PROTOCOL: THE ROLE OF HYPERGLYCEMIA, HYPERINSULINEMIA AND ELEVATED FREE FATTY ACIDS FOR CARDIAC FUNCTION IN PATIENTS WITH TYPE 2 DIABETES – THE HYPERCARD2 STUDY

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1. CONTENT

2. STUDY RATIONALE

2.1 BACKGROUND:

Metabolically, type 2 diabetes is characterized by elevated plasma glucose and insulin concentrations, increased free fatty acid concentrations, compromised whole body glucose uptake and oxidation and increased hepatic glucose production, and is associated with increased cardiovascular mortality. The more dysregulated the metabolic state, the greater the cardiovascular risk (1).

Myocardial (diastolic) dysfunction is characteristic of patients with type 2 diabetes and this has been associated with myocardial insulin resistance, reduced glucose and increased lipid oxidation compared to normal glucose tolerant subjects (2). Since ATP generation from lipid oxidation requires more oxygen than when energy is sourced from glucose oxidation this may explain why patients with diabetes have highly increased cardiovascular mortality compared to non-diabetic controls: during ischemia energy metabolism may be compromised in diabetic hearts (3,4).

The pathophysiology of type 2 diabetes is generally considered to be one of insulin resistance first, beta-cell failure later, leaving patients in a state of relative insulin deficiency despite elevated plasma insulin concentration (1). Insulin administration relieves the relative insulin deficiency and increases glucose uptake in insulin sensitive tissues and inhibits lipolysis, thereby decreasing plasma glucose and free fatty acid concentrations. In addition, insulin stimulates glucose and inhibits lipid oxidation, thus treatment with conventional antiglycemic agents such as insulin, insulin secretagogues or insulin sensitizers work to normalize the dysregulated metabolic profile of the T2D individual (5).

However, despite this (partial) metabolic correction, tight glycemic control with conventional antiglycemic agents does not protect against cardiovascular death (6,7). In fact, when ambitious glycemic goals are set as in the ACCORD trial, cardiovascular mortality is increased in patients with T2D and high cardiovascular risk (8). In the setting of acute myocardial stress such as in acute myocardial infarction or admission to an intensive care unit, insulin treatment of hyperglycemia also does not improve outcome and is in some studies associated with increased mortality (9–12). Thus, insulin action on the diabetic heart may not necessarily be beneficial.

In this setting the results of the EMPA-REG trial were overwhelming. The addition of Empagliflozin, an SGLT2 inhibitor (SGLT2i), as add on to background antiglycemic therapy, reduced the risk of cardiovascular death markedly and very early after the initiation of treatment compared to placebo (13). A finding that was replicated in the CVD-REAL Nordic study (14) which examined the efficiency of SGLT2 inhibitor treatment in more than 22000 patients started on SGLT2i treatment compared to matched controls started on other antiglycemic treatments. In addition, CVD-REAL Nordic could demonstrate similar cardiovascular risk reduction in in patients without established cardiovascular disease.

Empagliflozin lowers glucose concentrations by inducing renal glucose excretion (15). Within 4 weeks of treatment this results in reduced tissue glucose uptake and oxidation, increased hepatic glucose production, increasing fatty acid mobilization and oxidation as well as increased ketogenesis, possibly due to a decreased insulin/glucagon ratio (16,17 ,).

Therefore, with the introduction of SGLT2i we now have two fundamentally different treatments for hyperglycemia in type 2 diabetes: Conventional antiglycemic agents, which lower plasma glucose and corrects the characteristic metabolic aberrations of the disease, through insulin action, and SGLT2i which lower plasma glucose but amplifies the rest of the dysmetabolic profile characteristic of the type 2 diabetic patient. The first treatment does not prevent cardiovascular death and may even prove harmful if glycemic control is too tight; the other treatment convincingly lowers cardiovascular mortality.

It has been suggested that the metabolic alterations induced by empagliflozin may improve myocardial function and reduce the risk of complications during myocardial ischemia in patients with type 2 diabetes (3,18). Support for the importance of free fatty acids for myocardial function in conditions with increased myocardial insulin resistance and lipid oxidation such as in type 2 diabetes comes from a couple of studies where acute lowering of free fatty acids with the niacin derivative, acipimox, caused cardiac dysfunction (19,20), and recently it has been suggested that insulin resistance, rather than a pathophysiological feature of type 2 diabetes, is defense mechanism that protects against intracellular nutrient overload induced by hyperinsulinemia and hyperglycemia (21-23).

2.2 QUESTION:

What is the role of hyperglycemia, hyperinsulinemia and elevated free fatty acids for cardiac function in patients with type 2 diabetes?

2.3 HYPOTHESIS:

We hypothesize that hyperinsulinemia and hyperglycemia are factors that may negatively influence cardiac function, while an increased availability off free fatty acids are important for maintaining cardiac function in patients with type 2 diabetes. If so the metabolic phenotype of type 2 diabetes may be a necessary adaptation for optimal cardiac function in a hyperinsulinemic and hyperglycemic environment and thus treating hyperglycemia in an insulin independent manner that does not suppress lipid oxidation could improve myocardial function and protect the heart when stressed.

2.4 ENDPOINTS:

2.4.1 PRIMARY ENDPOINT:

Myocardial diastolic function (Cardiac MRI): Change in left ventricular peak filling rate (ΔLVPFR)

Change is defined as LVPFRtreatment - LVPFRbaseline. Baseline is defined as the visit during the wash-out period preceding the treatment period. Thus, if the patient is randomized to insulin first. Baseline for insulin treatment is visit 1, and baseline for empagliflozin treatment is visit 3.

2.4.2 KEY SECONDARY ENDPOINT:

Myocardial diastolic function: Change in left atrial passive emptying fraction

2.4.3 SECONDARY ENDPOINTS:

Myocardial systolic function (Cardiac MRI): Change in left ventricular ejection fraction (ΔLVEF)

The study design does not allow for blinding, but evaluation of primary and secondary outcomes will be performed by specialists in the field of MRI blinded to the treatment og the patients.

2.4.4 DESCRIPTIVE ENDPOINTS:

CARDIOVASCULAR ENDPOINTS:

a)   VO2max – estimated from Aastrands two-point test.

b)   Change in central blood volume and hematocrite.

METABOLIC ENDPOINTS:

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a)   Basal and postprandial AUC FFA and glycerol turnover

b)   Endogenous glucose production and tissue glucose disposal (metabolic clearance of glucose)

c)   Fasting and postprandial energy expenditure and respiratory quotient.

d)   Glucagon/insulin ratio

e)   Insulin sensitivity (Average glucose metabolic clearance rate during OGTT/average insulin concentration)

f)   Beta-cell function (prehepatic insulin secretion rate (determined by deconvolution of C-peptide data) correlated to ambient glucose concentrations, glucose sensitivity).

g)   Adlibitum food intake

2.5 AIM:

The aim of the present study is to elucidate the physiological importance of hyperglycemia, hyperinsulinemia and elevated free fatty acids for myocardial function during rest and chronotropic stress in patients with type 2 diabetes. Secondarily, the study aims to characterize metabolic effects of insulin dependent and independent glucose lowering in patients with type 2 diabetes.

3. METHOD

The study is a 20-week prospective, interventional, comparator controlled, open label, 2-arm cross-over study where subjects are randomized to NPH insulin or Empagliflozin (25 mg once daily) treatment first for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over to 5±1 weeks treatment with the remaining study drug. For 7 weeks preceding randomization but after inclusion, patients undergo a program of washout of pre-existing antiglycemic treatment (except metformin) and run-in of empagliflozin (see below).

As described previously, insulin and empagliflozin represents two metabolically opposing methods for lowering plasma glucose concentrations. By titrating insulin treatment to match the glycemic control found with empagliflozin in the same patients, the result is two distinct metabolic phenotypes: one with hyperinsulinemia and suppressed levels of FFAs (NPH insulin treatment), and one with reduced insulin levels and increased levels of FFAs (Empagliflozin treatment) - but both with the same levels of plasma glucose control (figure 1).

FFA / Glucose / Insulin
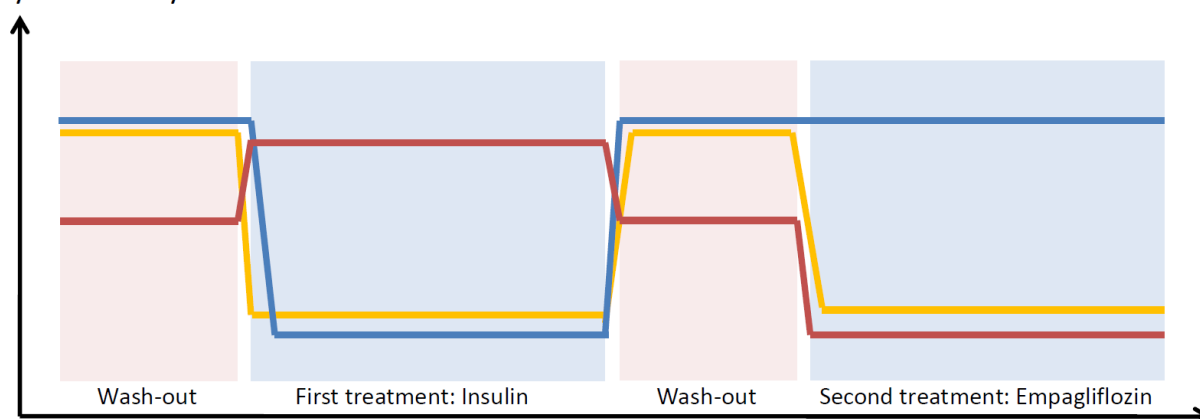
Figure 1. Schematic representation of the metabolic changes expected with the two study drug treatments in a patient randomized to insulin first. Insulin treatement is characterized by low glucose, low FFAs and high insulin concentrations;Empagliflozin treatment by low glucose, high FFAs and low insulin.

