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Cardiovascular, renal and gastrointestinal effects of incretin-based therapies: an acute and 12-week randomised, double-blind, placebo-controlled, mechanistic intervention trial in type 2 diabetes

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ABSTRACT

Introduction: Incretin-based therapies, that is, glucagon-like peptide (GLP)-1 receptor agonists and dipeptidyl peptidase (DPP)-4 inhibitors, are relatively novel antihyperglycaemic drugs that are frequently used in type 2 diabetes management. Apart from glucose-lowering, these agents exhibit pleiotropic actions that may have favourable and unfavourable clinical consequences. Incretin-based therapies have been associated with heart rate acceleration, heart failure, acute renal failure and acute pancreatitis. Conversely, these agents may reduce blood pressure, glomerular hyperfiltration, albuminuria and hepatic steatosis. While large-sized cardiovascular safety trials can potentially identify the clinical significance of some of these pleiotropic actions, small-sized mechanistic studies are important to understand the (patho) physiological rationale of these findings. The current protocol describes a mechanistic study to assess cardiovascular, renal and gastrointestinal effects, and mechanisms of incretin-based therapies in type 2 diabetes.

Methods and analyses: 60 patients with type 2 diabetes will undergo acute and prolonged randomised, double-blind, intervention studies. The acute intervention will consist of intravenous administration of the GLP-1 receptor agonist exenatide or placebo. For the prolonged intervention, patients will be randomised to 12-week treatment with the GLP-1 receptor agonist liraglutide, the DPP-4 inhibitor sitagliptin or matching placebos. For each examined organ system, a primary end point is defined. Primary cardiovascular end point is change in resting heart rate variability assessed by beat-to-beat heart rate monitor and spectral analyses software. Primary renal end point is change in glomerular filtration rate assessed by the classic inulin clearance methodology. Primary gastrointestinal end points are change in pancreatic exocrine function assessed by MRI-techniques (acute intervention) and faecal elastase-1 levels (12-week intervention). Secondary end points include systemic haemodynamics, microvascular function, effective renal plasma flow, renal tubular function, pancreatic volume and gallbladder emptying-rate.

Medical ethics and dissemination: The study is approved by the local Ethics Review Board (VU University Medical Center, Amsterdam) and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Trial registration number: NCT01744236.

Strengths and limitations of this study

- This study includes three randomised, placebo-controlled, double-blind clinical trials to assess both acute and prolonged (12 weeks) effects of incretin-based therapies on the cardiovascular, renal and gastrointestinal system.
- Multiple secondary end points are studied in order to provide an integrative view on the examined organ systems.
- The study may not be sufficiently powered to draw conclusions on secondary end points.
- The duration of the prolonged intervention study is 12 weeks and can, therefore, not be considered as a long-term exposure to the study drugs.

INTRODUCTION

The global prevalence of type 2 diabetes has reached alarming proportions, which is strongly related to ageing and the obesity pandemic. Long-term intensive glycaemic control has reduced the incidence of its microvascular and macrovascular complications.[1 2] However, approximately 25% of patients with type 2

diabetes do not reach glycaemic targets with currently available antihyperglycaemic treatment options.[3] Intensified treatment schedules may be associated with adverse effects, including weight gain and hypoglycaemia. Thus, besides improving and implementing public health initiatives and preventive treatment strategies, the development of novel antihyperglycaemic agents is needed. Over the past decade, several antihyperglycaemic drug classes have been introduced into the market. However, as with every novel drug, there are potential safety risks that need exploration in clinical trials. In spite of stringent regulatory rules that enforce the conduction of large-scaled registration programmes, rare adverse effects may only become apparent after marketing authorisation, when a large group of patients has been treated for a longer period of time. In addition to ongoing long-term cardiovascular safety trials for all novel antihyperglycaemic drugs,[4] there is a growing need to improve the postmarketing monitoring of potential risks and benefits of these drugs.

The 7th-Framework Programme funded European project 'Safety Evaluation of Adverse Reactions in Diabetes' (SAFEGUARD) is a pharmacovigilance project designed to assess, quantify and understand safety aspects of antihyperglycaemic drugs in type 2 diabetes, with a focus on incretin-based therapies.[5] The SAFEGUARD-project consists of eight work packages, including pharmacovigilance database studies, observational database studies, meta-analyses and mechanistic studies. Here, we describe one of the protocols of the mechanistic work package that explores some of the pleiotropic actions that have been associated with the use of incretin-based diabetes therapies.

GLP-1 and DPP-4

Incretin-based therapies are based on the gut-derived incretin hormone glucagon-like peptide (GLP)-1. GLP-1 is released by intestinal L-cells on food ingestion and regulates glucose homeostasis by influencing pancreatic islet-cell function, including glucose-dependent stimulation of insulin and suppression of glucagon secretion.[6] However, native GLP-1 is rapidly metabolised by the serine protease dipeptidyl peptidase (DPP)-4, leading to a plasma half-life of ~2 min. In type 2 diabetes, the effect of this incretin on endocrine pancreatic function is impaired. However, when native GLP-1 is administered at pharmacological doses, it lowers fasting and postprandial glucose, improves islet-cell function, delays gastric emptying and induces bodyweight loss.[7] Therefore, GLP-1 was regarded as an attractive therapeutic option for type 2 diabetes.[8] To date, two incretin-based drug classes have been developed and marketed: injectable DPP-4-resistant GLP-1 receptor agonists which mimic the effects of native GLP-1, and oral DPP-4 inhibitors which prolong the actions of endogenously secreted GLP-1.[9] Both incretin-based drug classes improve glycaemic control in patients with type 2 diabetes, with minor risk of hypoglycaemia in clinical practice.[2 9]

Pleiotropic effects of incretin-based therapies

Interestingly, the receptor for GLP-1 has been identified in many non-pancreatic organ systems, such as the heart, blood vessels, kidneys, gastrointestinal system and central nervous system.[10 11] It was, therefore, not unexpected to find pleiotropic effects of GLP-1 and related therapies. Although some of the pleiotropic actions of incretin-based therapies may be beneficial, these could also cause adverse effects. For example, clinical use of incretin-based therapies has been associated with heart rate acceleration,[12] heart failure,[13] sporadic cases of acute renal failure[14] and acute pancreatitis.[15–17] However, to date, a causal relationship between the use of incretin-based therapies and the occurrence of adverse effects is unclear (as type 2 diabetes per se is associated with these conditions), and the underlying mechanisms remain hitherto largely unexplored.

