributed to their insulin resistance (**Figure 1**). To further unravel this

hypothesis, future studies should focus on studying the presence of B cells and their activation status in WAT biopsies in South Asians. Moreover, determining whether the higher gene expression of B cell markers is a contributor to the development of T2DM in South Asians or merely a result of their T2DM requires research in a healthy population. Indeed, if increased B cell markers are already present in healthy South Asians, it would suggest that these markers are more likely a contributing factor rather than a consequence of T2DM.

Another immune cell involved in the development of insulin resistance is the monocyte (**Figure 1**). Monocytes have been shown to play a role in the development of atherosclerosis and insulin resistance (13, 14). Monocytes contribute to the initiation, progression, and thrombus formation of atherosclerosis. Especially the $CD16^{+}$ monocytes are associated with the development of obesity and related diseases (15, 16). A recent paper showed that South Asians with intermediate risk for cardiovascular disease have more circulating classical monocytes ($CD14^{++}CD16^{-}$) than their Europid counterparts (17). This is of specific interest as previous research showed beneficial changes in different monocyte subsets in people living with obesity after 1.5 years of an intensive combined lifestyle intervention (CLI). This intervention included group therapy with nutritional advice, physical activity, and structural behavioral changes using a cognitive behavioral therapy-based approach (15). Therefore, an intensive CLI that decreases relative classical monocyte levels and reduces the expression of CD16 in intermediate and non-classical monocytes (15) could benefit South Asians. As the function of the monocytes was not assessed in the study, exposing the monocytes to a stimulus like lipopolysaccharide (LPS) could provide more information on possible CLI-induced changes in the function of the different monocyte subsets (15). In addition to the subtypes of monocytes, changes in the metabolism of these cells can also play a role in developing obesity-related diseases in South Asians. A recent study found that the monocytes of insulin-sensitive individuals are more dependent on non-oxidative glycolysis, compared to oxidative metabolism in insulin-resistant individuals (13). Research focusing on measuring the quantity and quality of monocytes, using flow cytometry and metabolic signatures to measure monocyte metabolism in different states of metabolic status, could provide more insight into the pro-inflammatory status of South Asians. While this proposed study design focuses more on specific cell types, an organ-on-a-chip system with microfluidics can model vascular disease e.g., thrombosis, atherosclerosis, and inflammation (18). A system designed to mimic the pathology of atherosclerosis development in South Asians, incorporating their monocytes and comparing the development of vascular disease with a model using monocytes from Europids, could provide more insight into the role of the monocytes in the development of atherosclerosis in South Asians.

A relevant question is whether treatments targeting inflammation, such as the B cells or monocytes, result in the prevention and/or improvement of obesity-related diseases in South Asians. Concerning potential treatments, a pharmacological intervention could be the anti-inflammatory drug salsalate. Salsalate is a salicylate known for treating inflammatory diseases like arthritis (19). It induces anti-inflammatory effects by inhibiting cyclooxygenase (COX) enzymes and the nuclear factor-κB (NF-κB) cascade, decreasing the release of pro-inflammatory cytokines (20, 21). Of specific interest is the fact that it decreases the chemokine monocyte chemoattractant protein-1 (MCP-1), which mediates the release of monocytes from the bone marrow and guides monocytes into VAT (22). In Europids with T2DM, treatment with salsalate decreases inflammatory mediators, lowers circulating leukocytes, neutrophils, and lymphocyte counts, and results in the improvement of glycemia (23). Since South Asians have a phenotype that includes decreased insulin sensitivity and more pro-inflammatory monocytes compared to Europids, salsalate could be particularly beneficial for South Asians. In addition to the improvement of glucose regulation, salsalate may also exert its beneficial effect via the activation of BAT. A previous study from our group showed that, in mice, salsalate prevents weight gain, and deterioration of glucose metabolism by improving fasting insulin levels and glucose tolerance during intravenous glucose tolerance tests, and activates BAT (24), making it of interest in South Asians as they have dyslipidemia and lower BAT volume. Altogether, this compound may be of specific interest to the South Asian population and this type of treatment may even be considered as a general first-line treatment in South Asians with prediabetes, with the aim to delay progression towards T2DM.

Finally, an important question to consider is why South Asians exhibit a more pro-inflammatory phenotype compared to Europids. One theory is that this pro-inflammatory phenotype could have evolved since it had a survival advantage in the past, possibly due to the high risk of exposure to infections in South Asia (25). A more pro-inflammatory profile could lead to faster and more effective eradication of various micro-organisms, thereby enhancing survival chances. On the other hand, South Asians living in the United Kingdom had a higher risk of mortality during the Coronavirus disease 2019 (COVID-19) pandemic compared to other ethnicities (26). However, many other factors could have influenced this higher mortality, such as socioeconomic position, racial discrimination in healthcare, and the presence of comorbidities (26).

Incretin hormones in the South Asian population

The incretin hormone glucagon-like peptide-1 (GLP-1) has gathered extensive interest over the years as it improves insulin sensitivity and stimulates satiety, and various GLP-1 receptor agonists have been shown to improve T2DM and result in weight loss

(27). However, the postprandial excursion of GLP-1 in South Asians has not been extensively studied. In **Chapter 3** we observed a biphasic peak of active GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) during the MMTT in South Asian females, while a single peak was observed in Europids. In addition to the peak at 30 minutes, levels of active GLP-1 and active GIP were higher towards the end of the MMTT in South Asian compared to Europid females. A similar biphasic pattern, albeit less pronounced, was evident for total GLP-1 and GIP in South Asian females compared to Europids. Since glucose levels followed the same biphasic pattern in South Asians compared to Europids, we propose a mechanism that could be driven by biphasic emptying of the stomach in South Asians (28) (**Figure 1**). This is plausible since gastric emptying rate is known to influence glucose concentration and differences in gastric emptying rates have been described to differ between various ethnicities (28). The biphasic glucose excursion during the MMTT in South Asian females could have led to an observed second peak of especially active GLP-1 and GIP. Further research is needed to determine if the differences observed are indeed related to differences in the rates and patterns of gastric emptying between healthy South Asians and Europids and if they contribute to an increased risk of developing obesity and related diseases in this population. This could be explored by measuring the gastric emptying rate during an MMTT using a gastric emptying scintigraphy.

Despite higher levels of active GLP-1 and active GIP towards the end of the MMTT, the insulin response was not different in South Asian females compared to Europid females. This could indicate a lower insulin response to active GLP-1 in South Asian females, for instance, due to a lower pancreatic GLP-1 receptor sensitivity. Whether the sensitivity of these receptors changes during the development of T2DM in South Asians needs to be assessed with long-term follow-up studies assessing the receptor sensitivity before, during, and after the development of T2DM. This could be studied by infusing GLP-1 intravenously, followed by assessing the response of insulin and glucose levels. In addition, the role and function of GLP-1 in satiety among South Asians could be assessed by using questionnaires about feelings of hunger and satiety, or by providing *ad libitum* meals to measure the amount of food intake with and without prior intravenous GLP-1 infusion.

In contrast to the South Asian females, in **Chapter 3**, we found a lower area under the curve (AUC) of total GLP-1 levels already in young and lean South Asian males compared to Europids. Similar to the South Asian females, this did seem to affect the response of insulin, as insulin levels, if anything, were even higher in South Asian males compared to Europid males. This could fit with an enhanced GLP-1 receptor sensitivity in South Asian males. Alternatively, GIP could have compensated for the lower GLP-1 with respect to

the stimulation of insulin release. It is interesting to speculate on the underlying causes of the lower AUC of total GLP-1 in South Asian males. This could be due to a lower release of GLP-1 by the intestinal L-cells (**Figure 1**) or increased degradation of GLP-1 by the enzyme dipeptidyl-peptidase-4 (DPP-4) in the circulation. DPP-4 activity in South Asians can be assessed *in vitro*. If DPP-4 activity is higher in South Asian compared to Europid males, then treatment with DPP-4 inhibitors would be beneficial. This treatment could increase the GLP-1 concentration after a meal, thereby potentially improving the induction of satiety and stimulating insulin release postprandially (29). However, DPP-4 inhibitors increase active GLP-1 levels rather than total GLP-1 (29), and active GLP-1 levels were not significantly different during an MMTT in young and healthy South Asian males. In addition, active GLP-1 represents the endocrine function of GLP-1, while total GLP-1 represents the neural function as well (30), therefore increasing total GLP-1 would potentially be more beneficial as it targets satiety, shifting energy balance towards a negative one. Whether active GLP-1 levels differ further between South Asians and Europids living with obesity needs to be assessed in future studies, preferably combed with an MMTT as well.

