GLP-1 receptor agonism to improve cardiometabolic health

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Chapter 1

General introduction and outline of this thesis

GENERAL INTRODUCTION

1. Obesity and type 2 diabetes

Over the past decades, the worldwide prevalence of overweight and obesity has increased dramatically. The World Health Organization defines overweight as a body mass index (BMI), which is the person's weight in kilograms divided by the square of his/her height in meters, greater than or equal to 25 kg/m^2 and obesity as a BMI greater than or equal to 30 kg/m^2. In 2016 it was estimated that since 1975 obesity prevalence had nearly tripled and in 2016 more than 1.9 billion adults were overweight of whom 650 million were obese[1]. The cause of this rise in overweight and obesity is a globally increasing imbalance between energy intake and energy expenditure. This problem is affecting more and more people as a consequence of excessive intake of high-caloric foods and low physical activity due to an increasingly sedentary lifestyle. By disturbing metabolic processes and inducing low grade systemic inflammation, overweight and obesity increase the risk to develop type 2 diabetes, which in turn can lead to cardiovascular diseases. In 2021, it was estimated that 537 million adults aged 20-79 years had diabetes, with type 2 diabetes being the most common type accounting for over 90% of all diabetes. This corresponds to a global prevalence of 10.5% in this age group. However, even though the numbers are already very high, the burden of diabetes is expected to further increase and global prevalence is expected to rise to 783 million people in 2045[2]. In the Netherlands, in 2019, 1.2 million people were living with diabetes, of whom 90.4% with type 2 diabetes[3].

2. Pathogenesis of type 2 diabetes

Type 2 diabetes is a chronic disease involving environmental and genetic factors that is characterized by a disturbed glucose regulation. Under physiological conditions insulin is secreted postprandially by β-cells of the pancreas, via several mechanisms in which rising blood glucose levels and also secretion of gut hormones including glucagon-like peptide-1 (GLP-1) play a role. This causes uptake of glucose in metabolically active tissues, suppression of endogenous glucose production by the liver and inhibition of intracellular lipolysis in adipocytes, thus resulting in storage of glucose and restoring blood glucose levels to the pre-prandial concentration. However, in type 2 diabetes the metabolic tissues do not respond to insulin as they should as a consequence of insulin resistance. Insulin resistance of metabolic organs is already present long before hyperglycemia occurs. However, in the early stage of insulin resistance, insulin secretion by the pancreas is increased to compensate for the increased insulin resistance in order to maintain glucose tolerance. Ultimately, as insulin resistance increases and β-cell function declines, compensatory insulin secretion fails to overcome insulin resistance, resulting in the development of type 2 diabetes with hyperglycemia[4,5].

In addition, insulin resistance plays an important role in the pathogenesis of combined dyslipidemia, which is characterized by high low-density lipoprotein (LDL) cholesterol and plasma triglyceride levels and low high-density lipoprotein (HDL) cholesterol levels via several mechanisms. On the one hand, insulin resistance of white adipose tissue and skeletal muscle results in attenuated lipoprotein lipase (LPL)-mediated catabolism of triglyceride-rich lipoproteins (TRLs), i.e. intestine-derived chylomicrons and liver-derived very-low-density lipoproteins (VLDL). On the other hand, the decreased insulin-mediated inhibition of intracellular lipolysis in white adipocytes subsequently results in increased release of free fatty acids (FFAs) from white adipose tissue that are transported to the liver where FFAs serve as substrate for hepatic production and secretion of triglyceride and cholesterol-containing VLDL. The hepatic insulin resistance further increases VLDL production, which under physiological conditions is suppressed by insulin[6]. The overproduction of TRLs in combination with reduced clearance of TRLs together cause hypertriglyceridemia and high LDL-cholesterol. Attenuated catabolism of TRLs is accompanied by reduced availability of phospholipid-containing surface remnants that are transferred by phospholipid transfer protein (PLTP) to feed the HDL pool. In addition, a high VLDL-TG concentration drives the neutral lipid transfer activity of cholesteryl ester transfer protein (CETP), that is involved in the transfer of triglycerides from VLDL to HDL, coupled to transfer of cholesteryl esters from HDL towards VLDL. This process results in formation of pro-atherogenic VLDL particles and triglyceride-enriched HDL particles that are subsequently hydrolysed and cleared by hepatic triglyceride lipase resulting in decreased HDL-cholesterol levels[7-11].

3. Visceral adipose tissue, ectopic fat and insulin resistance

Insulin resistance is thus essential in the pathophysiology of type 2 diabetes and the mechanisms resulting in insulin resistance have been studied extensively. Although numerous factors contribute to the development of insulin resistance, overweight and (mainly central) obesity are major risk factors[12-15]. Humans have white adipose tissue compartments in different areas of the body, of which the subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) compartments are most prominent. VAT has been causally linked to insulin resistance and has been shown to be a major contributor to metabolic risk[16-18]. In contrast, SAT does not seem to exert this effect and in line with that, it has been shown that obesity does not increase the risk for development of metabolic disorders in 10-30% of obese individuals[19,20]. This can be explained by the fact that the adipose tissue compartments function as the main lipid storage in our body. In healthy adipose tissue, in a situation of nutrient excess, adipogenesis is increased. However, ongoing exceeding of the buffer capacity of adipose tissue results in adipocyte dysfunction with hypertrophic as well as dying adipocytes and influx of immune cells[21]. Ultimately, this leads to liberation of FFAs from adipocytes and storage of FFAs as triglycerides

around and in other tissues. Storage of triglycerides within non-adipose tissues is called ectopic fat storage and causes disturbance of cellular function and insulin resistance.

VAT and increased ectopic fat play an important role in the development of local and whole-body insulin resistance and type 2 diabetes[21-24]. Ectopic fat accumulation occurs in many different organs that usually contain only a small amount of lipids, such as skeletal muscle, pancreas, heart and liver, resulting in disturbance of the function of these organs[12,13,25]. The detrimental effects of VAT are explained by its high lipolytic activity whereby the released FFAs and pro-inflammatory cytokines are directly delivered to the portal vein, contributing to the development of hepatic steatosis and hepatic insulin resistance[26-28]. In skeletal muscle cells, lipid accumulation results in impaired post-receptor signalling leading to attenuated GLUT-4 trafficking to the cell membrane and diminished glucose uptake into the cell[29]. Since skeletal muscle accounts for around 70% of insulin-mediated glucose uptake, insulin resistance of skeletal tissue is a major factor in the development of type 2 diabetes[30]. Lipid accumulation in the pancreas results in pancreatic steatosis, also called nonalcoholic fatty pancreas disease. It is a frequent clinical condition that might be associated with β-cell dysfunction[31], although other studies report no significant relationship[32,33]. In the heart, lipids can accumulate within cardiomyocytes (i.e. myocardial steatosis) or around the heart within adipocytes of para- and epicardial adipose tissue. Myocardial steatosis is an independent predictor of diastolic dysfunction in patients with type 2 diabetes[34]. Epicardial adipose tissue has been linked to the development and severity of coronary artery disease[35]. Furthermore, the thickness of the epicardial adipose tissue is correlated with whole body insulin resistance[36]. The sequence of events by which nutrient excess leads to cardiometabolic diseases is schematically depicted in **Figure 1**. In the next section, hepatic steatosis will be discussed more extensively.

3.1 Hepatic steatosis

Hepatic steatosis is a condition of ectopic fat accumulation that is characterized by accumulation of lipids within hepatocytes. In the absence of alcohol abuse, the disorder is referred to as nonalcoholic fatty liver disease (NAFLD), a disease strongly linked to obesity[37,38]. Hepatic steatosis is very disruptive for the function of the liver and, it has recently been shown that hepatic steatosis in fact forms a greater risk of incident type 2 diabetes than obesity and waist circumference as a measure of visceral obesity[39]. As described in section 2, as a consequence of hepatic steatosis, dyslipidemia is induced by VLDL overproduction. Furthermore, intracellular lipid accumulation results in reduced stimulation by insulin of glycogen synthase activity and decreased phosphorylation of the forkhead box O (FOXO) transcription factor, resulting in increased transcription of rate-controlling enzymes of gluconeogenesis[29,40].

Another possible mechanism via which hepatic steatosis can negatively affect metabolic health is via regulation of CETP production. This protein is mainly produced by Kupffer cells and can contribute to a pro-atherogenic lipoprotein profile, as described in section 2[41-43]. It is still largely unknown how CETP production by Kupffer cells is regulated. Interestingly, it has previously been shown that a reduction of hepatic triglyceride content related to pioglitazone treatment[44] or caloric restriction[45] is accompanied by a decrease in circulating CETP, which suggests a role for the hepatic triglyceride content in the regulation of CETP production. However, whether hepatic triglyceride content is indeed related to circulating CETP and whether these factors can also be affected by treatment with GLP-1 receptor agonists, a relatively new class of drugs to treat patients with type 2 diabetes, needs further investigation.

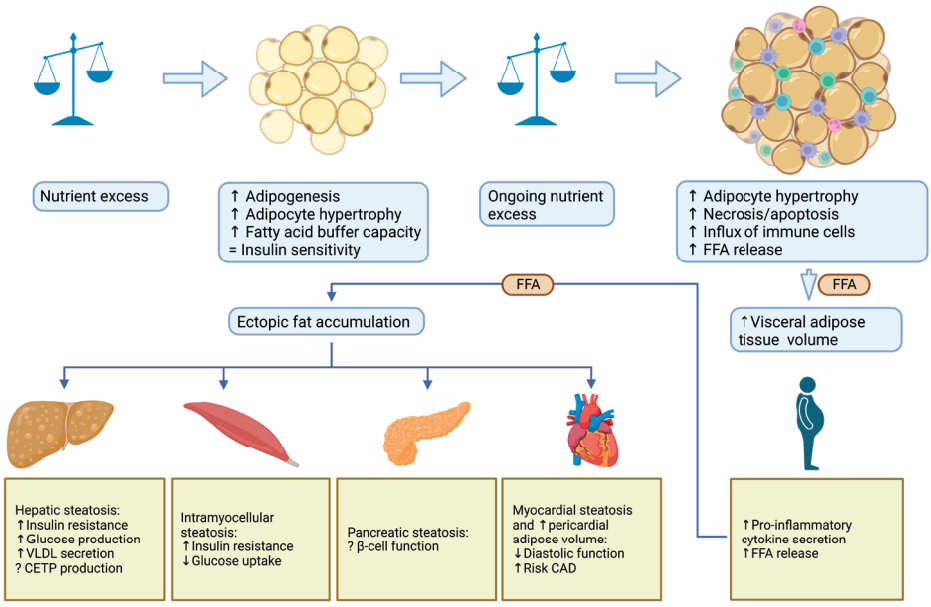

Figure 1. Schematic model of the relation between nutrient excess and cardiometabolic diseases. See section 3 for explanation. CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; FFA, free fatty acid; VLDL, very-low-density lipoprotein. Created with Biorender.com.

4. The disadvantageous phenotype of the South Asian population

Obesity and type 2 diabetes prevalence differ between ethnicities. Of special interest is the population of South Asians, in whom the risk to develop type 2 diabetes as well as cardiovascular diseases is exceptionally high. South Asians originate from the Indian subcontinent, a region that is represented predominantly by India, and is home to approximately one fifth of the world's population. In 2021, in the South-East Asia region

90 million adults aged 20-79 years had diabetes, with an annual mortality of 747,000 diabetes-related deaths[2]. Most South Asians living in the Netherlands originate from Surinam, a former Dutch colony, where many people from India migrated to between the end of the 19th century and the beginning of the 20th century. In 2011 it was estimated that almost 151,000 South Asians were living in the Netherlands, with most of them living in the Hague[46]. Strikingly, a study comparing South Asian Surinamese subjects with ethnic Dutch subjects, showed a prevalence of type 2 diabetes of 16.7% versus 4.2%, respectively, in subjects aged 35-44 years, and of 35.0% versus 8.2%, respectively, in subjects of 45-60 years old[47]. Furthermore, South Asians develop type 2 diabetes at a younger age and at a lower BMI than Western Europeans[48,49].

The pathophysiological mechanism resulting in the high prevalence of type 2 diabetes in the South Asian population, e.g. as compared to Western Europeans, is not completely understood, but several potential pathophysiological mechanisms have been proposed. An important factor is the body composition of South Asians compared to Western Europeans, with a higher percentage of body fat at comparable BMI[50,51]. Furthermore, adipose tissue is distributed differently, with fat primarily stored in VAT, as well as more other ectopic fat deposition[52,53]. In line, it has been shown that young, lean and healthy South Asian subjects have an increased prevalence of insulin resistance, which is associated with an increased hepatic triglyceride content[54]. In addition, South Asians have an increased size of adipocytes within SAT at smaller SAT volume, suggesting a lower storage capacity (i.e. adipose tissue dysfunction) and possible predisposition to overflow of fat to ectopic depots[52,55]. This probably also contributes to the dyslipidemic, pro-atherogenic lipoprotein profile often present in South Asians, with high levels of LDL-cholesterol and plasma triglycerides and low HDL-cholesterol levels[56,57]. Besides these mechanisms, other factors possibly also contribute to the increased metabolic risk of South Asians. Several studies have reported an unbalanced diet with high saturated fat and carbohydrate intake and low intake of fiber, which could predispose to the development of type 2 diabetes[58,59]. Furthermore, a relatively low resting energy expenditure and brown adipose tissue (BAT) volume, which burns triglycerides and glucose, in the South Asian populations might play a role[60]. Altogether, many pathogenic factors contribute to the disadvantageous metabolic phenotype of South Asians and further studies aimed to decrease the risk to develop disease are warranted.

5. The role of the endocannabinoid system in metabolism

The endocannabinoid system (ECS) is an extensive neuromodulatory system which is composed of endocannabinoids and their receptors cannabinoid receptor type 1 (CB_1) and type 2 (CB_2). Although its functions are still not fully elucidated, the ECS plays an important role in the regulation of appetite, energy balance and metabolism. Accumu-

lating evidence shows that, once obesity has developed, the endocannabinoid system can contribute to further increased obesity and insulin resistance[61]. Binding of endocannabinoids to their receptors results in various effects, including reduced energy expenditure and increased accumulation of VAT. Since activation of the endocannabinoid system can aggravate obesity, reduction of the tone of the endocannabinoid system could be an effective approach to reduce the obesity epidemic. Treatment of obese subjects with rimonabant, an inverse agonist for the CB_1 receptor, induced marked weight loss and improved lipid metabolism, but unfortunately was associated with psychiatric side effects resulting in discontinuation of its clinical use[62,63]. Others have investigated the effect of a small reduction of body weight on circulating endocannabinoid levels, showing variable results: while some studies report a decrease in endocannabinoid levels[64], other studies showed no effect[65,66]. The effects of prolonged caloric restriction and subsequent pronounced weight loss on endocannabinoid levels, however, are still unknown.

6. Treatment of type 2 diabetes

In order to minimize the micro- and macrovascular complications of type 2 diabetes, it is very important to pursue optimal regulation of glucose levels, but also of other cardiovascular risk factors such as cholesterol levels and blood pressure. Since insulin resistance plays a fundamental role in the pathogenesis of type 2 diabetes, and in most patients is caused by obesity, weight reduction is the cornerstone of treatment[67,68]. Indeed, prolonged caloric restriction can substantially improve insulin sensitivity accompanied by reduced hepatic triglyceride content and intramyocellular lipid accumulation[68,69]. Furthermore, insulin sensitivity can be improved by increased physical exercise[70]. However, in addition, pharmacological treatment is often necessary.

In pharmacological management of type 2 diabetes various glucose-lowering agents are currently being used. Usually, the first drug of choice is metformin, which has an excellent safety profile and acts on multiple tissues though various mechanisms, which are still incompletely understood. Important is the suppression of the mitochondrial respiratory chain complex 1 in the liver, resulting in a moderate increase of adenosine monophosphate (AMP) which by activation of AMP-activated protein kinase (AMPK) results in inhibition of gluconeogenesis. This results in reduction of enhanced basal hepatic glucose production[71]. In addition, the UK Prospective Diabetes Study (UKPDS) has shown, albeit in a relatively small number of patients, that the risk of cardiovascular events is decreased by intensive glucose-lowering management with metformin[72]. When glucose control is insufficient with diet and metformin, most commonly, a sulfonylurea is added, which augments insulin secretion by β-cells. Unfortunately, sulfonylureas often cause hypoglycemia and are associated with weight gain[4]. A relatively new therapeutic

modality is the group of sodium-glucose cotransporter 2 (SGLT2) inhibitors, which block reabsorption of glucose in the kidney, resulting in renal glucose disposal via glucosuria. This results in reduction of blood glucose levels and, by urinary loss of calories, induces moderate weight loss[73]. However, further studies are needed to investigate long-term effects on glycemic control and cardiovascular function.

Ultimately, if other glucose lowering agents fail, insulin therapy is often required. Unfortunately, treatment with insulin is frequently accompanied by significant weight gain due to its anabolic function, and hypoglycemia is often reported[4,74]. As can be appreciated from the above, most glucose-lowering agents, with the exception of SGLT2 inhibitors, do not reduce body weight or even increase body weight, which eventually results in increasing insulin resistance and requirement of the use of (more) medication such as insulin. Activation of glucagon-like peptide-1 (GLP-1) signalling pathways may be a solution to break this vicious cycle, as will be discussed in the next sections.

7. Treatment of type 2 diabetes with GLP-1 modulators

A relatively new class of drugs to treat patients with type 2 diabetes beneficially modulates GLP-1 signalling. GLP-1 is an incretin hormone that is released after food ingestion by enteroendocrine L-cells in the terminal ileum and colon. GLP-1 binds to receptors present on vagal afferent neurons and in many different tissues such as pancreas and brain. Its main effects are stimulation of glucose-dependent insulin secretion by pancreatic β-cells, inhibition of glucagon release by pancreatic α-cells, delay of gastric emptying and induction of satiety[75]. Native GLP-1 has a very short half-life of less than 2 minutes due to degradation by dipeptidyl peptidase 4 (DPP-4)[76]. Pharmacologically, GLP-1 degradation can be delayed by use of DPP-4 inhibitors. Although no significant side-effects have been reported, the effect of DPP-4 inhibition on insulin secretion and improvement of glucose regulation is only modest. This is probably explained by the fact that DPP-4 inhibitors do not increase plasma GLP-1 levels but merely prolong the effect of physiologically available GLP-1[77,78]. In favourable contrast, long-circulating GLP-1 receptor agonists exert similar effects as native GLP-1 but have much higher therapeutic efficacy because they are modified to be resistant to rapid degradation by DPP-4. Although it has been shown extensively that GLP-1 receptor agonists improve glycemic control and induce weight loss[79-81], the effects on ectopic fat and the cardiovascular system are still unclear.

7.1 Effects of GLP-1 receptor agonism on body weight and ectopic fat

It is well known that GLP-1 receptor agonists induce weight loss and reduce fat mass. Interestingly, this decrease in fat mass does not seem to occur homogeneously in the body. Several studies in animal models have shown that increased GLP-1 receptor

activation reduces hepatic steatosis and VLDL production[82,83] and reduces myocardial steatosis[84], independently of weight loss. However, in humans conflicting results on effects of GLP-1 receptor agonists on the different fat depots have been reported, with studies reporting mainly decrease of VAT[85,86] and epicardial adipose tissue[87], and others reporting no effect on VAT[88]. In addition, the GLP-1 receptor agonists liraglutide[89,90] and exenatide[91] have been shown to reduce hepatic steatosis. Since it is unclear to what extent different adipose tissue compartments are affected by weight loss induced by treatment with GLP-1 receptor agonists, this is an important topic of investigation. Indeed, as explained in section 3, reduction of VAT and ectopic fat would be far more beneficial from a perspective of cardiovascular risk reduction than reduction of SAT.

Finally, although it is well known that the GLP-1 receptor agonists reduce body weight, the exact mechanism behind this effect is still unclear. Although the weight loss can at least be partially explained by a reduction of food intake as a consequence of increased satiety, several studies have indicated that GLP-1 receptor agonists may beneficially impact energy expenditure as well[92-96]. In fact, studies in preclinical models suggest that increased thermogenesis in BAT by GLP-1 receptor agonists contributes to the weight loss[92,93]. However, as reported effects of GLP-1 receptor agonists on energy metabolism are conflicting, further studies to investigate effects including the effects of treatment duration are necessary.

7.2 Effects of GLP-1 receptor agonism on cardiovascular function

Type 2 diabetes predisposes to the development of coronary artery disease. Furthermore, type 2 diabetes is independently associated with a 4 to 5-fold increase in the risk of heart failure, even without presence of prior coronary heart disease[97]. Many patients with type 2 diabetes develop heart failure with preserved ejection fraction (HFpEF). Often, this is preceded by diabetic cardiomyopathy, which is a disorder of the heart muscle characterized by left ventricular diastolic dysfunction as a consequence of impaired relaxation and compliance[98,99]. Unfortunately, pharmacological options to treat or prevent development of diabetic cardiomyopathy remain limited.

Several studies have investigated the effect of GLP-1 receptor agonists on the cardiovascular system in patients with type 2 diabetes. Recently, it was shown in the LEADER trial that liraglutide reduces total cardiovascular mortality in patients with type 2 diabetes and with high cardiovascular risk, and similar effects have been reported for the GLP-1 receptor agonists semaglutide and dulaglutide[100,101]. This risk reduction is at least partially caused by anti-atherosclerotic effects of improved glucose and lipid levels. However, GLP-1 receptor agonists also have direct and indirect effects on the myocardium and multiple preclinical studies have shown therapeutic benefits of GLP-1

receptor agonists on cardiac function[102,103]. Using cardiac magnetic resonance imaging (MRI), effects of GLP-1 receptor agonists on both systolic and diastolic cardiac function and effects on myocardial tissue characteristics can be studied in detail, which has not been done before in humans.

OUTLINE OF THIS THESIS

The aim of thesis was to study the effects of liraglutide on cardiometabolic health, by assessing its effects on the various ectopic fat depots and on cardiovascular function in Dutch South Asian and Dutch Western European subjects. We investigated these effects using MRI and proton magnetic resonance spectroscopy (^{1}H-MRS). In addition, since liraglutide at least partly acts by induction of satiety, we studied effects of dietary restriction on these parameters.

Since South Asians have a high risk to develop type 2 diabetes, which may be related to their high ectopic fat accumulation, in **Chapter 2**, we describe the effects of treatment with liraglutide for 26 weeks on ectopic fat deposition and HbA1c in South Asian patients with type 2 diabetes. In this placebo-controlled, double blind, clinical trial we randomly assigned patients to treatment with liraglutide or placebo added to standard care. Using MRI and ^{1}H-MRS, we assessed effects on SAT, VAT, epicardial and paracardial adipose tissue volume and on myocardial and hepatic triglyceride content. In addition, we assessed the association between the decrease of ectopic fat and the improvement of glucoregulation.

Since liraglutide may be beneficial in the prevention of development of diabetic cardiomyopathy, in the same trial as described in Chapter 2, we investigated effects of liraglutide on cardiac function and myocardial tissue characteristics. The results of this study are described in **Chapter 3**.

In **Chapter 4**, we aimed to assess effects of treatment with liraglutide on energy expenditure and BAT using indirect calorimetry and MRI, respectively. We describe the effects of liraglutide treatment on energy metabolism after 4, 12 and 26 weeks of treatment. Furthermore, we measured the fat fraction in the supraclavicular BAT using chemical-shift water-fat MRI.

CETP is a protein that plays a role in plasma lipid transport and is produced by Kupffer cells. However, the regulation of CETP production by the Kupffer cells is still incompletely understood albeit that the hepatic triglyceride content possibly plays a role. Since it was

recently shown that GLP-1 receptor agonists can reduce hepatic triglyceride content, we assessed whether treatment with liraglutide for 26 weeks decreases CETP concentration in humans. The findings of this study are described in **Chapter 5**. In addition, in this chapter we investigated the relationship between the hepatic triglyceride content and circulating CETP in a large population-based cohort.

Chapter 6 focusses on the effects of prolonged caloric restriction on endocannabinoids in relation to ectopic fat accumulation and cardiac function. Elevated levels of endocannabinoids can contribute to accumulation of VAT and insulin resistance. It has been shown that a very low calorie diet (VLCD; 450 kcal/day) substantially reduces hepatic and myocardial steatosis and improves myocardial function in obese patients with type 2 diabetes *without* coronary artery disease[104]. We now aimed to assess the effects of pronounced weight loss after prolonged caloric restriction on plasma endocannabinoid levels in patients with type 2 diabetes *with* established coronary artery disease, a group with even more increased cardiovascular risk. In addition, we investigated the effects on ectopic fat accumulation and cardiac function. In this chapter we describe the results of a prospective intervention study in which patients with type 2 diabetes and established coronary artery disease followed a 16 week very low calorie diet.

Chapter 7 provides a general discussion and summary of the findings of the studies described in this thesis.

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Chapter 2

A double-blind, placebo-controlled, randomised trial to assess the effect of liraglutide on ectopic fat accumulation in South Asian type 2 diabetes patients

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ABSTRACT

Background: South Asians have a high risk to develop type 2 diabetes, which may be related to substantial ectopic fat deposition. Since glucagon-like peptide-1 analogues can reduce ectopic fat accumulation, the aim of the present study was to assess the effect of treatment with liraglutide for 26 weeks on ectopic fat deposition and HbA1c in South Asian patients with type 2 diabetes.

Methods: In a placebo-controlled trial, 47 South Asian patients with type 2 diabetes were randomly assigned to treatment with liraglutide (1.8 mg/day) or placebo added to standard care. At baseline and after 26 weeks of treatment we assessed abdominal subcutaneous, visceral, epicardial and paracardial adipose tissue volume using MRI. Furthermore, myocardial and hepatic triglyceride content were examined with proton magnetic resonance spectroscopy.

Results: In the intention-to-treat analysis, liraglutide decreased body weight compared to placebo (-3.9 ± 3.6 kg *vs* -0.6 ± 2.2 kg; mean change from baseline (liraglutide *vs* placebo): -3.5 kg; 95%CI [-5.3, -1.8]) without significant effects on the different adipose tissue compartments. HbA1c was decreased in both groups without between group differences. In the per-protocol analysis, liraglutide did decrease visceral adipose tissue volume compared to placebo (-23 ± 27 cm^2 *vs* –2 ± 17 cm^2; mean change from baseline (liraglutide *vs* placebo): -17 cm^2; 95%CI [-32, -3]). Furthermore, HbA1c was decreased by liraglutide compared to placebo (-1.0 ± 0.8% (-10.5 ± 9.1 mmol/mol) *vs* (-0.6 ± 0.8% (-6.1 ± 8.8 mmol/mol), with a between group difference (mean change from baseline (liraglutide *vs* placebo): -0.6% (-6.5 mmol/mol); 95%CI [-1.1, -0.1 (-11.5, -1.5)]. Interestingly, the decrease of visceral adipose tissue volume was associated with the reduction of HbA1c (β: 0.165 mmol/mol (0.015%) per 1 cm^2 decrease of visceral adipose tissue volume; 95%CI [0.062, 0.267 (0.006, 0.024%)]).

Conclusions: While the intention-to-treat analysis did not show effects of liraglutide on ectopic fat and HbA1c, per-protocol analysis showed that liraglutide decreases visceral adipose tissue volume, which was associated with improved glycaemic control in South Asians.

BACKGROUND

South Asians are at high risk to develop type 2 diabetes in comparison with other populations, with an estimated prevalence of type 2 diabetes of 8.5% in the adult population[1]. Furthermore, South Asians tend to develop type 2 diabetes at a young age and at a low BMI[2]. Notably, at a BMI of 21 kg/m^2 South Asians show similar distributions of variables for glucose metabolism as white Caucasians at a BMI of 30 kg/m^2[3]. The underlying cause of the increased risk to develop type 2 diabetes remains largely unknown, but an increased amount of ectopic fat is likely to play a role[4]. It is well known that central obesity, but also increased accumulation of ectopic fat in liver[5] and muscle[6] play an important role in development of insulin resistance and type 2 diabetes[7]. Interestingly, several studies have shown that, compared to Europids with a similar BMI, South Asians have more visceral adipose tissue[8,9] and a higher intrahepatic triglyceride content[10,11]. Ectopic fat accumulation increases insulin resistance and metabolic risk[12,13], but may also contribute to remodelling of the heart and to diastolic dysfunction[14]. Therefore, interventions focussed on reducing ectopic fat accumulation could be an effective approach to reduce insulin resistance and improve glycaemic control in this population.

Glucagon-like peptide-1 (GLP-1) analogues are prescribed to patients with type 2 diabetes to improve glycaemic control and induce weight loss[15,16]. The reduction in body weight is primarily the result of a reduction in fat mass, but this reduction does not seem to occur homogeneously in different adipose tissue depots in the body[17,18]. Recently, it has been shown that liraglutide, a GLP-1 analogue, reduces hepatic steatosis in patients with non-alcoholic steatohepatitis[19]. Furthermore, previous studies investigating the effect of GLP-1 analogues on different fat depots, have shown that while both subcutaneous and visceral adipose tissue are reduced, the decrease of visceral adipose tissue[17,20], and epicardial fat[18,21] is even more pronounced. However, in another study mainly subcutaneous adipose tissue was reduced after treatment, while visceral adipose tissue was not affected[22]. Several studies have recently suggested that subcutaneous adipose tissue does not increase the risk to develop diabetes and might even possess protective properties[23,24]. Visceral adipose tissue, however, is causally linked to insulin resistance[25]. Apparently, conflicting data have been reported with respect to the effect of GLP-1 analogues on the various adipose depots in the general population. Since it is unclear to what extent different adipose tissue compartments are affected by weight loss induced by treatment with GLP-1 analogues, it is important to further investigate the effects of treatment with GLP-1 analogues on the different fat depots, especially since reduction of ectopic adipose tissue would be more beneficial than reduction of subcutaneous adipose tissue.

Since South Asians have a specific body fat distribution, with high amounts of visceral adipose tissue[8,9], effects of a GLP-1 analogue on ectopic fat depots, and subsequently effects on glycaemic control, could be pronounced especially in this population. Therefore, the aim of the present study was to assess the effect of treatment with liraglutide for 26 weeks on ectopic fat deposition and HbA1c in South Asian patients with type 2 diabetes.

METHODS

Study overview and study population

This study is a 26-week, prospective, randomised, double-blind, clinical trial. Patients from South Asian descent, i.e. individuals with two South Asian parents, with type 2 diabetes were recruited via advertisements and from the outpatient clinics of the Leiden University Medical Center (LUMC, Leiden, the Netherlands), general practitioners, and local hospitals. A screening visit was performed prior to inclusion to assess eligibility for participation. We included subjects with BMI ≥23 kg/m^2, aged 18-74 years, with an HbA1c ≥6.5% and ≤11.0% (≥47.5 and ≤96.4 mmol/mol). Concomitant treatment with metformin, sulfonylurea derivatives and insulin was optional, although the dosage of all glucose-lowering medication needed to be stable for at least 3 months prior to participation. Main exclusion criteria were use of other glucose-lowering therapy than mentioned above or presence of renal disease, 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. Furthermore, patients with any contra-indication for contrast-enhanced MRI were excluded. The trial was conducted in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all subjects before inclusion. The trial was approved by the local ethics committee and conducted at the LUMC, and was registered at clinicaltrials.gov (NCT01761318).

Study design

At baseline, participants were randomised to receive treatment with liraglutide (Victoza®) or placebo (both provided by Novo Nordisk A/S, Bagsvaerd, Denmark) by block randomisation with block size of 4 and stratification 1:1 for sex and insulin use. During the study, all participants, study investigators and outcome assessors were blinded to treatment allocation. The starting dose of the study medication was 0.6 mg per day, which was titrated in two weeks to a maximum dose of 1.8 mg per day, if tolerated. If necessary in case of adverse events, the dose was reduced. During trial participation,

a weekly telephone call was scheduled to discuss blood glucose management and adverse events, and at week 4 and week 12 participants visited the study center for routine blood tests and clinical measurements. In addition to study medication, participants received treatment according to current clinical guidelines to achieve optimal glycaemic control and regulation of blood pressure and cholesterol levels.

Data collection

After inclusion, participants visited the study center at baseline and after 26 weeks of treatment, after ≥6 h of fasting, for medical history assessment, standard physical examination, collection of venous blood samples and MRI. All blood samples were centrifuged and stored at -80°C until analysis. Plasma total cholesterol, HDL-cholesterol and triglyceride concentrations were measured on a Modular P800 analyser (Roche Diagnostics, Mannheim, Germany). LDL-cholesterol was calculated according to the Friedewald formula[26]. HbA1c was assessed with ion-exchange high-performance liquid chromatography (HPLC; Tosoh G8, Sysmex Nederland B.V., Etten-Leur, the Netherlands). Body composition and lean body mass was assessed using bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystart Ltd., Douglas, UK).

MRI for adipose tissue volume

A 3.0 Tesla MRI scanner (Ingenia, Philips Healthcare, Best, the Netherlands) was used, with a dStream Torso anterior coil and a FlexCoverage posterior coil in the table top (in total up to 32 coil elements for signal reception). To assess visceral and abdominal subcutaneous adipose tissue volumes, 2-point Dixon water-fat separated transverse images were obtained of the abdomen during one breath-hold, with the following parameters: repetition time (TR) 3.5 ms, first/second echo time (TE1/TE2) 1.19/2.3 ms, flip angle (FA) 10°, field of view (FOV) 500x365 mm^2, acquired voxel size 1.60x1.70 mm^2, slice thickness 4 mm, slice gap -2 mm, and number of slices 140.

For quantification of epicardial and paracardial fat, ECG-triggered fat-selective images, using a multi-shot turbo spin-echo sequence with spectral pre-saturation with inversion recovery (SPIR) for water suppression, were acquired in 4-chamber view orientation at end-diastole, during one breath-hold, with imaging parameters: TR/TE 1000/11 ms, FA 90°, FOV 280x223 mm^2, acquired voxel size 1.09x1.12 mm^2, and slice thickness 4 mm.

MR images were analysed in MASS Research Software V2018-EXP (Leiden University Medical Center, the Netherlands). For assessment of visceral and abdominal subcutaneous adipose tissue volume, three transverse slices were reformatted, at the level of the fourth and fifth lumbar vertebrae, with slice thickness of 10 mm and slice gap of 12 mm. In each slice, the outer borders of visceral and subcutaneous adipose tissue were

manually outlined, and the areas were automatically calculated based on pixel intensity thresholding. Subsequently, visceral and abdominal subcutaneous adipose tissue volume were quantified as the mean area in squared centimeters of all three slices. Similarly, epicardial and paracardial fat (between outer wall of the myocardium and visceral pericardium and between visceral and parietal pericardium, respectively) were assessed. Epicardial and paracardial fat were measured in 4 chamber view orientation, in the region surrounding the left and right ventricles, below the level of the atrioventricular valves.

Proton magnetic resonance spectroscopy for myocardial and hepatic triglyceride content

Myocardial and hepatic triglyceride content were examined with proton magnetic resonance spectroscopy (^{1}H-MRS)[27]. Spectra were acquired using single voxel point resolved spectroscopy (PRESS), with first order volume B0 pencil beam shimming, respiratory navigator (trigger and track), and multiply optimized insensitive suppression train (MOIST) suppression (bandwidth 190 Hz) for the water-suppressed acquisitions. Parameters were as follows: TR 3.5 or 9 seconds (water-suppressed and non-water-suppressed acquisition, respectively), TE 35 ms, bandwidth 1500 Hz and acquired samples 2048 (spectral resolution 0.73 Hz/sample). Cardiac ^{1}H-MRS additionally used ECG-triggering (R-top trigger delay 200 ms) and acquired in the midventricular septum (voxel size 40x15x25 mm^3, shim volume 50x25x35 mm^3, number of signal averages (NSA) of water-suppressed and non-water-suppressed acquisition 64 and 6, respectively). A high permittivity pad was placed on the thorax at the location of the heart to improve signal-to-noise ratio[28]. Hepatic ^{1}H-MRS was obtained in the liver parenchyma, avoiding the inclusion of blood vessels or subcutaneous fat (voxel size 20x20x20 mm^3, shim volume 35x35x35 mm^3, NSA of water-suppressed and non-water-suppressed acquisition 32 and 8, respectively). The voxels were planned at the same location for the baseline and follow-up measurements.

The spectral raw data were processed using an in-house developed script (MATLAB R2015a (MathWorks, Massachusetts, United States). The raw data were phase-, frequency- and eddy current-corrected, if required. Individual signal averages were analysed and signal averages exceeding the 95% confidence interval were considered outliers and were excluded. Reconstructed data were further analysed in the Java-based Magnetic Resonance User Interface (jMRUI v5.0; MRUI Consortium). For the water-suppressed signals, the Hankel-Lanczos filter was applied to remove residual water. The spectra were fitted using the AMARES algorithm, with the assumption of Gaussian line shapes. Prior knowledge for the fit included the following starting values: triglyceride-methyl (CH_3) 0.9 ppm, triglyceride-methylene (CH_2) 1.3 ppm, COO-CH_2 2.05 ppm, creatine 3.05 ppm,

trimethylamines (TMA) 3.25 ppm, with soft constraints for the linewidth of the fit of each signal. The first-order phase was fixed to zero. Myocardial and hepatic lipid-to-water ratios were quantified as the signal of triglyceride methylene divided by the unsuppressed water signal, multiplied by 100%[29].