Incretin-based therapies and the cardiovascular system

GLP-1 receptor agonists have been associated with resting heart rate acceleration (mean increase of 2–4 bpm),[12] an established risk factor for cardiovascular and all-cause mortality.[18] The mechanisms underlying this finding remain unclear, but alterations in cardiac autonomic nervous system balance may be involved.[19 20] Other potential mechanisms include changes in baroreceptor sensitivity, natriuresis and reduced systemic vascular resistance. Furthermore, although mechanistic trials with incretin-based therapies showed improvement in parameters associated with heart failure,[21 22] recent large-sized clinical trials revealed signs of increased hospitalisation for heart failure with the DPP-4 inhibitors saxagliptin and alogliptin, but not sitagliptin.[13 23 24]

Conversely, long-term GLP-1 receptor agonist administration decreases systolic and diastolic blood pressure in clinical trials.[12] Moreover, it improves endothelial dysfunction, measured as flow-mediated vasodilation,[25 26] decreases macrophage foam cell formation and atherosclerosis in animals,[27] and reduces carotid intimal-media thickness in humans.[28] Also, in animal models of myocardial infarction, native GLP-1 administration reduced infarction size[29] and improved postinfarction myocardial function.[30] Similar cardiovascular improvements have been noted in small-sized clinical studies.[31–33]

Incretin-based therapies and the renal system

Several case reports have described the occurrence of acute renal failure in patients with type 2 diabetes treated with incretin-based therapies.[34 35] However, associations between the use of incretin-based therapies and renal failure were not supported by a large-sized database analysis.[36] Moreover, to date, clinical studies have not given rise to concerns regarding renal adverse events.

More recently, incretin-based therapies have been associated with renoprotective properties. In animal models, administration of GLP-1 and associated therapies reduced systemic hypertension and albuminuria, and

ameliorated renal damage, as verified by histology.[37–40] In clinical studies, GLP-1 receptor agonists have been associated with decreased development and progression of albuminuria.[41 42] Moreover, the clinical use of the DPP-4 inhibitors saxagliptin and linagliptin were associated with a glucose-independent reduction in albuminuria by approximately 25%.[43–45] Collectively, these data suggest a renoprotective effect with the use of incretin-based therapies. However, the mechanisms by which these antihyperglycaemic drugs may improve renal outcome beyond glycaemic control are unclear, but may involve improvements in body weight, blood pressure, renal haemodynamics and albuminuria or lipid profiles.[40 46]

Incretin-based therapies and the gastrointestinal system

GLP-1 receptor agonists and DPP-4 inhibitors have been associated with acute pancreatitis,[15–17] and histological inflammatory changes of the pancreas have been shown in rodents.[47] Also, it has been hypothesised that incretin-based therapies increase the risk of chronic pancreatitis or pancreatic cancer.[48] However, available data are conflicting since many other studies fail to find such adverse effects with incretin-based therapies.[49–51] As type 2 diabetes, and frequently associated comorbidities, is by itself associated with pancreatitis, it is difficult to distinguish the sole contribution of incretin-based therapies.

In contrast, beneficial gastrointestinal effects of GLP-1 have been described, contributing to improved glycaemic control trough decreased (postprandial) glycaemic excursions.[52] Moreover, mounting evidence suggests therapeutic effects of incretin-based therapies on gastrointestinal diseases, including non-alcoholic fatty liver disease and irritable bowel syndrome.[53 54] Mechanistically, GLP-1 peptide infusion decreases stomach and bowel motility,[55] and secretion of gastric acid and exocrine pancreatic enzymes.[56–58] Many of these effects are likely to be mediated through changes in vagal nerve function.[56] In addition, the GLP-1 receptor agonist exenatide reduces gallbladder emptying rate.[59] Hepatic effects of incretin-based therapies are still unclear, but may include reduced endogenous glucose and lipid production.[60]

Rationale and aim

Since a decade, GLP-1 receptor agonists and DPP-4 inhibitors have been implemented for glucose-lowering treatment of type 2 diabetes, and are now widely used in clinical practice. However, there remain many gaps in our understanding about various pleiotropic effects of these antihyperglycaemic drugs. Importantly, incretin-based therapy use has been associated with potential adverse events, among others, affecting the cardiovascular, renal and gastrointestinal systems. While large-sized randomised trials and database studies patients with type 2 diabetes will provide evidence on their clinical risks, mechanistic studies are needed to identify the underlying mechanisms of the adverse effects. Therefore, the current study aims to investigate the acute and prolonged effects of incretin-based therapies on the cardiovascular, renal and gastrointestinal system of patients with type 2 diabetes.

METHODS AND ANALYSES

Study design

In order to assess acute and prolonged effects of incretin-based therapies on the cardiovascular, renal and gastrointestinal system, the current study integrates three double-blind, randomised, placebo-controlled trials in patients with type 2 diabetes. In the main parallel-group study, 60 patients will be randomised and treated for 12 weeks with the GLP-1 receptor agonist liraglutide, the DPP-4 inhibitor sitagliptin or matching placebos (figure 1). After the baseline measurements of the 12-week intervention study, two acute intervention-studies are performed to assess the effect of intravenous administration of the GLP-1 receptor agonist exenatide versus placebo: 1 parallel-group trial in all 60 patients to assess acute cardiovascular and renal effects, and 1 cross-over trial in a subset of 12 male patients to assess acute pancreatic effects. Placebo is chosen as comparator for all three trials, since this allows for the study of drug-induced changes per se, instead of comparing changes with an active comparator.

All examinations will be performed at the Clinical Research Unit (CRU) of the Department of Internal Medicine/Diabetes Centre of the VU University Medical Center in Amsterdam, the Netherlands.

Outcome measures

Outcome measures will be studied after acute intravenous drug-administration, and after 12 weeks of prolonged drug-intervention. In addition, predefined outcome measures will be evaluated after 2 and 6 weeks of prolonged intervention (safety visits; table 1). Outcome measures will be compared between the intervention and placebo groups.

Cardiovascular outcomes

Primary outcome measure is resting heart rate variability (HRV) assessed with a beat-to-beat heart rate monitor and spectral analyses software. Secondary outcome measures include systemic haemodynamics (heart rate, blood pressure, cardiac output, vascular resistance), arterial stiffness and microvascular function (see online supplementary table A).

Renal outcomes

Primary outcome measure is glomerular filtration rate (GFR), assessed by using the inulin clearance technique, based on timed urine sampling. Secondary outcome measures include para-amino hippuric acid (PAH)-measured effective renal plasma flow, tubular function, and glomerular and tubular damage markers (see online supplementary table B).

