BMJ Open

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

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-054100
Article Type:	Protocol
Date Submitted by the Author:	04-Jun-2021
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Keywords:	Coronary heart disease < CARDIOLOGY, Ischaemic heart disease < CARDIOLOGY, Myocardial infarction < CARDIOLOGY, Diabetic nephropathy & vascular disease < DIABETES & ENDOCRINOLOGY, DIABETES & ENDOCRINOLOGY

TITLE

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

Short title: The HyperCarD2 study

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WORD COUNT: Max 4000 words

ABSTRACT

Introduction: Type 2 diabetes (T2D) is characterised by elevated plasma glucose, free fatty acid (FFA) and insulin concentrations, and this metabolic profile is linked to diabetic cardiomyopathy, a diastolic dysfunction at first, and increased cardiovascular disease (CVD) risk. Shifting cardiac metabolism towards glucose utilisation has been suggested to improve cardiovascular function and CVD risk, but insulin treatment increases overall glucose oxidation and lowers lipid oxidation, without reducing CVD risk, whereas SGLT-2 inhibitors (SGLT-2i) increase FFA, ketone body concentrations and lipid oxidation while decreasing insulin concentrations and CVD risk. The aim of the present study is to elucidate the importance of different metabolic profiles obtained during treatment with an SGLT2i versus insulin for myocardial function in patients with T2D.

Methods and analyses: Randomised, cross-over study, where 20 patients with T2D and BMI >28 kg/m2 receive 25 mg empagliflozin qd or NPH insulin bid first for 5 weeks followed by a 3-week washout before crossing over to the remaining treatment. Insulin treatment is titrated to achieve similar glycaemic control as with empagliflozin. In those randomised to insulin first, glycaemia during an initial empagliflozin run-in period prior to randomisation serves as target glucose. Metabolic and cardiac evaluation is performed before and at the end of each treatment period.

The primary endpoint is change (treatment – washout) in left ventricular peak filling rate, as assessed by cardiac MR (CMR) with and without acute lowering of plasma FFAs with acipimox. Secondary and explorative endpoints are changes in left atrial passive emptying fraction, left ventricular ejection fraction, central blood volume and metabolic parameters.

Ethics and dissemination: This study is approved by the Danish Medicines Agency, the Danish Data Protection Agency and the Capital Region Ethics Committee. The trial is conducted in accordance with ICH-GCP guidelines and the Helsinki Declaration. Trial registration number: EudraCT: 2017-002101

STRENGTHS AND LIMITATIONS

- Comparison with NPH Insulin, which has opposite metabolic effects to empagliflozin, provides a strong basis for detecting metabolic effects on cardiac function.
- Repeated CMR, during depletion of plasma FFAs with acipimox during treatments and washouts allows for dissection of the individual roles of hyperglycaemia, hyperinsulinemia and elevated free fatty acids on cardiac function in T2D.
- Cross-over over design is more difficult to perform, but provides greater statistical power
- Effects of metabolic changes on cardiac function are limited to the 5-week intervention period, which excludes effects arising from longer-term treatment.

INTRODUCTION

Type 2 diabetes (T2D) is characterised by hyperglycaemia, hyperinsulinemia, increased free fatty acids and impaired tissue glucose uptake and oxidation [1]. T2D is associated with an increased cardiovascular morbidity, and the more dysregulated the metabolic state, the greater the cardiovascular risk [2–5]. T2D develops when insulin secretion can no longer compensate for the ambient insulin resistance, and therefore previous treatments has focused on increasing insulin signalling by either exogenous insulin administration, stimulation of endogenous insulin secretion or enhancing insulin sensitivity [1].

Diabetic cardiomyopathy (DCM), is an early "silent" complication to T2D, independent of hypertension and/or coronary heart disease. It is characterised by left ventricular (LV) hypertrophy and diastolic dysfunction [6] and has been linked to the increased cardiovascular risk in T2D [7]. DCM may be accurately described by measuring left ventricular peak filling rate (LVPFR) and left ventricular ejection fraction (LVEF) using cardiac magnetic resonance imaging (CMR) [8,9]. Both diastole and systole are energy requiring processes and sensitive to changes in energy availability [10,11]. Interestingly, cardiac metabolism in patients with T2D is altered and depends more on lipid oxidation and less on glucose oxidation compared to non-diabetic controls [12,13]. It has been argued that glucose oxidation is a better source of energy for the heart than lipid oxidation, especially during stress such as myocardial ischemia, because this yields more ATP pr. unit oxygen [14]. However, manipulating cardiac metabolism towards glucose oxidation, by administering glucose-insulin (-potassium) infusions in patients with hyperglycaemia and myocardial infarction has been attempted, but did not improve survival in neither diabetic nor non-diabetic patients [15–17]. In intensive care unit patients, strict glycaemic control using insulin has been associated with increased mortality [18], and in patients with T2D and increased CVD risk, intensive glycaemic control has not reduced CVD risk compared to conventional glycaemic control [19–22] and in the ACCORD study, which involved aggressive insulin treatment resulted in excess mortality [23]. Thus, insulin treatment does not prevent cardiovascular events in patients with T2D nor improve prognosis when such occur [24].

SGLT2-inhibition (SGLT2i), on the other hand, is a newer treatment principle in T2D, which has proven effective in attenuating the risk of myocardial infarctions, worsening of heart failure, cardiovascular mortality and all-cause mortality in patients with T2D [25–27]

SGLT2i increases renal glucose excretion thereby lowering plasma glucose and insulin levels and increasing glucagon release, lipolysis and ketogenesis [28,29]. Additionally, tissue glucose uptake and oxidation is reduced and hepatic glucose production increased [30]. The exact cardioprotective mechanisms of SGLT2i are not yet understood, but has been proposed to be linked to improved haemodynamics [31], inhibition of myocardial Na^+/H^+ exchange [32,33] or reductions in inflammatory activity [34,35].

An early and interesting hypothesis proposed that changes in cardiac metabolism may be responsible for the cardioprotective effect of SGLT2i. The lowered glucose and insulin

concentrations, persistent hyperketonaemia and elevated free fatty acids, caused by SGLT2i treatment, leads to reduced glucose uptake, increased ketone body uptake and oxidation and unchanged uptake of free fatty acids in the heart while overall lipid oxidation is increased [36,37]. This altered energy metabolism may improve myocardial function, especially during myocardial stress [38–40]. The SGLT2i induced myocardial fuel switch from glucose to fatty acids and ketone bodies, has been suggested to ameliorate adverse cardiac remodelling and heart failure in nondiabetic porcine models [41], and it is noteworthy that eliminating the availability of free fatty acids to insulin resistant hearts can lead to cardiac dysfunction in rodents and in humans, suggesting an important role for lipid metabolism in cardiac function [42–45]. Cardiovascular endpoint trials with SGLT2 inhibitors have shown effects within weeks after initiation of treatment, coinciding with the metabolic effects of the treatment [25,37]

Altogether, SGLT2 inhibitors "amplify" some components of the dysmetabolic profile of T2D and works opposite the metabolic effects of insulin. This raises the question of how cardiac function in patients with T2D depends on lipid and glucose oxidation in the resting state and during stress, and how increasing or lowering blood glucose, free fatty acids, ketone bodies and insulin concentrations influence cardiac function.