Statistical analyses

The main outcome measure of this study was the effect of liraglutide on cardiac function and sample size calculation was based on this outcome measure as described previously[30]. In this manuscript, we report on secondary outcome measures. Data are shown as means ± SD, or as median (interquartile range) when not normally distributed. Within-group changes were assessed using paired t-tests. We performed an ANCOVA to assess between-group differences with treatment included as fixed effect and the baseline value as a covariate. The intention-to-treat analysis included data of all participants who were randomised and started study medication. The per-protocol analysis included only participants who adhered to the assigned medication, i.e. used ≥80% of prescribed study medication. A P-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL).

RESULTS

Population characteristics

As shown in the trial flow diagram in **Figure 1**, 51 patients were included after screening, of whom 4 were excluded before randomisation. Between July 2015 and December 2016, 22 patients were randomised to receive liraglutide and 25 to receive placebo. All randomised patients finished the study and were included in the intention-to-treat analysis. During the study, 19 participants (86.4%) of the liraglutide group and 24 participants (96.0%) of the placebo group used the standard dose of 1.8 mg/day, while in the rest of the participants the maximally tolerated dose was 1.2 mg/day. In the liraglutide group, participants used on average 95.4 ± 8.1% of the prescribed cumulative dose, and in the placebo group the participants used 98.7 ± 5.2%. One participant of the liraglutide group used <80% of the prescribed cumulative dose, and of two participants (one allocated to receive placebo and one to receive liraglutide) adherence could not be calculated, due to missing (empty) medication pens. These participants were included in the intention-to-treat analysis but not in the per-protocol analysis. One serious adverse event (admission for symptoms of acute coronary syndrome) occurred in the placebo group. In the liraglutide group compared to the placebo group, more participants reported nausea (73 *vs* 40%) and vomiting (27 *vs* 8%) at least once during study participation.

As shown in **Table 1**, baseline characteristics of the participants in both treatment groups were balanced. Individuals were 55 ± 11 years old in the liraglutide group, *vs* 55 ± 9 years in the placebo group, with a body weight of 81.9 ± 11.0 *vs* 77.8 ± 12.4 kg and BMI of 30.4 ± 3.8 *vs* 28.6 ± 4.0 kg/m^2, respectively.

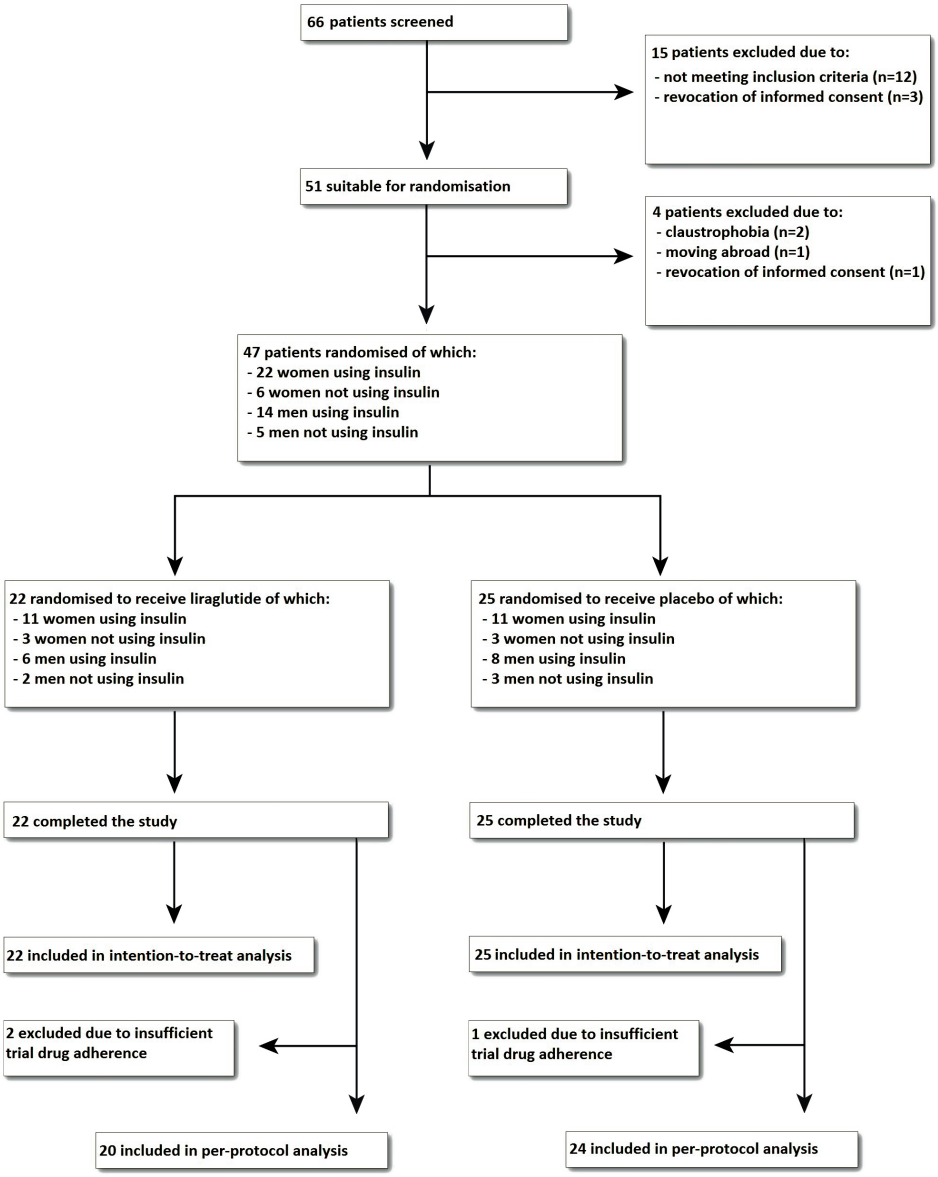

Figure 1. Trial flow diagram.

Table 1. Baseline characteristics of study participants.

Characteristic	Liraglutide (n= 22)	Placebo (n=25)
Demographics		
Age (year)	55 ± 11	55 ± 9
Sex (no. (%))		
Male	8 (36%)	11 (44%)
Female	14 (64%)	14 (56%)
Diabetes duration (years)	19 ± 10	17 ± 10
Concomitant drug use		
Metformin (no. (%))	22 (100%)	23 (92%)
Metformin dose (g/day)	1.8 ± 0.7	1.7 ± 0.6
Sulfonylurea (no. (%))	3 (14%)	5 (20%)
Insulin (no. (%))	17 (77%)	19 (76%)
Insulin dose (units/day)	77 ± 34	67 ± 30
Lipid-lowering drugs (statin and/or other), no. (%)	17 (77%)	20 (80%)
Clinical parameters		
Body weight (kg)	81.9 ± 11.0	77.8 ± 12.4
BMI (kg/m^2)	30.4 ± 3.8	28.6 ± 4.0
Waist circumference (cm)	104 ± 8	98 ± 10
Hip circumference (cm)	104 ± 7	104 ± 9
Waist-hip ratio	1.00 ± 0.07	0.95 ± 0.09
Lean body mass (kg)	51.6 ± 10.6	48.9 ± 11.2
Lean body mass (%)	62.8 ± 8.4	63.1 ± 9.8
Metabolic factors		
HbA1c (mmol/mol)	65 ± 10	70 ± 12
HbA1c (%)	8.1 ± 0.9	8.6 ± 1.1
Total cholesterol (mmol/L)	3.95 ± 0.65	4.46 ± 1.10
HDL-cholesterol (mmol/L)	1.24 ± 0.33	1.21 ± 0.30
LDL-cholesterol (mmol/L)	2.00 ± 0.65	2.21 ± 0.97
Triglycerides (mmol/L)	1.55 ± 0.86	2.08 ± 1.80
Adipose tissue compartments		
Subcutaneous AT (cm^2)	315 ± 97	326 ± 141
Visceral AT (cm^2)	187 ± 57	149 ± 49
Epicardial AT (cm^2)	10 ± 3	9 ± 3
Paracardial AT (cm^2)	12 ± 4	9 ± 4
Hepatic TGC (%)	6.9 ± 6.3	11.8 ± 10.9
Myocardial TGC (%)	0.9 ± 0.4	1.0 ± 0.6

Results are presented as n (%) or mean ± SD. n=47. Missing data in liraglutide group: n=1 for epicardial adipose tissue volume and paracardial adipose tissue volume. Missing data in placebo group: n=1 for lean body mass (kg and %), epicardial adipose tissue volume and myocardial triglyceride content. AT: adipose tissue, TGC: triglyceride content.

Effects of liraglutide on body weight and ectopic fat in the intention-to-treat analysis

Results of the intention-to-treat analysis are shown in **Table 2**. Treatment with liraglutide for 26 weeks decreased body weight, while body weight in participants treated with placebo was not affected (-3.9 ± 3.6 kg *vs* -0.6 ± 2.2 kg; mean change from baseline (liraglutide *vs* placebo): -3.5 kg; 95%CI [-5.3, -1.8]). Part of this weight loss was explained by a decrease in lean body mass that occurred in the liraglutide group but not in the placebo group (-2.3 ± 2.3 kg *vs* 0.4 ± 2.9 kg; mean change from baseline (liraglutide *vs* placebo): -2.7 kg; 95%CI [-4.3, -1.1]). Notably, waist circumference was decreased by liraglutide, while hip circumference was unaffected. Furthermore, although liraglutide decreased body weight, no effect was present on the investigated separate adipose tissue compartments, with the exception of a tendency to a decreased visceral adipose tissue volume in the liraglutide group compared to the placebo group (-20 ± 29 cm^2 *vs* -2 ± 17 cm^2; mean change from baseline (liraglutide *vs* placebo): -13 cm^2; 95%CI [-27, 1]).

Effects of liraglutide on HbA1c and lipid levels in the intention-to-treat analysis

In the intention-to-treat analysis HbA1c was decreased in the liraglutide group (-8.5 ± 11.2 mmol/mol; -0.8 ± 1.0%), but also in the placebo group (-6.8 ± 9.3 mmol/mol; -0.6 ± 0.8%), without between group differences (mean change from baseline (liraglutide *vs* placebo): -4.0 mmol/mol (-0.4%); 95%CI [-9.7, 1.6 (-0.9, 0.1%)]. To improve glycaemic control metformin was started for 1 participant and sulfonylurea derivatives were started in 3 participants of the placebo group according to clinical guidelines. The mean insulin dose was not significantly changed compared to baseline in the liraglutide and the placebo group (-11 ± 34 units/day *vs* 1 ± 23 units/day; mean change from baseline (liraglutide *vs* placebo): -12 units/day; 95%CI [-31, 8]). Furthermore, while glycaemic control was improved in both groups, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride were not affected.

Effects of liraglutide on ectopic fat and HbA1c in the per-protocol analysis

Results of the per protocol analysis are shown in **Table 3**. In this analysis, 3 patients who used <80% of the prescribed cumulative dose were excluded from analysis, of whom 2 were randomised to receive liraglutide and 1 to receive placebo. As in the intention-to-treat analysis, treatment with liraglutide decreased body weight and lean body mass. Furthermore, as shown in **Figure 2**, visceral adipose tissue volume was decreased by liraglutide, but not by placebo (-23 ± 27 cm^2 *vs* 2 ± 17 cm^2; mean change from baseline (liraglutide *vs* placebo): -17 cm^2; 95%CI [-32, -3]). Other adipose tissue compartments were not affected by treatment with liraglutide. HbA1c was decreased in the liraglutide

group (-10.5 ± 9.1 mmol/mol; -1.0 ± 0.8%) to a greater extent than in the placebo group (-6.1 ± 8.8 mmol/mol; -0.6 ± 0.8%), with a between group difference (mean change from baseline (liraglutide *vs* placebo) of: -6.5 mmol/mol (-0.6%); 95%CI [-11.5, -1.5 (-1.1, -0.1%)]. Interestingly, an association was present between the decrease of subcutaneous adipose tissue volume and HbA1c after treatment (β: 0.075 mmol/mol (0.007%) per 1 cm^2 decrease of subcutaneous adipose tissue volume; 95%CI [0.004, 0.146 (0.000, 0.013%)]) (**Figure 3A**). A similar but stronger association was present between the decrease of visceral adipose tissue volume and the reduction of HbA1c after treatment (β: 0.165 mmol/mol (0.015%) per 1 cm^2 decrease of visceral adipose tissue volume; 95%CI [0.062, 0.267 (0.006, 0.024%)]) (**Figure 3B**). No association was present between other adipose tissue compartments and HbA1c.

Table 2. Clinical parameters, metabolic factors and adipose tissue compartment changes from baseline after 26 weeks of treatment in the intention-to-treat analysis.

Characteristic	Mean ± SD change from baseline to 26 weeks		Mean [95%CI] changes from baseline (liraglutide *vs* placebo)	P value
	Liraglutide (n=22)	Placebo (n=25)		
Clinical parameters				
Body weight (kg)	-3.9 ± 3.6	-0.6 ± 2.2	-3.5 [-5.3, -1.8]	<0.001
BMI (kg/m^2)	-1.5 ± 1.4	-0.2 ± 0.8	-1.4 [-2.0, -0.7]	<0.001
Waist circumference (cm)	-5 ± 4	0 ± 4	-5 [-8, -2)	<0.001
Hip circumference (cm)	-4 ± 5	-2 ± 3	-2 [-5, 0]	0.067
Waist-hip ratio	-0.01 ± 0.04	0.02 ± 0.05	-0.01 [-0.04, 0.01]	0.312
Lean body mass (kg)	-2.3 ± 2.3	0.4 ± 2.9	-2.7 [-4.3, -1.1]	0.001
Lean body mass (%)	0.2 ± 1.7	0.8 ± 2.7	-0.6 [-1.9, 0.8]	0.403
Metabolic factors				
HbA1c (mmol/mol)	-8.5 ± 11.2	-6.8 ± 9.3	-4.0 [-9.7, 1.6]	0.156
HbA1c (%)	-0.8 ± 1.0	-0.6 ± 0.8	-0.4 [-0.9, 0.1]	0.156
Total cholesterol (mmol/L)	0.24 ± 1.09	-0.42 ± 0.82	0.52 [-0.05, 1.09]	0.073
HDL-cholesterol (mmol/L)	-0.04 ± 0.12	-0.05 ± 0.12	0.02 [-0.05, 0.09]	0.657
LDL-cholesterol (mmol/L)	0.15 ± 0.74	-0.14 ± 0.74	0.22 [-0.20, 0.63]	0.296
Triglycerides (mmol/L)	0.28 ± 1.25	-0.38 ± 1.30	0.40 [-0.24, 1.04]	0.214
Adipose tissue compartments				
Subcutaneous AT (cm^2)	-24 ± 37	-10 ± 37	-15 [-37, 6]	0.158
Visceral AT (cm^2)	-20 ± 29	-2 ± 17	-13 [-27, 1]	0.074
Epicardial AT (cm^2)	0 ± 2	1 ± 1	-1 [-2, 0]	0.232
Paracardial AT (cm^2)	-1 ± 3	0 ± 3	-1 [-2, 1]	0.494
Hepatic TGC (%)	-1.2 ± 4.1	-3.3 ± 5.4	0.4 [-1.9, 2.8]	0.704
Myocardial TGC (%)	0.1 ± 0.5	-0.1 ± 0.6	0.2 [-0.1, 0.5]	0.157

Results are presented as n (%) or mean ± SD. n=47. Missing data in the liraglutide group: n=3 for epicardial adipose tissue volume and paracardial adipose tissue volume, n=1 for myocardial TGC. Missing data in placebo group: n=1 for lean body mass (kg and %), n=3 for epicardial adipose tissue volume, n=2 for paracardial adipose tissue volume, and n=1 for myocardial TGC. AT: adipose tissue, TGC: triglyceride content.

Table 3. Clinical parameters, metabolic factors and adipose tissue compartment changes from baseline after 26 weeks of treatment in the per-protocol analysis.

Characteristic	Mean ± SD change from baseline to 26 weeks		Mean [95%CI] changes from baseline (liraglutide *vs* placebo)	P value
	Liraglutide (n=20)	Placebo (n=24)		
Clinical parameters				
Body weight (kg)	-4.3 ± 3.4	-0.6 ± 2.2	-4.0 [-5.8, -2.3]	<0.001
BMI (kg/m^2)	-1.6 ± 1.4	-0.2 ± 0.9	-1.5 [-2.2, -0.8]	<0.001
Waist circumference (cm)	-5 ± 4	0 ± 4	-5 [-8, -2)	0.001
Hip circumference (cm)	-4 ± 5	-2 ± 3	-2 [-5, 0]	0.068
Waist-hip ratio	-0.01 ± 0.04	0.02 ± 0.05	-0.01 [-0.04, 0.02]	0.394
Lean body mass (kg)	-2.4 ± 2.4	0.4 ± 3.0	-2.8 [-4.5, -1.1]	0.002
Lean body mass (%)	0.4 ± 1.6	0.8 ± 2.7	-0.4 [-1.8, 1.0]	0.605
Metabolic factors				
HbA1c (mmol/mol)	-10.5 ± 9.1	-6.1 ± 8.8	-6.5 [-11.5, -1.5]	0.011
HbA1c (%)	-1.0 ± 0.8	-0.6 ± 0.8	-0.6 [-1.1, -0.1]	0.011
Total cholesterol (mmol/L)	0.06 ± 0.98	-0.37 ± 0.78	0.28 [-0.26, 0.81]	0.305
HDL-cholesterol (mmol/L)	-0.04 ± 0.12	-0.06 ± 0.11	0.02 [-0.05, 0.10]	0.510
LDL-cholesterol (mmol/L)	0.04 ± 0.66	-0.07 ± 0.68	0.08 [-0.32, 0.48]	0.689
Triglycerides (mmol/L)	0.12 ± 1.13	-0.38 ± 1.32	0.13 [-0.47, 0.74]	0.663
Adipose tissue compartments				
Subcutaneous AT (cm^2)	-26 ± 38	-11 ± 37	-15 [-38, 7]	0.182
Visceral AT (cm^2)	-23 ± 27	-2 ± 17	-17 [-32, -3]	0.020
Epicardial AT (cm^2)	0 ± 2	1 ± 1	-1 [-2, 0]	0.139
Paracardial AT (cm^2)	-1 ± 3	0 ± 3	-1 [-3, 1]	0.467
Hepatic TGC (%)	-1.9 ± 3.6	-3.2 ± 5.5	-0.3 [-2.6, 2.0]	0.807
Myocardial TGC (%)	0.1 ± 0.5	-0.1 ± 0.6	0.2 [-0.1, 0.5]	0.157

Results are presented as n (%) or mean ± SD. n=44. Missing data in the liraglutide group: n=3 for epicardial adipose tissue volume and paracardial adipose tissue volume. Missing data in placebo group: n=1 for lean body mass (kg and %), n=3 for epicardial adipose tissue volume, n=2 for paracardial adipose tissue volume, and n=1 for myocardial triglyceride content. AT: adipose tissue, TGC: triglyceride content.

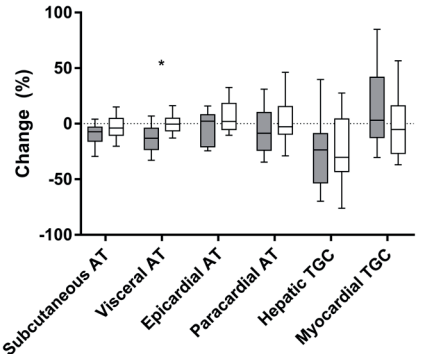

Figure 2. The effect of liraglutide and placebo on different adipose tissue compartments. Percentual changes are depicted after 26 weeks of treatment with liraglutide (n=24) and placebo (n=20) compared to baseline. Box and whiskers show 25th and 75th percentile and 10th and 90th percentile, respectively. Missing data in liraglutide-group: n=3 for epicardial AT and paracardial AT. Missing data in placebo-group: n=3 for epicardial AT, n=2 for paracardial AT and n=1 for Myocardial TGC. AT: adipose tissue, TGC: triglyceride content. * P<0.05.

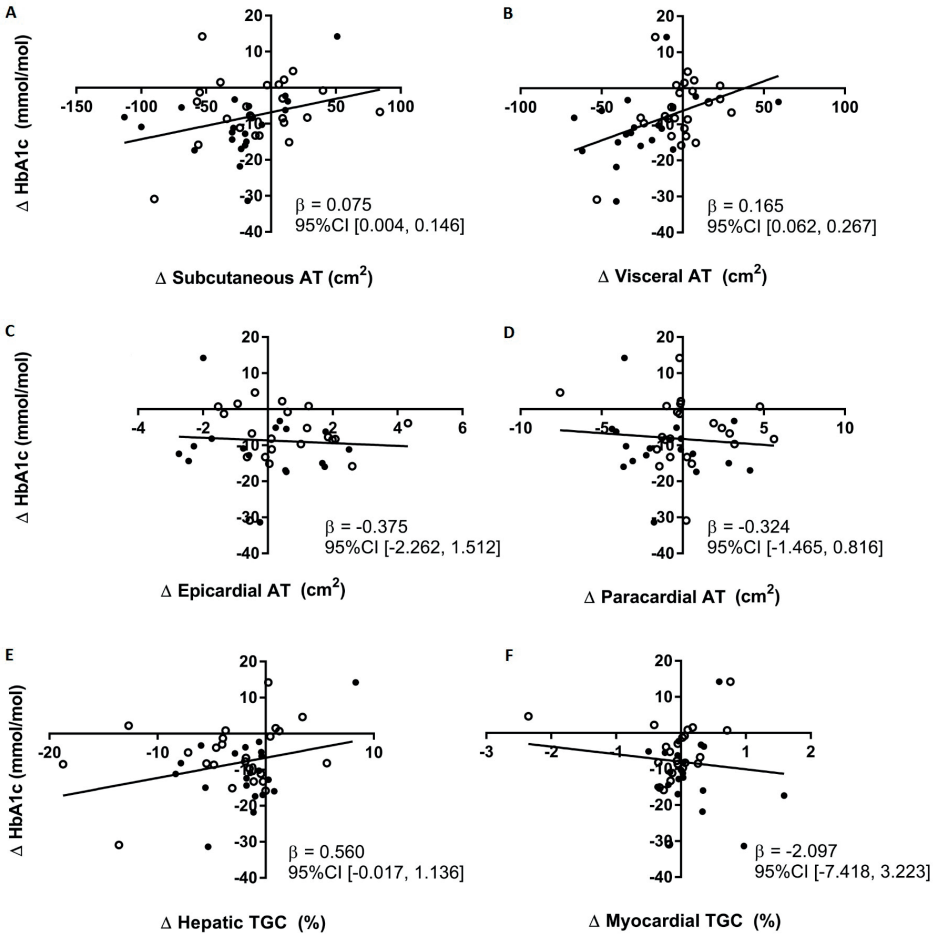

Figure 3. Associations between the change of adipose tissue compartments and HbA1c after treatment. Subcutaneous AT in relation to HbA1c, n=44 (A), Visceral AT in relation to HbA1c, n=44 (B), Epicardial AT in relation to HbA1c, n=38 (C), Paracardial AT in relation to HbA1c, n=39 (D), Hepatic TGC in relation to HbA1c, n=44 (E) and Myocardial TGC in relation to HbA1c, n=43 (F). Regression lines are shown for placebo (open symbol) and liraglutide (closed symbol) combined. AT: adipose tissue, TGC: triglyceride content.

DISCUSSION

In this double-blind, randomised placebo-controlled trial in South Asian patients with type 2 diabetes, we observed that liraglutide decreased body weight. Although this was not accompanied by effects on specific adipose tissue compartments in the intention-to-treat analysis, liraglutide did decrease visceral adipose tissue volume and HbA1c compared to placebo in the per-protocol analysis. In fact, the reduction in visceral adipose tissue was associated with an improved HbA1c. These data imply that GLP-1 analogues such as liraglutide are an effective treatment option for South Asian patients

with type 2 diabetes that might improve glycaemic control by reducing visceral adipose tissue volume.

We are the first to investigate the effects of liraglutide on ectopic fat deposition in a group of South Asians participants. Although the intention-to-treat analysis did not reveal a significant effect of liraglutide on ectopic fat, a trend towards a reduction of visceral adipose tissue volume was observed. This was likely caused by non-adherence of a few participants to the study protocol, as the per-protocol analysis did show that liraglutide decreased visceral adipose tissue. These data are in accordance with results published by Jendle et al.[17], who reported a dose-dependent reduction of visceral adipose tissue and a relatively small reduction of subcutaneous adipose tissue after treatment with 0.6, 1.2 or 1.8 mg liraglutide per day for 26 or 52 weeks in a mixed population. In line, Ishii et al.[20] reported that by treatment of Japanese individuals with liraglutide (0.9 mg/day for 26 weeks) reduced visceral adipose tissue volume without effects on subcutaneous adipose tissue. On the other hand, Suzuki et al.[22] reported that treatment of Japanese individuals with liraglutide (0.9 mg/day for 26 weeks) reduced subcutaneous adipose tissue volume without effects on visceral adipose tissue volume. Furthermore, in a study performed by Morano et al.[18], treatment of patients with type 2 diabetes with liraglutide (1.2 mg/day for 12 weeks) or exenatide, another GLP-1 analogue, resulted in a reduction of epicardial fat volume as assessed by ultrasonography. Iacobellis et al.[21] reported a similar reduction in epicardial fat volume after treatment with liraglutide (up to 1.8 mg/day for 3 and 6 months) and it was recently reported that epicardial adipose tissue expresses the GLP-1 receptor[31]. This is of clinical importance, since it was recently shown that inflammatory activity of epicardial adipose tissue volume might induce myocardial remodelling and dysfunction[32]. In the current study, a reduction of either epicardial or paracardial adipose tissue volume was not observed after treatment with liraglutide in South Asians as assessed by MRI, which is considered the gold standard for assessment of body fat, including epicardial fat[33]. The reason for the discrepancy with previously published results is unclear but may reflect an ethnic-specific response to liraglutide. There are indications for this from a study comparing the effect of very low calorie diet in middle-aged South Asians to Western Europeans. While the very low calorie diet equally reduced body weight in both groups, the diet reduced pericardial adipose tissue, which includes epicardial adipose tissue, in the Western Europeans only[34]. Similarly, we did not reproduce the results of Armstrong et al.[19], who reported a histologically assessed reduction of hepatic steatosis in patients with steatohepatitis after treatment with liraglutide (1.8 mg/day for 48 weeks) and of Dutour et al.[35] who reported reduction of hepatic steatosis in obese subjects with type 2 diabetes after treatment with exenatide (20 µg/day for 26 weeks). However, the patients in those studies had considerably higher severity of steatosis comparted to our participants who had a more modest hepatic

triglyceride content. These data may indicate that the potency of liraglutide to reduce hepatic steatosis is dependent on hepatic triglyceride content, although an ethnic-specific response to liraglutide cannot be ruled out.

In our trial, in the intention-to-treat analysis, treatment with both liraglutide and placebo resulted in reduction of HbA1c. Importantly, both groups were treated according to current clinical guidelines. Therefore, if necessary, the dose of glucose lowering medication, including insulin, was increased or new medication was started in both groups, which can thus explain the effect of placebo on HbA1c. These results are in line with previously published studies reporting no significant superiority of GLP-analogues over standard treatment[36-38]. However, in contrast to the intention-to-treat analysis, in the per-protocol analysis, treatment with liraglutide significantly reduced HbA1c compared to placebo. Interestingly, a previously published meta-analysis showed that the HbA1c-lowering effect of GLP-1 analogues is greater in studies with ≥50% Asian participants than in studies with <50% Asians[39]. Therefore, possibly, in South Asian patients with type 2 diabetes treatment with liraglutide exerts more substantial or diverse effects, resulting in a greater reduction of HbA1c. An explanation for this observation could be differences in either insulin sensitivity or beta-cell function between South Asians and other ethnic groups. Importantly, since the change in visceral adipose tissue and the change in HbA1c show a strong association, it is likely that the reduction of visceral adipose tissue contributed to the improved glycaemic control.

Based on our data and current literature, we can speculate on the mechanism behind the liraglutide-induced reduction of visceral adipose tissue in our per-protocol analysis. It has previously been shown that GLP-1 increases the expression of lipolytic markers while reducing expression of lipogenic and adipogenic genes in adipose tissue, with distinct effects on subcutaneous and visceral adipose tissue[40]. In another study, expression of brown adipose tissue-related genes was upregulated in subcutaneous adipose tissue of rats after treatment with liraglutide[41]. In line, it was recently shown that liraglutide-induced weight reduction resulted in a greater reduction of visceral adipose tissue volume than lifestyle counselling at similar weight reduction[42]. Another possible explanation for a specific reduction in visceral adipose tissue may be related to central effects of GLP-1. In rodents, activation of central GLP-1 receptors contributes substantially to improved insulin sensitivity[43] as related to an increase in sympathetic outflow[44]. Sympathetic innervation of visceral and subcutaneous adipose tissue, the principal initiator for lipolysis in white adipose tissue, is partially separated[45]. Therefore, central action of GLP-1 analogues might induce specific lipolysis in visceral adipose tissue as compared to subcutaneous adipose tissue.

It has previously been proposed that the subcutaneous adipose tissue compartment is less developed in South Asians compared to white people, resulting in a reduced storage capacity of this compartment causing more storage of fat in ectopic sites[46]. Furthermore, South Asians have an increased subcutaneous adipose tissue adipocyte size compared to white Caucasians, probably related to limited expansion of this depot, further contributing to overflow of fatty acids to ectopic depots[11]. Our results implicate that GLP-1 analogues could be an effective treatment option for South Asian patients with type 2 diabetes, possibly through improving insulin sensitivity via a specific reduction in visceral adipose tissue. If reduction in visceral adipose tissue is indeed causal for the improvement of HbA1c, GLP-1 analogues are likely to be also beneficial for other patients with high amounts of ectopic fat. All in all, it is clear that liraglutide and other GLP-1 analogues decrease body weight related to a specific decrease in visceral adipose tissue. Further research is warranted to determine treatment effects in different ethnic groups and in subjects with different body compositions.

The main strength of this study is the randomised, double-blind, placebo-controlled trial design. In addition, the study design in which participants were treated according to current clinical guidelines increases the external validity of our results. Moreover, we had no drop-out and study drug compliance was generally high. Furthermore, we performed a per-protocol analysis excluding participants with a low drug adherence or missing data on drug adherence. Limitations are that our study was powered on other outcome measures than the outcomes reported here, and the relatively small group size.

CONCLUSIONS

In summary, in this randomised, placebo-controlled trial, we showed that liraglutide decreases body weight, which is partially caused by a reduction of visceral adipose tissue, and improves HbA1c in South Asian type 2 diabetes patients. Interestingly, the reduction of visceral adipose tissue was associated with a reduction in HbA1c. Collectively, these data indicate that GLP-1 analogues might be useful therapeutic means to improve glycaemic control by reducing visceral adipose tissue volume in South Asian type 2 diabetes patients.

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Chapter 3

Effect of liraglutide on cardiovascular function and myocardial tissue characteristics in type 2 diabetes patients of South Asian descent living in the Netherlands: a double-blind randomized placebo-controlled trial

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ABSTRACT

Background: The glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide may be beneficial in the regression of diabetic cardiomyopathy. South Asian ethnic groups in particular are at risk of developing type 2 diabetes.

Purpose: To assess the effects of liraglutide on left ventricular (LV) diastolic and systolic function in South Asian type 2 diabetes patients.

Study Type: Prospective double-blind randomized placebo-controlled trial.

Population: 47 type 2 diabetes patients of South Asian ancestry living in the Netherlands, with or without ischemic heart disease, who were randomly assigned to 26-week treatment with liraglutide (1.8 mg/day) or placebo.

Field strength/Sequence: 3T (bSSFP cine MRI, 2D and 4D velocity-encoded MRI, ^{1}H-MRS, T1 mapping).

Assessment: Primary endpoints were changes in LV diastolic function (early deceleration peak (Edec), ratio of early and late peak filling rate (E/A), estimated LV filling pressure (E/Ea)) and LV systolic function (ejection fraction). Secondary endpoints were changes in aortic stiffness (aortic pulse wave velocity (PWV), myocardial steatosis (myocardial triglyceride content) and diffuse fibrosis (extracellular volume (ECV)).

Statistical Tests: Data were analyzed according to intention-to-treat. Between-group differences were reported as mean (95%CI) and were assessed using ANCOVA.

Results: Liraglutide (n=22) compared with placebo (n=25) did not change Edec (+0.2 mL/s^2x10^{-3} (-0.3;0.6)), E/A (-0.09 (-0.23;0.05)), E/Ea (+0.1 (-1.2;1.3)) and ejection fraction (0% (-3;2)), but decreased stroke volume (-9 mL (-14;-5)) and increased heart rate (+10 bpm (4;15)). Aortic PWV (+0.5 m/s (-0.6;1.6)), myocardial triglyceride content (+0.21% (-0.09;0.51)) and ECV (-0.2% (-1.4;1.0)) were unaltered.

Data Conclusion: Liraglutide did not affect LV diastolic and systolic function, aortic stiffness, myocardial triglyceride content or extracellular volume in Dutch South Asian type 2 diabetes patients with or without coronary artery disease.

INTRODUCTION

Type 2 diabetes is associated with a two- to five-fold increased risk of heart failure[1]. Diabetic cardiomyopathy, which is characterized by left ventricular (LV) diastolic dysfunction, may eventually progress to heart failure with preserved ejection fraction[1]. A potential anti-hyperglycemic agent with cardioprotective effects is the glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide[2].

Recently, the LEADER trial has demonstrated a reduced total cardiovascular mortality as a result of liraglutide in patients with type 2 diabetes and high cardiovascular risk, presumably because of a lower risk of ischemic events[3]. Similar reductions in cardiovascular mortality have been reported in response to treatment with the GLP-1 receptor agonists semaglutide and dulaglutide[4]. However, it is largely unknown whether liraglutide in the management of type 2 diabetes is advantageous for heart function in asymptomatic diastolic dysfunction[2]. It is conceivable that the favorable metabolic impact of liraglutide on lipid profiles and inflammatory markers[5], in addition to the natriuretic and vasodilatory actions[6,7], has indirect beneficial effects on diastolic function. Liraglutide has been assumed to exert direct actions on the myocardium that may amend myocardial metabolism, although preclinical and clinical studies have not been conclusive[2]. The effects of liraglutide on diastolic function may be mediated by regression of type 2 diabetes-related myocardial steatosis, diffuse fibrosis and aortic stiffening[1,8]. Notably, clinical studies have consistently reported an increase in heart rate in individuals using liraglutide[2,5,9]. In this regard, the actual effect of liraglutide on heart function, taken into account the wide range of cardiovascular actions, is uncertain.

South Asian ethnic groups in particular are at increased risk of developing type 2 diabetes[10]. South Asians appear to have a strong genetic predisposition for insulin resistance, while differences in lifestyle factors seem to have a smaller role in the increased risk of type 2 diabetes as compared with other ethnic groups[11]. The impaired insulin sensitivity in South Asians has been related to the relatively high total body fat percentage and high fat storage in the visceral compartments[12]. In addition, adipocytes may be dysfunctional, as reflected by the increased release of free fatty acids, adipokines and proinflammatory cytokines among South Asian individuals[13,14]. Previously, it has been demonstrated that hyperglycemia is more detrimental for cardiac function in South Asians than in Europeans[15]. As the pathogenesis of type 2 diabetes but also the impact of type 2 diabetes on cardiac function appears to be different, the cardiometabolic effects of liraglutide in the treatment of type 2 diabetes may be more pronounced in South Asians compared with individuals of other ethnicities.

In this study, we aimed to assess the effects of 26-week liraglutide treatment among South Asian type 2 diabetes patients on LV diastolic and systolic function and, secondary, myocardial steatosis and diffuse fibrosis. We used cardiovascular magnetic resonance, as this imaging modality enables the measurement of LV diastolic and systolic function and aortic stiffness[16,17] and also the assessment of myocardial tissue characteristics[18,19].

MATERIALS AND METHODS

Study Design And Participants

This study is a 26-week double-blind randomized controlled trial (ClinicalTrials.gov NCT02660047)[20]. Written informed consent was obtained prior to inclusion. This study complied with the revised Declaration of Helsinki and was approved by the institutional research board and the Central Committee on Research Involving Human Subjects.