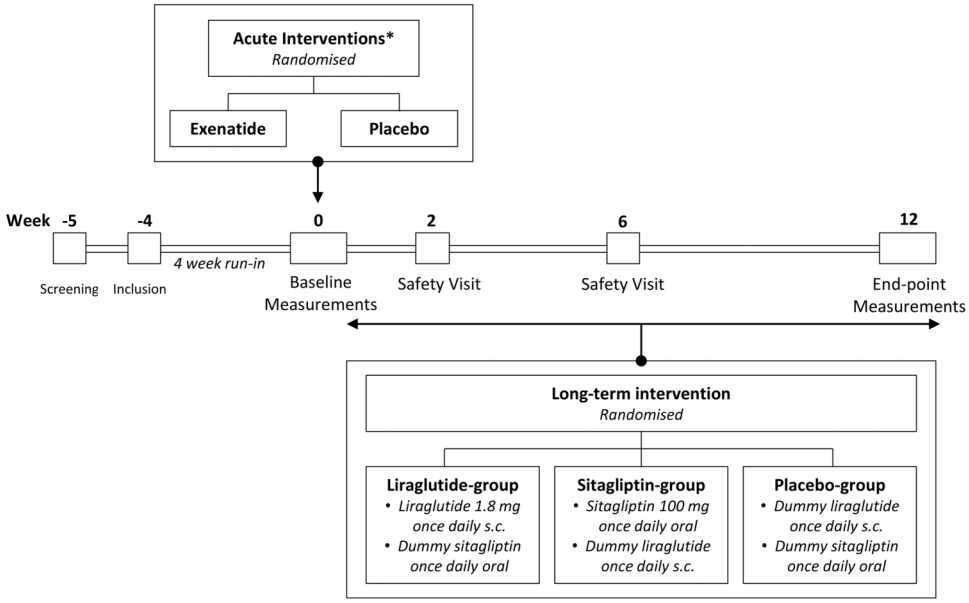

Figure 1 Study design: Subjects will be screened for eligibility. Included subjects will undergo a run-in period of at least 4 weeks after which the baseline end point measurements for the 12-week study and the acute intervention studies are performed*. Subsequently, subjects are randomised for the prolonged intervention. After 2 and 6 weeks, a safety visit is performed. At week 1 and 9, telephone follow-up is performed (not depicted). After 12 weeks of treatment, the end point measurements are performed. *For the acute cardiovascular and renal study, subjects are randomised to exenatide or placebo. For the acute pancreatic study, 12 patients will receive intravenous exenatide and placebo in a randomised cross-over design.

Gastrointestinal outcomes

Primary outcome measure is pancreatic exocrine function. For the acute intervention study, this is measured by exocrine secreted volume assessed by secretin-enhanced MR cholangiopancreatography. For the 12-week study, this is measured by faecal elastase-1 levels. Secondary outcome measures for the 12-week study consist of exocrine pancreatic function, pancreatic structure/volume, gallbladder emptying, gastric emptying, hepatic fat content and gastrointestinal damage markers (see online supplementary table C).

Other and exploratory outcome measures

Additional outcome measures include body anthropometrics, postprandial glucose excursions, glycated haemoglobin, plasma lipids and body fat content (see online supplementary table D). Moreover, urine, faeces and blood (plasma and serum) are collected and stored at −80°C for at least 15 years, to allow for determination of additional biomarkers for potential future research questions.

Participants

Volunteers with type 2 diabetes will be recruited using established recruitment methods: (1) participants in the previous studies of the VU University Diabetes Centre will be contacted (if informed consent was obtained); (2) advertisements in local newspapers, folders and posters; (3) affiliated healthcare workers (internal medicine, general practitioners) will inform patients of the existence of this study; and (4) websites. After providing extensive printed and oral information, a hand-signed informed consent form will be attained by a clinical research physician. Eligibility will be assessed during a screening visit, comprising of a medical interview and physical, blood, urine and ECG examination. Postvoiding bladder residue will be assessed using ultrasonic bladder scan. Inclusion and exclusion criteria are listed in box 1. After inclusion, participants will receive an unique study number.

Intervention

Acute intervention studies

The GLP-1 receptor agonist exenatide (AstraZeneca, London, UK) or placebo (isotonic 0.9% saline) will be infused for 5 h. One dose of exenatide 10 µg will be diluted in 46 mL isotonic 0.9% saline and 4 mL of the participant's blood to prevent binding of the drug to the infusion material. For placebo, no study drug will be added. The solution will be administered with a calibrated syringe pump at an infusion-rate of 50 ng/min for 30 min; this is then followed by 25 ng/min infusion for the remainder of the test procedures to target the steady-state plasma concentrations of exenatide within the therapeutic range (100–150 pg/mL).[61]

Twelve-week intervention study

The GLP-1 receptor agonist liraglutide (Novo Nordisk A/S, Bagsværd, Denmark) and matching liraglutide-placebo prefilled pens for subcutaneous use will be provided by Novo Nordisk. A dose increment scheme is employed, in which patients will inject liraglutide/-placebo at a dose of 0.6 mg once daily during the first week, 1.2 mg once daily during the second week, and

Table 1 Trial visits and end points

	Baseline						Safety		Prolonged treatment 12-week			
End point	**CV/R**	**CV/R acute**	**CV/R acute PP**	**GI**	**MRI**	**MRI acute***	**2-week**	**6-week**	**CV/R**	**CV/R PP**	**GI**	**MRI**
Cardiovascular												
Resting heart rate variability	X	X	X						X	X		
Systemic haemodynamics	X	X	X						X	X		
Arterial stiffness	X	X	X						X	X		
Microvascular function	X	X	X						X	X		
Renal												
Glomerular filtration rate	X	X							X			
Estimated glomerular filtration rate	X	X					X	X	X			
Effective renal plasma flow	X	X							X			
Fractional electrolyte excretion	X	X							X			
Urine osmolality	X	X							X			
Urine pH	X	X							X			
Glomerular damage parameter (albumin)	X	X							X			
Tubular damage parameters (KIM-1, NGAL)	X	X							X			
Body water percentage	X	X							X			
Gastrointestinal												
Faecal elastase-1				X							X	
Faecal chymotrypsin				X							X	
Lipase/amylase-levels	X	X	X				X	X	X			
13C-MTG breath test				X							X	
Pancreatic bicarbonate secretion					X	X						X
Pancreatic structure/volume					X							X
Trypsinogen blood/urine	X						X		X			
Gallbladder emptying				X							X	
Gastric emptying				X							X	
Hepatic fat content					X							X
Plasma albumin/AST/ALT/yGT/ALP	X							X	X			
GI damage parameters (I-FABP, L-FABP, calprotectin)	X						X		X			
Microbiome				X							X	
General												
HbA1c	X									X		
Fasting glucose	X								X			
Postprandial glucose			X							X		
Fasting lipid spectrum	X								X			
Anthropometrics (length/height/waist/hip)				X			X	X			X	
Body fat percentage				X							X	
Subcutaneous and visceral fat volume					X							X

*The acute pancreatic MRI study is performed in a subset of 12 patients.
13c-MTG, 13C-labeled mixed triglyceride breath test; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase CV/R, cardiovascular and renal testing day; GI, gastrointestinal testing day; HbA1c, glycated haemoglobin; I-FABP, intestinal fatty acid binding protein; L-FABP, liver-type fatty acid binding protein; PP, postprandial.

1.8 mg once daily during the remainder of the study. Based on the participants' tolerance to the trial product, the time interval between the dose increments can be extended, and drug dose can be reduced when regarded to be clinically necessary. Subjects are instructed to inject liraglutide/-placebo in the abdominal region, and always at the same time of the day (preferably evening).