This allows to dissect the role of hyperglycemia and hyperinsulinemia for myocardial function during rest and chronotropic stress, and additionally, by repeating cardiac assessment during hormone sensitive lipase inhibition with the niacin derivative, Acipimox (Olbetam®), the role of elevated FFAs for myocardial function in type 2 diabetes can be evaluated.

Study Outline
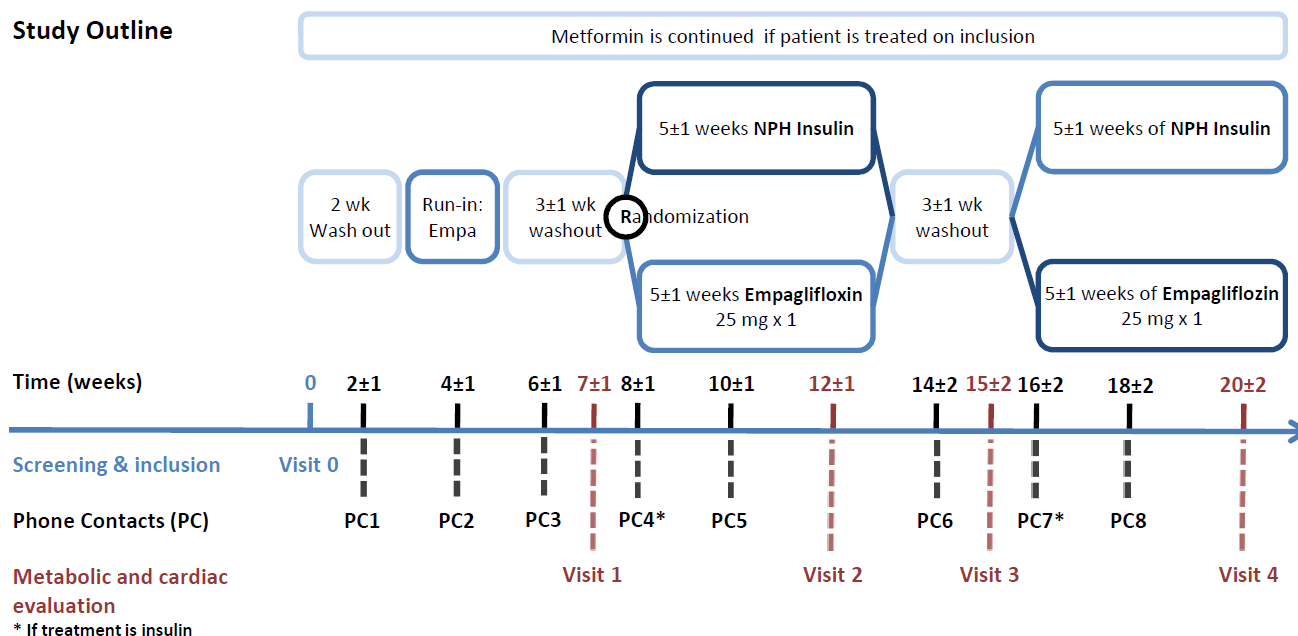
Figure 2. Study design outline.

3.1.1 INFORMED CONSENT, SCREENING AND INCLUSION (VISIT 0):

Patients are given oral as well as written information and time to consider participation in the study. Prior to the meeting the patient has been informed of the possibility of bringing an assessor (danish: bisidder). Time and date for a new meeting where the patient can provide his/her informed written consent is set within 1-2 weeks. In case the patient gives his/her informed consent immediately the screening procedure follows as described below. Informed consent is documented by means of a written, signed and dated informed consent form.

SCREENING: Once written informed consent is obtained the screening procedure follows. Medical history is recorded and screening blood samples are drawn and patients are screened according to in- and exclusion criteria. Screening blood tests include: Hematology (hemoglobin, thrombocytes, hematocrit, leukocytes, lever and renal function tests (creatinine, eGFR (Cockroft-Gault formula), Alkaline fosfatases, alanine aminotransferases, lactatedehydrogenase, bilirubin, amylase, sodium, potassium), fasting P-glucose, C-peptide, HbA1c, TSH, Urinary Albumin/creatinine massratio, and in fertile women, U-hCG. In addition, an ECG, blood pressure and pulserate is recorded along with anthropometric data (height, weight, hip and waist circumference) and a full echocardiography.

For use during metabolic testing, where patients are required to exercise at 50% of VO2 max, maximum oxygen uptake is estimated using the Åstrøm's two-point test performed on a cycle ergometer during indirect calorimetry. From measurements of VO_2 at two sub-maximal pulse rates VO2max is estimated by linear extrapolation to the theoretical maximum pulse rate (220-age)(24).

Afterwards, patients are supplied with a glucosemeter and the appropriate number of strips to comply with scheduled BG measurements of the study. Patients are instructed in the use of empagliflozin and informed of potential adverse event associated with wash-out and empagliflozin (run-in) treatment and how and when to contact study investigator.

Estimated duration of screening procedure: 120-160 min.

3.1.2 WASH-OUT PERIOD:

Following inclusion, patients are instructed to discontinue all their usual antiglycemic agents except metformin (which is continued throughout the study). Patients are instructed in measuring fasting blood glucose (BG) daily for the following 2±1 weeks. All BG concentrations are stored in the glucosemeter and uploaded to local database on the next visit. In case of fasting BG concentrations > 13 mmol/L, the patient is instructed to contact the investigator. An extra phone contact is arranged after 2 day, and if on average fasting BG >13 mmol/L, an additional safety visit is planned. If fasting plasma glucose is still > 13 mmol/L the patient is withdrawn from the study (see paragraph on risks and inconveniences, 7.1 washout). Patients are contacted by the study investigator by phone halfway through the washout period (Phone Contact (PC) 1). All additional phone contacts are recorded in the CRF.

3.1.3 RUN-IN PERIOD:

After successfully completing 2 weeks of washout, the patient is started on Empagliflozin 25 mg once daily in the morning. The patient measures BG twice daily (fasting and before evening meal). Patients are instructed to contact the study investigator in case of adverse events or intolerability. Halfway through the run-in period a phone contact is scheduled.

3.1.4 SECOND WASHOUT PERIOD:

 A 3-week washout period (±1 week) follows the run-in period, during which patients measure fasting BG daily. The same safety issues apply as in the previous washout period. Cardiometabolic assessment is performed during the final week of washout and antiglycemic therapy is begun after the 3rd day of cardiometabolic testing to minimize the time spent in moderate hyperglycemia. In case of withdrawal before randomization patients can be replaced.

3.1.5 RANDOMIZATION:

20 patients with type 2 diabetes are randomized consecutively by lottery in blocks of 3-5 to either NPH Insulin or Empagliflozin treatment first. All patient will receive both treatments during the trial. Randomization is performed on the metabolic experimental day at the end of the second washout period (Visit 1).

3.1.6 FIRST TREATMENT PERIOD:

Patients are treated with either oral empagliflozin 25 mg once daily (morning dosage) or subcutaneous NPH Insulin twice daily. Patients measure BG fasting and before evening meal. If randomized to insulin first the patient will be contacted by phone every 1-3 days for insulin titration after study drug initiation until target blood glucose is reached (see paragraph 3.2.3) (PC4). All patients receive a phone call mid period to increase compliance and inquire to adverse events (PC5). If insulin treated and BG concentrations are outside target concentrations, further insulin titration is performed on a daily basis until target BG is reached (see paragraph 3.2.3). At the end of the first study drug treatment period, cardiometabolic assessment is performed. Study drug treatment is initiated after the 3rd experimental day of visit 1.

3.1.7 THIRD WASHOUT PERIOD:

The two study drug periods are separated by 3±1 weeks of washout to exclude any metabolic carry-over effect (25,26). Patients measure blood glucose concentrations as during previous washout periods and the same safety measures apply (see 3.1.2). Cardiometabolic assessment is performed in the third week of washout.