Patients were recruited from the outpatient clinic of the Leiden University Medical Center (Leiden, the Netherlands), local hospitals and general practices in Leiden and The Hague, and by advertisements in local newspapers. Individuals aged 18-75 years of South Asian ancestral origin with type 2 diabetes treated with metformin, sulfonylurea derivatives and/or insulin for at least 3 months in stable dose were eligible for participation. South Asian descent was defined as both biological parents and their ancestors being South Asian (i.e. South Asian Surinamese, Indian, Pakistani, Bangladeshi or Sri Lankan origin). Inclusion criteria were: BMI ≥23 kg/m^2; HbA1c ≥6.5 and <11.0% (≥47.5 and <96.5 mmol/mol); estimated glomerular filtration rate (eGFR) >30 mL/min/1.73 m^2; blood pressure <180/110 mmHg. Main exclusion criteria were: use of GLP-1 receptor agonists; dipeptidyl peptidase 4 inhibitors or thiazolidinediones within the past 6 months; heart failure New York Heart Association (NYHA) class III-IV; acute coronary or cerebrovascular accident in the preceding 30 days; pancreatitis or medullary thyroid carcinoma; gastric bypass surgery; pregnant or lactating women; any contra-indication for magnetic resonance imaging (MRI). Due to the insufficient number of eligible patients, several criteria were adjusted (initial inclusion criteria: age 18-70 years; HbA1c ≥7.0 and <10.0% (≥53 and <86 mmol/mol); eGFR >60 mL/min/1.73 m^2; blood pressure <150/85 mmHg; no history of cardiovascular disease).

Randomization, Blinding And Intervention

Patients were randomized to once-daily subcutaneous injections of liraglutide (Victoza®, Novo Nordisk A/S, Bagsvaerd, Denmark) or placebo added to standard care during 26 weeks (randomization with block size 4, with 1:1 stratification for sex and insulin use). A randomization code list was generated by the institutional research pharmacist. If necessary to prevent hypoglycemia, the concomitant glucose-lowering medication was

adjusted at study entry. Starting dose of the trial medication was 0.6 mg/day, which was increased every 7 days up to 1.8 mg/day. The dose was reduced upon poor tolerance. Investigators and patients were blinded to treatment allocation. Furthermore, the MRI data were stripped of any information on the participant's identity and measurement date.

Study Procedures

Study days at baseline and after 26 weeks consisted of clinical measurements and MRI. Baseline and follow-up measurements were both scheduled either in the morning or evening. Patients were asked to fast overnight or for 6 hours, when measurements were in the morning or evening, respectively. To prevent hypoglycemia during fasting, insulin dose was adjusted and other anti-diabetic medications were temporarily discontinued. Patients were instructed to adhere to their usual diet and physical activity. During the trial, patients received a weekly telephone call for glycemic control based on their self-monitored blood glucose levels. At week 4 and 12, routine blood tests and clinical measurements were performed. Glycemic control and blood pressure management was according to the current guidelines [21,22]. Patients were asked for adverse events once a week. Study drug pens were collected during the trial as a surrogate marker of compliance.

MRI Protocol

MRI scans were acquired on a 3 Tesla MR scanner (Ingenia, Philips Healthcare, Best, the Netherlands). For contrast-enhanced MRI, 0.15 mmol gadoterate meglumine (0.5 mmol/mL Dotarem; Guerbet, Villepinte, France) per kilogram of body weight was administered intravenously. LV systolic and diastolic function parameters were assessed by short-axis and 4-chamber cine balanced steady-state free precession (bSSFP) and whole-heart gradient-echo 4D velocity-encoded MRI, with retrospective ECG (electrocardiography) gating. To determine aortic stiffness, the aortic pulse wave velocity (PWV) was calculated from a scout view of the aorta and two 2D velocity-encoded scans at the ascending and abdominal aorta. Myocardial steatosis was quantified as the myocardial triglyceride content, examined by proton-magnetic resonance spectroscopy (^{1}H-MRS) in the mid-ventricular septum and expressed as the amplitude of triglyceride methylene divided by the amplitude of unsuppressed water, multiplied by 100%. Myocardial diffuse fibrosis was assessed using native and post-contrast modified Look-Locker inversion (MOLLI) recovery T1 mapping. Native T1 and the extracellular volume (ECV) were measured in the mid-ventricular septum. To identify ischemic scarring, late gadolinium enhancement (LGE) MRI was acquired. If septal delayed enhancement was present, myocardial triglyceride content data was excluded and diffuse fibrosis was measured outside the region with scar. LGE-MRI was assessed visually by a radiologist (H.J.L.) and clinical investigator (E.H.M.P.) with 25 and 4 years of experience in cardiovascular MRI, respectively. A detailed description of the MRI protocol is provided as **Supplementary Material**.

Study Endpoints

Primary endpoints were LV diastolic function (peak deceleration slope of the transmitral early peak filling rate (Edec), ratio of transmitral early and late peak filling rate (E/A), early peak diastolic mitral septal tissue velocity (Ea), estimated LV filling pressure (E/Ea)) and LV systolic function (ejection fraction, stroke volume, cardiac output, cardiac index, peak ejection rate). Secondary endpoints included myocardial triglyceride content, ECV, aortic PWV, LV dimensions and clinical parameters (heart rate, blood pressure, body weight and HbA1c).

Statistical Analysis

Statistical analyses were performed with SPSS version 23 (IBM Corporation, Chicago, Illinois, USA), according to intention-to-treat. Within-group differences from baseline to 26 weeks were reported as means ± SD. Between-group differences for liraglutide vs. placebo were analyzed using ANCOVA with the baseline values as covariate to reduce within- and between-group variability and were reported as means (95%CI). Statistical tests were 2-sided and $P<0.05$ was considered significant. The power calculation is described in the **Supplementary Material**.

RESULTS

Baseline Characteristics

Patients were recruited between July 16, 2015 and December 6, 2017. A total of 47 patients were randomized to liraglutide (n=22) or placebo (n=25) (**Figure 1**). Between October 7, 2015 and March 9, 2018, all participants completed the trial. There were no clinically relevant differences between the treatment groups regarding demographics and clinical, laboratory and MRI parameters (**Table 1**). The total study population (40% men) had a mean (SD) age of 55 ± 10 years, a diabetes duration of 18 ± 10 years and HbA1c of 8.4 ± 1.0% (68 ± 11 mmol/mol), whilst 77% of the patients was using insulin.

Drug Compliance And Clinical Parameters

Study drug compliance was high (95 ± 8% and 99 ± 5% for liraglutide and placebo treatment, respectively) and the dose could be titrated up to 1.8 mg/day in most patients (in 86% and 96% of the patients treated with liraglutide and placebo, respectively). For glycemic control, in some patients in the placebo group, concomitant medication was started (metformin (n=1) or sulfonylurea derivates (n=3)) or the insulin dose was adjusted (1 ± 23 and -11 ± 34 units/day in the placebo and liraglutide group, respectively). For blood pressure management, in some patients in the placebo and liraglutide group, antihypertensive mediation was started or the dose was elevated (n=5 vs. n=3) or the dose was reduced (n=1 vs. n=2).

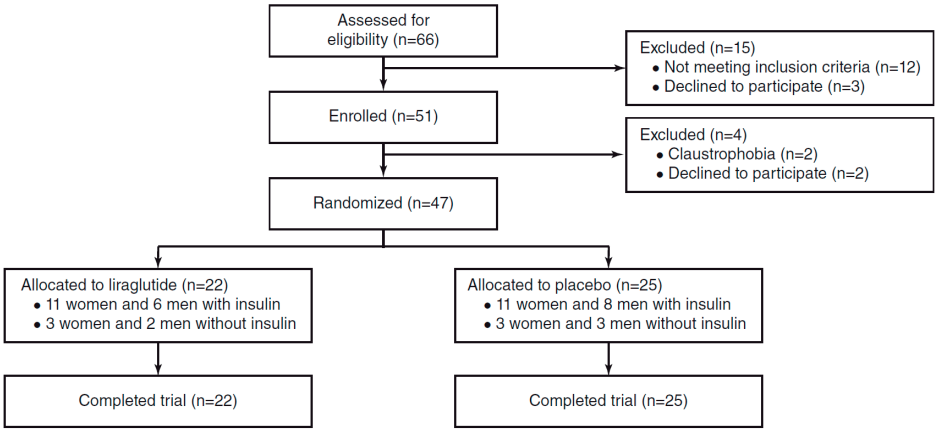

Figure 1. Trial profile.

Table 1. Baseline Characteristics

	Liraglutide (n=22)	Placebo (n=25)
Demographic and clinical characteristics		
Age, years	55 (11)	55 (9)
Men, no.	8 (36%)	11 (44%)
Diabetes duration, years	19 (10)	17 (10)
Diabetes complications, no.	15 (68%)	16 (64%)
Coronary artery disease, no.		
Non-significant coronary artery stenosis	4 (18%)	0 (0%)
Percutaneous coronary intervention	2 (9%)	3 (12%)
Coronary artery bypass grafting	1 (5%)	2 (8%)
Smoking, no.		
Currently	2 (9%)	5 (20%)
Previously	6 (27%)	0 (0%)
Never	14 (64%)	20 (80%)
Medication		
Metformin, no.	22 (100%)	23 (92%)
Sulfonylurea derivatives, no.	3 (14%)	5 (20%)
Insulin, no.	17 (77%)	19 (76%)
Metformin dose, g/day	1.8 (0.7)	1.7 (0.6)
Insulin dose, units/day	77 (34)	67 (30)
Lipid-lowering drugs, no.	17 (77%)	20 (80%)
Anti-hypertensive drugs, no.	16 (73%)	18 (72%)
Beta-blockers, no.	8 (36%)	9 (36%)
Diuretics, no.	9 (41%)	8 (32%)
ACE-inhibitors, no.	6 (27%)	7 (28%)

Table 1. Baseline Characteristics (*continued*)

	Liraglutide (n=22)	Placebo (n=25)
Angiotensin II receptor-blockers, no.	7 (32%)	9 (36%)
Calcium-antagonists, no.	2 (9%)	5 (20%)
Clinical parameters		
Weight, kg	82 (11)	78 (12)
BMI, kg/m^2	30.4 (3.8)	28.6 (4.0)
Waist circumference, cm	104 (8)	98 (10)
Waist-hip ratio	1.00 (0.07)	0.95 (0.09)
Heart rate, bpm	73 (13)	77 (11)
Systolic blood pressure, mmHg	149 (25)	141 (18)
Diastolic blood pressure, mmHg	85 (11)	85 (10)
Laboratory parameters		
HbA1c, %	8.1 (0.9)	8.6 (1.1)
HbA1c, mmol/mol	65 (10)	70 (12)
Triglycerides, mmol/L	1.6 (0.9)	2.1 (1.8)
Total cholesterol, mmol/L	4.0 (0.6)	4.5 (1.1)
HDL-cholesterol, mmol/L	1.2 (0.3)	1.2 (0.3)
LDL-cholesterol, mmol/L	2.0 (0.7)	2.2 (1.0)
LV diastolic function		
Edec, mL/s^2 x10^{-3}	-2.5 (1.3)	-2.7 (1.2)
E, mL/s	305 (99)	328 (118)
A, mL/s	316 (75)	306 (58)
E/A	0.99 (0.31)	1.11 (0.43)
E, cm/s	34 (9)	37 (9)
Ea, cm/s	5.3 (2.1)	5.7 (1.9)
E/Ea	7.4 (3.9)	7.4 (3.3)
LV systolic function		
Stroke volume, mL	70 (12)	67 (15)
Ejection fraction, %	56 (8)	57 (7)
Cardiac output, L/min	4.7 (0.9)	4.7 (1.1)
Cardiac index, L/min/m^2	2.4 (0.4)	2.5 (0.4)
Peak ejection rate, mL/s	338 (82)	345 (84)
LV structure		
End-diastolic volume, mL	128 (25)	120 (36)
End-systolic volume, mL	57 (21)	53 (24)
Mass, g	98 (22)	96 (24)
Aortic stiffness		
Aortic pulse wave velocity, m/s	8.8 (2.4)	8.3 (2.4)
Myocardial tissue characteristics		

Table 1. Baseline Characteristics (*continued*)

	Liraglutide (n=22)	Placebo (n=25)
Myocardial triglyceride content, %	0.92 (0.43)	1.00 (0.58)
Native T1 relaxation time, ms	1264 (45)	1254 (33)
Extracellular volume, %	25.9 (3.1)	27.0 (2.6)

Data are presented as mean (SD) or no. (%). Diabetes complications: retinopathy, neuropathy, nephropathy or macrovascular complications. A: late transmitral peak filling rate; E: early transmitral peak filling rate; Ea: early peak diastolic mitral septal tissue velocity; E/Ea: estimation of LV filling pressure; Edec: early deceleration peak.

In both the liraglutide and placebo group there was a decrease (mean ± SD) after 26 weeks in HbA1c (-0.8 ± 1.0 vs. -0.6 ± 0.8% (-9 ± 11 vs. -7 ± 9 mmol/mol) and systolic blood pressure (-14 ± 18 vs. -7 ± 15 mmHg), but not in diastolic blood pressure (-3 ± 11 vs. -3 ± 9 mmHg). However, between-group differences for liraglutide vs. placebo in HbA1c (-0.4% (95%CI: -0.9 to 0.2); -4 mmol/mol (95%CI: -10 to 2), *P*=0.16) and systolic blood pressure (-3 mmHg (95%CI: -9 to 3, *P*=0.36)) were non-significant. Liraglutide compared with placebo decreased body weight (-3.9 ± 3.6 vs. -0.6 ± 2.2 kg; between-group difference: -3.5 kg (95%CI: -5.3 to -1.8, *P*<0.001)) and increased heart rate (9 ± 11 vs. -2 ± 8 bpm; between-group difference: 10 bpm (95%CI: 4 to 15, *P*=0.001)).

LV Function, Aortic Stiffness And Myocardial Tissue Characteristics

LV diastolic function parameters were unaltered by liraglutide. Also, LV systolic function was unaffected upon liraglutide, as ejection fraction and peak ejection rate were unchanged and, despite the decreased stroke volume, cardiac output and cardiac index were preserved (**Table 2 and Figure 2**). The decrease in stroke volume was in parallel with the reductions in end-diastolic and end-systolic volume, which persisted when adjusting for body surface area. Whereas liraglutide significantly reduced end-diastolic volume, the decrease in LV mass was not significant. Also, liraglutide did not change aortic stiffness, myocardial triglyceride content or diffuse fibrosis. A total of 6 patients had delayed enhancement at baseline, but in only one patient the ventricular septum was involved. In all patients, the extent of delayed enhancement was unchanged at follow-up. Details on missing values are provided as **Supplementary Material**.

Adverse Events

There was one serious adverse event in the placebo group (admission for acute coronary syndrome symptoms without requiring further treatment). More patients with treatment with liraglutide compared with placebo had complaints of nausea (73% vs. 40%) and vomiting (27% vs. 8%). There were no cases of severe hypoglycemia.

Table 2. Study Endpoints: Mean Change Over 26 Weeks

	Mean change (SD) from 0 to 26 weeks		Mean change (95%CI) from 0 to 26 weeks	
	Liraglutide (n=22)	**Placebo (n=25)**	**(Liraglutide vs. Placebo)**	***P* value**
Primary				
LV diastolic function				
Edec, mL/s^2 x10^{-3}	0.2 (1.1)	0.1 (0.7)	0.2 (-0.3 to 0.6)	0.46
E, mL/s	-36 (84)	-18 (55)	-24 (-60 to 12)	0.18
A, mL/s	17 (77)	-2 (45)	18 (-21 to 56)	0.35
E/A	-0.11 (0.24)	-0.05 (0.24)	-0.09 (-0.23 to 0.05)	0.21
E, cm/s	-2 (7)	-1 (7)	-2 (-6 to 1)	0.20
Ea, cm/s	-0.1 (1.1)	-0.1 (1.1)	-0.1 (-0.7 to 0.5)	0.73
E/Ea	-0.4 (2.4)	-0.3 (2.6)	0.1 (-1.2 to 1.3)	0.89
LV systolic function				
Ejection fraction, %	0 (5)	0 (3)	0 (-3 to 2)	0.86
Stroke volume, mL	-10 (9)	0 (7)	-9 (-14 to -5)	<0.001
Cardiac output, L/min	-0.2 (0.5)	-0.1 (0.5)	-0.1 (-0.4 to 0.2)	0.44
Cardiac index, L/min/m^2	-0.1 (0.3)	-0.1 (0.3)	0.0 (-0.2 to 0.1)	0.87
Peak ejection rate, mL/s	-9 (60)	-7 (45)	-3 (-34 to 27)	0.83
Secondary				
LV structure				
Mass, g	-4 (9)	0 (7)	-4 (-9 to 0)	0.07
End-diastolic volume, mL	-19 (13)	-1 (11)	-17 (-24 to -10)	<0.001
End-systolic volume, mL	-9 (9)	-1 (7)	-7 (-11 to -3)	0.001
Aortic stiffness				
Aortic pulse wave velocity, m/s	0.2 (2.1)	-0.2 (1.7)	0.5 (-0.6 to 1.6)	0.35
Myocardial tissue characteristics				
Myocardial triglyceride content, %	0.14 (0.47)	-0.09 (0.56)	0.21 (-0.09 to 0.51)	0.16
Native T1 relaxation time, ms	-6 (36)	6 (26)	-7 (-21 to 7)	0.35
Extracellular volume, %	0.5 (2.6)	0.4 (1.3)	-0.2 (-1.4 to 1.0)	0.76

Abbreviations as in Table 1.

DISCUSSION

In this double-blind, randomized controlled trial in type 2 diabetes patients of South Asian descent living in the Netherlands, with or without coronary artery disease, 26-week treatment with 1.8 mg/day liraglutide had no effect on LV diastolic and systolic function, aortic stiffness, myocardial triglyceride content or extracellular volume, as compared with placebo when added to standard care. Our results imply that liraglutide does not amend cardiovascular remodelling in diabetic cardiomyopathy in a Dutch

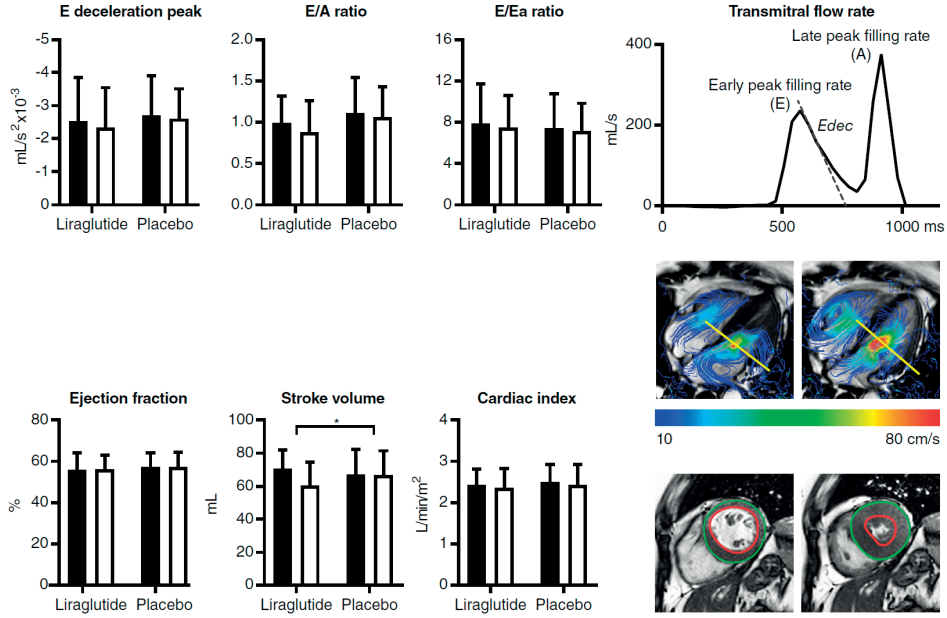

Figure 2. Liraglutide does not alter left ventricular (LV) diastolic and systolic function in South Asian type 2 diabetes patients with or without coronary artery disease and without advanced heart failure. LV diastolic and systolic outcome measures (mean ± SD) before (black bars) and after (white bars) treatment with liraglutide and placebo are presented. An example of a transmitral flow rate curve, 4D velocity-encoded and short-axis cine magnetic resonance is provided for illustration. E/A: ratio of transmitral early and late peak filling rate; E/Ea: estimation of LV filling pressure; Ea: early peak diastolic mitral septal tissue velocity; Edec: early deceleration peak.

South Asian type 2 diabetes population including patients with pre-existing ischemic heart disease, at least not upon a treatment period of 26 weeks.

Mechanisms

Whether liraglutide exerts direct actions on the ventricles, such as enhancement of coronary blood flow and myocardial glucose uptake, has been debated[2]. The GLP-1 receptor has been demonstrated to be present on the sinoatrial node and atrial cardiomyocytes[23], but its function as well as its presence on ventricular cardiomyocytes and blood vessels in humans is still uncertain. Furthermore, it has been suggested that the cardioprotective effects of native GLP-1 as described in earlier studies may be related to actions of degradation products of GLP-1, which are not produced by GLP-1 analogues[2]. We hypothesized that liraglutide may reverse diabetic cardiomyopathy, partly as a result of its indirect cardiovascular actions[5-7]. However, based on our findings, at least large immediate effects on LV function in South Asian type 2 diabetes patients can be excluded.

The reductions in LV end-diastolic volume and stroke volume in our study were not explained by liraglutide-induced body weight loss. It is conceivable that the decreased end-diastolic volume and stroke volume were related to the increased heart rate and consequent reduced ventricular filling time. Notably, the elevation in heart rate in our study was relatively large compared with other trials with the same dose and of similar duration[2,5,9]. Proposed mechanisms for the heart rate acceleration upon treatment with GLP-1 receptor agonists include enhancement of the sympathetic activity[24] and inhibition of the cardiac vagal neurons[25] as well as direct sinoatrial node stimulation[9]. Our study population included patients with prevalent coronary artery disease. It has been suggested that individuals with pre-existing cardiac disease may be more susceptible to heart rate acceleration upon GLP-1 receptor agonists[2]. Furthermore, South Asians may have an altered balance in the autonomic nervous system[26], which may have contributed to the profound heart rate elevation by liraglutide treatment in our study population.

Previous Studies

Only a few previous studies, including two open-label randomized controlled trials[27,28] and one small double-blind randomized controlled trial[29], assessed the effect of liraglutide on diastolic function in type 2 diabetes, during an intervention period of 4 to 6 months. One study demonstrated an improvement in myocardial relaxation in response to liraglutide, with amelioration of aortic stiffening[28], whereas others reported no improvement of diastolic function[27,29]. Large trials on the impact of liraglutide on systolic function have been previously performed in heart failure with reduced ejection fraction, where no effect was reported[30,31]. Regarding the impact of GLP-1 receptor agonists on myocardial tissue characteristics, most research has been limited to preclinical studies. In animal models of type 2 diabetes, liraglutide has been shown to reduce cardiac fibrosis[32], possibly by inhibition of the endoplasmic reticulum (ER) stress pathway via activation of the AMP-activated protein kinase (AMPK) system[33,34]. Activation of AMPK, which acts as a regulator of cellular energy status, has also been proposed as the underlying mechanism for improved cardiac function in type 2 diabetes after liraglutide as observed in preclinical research[33]. Furthermore, GLP-1 receptor agonists have been shown to relieve the intramyocardial lipid deposition in diabetic mice, in association with ameliorated levels of plasma cholesterol[35]. However, attenuation of myocardial steatosis by treatment with GLP-1 receptor agonists in type 2 diabetes has not been confirmed in human studies[36].

In a recent double-blind randomized controlled trial on the effect of liraglutide on cardiac function in European type 2 diabetes patients[37], liraglutide decreased the LV filling pressure, presumably through natriuresis and vasorelaxation, whereas myocardial relaxation was unaltered. Apart from ethnicity, the present South Asian cohort was distinct

from this European study group regarding sex (40% vs. 59% men), diabetes duration (18 ± 10 vs. 11 ± 7 years), insulin use (77% vs. 65%) and ischemic heart disease (17% vs. 0%). As there have been no large-scale clinical studies, it remains unknown whether certain patient characteristics have a modifying role in the cardiovascular actions of liraglutide.

Strengths And Limitations

The most important strengths of the present study are related to its double-blind, randomized controlled design, the absence of drop-outs and high study drug compliance. Liraglutide was added to standard care, mimicking the real-world setting. There are some limitations which need to be addressed. This trial comprised South Asian individuals living in a high-income country and included predominantly South Asian Surinamese, who originate from the northern part of India. Extrapolation of our results to other South Asian ethnic groups should be performed with caution. Furthermore, we did not use echocardiography, which is the routine clinical approach for evaluating diastolic function. Nonetheless, MRI is widely used in clinical studies for the assessment of diastolic function and, importantly, it has been validated with echocardiography[38]. It has to be noted that in individuals with high heart rate (>100 bpm), early and late diastolic filling cannot be separated. As a consequence, two participants in the liraglutide group had missing data for diastolic function at follow-up, which might have introduced bias. The LV diastolic function parameters in our study population, as well as aortic pulse wave velocity, were approximately one standard deviation from the mean in healthy individuals[39]. However, in contrast to the clear impairments in LV diastolic function, the myocardial triglyceride content was 0.92-1.00% in this type 2 diabetes cohort, whereas the values in healthy controls are approximately 0.58% and 0.84% among Europeans and South Asians, respectively[39]. The type 2 diabetes patients in the present study did not demonstrate abnormalities in extracellular volume, possibly as a result of angiotensin-converting enzyme (ACE) inhibitors which may relieve fibrotic remodelling[39]. Hence, we cannot exclude a beneficial effect of liraglutide on extracellular volume in type 2 diabetes patients with marked cardiac fibrosis. Also, we cannot preclude cardiovascular benefits after prolonged (>26 weeks) therapy with liraglutide. Nevertheless, in animal studies, improved myocardial function has been reported already after brief (1 week) treatment with liraglutide[33].

Implications

In our study, liraglutide did not enhance heart function and may therefore have no specific role in the prevention of heart failure with preserved ejection fraction in South Asian type 2 diabetes patients. In contrast, recent studies have indicated that the sodium-glucose co-transporter 2 (SGLT2) inhibitors empagliflozin, canagliflozin and dapagliflozin have a benefit on the incidence of heart failure[4], potentially because of di-

rect improvement of myocardial relaxation in addition to diuretic effects[40]. Conversely, the previously reported reduced cardiovascular mortality rate in response to liraglutide among patients with type 2 diabetes and high cardiovascular risk is probably primarily related to slowed progression of atherosclerosis[3,41]. Following the results from recent cardiovascular outcome trials[3,4], SGLT2 inhibitors have been recommended as part of type 2 diabetes management among individuals with co-existing heart failure or at risk of heart failure, and either GLP-1 receptor agonists or SGLT2 inhibitors should be considered in type 2 diabetes patients with established atherosclerotic disease and no specific concerns of heart failure[22]. Our study did not demonstrate regression of LV diastolic dysfunction in response to liraglutide. Nonetheless, because of its presumed anti-atherosclerotic actions, GLP-1 receptor agonists remain worth considering especially in South Asian type 2 diabetes patients given their disadvantageous cardiometabolic profile and high risk of ischemic heart disease[10].

Conclusion

In conclusion, in this 26-week double-blind randomized placebo-controlled trial in Dutch South Asian type 2 diabetes patients with or without coronary artery disease, liraglutide had no effect on LV diastolic and systolic function, nor on aortic stiffness, myocardial triglyceride content and extracellular volume. A previous study reported a reduced LV filling pressure after liraglutide therapy in a European cohort of type 2 diabetes patients without ischemic heart disease, who were predominantly men, with a shorter diabetes duration and less use of insulin as compared with the South Asian type 2 diabetes patients in the present study[37]. Further research should reveal whether the cardiovascular impact of liraglutide might be dependent on patient characteristics such as sex, ethnicity, diabetes duration, co-medication or history of ischemic heart disease.

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SUPPLEMENTARY MATERIAL

MRI acquisition and analysis

LV Systolic And Diastolic Function

MRI scans were acquired on a 3 Tesla magnetic resonance scanner (Ingenia, Philips Healthcare, Best, the Netherlands), using a dStream Torso anterior coil and a FlexCoverage posterior coil in the table top, resulting in up to 32 coil elements for signal reception. LV dimensions and systolic and diastolic function were quantified by standard short-axis and 4-chamber long-axis cine balanced steady-state free precession (bSSFP) and free-breathing whole-heart gradient-echo 4D velocity-encoded MR, with retrospective electrocardiography (ECG) gating. Typical imaging parameters of the bSSFP cines were: echo/repetition time (TE/TR) 1.5/3.0 ms, flip angle (FA) 45°, field-of-view (FOV) 350x350 mm^2 (4-chamber) and 400x352 mm^2 (short-axis), acquired voxel size 2.0x1.6 mm^2 (4-chamber) and 1.5x1.5 mm^2 (short-axis), slice thickness 8 mm, number of phases 30 (4-chamber) and 35 (short-axis). For short-axis bSSFP cine, the complete LV was imaged using 14-16 slices. 4D velocity-encoded (venc 150 cm/s) MR was acquired in parallel with the 4-chamber view, with typical imaging parameters: TE/TR 4.6/9.0 ms, FA 10°, FOV 360x360 mm^2, acquired voxel size 3.0x3.0 mm^2, slice thickness 3 mm, number of slices 41, number of phases 30, sensitivity encoding (SENSE) factor 2.

LV function and dimensions were assessed with MASS version 2015-EXP (Leiden University Medical Center, Leiden, the Netherlands). For LV diastolic function, the flow rate curves over the mitral valve were derived from the 4D velocity-encoded scans by retrospective mitral valve tracking, perpendicular to the streamlines of inflow across the mitral valve, at the location of peak flow velocity[1,2]. Subsequently, the ratio of the transmitral early (E) and late (A) peak filling rate (E/A ratio), the peak deceleration slope of the E wave (Edec) and the transmitral early peak velocity were calculated. The transmitral filling rate curves were corrected for the through-plane background velocity of the LV myocardial wall. The early peak diastolic mitral septal tissue velocity (Ea) was measured on 4-chamber cines. The mitral septal tissue velocity curves were calculated from the displacement of the semi-automatically tracked mitral valve, at the insertion to the LV septum, relative to the LV apex, throughout the cardiac cycle. The estimated LV filling pressure was defined as the ratio of the transmitral early peak velocity and Ea[3]. For LV dimensions and systolic function, LV endocardial and epicardial contours were semi-automatically drawn in the short-axis cines in the end-diastolic and end-systolic phase, to quantify the end-diastolic LV mass, LV end-diastolic and end-systolic volumes and subsequently LV stroke volume, ejection fraction, cardiac output and cardiac index.

The peak ejection rate was derived from the systolic flow rate curves over the aortic valve after retrospective valve tracking[4].

Aortic Stiffness

For aortic PWV a double-oblique sagittal scout view of the aorta and free-breathing through-plane 2D velocity-encoded MR scans were obtained (one transecting the proximal ascending aorta, with venc 200 cm/s, and one transecting the abdominal aorta above the bifurcation, with venc 150 cm/s). Typical imaging parameters were: TE/TR 2.5/4.4 ms, FA 20°, FOV 350x282 mm^2, slice thickness 8 mm, acquired voxel size 2.8x2.8 mm^2, temporal resolution 10 ms. Aortic PWV analyses were performed as previously described, using MASS version 2015-EXP (Leiden University Medical Center, Leiden, the Netherlands) and custom-made software for further analysis of the aorta velocity-time curves[5]. Aortic PWV was calculated by dividing the distance between ascending and abdominal aorta by the transit time of the onset of the systolic wave front.

Myocardial Tissue Characteristics

Myocardial triglyceride content was measured by proton-magnetic resonance spectroscopy (^{1}H-MRS), in a voxel of 40x15x25 mm^3 in the mid-ventricular septum, with ECG-triggering, using a respiratory navigator. Typical parameters were: TE 35 ms, TR 3.5 or 9 seconds (water-suppressed and non-water suppressed acquisition, respectively), acquired samples 2048 (spectral resolution 0.73 Hz/sample), number of signal averages 64 or 6 (water-suppressed and non-water suppressed acquisition, respectively). The signal-to-noise ratio was increased by a high permittivity pad on the thorax[6]. The myocardial triglyceride content was quantified using in-house developed software to assess the individual 64 water-suppressed and 6 non-water suppressed signals and the Java-based MR user interface (jMRUI v5.0; MRUI Consortium) to fit the averaged signal[7,8]. Prior knowledge for the fit included the following starting values: triglyceride methyl (CH_3) 0.9 ppm, triglyceride methylene $(CH_2)^n$ 1.3 ppm, $COO\text{-}CH_2$ 2.05 ppm, creatine 3.05 ppm, trimethylamines (TMA) 3.25 ppm. Myocardial lipid-to-water ratios were calculated as the signal of triglyceride methylene divided by the unsuppressed water signal, multiplied by 100%[9].

The extracellular volume (ECV) as a measure of myocardial diffuse fibrosis was determined using native and post-contrast T1 mapping (5s(3s)3s and 4s(1s)3s(1s)2s modified Look-Locker inversion recovery (MOLLI) scheme, respectively), obtained in short-axis orientation at the mid-ventricular level. Post-contrast T1 mapping was acquired 20-25 minutes after contrast administration. Typical imaging parameters were: TE/TR 1.1/2.3 ms, FA 20°, FOV 350x300 mm^2, slice thickness 8 mm, acquired voxel size 2.1x2.1 mm^2,

SENSE factor 2. ECV and T1 relaxation times were obtained using [blinded], in the mid-ventricular septum, after manual correction for motion of the T1 images

Late gadolinium enhanced (LGE) MRI was acquired 15-20 minutes after contrast administration, with an ECG-triggered 3D whole-heart gradient-echo phase-sensitive inversion recovery sequence, with respiratory navigating, as previously described[10]. LGE MRI was assessed visually by an experienced radiologist and a clinical investigator.

Power calculation

The power calculation for LV diastolic and systolic function, based on previous data on MRI-derived LV function in type 2 diabetes[11], showed that a total of 25 patients in each group would be needed to detect a change upon liraglutide of approximately 15-20% and 10-20% in diastolic and systolic function parameters, respectively, with at least 90% power (α=0.05) and estimated drop-out rate of 10%. Post-hoc power calculation for the secondary endpoints demonstrated a power of at least 90% (α=0.05) to detect an absolute change upon liraglutide of 0.40% in myocardial triglyceride content, 2.5% in ECV and 2.5 m/s in aortic PWV.

Missing data

At baseline, the number of missing values in the liraglutide group was: n=1 for LV diastolic function, and in the placebo group: n=1 for LV diastolic function and myocardial tissue characteristics. For the assessment of the difference between baseline and follow-up, the number of missing values in the liraglutide group was: n=3 for E, A, E/A ratio, Ea and LV filling pressure (n=1 excluded because of mitral valve stenosis, n=2 missing due to a heart rate of ≥100 bpm with fusion of the E/A peak at follow-up), n=4 for Edec peak (n=1 missing due to a heart rate of 96 bpm with partial fusion of the E/A peak at follow-up), n=1 for myocardial triglyceride content (excluded due to insufficient quality), and in the placebo group: n=1 for LV diastolic function (excluded because of mitral valve stenosis) and peak ejection rate (missing due to imaging time constraints), n=1 for myocardial triglyceride content (excluded due to ischemic scarring of the septum) and native T1 (excluded due to insufficient quality), n=2 for post-contrast T1 and extracellular volume (missing due to imaging time constraints). In both the liraglutide and placebo group n=1 was missing for study drug compliance.

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Chapter 4

Liraglutide decreases energy expenditure and does not affect the fat fraction of supraclavicular brown adipose tissue in patients with type 2 diabetes

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ABSTRACT

Background and Aims: Several studies have shown that glucagon-like peptide-1 (GLP-1) analogues can affect resting energy expenditure, and preclinical studies suggest that they may activate brown adipose tissue (BAT). The aim of the present study was to investigate the effect of treatment with liraglutide on energy metabolism and BAT fat fraction in patients with type 2 diabetes.

Methods and Results: In a 26-week double-blind, placebo-controlled trial, 50 patients with type 2 diabetes were randomized to treatment with liraglutide (1.8 mg/day) or placebo added to standard care. At baseline and after treatment for 4, 12 and 26 weeks, we assessed resting energy expenditure (REE) by indirect calorimetry. Furthermore, at baseline and after 26 weeks, we determined the fat fraction in the supraclavicular BAT depot using chemical-shift water-fat MRI at 3T. Liraglutide reduced REE after 4 weeks, which persisted after 12 weeks and tended to be present after 26 weeks (week 26 *vs* baseline: liraglutide -52 ± 128 kcal/day; P=0.071, placebo +44 ± 144 kcal/day; P=0.153, between group P=0.057). Treatment with liraglutide for 26 weeks did not decrease the fat fraction in supraclavicular BAT (-0.4 ± 1.7%; P=0.447) compared to placebo (-0.4 ± 1.4%; P=0.420; between group P=0.911).

Conclusion: Treatment with liraglutide decreases REE in the first 12 weeks and tends to decrease this after 26 weeks without affecting the fat fraction in the supraclavicular BAT depot. These findings suggest reduction in energy intake rather than an increase in REE to contribute to the liraglutide-induced weight loss.

INTRODUCTION

Together with the occurrence of obesity, type 2 diabetes prevalence has increased substantially over the past decades and was recently estimated to affect 422 million patients worldwide[1]. Diet and lifestyle interventions and treatment with oral glucose-lowering drugs are often insufficiently effective in decreasing blood glucose levels. As a result, many patients eventually require treatment with insulin to improve glycemic control. Unfortunately, insulin therapy commonly results in weight gain due to anabolic effects, which further increases insulin resistance[2,3]. Therefore, development of new therapeutic strategies for type 2 diabetes that do not induce weight gain is important.