The DPP-4 inhibitor sitagliptin (Merck, Whitehouse Station, New Jersey, USA) and matching sitagliptin-placebo will be encapsulated by an independent GMP-certified clinical research organisation (ACE Pharmaceuticals BV, Zeewolde, the Netherlands). Sitagliptin/-placebo 100 mg will be taken orally once daily for 12 weeks, at the same time as the liraglutide/-placebo injection. No dose escalation is needed for sitagliptin/-placebo.

Participants randomised to the *liraglutide-arm* will receive sitagliptin-placebo capsules, while participants

Box 1 Inclusion and exclusion criteria

Inclusion criteria

- Type 2 diabetes
  - Stable dose of oral antihyperglycaemic drugs (metformin and/or sulfonylurea) for at least 3 months prior to inclusion
  - HbA1c 6.5–9.0% DCCT or 48–75 mmol/mol IFCC
- Age between 35 and 75 years
- Females must be postmenopausal (defined as: no menses >1 year)
- Caucasian
- Body mass index 25–40 kg/m^2

Exclusion criteria

- Use of the following medication: thiazolidinediones, GLP-1 receptor agonists, DPP-4 inhibitors, insulin, glucocorticoids, non-steroidal anti-inflammatory drugs, antimicrobial agents, chemotherapeutics or immune suppressants. Patients on diuretics will only be excluded when these drugs (eg, hydrochlorothiazide) cannot be stopped for the duration of the study
- History of pancreatic disease or impaired pancreatic exocrine function (defined as: use of pancreatic enzymes)
- Active liver disease or a threefold elevation of liver enzymes (AST/ALT) at screening
- (History of) malignancy (with the exception of basal cell carcinoma)
- Estimated-GFR <60 mL/min/1.73 m^2; Current urinary tract infection and active nephritis
- Recent (<6 months) history of cardiovascular disease, including acute coronary syndrome, stroke, transient ischaemic neurological disorder; Chronic heart failure (New York Heart Association grade II-IV) or atrial fibrillation
- Alcohol abuse, defined as >4 units day
- Allergy to any of the test agents
- Complaints compatible with or established gastroparesis and/or neurogenic bladder
- History of or present (severe) mental illness
- Inability to understand the study protocol and/or to give written informed consent
- Contraindications for MRI; claustrophobia or presence of metal objects/implants

randomised to the *sitagliptin-arm* will receive liraglutide-placebo prefilled pens. Participants randomised to the *placebo-arm* will receive liraglutide-placebo prefilled pens and sitagliptin-placebo capsules.

Patients using sulfonylurea will be instructed to frequently check their blood glucose levels, and in case of clinically significant hypoglycaemia, sulfonylurea dose will be decreased based on the discretion of a research physician. In general, patients and caregivers will be instructed not to change comedication without clinical need.

Randomisation

Randomisation will be performed by an institutional trial pharmacist. For the acute studies, block randomisation will be performed with an allocation ratio of 1:1, and a block-size of 6 (acute cardiovascular and renal study) and 4 (acute pancreatic study). For the 12-week study, block randomisation will be performed with an allocation ratio of 1:1:1, and a block-size of 6. Stratification will be applied, thereby evenly dividing the number of patients receiving acute intravenous administration of exenatide or placebo among the three prolonged intervention groups (figure 2).

Blinding

Study medication will be provided by the trial pharmacist, and all study personnel and participating patients will remain blinded with regards to the study medication. The blind shall not be broken, unless information concerning the study medication is considered medically necessary. If the investigator is unblinded, study medication will be stopped and the participant will be withdrawn from the study. The blind will be broken by the institutional trial pharmacist after the last patient has completed the last study visit and all data are entered into the online clinical trial database.

Compliance

For the 12-week study, participants will receive extensive printed and oral study, and drug-related instructions by a clinical research physician. The research physician will be in contact with the participants after 1, 2 and 6 weeks of intervention, during which safety and drug accountability will be monitored.

Data collection and study procedures

Data will be collected during designated end point visits at baseline and after 12 weeks of treatment: 1 for the cardiovascular and renal end points, 1 to collect gastrointestinal data, and 1 visit to perform the MRI (table 1). These visits will be planned in no particular order. During the baseline cardiovascular and renal end point visit, the acute study will be performed. At least 24 h will be observed between the acute intervention and the next visit, to allow for washout of the study drug. Patients participating in the acute pancreatic study will undergo two additional MRI prior to the start of the 12-week intervention.

Cardiovascular and renal study procedures

Two days prior to this study visit, participants will be asked to adhere to an standardised sodium chloride (9–12 g/day) and protein (1.5–2.0 mg/kg/day) intake in order to minimise diet-induced variation in renal physiology.[76 77] In addition, they will be asked to refrain from vigorous physical activity and alcohol ingestion for at least 24 h, and from consuming caffeine for at least 12 h. After an overnight fast, participants will be instructed to drink 500 mL of water. Intake of all morning medications, except for metformin, will be delayed until conclusion of the examination day. After arrival at the CRU at 7:30, intravenous catheters will be placed in both arms. Blood and urine will be collected, and the participant will assume a semirecumbent position. Then, resting HRV will be measured using a

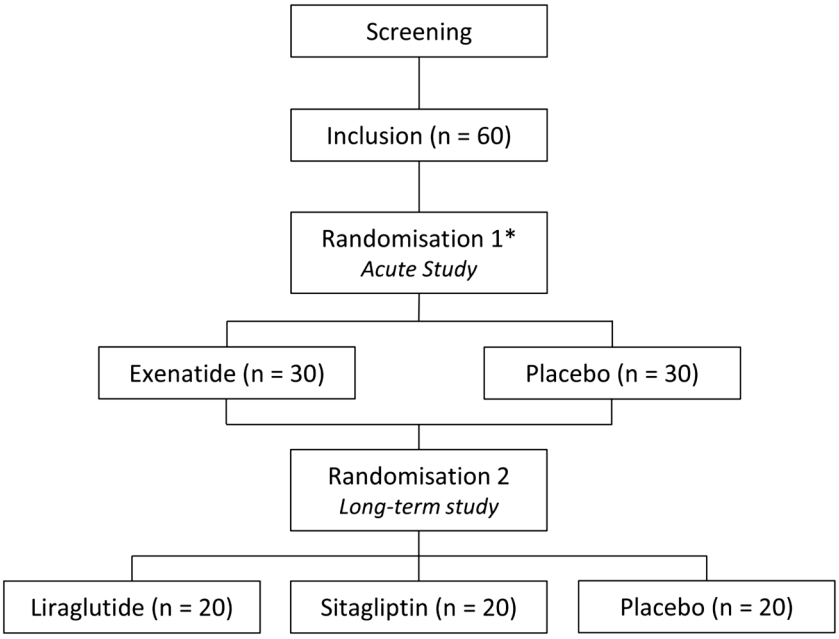

Figure 2 Flow chart of the acute and prolonged intervention study. *For the acute cardiovascular and renal intervention study, patients are randomised to receive exenatide or placebo (parallel-group design). For the acute pancreatic intervention study, patients receive exenatide and placebo, in a randomised order (cross-over design).

beat-to-beat heart rate monitor. Systemic haemodynamic parameters will be assessed using an oscillometric blood pressure measurement device and beat-to-beat blood pressure monitor. Arterial stiffness will be measured using applanation tonometry, and microvascular function with capillary videomicroscopy and laser Doppler fluxmetry. Bio-impedance analysis (BIA) will be performed to measure body water and fat content.