3.1.8 SECOND TREATMENT PERIOD:

Patients are treated with either empagliflozin or insulin. BG measured as in 3.1.6. Patients treated with insulin in this period patients will be contacted by phone every 1-3 days for insulin titration after study drug initiation until target blood glucose is reached (see paragraph 3.2.3) (PC7). All patients receive a phone call mid period to increase compliance and inquire to adverse events (PC8). If insulin treated and BG concentrations are outside target concentrations, further insulin titration is performed on a daily basis until target BG is reached (see paragraph 3.2.3). At the end of the second study drug treatment period, cardiometabolic assessment is performed.

3.2 INVESTIGATIONAL PRODUCTS:

Only drugs already registered for treating type 2 diabetes are used in this Study. Patients will be provided with the study medication and receive a thorough instruction in the administration of, and the risks and side effects associated with use of the study drugs. Compliance will be controlled by collecting the used insulin pens and empty drug packaging.

3.2.1 ADDITIONAL LABELLING:

The additional labels (tillægsetiketter) will contain the following information:

- Name, address, phone number for sponsor-investigator (address and phone number can be omitted, if has received a patient card or equivalent)
- Name of the study (HyperCarD2)
- Patient ID-number

A copy of the additional label will be inserted into the trial master file at Hvidovre Hospital, where the additional labelling will be performed under double control, which is documented. The personel on site has prior experience with and been trained in the additional labelling procedure.

3.2.2 EMPAGLIFLOZIN TREATMENT:

25 mg of Empagliflozin (Jardiance®) once daily, administered orally before 8 in the morning. Patients measure morning fasting and preprandial evening blood glucose concentrations and these are recorded for later insulin titration. Patients are instructed to contact the study investigator in case of adverse events or intolerability. Halfway through the run-in period a phone contact is scheduled. Treatment duration: 5±1 weeks.

3.2.3 NPH INSULIN (100 IU/ML) TREATMENT:

Patients are treated with NPH insulin (Insulatard®) at a starting dose of 0.2 IU/kg body weight/day administered by subcutaneous injection twice daily. Patients measure BG twice a day and NPH Insulin dosage is up titrated daily by 0.05 IU/kg body weight/day until average blood glucose over three consecutive days is within ±1 mmol/L of the individual glycemic target as defined below. This is done in co-operation with the investigator via phone contacts (PC4/7, fig. 2 and table 2). Phone contacts are repeated every 1-3 days

until treatment target has been reached. All phone contacts required to reach the individual glycemic target are recorded in the CRF. Treatment duration: 5±1 weeks.

This insulin titration regimen is frequently used in our clinic and is in our experience not associated with a greater risk of hypoglycaemia than what is generally found with NPH insulin treatment. During the second week of insulin treatment a second PC is planned and in case patients do not meet the target by ±1.5 mmol/L on average over the preceding 3 days, insulin dosage may be further adjusted (PC5/8, fig. 2 or table 2).

TARGET BLOOD GLUCOSE CONCENTRATION: The average fasting and evening glucose concentrations during the last week of the run-in period serve as glycemic target for NPH insulin treatment in the patients randomized to insulin first. Average fasting and preprandial evening BG values of week 3 and 4 of the first study drug period serve as glycemic target for NPH insulin treatment in those patients randomized to empagliflozin first.

3.3 OVERVIEW OVER METABOLIC AND CARDIAC FUNCTION STUDIES:

A total of 4 experimental visits are planned, each visit consisting of 1 full metabolic study day and 1-2 -hour cardiac MRI study days. The metabolic and cardiac function studies are the same with each visit. Visit 1 is planned at the third week of the second washout period, visit 2 at the last week of the first study drug period. Visit 3 in the final week of the third washout period and visit 4 in the final week of the second treatment period (figure 2).

3.3.1 METABOLIC STUDY DAY OVERVIEW

- DXA-scan and fasting safety and efficacy blood samples
- Determination of 3-hour basal metabolism.
    - Infusion of glucose and glycerol tracers
    - Basal muscle and fat biopsies
    - Basal energy expenditure and determination of respiratory quotient
- 5-hour OGTT
    - with oral glucose tracer and
    - continued intravenous glucose and glycerol tracer.
    - Fat- and muscle biopsies at maximum insulin stimulation
- Exercise test and determination of VO2max
- Ad libitum meal
- 24h Holter and BP monitoring.

3.3.2 CARDIAC MAGNETIG RESONANSE IMAGING (CMRI):

- Fasting blood samples, repeated regularly during experiment.
- Echocardiography
- CMRI Rest
    - Without enhancement
    - With enhancement
- CMRI Stress
    - Unenhanced sequence repeated during pharmacological chronotropic stress with glycopyrrolate infusion.

3.3.3 CMRI; ACIPIMOX:

- Same protocol as 3.3.2 without enhancement, but performed during pharmacological suppression of plasma free fatty acids with acipimox.

3.4 METABOLIC STUDY DAY:

3.4.1 DUAL ENERGY X-RAY ABSORPTIOMETRY (DXA) SCAN:

Patient meets in the morning at 0730 after an overnight fast at the Dept. of Endocrinology 541, Hvidovre Hospital where a DXA-scan is performed to determine body composition. Duration: ~20 min. The DXA-scan results will be stored in the CRF.

3.4.2 BASAL BIOCHEMISTRY AND ADMINISTRATION OF INVESTIGATIONAL DRUG:

The rest of the experiments are performed at The Unit for Endocrine and Metabolic Research 159, Hvidovre Hospital. Two catheters, one in each arm are inserted for infusion of tracers and for repeated drawing of arterialized blood samples respectively. Safety blood samples (haematology (including hematocrit), liver and renal function tests) are drawn.

Blood glucose data are uploaded to local data base and drug accountability check is performed. Anthropometric data are recorded. A 12-lead ECG is recorded. These data are recorded in the CRF

Baseline blood samples are drawn (P-glucose, insulin, C-peptide, HbA1c, glucagon, free fatty acids (FFA), triglycerides, ketone bodies (beta-hydroxybuturate, acetoacetate), urate, cortisol, adrenocorticotropic hormone, Growth hormone (GH), IGF-1, creatinine, blood urea nitrogen, pro-BNP, pro-ANP and markers of cardiac function, tracers (background), gut hormones, inflammatory markers and metabolomics). The patient empties bladder and urine is sent for determination of albumin/creatinine-ratio.

The investigational drug and the patients concurrent medicine is administered as previously described at 0800 with 500 mL oral water. This is recorded in the CRF.

3.4.3 BASAL METABOLISM:

Primed infusions of stable glucose ([6,6-D_2]-glucose) and glycerol ([1,1,2,3,3-D_5]-glycerol) tracers are initiated (T=-180 min). Blood is sampled for glucose, insulin, C-peptide, glucagon, FFAs, triglycerides and ketone bodies, tracers/traces, gut hormones, GH and metabolomics at -30, -15 and -2 min. The patient empties bladder, urine is weighed and samples are taken for determination of tracer concentrations and urinary nitrogen excretion. Tracer priming doses and infusion rates as well as plasma glucose concentrations are recorded in the CRF. Duration: ~3 hours.

3.4.4 5-HOUR OGTT:

The patient ingests anhydrous glucose (72 g) with added [U-$^{13}C_6$]-glucose tracer (3 g) dissolved in 250 mL of water over 5 minutes (T=0 min). Intravenous tracer infusions continue unchanged. Blood is sampled regularly for 5 hours for determination of glucose, insulin, C-peptide, FFAs, triglycerides, tracers/tracees, ketone bodies, gut hormones and GH. The patient empties bladder regularly during and at the end of the OGTT. Urine is sampled for nitrogen excretion and tracers/traces. Glucose concentrations are recorded in the CRF.

3.4.5 MUSCLE AND FAT BIOPSIES:

Fat and muscle biopsies are obtained at t= 0 min and t= 90 min, i.e. in the fasting and the maximally insulin stimulated state respectively. Local analgesia is applied before biopsy sampling. The procedure is documented in the CRF.

3.4.6 INDIRECT CALORIMETRY:

30 min ventilated hood indirect calorimetry is performed at t=-60 min, t=60 min, t=120 min and t= 240 min for determination of fasting and postprandial energy expenditure. The results are recorded in the CRF.

3.4.7 EXERCISE TEST (50% VO2MAX):

At T=300 min, the patient is placed on the cycle ergometer with a chest mounted pulse rate monitor and a mask mounted indirect calorimeter. The patient is exercised at 60 W for 4 minutes after which work load is increased until an oxygen consumption of 50% of the estimated VO2max is reached. Work load is adjusted continuously to maintain oxygenconsumption at 50% of max. Blood is sampled for glucose, insulin, FFA, triglycerides, ketone bodies and tracer/tracee concentrations. To estimate VO2max, work load is increased by 50W for a pulse rate increase of 30 after 30 min. When pulse is steady for 2 min, oxygen consumption and pulse rate are recorded and the test is stopped. Workloads and calorimetric data are recorded in the CRF. Duration: ~40 min.