The sex differences observed in postprandial GLP-1 regulation in South Asian males and females compared to Europids suggests the need for a more tailored made approach for using GLP-1 receptor agonists in this population, possibly even stratified by sex. For South Asian females, treatment with GLP-1 receptor (GLP-1R) agonists could be beneficial as GLP-1 R agonists have been shown to slow gastric emptying rate, thereby reducing postprandial glucose levels (31). Although the biphasic curve of glucose and active GLP-1 and GIP did not affect insulin excursions in our population of female South Asians, the AUC of glucose was already higher in healthy and lean South Asian compared to Europid females. This suggests that if South Asian females were to become more metabolically compromised, treatment with GLP-1R agonist might reduce glucose excursion by slowing the gastric emptying rate and thereby potentially improving insulin excursions postprandially. In **Chapter 3** we found a lower AUC of total GLP-1 in the South Asian males compared to the Europid males. People living with obesity typically have lower postprandial GLP-1 levels compared to individuals without obesity (32). The South Asian males in our cohort had a significantly higher BMI and higher cholesterol levels compared to Europid males, indicating a more metabolic comprised phenotype, more similar to people living with obesity. If the differences observed in the AUC of total GLP-1 between South Asian males and Europids are also observed or even more pronounced in South Asians living with obesity, this would support the use of GLP-1R agonists in the male South Asian population as well. Interestingly, the effects of the GLP-1R agonist liraglutide on weight loss and glucose regulation have been studied in South Asians compared to Europids with T2DM in the cohort described in **Chapters 2**

and **7**. South Asians and Europids with T2DM had a similar improvement in the glycemic index after treatment with liraglutide, indicating that both ethnicities with overweight and obesity and type 2 diabetes mellitus indeed benefited from this intervention (33, 34). Nonetheless, considering our finding of lower AUC of total GLP-1 already in young South Asian males likely resulting in lower activation of GLP-1 receptor pathways in peripheral tissues, at least South Asians males could potentially benefit from earlier treatment with GLP-1R agonists to prevent or delay the development of T2DM.

Leptin in the South Asian population

In addition to the differences observed in **Chapter 3**, which focused on incretin hormones and glucagon, we described in **Chapter 4** that leptin levels were significantly higher in both South Asian males and females compared to Europids. The average baseline leptin levels in South Asian and Europid males were 83 ± 54 *vs.* 22 ± 21 and females 410 ± 236 *vs.* 183 ± 128 ng/mL. Of note, these levels in lean South Asians, especially females, are in the range of leptin levels observed in Europid individuals living with obesity (35-38).

The increased fat percentage in South Asians in our cohort could be the reason for the significantly higher leptin levels, as we saw a positive correlation between leptin and fat percentage and fat mass in South Asians only (**Figure 1**). In addition, the location of the fat mass influences leptin concentrations. It has been described that subcutaneous adipose tissue (SAT) secretes more leptin than other fat depots like visceral adipose tissue (VAT) (39). However, this is contrary to the idea that South Asians have less SAT and store more fat ectopically. Whether South Asians have higher leptin expression in SAT compared to Europids, could be further examined by studying leptin expression in fat biopsies from subcutaneous depots. Furthermore, measuring leptin levels in cohorts with similar fat percentages and location of this fat could give a more definite answer if South Asians have indeed higher circulating leptin levels independently of fat percentage; however, matching South Asians with Europids for clinical studies remains a challenge, as described below.

Interestingly, the pro-inflammatory phenotype of South Asians may influence leptin levels as well (40) (**Figure 1**). Chronic inflammation can impair leptin action by interfering with leptin receptor signaling, resulting in leptin resistance in the hypothalamus (40). Furthermore, as leptin receptors are present on different immune cells, such as monocytes, leptin binding to these receptors can result in low-grade inflammation. The hyperleptinemia observed in South Asians could, in turn, aggravate inflammation (40). The interplay between leptin and inflammation could lead to a higher tendency to develop obesity and obesity-related diseases in South Asians (**Figure 1**). Another

way to further study the interaction between leptin and the immune system is to expose monocytes to leptin *in vitro* to measure their sensitivity with respect to secretion of cytokines. A difference in sensitivity in leptin in other cell types like neurons and adipocytes could further explain the observed hyperleptinemia in South Asians. As a result of decreased leptin sensitivity in neurons and adipocytes, higher leptin levels are required to maintain a stable energy balance. *In vitro* studies can be done to assess the sensitivity of leptin receptors in South Asians of the neurons in the hypothalamus, adipocytes, and monocytes. Most studies determine leptin resistance based on the circulating leptin levels or focus on leptin signaling due to mutation of the leptin receptor gene, altered leptin transport across the blood-brain barrier, or decreased leptin receptor expression, which are all associated with the development of obesity (41, 42). Therefore, in addition to *in vitro* studies assessing leptin receptor sensitivity, measuring leptin receptor mRNA in neurons and protein levels or evaluating leptin receptors after prolonged fasting could provide more insight into the leptin receptors' sensitivity (41). However, these measurements in the South Asian population compared with Europids could not provide information on the location of leptin sensitivity. The leptin receptors are mainly located in hypothalamic neurons and are also expressed in smaller amounts in other tissues like adipocytes and skeletal muscle (39, 43-45). To pinpoint the location of leptin sensitivity, white fat biopsies, and muscle biopsies could be taken from South Asians, and leptin receptor sensitivity could be measured there.

In addition to the focus on the difference in the regulation of leptin in South Asians, leptin levels could potentially serve a more clinical function. Specifically in South Asians, our study showed that leptin represented the metabolic status of adiposity better than BMI. We found that the fat percentage was significantly higher in South Asians compared to Europids despite their normal BMI, and only the fat percentage positively correlated with leptin levels, not with BMI, in South Asian males and females. Therefore, leptin could serve as a metabolic biomarker for adiposity in this population. Standard measurements for predicting metabolic status, such as BMI and waist circumference, often misrepresent the actual metabolic status of South Asians. If leptin proves to be a more accurate marker, it could improve the assessment of their metabolic status. Indeed, the use of leptin as a predictive marker for metabolic syndrome has been described before (46). To evaluate leptin as a potential biomarker in South Asians, measurements of circulating leptin levels must be taken at baseline and followed longitudinally in large cohorts to monitor the development of obesity and obesity-related diseases.

Cortisol in the South Asian population

Besides GLP-1 and leptin, another hormone that may be of interest to South Asians is cortisol. As described in **Chapter 1,** the metabolic phenotype of South Asians, characterized by higher abdominal fat mass, increased insulin resistance, and lower muscle mass, is similar to symptoms seen in individuals with hypercortisolism, an extreme example of which is Cushing's syndrome (47). Our preliminary data described in **Chapters 3** and **4** showed that circulating plasma cortisol levels are lower in young and lean South Asian versus Europid females (4.2 ± 2.0 *vs.* 6.3 ± 2.1 µmol/L; P = 0.011). Previous research also found lower circulating cortisol levels in middle-aged (40-67 years) South Asian men compared to Europids (48).