Objective

The primary objective of the present study is to evaluate myocardial function in patients with T2D and high risk of CV events using advanced cardiac magnetic resonance imaging (CMR) scans during rest, chronotropic stress and under depletion of plasma free fatty acids before and after 5 weeks of empagliflozin treatment (high free fatty acid and ketone body concentrations, high lipid oxidation and low insulin concentrations) and before and after 5 weeks of human insulin treatment titrated to yield glycaemic control similar to the empagliflozin treatment period (low free fatty acid and ketone body concentrations, high insulin concentrations and glucose oxidation).

Hypothesis

We hypothesise that hyperinsulinemia and hyperglycaemia are conditions that negatively affect cardiac function in T2D, while the availability of free fatty acids and ketone bodies and switching metabolism towards lipid oxidation improves cardiac diastolic and systolic function. Thus, we expect that lowering plasma glucose insulin-independently, and increasing fatty acid concentrations, lipid oxidation and ketone body availability with empagliflozin treatment, improves myocardial function in patients with T2D, and that depleting plasma of free fatty acids during empagliflozin treatment will impair cardiac function.

METHODS AND ANALYSES

Design

This is a 20-week prospective, investigator-initiated, comparator controlled, open label, 2-arm cross-over, human study where subjects are randomised in blocks of 3-5 to NPH insulin or Empagliflozin treatment (25 mg once daily) for 5±1 weeks, followed by 3±1 weeks wash-out and

cross-over of treatment for 5±1 weeks (figure 1). For 7 weeks preceding randomisation, but after inclusion, patients undergo a program of 2 weeks of washout of pre-existing antiglycaemic treatment (except metformin), 2 weeks of empagliflozin run-in (used for glycaemic target and titration of treatment in participants randomised to insulin first, see below) followed by 3 weeks of wash-out. During run-in and treatment periods, participants measure blood glucose twice daily (fasting and before evening meal), and during washouts patients measure fasting blood glucose. After the screening visit (V0) there are four study visits (V1-4) – before and at the end of each treatment period. Each visit consists of three study days – a metabolic study day (MET) and two CMR study days. Randomisation is performed at V1 after the metabolic study day.

Participants

Twenty subjects older than 18 years diagnosed with T2D, a BMI ≥28 kg/m^2, HbA1c ≤ 9%, fasting C-peptide >500 pmol/L and unchanged antiglycaemic treatment for 12 weeks prior to screening, and who are at a risk of cardiovascular disease (CVD), are eligible for the study. High CVD risk is modified from the EMPA-REG protocol [46]. Inclusion, exclusion and withdrawal criteria are listed in Box 1.

Recruitment

Participants are recruited from the Department of Endocrinology and Cardiology at Hvidovre Hospital and are identified by reviewing laboratory results and patient files. Potential participants will be contacted by means of a recruitment letter, in which they are informed of the opportunity to participate in a scientific research project. We also will advertise for participant in local newspapers and on the internet as well as social media (e.g. www.forsøgsperson.dk; www.sundhed.dk and www.facebook.com).

Outcomes

The primary outcome is change in myocardial diastolic function. This was chosen because firstly, diastole is a highly energy requiring process [10,11], and secondly, because diastolic dysfunction (with or without LV hypertrophy) is the notable early manifestation of diabetic cardiomyopathy [6]. Thus, if changes in overall energy metabolism are to affect cardiac function in patients with T2D, it may well occur in diastole at the earliest. Diastolic cardiac function can be accurately assessed using CMR by measuring left ventricular peak filling rate (LVPFR) and left atrial passive emptying fraction (LAPEF) [47]. Our primary outcome measure is change ($LVPFR_{treatment} - LVPFR_{wash\text{-}out}$) in LVPFR (ΔLVPFR). All endpoints are listed in Box 2.

Randomisation and intervention

Participants are randomised consecutively by lottery in blocks of 3-5 to treatment with either subcutaneous NPH insulin (Insulatard®) twice daily or oral empagliflozin (Jardiance®) 25 mg once daily first. All patient will receive both treatments during the trial. Randomisation is performed at V1. NPH insulin is initiated at a dose of 0.2 IU/kg body weight/day and is titrated daily over phone (phone contacts, figure 1) by 0.05 IU/kg body weight/day until average blood glucose over three consecutive days is within ±1 mmol/L of the individual glycaemic target. In participants

randomised to insulin first, the glycaemic target is average fasting and evening glucose concentrations during the second week of empagliflozin run-in. In patients randomised to insulin second, the glycaemic target is average fasting and pre-prandial evening BG values of week 3 and 4 during the first (empagliflozin) treatment period.

As previously discussed, insulin and empagliflozin represents two metabolically opposing methods for lowering plasma glucose concentrations. By titrating insulin treatment to match the glycaemic control found with empagliflozin in the same participants, 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 glycaemic control (Figure 2). NPH Insulin has been chosen over more modern human insulin analogues, as it is not albumin bound and can be measured in an ordinary insulin assay.

Safety considerations

During washout periods blood glucose concentrations will increase – that is a separate point of the study, but severely dysregulated diabetes is an exclusion criterium to ensure participant safety. The risk of severe hyperglycaemia is reduced in several ways in the study:

- Existing metformin treatment is continued throughout the whole study as background antiglycaemic treatment.
- In case of fasting BG concentrations of more than 13 mmol/L, patients are instructed to contact 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 hyperglycaemic events or other adverse events.
- As soon as the final day (CMR with acipimox) of a washout visit (visit 1 or 3) is completed, antiglycaemic treatment according to study drug sequence is commenced to minimise time spent in hyperglycaemia.

In case of fasting 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 antihyperglycaemic treatment is initiated.

Screening visit (V0)

Once oral and written informed consent is obtained by the study investigator, the screening procedure follows. Medical history is recorded, screening blood samples drawn, and an ECG, recording of blood pressure, pulse rate and registration of anthropometric data are performed, and patients are screened according to in- and exclusion criteria. A standard transthoracic echocardiography is performed, and VO2max is estimated (Box 3).

Study visits

All study visits consist of three study days – a metabolic study day and two CMR study days (Box 4).

The metabolic study day

The metabolic study is conducted at the Department of Endocrinology, Hvidovre Hospital, to document the metabolic effects of each study drug.
Participants meet in the morning after an overnight fast. Anthropometric data, blood pressure, pulse rate and an ECG are recorded, and two catheters, one in each arm are inserted for infusion of tracers and for repeated drawing of arterialised blood samples respectively. Baseline and safety blood samples are taken (Box 5), the participant empties bladder and the investigational drug (V2, V4) and the participants usual medications are administered at 0800h. Body composition is determined by Dual energy x-ray absorptiometry scan (DXA).

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 at -30, -15 and -2 min to characterise glucose, lipid and amino acid metabolism. The patient empties bladder, urine is weighed, and samples are taken for determination of tracer concentrations and urinary nitrogen excretion, and the 5h-OGTT is initiated.