3.4.7 AD LIBITUM MEAL AND VISUAL ANALOGUE SCALE HUNGER AND SATIETY SCORES:

SGLT2 inhibition is associated with a lower weight reduction than predicted from the urinary energy loss. SGLT2 inhibition does not change resting energy expenditure or blunt the thermogenic effect of feeding, suggesting that energy intake is increased (27). With this in mind, and because patients need a meal before they can be sent home, the metabolic assessment day is ended with an ad libitum meal, consisting of thoroughly mixed pasta Bolognese (with a fixed nutrient composition and energy content). Patients are placed in a quiet corner and instructed to eat until full. Two glasses of water (total 300 mL) are allowed with the meal. The meal is weighed before and after serving and the difference defined as the ad libitum meal intake. Duration ~30 min. Throughout the day patients are asked to score their hunger, satiety and sensation of fullness on a visual analogue scale. Ad libitum meal size and visual analogue scale scores are recorded in the CRF. Duration: ~30 min.

3.4.8 HOLTER MONITORING AND DIURNAL BLOOD PRESSURE MEASUREMENT:

Patients are fitted with a Holter monitor and a diurnal blood pressure monitor at the end of the day, which is returned after 24 hours.

3.5 CARDIAC MRI:

Cardiac MRI is planned to take place on two different days with each visit. On the first day CMRI is performed according to standard procedure, on the second day CMR is done without enhancement and while FFA concentrations are reduced by up to 80% using acipimox administered 0700 in the morning and repeated at 0900. The order of the experiments cannot be randomized because of concern for metabolic carry-over effects of FFA lowering. The CMRI protocol described here is not fundamentally different from that used in standard work up for ischemic heart disease.

3.5.1 CARDIAC MRI MINUS ACIPIMOX:

The patient meets fasting at 0745 at Rigshospitalet, for an extensive cardiac MRI assessment on a 1.5 Tesla MR scanner. Morning medication, including investigational medicinal product is administered at 0800 together with 500 mL water. Insulin is administered subcutaneously now, if the patient is treated with insulin. Anthropometric data are recorded. Two intravenous catheters are inserted into an antecubital and the contralateral dorsal hand vein for infusion (of gadolinium contrast and glycopyrrolate) and for blood sampling respectively.

BLOOD SAMPLES: are drawn regularly throughout the experimental day for analyses of glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, ketone bodies, hematocrit and markers of cardiac function.

ECHOCARDIOGRAPHY: Prior to CMRI a transthoracic echocardiography will be performed. The echocardiography will include all relevant echocardiographic parameters, with special reference to parameters supplementing the MRI scan.

CARDIAC MRI, REST: Following scout images, chamber function will be evaluated by 2D cine imaging. Biventricular and biatrial function will be evaluated from a transversal stack and from a short axis (double oblique) stack including the atria. Flow in the pulmonary artery and the ascending and descending aorta will be evaluated with phase-contrast flow sequences. An additional flow sequence will be obtained corresponding to the distal part of the descending aorta in order to determine aortic distensibility and flow-velocity. Myocardial perfusion will be evaluated during gadolinium infusion. Furthermore, CMRI data accurately determine the time of gadolinium contrast entry into the right and the left ventricle. Since cardiac output is also known, the transit time multiplied by cardiac output estimates central blood volume.

CARDIAC MRI, PHARMACOLOGICAL STRESS: The unenhanced Cardiac MRI protocol is repeated. This time during pharmacological stress with a primed continuous infusion of glycopyrrolate, which is designed to demasque diastolic dysfunction (29). 20 minutes after infusion is stopped, the pharmacological effect will subside.

Infusions of gadolinium contrast enhancer and glycopyrrolate are documented in the CRF. Glucose concentrations are recorded.

Duration: ~3 hours

3.5.2 CARDIAC MRI WITH ACIPIMOX:

The protocol of day 2 without enhancement is repeated, but this time the patient ingests 250 mg of Acipimox (Olbetam®) at ~2 hours before the experiment. The administration is repeated at just before Cardiac MRI. The early and repeated administration of acipimox is required to assure adequate suppression of hormone sensitive lipase activity and depletion of plasma FFAs (28).

Infusion of glycopyrrolate is documented in the CRF. Glucose concentrations are recorded.

STUDY DRUG INITIATION: After visits 1 and 3 the 1st and 2nd study drug periods are initiated respectively. The patients will receive information during all three experimental days, but after the 3rd experimental day information is repeated. Thus, patients will receive information on how to use the relevant study drug and what potential adverse reactions to expect and how to handle them. In case of insulin treatment, it is made sure that patients are comfortable with subcutaneous injection and the algorithm for titration of insulin treatment is recapitulated. Patients are again instructed in the requirements for blood glucose measurement.

STUDY DRUG CESSATION: After visit 2, study drug treatment is paused for a 3-week washout. Information on how to measure BG and when to contact investigator in the washout period and the symptoms of hyperglycemia are repeated.

USUAL ANTIGLYCEMIC MEDICATION: After visit 4, patients are taken off the final study drug and instructed in continuing their regular antiglycemic medication the next day.

3.6 TOOL INFUSIONS AND DRUGS:

3.6.1 METABOLIC STUDY:

Glucose and glycerol tracers are ordered from Cambridge Isotope Laboratories, Andover, MA, USA and are manufactured to be used in human research. Tracers for intravenous use will be dissolved, sterile filtered and aliquoted in vials at the Capital Region Pharmacy, Herlev, and kept at -20 grades Celsius until they are to be used. The oral glucose tracer is kept in a cool and dry location. On the day of experiments, the required amount of tracer is weighed and dissolved in the oral glucose solution. The use of tracers is required to precisely determine the turnover of glucose and lipids in the fasting and in addition glucose absorption from the intestine in the postprandial state.

3.6.2 CARDIAC MRI:

Gadolinium contrast enhancer and glycopyrrolate comes preformulated for intravenous use, and will be purchased directly from the Capital Region Pharmacy. Administration does not deviate from whereas dosage will be half of that used in standard operating procedures during cardiac ischemia work up. Acipimox will be purchased from the Capital Region Pharmacy.

3.7 STUDY FLOW CHART:

Visits 1-4 contain the same elements and therefore below a generic visit X is described. A few minor exceptions are denoted in the relevant fields with explanations in the foot notes.

Table 1.

VISITS				
Visit number	Visit 0	Visit X [1]		
Visit day		X.1 Metabolic	X.2 CMRI Placebo	X.3 CMRI Acipimox
Time (weeks)	0	Y ± 1 [2]	Y ± 1	Y ± 1
General				
Screening & informed consent	X			
Randomization		X [3]		
Demography	X			
Medical history	X			
Concomitant medication	X	X		
Alcohol/Smoking	X			
Cardiometabolic assessment				
General assessment				
Anthropometric measures [4]	X	X	X	X

ECG	X	X		
Metabolic assessment				
Blood glucose data upload		X		
DXA-scan		X		
Basal metabolism		X		
Regular blood samples			X	X
Muscle and subcutaneous fat biopsy		X		
Indirect calorimetry		X		
OGTT		X		
Exercise test (50% VO$_2$max)		X		
VO$_2$max	X	X		
Ad libitum meal		X		
Cardiac assessment				
Echocardiography	X		X	X
Cardiac MRI rest			X	X
Cardiac perfusion			X	
Cardiac MRI stress			X	X
Diurnal blood pressure		X		
Holter monitoring		X		
Safety				
Adverse events		X	X	X
Blood tests	X	X		
U-Albumine/Creatinine ratio	X	X		
U-HCG [5]	X	(X)		
Upload of BG to database		X		
Study Medication				
Dispensing visit	X			X[6]
Drug accountability		X	X	X
Study drug initiation				X[6]

[1] All cardiometabolic visits 1-4 are similar. Thus X=(1,2,3,4)
[2] Week number Y=(7,12,15,20) for visit number 1-4.
[3] Only visit 1
[4] Height, weight, waist and hip circumference, blood pressure and pulse rate
[5] Urinary hCG is measured at visit 0 in women with childbearing potential. Is repeated on subsequent visits at the discretion of the investigator
[6] Study drug initiation only visit 1 and 3

In addition to physical visits, patients are also contacted regularly over phone to secure the highest degree of compliance and safety during the washout and study drug periods. The different phone contacts and the planned content are described in table 2.

PHONE CONTACTS								
Contact number	PC1[1]	PC2	PC3	PC4[2]	PC5	PC6	PC7[3]	PC8
Drug status	Washout	Empa run-in	Washout	1st study drug period	1st study drug period	Washout	2nd study drug period	2nd study drug period
Time (weeks)	-6	-4	-2	0	2	6	8	10
Safety								
Adverse events	X	X	X	X	X	X	X	X
Hyperglycemia	X	X	X	X	X	X	X	X
Hypoglycemia		X		X	X		X	X
Study medication								
Study drug dose titration				X	X [2]		X	X [3]
Compliance	X	X	X	X	X	X	X	X
Protocol adherence								
Blood glucose measurements	X	X	X	X	X	X	X	X

[1] Not performed in diet or metformin only treated patients
[2] Performed if randomized to insulin first
[3] Performed if randomized to insulin second

4. TIMELINE

Approval from the relevant agencies is expected in the fall 2017. First patient in is expected December 2017. Last patient out is expected December 2020 after which the study will be unregistered with the Danish medicines agency and the Capital Region Municipal Ethical Committee within 90 days. Data analyses are expected to be completed Spring 2021 and no later than December 2021 will data be uploaded to the EudraCT database.