A relatively new class of drugs without adverse effects on body weight are glucagon-like peptide-1 (GLP-1) analogues. GLP-1 is a peptide hormone that is released by the intestinal enteroendocrine L-cells in response to food intake. Binding of GLP-1 to its receptor results in numerous effects, including stimulation of insulin-dependent insulin secretion and reduction of glucagon release and food intake[4-6]. Liraglutide is a long-acting GLP-1 analogue that is well tolerated and improves glycemic control in patients with type 2 diabetes[7,8]. Furthermore, liraglutide is effective in reducing body weight rapidly in a dose-dependent manner, with sustained weight loss up to 2 years[8,9].

Although the weight loss can be partly explained by a reduction of food intake, several studies have shown that GLP1 analogues affect energy metabolism as well. Treatment with the GLP-1 analogues exenatide and liraglutide for one year increased energy expenditure in patients with type 2 diabetes[10]. In line, the GLP-1 analogue exendin-4 increased fatty acid oxidation in mice[11]. In fact, in rodents, GLP-1 analogues increased brown adipose tissue (BAT) thermogenesis and adipocyte browning, which suggests that GLP-1 analogues may cause weight loss at least partly by activation of BAT[10,11]. In contrast, other studies failed to demonstrate increased energy expenditure after treatment with GLP-1 analogues[12-14]. Differences in treatment duration, with some studies investigating effects after several weeks and others after treatment for a year, make it difficult to compare the studies. Therefore, further research is needed to investigate the short- and long-term effects of GLP-1 analogues on energy metabolism.

Although positron emission tomography with [^{18}F]-fluorodeoxyglucose integrated with computed tomography ([^{18}F]FDG PET-CT) is the gold standard to study BAT in humans, it has several limitations. It only provides information on glucose uptake in BAT, even though it is known that BAT predominantly burns free fatty acids derived from triglycerides[15]. Furthermore, PET-CT has a relatively low reproducibility at ambient temperatures and subjects are exposed to ionising radiation and a radiotracer[16]. An alternative

method to study BAT is by assessing the fat fraction using MRI[17-20]. MRI is non-invasive, does not use ionising radiation and investigates intrinsic morphological differences between BAT and white adipose tissue by measuring the fat fraction.

The aim of the present study was to investigate the effect of treatment with liraglutide on resting energy expenditure (REE) after 4, 12 and 26 weeks in patients with type 2 diabetes. Furthermore, we aimed to assess the effect of liraglutide on BAT fat fraction in the supraclavicular BAT depot.

METHODS

Study overview and study population

This study used data from the MAGNA VICTORIA (MAGNetic resonance Assessment of VICTOza efficacy in the Regression of cardiovascular dysfunction In type 2 diAbetes mellitus) study, a prospective, randomized, double-blind, clinical trial, which primarily investigated the effects of liraglutide on cardiac function. Overweight and obese (BMI ≥25 kg/m^2) patients with type 2 diabetes were recruited from November 2013 until March 2016 via advertisements and from the outpatient clinics of the Leiden University Medical Center (LUMC, Leiden, the Netherlands), general practitioners, and local hospitals. We included patients aged 18-69 years, treated with metformin, and with a HbA1c ≥7.0 and ≤10.0% (53-86 mmol/mol). Concomitant treatment with sulfonylurea derivatives and insulin was optional, but the dosage of all glucose-lowering medication needed to be stable for at least 3 months prior to participation. Exclusion criteria were use of other glucose-lowering therapy than mentioned above, presence of renal, hepatic or cardiovascular disease, and contra-indications for MRI. The trial was approved by the local ethics committee and performed in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all subjects before participation. The trial was conducted at the LUMC, and was registered at clinicaltrials.gov (NCT01761318).

Study design and data collection

At baseline, participants were randomized with 1:1 stratification for sex and insulin use (block size of 4) to receive treatment with liraglutide (Victoza®, Novo Nordisk A/S, Bagsvaerd, Denmark) or placebo. Participants visited the study center at baseline and after 26 weeks of treatment, after ≥6 h of fasting, for medical history assessment, standard physical examination, measurement of body composition and energy expenditure, collection of venous blood samples and MRI. After 4 and 12 weeks of treatment additional venous blood samples were collected and additional measurements of body composi-

tion and energy expenditure were performed. The starting dose of the study medication was 0.6 mg per day, which was titrated in two weeks to a maximum dose of 1.8 mg per day, if tolerated. In addition to study medication, participants received treatment according to current clinical guidelines to achieve optimal glycemic control and regulation of blood pressure and cholesterol levels. Furthermore, participants were instructed not to change their activity level or diet during study participation. Body composition was assessed using bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystat Ltd., Douglas, UK). REE, respiratory quotient and substrate oxidation rates were determined with indirect calorimetry using a ventilated hood system (Oxycon Pro™, CareFusion, Heidelberg, Germany). In addition, REE was corrected for lean body mass (LBM). All blood samples were centrifuged and plasma was stored at -80°C until analysis. Plasma cholesterol and triglyceride concentrations were measured on a Modular P800 analyser (Roche Diagnostics, Mannheim, Germany). LDL-cholesterol was calculated according to Friedewald's formula[21]. Due to changes in laboratory procedures during the study, in a subset of participants HbA1c was assessed with boronate affinity high-performance liquid chromatography (Primus Ultra, Siemens Healthcare Diagnostics, Breda, the Netherlands), while in the other patients HbA1c was assessed with ion-exchange HPLC (Tosoh G8, Sysmex Nederland B.V., Etten-Leur, the Netherlands).

MRI scans of BAT

MRI scans of BAT were performed in a random subset of subjects who were positioned head-first and in supine position at room temperature on a 3.0 Tesla Ingenia whole-body MR system (Philips Medical Systems, Best, the Netherlands). The right supraclavicular BAT depot was studied and a dielectric pad was placed on the chest in the right supraclavicular region. The body coil was used for transmission and reception was achieved with a dStream Torso anterior coil (Philips Medical Systems, Best, the Netherlands), the FlexCovarage posterior coil (Philips Medical Systems, Best, the Netherlands) in the table (combined resulting in up to 32 channels) and the Base of the HeadNeck coil (20 channels). A 3D 6-point DIXON scan was used to acquire 83 coronal slices in the supraclavicular region, with parameters set at: first echo time (TE) = 1.42 ms, delta TE = 1.2 ms, repetition time (TR) = 8.8 ms, flip angle 3°, a field of view of = 500 x 404 mm (right/left (RL) and foot/head (FH)), and voxel sizes of = 1.2 x 1.2 x 1 mm. Fat fraction maps were reconstructed off-line using an in-house developed water-fat separation algorithm in MATLAB (MathWorks, Natick, USA) considering the multi-peak fat spectrum and monoexponential effective transverse relaxation time (T2*) together with a region-growing scheme to mitigate strong main field inhomogeneity effects[22-25]. A higher fat fraction indicates less active 'whitened' BAT, while a lower fat fraction suggests more active BAT[17,18,26]. After reconstruction of the fat fraction map, a manual region of interest (ROI) was drawn precisely outlining the supraclavicular BAT depot on the first 40

slices posterior of the sternoclavicular joint using MASS research software V2017-EXP (LUMC, Leiden, the Netherlands). We chose to segment the supraclavicular area as this area was previously confirmed as BAT using [^{18}F]FDG PET-CT scans and histology[17,27,28]. The anatomical landmarks confining the supraclavicular BAT depot used were the sternoclavicular joint inferiorly, the acromion laterally, the trapezius superiorly and the sternocleidomastoid muscle medially. Care was taken not to include non-adipose tissue such as major blood vessels, bone, bone marrow and muscle, resulting in segmentation of the depot as shown in **Figure 2**. Within the ROIs fat fraction was measured to calculate the median fat fraction of the supraclavicular BAT depot in each participant using MASS software. The volume was calculated by multiplying the area in the ROI by the slice thickness. The observer was blinded for treatment group but not blinded for moment of acquisition (baseline or follow-up).

Statistical analyses

Data in tables are shown as means ± SD, and as mean change (95% CI). Data in graphs are presented as means ± SEM. Within-group changes from baseline to week 26 of clinical parameters and metabolic factors and of the fat fraction were assessed using paired *t*-tests. We performed an ANCOVA to assess between-group differences. A generalized least squares (GLS) model with a continuous autoregressive model of order 1, with treatment arm and time as factor, was used to compare change of energy expenditure and body composition during study participation after 0, 4, 12 and 26 weeks of treatment. A *P*-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, USA) and RStudio version 1.1.383 (RStudio, Boston, USA).

RESULTS

Population characteristics

Fifty participants were included, of whom 24 were randomized to receive liraglutide and 26 to receive placebo, as shown in the trial flow diagram in **Figure 1**. One participant of the liraglutide group was withdrawn from the study before starting treatment due to claustrophobia and was not included in the analyses. Furthermore, one participant of the liraglutide group was withdrawn from the study due to misdiagnosis of type 2 diabetes, and one participant of the placebo group was lost to follow-up due to imprisonment. All data collected of these two participants was used in analyses of the current study, but could not be included in the comparison between baseline and week 26. As shown in **Table 1**, characteristics of the participants in both treatment groups were comparable at baseline. The mean age was 59.9 ± 6.2 years in the liraglutide group, and

59.2 ± 6.8 years in the placebo group, with a body weight of 98.4 ± 13.8 *vs* 94.5 ± 13.1 kg. Diabetes duration and treatment was similar between groups and HbA1c was not different between the two groups.

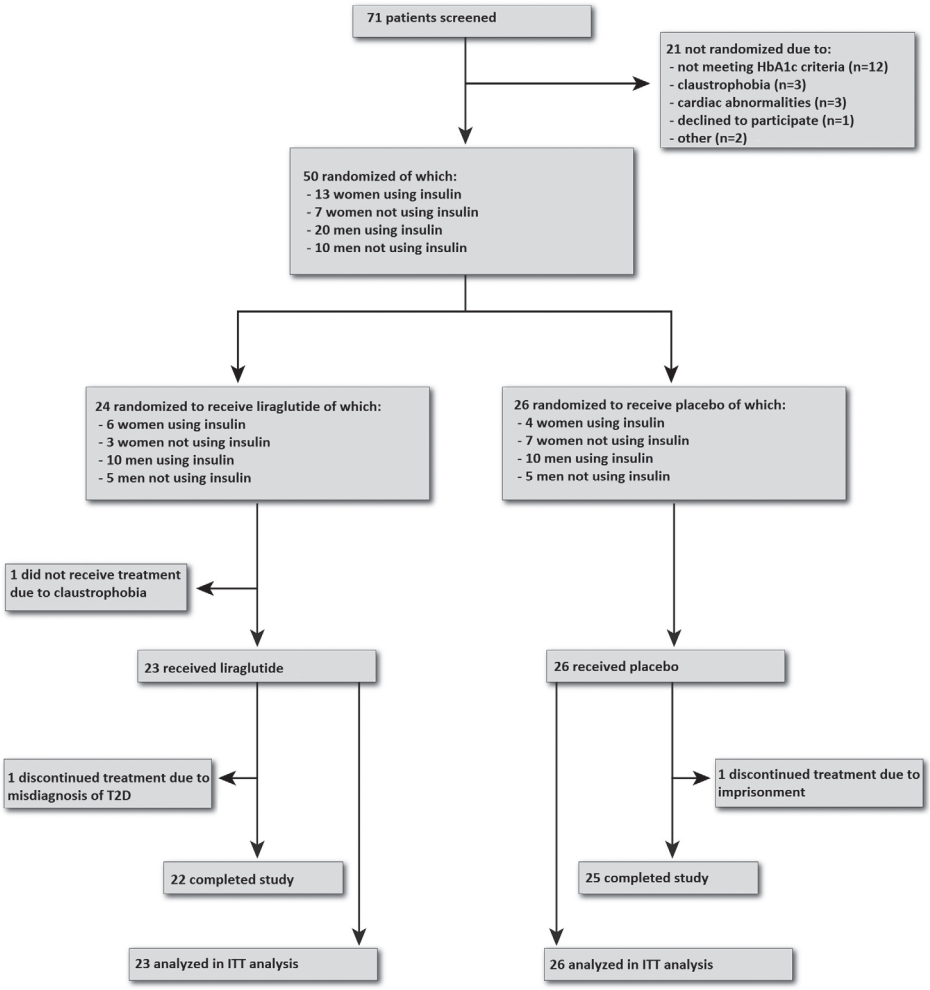

Figure 1. Trial flow diagram. ITT=intention-to-treat.

Treatment with liraglutide decreases body weight and lean body mass

Treatment with liraglutide for 26 weeks decreased body weight (-4.3 ± 3.8 kg; P<0.001) compared to placebo (+0.1 ± 2.5 kg; P=0.827; between group P<0.001), as shown in **Table 2**. Furthermore, LBM was decreased after treatment with liraglutide in contrast to treatment with placebo (-2.1 ± 2.9 kg; P=0.003, *vs* -0.2 ± 1.6 kg; P=0.455; between group P=0.012). HbA1c was improved after treatment with liraglutide (-1.1 ± 1.0% (-11.6

Table 1. Baseline characteristics of study participants.

Characteristic	Liraglutide (n = 23)	Placebo (n = 26)
Demographics		
Age (years)	59.9 (6.2)	59.2 (6.8)
Sex (no. (%))		
Male	14 (61%)	15 (58%)
Female	9 (39%)	11 (42%)
Diabetes duration (years)	11.3 (6.4)	10.9 (7.1)
Clinical parameters		
Bodyweight (kg)	98.4 (13.8)	94.5 (13.1)
BMI (kg/m^2)	32.6 (4.4)	31.6 (3.4)
Waist-hip ratio	1.03 (0.06)	1.03 (0.08)
Lean body mass (%)	63.9 (10.2)	63.4 (8.8)
Lean body mass (kg)	62.2 (9.4)	60.0 (12.1)
Metabolic factors		
Glucose (mmol/L)	8.7 (2.7)	7.3 (1.7)
HbA1c (%)	8.3 (1.1)	8.1 (0.9)
HbA1c (mmol/mol)	66.7 (11.5)	64.7 (10.2)
Total cholesterol (mmol/L)	4.82 (1.02)	4.80 (1.02)
HDL-cholesterol (mmol/L)	1.22 (0.25)	1.30 (0.39)
LDL-cholesterol (mmol/L)	2.60 (0.86)	2.55 (0.91)
Triglycerides (mmol/L)	2.19 (1.51)	2.10 (1.09)
Concomitant drug use		
Lipid lowering drugs (no. (%))	21 (91%)	19 (73%)
Metformin dose (g/day)	2.1 (0.7)	2.0 (0.5)
Sulfonylureas (no. (%))	6 (26%)	8 (31%)
Insulin (no. (%))	15 (65%)	17 (65%)
Insulin (units)	70 (46)	69 (58)

Results are presented as n (%) or mean (SD). n=49. Missing data in liraglutide-group: n=1 for LDL-cholesterol.

± 11.1 mmol/mol); P<0.001), but also after treatment with placebo (-0.7 ± 0.9% (-7.7 ± 9.4 mmol/mol); P<0.001; between group P=0.265). Furthermore, while HDL-cholesterol was not affected, total cholesterol, LDL-cholesterol and triglycerides were decreased in both groups (data not shown).

Table 2. Clinical parameters and metabolic factors change from baseline to after 26 weeks of treatment.

Characteristic	Mean (SD) change from baseline to 26 weeks		Mean (95% CI) changes from baseline (liraglutide vs. placebo)	P value
	Liraglutide (n=22)	Placebo (n=25)		
Clinical parameters				
Bodyweight (kg)	-4.3 (3.8)	0.1 (2.5)	-4.5 (-6.4 to -2.6)	**<0.001**
BMI (kg/m^2)	-1.5 (1.3)	0.1 (0.8)	-1.5 (-2.2 to -0.9)	**<0.001**
Waist-hip ratio	0.01 (0.04)	0.01 (0.04)	0.00 (-0.02 to 0.03)	0.692
Lean body mass (%)	0.5 (3.0)	-0.2 (2.2)	0.7 (-0.8 to 2.2)	0.333
Lean body mass (kg)	-2.1 (2.9)	-0.2 (1.6)	-1.7 (-3.1 to -0.4)	**0.012**
Metabolic factors				
Glucose (mmol/L)	-1.7 (2.2)	-0.6 (2.2)	-0.5 (-1.7 to 0.7)	0.434
HbA1c (%)	-1.1 (1.0)	-0.7 (0.9)	-0.3 (-0.8 to 0.2)	0.265
HbA1c (mmol/mol)	-11.6 (11.1)	-7.7 (9.4)	-2.9 (-8.1 to 2.3)	0.265
Total cholesterol (mmol/L)	-0.72 (0.89)	-0.46 (0.56)	-0.22 (-0.59 to 0.15)	0.231
HDL-cholesterol (mmol/L)	-0.02 (0.14)	0.05 (0.25)	-0.08 (-0.20 to 0.05)	0.222
LDL-cholesterol (mmol/L)	-0.44 (0.51)	-0.24 (0.52)	-0.17 (-0.44 to 0.10)	0.218
Triglycerides (mmol/L)	-0.50 (1.12)	-0.60 (0.98)	0.23 (-0.11 to 0.56)	0.177

Results are presented as mean (SD). n=47. Missing data in liraglutide-group: n=1 for LDL-cholesterol and glucose.

Treatment with liraglutide decreases REE without affecting glucose and lipid oxidation rates

As shown in **Figure 3**, liraglutide reduced REE after 4 weeks, which persisted after 12 and tended to be present after 26 weeks (week 26 *vs* baseline: liraglutide -52 ± 128 kcal/day; P=0.071, placebo +44 ± 144 kcal/day; P=0.153, between group P=0.057). Similarly, body weight was already decreased after 4 weeks of treatment with liraglutide. After correction for LBM, an important contributor to REE, liraglutide did not significantly decrease REE after 12 and 26 weeks. However, the decrease of REE after treatment with liraglutide for 4 weeks was still present after correction for LBM. No changes in glucose and lipid oxidation rates were observed after treatment with either liraglutide or placebo.

Treatment with liraglutide does not affect fat fraction of the supraclavicular BAT depot

We measured the fat fraction in the supraclavicular BAT depot in 22 participants, 10 of the liraglutide group and 12 of the placebo group. Characteristics of demographics, clinical parameters and metabolic factors of the subset of subjects in which we investigated BAT were representative of the total study group (not shown). Two MRI scans, one baseline measurement of the liraglutide group and one follow up measurement of the placebo group, were excluded from analyses due to presence of multiple artefacts. At

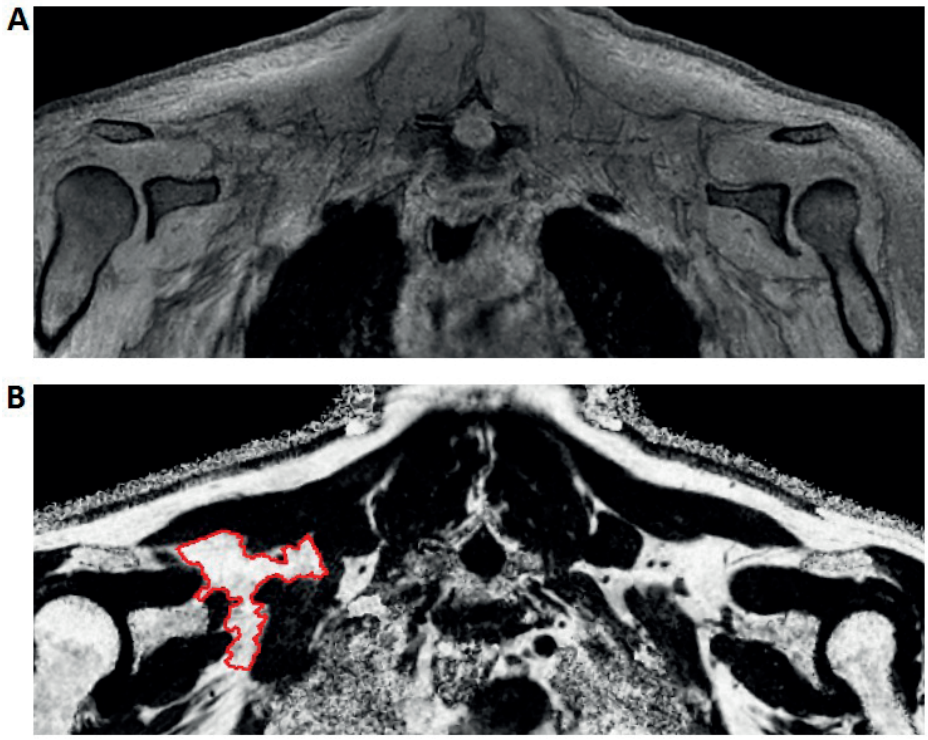

Figure 2. MRI 6-point DIXON scan showing the supraclavicular fat depot. Coronal first echo image (A). Shown in red (B) is an example of a manually drawn region of interest.

baseline, the fat fraction in the liraglutide group was 91.4 ± 1.7% and was measured in a volume of 62 ± 28 mL, while in the placebo group a fat fraction of 91.5 ± 1.7%, measured in a volume of 61 ± 22 mL. After treatment, the fat fraction in the liraglutide group was 90.7 ± 2.8%, measured in a volume of 59 ± 14 mL, and in the placebo group 91.1 ± 2.5%, measured in 63 ± 12 mL. Treatment with liraglutide for 26 weeks did not decrease fat fraction (-0.4 ± 1.7%; P=0.447) compared to placebo (-0.4 ± 1.4%; P=0.420; between group P=0.911), as shown in **Figure 4**.

DISCUSSION

In this study we observed that treatment of patients with type 2 diabetes with liraglutide decreased body weight, which was accompanied by decreased REE in the first 12 weeks and a tendency to a decreased REE after 26 weeks compared to placebo without affecting substrate oxidation rates. Furthermore, liraglutide did not affect the fat fraction of the supraclavicular BAT depot after 26 weeks. These findings imply that treatment with

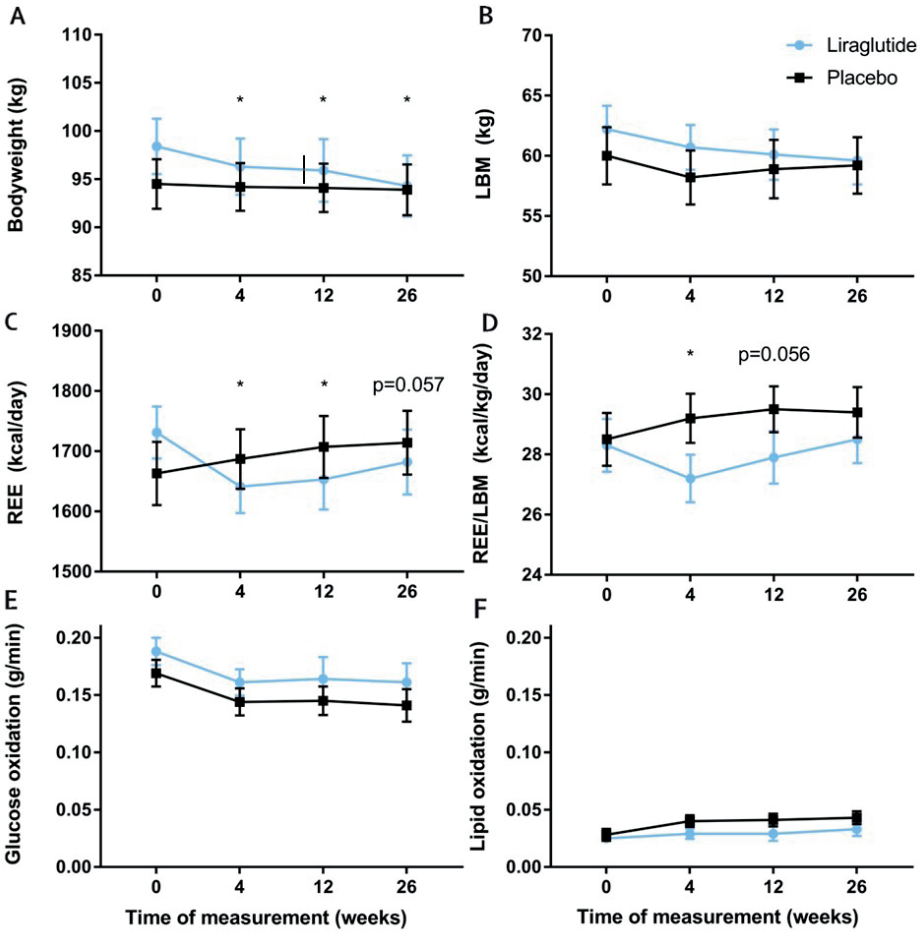

Figure 3. The effect of liraglutide and placebo on bodyweight and energy expenditure after 4, 12 and 26 weeks of treatment. Results are shown for bodyweight (A), lean body mass (LBM) (B), resting energy expenditure (REE) (C), REE/LBM (D), glucose oxidation (E) and lipid oxidation (F). Data are means ± SEM, *P < 0.05 between groups at same time point vs baseline. Liraglutide n=23, placebo n=26.

liraglutide induces weight loss by decreasing energy intake rather than by increasing energy expenditure and/or BAT activity.

In this study we investigated the effects of treatment with liraglutide on REE after short and long-term treatment. The initial decrease of REE is in line with previous results published by van Can et al.[12], who showed a reduction of REE after treatment with liraglutide (3.0 mg per day) for 5 weeks. In contrast, Horowitz et al.[14] did not find an effect of treatment with liraglutide (1.8 mg per day) for 4 weeks on REE. Harder et al.[13] treated with a relatively low daily dose of 0.6 mg for 8 weeks and also concluded that REE was unaffected. Effects of long-term treatment with liraglutide on REE are still largely un-

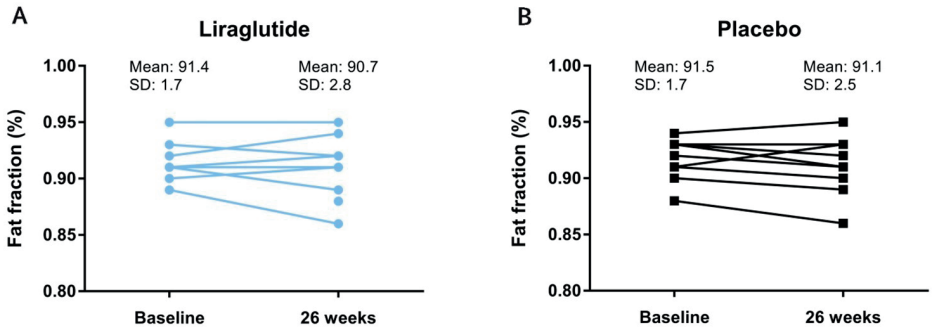

Figure 4. The effect of liraglutide and placebo on median fat fraction of the supraclavicular fat depot. Fat fraction at baseline and after 26 weeks of treatment with liraglutide (A) or placebo (B). Liraglutide: n=10, placebo: n=12, missing data in liraglutide-group: n=1 at baseline, in placebo-group n=1 after treatment.

known. Beiroa et al.[10], in contrast to our findings, showed that REE corrected for LBM was increased after treatment for 1 year with liraglutide (1.2 mg per day) and also after treatment with the GLP-1 analogue exenatide. We find no evidence for an increase of REE after prolonged treatment and REE seems to be decreased rather than increased. However, importantly, we used a higher dose of liraglutide and treated participants for only 26 weeks, which makes it difficult to compare the results.

We expected, as previously shown in mice, that long-term treatment with liraglutide would increase REE[10], and would increase lipid oxidation[11]. Furthermore, as mentioned above, studies show conflicting results about the short-term effects of liraglutide on REE. In general, weight loss is the result of a negative energy balance, caused by an imbalance between energy intake on one side and energy expenditure on the other side. Since we instructed participants not to change their physical activity and studied the participants in a fasted state to avoid effects of diet-induced thermogenesis, and REE was not increased in our study, logically, the weight-loss inducing effect of liraglutide should rather be attributed to a decrease in energy intake, a well-known effect of GLP-1 analogues[6,14,29]. Interestingly, we show that, initially, REE is decreased after treatment with liraglutide, which could possibly be explained as an adaptation of REE in response to the decreased food intake. This is supported by previous studies showing that treatment with a very low calorie diet can decrease energy expenditure[30,31]. In part, the decreased REE after weight loss can be accounted for by the loss of LBM, as was also shown in our study. However, we show that after correction for LBM liraglutide decreased REE after treatment for 4 weeks, which is in line with several other studies showing a decreased REE after weight loss after correction for LBM[32,33]. A possible explanation for this effect could be that, in response to a negative energy balance, metabolic efficiency is increased resulting in a decrease of REE to limit loss of body weight.

We are the first to investigate the effect of treatment with liraglutide on BAT in humans. It has previously been shown in rodents that central infusion with GLP-1 analogues stimulates BAT thermogenesis and browning of white adipose tissue[10,11]. Furthermore, Kooijman et al.[11] reported a 67% decrease of lipid content of BAT and increased uptake of plasma triglyceride-derived fatty acids in BAT. Since BAT is an important regulator of energy expenditure BAT is generally regarded as a promising target to combat obesity[27,34,35]. Indeed, in mice, it has been shown that activation of BAT with a β_3-adrenergic receptor agonist induces weight loss[36] and also after treatment with GLP-1 analogues in the aforementioned studies describing activation of BAT, body weight was reduced[10,11]. This suggests that BAT activation may be contributing to the weight-reducing effects of GLP-1 in humans.

If liraglutide would activate BAT in humans, we reasoned we should observe a decrease of the fat fraction in the supraclavicular BAT depot and/or a change in the volume of the BAT repository, since activation of BAT results in combustion of intracellular lipid stores[37]. Indeed, short-term cold exposure decreases the fat fraction of supraclavicular BAT[38]. In our study in patients with type 2 diabetes treatment with liraglutide did not decrease the fat fraction of the supraclavicular BAT depot. This may suggest that our treatment regime did not activate BAT at this location or that the dose or duration of treatment was insufficient to cause a detectable change in fat fraction in the investigated fat depot, e.g. by restoration of cellular triglyceride stores by the influx of triglyceride-derived fatty acids. Previous studies in rodents showed a reduction in fat fraction in BAT after central infusion with GLP-1 analogues[10,11], which is different from the usual subcutaneous administration and very likely affects pharmacokinetic parameters of liraglutide. Furthermore, it is known that metformin promotes clearance of triglycerides by BAT in mice, pointing to increased BAT activation, which means that, since all subjects were using metformin before and during the study, possibly BAT is already maximally activated in our subjects, leaving no room for further reduction of the fat fraction. Another possible explanation for the lack of an effect on the fat fraction could be the result of the fact that participants were studied for 6 months. Therefore, baseline and follow up scan were performed in a different season. Cold exposure is known to activate BAT and there is a seasonal variation in the ability to demonstrate the presence and activity of BAT using PET-CT scans, with a higher presence in winter than summer[34,39]. However, correction of the data for season did not change our results. Optimisation of the MRI technique and comparison of results with studies using alternative methods to investigate BAT are therefore warranted.

The main strength of this study is that we measured REE after 4, 12 and 26 weeks of treatment with liraglutide, which provides us detailed information on the effects of

short and long-term treatment. Furthermore, the study design of our randomized placebo-controlled trial, in which participants were treated according to current clinical guidelines in addition to the study medication, increases the generalisability of our results. A possible limitation is the relatively new MRI-method we used for assessment of effect of treatment on BAT. Further optimization of our technique could improve sensitivity. Another limitation is that, for practical reasons, we could only assess the effect on BAT with MRI after 26 weeks and not on other study visits. Finally, a possible limitation is that we did not keep dietary records and therefore could not investigate the effect of liraglutide treatment on food intake. However, it is well known that GLP-1 analogues decrease food intake[12,14]. Furthermore, measurement of long-term dietary intake in a usual care setting has previously been shown to be very inaccurate[40].

In conclusion, in this randomised placebo-controlled trial, we showed that treatment with liraglutide decreases REE after 4 weeks, which persists until 12 weeks of treatment and tends to be still present after 26 weeks of treatment. Furthermore, we showed that the fat fraction in the supraclavicular BAT depot was not changed after treatment, which may indicate that BAT in the supraclavicular region is not affected by liraglutide. All in all, our findings provide further insight into the mechanism of weight loss of GLP-1 analogues and indicate that liraglutide does not affect energy metabolism in such a way that it can contribute to weight loss.

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Chapter 5

Hepatic triglyceride content does not affect circulating CETP: lessons from a liraglutide intervention trial and a population-based cohort

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ABSTRACT

Cholesteryl ester transfer protein (CETP) is mainly expressed by Kupffer cells in the liver. A reduction of hepatic triglyceride content (HTGC) by pioglitazone or caloric restriction is accompanied by a decrease in circulating CETP. Since GLP-1 analogues also reduce HTGC, we assessed whether liraglutide decreases CETP. Furthermore, we investigated the association between HTGC and CETP in a population-based cohort.

In a placebo-controlled trial, 50 patients with type 2 diabetes were randomly assigned to treatment with liraglutide or placebo added to standard care. In this trial and in 1,611 participants of the Netherlands Epidemiology of Obesity (NEO) study, we measured HTGC and circulating CETP by proton magnetic resonance spectroscopy and ELISA, respectively. The HTGC was decreased in the liraglutide group (-6.3%; 95%CI of difference [-9.5, -3.0]) but also in the placebo group (-4.0%; 95%CI[-6.0,-2.0]), without between-group differences. CETP was not decreased by liraglutide (-0.05 µg/mL; 95%CI[-0.13,0.04]) or placebo (-0.04 µg/mL; 95%CI[-0.12,0.04]). No association was present between HTGC and CETP at baseline (β: 0.002 µg/mL per %TG, 95%CI[-0.005,0.009]) and between the changes after treatment with liraglutide (β: 0.003 µg/mL per %TG, 95%CI[-0.010,0.017]) or placebo (β:0.006 µg/mL per %TG, 95%CI[-0.012,0.024]). Also, in the cohort no association between HTGC and CETP was present (β:-0.001 µg/mL per SD TG, 95%CI[-0.005,0.003]).

A reduction of HTGC after treatment with liraglutide or placebo does not decrease circulating CETP. Also, no association between HTGC and CETP was present in a large cohort. These findings indicate that circulating CETP is not determined by HTGC.

INTRODUCTION

Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl esters from HDL towards triglyceride-rich lipoproteins, mainly VLDL, coupled to a net flux of triglycerides from VLDL to HDL[1]. CETP thereby causes a proatherogenic lipoprotein profile, with increased atherogenicity of VLDL particles[2]. Recently, it has been shown that circulating levels of CETP are mainly determined by Kupffer cells, which are the resident macrophages of the liver[3,4], and that adipose tissue does not relevantly contribute to serum CETP concentration[5].

Although Kupffer cells in the liver have been identified as the main source of circulating CETP, the regulation of hepatic CETP production is incompletely understood. Previous human studies showed that treatment with the peroxisome proliferator-activated receptor (PPAR)-γ agonist pioglitazone[6] and prolonged caloric restriction[7] both reduced hepatic triglyceride content, which was accompanied by a decrease in plasma CETP concentration. Interestingly, it was recently shown that treatment with liraglutide, a human glucagon-like peptide 1 (GLP-1) analogue, also reduces hepatic steatosis and can even lead to histological resolution of non-alcoholic steatohepatitis (NASH)[8]. GLP-1 analogues are prescribed to patients with type 2 diabetes to achieve glycemic control, whereby they also induce weight loss[9,10]. However, the exact mechanism of action by which GLP-1 analogues ameliorate hepatic steatosis and NASH is still unclear. In rodents, GLP-1 analogues were shown to improve diet-induced hepatic steatosis and reduce hepatic macrophage recruitment[11,12]. Notably, the GLP-1 analogue exendin-4 not only decreased hepatic triglycerides but also hepatic *CETP* gene expression and circulating CETP concentration in human CETP expressing transgenic mice[12]. Since we previously showed that hepatic *CETP* expression and plasma CETP concentration were strongly related with the hepatic Kupffer cell content[3], the reduction in CETP observed with exendin-4 treatment may be explained by a reduction in Kupffer cells. However, in humans, the effects of GLP-1 analogues on CETP production are still unknown.

The aim of the present study was to assess whether a liraglutide-induced reduction in hepatic triglyceride content would be accompanied by a reduction in circulating CETP concentration in patients with type 2 diabetes. In addition, we also investigated the association between hepatic triglyceride content and circulating CETP concentration in a population-based cohort of 1,611 participants.

MATERIALS AND METHODS

Randomised controlled trial

Study overview and study population

This study used data from the MAGNA VICTORIA (MAGNetic resonance Assessment of VICTOza efficacy in the Regression of cardiovascular dysfunction In type 2 diAbetes mellitus) study, a prospective, randomised, double-blind, clinical trial. The primary outcome measure of this study was the effect of liraglutide on cardiac function[13]. In the current manuscript, we report on secondary and other endpoints. Overweight and obese (BMI ≥25 kg/m^2) patients with type 2 diabetes were recruited from November 2013 until March 2016 via advertisements and from the outpatient clinics of the Leiden University Medical Center (LUMC, Leiden, the Netherlands), general practitioners, and local hospitals. We included patients aged 18-69 years, treated with metformin, and with a glycated haemoglobin (HbA1c) ≥7.0 and ≤10.0% (53-86 mmol/mol). Concomitant treatment with sulfonylurea derivatives and insulin was optional, although the dosage of all glucose-lowering medication needed to be stable for at least 3 months prior to participation. Exclusion criteria were use of other glucose-lowering therapy than mentioned above, presence of renal, hepatic or cardiovascular disease, and contra-indications for magnetic resonance imaging (MRI). The trial was approved by the ethics committee of the LUMC and performed in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all subjects before participation. The trial was conducted at the LUMC, and was registered at clinicaltrials.gov (NCT01761318, date of registration 04/01/2013).