5h-OGTT

The patient ingests anhydrous glucose (72 g) with added [U-13C6]-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 characterisation of postprandial glucose, lipid and amino acid metabolism (Box 4 and 5). The patient empties bladder regularly during and at the end of the OGTT. Urine is sampled for nitrogen excretion and tracers/tracees.

Fat and muscle biopsies: Biopsies are obtained during the basal (T=-60 min) and the maximally insulin stimulated (T=60 min) state. Muscle biopsies are considered proxies for cardiomyocyte metabolic status. 30 min ventilated hood indirect calorimetry (Vyaire Vyntus® CPX) is performed during the basal period (prior to biopsies) t= -90 min and postprandially at t=60 min for determination of fasting and postprandial energy expenditure and respiratory quotient.

Exercise test (50% VO2max)

At T=300 min, the participant is exercised at 60 W for 4 minutes after which work load is increased until oxygen consumption is 50% of estimated VO2max. Pulse rate is recorded with a chest mounted pulse rate monitor and oxygen consumption with a mask mounted indirect calorimeter. Blood is sampled to characterise glucose, lipid and amino acid metabolism (Box 4 and 5). After 30 min, VO2max is estimated by increasing workload by 50W until a pulse rate increase of 30. When pulse is steady for 2 min, oxygen consumption and pulse rate are recorded, and the test is stopped. VO2max is estimated by linear extrapolation to the theoretical maximum pulse rate (220-age).

Ad libitum meal test

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 [37]. Therefore, the metabolic study day is ended with an ad *libitum meal*, consisting of thoroughly mixed pasta bolognese (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 ad libitum meal intake defined as the difference. Throughout the day patients are asked to score their hunger, satiety and sensation of fullness on a visual analogue scale [48].

Cardiac evaluation

Two CMR days are performed during each visit (V1-V4). In addition, diurnal blood pressure and Holter monitoring are performed. CMR is conducted at the Department of Cardiology, Rigshospitalet, Copenhagen, whereas Holter monitoring and diurnal blood pressure monitoring are performed at the Department of Cardiology, Hvidovre Hospital.

Participants meet fasting and morning medication, including investigational medicinal product (V2 and V4), is administered. Anthropometric data are recorded, and two intravenous catheters are inserted into an antecubital and the contralateral dorsal hand vein for infusion of adenosine, gadolinium contrast and glycopyrrolate and for blood sampling respectively (Box 5). Prior to CMR a transthoracic echocardiography is performed (Box 3)

CMR is performed on a 1.5 Tesla scanner (Siemens Aera; Siemens; Erlangen; Germany) with the patient lying supine on the back, using an 18-channel cardiac coil with continuous ECG gating.

Cine 2-, 3- and 4-chamber images, complete transverse and short axis cine stacks covering the whole heart are acquired. All images are obtained during end-expiratory breath-holds. Myocardial perfusion images during rest and stress are obtained at the basal, mid-ventricular and apical cardiac short-axis level. Rest perfusion images of the myocardium is acquired using an intravenous bolus of gadolinium contrast (Gadovist®, Bayer AG, Germany) 0.075 mmol/kg bodyweight. The time of gadolinium contrast entry into the right and the left ventricle is accurately determined, and this transit time of gadovist multiplied by cardiac output is used to calculate the pulmonary and central (pulmonary + cardiac) blood volume.

Myocardial stress perfusion images are obtained with an i.v dose of 0.075 mmol/kg of gadovist during and 10 min after an intravenous adenosine (140 ug/min) administrated for maximum 4 minutes). This is followed by evaluation of cardiac function during chronotropic stress, where short axis cine stack will be reacquired 10 minutes after the administration of intravenous glycopyrrolate (4 µg/kg, max. 400µg, given as a bolus). This approach has been shown to unmasque subclinical diastolic dysfunction as has been demonstrated in normal healthy elderly [47].

Cardiac MRI, Acipimox

CMR scans will follow the same procedure as described above, but participants are instructed to ingest 250 mg acipimox p.o. twice, 4 hours before and right before the scan, to determine

myocardial function. This repeated administration of acipimox is required for adequate suppression of hormone sensitive lipase activity and depletion of plasma FFAs (28). This has been shown to gradually impair cardiac function [49], and is done to disclose any coupling between FFA availability and cardiac function.

CMR image analysis

Is performed using Circle42 (Circle Cardiovascular Imaging Inc., Calgary Canada, v5.5.1). LV volumes, LV mass, LV ejection fraction (LVEF) and LV peak filling rate are determined by tracing of the endo- and epicardial contours in end-diastolic and end-systolic phases. The papillary muscles are excluded from the myocardium. On native and post-contrast T1-mappings, endocardial and epicardial borders are traced, and the mean extra cellular volume (ECV) is calculated from areas outside late gadolinium enhancement (LGE) lesions. For determination of the ECV within an LGE lesion, myocardium without LGE in the segment is excluded. Myocardial perfusion scans are inspected for perfusion defects. Regions with infarctions, sub-endocardial perfusion defects or dark-rim artefacts will be excluded. Blinded to clinical data, the analyses will be reviewed and finalised by two CMR specialist.

Diurnal blood pressure and Holter monitoring

Between study days during each visit, diurnal blood pressure is recorded (ScottCare, ABP 320, Cleveland, OH)) for 24 hours with 15 minutes intervals between 6.00 to 22.00- and 60-min intervals during night-time. Cardiac rhythm is evaluated with Holter monitoring (SCOTTCARE, CHROMA, model RZ153C, Cleveland, OH) for 48 hours.

Patient and public involvement

No patient involved.

ANALYSES

Blood and tissue samples

Subcutaneous fat- and muscle biopsies: Local analgesia is applied before sampling with a Bergströms cannula. Samples are immediately frozen in liquid nitrogen and stored at -80°C. Blood and urine samples: Samples are spun, aliquoted and stored at –20°C (GLP-1, PYY, Glucagon) or –80°C for later analysis. Bedside plasma glucose measurements are performed using the glucose oxidase technique (YSI model 2300 STAT Plus; YSI,Yellow Springs, OH). Home blood glucose measurements are carried out on Contour XT (Ascensia Diabetes Care Holdings AG, Basel, Switzerland). Safety blood- and urine samples are analysed on the same day at the Department of Clinical Biochemistry, Hvidovre Hospital.

Statistical methods

Sample size calculation

Measures of myocardial function are highly reproducible when assessed using CMR, and interstudy and cohort coefficients of variation are in the range of 3-5% [50–52]
Using the same CMR protocol as the present, 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) [52]. 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.

Statistical analysis plan

The primary and secondary endpoints are analysed assuming no period effect or treatment-period interaction. This assumption is reasonable, given results from similar studies, where no such interactions or effects have been reported [53]. Normally distributed data are presented using standard descriptive statistics, and reported as mean (SD) for normally distributed and median (Q1;Q3) for non-normally distributed data. Likewise, comparisons of normally distributed data is done using the paired Student's t-test for all completers, whereas Wilcoxon's paired signed rank test will be used if data is non-normally distributed.