5. STATISTICAL CONSIDERATIONS

Patients will be recruited until 20 patients have been randomized. The study is designed as an open-label cross-over trial with patients randomized to insulin or empagliflozin first, i.e. 10 patients in each arm. To eliminate a carry-over effect of treatment, an up to 3-week wash-out period is planned (25-27). Assuming no period effect or treatment-period interaction, normally distributed data will be compared using the paired Student's t-test for all completers. Wilcoxon's paired signed rank test will be used if data is non-normally distributed. Thus, patients will act as own controls, thereby increasing the statistical power of the study.

The primary aim of the study is to describe the effects of hyperglycemia, hyperinsulinemia and free fatty acids on diastolic myocardial function. The primary endpoint is change in left ventricular peak filling rate (ΔLVPFR). The key secondary endpoint, left atrial passive emptying fraction, evaluates another aspect of left ventricular diastolic function.

The null-hypothesis is that change from baseline in myocardial diastolic function is the same with insulin or empagliflozin treatment at comparable levels of glycemic control, and that effects of plasma depletion of free fatty acids on myocardial diastolic function is independent of antiglycemic treatment.

• • •

5.1 CALCULATION OF SAMPLE SIZE:

In general, measures of myocardial function are highly reproducible when assessed using CMRI, and interstudy and cohort coefficients of variation are in the range of 3-5% (29,30).

Using the same CMRI protocol as the one outlined here, Ahtarovski et al found a mean difference of 92 ml/s in Left Ventricular Peak Filling Rate between healthy young (585±62 ml/s) and healthy elderly subjects (493±55 ml/s) (31). We assume that T2D patients have LVPFR corresponding to healthy elderly subjects, and we assume that empagliflozin treatment improves LVPFR by 30 ml/s (ΔLVPFR=30 ml/s) from baseline and that insulin treatment does not improve LVPFR (ΔLVPFR=0 ml/s).

Conservatively setting the standard deviation of between treatment differences of ΔLVPFR at 30 ml/s, a number of 20 patients would be adequate to determine a 30 ml/s difference between the two treatments with a power of 93% and a two-sided significance level of 0.01, when evaluating data with the paired student's t-test.

http://hedwig.mgh.harvard.edu/sample_size/js/js_crossover_quant.html

The same robustness of the study applies to secondary endpoints of cardiac function and descriptive endpoints of metabolism.

5.2 MISSING OR INVALID DATA:

5.2.1 BLOOD GLUCOSE MONITORING:

Patients are required to measure and record fasting BG concentrations daily during washout periods and fasting and evening preprandial glucose concentrations daily during treatment periods. Data will be used to ascertain that glucose control is similar in the two treatments periods. In this protocol, less than 3 morning and preprandial evening (for treatment periods) BG measurements on three individual days pr. week on average is considered insufficient data to reliably establish glycemic levels in a given period, and the patient is removed from analyses.

5.2.2 GLUCOSE REGULATION:

The two treatment periods should be characterized by similar glycemic control. To compare glycemic regulation, the median glucose concentration during the two treatment periods are compared. If differing more than ± 1.5 mmol/L, patients are withdrawn from the primary analyses.

5.2.3 ACIPIMOX EFFECTS:

Cardiac MRI, with acipimox is performed to evaluate cardiac function when plasma FFAs are depleted. Acipimox leads to a very swift and dramatic reduction (~70-80%) in plasma FFAs, suppressing release from fatty tissues by inhibiting hormone sensitive lipase (28). If FFA concentrations are not significantly reduced compared to the ordinary Cardiac MRI day, then the premise for the experiment is not met and the patient is withdrawn from analyses regarding effects of FFA depletion on cardiac function.

5.2.4 METABOLIC STUDIES:

In metabolic studies where blood needs to be sampled at given time points it is not uncommon that single data points are missing. Often due to venous catheter malfunction or other unforeseen event that prevents the timely drawing of blood. In case of such missing data one of two methods for imputing data will be applied. In case the missing data point is located on a curve which is well defined before and after the point

in question, the missing data is replaced by the mean of the two surrounding data point values. In case the missing data point is the last in a row of continuous data, the previous data value is carried forward.

6. SUBJECTS

Elegible for inclusion are male patients aged ≥18 years with T2DM who are either drug naïve (no anti-diabetes agents for ≥12 weeks prior to randomization) with HbA1c ≤10.0% or taking any background anti-diabetes therapy (except insulin) with HbA1c ≤9.0% despite diet and exercise counseling.

6.1 INCLUSION CRITERIA:

- Age ≥ 18 years
- BMI ≥ 28 kg/m2
- HbA1c ≤ 9.0% (≤10% in diet or metformin only treated patients)
- Fasting C-peptide ≥ 500 pmol/L
- Unchanged antiglycemic treatment 12 weeks prior to inclusion as assessed by investigator

6.2 EXCLUSION CRITERIA:

- Insulin treatment within 3 months of informed consent
- Type 1 diabetes
- Psychiatric disorder or mental retardation
- Drug or alcohol abuse within 3 months from informed consent
- Poor compliance
- Anemia (hgb ≤ 6.4 mmol/L) or other blood dyscrasias causing hemolysis or unstable red blood cells
- Indication of liver disease, defined by serum levels of alanine amininotransferase, aspartate aminotransferase, or alkaline pho, sphatase above 3 x upper limit
- Impaired renal function (eGFR<45ml/min/1.73 m2)
- Treatment with anti-obesity drugs 3 months prior to informed consent
- Systemic steroid treatment within 6 weeks of informed consent
- Uncontrolled thyroid disease (prescribed changes in thyroid medication within the 6 weeks prior to informed consent)
- Any uncontrolled endocrine disorder except type 2 diabetes
- Bariatric surgery or other gastrointestinal disorders that compromises gastrointestinal absorption
- Peptic ulcer – verified by endoscopically
- Any form of planned surgery within 3 months of informed consent
- Acute coronary syndrome, stroke or TCI within 2 months prior to informed consent
- Persistent atrial fibrillation
- Inability to undergo experimental procedures including exclusion criteria for MRI scanning:
  - Implantable cardioverter defibrillator/pacemaker
  - Ferromagnetic clips
  - Claustrophobia.
- Contraindication to glycopyrrolate infusion:
  - Known closed-angle glaucoma
  - known severe prostate hyperplasia
  - Tachycardia (HR > 100 at rest)
  - Known bladder atony
  - Cardia insufficiency or non-congenital pylorus stenosis – verified endoscopically
  - Known gastroparesis

- Allergy towards any of the drugs or diagnostics used in the protocol (insulin, empagliflozin, acipimox, glycopyrrolate and gadolinium contrast enhancer).
- Any condition which in the opinion of the investigator may jeopardize subject safety or compliance with the protocol

6.3 WITHDRAWAL CRITERIA:

- Subjects may withdraw from the study without any notice or reason
- Pregnancy discovered during the experiment
- Unacceptable adverse reactions or reactions associated with the planned experiments, including severe glycemic dysregulation during washout periods (see paragraph 7.1)

7. RISKS AND INCONVENIENCES:

7.1 WASHOUT:

During washout periods patients are expected to experience increasing blood glucose concentrations. Severely dysregulated patients are not eligible for the study, however it cannot be excluded that patients will experience symptoms of hyperglycemia during washout periods, such as polyuria, thirst, fatigue and headache. The risk of severe hyperglycemia is reduced in several ways in the study:

- Existing metformin treatment is continued throughout the whole study as background antiglycemic treatment.
- Upon entry into the study patients are instructed to measure fasting BG daily, and contact the study investigator in case of BG concentrations of more than 13 mmol/L. Patients are given direct phone numbers and e-mail addresses for easy access to study personnel.
- Phone contacts by study investigator are planned in the second week of washout periods to follow up on the patient and enquire to hyperglycemic events or other adverse events.
- As soon as the final day (Cardiac MRI with acipimox) of a washout visit (visit 1 or 3) is completed, antiglycemic treatment according to study drug sequence is commenced to minimize time spent in hyperglycemia. Depending on when the final visit is planned during the 3rd washout week, treatment periods may differ slightly, but this is not expected to influence results in any way.

In case of BG > 13 mmol/L, the patient will be contacted daily for two additional days. If average fasting BG over the 3 days > 13 mmol/L that triggers an extra safety visit, where fasting plasma glucose (PG) is measured. If PG > 13 mmol/L on the day of the extra visit, then the patient is withdrawn from the study and antihyperglycemic treatment is initiated. Extra phone contacts and visits are recorded in the CRF.

7.2 INVESTIGATIONAL MEDICINAL PRODUCTS:

Patient are thoroughly informed on the actions and potential adverse reaction associated with the study drugs on inclusion in the study and again upon initiation. They are instructed in the correct use of the drugs and how to handle side effects. Patients are provided with investigator contact information so that they may contact investigator with additional questions regarding the investigational medical products.