A possible reason for the counterintuitively lower cortisol in South Asians compared to Europids is that they might have a higher sensitivity to cortisol (**Figure 1**). Cortisol binds to the nuclear glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), resulting in the transactivation and transrepression of various genes. Of note, GR sensitivity differs between people; 4.5% and 38.0% of the Europid population are carriers of two distinct polymorphisms of the GR associated with increased glucocorticoid sensitivity (49, 50). This coincides with higher abdominal circumference and a disadvantageous glucose metabolism. GR sensitivity can be assessed using a bioassay *in vitro* in peripheral blood mononuclear cells, which would be highly interesting to perform in South Asians. Another option could be that the prevalence of the GR polymorphism associated with increased sensitivity of the GR is higher in South Asians compared to Europids. Two polymorphisms known to be associated with increased sensitivity to corticosteroids are a BclI polymorphism (rs41423247) and N363S (rs56149945) (51, 52). However, a previous study found a lower frequency of the N363S polymorphism in South Asians compared to Europids (53). If the sensitivity to cortisol is indeed increased in South Asians compared to Europids, treatment with a GR antagonist or a mineralocorticoid receptor antagonist could be an option to counteract the cortisol-induced metabolic dysfunctions observed in obesity (54).

In a physiological situation, the hypothalamic-pituitary-adrenal (HPA) axis consists of the secretion of hypothalamic corticotrophin-release hormone (CRH) to stimulate adrenocorticotrophic hormone (ACTH) from the pituitary and the secretion of glucocorticoids by the adrenal cortex (55). Different factors can disrupt the HPA-axis, like prolonged emotional stress (56). Our preliminary data suggest that South Asian females have higher perceived stress scores (PSS) based on the PSS-14 questionnaire. As prolonged emotional stress normally results in hypercortisolism, the results of higher PSS in South Asians are again in contrast with the observed lower circulating cortisol levels, again pointing towards possibly higher GR sensitivity (56, 57).

Another explanation for the finding of lower circulating cortisol in South Asians compared to Europids in our and other studies is a disrupted cortisol rhythm in South Asians. Circulating cortisol levels differ throughout the day, with the highest peak occurring in the morning, rising before waking (58). Physical activity, diet, and sleep all influence the body's circadian rhythm. South Asians exercise less and have more sleep disturbances due to for example sleep apnea, described in **Chapter 1**, which could lead to a blunted rhythm in circadian cortisol with lower morning cortisol levels (59). Another option is that different ethnicities have their own (genetically determined) cortisol rhythm. A meta-analysis comparing the circadian rhythm of Blacks, Hispanics, and Whites found differences in the cortisol rhythm between each ethnicity. Blacks and Hispanics had a more blunted cortisol rhythm that increased towards bedtime, with especially a lower peak cortisol in the morning (60). A flatter circadian cortisol rhythm is associated with decreased insulin sensitivity and T2DM (61). Therefore, South Asian males and females could potentially have a flatter cortisol rhythm than Europids, contributing to their higher risk of developing T2DM. The cortisol rhythm in South Asians has not been extensively researched thus far, making this an interesting topic for future studies.

Sex hormones in the South Asian population

In **Chapter 3** and **4**, we found noticeable differences between males and females of South Asian descent**.** For instance, active GLP-1 and GIP increased towards the end of the MMTT only in South Asian females, while plasma cortisol and perceived stress were lower only in females. A potential influence could be differences in sex hormones. Such differences in South Asians have been described previously and could potentially contribute to their increased risk of developing obesity and obesity-related diseases (62, 63).

During menopause, estrogen levels decrease, leading to an increase in abdominal fat and contributing to the development of obesity (64). In South Asians, it has been described that the mean age of menopause is about 3 years lower than in Europids and that South Asian females can experience menopause-related symptoms differently (65, 66). During menopause, the risk of obesity-related diseases like CVD and T2DM increases due to the changes in estrogen, and earlier menopause is associated with increased development of obesity-related diseases (67). Together with the already present increased risk of developing metabolic diseases in the South Asian population, (see **Chapters 1** and **8**)**,** screening every South Asian female peri/post-menopausal for obesity-related diseases could potentially prevent such diseases or allow earlier treatment of these diseases, resulting in long-term health improvement. In addition, hormone replacement treatment (HRT) has been shown to significantly reduce the

risk of CVD and overall mortality in females when started before 60 years of age (68). HRT may also be an interesting option for South Asians that should be further studied.

Certain diseases affecting sex hormones are potentially more prevalent in South Asians, although the prevalence of these diseases varies across studies (69-71) (**Figure 1**). Examples include endometriosis and polycystic ovarium syndrome (PCOS) (72, 73). South Asian women with endometriosis often report less pain and a better quality of life but tend to have more advanced stages of the disease at diagnosis (72). Even though endometriosis was not associated with the development of T2DM in some studies, there are indications that there is a relation with insulin sensitivity, as women with endometriosis have an increased risk of gestational diabetes mellitus (74, 75). Additionally, there are indications that endometriosis is linked with abnormal metabolic measurements, like higher insulin levels (76). However, before we can speculate about the impact of endometriosis on insulin sensitivity in the South Asian population, the mechanisms underlying the pathology of endometriosis need to be further investigated. PCOS is associated with insulin resistance and hyperinsulinemia and was found to be more prevalent in South Asians (77). South Asian women with PCOS often present with anovulation at a younger age, more severe hirsutism, and a higher prevalence of acanthosis nigricans than Europids (71). Metformin, the first-line treatment for T2DM is also registered for people with PCOS (78). As South Asians already have an increased risk of developing T2DM with signs of insulin resistance when they are young and lean, starting metformin in South Asian females with PCOS could potentially decrease the risk of developing T2DM later in life (79, 80).

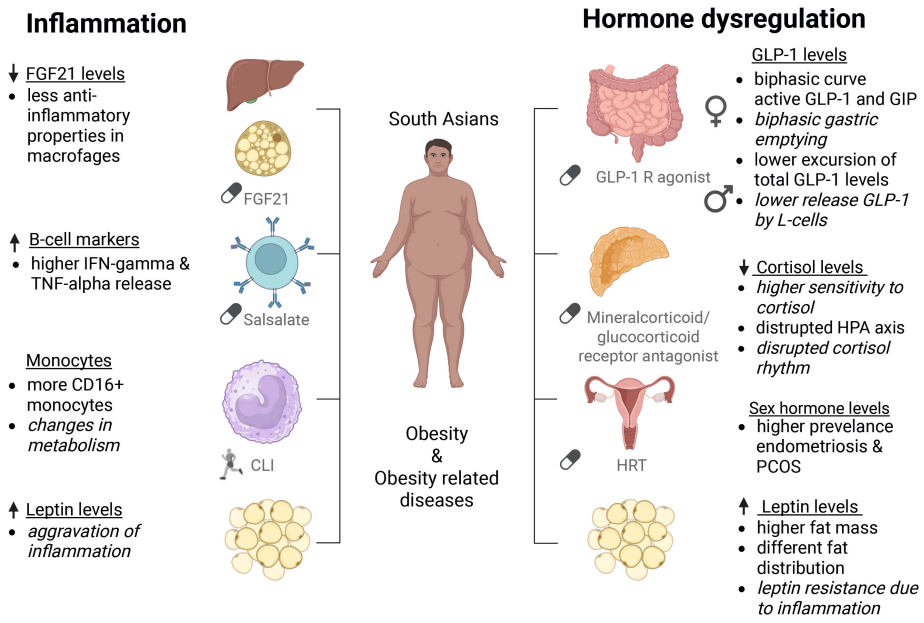


Figure 1. Inflammatory and hormonal factors that may underlie the high risk of developing obesity and associated diseases in the South Asian population. The pro-inflammatory phenotype in South Asians includes lower circulating FGF21 levels, higher B cell markers, more CD16+ monocytes, and increased circulating leptin levels. In addition, hormonal dysregulation in the South Asian population includes biphasic active GLP-1 and GIP excursions to a MMTT in females, lower circulating total GLP-1 levels in males, higher circulating leptin levels, lower cortisol levels, and a higher prevalence of diseases that influence sex hormones. Changes in the metabolism of monocytes causing a shift from non-oxidative glycolysis to oxidative metabolism, and the aggravation of inflammation by increased circulating leptin levels, are proposed hypotheses contributing to the inflammatory factors of the increased risk of South Asians to develop obesity and related diseases. We hypothesize that a lower release of GLP-1 by the intestinal L-cells, a higher sensitivity to cortisol with a disrupted cortisol rhythm, and leptin resistance due to inflammation contribute further to the increased risk in this population. Proposed treatment options for the underlying mechanisms contributing to the development of obesity and obesity-related diseases in the South Asian population are shown in grey. CLI, combined lifestyle intervention; FGF21, fibroblast growth factor 21; GLP-1, glucagon-like peptide 1; GLP-1R agonist, glucagon-like peptide-1 receptor agonist; HPA axis, hypothalamic-pituitary-adrenal; HRT, hormone replacement therapy; IFN-gamma, interferon-gamma; PCOS, polycystic ovarium syndrome; TNF-alpha; tumor necrosis factor-alpha.