Ethics and dissemination

The study is conducted according to ICH GCP guidelines E6 (R2) and registered with the Danish Medicines Agency (EudraCT no. 2017-002101-35, The Capital Region Ethical Committee (H-17018846) and the Danish Data Protection Agency (2012-58-0004; AHH-2017-093, I-Suite nr.: 06012). Our results, regardless of outcome, 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.

Timeframe

Screenings are performed from January 2018. Last patient, last visit is expected second half of 2021 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 by Winter 2022.

DISCUSSION

The profound and swift benefits of SGLT2i on cardiovascular risk in T2D have inspired the discussion of metabolism and its importance for cardiac function in patients with T2D [54–58]. Especially since, SGLT2 inhibitors have metabolic effects that by and large are opposite to those of insulin treatment. Thus, insulin treatment is associated with increased tissue glucose uptake and utilisation, but suppression of lipid mobilisation and oxidation as well as lowering of plasma concentrations of ketone bodies [59]. SGLT2 inhibitors increase lipid mobilisation and oxidation, increase plasma ketone body concentrations and reduce tissue glucose uptake [37,60]. Both treatments lower plasma glucose, but insulin treatment increases whereas SGLT2i treatment decreases plasma insulin concentrations. Whether such changes in metabolism affect cardiac function, is still unsettled, but forcing cardiac glucose uptake and utilisation through insulin treatment has been suggested by some to benefit and by others to impair cardiac function [45,61–

64], while yet others have suggested increased lipid and ketone body oxidation to be important for proper cardiac function in T2D [44,55,64]. Studies on SGLT2i treatments and the effects on cardiac function are beginning to emerge. In a recent study, 42 patients with T2D were randomised to 12 weeks of empagliflozin 10 mg or placebo once daily. SGLT2 inhibition was shown to rapidly improve diastolic cardiac function as evaluated with echocardiography [65]. In a placebo controlled cross-over design, after 4 weeks of empagliflozin treatment in patients with T2D, myocardial glucose uptake was reduced and fatty acid oxidation unaltered, but this did not significantly change myocardial oxygen consumption or cardiac efficiency, nor any measure of cardiac function [66]. In a Swedish study, 6 weeks dapagliflozin treatment showed unchanged cardiac fatty acid uptake, a trend toward reduced left atrial maximal volume, and reduced LV oxygen consumption and external work compared to placebo [67], and in the only study found, where an active comparator was used, 10 mg empagliflozin once daily for 12 weeks did not change cardiac lipid accumulation (as measured by MR spectrometry), cardiac function or cardiac metabolism compared to sitagliptin 50 mg daily [68].

In conclusion, existing studies in humans have shown divergent results regarding changes in cardiac diastolic function with little changes in cardiac metabolism. However, most studies have compared cardiac effects of SGLT2i to placebo, thus not accounting for the circumstances that characterised the EMPA-REG trial, where anti glycaemic treatment was intensified in the placebo group concurrently [25]. Thus, the CVD risk benefits of the study may have arisen from unfavourable metabolic consequences of the treatment in the placebo arm. In the one study with an active comparator empagliflozin was compared to sitagliptin, which not only affects the incretin system but also has less specific metabolic effect [69]. Therefore, to date our study, is the one to most directly pursue the coupling between metabolism and cardiac function, by choosing insulin as the comparator, and by including the effects of acute lowering of free fatty acid concentrations in plasma on cardiac function.

Disclosure summary

All authors have completed the ICMJE uniform disclosure form and declare: no support from any organisations for the submitted work; SM and NBJ have received research grants from Boehringer Ingelheim and JJH serves on advisory boards for Novo Nordisk, no other relationships or activities that could appear to have influenced the submitted work.

Funding

The study is funded by an investigator-initiated study grant from Boehringer Ingelheim.
Additional funding comes from Grosserer L. F. Foghts Fond, Charlottenlund, Denmark.
Grant numbers not applicable.

Contributions Author contributions

Box 1: Eligibility criteria
Inclusion criteria
Age ≥18 years BMI ≥ 28 kg/m^2 HbA1c ≤ 9% (≤10% in diet or metformin treated only) Fasting C-peptide ≥ 500 pmol/L Unchanged glycaemic treatment for 3 months prior to inclusion High cardiovascular risk as one of the following: • Previous myocardial infarction, stroke or peripheral arterial disease more than 2 months prior to informed consent • Evidence of multi-vessel coronary arterial disease (CAD) but without prior myocardial infarction, if more than 50% stenosis is present, if revascularised (CABG or PCI) more than 2 months prior or if 1 vessel is vascularised and the other with 50% stenosis. • Single vessel CAD without prior myocardial infarction if more than 50% stenosis is present, not revascularised and positive stress test for ischemia.
Exclusion criteria
Insulin treatment within 3 months from informed consent Type 1 diabetes Psychiatric disorder or mental retardation Drug or alcohol abuse within 3 months from informed consent Poor compliance Anaemia (Hb < 6.4 mmol/L) or other blood dyscrasias causing haemolysis or unstable erythrocytes. Indication of liver disease (ALT or Alkaline phosphatase elevation above 3x upper normal limit) Impaired renal function (eGFR<45 ml/min/1.73 m^2) Anti-obesity medication within 3 months from informed consent Systemic steroid treatment within 6 weeks from informed consent. Any uncontrolled endocrine disorder except T2D Bariatric surgery or other gastrointestinal conditions that may compromise gastrointestinal absorption Peptic ulcer – verified endoscopically Any form of surgery within 3 months of informed consent Acute myocardial infarction, stroke or peripheral arterial disease within 2 months of informed consent. Persistent or permanent atrial fibrillation Inability to undergo experimental procedures including exclusion criteria for CMR 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 Contraindications to adenosine: 2nd or 3rd degree atrioventricular block Severe hypotension (BP ≤ 90/50 mmHg) Long QT syndrome Unstable angina pectoris Decompensated heart failure Sinus node dysfunction Chronic obstructive pulmonary disease or asthma bronchiale (FEV1 ≤ 50% of expected) Allergy towards any of the drugs or diagnostics used in the protocol (insulin, empagliflozin, acipimox, glycopyrrolate, adenosine, gadolinium contrast enhancer). Any condition which in the opinion of the investigator may jeopardize subject safety or compliance with the protocol.
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 glycaemic dysregulation during washout periods.