Subsequently, the renal tests will start. A 10 min priming with inulin and para-aminohippurate sodium (PAH) will be followed by continuous infusion of these renal tracer substances. After 90 min of equilibration, urine will be collected by spontaneous voiding every 45 min for two time periods, while blood will be collected before and after each collection period. Samples will be used to measure GFR (inulin clearance), renal plasma flow (PAH clearance) and tubular function. Diuresis will be stimulated by oral intake of 10 mL/kg (maximal 1000 mL) tap water during the equilibration phase, followed by 200 mL of tap water per hour (figure 3).

Subsequently, during the baseline end point visit, but not during the end point visit after 12 weeks of treatment, the acute cardiovascular and renal intervention study with intravenous administration of exenatide or placebo will start. After an equilibration period of 60 min to ensure steady-state plasma exenatide concentrations,[61] urine will be collected by spontaneous voiding every 45 min for two time periods while blood will be collected before and after each collection period for determination of inulin and PAH. In addition, the cardiovascular tests will be repeated intermittently (see online supplementary table A and B). Resting HRV will be assessed after 30, 90 and 165 min. Blood pressure and systemic haemodynamics will be assessed after 30, 60, 90, 120 and 165 min of intervention. Arterial stiffness will be assessed after 30, 60, 90 and 160 min, and microvascular function after 110 min.

Finally, at the end of the renal protocol, a meal-test protocol will start, during which intravenous administration of exenatide or placebo is continued. A standardised high-fat mixed meal of 905.7 kcal (50 g fat, 75 g carbohydrates and 36.8 g protein) will be consumed within 15 min, and cardiovascular and metabolic changes in the postprandial state will subsequently be examined. The same meal-test protocol will be performed during the end point visit after 12 weeks of treatment without intravenous exenatide or placebo administration. Resting HRV will be measured after 30, 60 and 120 min, and blood pressure, systemic haemodynamics and arterial stiffness will be measured every 30 min post meal. Microvascular function will be measured 90 min after the start of the meal.

Gastrointestinal study procedures

During this visit, three experimental protocols will be performed simultaneously: (1) a ^{13}C-labelled mixed triglyceride (^{13}C-MTG) breath test (pancreatic digestive function), (2) a meal-stimulated gallbladder ultrasound examination (gallbladder emptying rate) and (3) an acetaminophen absorption kinetic test (gastric emptying rate). Two days prior to the study visit, participants are instructed not to take any product that is naturally enriched with ^{13}C such as corn products, cane sugar, pineapple and tequila. After an overnight fast, participants will be instructed to delay all morning medications, apart from metformin, until conclusion of the examination day, and arrive at the CRU at 7:30. Body anthropometrics, including weight, height, waist-circumference and hip-circumference are measured. Participants will then assume a semirecumbent position, after which BIA is used to assess body composition. An intravenous

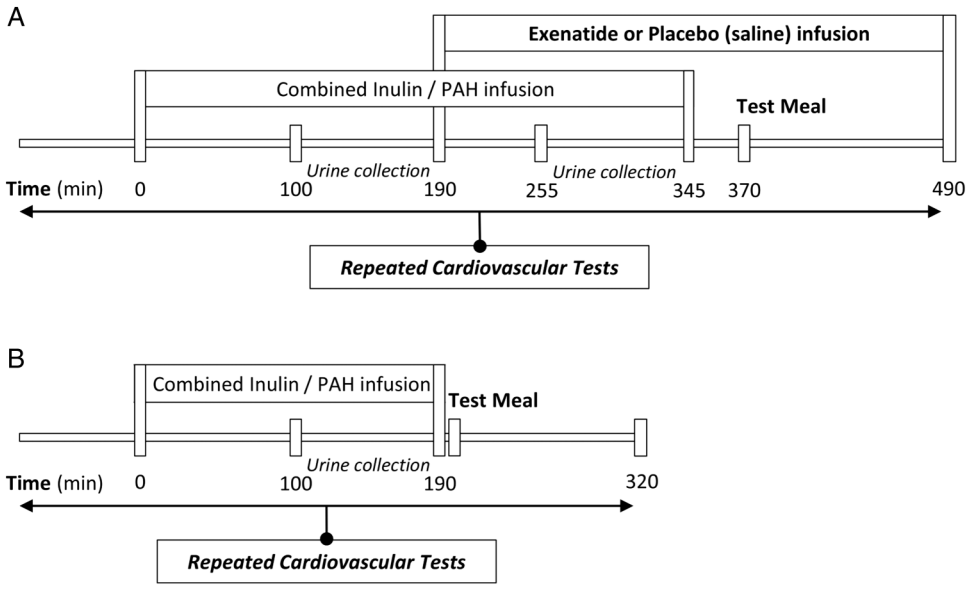

Figure 3 End point visit cardiovascular and renal tests. Schematic overview of the cardiovascular and renal end point visits. (A) At the baseline end point visit, the acute cardiovascular and renal intervention study is performed; (B) At the 12-week end point visit, no acute intervention study is performed. PAH, para-amino hippuric acid.

catheter is inserted into an antecubital vein for blood sampling. The gallbladder will be visualised by ultrasonographic imaging, recording the length, height and width of three measurements to calculate baseline gallbladder volume. Blood will be sampled to determine baseline serum acetaminophen, and reference breath samples will be collected using straw and gas collection tubes. Then, participants will be served a high-fat mixed meal (420 kcal, 22.4 g fat, 38.6 g carbohydrates and 14.6 g protein), containing the ^{13}C-MTG stable isotopes (Euriso-Top, Saint-Aubin Cedex, France) and a standardised acetaminophen solution (Daro, Remark Groep, Rogat, the Netherlands). This high-fat mixed meal triggers gallbladder contraction. Subsequently, gallbladder ultrasonographic imaging will be performed every 15 min for 3 h. Postprandially, blood will be drawn every 30 min for 3 h to examine serum acetaminophen levels, and at set times to assess glucose levels. Every 30 min, a breath sample will be collected for the ^{13}C-MTG breath test for 6 h.

MRI procedures

After an overnight fast, participants will be instructed to delay all morning medications. Participants will arrive at the CRU between 7:00 and 9:00, and an intravenous catheter will be inserted into an antecubital vein for secretin administration. A negative oral contrast agent (Lumirem, Guerbet, Gorinchem, the Netherlands) is given to enhance visualisation of the pancreatic duct system. The MRI protocol will consist of (1) structural sequences to assess organ anatomy, (2) spectroscopy sequences to assess hepatic lipid content and (3) MR cholangiopancreatography (MRCP) sequences for pancreatic duct morphology. Moreover, secretin (Secrelux, Sanochemia Pharmazeutika AG, Vienna, Austria) will be administered intravenously to induce pancreatic excretion, which is measured and quantified using MRCP sequences.