Challenges in researching the South Asian population

In the study described in **Chapters 3** and **4,** we compared lean and young South Asians and Europids who were mainly matched on BMI and age. However, in those cohorts, it was evident that South Asians had a higher fat percentage than Europids with the same BMI range. These differences in body composition may have influenced the results. For example, the higher fat percentage of South Asians could explain the difference in leptin levels observed in **Chapter 4**. In addition, in the cohort of individuals with T2DM, in **Chapters 2** and **7**, participants were included based on age (18-74 years), BMI ≥ 25 kg/m^2, and HbA_{1c} ≥ 6.5% and ≤ 11.0%). Despite the South Asians having a lower BMI and smaller waist circumference, their fat percentage was not significantly different. Furthermore, South Asians had a significantly longer duration of T2DM, which implies longer exposure to the disease and its associated treatments. Therefore, matching South Asians with Europids by BMI likely is not optimal. We could overcome this by having stricter BMI cut-offs. For example, the upper BMI limit for the lean cohorts could be decreased from 25 to 23 kg/m^2. However, since it is generally challenging to find a sufficient number of participants of South Asian descent for studies, it should be realized that lowering BMI criteria could further hamper inclusion. In addition, the average BMI in our cohort described in **Chapters 3** and **4** was 23 kg/m^2. Despite matching based on BMI, we already observed significant metabolic differences. Of note, these metabolic differences are part of the South Asian phenotype and are part of the driving factors behind our research. Another option would be matching based on waist circumference. An increased waist circumference is an indicator of more central obesity and is associated with type 2 diabetes, hypertension, and metabolic syndrome (81). However, waist circumference was not significantly different between South Asians and Europids in our cohort, despite the metabolic difference. However, a potential difference might have been masked by the height difference between both populations with the Europids being consistently taller compared to the South Asians. Therefore, matching on waist circumference is also not a viable option.

Other options for matching are based on fat mass, fat percentage, or a metabolic biomarker in blood. However, not every research facility has the possibility to measure fat mass or fat percentage. In addition, individuals willing to participate are often unaware of their fat mass, leading to significantly more screening and potentially more exclusion of participants from the study. Similarly, the circulating levels of a biomarker are also not known before screening. An intervention with a venous puncture to draw blood is necessary to acquire it, leading to an extra burden for individuals willing to participate in a study. Using fat mass and leptin measurements in the assessment of the metabolic status of individuals could be more useful clinically when assessing the potential risk of developing obesity and obesity-related diseases.

In addition to the challenge of matching South Asians based on their metabolic status, other factors could contribute to differences between cohorts in studies, including South Asians. For example, South Asians have a different lifestyle compared to Europids. South Asians are known to exercise less than Europids and smoke more (82). Both exercise and smoking influence many metabolic processes, such as lipid metabolism and systemic inflammation (83, 84). Therefore, individuals who engage in vigorous exercise and smokers are often excluded from research.

We experienced that the inclusion of South Asian males was especially challenging as they responded less to advertisements. Ideally, collaborating with researchers who are well-acquainted with the culture of South Asians, are integrated into their community, and potentially of South Asian heritage themselves could spread the word about new research and their potential benefit for the South Asian community and help recruit new study participants. Building databases with the contact information of willing participants of South Asian descent could improve accessibility for recruitment, although privacy issues remain a current challenge.

Novel therapeutic targets in the treatment of obesity

Many intervention options currently available to combat obesity are based on lifestyle interventions (85). These interventions are often difficult to adhere to and almost always result in weight regain after a prolonged period (86). Additionally, most treatment options are not specifically tailored to the underlying cause of the individual who is living with obesity. Therefore, effective preventative or treatment options with long-term health benefits should be developed considering the person's underlying mechanism(s) contributing to obesity.

Activating brown adipose tissue

In **Chapter 1**, we described a potential beneficial effect of activating BAT in decreasing ectopic fat mass and slightly increasing resting energy expenditure. This could be especially beneficial for individuals with low BAT activity, such as South Asians (87).

Optimizing the timing of BAT activation could lead to higher efficacy in improving metabolic health. In the cohort described in **Chapter 5**, we observed more effective cold-induced thermogenesis in the morning in males only. Still, we found that cold only increased FGF21 levels in the evening (88). This indicates that different sexes or people from different ethnicities could benefit more from personalized timing of exposure to cold. For example, males might benefit more from cold exposure in the morning to increase energy expenditure. In contrast, given their lower energy expenditure and lower FGF21 levels, South Asians could benefit from exposure both in the morning (to

increase energy expenditure) and evening (to increase FGF21 levels). To assess if cold exposure in the morning and evening can indeed increase energy expenditure and FGF21 levels, studies should be conducted on individuals exposed to cold for extended periods at different times of the day.

Furthermore, BAT contributes to diet-induced thermogenesis (DIT), which is influenced by the diurnal rhythm of BAT (89). DIT, especially fat oxidation, increases after breakfast, moderately increases after lunch, and does not increase after dinner. DIT increases more after breakfast and lunch in people with high BAT activity than in individuals with low BAT (89). Combining cold exposure and higher relative food intake in the morning compared to other times of the day could increase thermogenesis and fat oxidation even more, especially in males. A study with cold exposure in unfasted conditions throughout the day while measuring energy expenditure could provide more information on the effectiveness of these combined interventions.

In addition to cold exposure, pharmacological methods to active BAT have also been studied. As described in **Chapter 6**, we found that a single intravenous dose of the ADRB2 agonist salbutamol activates BAT, as indicated by increased glucose uptake assessed via an [^{18}F]FDG PET/CT scan, which was accompanied by an increase in resting energy expenditure. To assess the benefits of pharmacological activation of BAT, long-term studies need to be conducted to determine if chronic ADRB2 agonism can continuously activate BAT and to evaluate the duration of the side effects, as we noticed an increase in heart rate and blood pressure after a single dose of salbutamol. Ideally, targeting the ADRB2 on brown adipocytes by nanotechnology could prevent such cardiovascular side effects. Different mechanisms using nanotechnology to improve the efficacy of salbutamol have been studied to treat asthma, such as the use of a nanocarrier attached to salbutamol to increase the interaction with the pleura of the longs or liposomes to increase the retention of salbutamol in the longs (90, 91). The use of these nanoparticles with incorporated salbutamol could possibly be constructed to target specifically the ADRB2 on brown adipocytes.