Box 2: Endpoints	
Primary end point	Change in left ventricular peak filling rate (ΔLVPFR)
Secondary endpoints	Change in left atrial passive emptying fraction (ΔLAPEF) Change in left ventricular ejection fraction (ΔLVEF)
Explorative endpoints include	Cardiovascular: Change in VO2max and exercise tolerance test variables Change in central blood volume and haematocrite Change in heart rate variability Change in left ventricular volume Metabolic:

	Basal and postprandial AUC Free Fatty Acids and glycerol turnover Endogenous glucose production and tissue glucose disposal (metabolic clearance of glucose) Fasting and postprandial energy expenditure and respiratory quotient Glucagon-Insulin ratio Insulin sensitivity (AUC glucose metabolic clearance / AUC insulin concentration) Beta-cell function (prehepatic insulin secretion rate, correlated to ambient glucose)

Box 3: Screening procedures	
Blood samples	Haematology (haemoglobin, thrombocytes, haematocrit, leukocytes), liver and renal function tests (creatinine, eGFR (Cockroft-Gault formula), alkaline phosphatases, alanine aminotransferases, lactate dehydrogenase, bilirubin, amylase, sodium, potassium), fasting P-glucose, C-peptide, HbA1c, TSH, Urinary Albumin/creatinine mass ratio, and in fertile women, U-hCG.
Echocardiography	Parasternal long axis view, parasternal short axis view at aortic, mitral and apex levels, apical 4-chamber view, LVEF, E/E', E', LVEDV/BSA.
Estimation of VO2max	Maximum oxygen uptake is estimated using Åstrøm's two-point test performed on a cycle ergometer during indirect calorimetry. From measurements of VO2 at two sub-maximal pulse rates VO2max is estimated by linear extrapolation to the theoretical maximal pulse rate (220-age) [70].

Box 4: Visit overview		
Metabolic study day	Cardiac MR	Cardiac MR, Acipimox
- 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.	- Fasting blood samples, before and after CMR. - Echocardiography - CMR Rest -- Without enhancement -- With enhancement and adenosine infusion - CMR Stress -- Unenhanced repeated during pharmacological chronotropic stress with glycopyrrolate infusion. - 24h ambulant blood pressure	Same protocol as Cardiac MR day, but during pharmacological suppression of hormone sensitive lipase activity and depletion of plasma free fatty acids. - 48h Holter monitoring.

Box 5: Blood samples on metabolic and CMR study days include	
Metabolic study day	Blood samples: glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, total amino acids and ketone bodies, tracers/tracees, gut hormones. HbA1c, urate, blood urea nitrogen, cortisol, is sampled at baseline.
Cardiac MRI days	Markers of cardiac function, including pro-ANP and pro-BNP, glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, ketone bodies, haematocrit are drawn before and after CMR.

Figure legends

Figure 1. Study outline. Included patients undergo a 7 week program of washout of pre-existing antiglycemic treatment (except metformin) and run-in of empagliflozin. Herafter they are randomized to treatment for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over to 5±1 weeks treatment with the remaining study drug.

Figure 2. Metabolic profile of the two study drugs. Schematic representation of the metabolic changes expected with the two study drug treatments in a patient randomized to insulin first. Insulin treatment is characterized by low glucose, low FFAs and high insulin concentrations; empagliflozin treatment by low glucose, high FFAs and low insulin.

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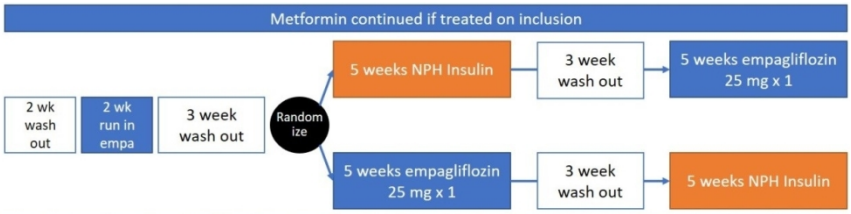

Time ± 1 (weeks)	0	2		4		6	7			8		10		12		14	15		16		18		20
Visits	V 0						V 1							V 2			V 3						V 4
Phone contacts (PC)		PC 1		PC 2		PC 3				PC 4		PC 5				PC 6			PC 7		PC 8		

Figure 1. Study outline

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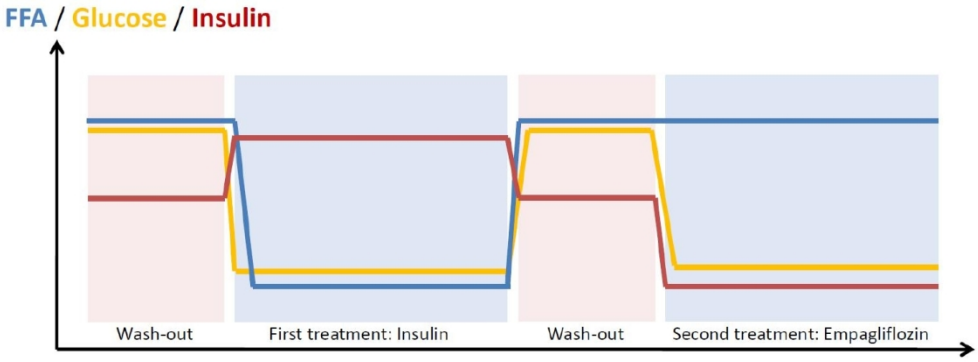

Figure 2. Metabolic profile of the two study drugs

407x150mm (96 x 96 DPI)

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

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-054100.R1
Article Type:	Protocol
Date Submitted by the Author:	21-Mar-2022
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Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Diabetes and endocrinology
Keywords:	Coronary heart disease < CARDIOLOGY, Ischaemic heart disease < CARDIOLOGY, Myocardial infarction < CARDIOLOGY, Diabetic nephropathy & vascular disease < DIABETES & ENDOCRINOLOGY, DIABETES & ENDOCRINOLOGY

TITLE

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

Short title: The HyperCarD2 study

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WORD COUNT: Max 4000 words

ABSTRACT

Introduction: Type 2 diabetes (T2D) is characterised by elevated plasma glucose, free fatty acid (FFA) and insulin concentrations, and this metabolic profile is linked to diabetic cardiomyopathy, a diastolic dysfunction at first, and increased cardiovascular disease (CVD) risk. Shifting cardiac metabolism towards glucose utilisation has been suggested to improve cardiovascular function and CVD risk, but insulin treatment increases overall glucose oxidation and lowers lipid oxidation, without reducing CVD risk, whereas SGLT-2 inhibitors (SGLT-2i) increase FFA, ketone body concentrations and lipid oxidation while decreasing insulin concentrations and CVD risk. The aim of the present study is to elucidate the importance of different metabolic profiles obtained during treatment with a SGLT2i versus insulin for myocardial function in patients with T2D.

Methods and analyses: Randomised, cross-over study, where 20 patients with T2D and BMI >28 kg/m2 receive 25 mg empagliflozin qd or NPH insulin bid first for 5 weeks followed by a 3-week washout before crossing over to the remaining treatment. Insulin treatment is titrated to achieve similar glycaemic control as with empagliflozin. In those randomised to insulin first, glycaemia during an initial empagliflozin run-in period prior to randomisation serves as target glucose. Metabolic and cardiac evaluation is performed before and at the end of each treatment period.

The primary endpoint is change (treatment – washout) in left ventricular peak filling rate, as assessed by cardiac MR (CMR) with and without acute lowering of plasma FFAs with acipimox. Secondary and explorative endpoints are changes in left atrial passive emptying fraction, left ventricular ejection fraction, central blood volume and metabolic parameters.

Ethics and dissemination: This study is approved by the Danish Medicines Agency, the Danish Data Protection Agency and the Capital Region Ethics Committee. The trial is conducted in accordance with ICH-GCP guidelines and the Helsinki Declaration. Trial registration number: EudraCT: 2017-002101.