Study design and data collection

At baseline, participants were randomised to receive treatment with liraglutide (Victoza®, Novo Nordisk A/S, Bagsvaerd, Denmark) or placebo by block randomisation with block size of 4 and stratification 1:1 for sex and insulin use. During the study, all participants, study investigators and outcome assessors were blinded to treatment allocation. Participants visited the study center at baseline and after 26 weeks of treatment, after ≥6 h of fasting, for medical history assessment, standard physical examination, collection of venous blood samples and MRI. After 4 and 12 weeks of treatment additional venous blood samples were collected. The starting dose of the study medication was 0.6 mg per day, which was titrated in two weeks to a maximum dose of 1.8 mg per day, if tolerated. In addition to study medication, participants received treatment according to current clinical guidelines to achieve optimal glycemic control and regulation of blood pressure and cholesterol levels. Body composition was assessed using bioelectrical impedance analysis (BIA; Bodystat 1500, Bodystat Ltd., Douglas, UK). All blood samples were centrifuged

and stored at -80°C until analysis. Plasma cholesterol and triglyceride concentrations were measured on a Modular P800 analyser (Roche Diagnostics, Mannheim, Germany). LDL-cholesterol was calculated according to Friedewald's formula[14]. Due to changes in laboratory procedures during the study, in a subset of participants HbA1c was assessed with boronate affinity high-performance liquid chromatography (Primus Ultra, Siemens Healthcare Diagnostics, Breda, the Netherlands), while in the other patients HbA1c was assessed with ion-exchange high-performance liquid chromatography (HPLC) (Tosoh G8, Sysmex Nederland B.V., Etten-Leur, the Netherlands). Plasma CETP concentrations were determined by ELISA slightly modified from Niemeijer-Kanters et al[15]. In short, plates were coated with a combination of monoclonal antibodies TP1 (5 µg/ml) and TP2 (2.5 µg/mL; both from Ottawa Heart Institute Research Corporation, Ottawa, Canada) during an overnight incubation at 4°C. The next day plates were blocked with 1% BSA (Sigma Aldrich, Zwijndrecht, the Netherlands) for 2 hours at room temperature. EDTA-plasma samples were 80-fold diluted in Assay buffer containing 1% BSA and 0.1% Triton-X100 (Biorad, Veenendaal, the Netherlands) and incubated for 2 hours at 37°C. Autocal (Instruchemie, Delfzijl, the Netherlands) was used as a standard. Subsequently, plates were incubated with the secondary antibody TP20 labeled with digoxigenin (0.33 µg/mL; Ottawa Heart Institute Research Corporation, Ottawa, Canada) for 2 hours at 37°C, followed by 1 hour of incubation with anti-digoxigenin-POD, Fab fragments coupled to peroxidase (0.0375 U/mL; Roche Molecular Biochemicals, Mannheim, Germany) at room temperature. Finally, plates were incubated with TMB for 15 minutes and, after termination of the reaction with H_2O_2, absorbance was read at 450 nm. The interassay variance was <10% and the intraassay variance was <5%.

Proton magnetic resonance spectroscopy

Hepatic triglyceride content was assessed with proton magnetic resonance spectroscopy (^{1}H-MRS) on a 3 Tesla Ingenia whole-body MR system (Philips Medical Systems, Best, the Netherlands). Subjects were scanned in supine position after at least 6 hours of fasting. The body coil was used for transmission and reception was achieved with a 16-element anterior, and a 12-element posterior array. The 20 x 20 x 20 mm^3 voxel of interest (VOI) was placed in the liver while carefully avoiding contamination of bile ducts and blood vessels. VOI localization was achieved using a PRESS (point resolved spectroscopy) sequence, with echo time of 35 ms and repetition time of 9 s for the unsuppressed spectra and 3.5 s for the water-suppressed spectra. First order pencil beam B_0 shimming, with nine projections, was performed in the spectroscopic VOI. Four signal averages (NSA) were acquired without water suppression, and 32 NSA with water suppression using the MOIST (Multiply Optimized Insensitive Suppression Train) sequence. Spectra were acquired during free-breathing at end-expiration with pencil beam navigator-based respiratory triggering technique. The navigator voxel was placed

at the lung-liver interface. Further movement artefacts were minimalized by applying motion tracking that corrected the voxel location according to the navigator position. The excitation bandwidth was 1500 Hz and 1024 samples were acquired resulting in spectral resolution of 1.46 Hz/sample. The raw spectral data were processed by an in-house developed program that performed channel weighting, phase correction and frequency drift correction. After these steps, signal averages that were > 2.5 times the standard deviation were excluded. Lastly the remaining averages were summed after which the spectra were fitted in the time-domain using the Java-based MR User Interface ((jMRUI version 5.0; Katholieke Universiteit Leuven, Leuven, Belgium). Before fitting the spectrum residual water signal for the water-suppressed spectra was removed using a Hankel-Lanczos singular value decomposition (HLSVD) filter. The advanced method for accurate, robust and efficient spectral fitting (AMARES) algorithm was used to fit the resonances to a Gaussian line shape. Hepatic triglyceride content was calculated by dividing sum of triglyceride methyl (CH_3) and triglyceride methylene (CH_2) by water + CH_3 + CH_3 * 100%. All spectra were blinded before analysis.

Statistical analyses

Data are shown as means ± SD, or as median (interquartile range) when not normally distributed. Within-group changes were assessed using paired *t*-tests. We performed an analysis of covariance (ANCOVA) to assess between-group differences. Linear regression analyses were performed to determine associations between hepatic triglyceride content and plasma CETP concentration, and between the change (Δ) in hepatic triglyceride content and the change in plasma CETP level. β and corresponding 95% CI were reported. A *P*-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS version 23.0 for Windows (IBM Corporation, Chicago, IL).

Population-based study

Study overview and study population

The study population was part of the Netherlands Epidemiology of Obesity (NEO) study, which is a population-based prospective cohort study of 6,671 men and women between 45 and 65 years, with an oversampling of persons with a BMI of 27 kg/m^2 or higher. Participants were recruited from September 2008 until September 2012, and visited the NEO study center after an overnight fast of at least 10 h for extensive baseline measurements, including venous blood sampling and anthropometry. In a random subgroup of 2,082 participants hepatic triglyceride content was available. The present study is a cross-sectional analysis of the baseline measurements of the NEO study. We excluded participants with missing data on serum CETP concentration (n=16), or high alcohol intake according to criteria from the World Gastroenterology Organisation[16], i.e.

>30 g/day for men and >20 g/day for women. Therefore, the present study population comprised 1,611 NEO study participants who underwent ^{1}H-MRS of the liver. The NEO study was approved by the medical ethics committee of the LUMC and all participants gave written informed consent. More detailed information about the study design and data collection has been described previously[17].

Data collection

Hepatic triglyceride content was quantified by ^{1}H-MRS on a 1.5 Tesla MR system (Philips Medical Systems, Best, the Netherlands). CETP concentrations were measured with ELISA kits according to the manufacturer's instructions (DAIICHI CETP ELISA, Daiichi, Tokyo, Japan), in serum that had undergone one previous freeze-thaw cycle. Fasting concentrations of ALT and AST were measured with a Cobas Integra 800 analyzer (Roche Diagnostics, Mannheim, Germany). More detailed information on covariates can be found in the supplementary information.

Statistical analyses

Linear regression analyses were performed to examine the association between hepatic triglyceride content and serum CETP concentration. All results from the NEO study were based on analyses weighted towards a reference BMI distribution of the general population, and therefore apply to a population-based study without oversampling of individuals with overweight or obesity (see supplementary information). As hepatic triglyceride content was not normally distributed, this variable was transformed to the natural logarithm. For the purpose of interpretation, beta coefficients from linear regression analyses were multiplied by ln(1.1), and the difference in serum CETP concentration with corresponding 95%CI was expressed per 10% relative increase in hepatic triglyceride content. The crude model (Model 1) was adjusted for age and sex (Model 2), and subsequently ethnicity, smoking status, alcohol intake and physical activity were added as confounding variables (Model 3). Analyses were performed using STATA Statistical Software (Statacorp, College Station, Texas, USA), version 12.0.

RESULTS

Randomised controlled trial

Population characteristics

Participants were included between December 2013 and September 2015 with the last participant visiting in March 2016. Fifty participants were included, of whom 24 were randomised to receive liraglutide and 26 to receive placebo. One participant of the

liraglutide group was withdrawn from the study before starting treatment due to claustrophobia and was not included in the analyses. Another participant of the liraglutide group did not finish the study due to misdiagnosis of type 2 diabetes and one patient of the placebo group was lost to follow-up due to imprisonment, but the baseline measurements of both participants were used for analyses. Three serious adverse events occurred that were not related to study drug use. As shown in **Table 1,** baseline characteristics of the participants in both treatment groups were comparable. Individuals were 59.9 ± 6.2 years old in the liraglutide group, *vs* 59.2 ±6.8 years in the placebo group, with a body weight of 98.4 ± 13.8 *vs* 94.5 ± 13.1 kg and BMI of 32.6 ± 4.4 *vs* 31.6 ± 3.4 kg/m^2, respectively. Of all 49 participants included in the study, 40 participants used lipid lowering drugs at the start of the study.

A decrease in hepatic triglyceride content was not accompanied by a change in circulating CETP

Treatment with liraglutide for 26 weeks decreased body weight in contrast to treatment with placebo (-4.3 ± 3.8 kg *vs* 0.1 ± 2.5 kg; mean change from baseline (liraglutide *vs* placebo): -4.5 kg; 95%CI [-6.4, -2.6]), as shown in **Table 2**. Furthermore, treatment with liraglutide decreased hepatic triglyceride content (-6.3%; 95%CI of difference [-9.5, -3.0]), but hepatic triglyceride content was also decreased in the placebo group (-4.0%; 95%CI [-6.0,-2.0]), without between-group differences (mean change from baseline (liraglutide *vs* placebo): -2.1%; 95%CI [-5.3, 1.0]). Interestingly, this decrease in hepatic triglycerides in both treatment groups was not accompanied by a decrease in circulating CETP after 26 weeks of intervention. Also, after 4 and 12 weeks of intervention, CETP was not affected in both the liraglutide group and the placebo group (not shown). **Figure 1** shows that hepatic triglyceride content was not associated with CETP at baseline (β: 0.002 µg/mL per 1% increase in hepatic triglyceride content; 95%CI [-0.005, 0.009]). Furthermore, there was no association between the changes of both variables after treatment with liraglutide (β: 0.003 µg/mL; 95%CI [-0.010, 0.017]) or placebo (β: 0.006 µg/mL; 95%CI [-0.012, 0.024]). At baseline, circulating CETP concentration was lower in participants using lipid lowering drugs (0.80 ± 0.23 µg/mL; 82% of participants) than in participants not using lipid lowering drugs (0.93 ± 0.27 µg/mL; 18% of participants), mean difference 0.14 µg/mL; 95%CI of difference [-0.08, 0.35]). Furthermore, while HDL-cholesterol was not affected, total cholesterol, LDL-cholesterol and triglycerides were decreased in both groups. Finally, HbA1c was improved after treatment with liraglutide (-1.1%; 95%CI of difference [-1.5, -0.6]), but also after treatment with placebo (-0.7% 95%CI of difference [-1.1, -0.3]); without between-group differences (mean change from baseline (liraglutide *vs* placebo): -0.3%; 95%CI [-0.8, 0.2]).

Table 1. Baseline characteristics of participants from the MAGNA VICTORIA study

Characteristic	Liraglutide (n = 23)	Placebo (n = 26)
Demographics		
Age (years)	59.9 ± 6.2	59.2 ± 6.8
Sex (n (%))		
Men	14 (61%)	15 (58%)
Women	9 (39%)	11 (42%)
Diabetes duration (years)	11.3 ± 6.4	10.9 ± 7.1
Alcohol use (no. (%))	9 (39%)	10 (39%)
Clinical parameters		
Body weight (kg)	98.4 ± 13.8	94.5 ± 13.1
BMI (kg/m^2)	32.6 ± 4.4	31.6 ± 3.4
Total fat mass (%)	36.1 ± 10.3	36.6 ± 8.8
Hepatic triglyceride content (%)	18.1 ± 11.2	18.4 ± 9.4
Fasting concentrations		
CETP (µg/mL)	0.84 ± 0.22	0.81 ± 0.26
Total cholesterol (mmol/L)	4.82 ± 1.02	4.80 ± 1.02
HDL-cholesterol (mmol/L)	1.22 ± 0.25	1.30 ± 0.39
LDL-cholesterol (mmol/L)	2.60 ± 0.86	2.55 ± 0.91
Triglycerides (mmol/L)	2.19 ± 1.51	2.10 ± 1.09
Glucose (mmol/L)	8.7 ± 2.7	7.3 ± 1.7
HbA1c (%)	8.3 ± 1.1	8.1 ± 0.9
HbA1c (mmol/mol)	66.7 ± 11.5	64.7 ± 10.2
AST (IU/L)	31 ± 11	35 ± 21
ALT (IU/L)	15 ± 7	13 ± 5
Concomitant drug use		
Lipid lowering drugs (no. (%))	21 (91%)	19 (73%)
Metformin (g/day)	2.1 ± 0.7	2.0 ± 0.5
Sulfonylureas (no. (%))	6 (26%)	8 (31%)
Insulin (no. (%))	15 (65%)	17 (65%)
Insulin (units)	70 ± 46	69 ± 58

Results are presented as n (%) or mean ± SD. n=49. Missing data: n=2 for alcohol use and n=2 for hepatic triglyceride content in placebo-group. ALT: alanine transaminase, AST: aspartate transaminase, CETP: cholesteryl ester transfer protein, HbA1c: glycated haemoglobin.

Table 2. Body weight, hepatic triglyceride content and metabolic factors change from baseline to after 26 weeks of treatment in the MAGNA VICTORIA study

Characteristic	Mean ± SD change from baseline to 26 weeks		Mean [95%CI] changes from baseline (liraglutide *vs* placebo)	P value
	Liraglutide (n=22)	Placebo (n=25)		
Clinical parameters				
Body weight (kg)	-4.3 ± 3.8	0.1 ± 2.5	-4.5 [-6.4, -2.6]	<0.001
BMI (kg/m^2)	-1.5 ± 1.3	0.1 ± 0.8	-1.5 [-2.2, -0.9]	<0.001
Hepatic triglyceride content (%)	-6.3 ± 7.1	-4.0 ± 4.6	-2.1 (-5.3, 1.0)	0.174
Metabolic factors				
CETP (µg/mL)	-0.05 ± 0.20	-0.04 ± 0.18	0.00 [-0.10, 0.11]	0.977
Total cholesterol (mmol/L)	-0.72 ± 0.89	-0.46 ± 0.56	-0.22 [-0.59, 0.15]	0.231
HDL-cholesterol (mmol/L)	-0.02 ± 0.14	0.05 ± 0.25	-0.08 [-0.20, 0.05]	0.222
LDL-cholesterol (mmol/L)	-0.44 ± 0.51	-0.24 ± 0.52	-0.17 [-0.44, 0.10]	0.218
Triglycerides (mmol/L)	-0.50 ± 1.12	-0.60 ± 0.98	0.23 [-0.11, 0.56]	0.177
Glucose (mmol/L)	-1.7 ± 2.2	-0.6 ± 2.2	-0.5 [-1.7, 0.7]	0.434
HbA1c (%)	-1.1 ± 1.0	-0.7 ± 0.9	-0.3 [-0.8, 0.2]	0.265
HbA1c (mmol/mol)	-11.6 ± 11.1	-7.7 ± 9.4	-2.9 [-8.1, 2.3]	0.265
AST (IU/L)	-6 ± 11	-12 ± 22	2 [-3, 6]	0.459
ALT (IU/L)	16 ± 12	14 ± 10	1 [-5, 7]	0.778

Results are presented as mean ± SD. n=47. Missing data: n=2 for hepatic triglyceride content in the placebo group, n=1 for hepatic triglyceride content, n=1 for LDL-cholesterol and n=1 for glucose in the liraglutide group. ALT: alanine transaminase, AST: aspartate transaminase, CETP: cholesteryl ester transfer protein, HbA1c: glycated haemoglobin.

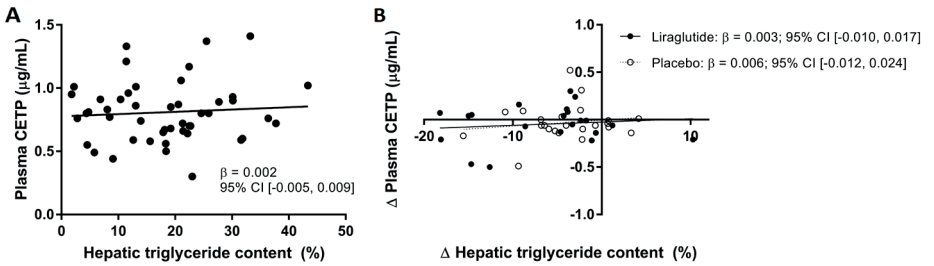

Figure 1. Associations between hepatic triglyceride content and plasma CETP levels in the MAGNA VICTORIA study. Hepatic triglyceride content in relation to plasma CETP level, n=47 (A), and change of hepatic triglyceride content in relation to change in plasma CETP level, liraglutide n=21, placebo n=23 (B). CETP: cholesteryl ester transfer protein.

Population-based study

Population characteristics

Demographic and clinical characteristics of the NEO study population are presented in **Table 3**. While mean BMI was lower in women than in men, mean total body fat was

higher in women. Hepatic triglyceride content ranged from 0.2% to 62.9% and was lower in women than in men. Serum CETP ranged from 0.88 to 5.02 µg/mL. Men had a lower serum CETP concentration (2.38 ± 0.62 µg/mL) than women (2.64 ± 0.64 µg/mL) (difference: -0.26; 95%CI [-0.17, -0.35]), and CETP concentration was lower in participants using lipid lowering drugs (2.13 ± 0.72 µg/mL) compared with participants not using lipid lowering drugs (2.56 ± 0.62 µg/mL) (difference -0.43; 95%CI [-0.54, -0.32]).

Table 3. Characteristics of participants from the NEO study population who underwent proton magnetic resonance spectroscopy of the liver to assess hepatic triglyceride content, stratified by sex.

Characteristic	Men	Women
Proportion of participants (%)	45	55
Ethnicity (% whites)	95	95
Age (year)	55.8 ± 6.5	55.1 ± 5.5
Alcohol use (% users)	89	82
Alcohol intake (g/day)	12.1 ± 9.5	5.6 ± 5.0
BMI (kg/m^2)	26.4 ± 3.5	25.4 ± 4.2
Total body fat (%)	24.2 ± 5.7	36.3 ± 6.1
Fasting concentrations		
CETP (µg/mL)	2.38 ± 0.62	2.64 ± 0.64
Total cholesterol (mmol/L)	5.56 ± 1.00	5.81 ± 1.03
HDL-cholesterol (mmol/L)	1.31 ± 0.36	1.72 ± 0.43
LDL-cholesterol (mmol/L)	3.62 ± 0.93	3.59 ± 0.95
Triglycerides (mmol/L)	1.38 ± 0.89	1.09 ± 0.68
ALT (IU/L)	28.4 ± 13.0	21.3 ± 7.7
AST (IU/L)	25.8 ± 7.3	22.9 ± 6.2
HbA1c (%)	5.4 ± 0.6	5.3 ± 0.3
HbA1c (mmol/mol)	35.5 ± 6.7	34.7 ± 3.6
Comorbidity and medication		
Hepatic triglyceride content (%)	3.4 (1.9, 7.7)	1.7 (1.1, 4.5)
Diabetes (% yes)	6	4
Impaired fasting glucose (% yes)	10	5
Oral glucose lowering drugs (% users)	3	1
Insulin (% users)	0.04	0.5
Oral glucose lowering drugs and insulin (% users)	0.8	0.1
Cardiovascular disease (% yes)	5	4
Lipid lowering drugs (% users)	12	5

Results were based on analyses weighted towards the BMI distribution of the general population (n=1,611) and presented as mean ± SD, median (interquartile range) or percentage. Missing data: n=2 for ethnicity, n=1 for total body fat, n=5 for plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, ALT and AST concentrations, n=15 for HbA1c concentration, n=6 for presence of diabetes, n=5 for presence of cardiovascular disease. ALT: alanine transaminase, AST: aspartate transaminase, BMI: body mass index, CETP: cholesteryl ester transfer protein, HbA1c: glycated haemoglobin, HDL: high-density lipoprotein, IU: international unit, LDL: low-density lipoprotein, NEO: Netherlands Epidemiology of Obesity.

Hepatic triglyceride content was not associated with circulating CETP in the general population

As shown in **Figure 2**, hepatic triglyceride content was not associated with serum CETP concentration in the NEO study, neither in men, women, lipid lowering drug users or non-users of lipid lowering drugs. As can be appreciated from **Suppl. table 1**, a 10% increase in hepatic triglyceride content was associated with a -0.001 µg/mL (95%CI [-0.005, 0.003]) difference in serum CETP. Similar associations around the null were observed after adjustment for confounding variables.

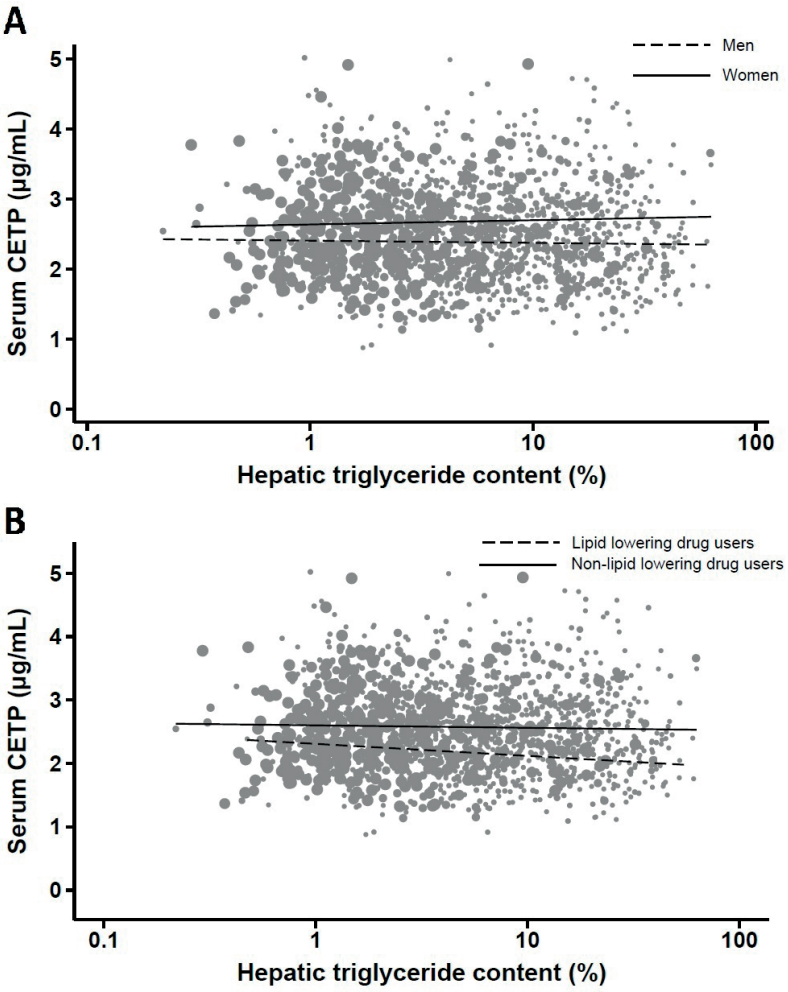

Figure 2. Associations between hepatic triglyceride content and serum CETP concentrations in the NEO study population. Crude associations between hepatic triglyceride content and serum CETP concentration, stratified by sex (A) and lipid lowering drug use (B). Results were based on analyses weighted towards the BMI distribution of the general population (n=1,611). CETP: cholesteryl ester transfer protein; NEO: Netherlands Epidemiology of Obesity.

DISCUSSION

In this double-blind, randomised placebo-controlled trial, we observed that a reduction in hepatic triglyceride content after treatment with liraglutide, but also with placebo, was not accompanied by a reduction in circulating CETP concentration. In line, we found no evidence for an association between hepatic triglyceride content and circulating CETP concentration in a large population-based cohort study (n=1,611). Our findings imply that plasma CETP concentration is not determined by hepatic triglyceride content.

Our observation that the hepatic triglyceride content decreased after liraglutide treatment is in accordance with results from previous studies in humans and rodents[8,11]. However, this decrease was also observed in the placebo group. Furthermore, although the GLP-1 analogue exendin-4 decreased hepatic *CETP* gene expression and CETP concentration in rodents[12], we found no effect of liraglutide treatment on the circulating CETP concentration. These data are corroborated by our finding that hepatic triglyceride content was not associated with circulating CETP concentration in the large population-based NEO cohort. There was also no association present after stratification by lipid lowering drug use, which implies that lipid lowering drugs do not mediate or influence the effects of hepatic triglyceride content on CETP concentration. Nevertheless, our data seem counterintuitive, as previous studies did report a decrease in CETP upon other interventions that reduce hepatic steatosis. For example, Jonker et al[18] showed that the PPARγ agonist pioglitazone decreased hepatic triglyceride content (from 6 to 4%) as well as CETP concentration (-12%), while the decrease of circulating CETP correlated with the decrease in hepatic triglyceride content. Notably, in their study both the hepatic triglyceride and circulating CETP concentration at baseline were lower compared to our study and the reduction of hepatic triglyceride content induced by pioglitazone was smaller than the decrease in the liraglutide group in the present study. In another study, patients with type 2 diabetes received a 16-week very low calorie diet, which resulted in dramatic weight loss and a large reduction in hepatic triglyceride content (from 21 to 3%) as well as CETP concentration (-18%)[7]. After gastric banding surgery, with associated substantial weight loss, similar large reductions in CETP concentration have been described[3,19].

Interestingly, hepatic triglyceride content was not only decreased in the liraglutide-treated group, but also in the placebo group. This is probably due to treatment of participants in both groups according to current clinical guidelines, which resulted in an intensified treatment with glucose-lowering drugs in the placebo-group, while in the liraglutide-group such treatment could be decreased. Both groups showed improved glucoregulation, evidenced by decreased HbA1c after treatment. It is thus likely that

intensified treatment of the placebo group decreased the rate of hepatic lipogenesis and consequently lowered intrahepatic triglyceride storage, resulting in increased hepatic insulin sensitivity that lowers glucose production[20,21]. Notably, similar to treatment with liraglutide, the reduction in hepatic triglycerides caused by placebo treatment was not accompanied by a reduction in circulating CETP.

It is interesting to speculate on the mechanism by which GLP-1 analogues may influence plasma CETP concentration in humans. Notably, in contrast to previous studies[7,18], our findings show that liraglutide and placebo-induced lowering of hepatic triglycerides is not accompanied by decreases in plasma CETP concentration. We have previously shown that the GLP-1 analogue exendin-4 decreased plasma CETP concentration, which was accompanied by a reduction in the number of hepatic macrophages[12], the main source of CETP production. The reduction in hepatic macrophage content by exendin-4 attributes to reduced macrophage recruitment from the circulation and/or enhanced macrophage elimination from the liver. Indeed, in rodents, exendin-4 decreased the hepatic gene expression of monocyte chemotactic protein-1[11,12], which mediates monocyte/macrophage recruitment from the circulation to tissue. Since Panjwani *et al.*[22] reported that the GLP-1 receptor is undetectable in isolated macrophages and hepatocytes, the effect of exendin-4 on hepatic macrophage content is unlikely mediated via the GLP-1 receptor. However, the exact action of exendin-4 on macrophage recruitment and elimination is unclear, and whether other GLP-1 analogues exert the same beneficial effects on hepatic macrophage content needs to be further investigated. It is possible that this is an effect specific for exendin-4 not shared by liraglutide. Furthermore, it is possible that, in contrast to in rodents, GLP-1 receptor agonists in general, or the applied dose of liraglutide specifically, fail to reduce the hepatic macrophage content in humans. Indeed, in the LEAN-trial, in which patients with NASH were treated with liraglutide, no effects were observed on lobular inflammation and overall non-alcoholic fatty liver disease (NAFLD) activity score[8]. Collectively, it is likely that liraglutide fails to affect hepatic macrophages to an extent that is sufficient to decrease CETP concentration.

In this light, it is interesting to note that we previously studied the association of metabolic liver inflammation with hepatic and circulating CETP[23]. We showed that metabolic liver inflammation, as a histologically determined component of NAFLD in obese individuals, did not associate with CETP measures (i.e. liver *CETP*, liver CETP positive cells and circulating CETP concentrations). These data are in line with the findings of the current study, as apparently, metabolic triggers of liver damage do not decrease CETP production. Interestingly, infection-related liver inflammation, as induced by Gram-negative bacteria, strongly decreases CETP production by the liver[24]. As both metabolic induced liver steatosis and inflammation[23] do not affect CETP production by Kupffer

cells, it seems that NAFLD does not mimic the strong effects of Gram-negative bacterial infections on the hepatic expression and production of CETP by Kupffer cells. This suggests that metabolically-induced and infection-related inflammation may have different effects on the expression and production of CETP by Kupffer cells.

Preclinical studies using mice and cultured cells have shown that the Liver X Receptor α (LXRα) plays a crucial role in regulation CETP expression. The natural ligands of LXRα are oxysterols[25,26]. In line, it has been shown that lipid lowering drugs decrease total hepatic cholesterol content and levels of oxysterols, thereby diminishing *LXR*-activation and CETP expression in CETP-transgenic mice[27]. Therefore, the lower levels of circulating CETP in participants using lipid lowering drugs in our intervention trial as well as in our cohort study are likely explained by attenuated CETP expression by reduced oxysterol-mediated LXRα signalling. It has previously been proposed that a decreased hepatic triglyceride content upon an intervention with caloric restriction or pioglitazone may reduce CETP concentration via this LXRα-dependent mechanism, as a reduction in triglycerides would be accompanied by a reduction in the natural LXRα agonists[7,18]. However, since in our trial a reduction of hepatic triglyceride content was not accompanied by a reduction of CETP concentration, our results imply that interventions on hepatic triglyceride content *per se* do not affect hepatic oxysterols and thereby *LXR*-mediated CETP production. With these new insights, we speculate that decreased CETP concentration previously found after interventions with pioglitazone and caloric restriction might be better explained by a reduction of the number of Kupffer cells than by a decreased hepatic oxysterol content.

The strengths of this study are the randomised placebo-controlled trial design, and the availability of data on hepatic triglyceride content and CETP concentration from a large population-based cohort. A limitation is that the study population of the NEO study was predominantly white and results may therefore not apply to other ethnical groups. Furthermore, the study design of the trial, in which in addition to study medication patients received treatment according to current clinical guidelines, is another possible limitation, since we were not able to investigate the effects of liraglutide only. Also, use of co-medication could interfere with the effects of liraglutide on CETP. Nevertheless, this study design increases the generalizability of our findings.

In summary, in a randomised placebo-controlled trial, we showed that liraglutide treatment and placebo intervention on top of standard treatment with glucose lowering drugs decrease hepatic triglyceride content without decreasing plasma CETP concentration. We confirmed the absence of an association between hepatic triglyceride content

and CETP in a large population-based cohort study. This implies that circulating CETP concentration is not determined by hepatic triglyceride content.

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SUPPLEMENTARY MATERIALS AND METHODS NETHERLANDS EPIDEMIOLOGY OF OBESITY (NEO) STUDY

Covariates

Questionnaires were sent to all participants and completed at home. The general questionnaire included questions on demographic, lifestyle and clinical information. Smoking status was categorized into never smoker, former smoker and current smoker. Menopausal status was classified as premenopausal, perimenopausal (menopausal during last year) or postmenopausal. Participants were classified as having pre-existing cardiovascular disease when a medical history of myocardial infarction, angina, congestive heart failure, stroke or peripheral vascular disease was reported via the questionnaire. Participant were classified as having diabetes when the disease was self-reported via the questionnaire or when anti-diabetic medication was used. Research nurses recorded current medication use. Alcohol intake was assessed with a semi-quantitative food frequency questionnaire (FFQ)[1], and calculated from the FFQ using the 2011 version of the Dutch food composition table (NEVO-2011).

Body weight and total body fat were determined with the Tanita bio-impedance balance (TBF-310, Tanita International Division, UK) without shoes and with subtraction of one kilogram (kg) to correct for the weight of clothing. BMI was calculated by dividing the weight in kilograms by the height in meters squared.

Fasting serum total cholesterol and TG concentrations were measured with enzymatic colorimetric assays (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany) and fasting serum HDL-cholesterol concentrations with third generation homogenous HDL-cholesterol methods (Roche Modular P800 Analyzer, Roche Diagnostics, Mannheim, Germany). Fasting LDL-cholesterol concentrations were calculated using the Friedewald equation[2]. HbA1c was assessed with Boronate affinity high-performance liquid chromatography (HPLC) (Primus Ultra, Siemens Healthcare Diagnostics, Breda, the Netherlands).

Statistical analyses: weighting

In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. Men and women aged between 45 and 65 years with a self-reported BMI of 27 kg/m^2 or higher were eligible to participate. From one nearby municipality (Leiderdorp, the Netherlands) all inhabitants aged between 45 and 65 years were invited to participate regardless of their BMI, in order to obtain a reference distribution for BMI. To correctly represent baseline associations in the general population[3], adjustments for the oversampling of individuals with a BMI ≥27 kg/m^2 were made in the analyses. This was done by weighting

all participants towards the BMI distribution of participants from the Leiderdorp municipality[4], whose BMI distribution was similar to the BMI distribution of the general Dutch population in the age range of 45-65 years[5]. In practice, this means that participants with a lower BMI were assigned larger weight factors than participants with a higher BMI in the analyses. All results are based on weighted analyses, and therefore apply to a population-based study without oversampling of individuals with overweight or obesity. As a consequence, the weighted characteristics of the population are expressed in proportions instead of absolute numbers.

Supplementary table 1. NEO study: Difference in serum CETP concentration per 10% relative increase in hepatic triglyceride content.

Model	Difference in serum CETP concentration (µg/mL)[a]	95%CI
1	-0.001	-0.005, 0.003
2	0.003	-0.002, 0.007
3	0.003	-0.001, 0.007

*Results were based on analyses weighted towards the BMI distribution of the general population (n=1,611). Model 1: unadjusted. Model 2: adjusted for age, sex. Model 3: adjusted for age, sex, ethnicity, smoking status, alcohol intake and physical activity. Missing data: n=2 for ethnicity, n=37 for physical activity. [a] (Beta coefficients from linear regression)*ln(1.1); difference per 10% relative increase in hepatic triglyceride content. BMI: body mass index, CETP: cholesteryl ester transfer protein, NEO: Netherlands Epidemiology of Obesity.*

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Chapter 6

Caloric restriction lowers endocannabinoid tonus and improves cardiac function in type 2 diabetes

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ABSTRACT

Background/Objectives: Endocannabinoids (ECs) are associated with obesity and ectopic fat accumulation, both of which play a role in the development of cardiovascular disease (CVD) in type 2 diabetes (T2D). The effect of prolonged caloric restriction on ECs in relation to fat distribution and cardiac function is still unknown. Therefore, our aim was to investigate this relationship in obese T2D patients with coronary artery disease (CAD).

Subjects/Methods: In a prospective intervention study, obese T2D patients with CAD (*n*=27) followed a 16 week very low calorie diet (VLCD; 450-1000 kcal/day). Cardiac function and fat accumulation were assessed with MRI and spectroscopy. Plasma levels of lipid species, including ECs, were measured using liquid chromatography-mass spectrometry.

Results: VLCD decreased plasma levels of virtually all measured lipid species of the class of *N*-acylethanolamines including the EC anandamide (AEA; -15%, p=0.016), without decreasing monoacylglycerols including the EC 2-arachidonoylglycerol (2-AG). Baseline plasma AEA levels strongly correlated with the volume of subcutaneous white adipose tissue (SAT; R^2=0.44, p<0.001). VLCD decreased the volume of SAT (-53%, p<0.001), visceral white adipose tissue (VAT) (-52%, p<0.001), epicardial white adipose tissue (-15%, p<0.001) and paracardial white adipose tissue (-28%, p<0.001). VLCD also decreased hepatic (-86%, p<0.001) and myocardial (-33%, p<0.001) fat content. These effects were accompanied by an increased left ventricular ejection fraction (54.8±8.7 to 56.2±7.9%, p=0.016).