In addition to targeting BAT with salbutamol, incorporating sex differences could benefit the pharmacological activation of BAT. Our study was performed only in young and lean males; these results should also be assessed in females. Differences in the effectiveness of salbutamol between males and females have been demonstrated as salbutamol inhalation decreased fat mass and increased lean mass only in healthy female athletes using salbutamol to prevent exercise-induced bronchoconstriction, and not in males (92). In addition, protein turnover was greater in response to the ADRB2 agonist formoterol in females than in males, which was attributed to the difference in sex

steroids (92, 93). Therefore, females could potentially experience a greater activation of BAT by an ADRB2 agonist than males. Furthermore, our cohort was young and lean, however, obesity is associated with lower expression of the ADRB2 gene in peripheral blood samples (94). Therefore, people living with obesity could have a reduced benefit of salbutamol to activate BAT. Future research should focus on the effectiveness of salbutamol to activate BAT in males and females living with obesity.

We noticed that half of the 10 males included in our study responded better to salbutamol with respect to glucose uptake by BAT compared to the other half. In previous studies using larger cohorts of individuals receiving salbutamol for asthma similar effects were noticed, with less effective treatment in about half of the population (95). Those studies identified two polymorphisms that results in amino acid changes (rs1042713, c.G46A, p.Gly16Arg; rs1042714, c.G79C, p.Gln27Glu) of the ADRB2 receptor that could explain this observation (96). These changes cause less effective ADRB2 receptors than other variants and have a prevalence of about 28% for the Gly16Arg polymorphism and 27% for the Gln27Glu polymorphism in the Europid population (96-98). Ideally, we would measure both ADRB2 polymorphisms in the saliva of all participants in our previous cohort described in **Chapter 6** and study whether the participants with lower effectiveness were carriers of these polymorphisms. This could lead to a more effective application of activating BAT as we would beforehand identify those individuals who would benefit from cold exposure or an ADRB2 agonist if they are not carriers of the polymorphisms. Furthermore, activating BAT via cold or pharmacological intervention could complement other treatment options that work through different pathways for treating obesity and obesity-related diseases.

Incretin hormone-based drugs as a treatment for obesity

As described in **Chapter 1**, GLP-1R agonists are pharmacological treatment options for obesity and T2DM that significantly affect weight loss and improve the glycemic index. Combining GLP-1R agonists with long-acting analogs of other hormones such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon is even more beneficial, and new potential treatment options are emerging using these mechanisms (99, 100).

In 2023, the dual GLP-1R and GIP receptor (GIPR) agonist tirzepatide received Food and Drug Administration (FDA) approval for type 2 diabetes, and subsequently for obesity (101). Combining GLP-1R and GIPR agonism enhances appetite suppression compared to GLP-1R agonism alone (99). The mean change in body weight at 72 weeks of treatment in people living with obesity with tirzepatide was -21% (99). The most common side effect of GLP-1R agonists is nausea (102). GIP-based therapy seems

to reduce this side effect when used in combination with GLP-1R agonists. Although the mechanism behind the anti-emetic effects of GIPR agonists remains unclear, it is hypothesized that the location of GIPRs in the brain may play a role (103). A systematic review comparing the effectiveness of tirzepatide in Asians (consisting of Chinese, South Koreans, and Indians) and non-Asians with T2DM showed that tirzepatide was more effective in reducing body weight in Asians. However, they experienced more gastrointestinal side effects (104). In non-Asians, tirzepatide was more effective in lowering fasting blood glucose levels and HbA_{1c} (104). A clinical trial comparing the effect of tirzepatide in South Asians with Europids could unravel the differential effect of tirzepatide between these ethnicities. The finding that tirzepatide improved glycemic parameters more effectively in Europids than in Asians could be due to possible longer exposure to T2DM in Asians. This would be similar to what we observed in our South Asian cohort described in **Chapter 7,** indicating a more metabolically compromised state. Comparing the effectiveness of a GLP-1R agonist to tirzepatide, especially when administered earlier in the disease progression of T2DM, could determine the most effective treatment of the two for South Asians with T2DM. This comparison should also assess whether combining GLP-1R and GIPR agonism within Tirzepatide results in fewer side effects compared to GLP-1R agonism and if adding GIPR agonism can lower ectopic fat in South Asians.

The most recent development in incretin-based pharmacological intervention for obesity is the triple GLP-1/GIP/glucagon receptor agonist, retatrutide (100). Adding a glucagon receptor- activating modality further decreases satiety and increases energy expenditure (105). A clinical study in individuals living with obesity showed that 48 weeks of retatrutide treatment can decrease body weight by up to 24% from baseline in the high dose group of 12 mg subcutaneously once weekly (100). These results are almost comparable to bariatric surgery, where individuals typically lose up to 30 % of their body weight within one year (106). Combining GLP-1R agonism with glucagon receptor agonism could especially benefit people with lower energy expenditure, such as the South Asian population.

As we showed in **Chapter 7**, the GLP-1R agonist liraglutide does not increase circulating levels of the satiety hormone growth differentiation factor 15 (GDF15), suggesting GLP-1 and GDF15 may have independent effects on energy metabolism. Therefore, combining GDF15-based therapy with liraglutide or another GLP-1R agonist could have additive effects on reducing satiety. In mice and rodents, this combination has indeed shown promising results thus far in reducing food intake and body weight, fasting glucose, insulin, and triglycerides (107). Similarly, combining GLP-1R agonists with FGF21 has benefited rodents, especially in treating metabolic dysfunction-associated

steatohepatitis (MASH), a disorder characterized by steatosis, hepatocyte ballooning, inflammation, and fibrosis (108). Many of these combinations of incretin hormones could especially benefit the South Asian population. As we described in **Chapters 1-3**, young and healthy South Asian males have lower GLP-1 levels after an MMT, lower energy expenditure, and lower levels of FGF21. However, whether this population benefits more from these treatment options compared to Europids remains to be investigated.

Concluding remarks and future perspectives

Obesity is a complex chronic disease with many physical and mental consequences. Although treatment options are improving, effective options with long-term efficacy are still sparse. To further combat obesity, understanding its underlying mechanisms, identifying and comprehending at-risk individuals and populations, and finding novel approaches for its treatment are therefore of utmost importance. In this thesis, we have addressed further underlying mechanisms contributing to the increased risk of South Asians developing obesity and obesity-related diseases and have studied potential novel approaches to target obesity.

Based on the results of this thesis, we conclude that a pro-inflammatory phenotype is part of the underlying mechanism of the unfavorable metabolic phenotype of South Asians. We anticipate that identifying if B cell markers are increased in healthy South Asians and measuring the quantity and quality of monocytes could provide insight into the increased risk of developing obesity and related diseases in South Asians. Another part of the underlying mechanism is their hormone dysregulation, with a biphasic excursion of active GLP-1 and GIP in South Asian females to an MMTT, with a lower AUC GLP-1 levels in males, and higher leptin levels in both sexes. Furthermore, there are indications that South Asians have an altered stress response, resulting in lower cortisol levels. In addition to the differences in circulating hormone levels, the sensitivity to these hormones could have be altered in South Asians. CLI for the modulation of immune cell subsets or anti-inflammatory medications like salsalate and GLP-1R agonist treatment could be especially beneficial in reversing the pro-inflammatory phenotype and hormone disruption in South Asians. More research is needed to test the effectiveness of these mediations in this population with the challenges considered of matching South Asians with Europids during the selection of participants. We propose using alternative methods of matching, like fat mass or fat percentage, or a metabolic biomarker like leptin instead of using BMI.

To combat obesity, effective and sustainable (non)-pharmacological treatment options are needed. When using cold exposure to activate metabolically favorable BAT, its diurnal rhythm must be considered. Our results that FGF21 only increased after

cold exposure in the evening indicate that the moment of BAT activation could be adapted to the personable phenotype of the individual. If exposure to cold is not feasible, using the ADRB2 agonist salbutamol to activate BAT might be indicated. Cold exposure and pharmacological options for the activation of BAT must be explored by chronic treatment of individuals with different metabolic statuses and ethnicities. Especially for the pharmacological treatments, finding options to specifically target the ADRB2 receptor on brown adipocytes could improve dyslipidemia and reduce ectopic fat deposition without major (cardiovascular) side effects. Furthermore, activation of BAT could be combined with current treatments for obesity, like GLP-1R agonists. Other combinations with GLP-1R agonists, such as long-acting analogs of FGF21 and GDF15, could further benefit people living with obesity. The latter is supported by our observation that GDF15 acts via a different pathway than the GLP-1R agonist liraglutide. Recent developments in treatment options that combine GLP-1 with GIP and glucagon-based therapies could, therefore, lead to novel combined therapies. These therapies may provide options to prevent obesity-related diseases, especially in South Asians. Improving access to diagnostics and treatment for all people living with obesity could improve the success rate of sustainable weight loss and improve metabolic parameters in people with obesity. Furthermore, providing a personalized approach with specific interventions for the phenotype of an individual living with obesity could lead to the first steps toward ending the obesity epidemic.