STRENGTHS AND LIMITATIONS

- Comparison with NPH Insulin, which has opposite metabolic effects to empagliflozin, provides a strong basis for detecting metabolic effects on cardiac function.
- Repeated cardiac MR, during depletion of plasma FFAs with acipimox during treatments and washouts allows for dissection of the individual roles of hyperglycaemia, hyperinsulinemia and elevated free fatty acids on cardiac function in T2D.
- A cross-over over design is vulnerable to dropout, but provides greater statistical power
- Effects of metabolic changes on cardiac function are limited to the 5-week intervention period, which excludes effects arising from longer-term treatment.

INTRODUCTION

Type 2 diabetes (T2D) is characterised by hyperglycaemia, hyperinsulinemia, increased free fatty acids and impaired tissue glucose uptake and oxidation [1]. T2D is associated with an increased cardiovascular morbidity, and the more dysregulated the metabolic state, the greater the cardiovascular risk [2–5]. T2D develops when insulin secretion can no longer compensate for the ambient insulin resistance, and therefore previous treatments has focused on increasing insulin signalling by either exogenous insulin administration, stimulation of endogenous insulin secretion or enhancing insulin sensitivity [1].

Diabetic cardiomyopathy (DCM), is an early "silent" complication to T2D, independent of hypertension and/or coronary heart disease. It is characterised by left ventricular (LV) hypertrophy and diastolic dysfunction [6,7] and has been linked to the increased cardiovascular risk in T2D [8] . DCM may be accurately described by measuring left ventricular peak filling rate (LVPFR) and left ventricular ejection fraction (LVEF) using cardiac magnetic resonance imaging (CMR) [9,10]. Both diastole and systole are energy requiring processes and sensitive to changes in energy availability [11,12]. Interestingly, cardiac metabolism in patients with T2D is altered and depends more on lipid oxidation and less on glucose oxidation compared to non-diabetic controls [13,14]. It has been argued that glucose oxidation is a better source of energy for the heart than lipid oxidation, especially during stress such as myocardial ischemia, because this yields more ATP pr. unit oxygen [15]. However, manipulating cardiac metabolism towards glucose oxidation, by administering glucose-insulin (-potassium) infusions in patients with hyperglycaemia and myocardial infarction has been attempted, but did not improve survival in neither diabetic nor non-diabetic patients [16–18]. In intensive care unit patients, strict glycaemic control using insulin has been associated with increased mortality [19], and in patients with T2D and increased CVD risk, intensive glycaemic control has not reduced CVD risk compared to conventional glycaemic control [20–23] and in the ACCORD study, which involved aggressive insulin treatment resulted in excess mortality [24]. Thus, insulin treatment does not prevent cardiovascular events in patients with T2D nor improve prognosis when such occur [25].

SGLT2-inhibition (SGLT2i), on the other hand, is a newer treatment principle in T2D, which has proven effective in attenuating the risk of myocardial infarctions, worsening of heart failure, cardiovascular mortality and all-cause mortality in patients with T2D [26–28]

SGLT2i increases renal glucose excretion thereby lowering plasma glucose and insulin levels and increasing glucagon release, lipolysis and ketogenesis [29,30]. Additionally, tissue glucose uptake and oxidation is reduced and hepatic glucose production increased [31]. The exact cardioprotective mechanisms of SGLT2i are not yet understood, but has been proposed to be linked to improved haemodynamics [32], inhibition of myocardial Na^+/H^+ exchange [33,34] or reductions in inflammatory activity [35,36].

An early and interesting hypothesis proposed that changes in cardiac metabolism may be responsible for the cardioprotective effect of SGLT2i. The lowered glucose and insulin

concentrations, persistent hyperketonaemia and elevated free fatty acids, caused by SGLT2i treatment, leads to reduced glucose uptake, increased ketone body uptake and oxidation and unchanged uptake of free fatty acids in the heart while overall lipid oxidation is increased [37,38]. This altered energy metabolism may rapidly improve myocardial function, especially during myocardial stress [39–42]. The SGLT2i induced myocardial fuel switch from glucose to fatty acids and ketone bodies, has been suggested to ameliorate adverse cardiac remodelling and heart failure in nondiabetic porcine models [43], and it is noteworthy that eliminating the availability of free fatty acids to insulin resistant hearts can lead to cardiac dysfunction in rodents and in humans, suggesting an important role for lipid metabolism in cardiac function [44–47]. Cardiovascular endpoint trials with SGLT2 inhibitors have shown effects within weeks after initiation of treatment, coinciding with the metabolic effects of the treatment [26,38]

Altogether, SGLT2 inhibitors "amplify" some components of the dysmetabolic profile of T2D and works opposite the metabolic effects of insulin. This raises the question of how cardiac function in patients with T2D depends on lipid and glucose oxidation in the resting state and during stress, and how increasing or lowering blood glucose, free fatty acids, ketone bodies and insulin concentrations influence cardiac function.

Objective

The primary objective of the present study is to evaluate myocardial function in patients with T2D and high risk of CV events using advanced cardiac magnetic resonance imaging (CMR) scans during rest, chronotropic stress and under depletion of plasma free fatty acids before and after 5 weeks of empagliflozin treatment (high free fatty acid and ketone body concentrations, high lipid oxidation and low insulin concentrations) and before and after 5 weeks of human insulin treatment titrated to yield glycaemic control similar to the empagliflozin treatment period (low free fatty acid and ketone body concentrations, high insulin concentrations and glucose oxidation).

Hypothesis

We hypothesise that hyperinsulinemia and hyperglycaemia are conditions that negatively affect cardiac function in T2D, while the availability of free fatty acids and ketone bodies and switching metabolism towards lipid oxidation improves cardiac diastolic and systolic function. Thus, we expect that lowering plasma glucose insulin-independently, and increasing fatty acid concentrations, lipid oxidation and ketone body availability with empagliflozin treatment, improves myocardial function in patients with T2D, and that depleting plasma of free fatty acids during empagliflozin treatment will impair cardiac function.

METHODS AND ANALYSES

Design

This is a 20-week prospective, investigator-initiated, comparator controlled, open label, 2-arm cross-over, human study where subjects are randomised in blocks of 3-5 to NPH insulin or Empagliflozin treatment (25 mg once daily) for 5±1 weeks, followed by 3±1 weeks wash-out and

cross-over of treatment for 5±1 weeks (figure 1). For 7 weeks preceding randomisation, but after inclusion, patients undergo a program of 2 weeks of washout of pre-existing antiglycaemic treatment (except metformin), 2 weeks of empagliflozin run-in (used for glycaemic target and titration of treatment in participants randomised to insulin first, see below) followed by 3 weeks of wash-out. During run-in and treatment periods, participants measure blood glucose twice daily (fasting and before evening meal), and during washouts patients measure fasting blood glucose. After the screening visit (V0) there are four study visits (V1-4) – before and at the end of each treatment period. Each visit consists of three study days – a metabolic study day (MET) and two CMR study days. Randomisation is performed at V1 after the metabolic study day.