Conclusions: Caloric restriction in T2D patients with CAD decreases AEA levels, but not 2-AG levels, which is paralleled by decreased lipid accumulation in adipose tissue, liver and heart, and improved cardiovascular function. Interestingly, baseline AEA levels strongly correlated with SAT volume. We anticipate that dietary interventions are worthwhile strategies in advanced T2D, and that reduction in AEA may contribute to the improved cardiometabolic phenotype induced by weight loss.

INTRODUCTION

The endocannabinoid (EC) system is a key player in lipid and glucose metabolism and in regulation of energy balance[1]. It consists of the cannabinoid receptors type 1 (CB1R) and type 2 (CB2R), their ligands, the endocannabinoids (ECs), and the enzymes responsible for synthesis and degradation of ECs. The two most well-studied ECs, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), belong to the class of *N*-acylethanolamines and monoacylglycerols, respectively. The ECs are phospholipid-derived lipids that can be produced by any cell type and organ and act mainly as paracrine mediators. Binding of ECs to the cannabinoid receptors results in various metabolic effects, including increased food intake, enhanced lipogenesis and reduced energy expenditure[2,3]. This is supported by several studies reporting strong associations between high plasma EC levels and triglycerides (TG), intra-abdominal obesity and insulin resistance in obese and T2D patients[4-7]. Furthermore, overactivity of the EC system can promote beta cell failure through activation of the Nlrp3-ASC inflammasome in infiltrating macrophages[8]. Thus, elevated levels of ECs can contribute to relative insulin deficiency and to accumulation of visceral adipose tissue (VAT) and obesity, and as such may have a causal role in the pathophysiology of T2D and CVD[3].

The prevalence of T2D, which is associated with the Western obesogenic lifestyle, is increasing[9]. T2D patients have a 2-fold increased risk to develop cardiovascular disease (CVD), their major cause of death[10]. Moreover, the 7-year incidence rate of myocardial infarction (MI) following a previous MI is increased more than 2-fold in T2D patients compared to non-diabetic subjects[11]. Many risk factors play a role in the development of cardiovascular complications, and a major role is attributed to ectopic lipid deposition. Ectopic lipid deposition stimulates inflammation and causes insulin resistance, probably as a consequence of accumulation of toxic metabolites including diacylglycerol and ceramides that interfere with the insulin signalling process[12]. For example, lipid accumulation can occur in myocardial tissue and in the pericardial fat depot, which is associated with impaired myocardial function and increased risk and degree of coronary atherosclerosis[13-16].

Many studies have targeted the EC system to improve metabolic health. Blockade of the CB1R with rimonabant caused weight loss and improved lipid metabolism in obese animal models and humans, but had unacceptable psychiatric side effects[17-20]. Alternatively, EC levels can be reduced by dietary interventions. For example, krill oil, which has a high polyunsaturated fatty acid (PUFA) content, reduces plasma 2-AG levels in obese humans, probably by reducing the availability of EC biosynthetic precursors[21]. Also, dietary long-chain PUFA reduces ectopic fat deposition in liver and heart and

susceptibility for inflammation in Zucker rats[22,23], supporting a connection between the EC system and ectopic lipid deposition. Studies in humans assessing the effect of weight loss on plasma EC levels show variable results: while some studies showed a decrease in EC levels[6,24], other studies showed no effect[25,26]. The effect of more pronounced weight loss by intensive caloric restriction on plasma EC levels is as yet unknown.

Previously our group showed that prolonged caloric restriction decreases ectopic fat deposition and improves myocardial function in obese T2D patients without CAD[27]. However, the effects of this intervention in T2D patients *with* established CAD are still unknown. Therefore, our aim was to investigate the effect of caloric restriction on plasma EC levels, ectopic lipid accumulation and cardiovascular function in obese patients with T2D and established CAD.

METHODS

Subjects

Obese (BMI >25 kg/m^2) T2D patients with documented coronary artery disease (CAD) were recruited via advertisements and from the outpatient clinic of the Leiden University Medical Center (LUMC), Leiden, the Netherlands. Established atherosclerosis or CAD were defined as a history of MI and/or percutaneous coronary intervention and/or a >50% stenosis in a coronary artery as documented by computed tomography angiography. Exclusion criteria were hepatic disease, glomerular filtration rate <60 mL/min, congenital heart disease and general contraindications to magnetic resonance (MR) scanning. Subjects underwent a medical screening including their medical history, physical examination, and blood chemistry tests. The study was approved by the local ethics committee and performed in accordance with the principles of the revised Declaration of Helsinki. Written informed consent was obtained from all subjects before participation. The study was registered in the Dutch Trial Register (NTR-2897).

Study design

The study was conducted at the LUMC, Leiden, the Netherlands. Patients were studied on two occasions: at baseline and after a 16-week dietary intervention period, during which a VLCD was prescribed. In order to assess the variability in study parameters without dietary intervention, 13 of the 27 patients were also studied 16 weeks prior to the start of the VLCD. Patients were instructed not to alter lifestyle habits during the study.

The VLCD consisted of Prodimed products (Prodimed® Benelux BV, Valkenswaard, the Netherlands), which are low in calories and have a relatively high protein content of

67% and a low fat content of 5%. All patients started with total meal replacement: 4-6 sachets a day (400-600 kcal/day) including a warm meal of Prodimed for three weeks, supplemented with a limited choice of vegetables. After these three weeks caloric intake was increased, by replacing a Prodimed at dinner time by meat or fish. Afterwards, when an additional 3% weight loss was achieved, the caloric intake was further expanded with one Prodimed being replaced with a normal meal. One week before the last study day, the diet was expanded, with a normal breakfast to achieve a caloric intake of 1000 kcal/day. Use of sulphonyl urea derivatives was discontinued the day the VLCD started and insulin therapy was adjusted according to glucose levels. Patients on insulin treatment were asked to measure their blood glucose levels 4 times a day throughout the study.

During the intervention period, patients visited the outpatient clinic weekly, and blood pressure and body weight were measured. Blood was drawn monthly to assess safety parameters. At each study day, anthropometric measurements, blood sampling and MR imaging and spectroscopy were performed after ≥ 5 hours of fasting. Blood was centrifuged at 4°C and EDTA plasma and serum were stored at -80°C until analyses.

Biochemical Assays

Serum concentrations of glucose, total cholesterol, HDL-cholesterol and TG were measured on a Modular P800 analyzer (Roche Diagnostics, Mannheim, Germany), NEFA were measured using a commercial kit (Wako Chemicals, Neuss, Germany), and insulin on an Immulite 2500 analyzer (Siemens, Breda, the Netherlands). LDL-cholesterol was calculated according to Friedewald's formula[28]. HbA_{1c} was measured on an HPLC system (Roche Diagnostics, Mannheim, Germany). High-sensitivity CRP (hs-CRP) levels were assessed using precoated 96-well multisport plates from Meso Scale Discovery (Gaithersburg, Maryland, USA). Concentrations of ALT and AST were measured with the Cobas Integra 800 analyzer (Roche Diagnostics, Mannheim, Germany). Plasma levels of several lipid species including ECs were quantified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described previously[29].

MR spectroscopy and Imaging

MR measurements were performed at a 1.5-Tesla MR-scanner (Gyroscan ACS-NT15; Philips Medical Systems, Best, the Netherlands).

Adipose tissue depots

The volume of pericardial fat, consisting of both epicardial and paracardial fat, was derived from fat-selective imaging using spectral presaturation with inversion recovery, as described before[30]. Abdominal VAT and subcutaneous adipose tissue (SAT) volumes

were quantified at the level of the fifth lumbar vertebra, using a turbo spin echo imaging sequence, as described before[15]. Volumes of VAT and SAT were quantified using MASS.

MR spectroscopy

Proton MR spectroscopy (^{1}H-MRS) was performed to quantify myocardial and hepatic TG content. Details on acquisition and post processing were described previously[31,32]. For the liver, voxel sites were matched at all study occasions.

Delayed enhancement

Delayed enhancement MRI for detection of myocardial scar was performed 15 minutes after injection of gadetorate meglumine (0.3 mL/kg, Dotarem; Guerbet, Bloomington, USA) as described[33].

Left ventricular dimensions and function

The heart was imaged in short-axis orientation to assess systolic function, as previously described[34]. Left ventricular (LV) end-diastolic and end-systolic contours were drawn, using in-house developed validated MASS software (LUMC, Leiden, the Netherlands). LV end-diastolic volume, end-systolic volume, ejection fraction, mass, cardiac output and stroke volume were calculated. Several function parameters were indexed to body surface area. LV mass was divided by LV end-diastolic volume to obtain the LV mass/LV end-diastolic volume ratio, also known as concentricity. LV diastolic function was studied from transmitral flow rate graphs, assessed from 3D three-directional velocity encoded MRI with retrospective valve tracking as previously described[35]. From the transmitral flow rate graphs, the following LV diastolic function parameters were determined using MASS software: maximal flow velocities and peak filling rate in early diastole and at diastolic atrial contraction. The ratio between peak filling at early diastole and diastolic atrial contraction was calculated. In addition, the downslope after early peak filling rate (deceleration) and the ratio between maximal flow velocity during early diastole and the through-plane velocity assessed in the myocardial wall, which is the estimate of the LV filling pressure, were assessed[36,37].

Pulse Wave Velocity

To evaluate aortic stiffness, aortic pulse wave velocity (PWV) was determined, using in-house developed and validated Matlab software (LUMC, Leiden, The Netherlands) as previously described[38]. A scout view of the aorta was obtained. Subsequently, two time-resolved velocity-encoded acquisitions perpendicular to the ascending aorta at the level of the pulmonary trunk and at the level of the aortic bifurcation were assessed, resulting in through-plane flow measurements. PWV was calculated with the formula: $\Delta x/\Delta t$. Δx is the length of the aorta between two measurement sites and Δt is the time

delay between the arrivals of the foot of the pulse wave at the respective measurements site. The distance between the measurement sites was determined manually with MASS. MASS and FLOW (LUMC, Leiden, the Netherlands) were used for data analysis.

Statistical analysis

Data are presented as mean ± SD or as median (interquartile range (IQR)) when not normally distributed. Based on the previous 16 week VLCD study we performed in obese patients with T2DM without cardiovascular complications, we performed a sample size calculation[27]. A p value <0.05 was considered statistically significant. Changes within participants were assessed using paired sample t-tests or Wilcoxon signed-rank test when appropriate. Linear regression analysis computed by Pearson's correlation was performed to identify correlations between plasma EC levels and various metabolic parameters. Statistical analyses were performed with SPSS for Windows, version 23.0 (SPSS Inc., Chicago, USA).

RESULTS

Clinical characteristics

While 32 patients were initially included to receive VLCD intervention, two patients left the study due to intolerance to the diet, one patient left because of worsening of Ménière's disease, and two patients were excluded due to non-adherence to the diet. Of the 27 participants who completed the study, results are shown in **Table 1**. Individuals were on average 62.2±6.0 years old, had a body weight of 98.5±16.0 kg, a BMI of 32.2±4.7 kg/m^2, and most of them were male (82%). VLCD induced an average weight loss of 16.5±5.5 kg (p<0.001), and BMI decreased on average with 5.4±1.7 kg/m^2 (p<0.001). VLCD improved glycemic control, as reflected by decreased fasting plasma glucose levels and decreased HbA_{1c} levels. VLCD decreased ALT, decreased total cholesterol and TG, and increased HDL-cholesterol, without changing NEFA. In addition, VLCD reduced systolic and diastolic blood pressures and heart rate.

Sixteen of these 27 patients had previously experienced a MI, 24 underwent percutaneous coronary intervention and three patients had a coronary artery bypass grafting. One patient had >50% occlusion of the coronary arteries on computed tomography angiography without a subsequent intervention. There were no differences in age, duration of T2D or anthropometric measurements between patients with and without a MI in medical history. At baseline 12 patients used insulin and all patients were on oral antihyperglycemic drugs. During the VLCD, insulin treatment was discontinued in 3 patients and in 3 patients all glucose lowering drugs could be discontinued. As shown

in **suppl. table 1**, clinical and metabolic parameters of the subgroup of 13 patients that was studied 16 weeks prior to the start of the VLCD did not change during this period. Also, MR parameters showed no significant variability before start of VLCD, as shown in **suppl. table 2**.

Table 1. Clinical and metabolic characteristics of obese type 2 diabetes patients with documented coronary artery disease before and after very low calorie diet.

	Before VLCD	After VLCD	*P* value
Clinical characteristics			
Age (years)	62.2 ± 6.0		
Males, n (%)	22 (82%)		
T2D duration (years)	11.0 ± 8.5		
Patients on insulin, n (%)	12 (44%)	9 (33%)	
Insulin dose (units/day)	79 ± 36	16 ± 12	
Body weight (kg)	98.5 ± 16.0	82.0 ± 14.0	<0.001
BMI (kg/m^2)	32.2 ± 4.7	26.8 ± 4.1	<0.001
Waist circumference (cm)	111 ± 12	93 ± 11	<0.001
Systolic blood pressure (mmHg)	146 ± 14	129 ± 12	<0.001
Diastolic blood pressure (mmHg)	83 ± 10	75 ± 9	0.002
Heart rate (beats/min)	68 ± 12	60 ± 8	0.002
Metabolic characteristics			
Glucose (mmol/L)	7.4 ± 1.7	6.3 ± 1.2	0.002
HbA_{1c} (%)	6.9 ± 0.9	5.8 ± 0.5	<0.001
HbA_{1c} (mmol/mol)	51 ± 10	40 ± 6	<0.001
Triglycerides (mmol/L)	1.80 ± 0.89	1.17 ± 0.43	<0.001
Total cholesterol (mmol/L)	4.18 ± 0.86	3.82 ± 0.67	0.018
HDL-cholesterol (mmol/L)	1.18 ± 0.28	1.32 ± 0.34	0.001
LDL-cholesterol (mmol/L)	2.18 ± 0.71	1.97 ± 0.60	0.088
NEFA (mmol/L)	0.61 ± 0.25	0.59 ± 0.29	0.836
ALT (IU/L)	30 ± 10	24 ± 11	0.024
AST (IU/L)	25 ± 7	22 ± 7	0.057
hs-CRP (mg/L)	1.7 (4.5)	1.1 (3.0)	0.107

Data are means ± SD or median (interquartile range), n=27. P value before vs. after diet based on a paired sample t-test or Wilcoxon signed-rank test. ALT: alanine transaminase, AST: aspartate transaminase, hs-CRP: high-sensitivity C-reactive protein, IU: International unit.

Endocannabinoid levels

The effects of the VLCD on plasma levels of the various ECs and lipid species are summarized in **Table 2**. VLCD decreased virtually all ECs from the group of *N*-acylethanolamines, without decreasing any ECs belonging to the group of monoacylglycerols. With respect to the most well studied CBR ligands, VLCD decreased plasma AEA levels (-13%; p=0.024),

without significantly decreasing plasma 2-AG levels (**Table 2**, **Fig. 1**). At baseline, plasma 2-AG levels did not correlate with either BMI, VAT and SAT volume (not shown). Interestingly, baseline plasma AEA levels correlated positively with BMI (R^2=0.21, p=0.017) and, while plasma AEA levels did not correlate with VAT volume, they strongly correlated positively with SAT volume (R^2=0.44, p<0.001) (**Fig. 2)**. Plasma levels of 2-AG, but not AEA, correlated with TG levels (R^2=0.41, p<0.001). Changes in plasma AEA and 2-AG levels as induced by the VLCD did not correlate with changes in adipose tissue volumes and parameters of cardiac function (**Suppl. table 3**).

Table 2. Plasma levels of lipid species before and after very low calorie diet.

	Before VLCD	After VLCD	*P* value
***N*-acylethanolamines (pmol/mL)**			
AEA	0.94 ± 0.41	0.82 ± 0.30	0.024
PEA	7.57 ± 2.03	6.26 ± 1.28	<0.001
SEA	6.36 ± 1.79	4.88 ± 0.87	<0.001
POEA	0.55 ± 0.47	0.34 ± 0.24	0.003
OEA	5.13 ± 1.57	4.74 ± 1.17	0.091
LEA	2.52 ± 0.64	2.13 ± 0.38	0.001
Alpha-LEA	0.19 ± 0.06	0.15 ± 0.03	0.003
DGLEA	0.16 ± 0.08	0.12 ± 0.04	<0.001
PDEA	0.09 ± 0.03	0.08 ± 0.02	0.105
EPEA	0.60 ± 0.37	0.37 ± 0.24	0.001
DEA	0.28 ± 0.08	0.25 ± 0.07	0.009
DHEA	5.51 ± 2.16	4.21 ± 2.04	<0.001
Monoacylglycerols (pmol/mL)			
2-AG	2.63 ± 1.25	2.42 ± 0.82	0.355
1-OG	425 ± 35	423 ± 38	0.828
2-OG	75 ± 30	68 ± 18	0.189
1-LG	79 ± 19	76 ± 14	0.378
2-LG	24 ± 11	21 ± 8	0.279

Data are mean ± SD, n=27. P value before vs. after VLCD based on a paired sample t-test. 1-LG: 1-linoleoyl glycerol, 1-OG: 1-oleoyl glycerol, 2-AG: 2-arachidonoyl glycerol, 2-LG: 2-linoleoyl glycerol, 2-OG: 2-oleoyl glycerol, AEA: anandamide, Alpha-LEA: N-α-linolenylethanolamide, DEA: N-docosatetraenoylethanolamide, DGLEA: dihomo-α-linolenoyl ethanolamide, DHEA: N-docosahexaenoylethanolamide, EPEA: eicosapentaenoyl ethanolamide, LEA: N-linoleoylethanolamide, OEA: N-oleoylethanolamide, PDEA: N-pentadecanoylethanolamide, PEA: N-palmitoylethanolamide, POEA: N-palmitoleoylethanolamide, SEA: N-stearoylethanolamide.

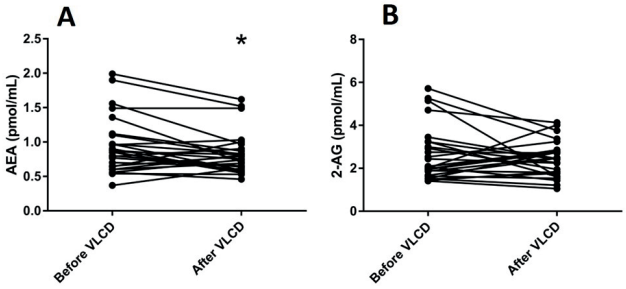

Figure 1. Plasma levels of AEA and 2-AG before and after VLCD. Change in AEA (A) and 2-AG (B). *p<0.05 vs. before VLCD, n=27.

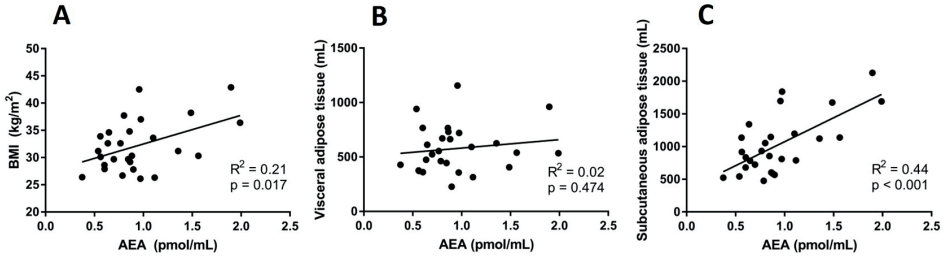

Figure 2. Correlations of AEA with BMI and adipose tissue volume. AEA plasma levels in relation to BMI (A), VAT volume (B), and SAT volume (C) as measured at baseline, n=27.

Adipose tissue distribution

VLCD reduced VAT volume (-52%, p<0.001) and SAT volume (-35%, p<0.001) (**Fig. 3**). Furthermore, VLCD substantially decreased hepatic TG content (-86%, p<0.001). VLCD also reduced epicardial fat volume (-15%, p<0.001) and paracardial fat volume (-28%, p<0.001). Delayed enhancement MRI revealed a septal MI in 5 patients, which prohibited myocardial TG measurements in these patients. Myocardial TG in the other patients decreased after VLCD (-33%, p<0.001). There were no differences in myocardial TG in patients with or without MI.

Cardiovascular parameters

The changes in cardiac dimensions and function before and after 16 weeks caloric restriction are shown in **Table 3**. While VLCD did not influence diastolic function, as the ratio of peak filling at early diastole and diastolic atrial contraction, the downslope after early peak filling rate, and the estimated filling pressure remained unchanged, VLCD did decrease LV mass and improved systolic cardiac function, as evidenced by an increased ejection fraction. VLCD also decreased cardiac output and increased end-diastolic volume and stroke volume. Furthermore, VLCD decreased aortic PWV from 7.9±1.9 m/s at baseline to 7.2±1.1 m/s (p=0.016) after VLCD.

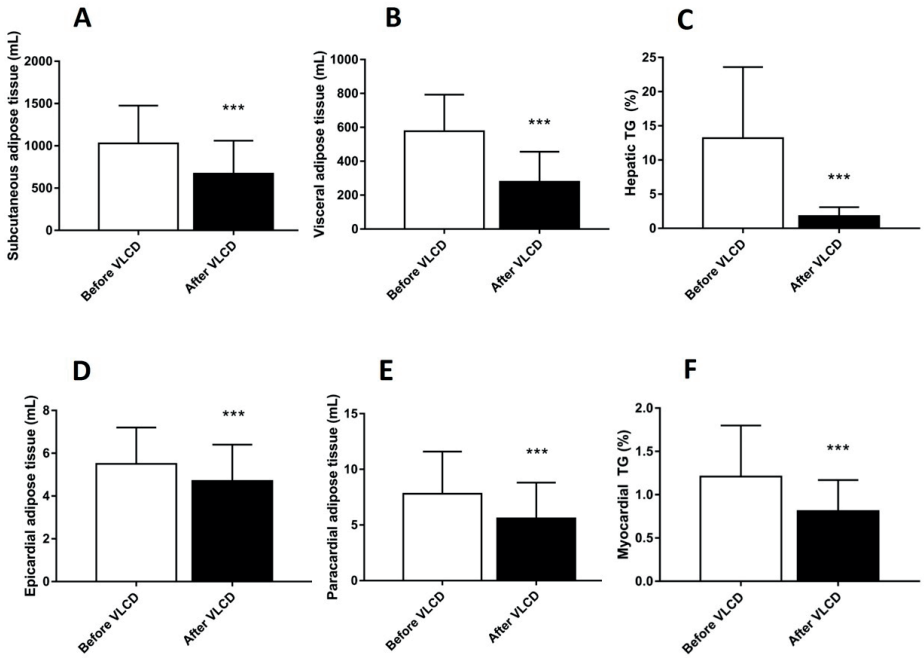

Figure 3. Volumes of various adipose tissue compartments before and after VLCD. Change in SAT volume (A), VAT volume (B), hepatic TG content (C), epicardial adipose tissue volume (D), paracardial adipose tissue (E) and myocardial TG content (n=22) (F). Results are expressed as mean ± SD. *** p<0.001 vs. before VLCD, n=27, except for F, n=22.

Table 3. Cardiac dimensions and systolic and diastolic cardiac function before and after very low calorie diet.

	Before VLCD	After VLCD	*P* value
Cardiac dimensions and function			
LV mass (g)	114 ± 27	104 ± 27	<0.001
LV mass index (g/m^2)	53 ± 11	53 ± 12	0.418
EDV (mL)	175 ± 38	183 ± 40	0.033
EDVI (mL/m^2)	82 ± 15	93 ± 17	<0.001
ESV (mL)	81 ± 29	82 ± 31	0.481
ESVI (mL/m^2)	38 ± 13	41 ± 15	<0.001
LV mass/EDV	0.66 ± 0.11	0.57 ± 0.09	<0.001
SV (mL)	94 ± 18	101 ± 17	0.007
SVI (mL/m^2)	44 ± 7	51 ± 6	<0.001
CO (L/min)	6225 ± 982	5619 ± 1410	0.029
CI (L/min/m^2)	2921 ± 390	2846 ± 643	0.571
EF (%)	54.8 ± 8.7	56.2 ± 7.9	0.016
E/A-peak ratio	0.95 ± 0.28	1.08 ± 0.27	0.063
E deceleration (mL/s^{2}x10^{-3})	-2.20 ± 0.96	-1.87 ± 0.53	0.108
E/Ea	8.9 ± 5.0	6.6 ± 4.4	0.076

Data are mean ± SD, n=27. P value before vs. after VLCD based on a paired sample t-test. A: diastolic atrial contraction, CO: cardiac output, CI: cardiac index, E: early diastolic filling phase, EDV: end-diastolic volume, E/Ea: estimate of LV filling pressure, EF: ejection fraction, ESV: end-systolic volume, I: indexed for body surface area, LV: left ventricular, SV: stroke volume.

DISCUSSION

In this study, we observed that a VLCD for 16 weeks decreased plasma levels of the CBR agonist AEA and other ECs we measured of the class of *N*-acylethanolamines in obese patients with T2D and established coronary atherosclerosis. Furthermore, VLCD resulted in reductions in SAT and VAT volume, reduced ectopic lipid accumulation, and improved cardiovascular function.

We show for the first time that vigorous caloric restriction with substantial weight loss and decrease of all adipose tissue depots can reduce AEA and other *N*-acylethanolamines in patients with T2D. In contrast, VLCD did not affect plasma levels of the EC 2-AG and other monoacylglycerols. Two previous studies have investigated the effect of small reductions of body weight on EC levels, both showing no effect[25,26]. Di Marzo et al[6] reported decreased plasma levels of AEA and 2-AG after a one year lifestyle modification programme including healthy eating and physical activity in obese men without T2D. Furthermore, the decrease in 2-AG in that study correlated positively with a decrease in VAT volume. In our study, however, effects of VLCD on 2-AG and correlations between changes in 2-AG and VAT were not observed, due to reasons unknown to us. Furthermore, in our study, AEA levels at baseline correlated strongly with SAT volume, but not with VAT volume, which to our knowledge was not described before.

It is interesting to speculate on the mechanism behind our observation that VLCD reduces AEA and other *N*-acylethanolamines. Potentially, the VLCD can have reduced the availability of biosynthetic precursors, thereby explaining decreased levels of *N*-acylethanolamines. Furthermore, the reduced volume of adipose tissue, being a source of EC production, could explain reduced AEA levels. Also, we cannot exclude that decrease or discontinuation of use of insulin injections and glucose lowering drugs has affected plasma levels of the measured lipid species. However, in our opinion, it is likely that VLCD influenced the activity or expression of enzymes responsible for degradation of ECs. After all, it is known that in adipocytes from normoglycemic non-obese subjects the expression of fatty acid amide hydrolase (FAAH), the enzyme responsible for degradation of mainly AEA and other *N*-acylethanolamines, is increased upon stimulation with insulin[39]. Furthermore, it has been shown *in vivo* that insulin functions as a negative regulator of AEA levels in non-diabetic subjects, but not in insulin-resistant individuals, probably due to lack of stimulation of expression of FAAH in adipocytes[40]. It is thus conceivable that the decrease in adipose tissue volume as induced by VLCD in our study improves insulin sensitivity of adipocytes thereby increasing FAAH expression and decreasing plasma levels of AEA and other *N*-acylethanolamines. This hypothesis is supported by our unpublished data showing a higher gene expression of FAAH in SAT in

normal glucose tolerant subjects than in subjects with T2D (Van Eyk, unpublished). The fact that FAAH degrades *N*-acylethanolamines but not monoacylglycerols[41], may explain why VLCD does not reduce monoacylglycerols including 2-AG.

We also detected a decreased LV mass and heart rate after VLCD, which can be considered beneficial, since both are recognized as important predictors for cardiovascular disease[42,43]. Furthermore, the ejection fraction increased after VLCD, which was not observed in other studies with a similar approach[27,44]. The improvement of ejection fraction in the current study is clinically very relevant, since LV ejection fraction is an important predictor of mortality in T2D patients[45]. Moreover, we show that parameters of LV function also improve in patients with complicated T2D, corroborating previous observations in uncomplicated T2D[27]. Other investigators observed an improved LV systolic function after weight loss, though based on other parameters than an improved ejection fraction[46,47]. However, those studies differed considerably from our study, as not all patients had T2D and/or CAD. Although the estimate of the LV filling pressure, did not change after the diet, the increased end-diastolic volume at similar estimates of LV filling pressure suggests an improved LV compliance[48]. Diastolic function did not change after VLCD in contrast to previous studies in uncomplicated T2D that found significant improvements in the ratio between peak filling at early diastole and diastolic atrial contraction after prolonged caloric restriction[27]. Our study showed a decrease in PWV, a surrogate marker for arterial stiffness and a powerful independent predictor of cardiovascular events, indicating a less stiff aorta[49]. No prior studies have been published on the effects of dietary intervention on PWV in complicated T2D.

Because ECs have various metabolic effects, including increase of food intake and lipogenesis and reduction of energy expenditure, the reduction of AEA may have contributed to the metabolic improvements and may have amplified the reduction of adipose tissue volume we observed. This way, the positive correlation between baseline plasma AEA levels and SAT volume suggests that decreased AEA levels might have contributed to reduced fat accumulation in SAT, although we were unable to detect a positive correlation between the changes of the variables, possibly related to insufficient power. Furthermore, Batetta et al[23] reported reductions of EC levels after dietary intervention with long-chain PUFA in Zucker rats, accompanied with reductions of ectopic lipid deposition in liver and heart. Therefore, the reduction in AEA may have contributed to the decrease in myocardial TG content, which is known to be associated with impaired myocardial function[15]. Also, it was recently shown that increased EC levels are associated with impaired coronary circulatory function and even more importantly, that gastric bypass-induced weight loss reduced EC levels and beneficially affected coronary

circulatory function[24,50], which suggests that the decrease in AEA might also contributed to the improved cardiac function we observed.

The associative nature of our study does not allow to establish a causal relationship between the reduction in EC levels and cardiometabolic phenotype, including weight loss, ectopic lipid deposition or measures of cardiac function. However, considering the remarkable beneficial effects of the CB1R inverse agonist rimonabant on body weight and lipid levels[19,20], it is likely that the EC system is an important player in metabolism. This makes a role for the EC system in the metabolic improvements we observed plausible. Future studies specifically targeting the EC system with dietary or pharmacological interventions can provide further knowledge and help entangle the role of ECs in the development of disease. Also, studying short term effects of caloric restriction, before weight loss has occurred, on EC levels could provide more information about the mechanism. Importantly, we show that prolonged caloric restriction, regardless of the role of ECs, improves cardiac function. Direct and indirect beneficial effects of caloric restriction and weight loss may have contributed to improved cardiac function, such as decreased blood pressure, improved glycemic control and improved PWV.

A strength of this study is that it is the first to investigate the effects of prolonged caloric restriction on EC levels in relation to cardiovascular function and ectopic fat distribution in overweight T2D patients *with* established CAD. Furthermore in a subgroup, it was established that study parameters did not change without dietary intervention. A limitation to this study is that patients followed a diet with very low caloric content and specific composition. It thus remains to be determined whether similar effects would be obtained by other interventions that induce similar weight loss. Another limitation is that due to practical issues patients were studied after ≥ 5 hours of fasting, which may be too short for some participants to reach baseline metabolic state. However, to reliably compare intra-individual data, we studied each patient at subsequent visits after the same fasting time. Finally, we investigated circulating ECs only, because EC concentrations, receptor expression and enzymes that play a role in synthesis and degradation of ECs within metabolic tissues could for obvious reasons not be investigated.

In conclusion, we showed that caloric restriction superposed on optimal pharmacological therapy for glucoregulation improves cardiovascular function in patients with advanced T2D. Since T2D is associated with increased cardiovascular mortality, the results of this study are highly clinically relevant. Considering the role of ECs in metabolism, we anticipate that the reduction in AEA may contribute to the improved metabolic phenotype induced by weight loss. However, mechanistic studies will have to be performed

to establish the causal role of dysregulation of the EC system in ectopic lipid deposition and CAD.

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SUPPLEMENTARY MATERIALS

Suppl. Table 1. Clinical and metabolic characteristics of a subgroup of patients four months before and at the start of the very low calorie diet.

	4 months before VLCD	At start of VLCD	P value
Clinical characteristics			
Age (years)	63.8 ± 4.8		
Males, n (%)	12 (92%)		
T2D duration (years)	7.8 ± 4.1		
Patients on insulin, n (%)	3 (23%)	4 (31%)	
Insulin dose (units/day)	68 ± 24	70 ± 22	
Body weight (kg)	94.9 ± 10.8	95.5 ± 11.7	0.316
BMI (kg/m2)	31.7 ± 3.9	31.9 ± 4.1	0.328
Systolic blood pressure (mmHg)	149 ± 14	143 ± 12	0.212
Diastolic blood pressure (mmHg)	87 ± 9	84 ± 11	0.129
Heart rate (beats/min)	67 ± 15	63 ± 13	0.276
Metabolic characteristics			
Glucose (mmol/L)	6.8 ± 1.4	7.4 ± 1.8	0.203
HbA1c (%)	7.2 ± 1.5	6.8 ± 1.1	0.422
HbA1c (mmol/mol)	55 ± 16	51 ± 12	0.422
Triglycerides (mmol/L)	2.01 ± 1.02	2.14 ± 1.00	0.600
Total cholesterol (mmol/L)	4.26 ± 0.76	4.42 ± 0.80	0.404
HDL-cholesterol (mmol/L)	1.15 ± 0.31	1.17 ± 0.30	0.604
LDL-cholesterol (mmol/L)	2.19 ± 0.64	2.27 ± 0.78	0.528
NEFA (mmol/L)	0.50 ± 0.20	0.54 ± 0.19	0.473

Data are mean ± SD, n=13. P value 4 months before vs. at the start of diet based on a paired sample t-test.

Suppl. Table 2. Cardiac dimensions and systolic and diastolic cardiac function of a subgroup of patients four months before and at the start of the very low calorie diet.

	4 months before VLCD	At start of VLCD	*P* value
Cardiac dimensions and function			
LV mass (g)	118 ± 27	116 ± 23	0.470
LV mass index (g/m^2)	57 ± 12	56 ± 11	0.452
EDV (ml)	178 ± 40	178 ± 40	0.897
EDVI (ml/m^2)	85 ± 15	85 ± 17	0.867
ESV (mL)	86 ± 31	86 ± 33	0.919
ESVI (mL/m^2)	41 ± 13	41 ± 15	0.994
LV mass/EDV	0.68 ± 0.17	0.67 ± 0.13	0.571
SV (mL)	92 ± 20	93 ± 20	0.695
SVI (mL/m^2)	44 ± 8	44 ± 8	0.739
CO (L/min)	5844 ± 834	5762 ± 956	0.701
CI (L/min/m^2)	2793 ± 281	2746 ± 355	0.645
EF (%)	52.4 ± 9.2	53.0 ± 10.0	0.443
E/A-peak ratio	0.86 ± 0.27	0.93 ± 0.26	0.304
E deceleration (mL/s^{2}x10^{-3})	-2.10 ± 1.02	-1.92 ± 0.94	0.653
E/Ea	7.2 ± 4.9	6.8 ± 4.4	0.769

Data are mean ± SD, n=13. P value before vs. after VLCD based on a paired sample t-test. A: diastolic atrial contraction, CO: cardiac output, CI: cardiac index, E: early diastolic filling phase, EDV: end-diastolic volume, E/Ea: estimate of LV filling pressure, EF: ejection fraction, ESV: end-systolic volume, I: indexed for body surface area, LV: left ventricular, SV: stroke volume.

Suppl. Table 3. Correlations between diet-induced changes in the endocannabinoids AEA and 2-AG and either adipose tissue volume or cardiac function.

	Δ plasma AEA		Δ plasma 2-AG	
	R^2	*P* value	R^2	*P* value
Adipose tissue depots				
Δ SAT volume	0.0625	0.209	0.0467	0.279
Δ VAT volume	0.0190	0.493	0.0493	0.265
Δ Epicardial adipose tissue volume	0.0036	0.768	0.0114	0.596
Δ Paracardial adipose tissue volume	0.0086	0.645	0.0035	0.769
Δ Hepatic TG content	0.0132	0.568	0.0328	0.365
Δ Myocardial TG content	0.0154	0.581	0.0829	0.194
Cardiac dimensions and function				
Δ LV mass	0.0017	0.841	0.0420	0.306
Δ EDV	0.0365	0.340	<0.0001	0.988
Δ ESV	0.1225	0.073	0.0058	0.707
Δ SV	0.0030	0.784	0.0029	0.788
Δ CO	0.0071	0.676	0.0199	0.482
Δ EF	0.0718	0.176	<0.0001	0.965
ΔE/Ea	0.0017	0.843	0.0524	0.260

Values result from correlation analyses using data from all individuals (n=27). CO: cardiac output, E/Ea: estimate of LV filling pressure, EDV: end-diastolic volume, EF: ejection fraction, ESV: end-systolic volume, LV: left ventricular, SV: stroke volume.