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CHAPTER 9

APPENDICES

SUMMARY

Obesity is a chronic disease defined by excessive fat deposits caused by a positive balance between energy intake and expenditure. It is a worldwide problem, with the prevalence of obesity having tripled since the early 1980s and is expected to rise further. Obesity imposes a significant burden on individuals, including stigma, physical limitations, and the difficulty of achieving and maintaining weight loss. It also can lead to many related diseases such as cardiovascular disease and type 2 diabetes mellitus (T2DM). Furthermore, certain ethnicities are more susceptible to developing obesity and related diseases, notably South Asians having a fourfold higher risk of developing T2DM compared to Europids. This thesis aims to i) unravel additional underlying causes of the disadvantageous metabolic profile of South Asians, and ii) comprehensively understand how different (non-)pharmacological interventions can modulate circulating levels of various hormones and regulate overall energy metabolism in humans with different comorbidities.

Chapter 1 introduced the epidemiology, the underlying, and sustaining factors of obesity, as well as a detailed description of the physiology of adipose tissue and energy metabolism. It introduces potential underlying mechanisms for the increased risk of developing obesity and cardiometabolic diseases in the South Asian population. Finally, it discusses the targets for preventing and treating obesity and related diseases.

Chapter 2 explored differences in circulating levels of inflammation-related proteins in South Asians and Europids with T2DM using a panel of 73 inflammation-related proteins from Olink Proteomics. The relative plasma levels of six proteins were higher, and six were lower in South Asians compared to Europids. Relative plasma levels of fibroblast growth factor 21 (FGF21) were most notably different and lower in South Asians, especially in females. To validate these findings, we measured circulating FGF21 concentrations in the serum samples of the same cohort and found a lower concentration of circulating FGF21 in both males and females. Lower FGF21 levels and concentrations in South Asians may align with their pro-inflammatory phenotype, given FGF21's known anti-inflammatory properties. Future research is needed to determine if decreased FGF21 levels and concentrations are a cause or consequence of the increased T2DM risk in South Asians.

In addition to inflammation, another factor contributing to the unfavorable metabolic profile of South Asians could be differences in hormone regulation. Therefore, in **Chapter 3,** we compared the effect of a single mixed meal tolerance test (MMTT) on the excursion of incretin hormones as well as glucagon in young and lean Dutch South

Asian and Dutch Europid males and females in relation to the excursions of glucose and insulin. After an overnight fast, we measured incretin hormones (i.e., total and active glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide-1 (GIP)), glucagon, glucose, insulin and parameters involved in lipid metabolism at baseline and six time points up to 240 minutes post-meal. While Europids exhibited a single glucose peak, South Asians showed an second glucose peak. Potentially as a consequence, especially South Asian females had a higher second peak of active GLP-1 and GIP compared to Europid females. Moreover, this was accompanied by a biphasic insulin response with a higher area under the curve (AUC) of insulin in males. These effects may result from biphasic gastric emptying in South Asians, though the consequences for the disadvantageous metabolic phenotype of South Asians remains to be determined. We further explored differences in hormone regulation between South Asians and Europids in **Chapter 4**, by analyzing the excursion of the hunger and satiety hormones peptide YY (PYY), ghrelin, and leptin in response to an MMTT in the same participants as in **Chapter 3**. While PYY levels did not differ before and during the MMTT between males and females of both ethnicities, South Asian males exhibited lower ghrelin levels before and during the MMTT, and both South Asian males and females had higher levels of leptin at before and during the MMTT compared to Europids. Baseline leptin levels correlated positively with fat mass and fat percentage in South Asians, suggesting leptin could potentially serve as a biomarker for body fat in South Asians.

Next, we aimed to understand how (non-)pharmacological interventions can modulate the concentration of different hormones and regulate overall metabolism in humans with different comorbidities. To do so, in **Chapter 5** we investigated the effect of cold exposure on FGF21 and growth differentiation factor 15 (GDF15) in healthy lean individuals and examined whether cold-induced changes in FGF21 and GDF15 levels before, during, and after 90 minutes of stable cold exposure differ between morning and evening. The potential metabolically beneficial effects of cold exposure are likely mediated through the activation of brown adipose tissue (BAT), partly through the secretion of hormones such as FGF21 and GDF15. We found that cold exposure increased FGF21 levels only in the evening, without affecting GDF15. The changes in FGF21 were not correlated with the change in cold-induced energy expenditure, indicating that the timing of cold exposure influences FGF21 levels independently of energy expenditure changes. These findings could be significant for improving metabolic health through cold exposure.

Cold exposure may not suit everyone and therefore pharmaceutical activation of BAT may be of interest. In rodents, BAT is activated via the adrenergic beta-3 receptor (ADRB3); however, in humans, ADRB2 is responsible for the noradrenergic activation

of human BAT, at least *in vitro.* Therefore, in **Chapter 6**, we aimed to investigate whether the ADRB2 agonist salbutamol intravenously activates human BAT *in vivo.* In a randomized double-blinded crossover trial involving healthy young males, we compared glucose uptake in BAT after intravenous salbutamol administration with or without propranolol, an ADRB1 and ADRB2 blocker. The activation of BAT was measured by the increase in glucose uptake in BAT using a dynamic [^{18}F]fluoro-D-deoxy glucose positron emission tomography/computed tomography ([^{18}F]FDG PET/CT scan). We found that intravenous salbutamol increased glucose uptake in BAT, an effect that was diminished when combined with propranolol. The increase in glucose uptake was positively associated with increased energy expenditure. Our findings demonstrate that ADRB2 activates human BAT; however, the long-term effects of pharmacological stimulation of BAT require further investigation.

We next investigated both the intervention options and potential metabolic differences in the South Asian population with Europids in **Chapter 7**. Here, we investigated whether GDF15 may be involved in satiety induction by GLP-1 receptor agonism in patients with T2DM, and whether GDF15 levels differ between South Asians versus Europids. In a randomized control trial, we measured GDF15 levels at baseline and after 26 weeks of daily treatment with the GLP-1 agonist liraglutide compared to placebo in South Asians and Europids with T2DM. At baseline, we found no significant difference in GDF15 levels between South Asians and Europids. Additionally, liraglutide did not modify GDF15 levels in either ethnicity. Our findings suggest that liraglutide induces satiety independent of the GFRAL/GDF15 pathway, suggesting that other mechanisms likely explain the weight loss induced by liraglutide.

Finally, in **Chapter 8**, we discussed all findings described in this thesis in the context of the latest scientific literature. We explored the potential implications of our findings for clinical practice and addressed remaining future challenges. Moreover, we elaborated on the role of inflammation and hormone dysregulation as an underlying role in the metabolic profile of South Asians, with a particular focus on GLP-1, the stress system, and sex hormones. We proposed new criteria to combat the challenges during the matching of cohorts of South Asians. Furthermore, we discussed the implications of our findings for the optimization of interventions by incorporating the timing for BAT activation, exploring the pharmacological activation of BAT, and considering the possible combinations of other hunger- and satiety-related factors like FGF21 and GDF15 with the current GLP-1 agonists. These findings could potentially lead to new therapeutic options for combating obesity and obesity-related diseases. Future research is essential to translate these discoveries into practical clinical applications.