7.2.1 NPH INSULIN:

The dominating adverse reaction related to insulin treatment is hypoglycemia. The patient receives thorough information on symptoms of hypoglycemia and how to handle them when insulin therapy is initiated. In addition the patient is instructed in how to avoid such hypoglycemic attacks. Local skin irritation is not a problem if correct injection technique is applied. Known adverse reactions are listed in the table below.

Very common (> 10%)	Hypoglycemia.
Not common (0,1-1%)	Edema. Difficulties accomodating, retinopathy.
Very rare (< 0,01%)	Peripheral neuropathy. Anaphylactic reaction. Visual impairment.

7.2.2 EMPAGLIFLOZIN:

The dominating adverse reaction is urinary tract infections and other infections of genitalia. Because of the short treatment duration in this study the risk for such adverse reactions are considered to be low. Empagliflozin is used either alone or in combination with metformin in this study. The risk of hypoglycemia during empagliflozin treatment is therefore considered negligible. Patients are informed of symptoms and how to react if they occur. The treatment is also slightly diuretic, and may for a shorter duration of time cause increased creatinine levels, which subsides with continued use and is reversed in case of cessation of the drug.

Very common (> 10%)	Hypoglycemia*.
Common (1-10%)	Pruritus. Balanitis, polyuria, urinary tract infection, vaginal candidiasis, Vulvovaginitis.
Not common (0,1-1%)	Hypovolemia.

* Hypoglycemia is seen with concurrent sulfonylurea or insulin treatment.

7.3 RECORDING OF ADVERSE EVENTS:

7.3.1 DEFINITIONS

Recording and reporting of adverse events/reactions is done in accordance with ICH GCP Guidelines.

Adverse Event (AE)	Any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An adverse event (AE) can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.
Adverse Event of Special Interest (AESI)	The term AESI relates to any specific AE that has been identified at the project level as being of particular concern for prospective safety monitoring and safety assessment within this trial, e.g. the potential for AEs based on knowledge from other compounds in the same class. AESIs need to be reported to the Pharmacovigilance Department of Boehringer Ingelheim within the same timeframe that applies to SAEs.
Adverse Reaction (AR)	A response to a drug which is noxious and unintended and which occurs at doses normally used in man for prophylaxis, diagnosis, or therapy of diseases or for modification of physiological function. The phrase, "response to a drug", means that a causal relationship between a medicinal product and an adverse event is at least a reasonable possibility, i.e., the relationship cannot be ruled out. .
Serious Adverse Event (SAE)	Any untoward medical occurrence that at any dose: - results in death, - is life-threatening, - requires inpatient hospitalization or prolongation of existing hospitalization, - results in persistent or significant disability/incapacity,

	- is a congenital anomaly/birth defect - Is an important medical events that may not result in death, be life-threatening, or require hospitalisation may be considered a serious adverse event when, based upon appropriate medical judgement, they may jeopardise the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.
Serious Adverse Reaction (SAR)	A SAE for which a causal relationship to the trial/study product is at least possible i.e. a causal relationship is conceivable and cannot be dismissed.
Suspected Unexpected Serious Adverse Reaction (SUSAR)	An SAE where severety and the adverse event is not previously reported and regarded as possibly or probably related to the trial/investigational product by the Investigator.

Reference documents for recording and reporting of adverse events are:

- Insulin: 'Summary of Product Characteristics' for Insulatard (EMEA/H/C/000441 -IG/0642; European Medicines Agancy, published 21/12/2015), herunder især sektionen '4.8 Undesirable effects'.
- Empagliflozin: 'Summary of Product Characteristics' for Jardiance (EMEA/H/C/002677 -II/0014; European Medicines Agancy, published 16/06/2014) , herunder især sektionen '4.8 Undesirable effects'

7.3.2 REPORTING OF AE, AR, SAE, SAR AND SUSAR

All events meeting the definition of an AE will be collected and reported from the first trial-related activity after the subject has signed the informed consent until the end of the trial.

All AEs occurring during the study will be recorded in the CRF. The following information will be recorded: description of event (diagnosis), date of debut and end date, severity, treatment, causality. Follow up if necessary will be described as well. Severity will be rated on a 3-point scale: 1=mild, 2=moderate, 3=severe.

In case of SAEs, SARs or SUSARs, investigator will immediately report to sponsor within 24 hours after learning of the event followed by a written report. All follow-up data will be described in detail in the SAE-form and hereafter the form will be send to the sponsor within 24 hour of obtaining the new knowledge. Previous non-serious AEs, which are upgraded by sponsor to SAEs follow the reporting of SAEs.

SARS: Once a year, sponsor will send a report on any SARs recorded in the study and in addition assess safety for patients included in the study and whether the study should continue. This report will be sent to the Danish Medicinal Agency (Lægemiddelstyrelsen) and the Capital Region Ethics Committee in accordance with EudraLex – Volume 10 Clinical Trial Guidelines.

SAES: If sponsor upgrades an SAE to an SAR the procedure decribed for SARs above will apply. AEs, SAEs and ARs are recorded in the CRF document. They will be reported in the final document (End of Trial Form) to the Danish Medicinal Agency (Lægemiddelstyrelsen) and the Capital Region Ethics Committee (if required) no later than 1 year after the end of the trial.

SUSARS: We expect no SUSARs in the present study. All investigational products are approved for the use in type 2 diabetes and has been investigated in several large-scale studies.

Sponsor will ensure that all information on SUSARs that are fatal or life-threatening is recorded and reported to the Danish Medicines Agency as soon as possible and no later than 7 days after the sponsor became aware of such possible side effects. Within 8 days after reporting the sponsor must notify the Danish Medicines Agency all relevant information about the sponsor and investigator's response to the alert and consequence for the study conduct.

All other SUSARs will be reported to the Danish Medicines Agency within 15 calendar days after the sponsor became aware of them.

AESIS: In this study the following events are considered AESIs:

Hepatic injury

A hepatic injury is defined by the following alterations of hepatic laboratory parameters after randomisation:

- an elevation of AST and/or ALT ≥ 3 fold ULN combined with an elevation of total bilirubin ≥ 2 fold ULN measured in the same blood sample
- an isolated elevation of ALT and/or AST ≥ 5 fold ULN

These laboratory findings constitute a hepatic injury alert and the patients showing these abnormalities need to be followed up according to medical judgement. In case of clinical symptoms of hepatic injury (icterus, unexplained encephalopathy, unex-plained coagulopathy, right upper quadrant abdominal pain, etc.) without laboratory results (ALT, AST, total bilirubin) available, the Investigator should make sure these parameters are analysed , if necessary in an unscheduled blood test.

Decreased renal function

Decreased renal function is defined by a creatinine value showing a ≥ 2 fold increase from baseline and is above the ULN. For the AESI "decreased renal function" the Investigator shall collect an unscheduled laboratory sample for creatinine as soon as possible and initiate follow-up laboratory tests of creatinine according to medical judgement.

Metabolic acidosis, ketoacidosis and diabetic ketoacidosis (DKA)

In case of metabolic acidosis, ketoacidosis and DKA further investigations should be done according to the medical judgment and the clinical course until a diagnosis is made and/or the patient is recovered.

DKA is defined by the diagnostic criteria in the table below, and as defined by the American Diabetes Association (ADA).

Investigators should note that not all criteria in the table below need to apply for the diagnosis of DKA, and clinical judgment should also be taken into consideration. Due to its mechanism of action, empagliflozin may potentially modify the clinical presentation of DKA which may occur at lower plasma glucose levels than stated in the table below.

Table 1 Diagnostic criteria for DKA

	DKA		
	Mild	Moderate	Severe
Plasma glucose (mg/dL)	>250	>250	>250
Arterial pH	7.25-7.30	7.00-7.24	<7.00
Serum bicarbonate (mEq/L)	15-18	10 to <15	<10
Urine ketones*	Positive	Positive	Positive
Serum ketones*	Positive	Positive	Positive
Effective serum osmolality (mOsm/kg)**	Variable	Variable	Variable
Anion gap***	>10	>12	>12
Alteration in sensoria or mental obtundation	Alert	Alert/drowsy	Stupor/coma

*   Nitroprusside reaction method

| ** | Calculation: 2[measured Na (mEq/L) + glucose (mg/dL)/18 |
| *** | Calculation: (Na+) – (Cl- + HCO3-) (mEq/L) |

All

(i)   SAEs,

(ii)   AESIs independent from their seriousness, if applicable

(iii)   non-serious AEs which are relevant for the reported SAE or AESI .

(iv)   pregnancies in female subjects and partners of male subject.

will be reported to the Pharmacovigilance Department of Boehringer Ingelheim.

7.4 EXPERIMENTAL DAYS:

7.4.1 METABOLIC STUDY DAY:

DXA-SCAN: is associated with a minimal X-ray dose of 0.008 mSv, equivalent to 1 extra day of background irradiation. Thus, the risk is minimal.