Chapter 7

General discussion and future perspectives

GENERAL DISCUSSION

The prevalence of type 2 diabetes is rising steadily and is becoming one of the largest health problems in the world[1,2]. Type 2 diabetes is a chronic disease characterized by disturbed glucose regulation and is strongly associated with dyslipidaemia. Insulin resistance is a key player in the development of type 2 diabetes and obesity is a well-known risk factor for the development of insulin resistance. Mechanistically, accumulation of ectopic fat, which is the process of storage of triglycerides in non-adipose tissues, such as liver, muscles, kidneys and pancreas is crucial for the development of insulin resistance[3,4]. Type 2 diabetes can result in cardiovascular diseases as a consequence of micro- and macrovascular damage, and therefore it is of utmost importance to pursue optimal management of blood glucose levels, but also of blood lipid levels and blood pressure[5]. The cornerstone in achieving this is improvement of lifestyle, with focus on increased physical exercise and, in most patients, weight loss. Furthermore, a change in composition of the diet with high daily intake of dietary fibre can improve fasting blood glucose levels and reduce cardiovascular risk[6,7]. However, additional pharmacological treatment is often unavoidable. Unfortunately, even under tight pharmacological control, the risk to develop cardiovascular complications remains high[8]. Furthermore, most of the registered glucose-lowering agents including metformin and insulin do not induce weight loss and even increase body weight. This is then often accompanied by further increase in insulin resistance resulting in requirement of addition of other drugs or intensification of current (insulin) therapy. Glucagon-like peptide-1 (GLP-1) receptor agonists are a relatively novel treatment option for patients with type 2 diabetes[9-11]. Although it has been shown over the past decade that GLP-1 receptor agonists improve glycemic control and in fact reduce body weight[12,13], the effects of GLP-1 receptor agonists on different adipose tissue compartments, ectopic fat deposition, and cardiac function remained unclear.

In this thesis, we have investigated the effects of GLP-1 receptor agonists on cardiometabolic health. To this end we performed a clinical trial in which we studied effects of the GLP-1 receptor agonist liraglutide on ectopic fat accumulation (**Chapter 2**) and on cardiovascular function (**Chapter 3**) in patients with type 2 diabetes. We were the first to specifically describe these effects in South Asian subjects, a population with high risk to develop type 2 diabetes and pronounced ectopic fat deposition[14-16]. Furthermore, we aimed to clarify the mechanism(s) behind liraglutide-induced weight loss by investigating the effect of liraglutide on energy expenditure and brown adipose tissue (BAT), which might be activated by GLP-1 receptor agonism[17] (**Chapter 4**). In addition, we investigated the relationship between hepatic triglyceride content and circulating cholesteryl ester transfer protein (CETP), a protein associated with a pro-atherogenic profile (**Chapter**

5). Finally, we investigated effects of prolonged caloric restriction on cardiometabolic health and studied the relationship with endocannabinoids, which can contribute to accumulation of visceral adipose tissue (VAT) and induction of insulin resistance (**Chapter 6**). In this chapter I will discuss the results obtained from the studies described in the previous chapters of this thesis. Furthermore, the implications of our work are discussed and ideas are described for future research to improve cardiometabolic health in type 2 diabetes.

1. GLP-1 receptor agonists in the treatment of type 2 diabetes

GLP-1 is a peptide hormone that is formed by posttranslational processing of proglucagon, and is mainly produced and released by enteroendocrine L-cells after food ingestion. GLP-1 delays gastric emptying, induces satiety, and stimulates glucose-dependent insulin secretion and inhibits glucagon release by the pancreas[18]. Together, these actions of GLP-1 prevent excessive hyperglycemia upon a meal. The physiological role of GLP-1 in energy metabolism is steadily being unraveled and recent studies have shined a new light on its function. Recently, it has been suggested that, since GLP-1 is quickly degraded by dipeptidyl peptidase 4 (DPP-4) expressed by endothelial cells in the gut, the main function of endogenous GLP-1 is to activate sensory afferent fibers of the vagal nerve. Via this way, nutritional abundance is signaled to the brain, with subsequent reduction of appetite and inhibition of parasympathetic outflow resulting in inhibition of gastric emptying[19]. Furthermore, the incretin effect, i.e. the augmented insulin secretion upon oral glucose intake caused by incretin hormones, which was historically thought to be the main "function" of GLP-1, has recently been studied more extensively and seems to play a less significant role than previously assumed. Interestingly, intravenous peripheral administration of a specific antagonist for the GLP-1 receptor, exendin(9-39)NH_2, resulted in only a small decrease in insulin secretion upon oral glucose intake, suggesting a minor contribution of GLP-1 to the incretin effect. In contrast, antagonism of glucose dependent insulinotropic peptide (GIP), another well-known incretin hormone produced by intestinal K cells, using GIP(3-30)NH_2, markedly reduced insulin secretion and even induced glucose intolerance[20,21]. Of course, it is important to note that GLP-1 receptor agonists that are used in the pharmacological treatment of type 2 diabetes and obesity result in supraphysiological GLP-1 receptor agonism, because these agonists are designed to be more resistant to degradation by DPP-4 compared to endogenous GLP-1. Therefore, physiological effects of endogenous GLP-1 are not necessarily equal to pharmacological effects. Indeed, in addition to the effects of endogenous GLP-1 on vagal nerve activation and subsequent induction of satiety and inhibition of gastric emptying, pharmacological treatment with GLP-1 receptor agonists has substantial direct effects on the pancreas to inhibit glucagon release and enhance glucose-induced insulin secretion resulting in more pronounced reduction of blood glucose levels[19,22]. The current

view of the proposed effects of GLP-1 on various organs is schematically depicted in **Figure 1**.

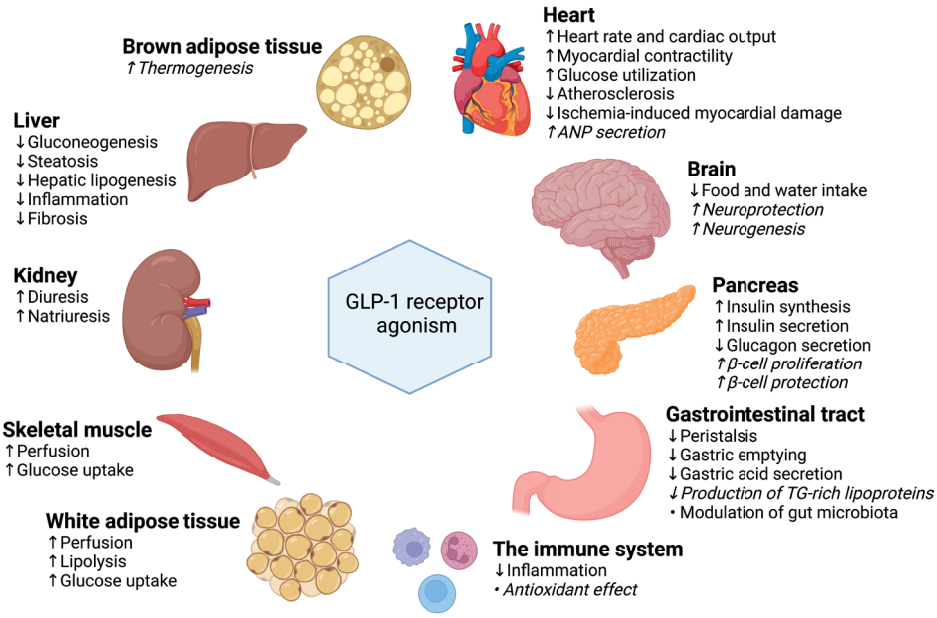

Figure 1. Schematic depiction of the proposed effects of GLP-1 on various organs. See section 1 for explanation. Effects not reported (as yet) in humans but only in pre-clinical models are italicized. ANP, atrial natriuretic peptide; TG, triglyceride. Created with BioRender.com.

In 2002, the first results of continuous subcutaneous infusion of native GLP-1 for a prolonged period of 6 weeks in patients with type 2 diabetes were published, showing inhibition of gastric emptying and reduction of body weight. Furthermore, infusion of GLP-1 improved insulin sensitivity and β-cell function and, consequently, improved glycemic control[23]. Since then, GLP-1 receptor agonists have been developed and have now taken an important position in pharmacological treatment of type 2 diabetes and, more recently, also in the treatment of obesity. Clinical trials with GLP-1 receptor agonists have shown that GLP-1 receptor agonism effectively induces weight loss and improves glycemic control[24,25] and in the studies we performed these effects were also present. We investigated this specifically in a South Asian population, in which the risk to develop type 2 diabetes as well as cardiovascular diseases is exceptionally high. We were the first to report results of a randomized placebo-controlled clinical trial in this group. Although studies on effects of GLP-1 receptor agonists in South Asians have not been extensively performed, a recent review of several observational studies with Indian subjects reported a larger reduction in HbA1c and body weight compared to the

'Liraglutide Effect and Action in Diabetes' (LEAD) program, a phase 3 controlled clinical trial program conducted in a western population[26]. However, the observational studies did not include a control arm, which may have affected the results. The degree of improvement of glycemic control and reduction of body weight in the trial we performed in a South Asian population was similar to those observed in other populations[27,28]. Also, when compared to the Western European subjects of our MAGNA VICTORIA trial no difference in liraglutide efficacy on glycemic endpoints was observed[29]. Importantly, however, the average BMI of our South Asian population was relatively low (29 kg/m^2), indicating that not only obese, but also overweight patients with type 2 diabetes from South Asian descent can similarly benefit from treatment with liraglutide or other GLP-1 receptor agonists.

Additional beneficial effects of GLP-1 receptor agonism on different aspects of cardiometabolic health have recently been revealed. For example, liraglutide was shown to reduce hepatic steatosis in patients with nonalcoholic steatohepatitis (NASH)[30]. In addition, treatment of obese patients with type 2 diabetes with exenatide or liraglutide for 2 weeks decreased postprandial dyslipidemia, which is an important aspect of diabetic dyslipidemia[31,32]. Moreover, treatment of subjects with type 2 diabetes with oral semaglutide for 12 weeks reduced postprandial triglycerides, apolipoprotein (Apo) B-48 and very-low-density lipoprotein (VLDL)[33]. Also, cardiovascular outcome trials have provided evidence for decreased cardiovascular and non-cardiovascular mortality, as well as renoprotective effects[34]. Our present findings resulting from studying the effects of GLP-1 receptor agonist liraglutide, provide further insight in potential mechanisms of GLP-1 receptor agonism in improving cardiometabolic health, as will be discussed in the next sections.

2. The mechanism behind weight loss induced by GLP-1 receptor agonism

In the MAGNA VICTORIA trials that we have performed, liraglutide (1.8 mg/day) reduced body weight in both South Asian and Western European patients with type 2 diabetes by 3.9 and 4.3 kg, respectively, which is in line with previous studies investigating effects of liraglutide (1.8 mg/day) on body weight, showing a similar weight reduction (i.e. 2.8-5.2 kg) after similar duration (i.e. 24-26 weeks) of treatment[27,35-37]. In general, weight loss is the consequence of a negative energy balance, caused by an imbalance between energy intake and expenditure. Effects of GLP-1 receptor agonists on energy intake have been studied extensively, showing decreased food intake by induction of satiety [38-41]. Studies in rats have shown that GLP-1 reduces appetite by activating specific regions in the hypothalamus and the amygdala[42]. Furthermore, delayed gastric emptying leads to gastric distension with activation of gastric mechanoreceptors, which induces satiation signals

and results in reduction of food intake[43-45]. Although it is thus well known that GLP-1 reduces food (energy) intake, it was still unclear how GLP-1 receptor agonism affects energy expenditure, the other side of the energy balance. Indeed, another mechanism by which GLP-1 was presumed to contribute to weight loss, is by increase of energy expenditure. In rodents, intracerebroventricular injection of GLP-1 receptor agonists induces weight loss mostly independent of its anorexigenic effects by increasing BAT thermogenesis[17,46,47]. In humans, one study showed increased energy expenditure after long-term (1 year) treatment with GLP-1 receptor agonists exenatide and liraglutide in addition to metformin[17], while most studies in humans showed no effect on energy expenditure during infusion with native GLP-1[48,49] or after prolonged (up to 12 weeks) treatment with GLP-1 receptor agonists[39,50,51]. In this thesis we describe effects of treatment with liraglutide on energy expenditure after both short and prolonged treatment, providing insight in effects over time, and are the first to relate this to effects on BAT in humans as measured by MRI (**Chapter 4**). In a population of Western European subjects with type 2 diabetes, we showed that liraglutide does not increase energy expenditure and even decreases energy expenditure after 4 and 12 weeks of treatment compared to energy expenditure at baseline. After 26 weeks, the resting energy expenditure was still decreased, although not significantly (p=0.056). A possible explanation for the decreased energy expenditure that we reported can be a reduction of diet-induced thermogenesis upon decreased food intake (and body weight) or an increased metabolic efficiency to adapt to the negative energy balance in order to limit loss of body weight. Interestingly, as mentioned above, an increased energy expenditure in obese type 2 diabetes patients who were treated with exenatide or liraglutide for 1 year has been reported previously[17] and we can speculate on a potential underlying mechanism. It is well-known that after prolonged treatment for approximately 1 year, GLP-1 receptor agonist-induced weight loss reaches a plateau phase[10,52]. Theoretically, when this phase is reached and energy balance is restored, the stimulating effect on energy expenditure by GLP-1 is unmasked. However, since body weight remains stable from that time, the clinical relevance of such a potentially increased energy expenditure induced by GLP-1 receptor agonism may be limited.

Furthermore, we investigated the effect of liraglutide on BAT. Activated BAT can combust free fatty acids and (to a lesser extent) glucose, resulting in increased energy expenditure[53]. Studies in rodents have shown that BAT activity is increased after intracerebroventricular injection of the GLP-1 receptor agonists liraglutide[17] and exendin-4[46]. As a consequence of combustion of intracellular triglycerides in brown adipocytes, BAT activation results in reduction of the fat fraction of BAT depots, which can be assessed using MRI[54]. In this thesis we investigated the effect of treatment of Western European patients with type 2 diabetes with liraglutide for 26 weeks on BAT and showed that treatment

does not affect the fat fraction in the supraclavicular BAT depot. No other studies have reported on effects of GLP-1 receptor agonists on BAT in patients with type 2 diabetes. Nonetheless, a lack of effect on the MRI-measured fat fraction was recently also shown in another trial with healthy non-obese subjects treated with exenatide for 12 weeks by Janssen et al[51]. Interestingly, in that study, an increased BAT volume and glucose uptake as measured by ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging was observed. This suggests that in healthy non-obese subjects treatment with exenatide and potentially other GLP-1 receptor agonists, has potential to increase metabolic activity of BAT, with increased uptake of glucose to replenish triglyceride stores via induction of lipogenesis. Such a lipogenic effect might counteract potential effects on the MRI-measured fat fraction of BAT, especially when BAT activation is only modest. The proposed effects of liraglutide on energy metabolism is schematically depicted in **Figure 2**.

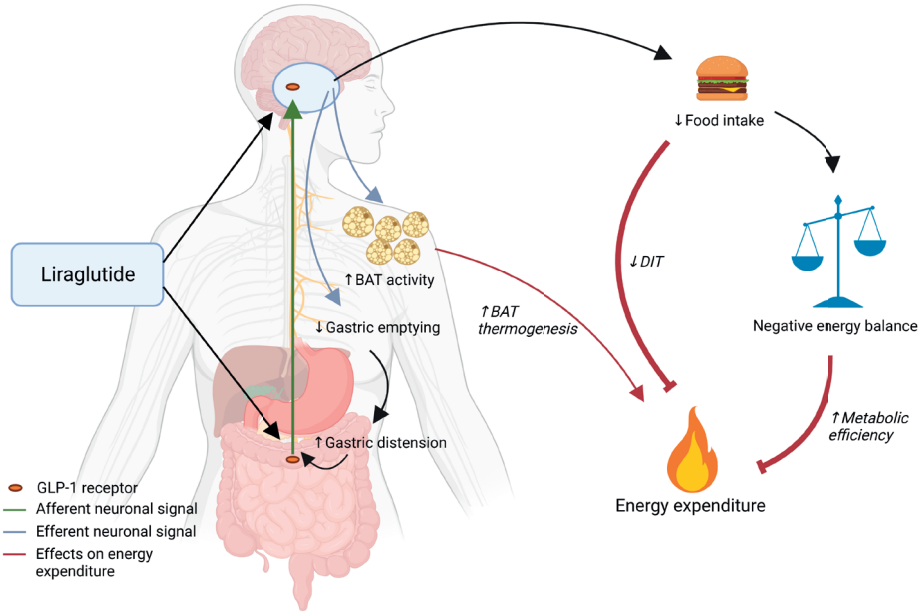

Figure 2. Schematic depiction of the proposed effects of liraglutide on energy metabolism. Liraglutide may exert effects on the central nervous system either directly, via activation of central GLP-1 receptors or indirectly, via activation of afferent fibers of the vagal nerve. This can immediately result in effects on the regulation of appetite and satiety or act via reduction of gastric emptying, which results in gastric distension and subsequently induction of satiation. Consequently, food intake is decreased, resulting in decreased diet-induced thermogenesis and promoting a negative energy balance. To counteract the negative energy balance, we propose that metabolic efficiency might be increased. In contrast, energy expenditure may be stimulated as a consequence of efferent neuronal signaling towards BAT, increasing BAT thermogenesis. The fact that the stimulating effect on energy expenditure by GLP-1 via BAT thermogenesis is surpassed by the effects caused by reduced food intake (as shown in the figure using wider arrows), might explain our observation of reduced energy expenditure upon treatment with liraglutide. BAT, brown adipose tissue; DIT, diet-induced thermogenesis; GLP-1, glucagon-like peptide-1 . Created with BioRender.com.

Of note, when comparing our results of effects of liraglutide on the MRI-measured fat fraction to other studies measuring effects of interventions on BAT, a few things are of importance. ^{18}F-FDG PET is the current gold standard for assessing BAT volume and activity and measurement of the fat fraction with MRI has been shown to correlate well to the glucose uptake rate measured using ^{18}F-FDG PET in healthy subjects, at least upon activation of BAT by cold exposure[55]. It should be realized though that cold exposure is potentially the most effective trigger to activate BAT and reduce its fat fraction. In addition, the uptake of glucose by BAT is greatly decreased in patients with type 2 diabetes[56], making it difficult to compare our results measured with MRI in subjects with type 2 diabetes to studies performed with ^{18}F-FDG PET in healthy subjects with normal insulin sensitivity. Furthermore, we must take caution in directly comparing effects of different GLP-1 receptor agonists. For example, exenatide is based on a natural occurring peptide bearing a 53% homology to human GLP-1 while liraglutide is an analogue of human GLP-1 which is modified to slow down its renal clearance[57]. In addition, we can speculate on the difference in observations between humans and rodents, the latter showing relatively large effects of GLP-1 receptor agonism on BAT. Rodents have a relatively high thermoneutral temperature point with increased cold-induced thermogenesis below a temperature of 29-33°C[58] while humans have a thermoneutral point of 21-23°C (lightly clothed)[59]. Therefore, in rodents a large relative amount of BAT and therefore energy combustion by BAT likely contributes to a larger extent to total resting energy expenditure than in humans, which may increase the potential of activation of BAT by GLP-1. Experiments investigating GLP-1 receptor agonism in rodents at thermoneutral temperature, which would reduce cold-induced thermogenesis by BAT, or in humans at low temperatures, which could promote this, could provide more information on the effectiveness of GLP-1 receptor agonists to activate BAT under different ambient temperatures. Finally, the effects in rodents were observed after intracerebroventricular injection of medication[17,46], resulting in high local concentrations to stimulate sympathetic outflow to BAT. Indeed, it has been shown that transfer of liraglutide to cerebrospinal fluid is minimal[60]. Therefore, although GLP-1 receptor agonists could theoretically affect the central nervous system via peripheral effects on the vagal nerve, this may limit therapeutic potential of the current generation of (peripherally administered) GLP-1 receptor agonists since effects on energy expenditure and BAT are largely mediated via central nervous system activation[61].

To conclude, although studies in rodents support a role for (central) GLP-1 receptor agonism in energy expenditure and BAT activity, liraglutide induces weight loss in humans by decreasing energy intake rather than by activating BAT or increasing energy expenditure. However, there seems to be potential for exenatide and possibly other

GLP-1 receptor agonists to activate BAT. Therefore, further development of GLP-1 receptor agonists targeting BAT activation could yield clinical benefit.

3. Effects of GLP-1 receptor agonists on cardiometabolic health

3.1 The role of visceral adipose tissue and ectopic fat in metabolic health

The main cause of type 2 diabetes is insulin resistance of various metabolic organs in combination with insufficient insulin secretory capacity of the pancreas to overcome this resistance. Although several factors can contribute to the development of insulin resistance, central obesity with concomitant accumulation of VAT, and increased fat accumulation in non-adipose tissue are major risk factors[3,4,62-65]. Under physiological conditions subcutaneous adipose tissue (SAT) functions as a reservoir for energy storage. However, when its storage capacity is exceeded, necrosis of adipocytes attracts immune cells resulting in induction of insulin resistance. The VAT contains more hypertrophic adipocytes than SAT and shows more macrophage infiltration and inflammation[66]. Furthermore, an increased rate of basal lipolysis in VAT compared to SAT results in increased release of free fatty acids (FFAs) to the portal circulation[67]. As a result of this, ectopic fat accumulation occurs in different organs, leading to disrupted function of those organs[3]. Hepatic steatosis results in insulin resistance of hepatocytes leading to impaired insulin-induced suppression of glycogenolysis and gluconeogenesis[68]. Furthermore, hepatic insulin resistance results in overproduction of VLDL, accelerating dyslipidemia[69]. In skeletal muscle cells, increased intramyocellular lipid accumulation results in accumulation of toxic lipid intermediates. This leads to defects in insulin signaling and subsequently decreased glucose transporter 4 (GLUT4) regulated glucose transport over the cell membrane and consequently insulin resistance[70,71]. In addition, excess of myocardial and epicardial fat may contribute to remodelling of the heart and contractile dysfunction[72]. Lastly, ectopic renal fat accumulation can cause podocyte lipotoxicity contributing to initiation and progression of albuminuria and chronic kidney disease[73,74].

As might be expected, in line with the above, a structured weight loss programme induced greater improvement of metabolic parameters in subjects with mainly reduction of VAT than in subjects with mainly reduction of SAT[75], and diet-induced weight loss in obese adolescents with excessive accumulation of hepatic triglyceride and normal glucose tolerance improved hepatic and skeletal muscle insulin sensitivity[76]. In **Chapter 6** we showed that treatment of obese patients with cardiovascular disease and type 2 diabetes with a 16-week very low calorie diet (VLCD) of 450-1000 kcal/day, besides reducing SAT volume, reduced VAT volume and ectopic fat accumulation in all compartments that we measured. Furthermore, this resulted in greatly improved glycemic control and im-

provement of dyslipidemia. Of note, these improvements were paralleled by decreased levels of the endocannabinoid anandamide (AEA). The endocannabinoid system (ECS) consists of endocannabinoids, their receptors and associated metabolic enzymes and increased ECS activity is associated with obesity and type 2 diabetes[77,78]. Indeed, treatment of obese subjects with rimonabant, an inverse agonist for the CB_1 receptor, induced marked weight loss and improved lipid metabolism, but increased the risk of psychiatric adverse events by binding to CB_1 receptors in the central nervous system, resulting in discontinuation of its clinical use[79-81]. Recently, it was shown that insulin resistance correlates with increased expression of the cannabinoid receptor type 1 (CB_1) in both SAT and VAT[82]. Furthermore, in the same study, *ex vivo* CB_1 inhibition with the antagonist AM281 resulted in reduction of lipolysis rates. Also, recently, high circulating levels of the endocannabinoid 2-arachidonoylglycerol (2-AG), but not AEA, were shown in obese and overweight patients with nonalcoholic fatty liver disease (NAFLD)[83]. We anticipate that in the study in which patients with type 2 diabetes were exposed to a VLCD, the reduction of activity of the endocannabinoid system as measured by a reduction of AEA, may have contributed to the improved metabolic phenotype induced by weight loss. Together, these findings underline the role of the ECS in metabolism, making it a promising target to improve metabolic health. Future studies can focus on nutritional interventions, which can affect the availability of biosynthetic precursors[84], or pharmacological agents targeting the ECS. Since the CB_1 receptor inverse agonist rimonabant failed because of psychiatric side effects[81], targeting the ECS should probably be strictly peripheral though. Recently published results on treatment with JM-00266, a non-brain penetrant CB_1 receptor inverse agonist, showing an improved glucose tolerance and insulin sensitivity in wild-type mice, but not in CB_1 receptor knockout mice, are very promising[85].

3.2 Effects of GLP-1 receptor agonism on VAT and ectopic fat

As has been discussed in section 3.1, interventions specifically targeting ectopic fat are likely a very effective therapeutic strategy to reduce insulin resistance and improve metabolic health in patients with type 2 diabetes. The effects of GLP-1 receptor agonism on VAT and ectopic fat will be discussed in this section. In **Chapter 3** we described the effects of treatment with liraglutide on ectopic fat accumulation in patients with type 2 diabetes from South Asian descent. South Asians develop type 2 diabetes more often and at a lower BMI than Western Europeans, which is related to a relatively high amount of VAT[86-88]. GLP-1 receptor agonism reduces body weight, and reductions of SAT[89], VAT[90-92], epicardial fat volume[93,94] and hepatic triglyceride content[30,95] have been reported in study populations of different compositions. We were the first to investigate the effects of liraglutide on different fat depots and ectopic fat in a group of South Asian patients with type 2 diabetes. In our per-protocol analysis we showed that liraglutide

compared to placebo added to standard care decreases the VAT volume, without significantly decreasing the volume of SAT and epicardial fat and the myocardial and hepatic triglyceride content. When comparing the effects on SAT and VAT to other studies in which subjects were treated with liraglutide, our results are mostly in line, showing a decrease of VAT[90-92]. However, Suzuki et al[89], using a relatively low dose of 0.9 mg/day liraglutide and studying Japanese type 2 diabetes patients, reported a decrease of SAT without an effect on VAT. And similarly, when comparing the South Asian to the Western European subjects of our MAGNA VICTORIA-trial, it must be noted that in the Western European treatment group, despite decreasing SAT volume, no effects on VAT or ectopic fat accumulation were observed[96]. The reason for the discrepancy in effect on different adipose tissue depots between the populations is unclear but an ethnic-specific effect of liraglutide or of weight loss itself might play a role. There are indications for an ethnic-specific effect from a study comparing the effect of VLCD in overweight non-diabetic South Asians to Western Europeans[97]. Although the VLCD equally reduced body weight in both groups, the diet reduced pericardial adipose tissue in the Western Europeans only. Importantly, the reduction of VAT in the populations of South Asians we studied was accompanied by and associated with a decrease in HbA1c, indicating improved glycemic control. This demonstrates that treatment with liraglutide and possibly other GLP-1 receptor agonists could be an effective treatment option for South Asian patients with type 2 diabetes and possibly in other populations with increased VAT accumulation.

We can only speculate on the mechanism behind GLP-1 induced reduction of VAT. GLP-1 may directly activate the GLP-1 receptor on adipocytes, although this is unlikely since most studies show no presence of the GLP-1 receptor on primary adipocytes[98-100]. Furthermore, improved perfusion of adipose tissue as a consequence of vasodilatation by GLP-1[101,102] can possibly affect its metabolic regulatory function. Whether GLP-1 may differently affect perfusion between VAT and other adipose tissues including SAT remains to be investigated. Another potential mechanism by which GLP-1 receptor agonism could specifically reduce VAT may relate to the autonomic nervous system. Mouse studies have shown that activation of central GLP-1 receptors improves insulin sensitivity as related to an increased sympathetic outflow[46,103]. Since sympathetic innervation of adipose tissue is an important activator of lipolysis and innervation of VAT and SAT is partially separated[104,105], this could theoretically result in specifically enhanced lipolysis in VAT as compared to SAT.

As mentioned earlier, hepatic steatosis is an important risk factor for disruption of glucose and lipid metabolism. South Asians have been shown to have a higher hepatic triglyceride content, even at young age and without overweight or obesity (BMI <25 kg/m^2)[15,106]. We investigated the effects of liraglutide on hepatic triglyceride content in

patients with type 2 diabetes of South Asian (**Chapter 2**) and Western European descent (**Chapter 5**). In the population of Western Europeans we studied, hepatic triglyceride content was decreased after treatment with liraglutide, but also after treatment with placebo in addition to standard care. This is in line with two other trials comparing effects of liraglutide vs insulin[107] and exenatide vs insulin or pioglitazone[108], showing equally reduced hepatic triglyceride content in all treatment groups. In contrast to our results, however, several trials in patients with NAFLD and type 2 diabetes under inadequate glycemic control on oral glucose-lowering drugs did show reductions of hepatic steatosis upon treatment with exenatide compared to insulin[95,109] and also in patients with NASH, treatment with liraglutide compared to placebo led to histological resolution of NASH with no worsening of fibrosis[30]. Importantly, none of these trials included patients already on insulin therapy, which proves a preserved β-cell function in these patients and might have affected results, since GLP-1 receptor agonism efficacy is affected by residual β-cell function[110] and, therefore, the effects on insulin secretion in patients with deteriorated β-cell function might be smaller. The observation that placebo in addition to standard care decreased hepatic triglyceride content in the Western European patients in our study similarly to liraglutide, might be explained by improved glycemic control after intensified treatment with glucose-lowering drugs in the placebo-group.

In the South Asian patients with type 2 diabetes no decrease of hepatic triglyceride content was observed after treatment with liraglutide or after intensified treatment, although weight loss was similar to the Western European group. We were the first to report on effects of GLP-1 receptor agonism on hepatic steatosis in this specific population. However, surprisingly and unexplained, the hepatic triglyceride content in the South Asian patients with type 2 diabetes we studied was already relatively low at baseline compared to the Western European patients, with mean hepatic triglyceride contents of 10% and 18%, respectively. Also, in other trials that showed reduction of hepatic triglyceride content upon treatment with GLP-1 receptor agonists as mentioned above, baseline hepatic triglyceride levels were considerably higher, indicating that the potency of liraglutide to reduce hepatic steatosis may be higher with increased hepatic steatosis. Studying of effects of GLP-1 receptor agonism on hepatic steatosis in subjects with more pronounced hepatic triglyceride content could provide further insight. Furthermore, an ethnic-specific effect, with impaired potential to reduce hepatic steatosis in the South Asian population, cannot be excluded. To conclude, although liraglutide does not improve hepatic steatosis in South Asian patients with type 2 diabetes, the reduction of VAT and improved glycemic control demonstrate that GLP-1 receptor agonism can be an effective treatment option.

3.3 The relationship between hepatic steatosis and CETP

In addition to effects of GLP-1 receptor agonism on hepatic triglyceride content, we investigated the possible relationship between hepatic triglyceride content and CETP, as described in **Chapter 5** of this thesis. CETP facilitates the transfer of triglycerides from VLDL to high-density lipoprotein (HDL), coupled to transfer of cholesteryl esters from HDL towards VLDL resulting in cholesterol enrichment of atherogenic VLDL particles and decreased HDL-cholesterol levels and may contribute to a pro-atherogenic profile[111,112]. Logically, pharmacological CETP inhibitors have been developed, but unfortunately most trials failed to show a beneficial cardiovascular effect[113-115]. Anacetrapib was shown to reduce the incidence of major coronary events by 9% compared to placebo[116], but the manufacturer decided not to pursue commercialization. Interestingly, recently, results were published on long-term safety and efficacy of anacetrapib after cessation of study treatment, showing that beneficial effects on major coronary events increased in follow-up, likely due to the accumulation of anacetrapib in adipose tissue resulting in a pharmacological action long after cessation of therapy[117]. Furthermore, the recently published data on the CETP inhibitor obicetrapib, a highly potent CETP inhibitor, showed very promising results by decreasing ApoB and reducing LDL-cholesterol concentration by up to 51%[118]. Although these observations refuelled interest in CETP and its role in dyslipidemia and atherosclerotic cardiovascular disease, regulation of the production of CETP is still not fully elucidated.

The circulating CETP concentration is mainly determined by Kupffer cells[119,120], suggesting a link between hepatic inflammation/steatosis and CETP production. To investigate a potential relationship between hepatic triglyceride content and circulating CETP, we studied a large population-based cohort with overweight subjects and did not observe an association between hepatic triglyceride content and circulating CETP. However, the mean hepatic triglyceride content in this population was low in both men (3.4%) and women (1.7%). Therefore, although a relationship between hepatic triglyceride content and CETP is not present in overweight subjects without hepatic steatosis, we cannot exclude a relationship in subjects with NAFLD. Further studies in subjects with different stages of NASH could help clarify this relationship.

Previous human studies with pioglitazone[121] and prolonged caloric restriction[122] have shown reduction of hepatic triglyceride content as well as plasma CETP concentration. Since GLP-1 receptor agonists also have previously been shown to reduce hepatic triglyceride content[30,95,109], we investigated the relationship between hepatic triglyceride content and CETP in patients with type 2 diabetes of Western European descent treated with liraglutide. In the trial we performed, the decrease in hepatic triglyceride content did not coincide with a decrease of circulating CETP concentration. It must be noted, as

mentioned in section 3.2, that in this trial the group treated with placebo in addition to standard care showed a similar decrease of hepatic steatosis. Importantly, in the LEAN-trial[30], although liraglutide induced histological resolution of NASH by liraglutide, no effects were observed on lobular inflammation. Combined with our results, this suggests that liraglutide fails to affect CETP production by Kupffer cells. This may seem surprising given that both pioglitazone[123] and GLP-1[124] have been shown to affect expression of the transcription factor liver X receptor alpha (LXRα), which is crucial in regulation of CETP expression[125,126]. However, in macrophages LXRα is mainly activated by oxidized sterols and the endogenous ligand desmosterol[127,128], while the effects of GLP-1 receptor agonism on these sterol derivatives has not been studied as yet. Studies comparing the effects of GLP-1 receptor agonists in human CETP transgenic mice on LXRα activation and its target genes including CETP could provide further insight in whether GLP-1 receptor activation may regulate CETP production by Kupffer cells.

3.4 Effects of GLP-1 receptor agonism on cardiovascular function

Type 2 diabetes is associated with a 4 to 5-fold increased risk to develop congestive heart failure, even without presence of prior coronary heart disease[129]. Although heart failure may result from coronary artery disease, in many patients with diabetes the cause of heart failure is diabetic cardiomyopathy, which is typically non-ischemic and preceded by diastolic dysfunction. Most patients with diabetes subsequently develop heart failure with preserved ejection fraction (HFpEF). The pathogenic process is multifactorial comprising disturbance of insulin signaling, oxidative stress, inflammation, autonomic dysfunction and formation of advanced glycation end-products (AGEs) as a consequence of hyperglycemia. Furthermore, diabetes is associated with increased myocardial triglyceride accumulation, which adversely affects cardiomyocyte function. Together, these processes induce increased cardiac stiffness and myocardial dysfunction[130,131]. Unfortunately, although the process of diabetic cardiomyopathy is increasingly unraveled, pharmacological options to treat or prevent development of heart failure remain limited.

In **Chapter 3** of this thesis, we describe effects of GLP-1 receptor agonists on cardiac function, based on the notion that direct actions of GLP-1 on the myocardium may improve cardiac function[132], and indirect effects of improved glycemic control and weight loss with concomitant reduction of myocardial steatosis can improve diastolic function[133,134]. Specifically, we evaluated effects of liraglutide on cardiac function in a randomized controlled trial in patients with type 2 diabetes of South Asian descent. While liraglutide improved VAT volume and glycemic control, liraglutide did not beneficially modulate diastolic function and myocardial tissue characteristics. These results are in line with evidence from other recent trials included in a recent meta-analysis,

showing that liraglutide does not improve left ventricular systolic and diastolic function[135]. Recently, similar results were reported for treatment with albiglutide[136], which makes us conclude that current-generation GLP-1 receptor agonists do not directly improve diabetic cardiomyopathy. This is in contrast to the relatively new therapeutic modality of sodium-glucose cotransporter 2 (SGLT2) inhibitors, which have recently been shown to reduce the risk of hospitalization for heart failure in patients with type 2 diabetes. Furthermore, SGLT2 inhibitors reduce progression of kidney disease and renal death[137-139]. These results have resulted in a recent change of international treatment guidelines, now providing a strong recommendation to initiate SGLT2 inhibitors early in patients with type 2 diabetes and heart failure and/or chronic kidney disease[140].

However, although GLP-1 receptor agonists do not improve diabetic cardiomyopathy, over the past years, several trials have shown the effectiveness of GLP-1 receptor agonists in reducing atherosclerotic myocardial infarction, stroke, and cardiovascular death (MACE) in patients with and without established atherosclerotic cardiovascular disease (ASCVD)[141]. This reduction of major cardiovascular events in patients with established ASCVD of approximately 14% is similar to the effect of SGLT2 inhibitors[139]. However, reduction of MACE in patients *without* established ASCVD upon treatment with SGLT2 inhibitors has not been shown. This might be explained by the fact that most trials primarily enrolled patients with high cardiovascular risk, resulting in underpowered analyses of patients without established ASCVD, although a more pronounced anti-atherosclerotic effect of GLP-1 receptor agonism is probably also important. The reduction of cardiovascular events by GLP-1 receptor agonism can largely be explained by the fact that GLP-1 receptor agonists can improve several risk factors for cardiovascular disease, including glycemic control, body weight, dyslipidemia and blood pressure[142]. Furthermore, the observation that the GLP-1 receptor is present on endothelial cells, monocytes, macrophages, and vascular smooth muscle cells suggests that GLP-1 can beneficially interfere with the atherogenesis process[143]. Indeed, in rodents, GLP-1 receptor agonism results in decreased formation, expansion, and vulnerability of atherosclerotic lesions[144]. These are important findings, since South Asians are at increased risk to develop atherosclerotic cardiovascular disease and even asymptomatic South Asians with type 2 diabetes show a higher prevalence and extent of coronary artery disease compared to matched Western European subjects[145]. Therefore, GLP-1 receptor agonists should be considered as treatment strategy in patients with high atherogenic cardiovascular risk such as the South Asian population.