NEDERLANDSE SAMENVATTING

Obesitas is een chronische ziekte die gekenmerkt wordt door een teveel aan lichaamsvet. Obesitas ontstaat door een positieve energiebalans, oftewel een disbalans tussen de energie-inname en -verbruik. Obesitas is een wereldwijd probleem en de prevalentie ervan is sinds het begin van de jaren 80 verdrievoudigd. De verwachting is dat dit alleen maar zal blijven stijgen. Obesitas vormt een aanzienlijke belasting voor individuen, niet alleen door fysieke beperkingen maar bijvoorbeeld ook het stigma waar mensen met obesitas vaak dagelijks mee te maken hebben. Daarnaast kan obesitas leiden tot een scala aan gerelateerde ziekten zoals hart- en vaatziekten, type 2 diabetes mellitus (T2DM) en 13 soorten kanker. Sommige etniciteiten zijn gevoeliger voor het ontwikkelen van obesitas en gerelateerde ziekten, waaronder Zuid-Aziaten, die een viervoudig hoger risico hebben om T2DM te ontwikkelen ten opzichte van Europeanen bij dezelfde ***body mass index*** (BMI). Het doel van dit proefschrift is om i) de onderliggende oorzaken van het ongunstige metabole profiel van Zuid-Aziaten te ontrafelen en ii) meer inzicht te krijgen in de werking van de stofwisseling en verschillende hormoonsystemen in obesitas-gerelateerde ziektes en de effecten van (non-)farmacologische interventies hierop.

In **Hoofdstuk 1** werden de epidemiologie en de onderliggende en onderhoudende factoren van obesitas besproken. Verder werd uitgebreid ingegaan op de werking van vetweefsel en de vetstofwisseling. Daarnaast werd een overzicht gegeven van mogelijke onderliggende mechanismen voor het verhoogde risico op obesitas en cardiometabole ziekten in de Zuid-Aziatische populatie. Ten slotte werden de huidige aangrijpingspunten voor het voorkómen en behandelen van obesitas en gerelateerde ziekten besproken.

In **Hoofdstuk 2** werden de verschillen in circulerende niveaus van ontstekingseiwitten bij Zuid-Aziaten en Europeanen met T2DM onderzocht in plasma. Hiervoor werd gebruik gemaakt van een panel met daarin 73 ontstekingseiwitten van Olink Proteomics. We vonden dat de relatieve concentraties van zes eiwitten hoger waren en zes lager bij Zuid-Aziaten ten opzichte van Europeanen. Een opvallend verschil was die van *fibroblast growth factor 21* (FGF21), waarvan de niveaus duidelijk lager waren bij Zuid-Aziaten, voornamelijk bij vrouwen, ten opzichte van Europeanen. Om deze bevindingen te valideren, hebben we circulerende FGF21-concentraties gemeten in het serum van hetzelfde cohort en vonden een lagere concentratie van circulerend FGF21 bij zowel Zuid-Aziatische mannen als vrouwen vergeleken met Europeanen. Omdat FGF21 ontstekingsremmende effecten kan hebben zouden deze juist lagere FGF21 concentraties bij Zuid-Aziaten kunnen passen bij het feit dat zij vaker hogere ontstekingswaarden hebben. Toekomstige studies zijn nodig om te bepalen of de

verlaagde FGF21- concentraties de oorzaak of het gevolg zijn van het verhoogde risico op T2DM bij Zuid-Aziaten.

Naast meer ontsteking dragen ook verschillen in hormonen bij aan het ongunstige metabole profiel van Zuid-Aziaten. Daarom werden in **Hoofdstuk 3** circulerende hormonen en na een gemengde maaltijd tolerantie test ('*mixed meal tolerance test*', MMTT) vergeleken tussen jonge en slanke Zuid-Aziatische en Europese mannen en vrouwen. Na een nacht vasten vergeleken we incretinehormonen (*glucagon-like peptide-1* (GLP-1), *glucose-dependent insulinotropic polypeptide* (GIP)), glucagon, glucose, insuline en lipiden voor en na een vloeibare maaltijd op zeven tijdspunten. We zagen een opvallend verschil in het verloop van het bloedsuiker in de Zuid-Aziaten vergeleken met de Europeanen, waarbij bloedsuiker in de Zuid-Aziaten twee pieken lieten zien en bij de Europeanen maar één. Verder zagen we dat de Zuid-Aziatische vrouwen hogere niveaus van actief GLP-1 en GIP hadden dan de Europese vrouwen tijdens het tweede gedeelte van de MMTT. Dit ging ook samen met een dubbele insulinepiek, wat zorgde voor een hogere oppervlakte onder de curve van insuline in Zuid-Aziatische mannen. Waarschijnlijk zijn deze verschillen het gevolg van een bifasische maaglediging bij Zuid-Aziaten, al is het nog onduidelijk hoe dit precies bijdraagt aan hun ongunstige metabole fenotype. In **Hoofdstuk 4** onderzochten we andere hormoonverschillen tussen Zuid-Aziaten en Europeanen door veranderingen in de honger- en verzadigingshormonen peptide YY (PYY), ghreline en leptine te meten gedurende een MMTT bij dezelfde deelnemers als in **Hoofdstuk 3**. PYY-niveaus verschilden niet tussen de etniciteiten maar de Zuid-Aziatische mannen hadden een lager ghrelineniveau voor en gedurende de MMTT. Daarnaast hadden zowel mannen als vrouwen hogere leptinewaarden voor en gedurende de MMTT. De nuchtere leptinewaarden correleerden positief met vetmassa en vetpercentage in de Zuid-Aziatische deelnemers. Dit suggereert dat leptine mogelijk een indicatie kan geven over de metabole gezondheid in Zuid-Aziaten.

Vervolgens probeerden we te begrijpen hoe (non-)farmacologische interventies de concentraties van verschillende hormonen en de vetstofwisseling kunnen beïnvloeden bij mensen met verschillende comorbiditeiten. Daarom onderzochten we in **Hoofdstuk 5** eerst mogelijk gunstige effecten van blootstelling aan kou. De mogelijke gunstige effecten van koudeblootstelling op de vetstofwisseling worden waarschijnlijk veroorzaakt door de activatie van bruin vetweefsel ('*brown adipose tissue*', BAT), gedeeltelijk door de uitscheiding van hormonen zoals FGF21 en *growth differentiation factor 15* (GDF15). Daarom onderzochten we het effect van koudeblootstelling op FGF21 en GDF15 bij gezonde, slanke personen. Ook onderzochten we of de veranderingen van FGF21 en GDF15 na koudeblootstelling voor, tijdens en na 90 minuten van stabiele kou verschilden tussen de ochtend en de avond. We vonden dat koudeblootstelling de

FGF21-niveaus alleen in de avond deed stijgen, zonder de GDF15-niveaus te beïnvloeden. De veranderingen in FGF21 hingen niet samen met de veranderingen in energieverbruik na koudeblootstelling, wat aangeeft dat het tijdstip van koudeblootstelling de FGF21-niveaus onafhankelijk beïnvloedt van de veranderingen in vetstofwisseling. Deze bevindingen kunnen relevant zijn voor het bevorderen van metabole gezondheid door koudeblootstelling.