For the acute pancreatic study, the same MRI protocol is used during concomitant infusion of exenatide or placebo. After arrival at the CRU between 7:00 and 9:00, infusion of the study drug or placebo is started. After an equilibration period of 60 min, the MRI protocol will start. At least 1 week gap is observed between the MRI visits.

Safety visits (weeks 2 and 6) and telephone follow-up (weeks 1 and 9)

At week 2 and 6, after an overnight fast, participants will be instructed to delay all morning medications, apart from metformin. Medical history is taken, and drug accountability is monitored and stimulated. Body anthropometrics and blood pressure are recorded. Fasting blood samples will be collected to assess renal and pancreatic safety parameters at week 2 and 6, whereas blood, urine and faeces will be stored at week 2 only. At week 1 and 9, medical history is taken and drug accountability is monitored and stimulated.

Early-term assessments

During all study visits and telephone follow-up, compliance and participant retention will be promoted. However, in case patients withdraw their participation, they will be asked to participate in early-term assessments aiming to study as many predefined outcome measures as possible, with a particular focus on primary outcomes.

Quality assurance: data management and monitoring

Outcome data will be collected on hard-copy case report forms (CRF). The CRF files will be anonymous, only indicating the participants study number. There will be double entry of all data into a secure online clinical trial database programme (OpenClinica LLC, V.3.3, Waltham, Massachusetts, USA), which complies with all regulations proposed by the International Conference on Harmonization of Good Clinical Practice (GCP). The final data set will be exported from OpenClinica, containing anonymous data, and will principally be available to study physicians and the principal investigator only.

An independent monitor, provided by the institutional Clinical Research Bureau of the VU University Medical Center, will oversee the progress of the clinical trial and ensure that it is conducted, recorded and reported in accordance with the protocol, standard operating procedures, GCP and the applicable regulatory requirements.

Sample size

Based on previous animal and clinical data on the effects of GLP-1 administration on HRV, the estimated GFR and duodenal aspiration measured exocrine pancreatic function;[56 62 63] assuming 2-sided significance level of 0.05 and a power (1-β) of 80%, we considered 15 participants per treatment arm to be sufficient to detect statistically significant changes in each of our outcome measures. To allow for a dropout rate of 15%, we decided to include a total of 60 patients with type 2 diabetes, with 20 per treatment arm for the 12-week study and 30 participants per treatment arm for the acute cardiovascular and renal intervention study. Using the same assumptions, we calculated that 12 patients would be sufficient for the acute cross-over pancreatic study. Sample size calculations were performed using an ANOVA-model with SAS-software (V.9.2, Cary, North Carolina, USA) for comparing liraglutide and sitagliptin to placebo. A detailed power analysis is available in the online supplementary material.

Statistical analyses

A per-protocol analysis is considered the most appropriate approach to examine biological effects of incretin-based therapies on different organs systems.

To test treatment effects versus placebo, we will perform multivariable linear regression models (for single measured end points) and linear mixed models (for repeatedly measured end points). All analyses will be corrected for potential between-group baseline differences. This strategy will be used for both acute and 12-week studies. Log-transformation will be applied before analysis of non-Gaussian distributed data, as assessed by visual inspection of histograms and Q-Q plots. For each end point, we will report the between-group difference with its 95% CI or SE of the mean and p value. All analyses will be performed using SPSS V.22.0 (IBM SPSS Inc, Chicago, Illinois, USA).

MEDICAL ETHICS AND DISSEMINATIONS

The original protocol and all amendments to this protocol were approved by the local Ethics Review Board (2012/391) and the National Central Committee on Research Involving Human Subjects (NL41701.029.12). The study will be conducted in accordance with the Declaration of Helsinki and GCP. All participants will provide written informed consent before participation. The study is registered at ClinicalTrials.gov (ID: NCT01744236).

All patient data will be handled confidentially and anonymously. Data acquired during the eligibility visit will be coupled to a screening-visit number. All data acquired during the study will be coupled to a participant study number. A code list, with identifier data, screening numbers and study numbers, will be stored securely on the institutional server and protected by passwords only known to the responsible study physicians and principal investigator.

During the study, participants will be monitored by safety visits and telephone follow-up. All antihyperglycaemic study drugs (exenatide, liraglutide and sitagliptin) have been approved by the Food and Drug Administration and the European Medicines Agency for the treatment of type 2 diabetes, and are considered to be safe. Known adverse effects are nausea, vomiting, diarrhoea and constipation (especially for exenatide and liraglutide) and nasopharyngitis (especially for sitagliptin). These adverse events are usually mild and transient. Both GLP-1 receptor agonists and DPP-4 inhibitors may induce hypoglycaemia, especially when combined with sulfonylurea treatment. As described above, care will be taken to frequently measure blood glucose levels and sulfonylurea dose will be decreased if regarded necessary. Given the short treatment duration in this cohort of patients with type 2 diabetes without serious complications, we expect heart failure and pancreatic risk to be low. Any reported adverse event will be recorded, while serious adverse events will additionally be reported to the Ethics Review Board. Standard clinical care will be provided to manage the adverse event. In case of possible damage as a result of participating in this trial, the loss is covered by institutional insurance.

The protocol has been developed by experts in the field of endocrinology, vascular medicine, nephrology, gastroenterology and radiology. Members of the SAFEGUARD consortium have been involved in the development of this study protocol, and will be involved in the drafting of the manuscripts. Novo Nordisk A/S has not been involved in the development of the study protocol, but will be allowed to comment on medical accuracy and confidentiality of manuscripts before publication. The Dutch Kidney Foundation was not involved in the development of the study protocol, nor will it be involved in the drafting of the manuscripts. The findings from this study will be disseminated through international peer-reviewed publications, at scientific conferences, and when considered publically interesting,

through mass media. Authorship eligibility will be determined using the guidelines of the International Committee of Medical Journal Editors.

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†Deceased. This paper is in memory of Professor Michaela Diamant, whose experience and expertise were crucial for the design of this study.

MMS and LT have contributed equally.

Contributors MMS, LT and MHAM drafted the manuscript. TH, MHHK, ICP, DLC, DHvR read the draft critically to make contributions and approved the final text. All authors were involved in the development of the study protocol We commemorate MD, the original primary principal investigator of this project, whose experience, expertise and capacity for inventive thought and understanding were crucial for the design of this protocol.

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Competing interests Before her passing away, on 9 April 2014, MD was a consultant for Abbott, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, GI Dynamics, Merck Sharp & Dohme, Novo Nordisk, Poxel Pharma and Sanofi. She was a speaker for AstraZeneca, Bristol-Myers Squibb, Eli Lilly, Novo Nordisk and Sanofi and through MD, the VU University Medical Center received research grants from Abbott, AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Medtronic, Merck Sharp & Dohme, Novo Nordisk and Sanofi. MD received no personal payments in connection to the aforementioned activities: all funds were directly transferred to the Diabetes Centre's non-profit Research Foundation. Through MHHK, the VU University Medical Center received research grants from Boehringer Ingelheim, Novo Nordisk and Sanofi.