3.5 A role for novel dual GLP-1/GIP receptor agonists

Although treatment with GLP-1 receptor agonists nowadays is widely recommended for treatment of obesity and type 2 diabetes, many patients unfortunately do not achieve

treatment goals. Therefore, optimization of pharmacological agents is very important. A possible way to improve therapy could be by using dual GLP-1/GIP receptor agonists. Indeed, already in 1993 it was shown that combined infusion of GLP-1 and GIP in healthy volunteers resulted in augmented β-cell responses compared to either hormone alone[146]. Recently, tirzepatide, a long-acting dual GLP-1/GIP receptor agonist has been developed and, interestingly, treatment of type 2 diabetes patients for 26 weeks improved markers for insulin sensitivity and β-cell function to a greater extent than dulaglutide, a GLP-1 receptor agonist[147]. Also, tirzepatide has proven to be superior to semaglutide with respect to improvement of glycemic control and reduction of body weight[148]. Furthermore, tirzepatide has recently shown spectacular effects in treatment of obesity[149] and NASH[150]. In fact, 72 weeks of treatment with tirzepatide (15 mg/week) decreased body weight of obese subjects with a mean body weight of 104.8 kg at baseline by an unprecedented 20.9%, which approximates the effect of bariatric surgery[149].

Dual agonists thus greatly enhance the therapeutic efficacy of GLP-1 receptor agonism, and several mechanisms may be involved. The GIP receptor is present on adipocytes and it is hypothesized that agonism can improve the lipid-buffering capacity of white adipose tissue by increasing adipose tissue perfusion, recruiting lipoprotein lipase (LPL) and enhancing insulin-stimulated glucose uptake[151]. This can subsequently result in reduction of ectopic fat accumulation. In addition, dual receptor agonism in mice has shown to cause more robust anorexigenic effects, possibly because, centrally, distinct neurons are activated and inhibiting food intake[152,153]. Interestingly, recent results from our group show that combined GIP/GLP-1 receptor agonism in mice, compared to single GLP-1 or GIP receptor agonism, diminishes inflammation and increases VLDL turnover, thereby attenuating atherosclerosis severity (Van Eenige et al, submitted). This may prove a useful therapeutic strategy to further decrease atherosclerotic cardiovascular risk in the South Asian population and other populations compared to GLP-1 receptor agonism alone, which warrants further investigation. Furthermore, very recently it was shown in mice that LY3437943, a triple agonist for the glucagon, GIP, and GLP-1 receptors is on equimolar basis even more effective than tirzepatide with respect to inducing a greater body weight reduction and increasing energy expenditure[154]. Although effects in humans are still to be studied, these results are a very promising basis for the future development of triple agonists in the treatment of obesity and type 2 diabetes.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The prevalence of type 2 diabetes keeps rising and type 2 diabetes is one of the largest health problems in the world. VAT and ectopic fat accumulation play a crucial role in the

pathogenesis of type 2 diabetes, and GLP-1 receptor agonists have potential to reduce body weight and ectopic fat accumulation and improve cardiometabolic health. In this thesis we provide insight in the mechanism behind liraglutide-induced weight reduction, showing that liraglutide reduces body weight by reducing energy intake, rather than by activating BAT or increasing energy expenditure. Furthermore, we studied effects of liraglutide on the various adipose tissue depots and ectopic fat deposition and demonstrated a specific reduction of VAT in South Asian patients with type 2 diabetes. This reduction was accompanied by a significant improvement of glycemic control. Lastly, we provided evidence that liraglutide does not improve cardiac function and myocardial tissue characteristics and thus does not improve diabetic cardiomyopathy.

The South Asian population is at high risk to develop cardiovascular disease and type 2 diabetes, which is incompletely understood but can partly be explained by pronounced VAT and ectopic fat deposition. Based on results of this thesis, we propose that GLP-1 receptor agonists might be useful therapeutic means to improve glycemic control by reducing volume of VAT, which has been causally linked to insulin resistance and has been shown to be a major contributor to metabolic risk, in South Asian type 2 diabetes patients. Possibly, this can also be applied to other patients with type 2 diabetes in combination with pronounced VAT accumulation. Furthermore, our results implicate that also patients with overweight (BMI between 25 and 30 kg/m^2), and not only with obesity (BMI ≥30 kg/m^2) can greatly benefit from treatment with GLP-1 receptor agonists, which is important since currently the lower BMI limit for reimbursement by health care providers lies at 30 kg/m^2. In addition, although SGLT2 inhibitors, with proven beneficial effects on heart failure and progression of kidney disease, might be a better therapeutic option for treatment of type 2 diabetes in most patients, GLP-1 receptor agonists, due to their anti-atherosclerotic effects, might be the best current option in patients with high risk for atherosclerotic complications, such as the South Asian population. We expect that long-term treatment with GLP-1 receptor agonists will further reveal its potential in decreasing atherosclerotic cardiovascular disease and overall improving cardiometabolic health in the forthcoming years. Until then, patient-centered decision making with careful identification of risk factors for heart failure and atherosclerosis is warranted.

Over the past years, GLP-1 receptor agonists have proven to be an effective treatment option for obesity and type 2 diabetes. Their successors that are currently being clinically developed, including tirzepatide, a dual GLP-1/GIP receptor agonist, and LY3437943, a next-generation triple GLP-1/GIP/glucagon receptor agonist, appear to be even more effective in reduction of body weight and improvement of glycemic control[149,154]. Interestingly, GLP-1/GIP/glucagon receptor triple agonism in mice increases energy expenditure, which is likely caused by additionally increasing hepatic fatty

acid oxidation as compared to dual GLP-1/GIP receptor agonism. Since dual GLP-1/GIP receptor agonists have been shown to cross the blood-brain barrier[155], and LY3437943 is possibly also able to do that, dual and triple agonism may also be more effective in activating BAT by enhancing sympathetic outflow from the hypothalamus compared to liraglutide. Taken together, if dual and triple agonists demonstrate not to cause more adverse reactions compared to GLP-1 receptor agonists, they may play a key role in the battle against reducing weight loss in overweight and obesity and improve associated cardiometabolic diseases including type 2 diabetes, NAFLD/NASH and atherosclerotic cardiovascular disease in the next decades.

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Chapter 8

Appendices

SUMMARY

Over the past decades, the worldwide prevalence of overweight and obesity has increased dramatically. By disturbing metabolic processes and inducing low grade systemic inflammation, overweight and obesity increase the risk to develop type 2 diabetes, which in turn can lead to cardiovascular diseases. Type 2 diabetes is a chronic disease that is characterized by a disturbed glucose regulation, and involves both environmental and genetic factors. Under physiological conditions, postprandially insulin is secreted by the pancreas via several mechanisms in which rising blood glucose levels and secretion of gut hormones including glucagon-like peptide-1 (GLP-1) play a role, the combined action of which results in uptake of circulating glucose by metabolic tissues and restores blood glucose levels to the pre-prandial concentration. However, in type 2 diabetes the metabolic tissues fail to adequately respond to insulin as a consequence of insulin resistance, resulting in hyperglycemia and contributing to dyslipidemia. Insulin resistance is thus essential in the pathophysiology of type 2 diabetes and increased accumulation of ectopic fat, which is storage of triglycerides in non-adipose tissues, plays an important role in the development of local and whole-body insulin resistance.

A relatively new class of drugs to treat patients with type 2 diabetes are GLP-1 receptor agonists. They mimic the action of GLP-1, which is an incretin hormone produced by L-cells within the intestine that binds to GLP-1 receptors present on vagal afferent neurons and in many different tissues such as pancreas and brain. GLP-1 receptor agonist are resistant to enzymatic breakdown, and therefore have strongly increased GLP-1 receptor signalling properties compared to endogenous GLP-1. Although it has been shown over the past decade that GLP-1 receptor agonists improve glycemic control and reduce body weight, the effects of GLP-1 receptor agonists on the various adipose tissue compartments and on cardiac function remained unclear. In this thesis, we investigated the effects of the GLP-1 receptor agonist liraglutide on these various aspects of cardiometabolic health.

Chapter 1 serves as a general introduction into obesity and its role in the pathogenesis of type 2 diabetes and cardiovascular diseases, with specific focus on the role of ectopic fat in the induction of insulin resistance. Furthermore, we elaborated on the disadvantageous phenotype of the South Asian population with a higher risk to develop type 2 diabetes and cardiovascular diseases at a relatively younger age and at lower BMI as compared to Western Europeans. Next, the role of the endocannabinoid system (ECS) in attenuating energy expenditure and aggravation of obesity was discussed as well as potential therapeutic strategies to reduce the tone of the ECS. Finally, treatment options for type 2 diabetes were discussed including GLP-1 receptor agonists, including their effects on ectopic fat and cardiovascular function.

Since South Asians have a high risk to develop type 2 diabetes, which may be related to high ectopic fat accumulation, in **Chapter 2**, we evaluated the effects of treatment with liraglutide for 26 weeks on various adipose tissue depots, ectopic fat deposition and HbA1c in Dutch South Asian patients with type 2 diabetes. In this placebo-controlled, double blind, clinical trial we randomly assigned patients to treatment with liraglutide or placebo added to standard care. Using MRI and ^{1}H-MRS, we assessed effects on subcutaneous adipose tissue (SAT), visceral adipose tissue (VAT), epicardial and paracardial adipose tissue volume and on myocardial and hepatic triglyceride content. While the intention-to-treat analysis did not show effects of liraglutide on ectopic fat and HbA1c, per-protocol analysis showed that liraglutide decreases visceral adipose tissue volume, which was associated with improved glycaemic control in South Asians. We concluded that this demonstrates that GLP-1 receptor agonists might be useful therapeutic means to improve glycemic control by reducing VAT volume in South Asian type 2 diabetes patients.

Furthermore, many patients with type 2 diabetes develop heart failure with preserved ejection fraction (HFpEF). Often, this is preceded by diabetic cardiomyopathy, which is a disorder of the heart muscle characterized by left ventricular diastolic dysfunction as a consequence of impaired relaxation and compliance. GLP-1 receptor agonists have direct and indirect effects on the myocardium and multiple preclinical studies have shown therapeutic benefits of GLP-1 receptor agonists on cardiac function. Since liraglutide may be beneficial in the prevention of development of diabetic cardiomyopathy, in **Chapter 3** we investigated effects of liraglutide on cardiac function by assessing effects on left ventricular diastolic and systolic function in the same group of Dutch South Asian patients with type 2 diabetes as described in Chapter 2. We showed that liraglutide compared with placebo does not affect left ventricular diastolic and systolic function, aortic stiffness, myocardial triglyceride content or extracellular volume. We concluded that liraglutide does not improve diabetic cardiomyopathy. Nonetheless, since GLP-1 receptor agonists have recently been reported to beneficially interfere with the atherogenesis process, GLP-1 receptor agonists should be considered as treatment strategy in patients with high atherogenic cardiovascular risk such as the South Asian population.

GLP-1 receptor agonists are well-known to reduce body weight, but the exact underlying mechanism is still unclear. Since GLP-1 receptor agonists induce satiety, the weight loss can at least be partially explained by a reduction of food intake. Nonetheless, several studies have indicated that GLP-1 receptor agonists may beneficially impact energy expenditure as well which may contribute to body weight reduction. In fact, studies in preclinical models suggest that GLP-1 receptor agonists increase thermogenesis in brown adipose tissue (BAT), thereby reducing the triglyceride content of BAT since active BAT combusts fatty acids into heat. In **Chapter 4**, we thus aimed to assess effects of treat-

ment with liraglutide on resting energy expenditure and fat content of BAT using indirect calorimetry and chemical-shift water-fat MRI, respectively. We describe the effects on energy expenditure after 4, 12 and 26 weeks of treatment, and measured the fat fraction in the supraclavicular BAT after 26 weeks. Interestingly, while treatment with liraglutide resulted in the expected decrease in body weight, liraglutide decreased energy expenditure in the first 12 weeks compared to baseline and still tended to decrease energy expenditure after 26 weeks without affecting the fat fraction in the supraclavicular BAT depot. We proposed that a possible explanation for this can be a decreased diet-induced thermogenesis upon decreased food intake or an increased metabolic efficiency to adapt to the negative energy balance in order to limit loss of body weight. We concluded that liraglutide induces weight loss in humans by decreasing energy intake rather than by activating BAT or increasing energy expenditure.

Hepatic steatosis is an important risk factor for disruption of glucose and lipid metabolism, and previous studies have shown that treatment with GLP-1 receptor agonists reduces hepatic steatosis. In **Chapter 5**, we investigated the possible relationship between hepatic triglyceride content and circulating levels of cholesteryl ester transfer protein (CETP). CETP facilitates the transfer of triglycerides from very-low-density lipoproteins (VLDL) to high-density lipoproteins (HDL), coupled to transfer of cholesteryl esters from HDL towards VLDL. This results in formation of pro-atherogenic VLDL particles and decreased HDL-cholesterol levels and therefore contributes to a pro-atherogenic lipid profile. CETP is mainly produced by Kupffer cells, but the regulation of CETP production by these cells is still incompletely understood albeit that previous publications suggested a role for the hepatic triglyceride content. Therefore, we assessed whether treatment with liraglutide for 26 weeks decreases the circulating CETP concentration. In a placebo-controlled trial, patients with type 2 diabetes of Western European descent were studied. Hepatic triglyceride content was decreased after treatment with liraglutide, but also after treatment with placebo added to standard care without between group differences. However, the reduction in hepatic triglyceride content was not accompanied by a reduction of circulating CETP in both groups. In addition, we investigated the relationship between the hepatic triglyceride content and circulating CETP in a large population-based cohort of 1,611 participants of the Netherlands Epidemiology of Obesity (NEO) study. Also in this population, no association between hepatic triglyceride content and CETP was present. We concluded that the hepatic triglyceride content does not determine circulating CETP. Furthermore, our results implicate that interventions reducing hepatic triglyceride content *per se* do not affect hepatic oxysterols and thereby liver X receptor alpha (LXRα)-induced CETP production.

GLP-1 receptor agonists thus mainly reduce body weight by reducing food intake, which may beneficially modulate the ECS. To explore the effects of reduced food intake on the ECS, **Chapter 6** aimed to evaluate the effects of prolonged caloric restriction on circulating endocannabinoids in relation to ectopic fat accumulation and cardiac function. In a prospective intervention study in obese patients with type 2 diabetes and coronary artery disease we showed that a 16-week very low calorie diet (VLCD) of 450-1000 kcal/day substantially reduced volume of VAT, SAT and pericardial fat and decreases myocardial and hepatic triglyceride content, which coincided with greatly improved glycemic control. These improvements were paralleled by circulating decreased levels of endocannabinoids of the class of *N*-acylethanolamines, including anandamide (AEA), without decreasing monoacylglycerols. Furthermore, VLCD decreased left ventricular (LV) mass, increased ejection fraction and increased end-diastolic volume at similar estimates of LV filling pressure, suggesting an improved LV compliance. Also, VLCD decreased aortic pulse wave velocity, a surrogate marker for arterial stiffness. We concluded that dietary interventions can effectively reduce ectopic fat accumulation and improve cardiovascular function in patients with type 2 diabetes. We anticipate that the reduction of activity of the endocannabinoid system, as measured by a reduction of AEA may have contributed to the improved metabolic phenotype induced by weight loss.

Finally, in **Chapter 7** we provided a general discussion of the findings of the studies described in this thesis. Specifically, the results described in this thesis provide insight in the mechanism behind liraglutide-induced weight reduction, showing that weight loss in humans is caused by decreasing energy intake rather than by activating BAT or increasing energy expenditure. Furthermore, we elucidated the effects of liraglutide on the various adipose tissue depots and ectopic fat deposition. We propose that GLP-1 receptor agonism might improve glycemic control by reducing VAT volume in South Asian type 2 diabetes patients. Lastly, we provided evidence that liraglutide GLP-1 agonism does not improve diabetic cardiomyopathy, but we speculate that GLP-1 agonism might be very effective in reducing atherogenic cardiovascular risk in populations at high-risk such as the South Asian population. Over the past years, GLP-1 receptor agonists have proven to be an effective treatment option for obesity and type 2 diabetes. Very interestingly, tirzepatide, a novel dual GLP-1/GIP receptor agonist has recently shown spectacular effects on body weight and glycemic control and next-generation triple agonists adding agonism of the glucagon receptor might appear to be even more effective. In the upcoming years long-term treatment with GLP-1 receptor agonists, alone and in combination with GIP and glucagon receptor agonism, will further reveal its potential in decreasing atherosclerotic cardiovascular disease and improving overall cardiometabolic health.

NEDERLANDSE SAMENVATTING

Gedurende de laatste decennia is wereldwijd het aantal mensen met overgewicht en ernstig overgewicht, oftewel obesitas, schrikbarend toegenomen. Overgewicht en obesitas verstoren diverse processen in de vet- en suikerstofwisseling (het 'metabolisme') en leiden tot laaggradige ontsteking in het lichaam. Hiermee vergroten overgewicht en obesitas het risico op het ontwikkelen van type 2 diabetes en hart- en vaatziekten. Type 2 diabetes is een chronische ziekte die wordt gekarakteriseerd door een verstoorde bloedsuikerregulatie ('glucosemetabolisme'). Verminderde gevoeligheid van weefsels voor het hormoon insuline ('insulineresistentie') is een essentiële factor voor de ontwikkeling van type 2 diabetes, en wordt veroorzaakt door stapeling van vet in vetcellen tussen organen ('visceraal vet') en in de organen zelf ('ectopisch vet'). Dergelijke vetstapeling ontstaat als de capaciteit van het onderhuidse ('subcutane') vet niet meer toereikend is. Ectopisch vet in organen zoals de lever, het hart of de spieren, beïnvloedt het vet- en glucosemetabolisme op een negatieve manier. Bij een gezond gewicht en normale insulinegevoeligheid, wordt er na de maaltijd insuline uitgescheiden door de alvleesklier ('pancreas') door directe stimulatie van zogenaamde β-cellen in de pancreas door glucose, maar bijvoorbeeld ook door afgifte van het darmhormoon glucagon-like peptide 1 (GLP-1) door zogenaamde L-cellen in het darmstelsel. Insuline stimuleert vervolgens de opname van glucose en vet in verschillende weefsels waardoor de bloedsuiker en -vetspiegel normaliseert. Bij patiënten met type 2 diabetes is er insulineresistentie, vaak ten gevolge van een verhoogde hoeveelheid visceraal vet en ectopisch vet, en lukt het niet goed om de opname van glucose en vetten te stimuleren, wat resulteert in een verhoogde bloedsuiker en -vetspiegel.

GLP-1 receptor agonisten worden toegepast als een relatief nieuwe behandelmethode voor patiënten met obesitas en type 2 diabetes. Ze bootsen het effect na van het darmhormoon GLP-1, dat wordt uitgescheiden door L-cellen in het darmstelsel. GLP-1 en GLP-1 receptor agonisten binden aan GLP-1 receptoren op zenuwbanen die informatie van de darm naar de hersenen stuurt ('afferente takken van de nervus vagus') en aan receptoren in allerlei weefsels, zoals de pancreas en de hersenen zelf. Dit leidt, naast stimulatie van uitscheiding van insuline door de pancreas, onder andere tot bewerkstelligen van een gevoel van verzadiging in de hersenen. GLP-1 receptor agonisten zijn in tegenstelling tot lichaamseigen GLP-1 minder gevoelig voor enzymatische afbraak en zijn daardoor beter en langer in staat om GLP-1 receptoren te activeren. Inmiddels is er enige jaren ervaring met behandeling met GLP-1 receptor agonisten en is duidelijk dat de medicatie leidt tot gewichtsafname en verbetering van bloedsuikerspiegels op de lange termijn. Het was nog niet duidelijk welke effecten GLP-1 receptor agonisten hebben op de verdeling van het ectopisch vet, dat zoals aangegeven een belangrijke rol

speelt in ontwikkeling van insulineresistentie, en welk effect GLP-1 receptor agonisten hebben op de hartfunctie. In dit proefschrift hebben we de resultaten beschreven van de onderzoeken die we hebben gedaan naar de effecten van liraglutide, een GLP-1 receptor agonist, op deze verschillende aspecten van de zogeheten cardiometabole gezondheid.

Hoofstuk 1 van dit proefschrift vormt een algemene introductie over obesitas en de rol in de ontwikkeling ('pathogenese') van type 2 diabetes en hart- en vaatziekten. Hier werd uitgebreid ingegaan op de rol van ectopisch vet bij ontwikkeling van insulineresistentie. Verder bespraken we het ongunstige metabole profiel van mensen van Zuid-Aziatische afkomst, die een verhoogd risico hebben om type 2 diabetes te ontwikkelen en daarnaast vaker hart- en vaatziekten krijgen ten opzichte van West-Europeanen. Opvallend is dat zij deze ziekten daarnaast op relatief jonge leeftijd en bij een relatief laag gewicht ontwikkelen. Vervolgens werd de rol van het endocannabinoïd systeem in de regulatie van het energieverbruik en de rol in obesitas besproken. Het endocannabinoïd systeem is een uitgebreid systeem van signaalstoffen en receptoren die aanwezig zijn in vrijwel het volledige menselijk lichaam. De functie is slechts gedeeltelijk opgehelderd, maar duidelijk is dat het endocannabinoïd systeem effecten heeft op de eetlust en op de balans tussen energieopslag en energieverbruik ('energiemetabolisme'). Verlaging van de activiteit van het endocannabinoïd systeem werd besproken als mogelijke strategie om overgewicht te bestrijden. Tenslotte werd in de algemene introductie ingegaan op verschillende behandelingen bij type 2 diabetes, waaronder GLP-1 receptor agonisten, inclusief de effecten die deze middelen hebben op ectopisch vet en hartfunctie.

Zoals genoemd hebben Zuid-Aziaten een hoger risico op het ontwikkelen van type 2 diabetes en hierbij lijkt een verhoogde stapeling van ectopisch vet een belangrijke rol te spelen. In **Hoofdstuk 2**, beschreven we de effecten van 26 weken behandeling met liraglutide in Nederlandse patiënten met type 2 diabetes van Zuid-Aziatische afkomst op de verschillende vetweefsels en op ectopisch vet. Tevens bespraken we de effecten op de bloedsuikerspiegel op de lange termijn. In de studie die we hebben verricht kregen patiënten willekeurig liraglutide of placebo in combinatie met de gebruikelijke behandeling bij type 2 diabetes. Vervolgens werd er aan het begin en aan het eind van de behandeling met MRI en MRI-spectroscopie, een speciale techniek waarmee de opgeslagen hoeveelheid vet in het hart en in de lever kan worden gemeten, onderzocht wat het effect was van liraglutide op de vetverdeling. De eerste analyse waarbij alle patiënten die behandeld werden waren meegenomen ('*intention-to-treat*' analyse) toonde geen effect van liraglutide op ectopisch vet en bloedsuiker ten opzichte van placebo in combinatie met conventionele behandeling. Een aantal patiënten had de medicatie echter niet goed toegediend of zich niet goed aan het protocol gehouden en daarom werd een extra analyse verricht zonder deze patiënten ('*per-protocol*' analyse). Deze analyse liet

zien dat liraglutide geen effect had op het ectopische vet, maar wel de hoeveelheid visceraal vet deed afnemen en dat deze afname samenhing met een verbetering van de bloedsuikerspiegel. We concludeerden dat liraglutide en mogelijk andere GLP-1 receptor agonisten een goede strategie kunnen zijn om de hoeveelheid visceraal vet te reduceren en de bloedsuikerspiegel te verbeteren bij patiënten van Zuid-Aziatische afkomst.

Veel patiënten met type 2 diabetes ontwikkelen hartfalen, een ziekte waarbij het hart niet in staat is om voldoende bloed rond te pompen. Het gaat hierbij vaak om hartfalen waarbij de hartspier zich niet goed genoeg kan ontspannen ('*heart failure with preserved ejection fraction* (HFpEF)'). Voordat hartfalen zich ontwikkelt, is er vaak eerst langzame achteruitgang van de mogelijkheid tot ontspanning en van de rekbaarheid ('compliantie') van de linker kamer van het hart, wat ook wel diabetische cardiomyopathie wordt genoemd. GLP-1 receptor agonisten hebben directe en indirecte effecten op de hartspier. Diverse studies in cellen en proefdieren hebben laten zien dat GLP-1 receptor agonisten de hartfunctie kunnen verbeteren. Omdat GLP-1 receptor agonisten wellicht dus gebruikt kunnen worden ter preventie van diabetische cardiomyopathie, hebben we in **Hoofdstuk 3** de effecten van liraglutide op hartfunctie onderzocht. We hebben hierbij gekeken naar effecten op het samentrekken ('systole') en het ontspannen ('diastole') van het de linker kamer van het hart in dezelfde groep Nederlandse Zuid-Aziatische patiënten als bestudeerd in hoofdstuk 2. We lieten zien dat liraglutide vergeleken met placebo geen effect heeft op de hartfunctie en ook niet op de stijfheid van de aorta en de hoeveelheid vet in de hartspier. We concludeerden dat liraglutide de ontwikkeling van diabetische cardiomyopathie niet direct lijkt te remmen. Echter, omdat recent is aangetoond dat GLP-1 receptor agonisten mogelijk voordelige effecten hebben op slagaderverkalking ('atherosclerose'), stellen we desalniettemin dat GLP-1 receptor agonisten een gunstig effect zouden kunnen hebben voor patiënten met een hoog risico op het ontwikkelen van hart- en vaatziekten, zoals de Zuid-Aziatische populatie.

Hoewel het bekend is dat behandeling met GLP-1 receptor agonisten leidt tot gewichtsafname, is het onderliggende mechanisme niet volledig opgehelderd. Het is duidelijk dat GLP-1 receptor agonisten leiden tot een gevoel van verzadiging en vermindering van voedselinname, en dit effect is daarom in ieder geval gedeeltelijk verantwoordelijk voor de gewichtsafname. Echter, verschillende studies hebben laten zien dat GLP-1 receptor agonisten ook het energieverbruik kunnen verhogen, wat daarmee ook kan bijdragen aan gewichtsafname. Studies in muizen hebben laten zien dat GLP-1 receptor agonisten de verbranding van glucose en vetten tot warmte in het bruine vetweefsel stimuleren. In tegenstelling tot wit vetweefsel, dat met name functioneert als opslag en buffer voor vet, draagt bruin vetweefsel juist bij aan het energieverbruik via deze warmteproductie ('thermogenese'). Thermogenese kan door verschillende factoren worden gestimuleerd,

waarvan blootstelling aan kou de meest belangrijke is. In **Hoofdstuk 4** hebben we de effecten van behandeling met liraglutide gedurende 26 weken op energieverbruik en op het bruine vetweefsel beschreven. Dit onderzoek werd uitgevoerd in West-Europese patiënten met type 2 diabetes, in wie na 4, 12 en 26 weken het energieverbruik in rust werd gemeten. Liraglutide leidde tot gewichtsverlies, maar opvallend genoeg deed liraglutide het energieverbruik afnemen in de eerste 12 weken vergeleken met het energieverbruik voor de start van de medicatie. Na 26 weken was deze afname van het energieverbruik minder uitgesproken. Verwacht werd dat de vetfractie van het bruine vetweefsel boven het sleutelbeen, zoals gemeten met MRI, zou afnemen bij stimulatie van thermogenese, maar deze vetfractie bleek na 26 weken behandeling niet veranderd. Een mogelijke verklaring voor onze bevindingen is dat door afname van voedselinname de warmteproductie die normaal gesproken wordt aangewakkerd door voedselinname ('*diet-induced thermogenesis*') is afgenomen. Tevens kan het zijn dat het lichaam in een fase van gewichtsafname die veroorzaakt wordt door verminderde voedselinname een hogere efficiëntie van verbranding heeft, waarmee het energieverbruik daalt en de gewichtsafname beperkt blijft. Samengevat liet de studie in dit hoofdstuk zien dat liraglutide in mensen leidt tot gewichtsafname door voedselinname te beperken en niet door bruin vetweefsel te activeren en het energieverbruik te verhogen.

Leververvetting is een belangrijke risicofactor voor verstoring van het glucose- en vetmetabolisme. Verschillende studies hebben laten zien dat behandeling met GLP-1 receptor agonisten de mate van leververvetting kan doen afnemen. In **Hoofstuk 5** beschrijven we een mogelijke relatie tussen de mate van leververvetting en de bloedspiegel van het eiwit *cholesteryl ester transfer protein* (CETP). CETP zorgt voor het transport van vetten ('triglyceriden') en cholesterol-bevattende verbindingen ('cholesterylesters') tussen de verschillende lipoproteïnen in het bloed. Lipoproteïnen zijn bolvormige deeltjes die bestaan uit vetten ('lipiden') en eiwitten ('proteïnen'), en worden onderscheiden in zeer lage-dichtheidslipoproteïnen ('*very low-density lipoproteïns*', VLDL), lage-dichtheidslipoproteïnen ('*low-density lipoproteins*', LDL) en hoge-dichtheidslipoproteïnen ('*high-density lipoproteins*', HDL), die alle in meer of minder mate triglyceriden en cholesterylesters transporteren in het bloed. De werking van CETP leidt tot vorming van meer cholesterol-verrijkte VLDL deeltjes die atherosclerose kunnen veroorzaken, en een verhoogde CETP activiteit is daarom ongewenst. CETP wordt geproduceerd door afweercellen van de lever, de zogenaamde 'Kupffer cellen', maar de regulatie van de productie van CETP door deze cellen is nog niet geheel begrepen. Eerdere studies suggereerden dat de mate van leververvetting een rol speelt omdat behandelingen die leververvetting verlaagden tevens de bloedspiegel van CETP deden afnemen. Wij hebben deze relatie verder onderzocht in de studie zoals hierboven reeds besproken, waarbij patiënten met type 2 diabetes van West-Europese afkomst gedurende 26 weken

met liraglutide of placebo werden behandeld. Interessant was dat behandeling met zowel liraglutide als met placebo in combinatie met conventionele therapie de mate van leververvetting deed afnemen. Deze afname ging echter niet gepaard met een afname van CETP in het bloed. Onderzoek in 1611 proefpersonen met overgewicht uit de Nederlandse Epidemiologie van Obesitas (NEO) studie liet ook geen relatie zien tussen de mate van leververvetting en de CETP concentratie in het bloed. We concludeerden daarom dat de mate van leververvetting niet de hoeveelheid CETP in het bloed bepaalt en dat daarnaast interventies die de hoeveelheid leververvetting doen afnemen niet per definitie de CETP productie door Kupffer cellen beïnvloeden.

Het is dus duidelijk geworden dat GLP-1 receptor agonisten het gewicht reduceren, met name door afname van voedselinname. Daarnaast heeft afname van gewicht en voedselinname mogelijk effecten op het endocannabinoïd systeem. Om de effecten van vermindering van voedselinname op het endocannabinoïd systeem te onderzoeken, hebben we in **Hoofdstuk 6** de effecten van een dieet met sterk verminderde inname van calorieën op endocannabinoïden in het bloed in relatie tot ectopisch vet en hartfunctie onderzocht. In een studie waarbij obese patiënten met type 2 diabetes en bekende aandoeningen van de kransslagaders (‘coronaire hartziekten’) gedurende 16 weken met een zeer-laag calorisch dieet van 450-1000 kcal/dag werden behandeld, werd een substantiële afname van zowel subcutaan als visceraal vet waargenomen. Tevens was er een afname van vet in en rondom het hart en een afname van de mate van leververvetting. Dit alles ging gepaard met een sterke verbetering van de bloedsuikerspiegels. Tevens was er een afname van specifieke endocannabinoïden waaronder anandamide. Tot slot werd gezien dat het dieet leidde tot een verbeterde compliantie van de linker kamer van het hart. We concludeerden dat een dieetinterventie met calorie-beperking effectief het ectopisch vet kan doen afnemen en de hartfunctie kan verbeteren in patiënten met type 2 diabetes en coronaire hartziekten. Daarnaast vermoeden we dat een afname van de activiteit van het endocannabinoïd systeem, gemeten als een afname van anandamide in het bloed, kan hebben bijgedragen aan de geobserveerde verbeteringen.

Hoofdstuk 7 vormt een algemene discussie van de verschillende bevindingen die beschreven zijn in dit proefschrift. Specifiek wordt ingegaan op het mechanisme achter gewichtsafname die wordt veroorzaakt door liraglutide, waarbij werd getoond dat de gewichtsafname in mensen het gevolg is van afname van energie-inname en niet van verhoogd energieverbruik. Verder lieten we ons licht schijnen op effecten van liraglutide op verschillende vetdepots en ectopisch vet. We droegen aan dat GLP-1 receptor agonisten in Zuid-Aziatische patiënten met type 2 diabetes de bloedsuikerspiegel kunnen verbeteren door middel van afname van visceraal vet. Ten slotte leverden we bewijs dat liraglutide geen directe gunstige effecten heeft op diabetische cardiomyopathie, maar

dat GLP-1 receptor agonisten mogelijk wel een gunstig effect kunnen hebben door bestrijding van atherosclerose en dat het daarom een zeer doeltreffende therapie kan zijn in de Zuid-Aziatische patiëntengroep. Gedurende de laatste jaren is bewezen dat GLP-1 receptor agonisten een effectieve behandeling zijn voor obesitas en type 2 diabetes. Zeer interessante resultaten zijn recent gepubliceerd over tirzepatide, een nieuw ontwikkeld medicijn dat naast GLP-1 receptoren ook receptoren activeert van een ander darmhormoon, namelijk het '*glucose dependent insulinotropic peptide*' (GIP). De resultaten zijn spectaculair: ten opzichte van GLP-1 receptor agonisten leidt tirzepatide tot veel grotere gewichtsafname en sterkere verbetering van bloedsuikerspiegels. Momenteel wordt zelfs alweer een opvolger van deze therapie ontwikkeld, waarbij naast activatie van GLP-1 en GIP receptoren ook receptoren voor het pancreashormoon glucagon worden geactiveerd, met zelfs nog meer uitgesproken effecten. In de komende decennia zal het potentieel van behandeling met GLP-1 receptor agonisten, alleen en in combinatie met GIP en glucagon receptor agonisme, in het bestrijden van atherosclerose en het verbeteren van cardiometabole gezondheid duidelijk worden.

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CURRICULUM VITAE

Hubertus Josephus van Eyk werd geboren op 27 oktober 1988 te Leiderdorp. In 2006 behaalde hij het gymnasiumdiploma aan het Stedelijk Gymnasium in Leiden, waarna hij startte met de studie Biomedische Wetenschappen aan de Universiteit Utrecht waarvan hij het eerste jaar met succes afrondde. In 2007 startte hij de studie Geneeskunde aan de Universiteit Leiden waarvan in 2011 het bachelordiploma werd behaald en in 2015 het masterdiploma (*cum laude)*. Tevens was hij sinds 2009 gestart met de Pre-master Biomedische Wetenschappen aan de Universiteit Leiden. Dit werd na succesvol afronden in 2011 voortgezet met de Master Biomedische Wetenschappen aan de Universiteit Leiden waarvan het diploma in 2015 werd behaald (*cum laude).* Tijdens de beide masters werden diverse onderzoeksstages verricht, op de afdeling Longziekten in het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van dr. E.F.A. van 't Wout en prof. dr. P.S. Hiemstra naar effecten van Pseudomonas aeruginosa-infectie op luchtweg epitheeldysfunctie en op de afdeling Endocrinologie in het LUMC onder begeleiding van dr. M.R. Boon en prof. dr. P.C.N. Rensen naar de rol van bruin vet in gezondheid en ziekte.

In 2014 startte hij met zijn promotietraject op de afdeling Endocrinologie onder begeleiding van dr. I.M. Jazet en prof. dr. P.C.N. Rensen, waarvan de resultaten in dit proefschrift beschreven zijn. Tijdens het promotietraject nam hij deel aan verscheidene cursussen op het gebied van epidemiologie en statistiek. De resultaten van het onderzoek werden gepresenteerd tijdens diverse nationale en internationale congressen en in 2018 mocht hij een *Young Investigator Fellowship* in ontvangst nemen van de *European Atherosclerosis Society*. Tevens werd hij uitgenodigd om zijn resultaten te presenteren tijdens de jaarlijkse bijeenkomst van de *North European Young Diabetologists*.

Vanaf 2018 werkte hij als arts-assistent niet in opleiding (ANIOS) op de afdeling interne geneeskunde in het Haaglanden Medisch Centrum in Den Haag. Sinds 2019 is hij in opleiding tot internist in het Alrijne ziekenhuis in Leiderdorp (opleider Drs. S. Anten, later Drs. L. Hardi) en het LUMC (opleider Prof. Dr. J.W. de Fijter, later Dr. N.M. Appelman-Dijkstra).

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