Koudeblootstelling is mogelijk niet voor iedereen geschikt en daarom kan activatie van BAT door middel van medicatie die deze route nabootst interessant zijn. Bij muizen wordt BAT geactiveerd via de adrenerge beta-3 receptor (ADRB3), maar bij mensen was dit veel minder duidelijk. Vanuit experimenten in een kweekschaaltje wisten we dat mogelijk de ADRB2 verantwoordelijk was voor activatie van BAT in mensen. Daarom onderzochten we in **Hoofdstuk 6** of toediening van de ADRB2-agonist salbutamol via de bloedvaten humaan BAT activeert *in vivo*. In een gerandomiseerd dubbelblind crossover onderzoek met gezonde jonge mannen vergeleken we de opname van glucose in BAT na intraveneuze toediening van salbutamol met en zonder propranolol, een medicijn dat zowel de ADRB1 als de ADRB2 blokkeert. We maten de activatie van BAT door de toename van glucose-opname in BAT met behulp van een positron emission tomography/computed tomography (PET/CT scan) met een radioactief gemerkt suiker (FDG). We vonden dat intraveneuze toediening van salbutamol de glucose-opname in BAT verhoogde en dat dit effect werd verminderd door combinatie met propranolol. De toename van glucose-opname hing samen met de toename in stofwisseling. Onze bevindingen laten zien dat ADRB2 humaan BAT activeert, maar de lange termijn effecten van deze mogelijke therapie vereisen meer onderzoek.

Vervolgens combineerden we de Zuid-Aziatische populatie en interventieopties in **Hoofdstuk 7**. Hier onderzochten we het onderliggende mechanisme waarmee de GLP-1 receptoragonist liraglutide verzadiging tot stand brengt in patiënten met T2DM. We richtten ons specifiek op of dit ging via verhoging van het verzadigingshormoon GDF15 -niveaus en onderzochten ook of niveaus van dit hormoon verschillen tussen Zuid-Aziaten en Europeanen. In een gerandomiseerd gecontroleerd onderzoek hebben we de GDF15-niveaus gemeten voor en na 26 weken behandeling met dagelijkse liraglutide in vergelijking met placebo bij Zuid-Aziaten en Europeanen met T2DM. Op baseline vonden we geen verschillen in GDF15-niveaus tussen beide etniciteiten. Onze bevindingen laten zien dat de GLP-1 receptoragonist liraglutide onafhankelijk werkt van GDF15, wat suggereert dat andere mechanismen het gewichtsverlies verklaren geïnduceerd door liraglutide.

Ten slotte bespraken we in **Hoofdstuk 8** de bevindingen die we in deze thesis beschrijven in relatie tot de laatste wetenschappelijke literatuur. We onderzochten de mogelijke implicaties van onze bevindingen voor de kliniek en benoemden de overgebleven toekomstige uitdagingen. Daarnaast gingen we dieper in op de rol van ontsteking en disbalans van hormonen als onderliggende factor in het metabole profiel van Zuid-Aziaten, met specifieke focus op GLP-1, het stresssysteem en geslachtshormonen. We stelden nieuwe criteria voor om het matchen van cohorten met Zuid-Aziaten te verbeteren. Verder bespraken we de implicaties van onze bevindingen voor het verbeteren van interventies door rekening te houden met het tijdstip van de dag voor de activatie van BAT en overwegen de mogelijke combinaties van andere honger- en verzadigingshormonen zoals FGF21 en GDF15 met de huidige GLP-1 receptor agonisten. Deze bevindingen kunnen mogelijk leiden tot nieuwe therapeutische opties voor het behandelen van obesitas en obesitas gerelateerde ziekten. Toekomstig onderzoek is essentieel om deze bevindingen te vertalen naar toepassingen voor praktische klinische zorg.

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*Shared first authorship

CURRICULUM VITAE

Carlijn Anne-Pauline Hoekx werd op 29 juli 1993 geboren in Gouda. In 2011 behaalde zij haar gymnasiumdiploma aan het categoraal gymnasium, het Coornhert Gymnasium in Gouda. Hierna begon zij met de studie geneeskunde aan de Universiteit Leiden. Gedurende haar studie werd haar interesse in de endocrinologie al gewekt, wat haar ertoe bracht een semi-coschap te volgen op de afdeling Kindergeneeskunde in het Juliana Kinderziekenhuis en een verlengd coschap Endocrinologie binnen de Interne Geneeskunde in het Leids Universitair Medisch Centrum (LUMC). Tijdens haar wetenschapsstage combineerde zij haar interesses en voerde zij haar eerste onderzoek uit onder begeleiding van dr. Sabine Hannema, kinderarts-endocrinoloog, in het LUMC, naar het effect van groeihormoon bij kinderen die een hematopoietische stamceltransplantatie hadden ondergaan, wat resulteerde in haar eerste publicatie.

Na het behalen van haar artsexamen in februari 2019 werkte Carlijn twee jaar als arts-assistent niet in opleiding (ANIOS) op de afdeling Kindergeneeskunde in het Isala Ziekenhuis in Zwolle en in het Prinses Máxima Centrum voor Kinderoncologie in Utrecht. In dit laatste ziekenhuis, waar klinische zorg en de nieuwste wetenschappelijke inzichten werden geïntegreerd, werd haar enthousiasme voor onderzoek verder aangewakkerd. Carlijn kon de endocrinologie niet loslaten en startte op 1 juni 2021 haar promotietraject onder begeleiding van dr. Mariëtte Boon, dr. Borja Martinez-Tellez en prof. dr. Patrick Rensen in het LUMC. Dit promotietraject werd gefinancierd door de VENI-subsidie van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), toegekend aan dr. Mariëtte Boon. Het laatste jaar van haar promotietraject bracht zij door in Sydney, Australië.

Gedurende haar promotietraject presenteerde Carlijn haar onderzoeksresultaten op diverse (inter)nationale congressen en werd zij geselecteerd om deel te nemen aan de EASO Early Career Network December Winter School in Sevilla. Daarnaast won zij de Prof. dr. J. Terpstra Young Investigator Award van de Nederlandse Vereniging voor Diabetes Onderzoek (NVDO) voor haar onderzoek naar honger- en verzadigingshormonen bij Zuid-Aziaten tijdens de Annual Dutch Diabetes Research Meeting (ADDRM). Haar publicatie “Stimulation of the beta-2-adrenergic receptor with salbutamol activates human brown adipose tissue”, die zij samen met dr. Maaike Straat schreef, werd bekroond met twee prijzen: Best Clinical Paper of the Year 2023 van de Nederlandse Vereniging voor Endocrinologie (NVE) en de Publicatieprijs van de Nederlandse Associatie voor de Studie van Obesitas (NASO). Verder zette Carlijn zich tijdens haar promotie in voor de belangen en verbondenheid van promovendi binnen

het LUMC als bestuurslid en hoofd evenementen van de LUMC Association for PhD Candidates (LAP).

In haar vrije tijd reist Carlijn de wereld over, renoveert zij samen met haar vriend hun appartement in Amsterdam en geniet zij volop van een variatie aan sporten. Recentelijk is Carlijn teruggekeerd naar Nederland na ongeveer een jaar in Sydney, Australië, te hebben gewoond en werkt nu bij The Body Clinic in Amsterdam als arts medisch afvallen en vanaf 1 juli start ze als ANIOS op de afdeling klinische genetica in het LUMC.

DANKWOORD

Met het afronden van mijn promotieonderzoek komt een turbulente, intensieve en ontzettend interessante reis ten einde. Een reis die ik nooit op deze manier had kunnen maken zonder de hulp en steun van de mensen om mij heen. Daarom sluit ik graag af met het bedanken van iedereen die mij de afgelopen jaren heeft bijgestaan.

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Beste Patrick, dankzij jouw scherpe oog voor detail is er een nieuwe wereld voor mij opengegaan. Het is inspirerend om van je te leren, op het gebied van wetenschap, presentaties en de organisatie rondom onderzoek. Beste Mariëtte, jouw enorme enthousiasme voor de wetenschap en altijd aanwezige positiviteit hebben mijn promotietraject gemaakt. Dank je wel dat je altijd voor mij klaarstond, van vroeg in de ochtend tot laat op de avond. Het is inspirerend om te zien hoe je een dappere keuze in je carrièrepad hebt gemaakt, die zo goed heeft uitgepakt. Dear Borja, you helped me incredibly throughout my PhD. You encouraged me and discussed statistics with me, and it is incredible to see what you have built and are building in the world of science. Thank you for your incredible support and advice.

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