CATHETERS AND BIOPSIES: There is a slight risk of local irritation when intravenous catheters are inserted. The low risk of infection is effectively minimized by correct disinfection of the skin prior to insertion of the catheter. Muscle and fat biopsies are standard procedures in metabolic research. It is performed in local analgesia which minimizes the immediate discomfort. Tenderness at the site of biopsy may be present afterwards and minor local hematoma may be present for 1-2 weeks.

TRACERS: The use of tracers for infusion is not associated with increased risk. Tracer infusion is a standard procedure in metabolic research and has been done many times before in our lab. The tracers used are stable and without the risk of radiation. Stable tracers have been used in experiments involving pregnant women and children.

BLOOD VOLUME: Over 20 weeks a maximum of 600 mL total of blood will be drawn. This is considered safe. In comparison blood donation involves drawing 500 mL of blood in 1 day.

7.4.2 CARDIAC MRI:

CARDIAC MRI: A standard procedure in the work up for ischemic heart disease. Is considered safe, when no contraindication is present. Paragraph, 6.2 exclusion criteria, lists the contraindications for MRI.

GLYCOPYRROLATE: An atropine analogue that does not cross the blood-brain barrier and thus is associated with fewer central side effects. Patients may experience dryness of mouth, flushing, problems emptying the bladder and constipation. Medical conditions contraindicating the use of glycopyrrolate are listed in 6.2 Exclusion Criteria.

GADOLINIUM CONTRAST ENHANCER: May cause nausea and headache when injected. It safe to use in the present dosage in patients with renal function down to eGFR 30 ml/min/1.73 m^2. Patients with eGFR < 45 ml/min/1.73 m^2 are excluded. No risk is expected with regards to gadolinium use in the present protocol.

7.4.5 TIME CONSUMPTION:

The experiment requires the patient to be available for a screening and informed consent visit and 4 visits consisting of 3 experimental day (9,3,3 hours respectively) over the full 20-week period.

7.4.6 ASSESSMENT OF BENEFITS VERSUS RISK:

Taken together,it is our assessment that the benefits associated with this study, i.e. a deepened understanding of the pathophysiology behind type 2 diabetes and potential long-term benefits in treatment of the disease and its complications, outweighs the potential risks and inconveniences associated with the study.

8. SAMPLING AND STORAGE OF BIOLOGICAL MATERIAL:

8.1 BIOLOGICAL MATERIAL AND ANALYSES:

8.1.1 BLOOD SAMPLES:

Drawn as specified in the methods section. Total blood volume drawn over the course of the 20-week study amounts to 600 ml total. Blood will be collected in prechilled EDTA-tubes, EDTA-tubes added DPP-IV inhibitor or heparin-tubes (TTR) and will be immediately centrifuged while clot-activator tubes will be left for 30 min before centrifugation. The supernatant will be aliquoted in cryotubes and stored at -20 or -80 centigrades for later batch analysis. Extra samples will be drawn for storage in the biobank.

Tracer analyses will be performed at the Clinical Metabolomics Core Facility at Rigshospitalet, amino acid and gut hormone analyses will be performed at Jens Juul Holst Lab at Department of Health Sciences, insulin and C-peptide and FFA/triglyceride concentrations as well as safety and efficacy samples will be measured at the Dept. of Biochemistry, Hvidovre Hospital. Plasma glucose concentrations will be measured bed-side using the glucose oxidase technique.

8.1.2 BIOPSY TISSUE:

Biopsies of abdominal subcutaneous fat and muscle from the vastus lateralis muscle will be obtained in the fasting and postprandial conditions as described previously. Tissue (total of approximately 200 mg fat and 200 mg muscle per visit) will be split in two portions. 1 portion is immediately frozen in liquid nitrogen for analyses of insulin signaling mechanisms and the other portion is frozen in isopentane for later histochemical analyses and characterization. Analyses of muscle- and fat biopsies will be performed at Erich Richters Laboratorium, Afdelingen for Molekylær Fysiologi, Institut for Idræt, Københavns Universitet.

8.1.3 URINE SAMPLES:

Collected urine will be weighed and samples of 20 mL at the different time points will be kept frozen for later analyses of tracer/tracee analyses and nitrogen content.

8.2 ESTABLISHMENT OF BIOBANKS:

8.2.1 ESTABLISHMENT OF A RESEARCH BIOBANK:

Plasma and serum will be analyzed as described above. During the data collection phase of the study the material will be kept in a research biobank at either -20 or -80 centigrades dependeing for later batch analyses once data collection has ended. Batch analyses is key to reliable and comparable results. The research biobank is located at Hvidovre Hospital and no one else but the doctors or the technical staff associated with study are allowed access to the material.

8.2.2 ESTABLISHMENT OF A BIOBANK:

Extra plasma and serum will be collected in a biobank, to allow for reanalysis of samples in case of erroneous analyses or analyses of potential new biomarkers that may contribute further to the understanding of type 2 diabetes. The biobank is located at Hvidovre Hospital and no one else but the doctors or the technical staff participating in the study are allowed access to the material.

Patients are informed of the handling and storage of biological material as described above before signing and dating informed consent. When signing informed consent, patients will be asked to specify, for which research purposes their biological material may be used. After 10 years' storage, the study ID file will be destoyed and samples of biological material in the Biobank are fully anononymized. Patients can request their biological material removed from the biobank before the 10 years if they so wish.

All samples of biological material in the biobank will be marked with study specific patient ID, there will be no information on the biosamples that directly link them to the patient. Relevant permits will be applied for with The Danish Data Protection Agency.

9. DATA MANAGEMENT:

All data regarding patients are confidential and kept in a double locked room. All patients are given a Study specific ID and will be registered by this ID on all data forms, samples and test results. Full name, social security number (CPR-nummer) and study specific ID will only be registered together in the study ID file, which will be kept separately. All health-related matters and sensitive personal data will be handled in accordance with the Danish "act on Processing of Personal Data" (Persondataloven).

Sponsor/investigator will provide direct access to source data/documents for trial related monitoring, audit, IRB/IEC review, and regulatory inspection. Sponsor has made an agreement with the GCP-unit, Region Hovedstaden, Copenhagen, Denmark, who will be monitoring the trial in accordance with the ICH guidelines for GCP. In case of AESIs the Boehringer Ingelheim Pharmacovigilance Office will be contacted as specified previously (7.3.2 Reporting of AESIs). A data processing agreement (Databehandleraftale) with Boehringer Ingelheim will be drawn and signed before the study is initiated.

Data as well as patient ID will be kept for 10 years, after which the ID file will be destroyed and data will be fully anonymous.

10. STUDY ECONOMY:

The study is initated by the participating doctors from Dept. of Endocrinology, Hvidovre Hospital and Dept. of Cardiology, Herlev Hospital and Rigshospitalet. None of the investigators have personal financial interest in the conduct or the outcome of the project and have no personal affiliation to the pharmaceutical industry. No commercial institution will influence the conduction of the study, results or the decision to publish results.

The study receives financial support from Boehringer Ingelheim GmBH, Germany, who manufactures and markets Jardiance (Empagliflozin). The support amounts to a total of 4 million DKK and reflects the cost of the study. Support is given as milestone payments and paid to a separate account managed by Hvidovre Hospital, from which expenses to the study can be covered if costs are documented.

11. REMUNERATION AND REIMBURSEMENT OF PATIENTS:

The study is designed to uncover pathophysiological aspects of type 2 diabetes without any intended patient gain from the treatment, and it is of lightly invasive character with frequent blood sampling, muscle and fat biopsies and cardiac MRI, which may be associated with discomfort. In addition, the patients are required to

invest time in the study which exceeds that of clinical routine visits in the outpatient clinic. Patients will therefore receive remuneration for lost time and earnings and reimbursement of transportation costs.

Patients are required to meet 13 times over 20 weeks, including 4 days of 9 hours duration and 8 days of 3 hours duration. For participation patients receive DKK 9375,- (taxed as B-income), which paid out on the final day of experiments. In case of withdrawal the patient receives DKK 375,- for completed visit 0 and DKK 2250,- for each completed visit 1-4. Transportations costs can be reimbursed to the extent of DKK 375,- pr study day.

12. RECRUITMENT OF PATIENT AND INFORMED CONSENT:

In the outpatient clinic at Hvidovre Hospital almost 2000 patients with type 2 diabetes are followed with regular clinical visits and diabetes care. Of these approximately 700 patients fulfill the inclusion criteria of a HbA1c below 75 mmol/mol without insulin treatment. Approximately 30% have known ischemic heart disease or previous stroke. Thus around 210 patient fulfill the inclusion criteria of the protocol.

12.1 RECRUITMENT AND FIRST CONTACT:

Potential candidates for study participation will be asked about their interest in participating in the study in during their clinical visits in the Endocrinolgy Outpatient Clinic 541, Endocrinology Day Hospital 244, Cardiology Outpatient Clinic 253 and Cardiology Day Hospital 257 at Hvidovre Hospital. Identification of these patients will be done by reviewing lab results and patient files. In addition, potential participants may be contacted by means of a recruitment letter, in which they are informed of the opportunity to participate in a scientific research project. Finally, it is part of the recruitment plan to ask general practitioners in Region H to forward recruitment materials to their patients, advertise for participant in local newspapers and on the internet as well as social media (e.g. forsøgsperson.dk and sundhed.dk; facebook.com).