Patient consent Obtained.

Ethics approval Institutional Ethics Review Board ('METC').

Provenance and peer review Not commissioned; externally peer reviewed.

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Online Appendix

Sample Size Calculation

At the time of the design of the study, no randomised controlled trials were available focusing on the effects of incretin-based therapies on the specified cardiovascular, renal and gastrointestinal endpoints in patients with type 2 diabetes. Consequently, sample size calculations were performed using expected effect sizes, based on pre-clinical experiments and small-sized studies using intravenous GLP-1 peptide administration in healthy and obese subjects. All power calculations were performed using an ANOVA-model with SAS-software (v.9.2, Cary, NC, USA), comparing the active antihyperglycaemic agents, i.e. liraglutide and sitagliptin, with placebo. No sample size calculations have been performed to compare the effects of liraglutide with sitagliptin.

Prolonged, 12-week, intervention study

Renal study

Based on one study, examining the effect of intravenous GLP-1 peptide administration on creatinine clearance in obese insulin-resistant subjects [1], we calculated that a sample size of 60 subjects with type 2 diabetes, with 20 subjects per treatment group, should be sufficient to detect a change of 15% in glomerular filtration rate (GFR) (i.e. a change in GFR from 60 mL/min to 69 mL/min, SD 8 mL/min), assuming α=0.05, a power (1-β) of 80% and a maximum drop-out rate of 15%.

Cardiovascular

Based on a rodent study using GLP-1 and a human type 2 diabetes study using metformin [2,3], we anticipate an effect size of roughly 1.0 on heart rate variability (HRV). Assuming α = 0.05, a power (1-β) of 80% and a maximum drop-out rate of 15%, 20 participants per treatment group should be sufficient to detect statistically significant differences between the two active agents and placebo.

Gastrointestinal

There is one study showing an acute inhibitory effect of GLP-1 peptide on pancreatic exocrine function in healthy males (~40% reduction), using gold standard aspiration techniques of pancreatic trypsin and lipase secretion [4]. To detect a similar reduction, with a SD of 35%, we anticipated on needing 13 patients per treatment arm (assuming α = 0.05; power of 80%; drop-out rate of 15%). With 60 patients, we should be able to detect a statistically significant difference using a somewhat less sensitive faecal elastase-1 test.

Acute intervention study

Cardiovascular and renal

Since the same subjects will be used for both the acute and 12-week studies, we will have 30 subjects per group for the acute intervention. We believe that the sample size calculations for the primary endpoints in the acute study, i.e. cardiovascular (acute change in HRV) and renal (acute change in GFR), can be extrapolated from the long-term intervention study. Therefore, 30 subjects per group should be sufficient to detect differences between the active agent and placebo.

Pancreatic

Using the same literature as for the 12-week intervention study [4], we calculated that for a crossover design, assuming α=0.05 and a power (1-β) of 80%, 12 patients are needed to observe a change of 40% (standard deviation 35%) in post-secretin pancreatic secretion volume.

Secondary endpoints (acute and 12-week study)

Current sample size analyses have only been performed to address the primary endpoints. Therefore, this trial may not be adequately powered to detect changes in secondary outcomes.

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Online Table A: Cardiovascular outcome measures

Outcome Measure	Measurement Technique	Timepoints
Primary outcome measure		
Heart rate variability	Resting state heart rate variability will be measured using a NexFin beat-to-beat ECG and blood pressure recording device (Nexfin©, BM Eye, Amsterdam, The Netherlands) for 5 minutes. Artefacts will be corrected using linear interpolation techniques. Spectral analysis will be performed using Kubios software 2.1 (University of Eastern Finland, Kuopio, Finland) to derive the ratio between low-frequency and high-frequency domains (LF/HF-ratio), a validated measure of cardiac sympathovagal balance. Additionally, time-domain and nonlinear aspects of heart rate variability will be assessed.	*Baseline* *Acute:* 30, 90 and 155 minutes *Acute meal*: 30, 60 and 120 min *Longterm* *Long-term meal:* 30, 60 and 120 min
Secondary outcome measures		
Hemodynamic variables	Heart rate, systolic and diastolic blood pressure will be measured using an oscillometric device (DinaMap®, GE Healthcare, Little Chalfont, United Kingdom) with adequate cuff size. Measurements will be performed on the non-dominant arm in triplicate, and the mean of the last 2 measurements will be used for analysis. Stroke volume, cardiac output and vascular resistance will be measured for 30 seconds using a NexFin device.	*Baseline* *Acute:* 30, 60, 90, 120 and 150 minutes *Acute meal*: 30, 60, 90 and 120 minutes *Longterm* *Longterm meal*: 30, 60, 90 and 120 minutes
Capillary density and recruitment	Nailfold capillary density will be measured on the middle finger of the non-dominant hand using the VCS Video Capillaroscopy System (KK Technology, Honiton, England). Two separate visual fields of 1 mm^2 will be recorded before and after 4 minutes of arterial occlusion (300 mmHg).	*Baseline* *Acute:* 110 minutes *Acute meal*: 90 minutes *Longterm* *Longterm meal*: 90 minutes
Capillary fluxmotion	A laser Doppler fluxmetry system (Periflux 4000; Perimed, Stockholm, Sweden) will be used to measure skin blood flow on the second phalanx of the middle finger on the dominant hand. Total skin perfusion and determinants of skin perfusion (endothelial, neurogenic, smooth muscle, respiratory and heart beat) will be measured by wavelet analysis of 30 minute recordings using Matlab 7.8.0 (Mathworks, Natick, MA, USA).	*Baseline* *Acute:* 110 minutes *Acute meal*: 90 minutes *Longterm* *Longterm meal*: 90 minutes
Vascular Stiffness	Pulse wave analysis will be performed on the radial artery of the non-dominant arm using the SphygmoCor© applanation tonometry system (Atcor Medical, Westy Ryde, Australia). The mean of 2 measurements with a high quality index will be used for analysis. The augmentation index will be used to analyse vascular stiffness.	*Baseline* *Acute:* 30, 60, 90, 120 and 150 minutes *Acute meal*: 30, 60, 90 and 120 minutes *Longterm* *Longterm meal*: 30, 60, 90 and 120 minutes