Participants

Twenty subjects older than 18 years diagnosed with T2D, a BMI ≥28 kg/m^2, HbA1c ≤ 9%, fasting C-peptide >500 pmol/L and unchanged antiglycaemic treatment for 12 weeks prior to screening, and who are at a risk of cardiovascular disease (CVD), are eligible for the study. High CVD risk is modified from the EMPA-REG protocol [48]. Inclusion, exclusion and withdrawal criteria are listed in Box 1.

Recruitment

Participants are recruited from the Department of Endocrinology and Cardiology at Hvidovre Hospital and are identified by reviewing laboratory results and patient files. Potential participants will be contacted by means of a recruitment letter, in which they are informed of the opportunity to participate in a scientific research project. We also will advertise for participant in local newspapers and on the internet as well as social media (e.g. www.forsøgsperson.dk; www.sundhed.dk and www.facebook.com).

Outcomes

The primary outcome is change in myocardial diastolic function. This was chosen because firstly, diastole is a highly energy requiring process [11,12], and secondly, because diastolic dysfunction (with or without LV hypertrophy) is the notable early manifestation of diabetic cardiomyopathy [49]. Thus, if changes in overall energy metabolism are to affect cardiac function in patients with T2D, it may well occur in diastole at the earliest. Diastolic cardiac function can be accurately assessed using CMR by measuring left ventricular peak filling rate (LVPFR) and left atrial passive emptying fraction (LAPEF) [50]. Our primary outcome measure is change ($LVPFR_{treatment}$ − $LVPFR_{wash\text{-}out}$) in LVPFR (ΔLVPFR). All endpoints are listed in Box 2.

Randomisation and intervention

Participants are randomised consecutively by lottery in blocks of 3-5 to treatment with either subcutaneous NPH insulin (Insulatard®) twice daily or oral empagliflozin (Jardiance®) 25 mg once daily first. All patient will receive both treatments during the trial. Randomisation is performed at V1. NPH insulin is initiated at a dose of 0.2 IU/kg body weight/day and is titrated daily over phone (phone contacts, figure 1) by 0.05 IU/kg body weight/day until average blood glucose over three consecutive days is within ±1 mmol/L of the individual glycaemic target. In participants

randomised to insulin first, the glycaemic target is average fasting and evening glucose concentrations during the second week of empagliflozin run-in. In patients randomised to insulin second, the glycaemic target is average fasting and pre-prandial evening BG values of week 3 and 4 during the first (empagliflozin) treatment period.
As previously discussed, insulin and empagliflozin represents two metabolically opposing methods for lowering plasma glucose concentrations. By titrating insulin treatment to match the glycaemic control found with empagliflozin in the same participants, 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 glycaemic control (Figure 2). NPH Insulin has been chosen over more modern human insulin analogues, as it is not albumin bound and can be measured in an ordinary insulin assay.

Safety considerations
During washout periods blood glucose concentrations will increase – that is a separate point of the study, but severely dysregulated diabetes is an exclusion criterium to ensure participant safety. The risk of severe hyperglycaemia is reduced in several ways in the study:

- Existing metformin treatment is continued throughout the whole study as background antiglycaemic treatment.
- In case of fasting BG concentrations of more than 13 mmol/L, patients are instructed to contact 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 hyperglycaemic events or other adverse events.
- As soon as the final day (CMR with acipimox) of a washout visit (visit 1 or 3) is completed, antiglycaemic treatment according to study drug sequence is commenced to minimise time spent in hyperglycaemia.

In case of fasting 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 antihyperglycaemic treatment is initiated.

Screening visit (V0)
Once oral and written informed consent is obtained by the study investigator, the screening procedure follows. Medical history is recorded, screening blood samples drawn, and an ECG, recording of blood pressure, pulse rate and registration of anthropometric data are performed, and patients are screened according to in- and exclusion criteria. A standard transthoracic echocardiography is performed, and VO2max is estimated (Box 3).

Study visits
All study visits consist of three study days – a metabolic study day and two CMR study days (Box 4).

The metabolic study day

The metabolic study is conducted at the Department of Endocrinology, Hvidovre Hospital, to document the metabolic effects of each study drug.

Participants meet in the morning after an overnight fast. Anthropometric data, blood pressure, pulse rate and an ECG are recorded, and two catheters, one in each arm are inserted for infusion of tracers and for repeated drawing of arterialised blood samples respectively. Baseline and safety blood samples are taken (Box 5), the participant empties bladder and the investigational drug (V2, V4) and the participants usual medications are administered at 0800h. Body composition is determined by Dual energy x-ray absorptiometry scan (DXA).

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 at -30, -15 and -2 min to characterise glucose, lipid and amino acid metabolism. The patient empties bladder, urine is weighed, and samples are taken for determination of tracer concentrations and urinary nitrogen excretion, and the 5h-OGTT is initiated.

5h-OGTT

The patient ingests anhydrous glucose (72 g) with added [U-13C6]-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 characterisation of postprandial glucose, lipid and amino acid metabolism (Box 4 and 5). The patient empties bladder regularly during and at the end of the OGTT. Urine is sampled for nitrogen excretion and tracers/tracees.

Fat and muscle biopsies: Biopsies are obtained during the basal (T=-60 min) and the maximally insulin stimulated (T=60 min) state. Muscle biopsies are considered proxies for cardiomyocyte metabolic status. 30 min ventilated hood indirect calorimetry (Vyaire Vyntus® CPX) is performed during the basal period (prior to biopsies) t= -90 min and postprandially at t=60 min for determination of fasting and postprandial energy expenditure and respiratory quotient.

Exercise test (50% VO2max)

At T=300 min, the participant is exercised at 60 W for 4 minutes after which work load is increased until oxygen consumption is 50% of estimated VO2max. Pulse rate is recorded with a chest mounted pulse rate monitor and oxygen consumption with a mask mounted indirect calorimeter. Blood is sampled to characterise glucose, lipid and amino acid metabolism (Box 4 and 5). After 30 min, VO2max is estimated by increasing workload by 50W until a pulse rate increase of 30. When pulse is steady for 2 min, oxygen consumption and pulse rate are recorded, and the test is stopped. VO2max is estimated by linear extrapolation to the theoretical maximum pulse rate (220-age).

Ad libitum meal test

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 [38]. Therefore, the metabolic study day is ended with an ad *libitum meal*, consisting of thoroughly mixed pasta bolognese (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 ad libitum meal intake defined as the difference. Throughout the day patients are asked to score their hunger, satiety and sensation of fullness on a visual analogue scale [51].

Cardiac evaluation

Two CMR days are performed during each visit (V1-V4). In addition, diurnal blood pressure and Holter monitoring are performed. CMR is conducted at the Department of Cardiology, Rigshospitalet, Copenhagen, whereas Holter monitoring and diurnal blood pressure monitoring are performed at the Department of Cardiology, Hvidovre Hospital.

Participants meet fasting and morning medication, including investigational medicinal product (V2 and V4), is administered. Anthropometric data are recorded, and two intravenous catheters are inserted into an antecubital and the contralateral dorsal hand vein for infusion of adenosine, gadolinium contrast and glycopyrrolate and for blood sampling respectively (Box 5). Prior to CMR a transthoracic echocardiography is performed (Box 3)

CMR is performed on a 1.5 Tesla scanner (Siemens Aera; Siemens; Erlangen; Germany) with the patient lying supine on the back, using an 18-channel cardiac coil with continuous ECG gating.