12. INFORMED CONSENT:

All potential candidates expressing interest in study participation will be contacted by the investigator to schedule an information meeting. Participants are informed of the possibility of bringing an assessor along to the meeting.

Prior to the meeting the patient will receive written information on the study as well as the supplementary file, „Forsøgspersoners rettigheder i et sundhedsvidenskabeligt forsøg", in due time such that it is in the hands of the potential participant a minimum of two days before the planned meeting. The contact information of the investigator will be contained in the information material, so the potential participant has the opportunity to contact the investigator with questions before, during or after the information meeting/study.

The oral information will be given at the information meeting by the investigator or in case of his absence by one the other doctors associated with study. The information meeting will take place at the Unit for Endocrine and Metabolic Research 159, Hvidovre Hospital, in a separate room, which allows for undisturbed conversation. During the meeting, it will be stressed that this is a request for participation in a study, that participation does not involve any treatment benefits, that participation is voluntary and that the participant can withdraw his/her consent at any time without the need for any justification. Withdrawal of consent will not influence usual diabetes care for the patient in the present or the future. Information is given without the use of medical terms and in a language adjusted to the individual receiving the information. Information will include aim, methods and potential risks and inconveniences of the study.

The potential participant will be informed that biological material will be stored in a biobank and that he/she can decide which research this material can be used for. Further, the potential participant will be informed that we intend to store the biological material for 10 years after which samples will be fully anonymized by

destruction of the study ID file. Should the potential participant so wish the biological material will be destroyed earlier.

The potential participant is informed of the opportunity to receive any new information relating to the health of the potential participant that may appear during the trial, if he/she wishes. Furthermore, information is given on opportunity to receive an overview of personal data by the end of the study. Finally, the potential participant is informed on the reimbursement and remuneration in the study and that all payments are tax liable.

The time from information meeting to written informed consent will be individualized up to 1 week. Only the participating doctors are authorized to collect written informed consent from participants. After the consent form has been dated and signed, it will be kept by the investigator in a locked filing cabinet. The participant is given a copy of the informed consent form to keep.

13. PUBLICATION OF RESULTS:

Positive, negative as well as inconclusive results will be published in relevant scientific journals. In addition we will seek to disseminate results through presentations at scientific meetings. Publication will take place as soon as scientifically feasible. No later than 12 months after unregistering of the study, will results be made available at www.clinicalregister.eu.

First authorship of future publications will be awarded to the doctor conducting the trial. Last authorship of future publications will awarded to Nils Bruun Jørgensen, MD PhD.

14. ETHICAL CONSIDERATIONS:

The study will be conducted according to ICH CGP guidelines E6(R2) and will be registered with Danish Medicines Agency (Lægemiddelstyrelsen), The Capital Region Ethical Committee (Regionale Videnskabsetiske komite) and the Danish Data Protection Agency (Datatilsynet). All experimental procedures, examinations and analyses have previously been approved.

The present study will not benefit the individual participant. The results however, may benefit patients in the future by providing new knowledge on the pathophysiology of type 2 diabetes and its effects on cardiac function. If we can confirm our primary hypothesis, then the results may have widespread implications, and may contribute to a change in type 2 diabetes treatment away from a focus on increasing insulin secretion/replacing insulin towards treatments that lower glucose availability and insulin concetrations. If the hypothesis can be discarded then uncertainty regarding insulin treatment in type 2 diabetes patients can be eliminated. Currently, there are no other studies conducted or registered that investigates the current problem, which this study is aiming to address, and so it is our judgement that the potential benefits are far greater than the risks and inconveniences associated with this study.

14.1 REGARDING INFORMED CONSENT:

All participants are given oral and written information by one of the participating doctors. Before screening, a dated and signed consent form will be present. Information is given by a doctor not providing the patients regular diabetes care. Patients are provided with investigator contact information so that they may contact investigator with additional questions regarding the study.

14.2 REGARDING RISKS AND INCONVENIENCES:

14.2.1 HYPERGLYCEMIA:

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During washout periods patients are expected to experience increasing blood glucose concentrations. Severely dysregulated patients are not eligible for the study, however it cannot be excluded that patients will experience symptoms of hyperglycemia during washout periods, such as polyuria, thirst, fatigue and headache. The risk of severe hyperglycemia is reduced in several ways in the study:

- Existing metformin treatment is continued throughout the whole study as background antiglycemic treatment.
- Upon entry into the study patients are instructed to measure fasting BG daily, and contact the study investigator in case of BG concentrations of more than 13 mmol/L. Patients are given direct phone numbers and e-mail addresses for easy access to study personnel.
- Phone contacts by study investigator are planned in the second week of washout periods to follow up on the patient and enquire to hyperglycemic events or other adverse events.
- As soon as the final day (Cardiac MRI with acipimox) of a washout visit (visit 1 or 3) is completed, antiglycemic treatment according to study drug sequence is commenced to minimize time spent in hyperglycemia. Depending on when the final visit is planned during the 3rd washout week, treatment periods may differ slightly, but this is not expected to influence results in any way.

In case of BG > 13 mmol/L, the patient will be contacted daily for two additional days. If average fasting BG over the 3 days > 13 mmol/L that triggers an extra safety visit, where fasting plasma glucose (PG) is measured. If PG > 13 mmol/L on the day of the extra visit, then the patient is withdrawn from the study and antihyperglycemic treatment is initiated. Extra phone contacts and visits are recorded in the CRF.

14.2.2 INVESTIGATIONAL MEDICINAL PRODUCTS:

Patient are thouroughly informed on the actions and potential adverse reaction associated with the study drugs on inclusion in the study and again upon initiation. They are instructed in the correct use of the drugs and how to handle side effects. Patients are provided with investigator contact information so that they may contact investigator with additional questions regarding the investigational medical products.

EMPAGLIFLOZIN (JARDIANCE®): The dominating adverse reaction is urinary tract infections and other infections of genitalia. Because of the short treatment duration in this study the risk for such adverse reactions are considered to be low. Empagliflozin is used either alone or in combination with metformin in this study. The risk of hypoglycemia during empagliflozin treatment is therefore considered negligible. Patients are informed of symptoms and how to react if they occur. The treatment is also slightly diuretic, and may for a shorter duration of time cause increased creatinine levels, which subsides with continued use and is reversed in case of cessation of the drug.

NPH INSULIN (INSULATARD®): The dominating adverse reation related to insulin treatment is hypoglycemia. The titration algorithm used here is also used in our outpatient clinic. In our experience glucose levels are quickly controlled without any increased risk of hypoglycaemia compared to other NPH insulin titration algorithms. Patients are instructed in the symptoms of hypoglycaemia and how to manage it. To reduce the risk associated with hypoglycaemia, patients are instructed to measure blood glucose fasting and preprandial evening during study drug treatment, and when symptoms of hypoglycaemia are present. Furthermore, treatment target is defined by the glycemic response to empagliflozin from a hyperglycemic state, and the average blood glucose is expected to be around 7-10, corresponding to an HbA1c of ~7.0-8.5% (35). The risk of hypoglycaemia at this level of glucose control for 5 weeks in 20 patients is minimal when treated with intermediate acting insulin under close supervision. Local skin irritation is not a problem if correct injection technique is applied.

14.2.3 VENOUS CATHETERS AND BIOPSIES:

There is a slight risk of local irritation when intravenous catheters are inserted. The low risk of infection is effectively minimized by correct disinfection of the skin prior to insertion of the catheter. Muscle and fat biopsies are standard procedures in metabolic research. It is performed in local analgesia which minimizes the immediate and later discomfort. Tenderness at the site of biopsy may be present afterwards and minor local hematoma may be present for 1-2 weeks.

14.2.4 BLOOD SAMPLING:

Over 20 weeks a maximum of 600 mL total of blood will be drawn. This is considered safe. In comparison blood donation involves drawing 500 mL of blood in 1 day. Prior to inclusion, patients will be informed of the extent of the planned blood sampling.

14.2.5 INFUSIONS DURING CARDIAC MRI:

Being an atropine analogue that does not cross the blood-brain barrier, glycopyrrholate infusion is associated with fewer central side effects. Patients may however experience dryness of mouth, flushing, problems emptying the bladder and constipation. Medical conditions which contraindicate the use of glycopyrrholate are ground for exclusion. Gadolinium contrast enhancer may cause nausea and headache when injected. Allergy to gadolinium contrast enhancer is listed as an exclusion criteria.

14.3 REGARDING DATA MANAGEMENT:

All data regarding patients are confidential and kept in a double locked room. All patients are given a Study specific ID and will be registered by this ID on all data forms, samples and test results. Full name, social security number (CPR-nummer) and study specific ID will only registered together in the study ID file, which will be kept separately.

15. PATIENT INSURANCE:

Like all other clinical trials performed in the Danish health care service, patients are covered by the Danish Patient Association Compensation (Patienterstatningen).

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