Online Table B: Renal outcome measures

Outcome Measure	Measurement Technique	Timepoints
Primary outcome measure		
Glomerular filtration rate	A standard-method renal clearance based on timed urine sampling using inulin (Inutest®, Fresenius Kabi Austria GmbH, Graz, Austria) will be used. First, a 10-minute priming infusion of inulin 45 mg/kg body weight) will be administered followed by a continuous infusion of 22.5 mg/min. After a 90 minutes equilibration period, subjects undergo 2 urine collection periods of 45 minutes. Urine and blood samples (collected before and after each urine collection period) will be analysed for inulin measured by colorimetric assay after preparation with p-dimethylamino-benzaldehyde.	*Baseline* *Acute* *Longterm*
Secondary outcome measures		
Effective renal plasma flow	A similar protocol as for the inulin-clearance is used, but now with infusion of PAH (Aminohippurate sodium 'PAH' 20%, Merck Sharp &Dohme International, Merck & Co., Inc., Whitehouse Station, NJ, USA) with a priming infusion of 6 mg/kg body weightand a continuous infusion of 11 mg/min. Samples will be analysed for PAH by colorimetric assay after preparation with trichloroacetic acid and indole-3-acetic acid.	*Baseline* *Acute* *Longterm*
Electrolyte excretion	Urine and blood samples (collected before and after each urine collection period) will be analysed for electrolytes. Sodium and potassium will be measured by an indirect ion-selective electrode (ISE) method. Urea will be assessed by enzymatic colorimetric tests on a Modular P auto analyser. Fractional and absolute electrolyte excretion will be used for analyses.	*Baseline* *Acute* *Longterm*
Urine osmolality	Urinary osmolality will be determined by freezing-point depression with a micro-osmometer (Fiske, Norwood, MA, USA).	*Baseline* *Acute* *Longterm*
Urine pH	Urinary pH levels will be measured with the hand-held VARIO® 2V00 pH-meter and SenTix V electrode (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) after calibration with standard pH solutions.	*Baseline* *Acute* *Acute meal* *Longterm*
Glomerular damage marker	Urinary albumin excretion will be measured by immunonephelometric methods	*Baseline* *Acute* *Acute meal* *Longterm*
Tubular damage marker	Urinary Kidney Injury Molecule-1 (KIM-1) and Neutrophil gelatinase associated lipocalin (NGAL) will be measured by ELISA.	*Baseline* *Acute* *Acute meal* *Longterm*

Online Table C: Gastrointestinal outcome measures

Outcome Measure	Measurement Technique	Timepoints
Primary outcome measure		
Pancreatic exocrine function	Faecal elastase-1 levels will be measured using ELISA-techniques.	*Baseline* *Safety visit*: 2 weeks *Longterm*
Secondary outcome measures		
Pancreatic exocrine enzyme secretion	Faecal levels of chymotrypsin will be measured using colorimetric-techniques.	*Baseline* *Safety visit*: 2 weeks *Longterm*
Pancreatic exocrine digestive function	A ^{13}C-mixed triglyerides (MTG) breath test will be performed. After baseline breath samples are collected, 250 mg ^{13}C-MTC (Euriso-Top, Saint-Aubin Cedex, France) is given. Breath samples will be collected every 30 minutes for 6 hours. ^{13}C-levels will be measured using isotope ratio mass spectrometry. Total ^{13}C-recovery and recovery speed will be used for analysis.	*Baseline* *Longterm*
Pancreatic and other abdominal organ structure	Standard abdominal MRI sequences will be performed to assess abdominal organ structure and pancreatic volume using a 1.5T Avanto scanner (Siemens Healthcare, Erlangen, Germany).	*Baseline* *Longterm*
Pancreatic exocrine bicarbonate secretion	Magnetic resonance cholangiopancreatography and structural MRI-images will be performed after secretin-infusion to assess bicarbonate and volume secretion. From these scans, total secreted volume, secretion speed and pancreatic duct thickness will measured.	*Baseline* *Longterm*
Pancreatic plasma enzyme secretion	Plasma levels of amylase and lipase will be measured using standard techniques according to the international federation of clinical chemistry. Serum and urinary trypsinogen levels will be measured by ELISA.	*Baseline* *Acute:* 30, 60 and 120 minutes *Acute meal*: 30, 60 and 120 minutes *Safety visit*: 2 and 6 weeks *Longterm*
Gallbladder emptying	Gallbladder ultrasonography will be performed in the fasting state, and every 15 minutes for 3 hours after a high-fat meal using a Acuson Sequoia machine (Siemens Healthcare, Munich, Germany) and a curved 4C1 abdominal transducer (Siemens). Gallbladder length, depth and height will be measured at each time point. Volume and gallbladder ejection fraction will be calculated.	*Baseline* *Longterm*
Gastric emptying	After drawing a blank blood sample, 1.5 mg soluble acetaminophen (Daro, Remark Groep, Rogat, the Netherlands) is given, and blood is collected every 30 minutes for 3 hours. Serum acetaminophen levels will be measured, and the total recovery and maximal recovery speed used for analysis.	*Baseline* *Longterm*
Hepatic fat content	Magnetic spectroscopy (H-MRS) will be performed in 3 regions ofthe liver, and the mean will be used for analysis.	*Baseline* *Longterm*
Hepatic function and enzyme	Blood will be drawn, and albumin will be measured by colorimetric techniques,	*Baseline*

secretion	and AST, ALT, yGT and ALP by standardized enzymatic techniques according to the international federation of clinical chemistry.	*Safety visit*: 6 weeks *Longterm*
Gastrointestinal damage markers	Faecal calprotectin will be measured using a Phadia EliA-technique. Serum levels of I-FABP and L-FABP will be measured using ELISA	*Baseline* *Safety visit*: 2 weeks *Longterm*

Online Table D: General outcome measures

Outcome Measure	Measurement Technique	Timepoints
Secondary outcome measures		
Anthropometrics	Weight is measured using a calibrated Kern MPE digital weighing scale, while participants wear light clothing, with empty pockets, and no shoes. Length will be measured using a stadiometer, with the subject standing upright without shoes on. Waist circumference is measured using a tape measure in the midst of the crista iliaca and the most lower palpable rib in the standing position. Hip circumference is measured on the widest portion of the buttocks.	*Baseline* *Safety visit*: 2 and 6 weeks *Longterm*
Body fat content	Body impedance analysis will be performed using a Maltron BF-906 device (Rayleigh, Essex, UK).	*Baseline* *Longterm*
HbA_{1c}	HbA_{1c} levels will be determined using high performance liquid chromatography	*Baseline* *Longterm*
Fasting and meal glucose levels	Fasting glucose levels will be measured from venous blood samples using the Gluco Quant-hexokinase method on a Modular P (Roche Diagnostics, Basel, Switzerland) within an hour of drawing blood. All other glucose levels will be measured from venous blood samples using a YSI 2300 STAT Glucose analyzer (YSI Life Sciences, Yellow Springs, Ohio, USA).	*Baseline* *Acute* *Acute meal* *Longterm* *Longterm meal*
Fasting lipid spectrum	Triglycerides and total cholesterol will be determined using an enzymatic colorimetric method, and high-density lipoprotein (HDL)-cholesterol will be assessed using the 3rd generation HDL-C plus method. Low-density lipoprotein (LDL) cholesterol is calculated by Friedewald formula.	*Baseline* *Longterm*