Cine 2-, 3- and 4-chamber images, complete transverse and short axis cine stacks covering the whole heart are acquired. All images are obtained during end-expiratory breath-holds. Myocardial perfusion images during rest and stress are obtained at the basal, mid-ventricular and apical cardiac short-axis level. Rest perfusion images of the myocardium is acquired using an intravenous bolus of gadolinium contrast (Gadovist®, Bayer AG, Germany) 0.075 mmol/kg bodyweight. The time of gadolinium contrast entry into the right and the left ventricle is accurately determined, and this transit time of gadovist multiplied by cardiac output is used to calculate the pulmonary and central (pulmonary + cardiac) blood volume.

Myocardial stress perfusion images are obtained with an i.v dose of 0.075 mmol/kg of gadovist during and 10 min after an intravenous adenosine (140 ug/min) administrated for maximum 4 minutes). This is followed by evaluation of cardiac function during chronotropic stress, where short axis cine stack will be reacquired 10 minutes after the administration of intravenous glycopyrrolate (4 µg/kg, max. 400µg, given as a bolus). This approach has been shown to unmasque subclinical diastolic dysfunction as has been demonstrated in normal healthy elderly [50].

Cardiac MRI, Acipimox

CMR scans will follow the same procedure as described above, but participants are instructed to ingest 250 mg acipimox p.o. twice, 4 hours before and right before the scan, to determine

myocardial function. This repeated administration of acipimox is required for adequate suppression of hormone sensitive lipase activity and depletion of plasma FFAs (28). This has been shown to gradually impair cardiac function [52], and is done to disclose any coupling between FFA availability and cardiac function.

CMR image analysis

Is performed using Circle42 (Circle Cardiovascular Imaging Inc., Calgary Canada, v5.5.1). LV volumes, LV mass, LV ejection fraction (LVEF) and LV peak filling rate are determined by tracing of the endo- and epicardial contours in end-diastolic and end-systolic phases. The papillary muscles are excluded from the myocardium. On native and post-contrast T1-mappings, endocardial and epicardial borders are traced, and the mean extra cellular volume (ECV) is calculated from areas outside late gadolinium enhancement (LGE) lesions. For determination of the ECV within an LGE lesion, myocardium without LGE in the segment is excluded. Myocardial perfusion scans are inspected for perfusion defects. Regions with infarctions, sub-endocardial perfusion defects or dark-rim artefacts will be excluded. Blinded to clinical data, the analyses will be reviewed and finalised by two CMR specialist.

Diurnal blood pressure and Holter monitoring

Between study days during each visit, diurnal blood pressure is recorded (ScottCare, ABP 320, Cleveland, OH)) for 24 hours with 15 minutes intervals between 6.00 to 22.00- and 60-min intervals during night-time. Cardiac rhythm is evaluated with Holter monitoring (SCOTTCARE, CHROMA, model RZ153C, Cleveland, OH) for 48 hours.

Patient and public involvement

No patient involved.

ANALYSES

Blood and tissue samples

Subcutaneous fat- and muscle biopsies: Local analgesia is applied before sampling with a Bergströms cannula. Samples are immediately frozen in liquid nitrogen and stored at -80°C. Blood and urine samples: Samples are spun, aliquoted and stored at –20°C (GLP-1, PYY, Glucagon) or –80°C for later analysis. Bedside plasma glucose measurements are performed using the glucose oxidase technique (YSI model 2300 STAT Plus; YSI,Yellow Springs, OH). Home blood glucose measurements are carried out on Contour XT (Ascensia Diabetes Care Holdings AG, Basel, Switzerland). Safety blood- and urine samples are analysed on the same day at the Department of Clinical Biochemistry, Hvidovre Hospital.

Statistical methods

Sample size calculation

Measures of myocardial function are highly reproducible when assessed using CMR, and interstudy and cohort coefficients of variation are in the range of 3-5% [53–55]

Using the same CMR protocol as the present, 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) [55]. 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. In case of a 20% dropout rate, power would still be acceptable (83%, p=0.01).

Statistical analysis plan
The primary and secondary endpoints are analysed assuming no period effect or treatment-period interaction. This assumption is reasonable, given results from similar studies, where no such interactions or effects have been reported [56]. Normally distributed data are presented using standard descriptive statistics, and reported as mean (SD) for normally distributed and median (Q1;Q3) for non-normally distributed data. Likewise, comparisons of normally distributed data is done using the paired Student's t-test for all completers, whereas Wilcoxon's paired signed rank test will be used if data is non-normally distributed.

Ethics and dissemination
The study is conducted according to ICH GCP guidelines E6 (R2) and registered with the Danish Medicines Agency (ref. id: 2017061587), The Capital Region Ethical Committee (H-17018846) and the Danish Data Protection Agency (2012-58-0004; AHH-2017-093, I-Suite nr.: 06012). Our results, regardless of outcome, 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.

Timeframe
Screenings are performed from January 2018. Last patient, last visit is expected second half of 2021 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 by Winter 2022.

DISCUSSION
The profound and swift benefits of SGLT2i on cardiovascular risk in T2D have inspired the discussion of metabolism and its importance for cardiac function in patients with T2D [57–61]. Especially since, SGLT2 inhibitors have metabolic effects that by and large are opposite to those of insulin treatment. Thus, insulin treatment is associated with increased tissue glucose uptake and utilisation, but suppression of lipid mobilisation and oxidation as well as lowering of plasma concentrations of ketone bodies [62]. SGLT2 inhibitors increase lipid mobilisation and oxidation, increase plasma ketone body concentrations and reduce tissue glucose uptake [38,63]. Both treatments lower plasma glucose, but insulin treatment increases whereas SGLT2i treatment decreases plasma insulin concentrations. Whether such changes in metabolism affect cardiac function, is still unsettled, but forcing cardiac glucose uptake and utilisation through insulin

treatment has been suggested by some to benefit and by others to impair cardiac function [47,64–67], while yet others have suggested increased lipid and ketone body oxidation to be important for proper cardiac function in T2D [46,58,67]. Studies on SGLT2i treatments and the effects on cardiac function are beginning to emerge. In a recent study, 42 patients with T2D were randomised to 12 weeks of empagliflozin 10 mg or placebo once daily. SGLT2 inhibition was shown to rapidly improve diastolic cardiac function as evaluated with echocardiography [68]. In a placebo controlled cross-over design, after 4 weeks of empagliflozin treatment in patients with T2D, myocardial glucose uptake was reduced and fatty acid oxidation unaltered, but this did not significantly change myocardial oxygen consumption or cardiac efficiency, nor any measure of cardiac function [69]. In a Swedish study, 6 weeks dapagliflozin treatment showed unchanged cardiac fatty acid uptake, a trend toward reduced left atrial maximal volume, and reduced LV oxygen consumption and external work compared to placebo [70], and in the only study found, where an active comparator was used, 10 mg empagliflozin once daily for 12 weeks did not change cardiac lipid accumulation (as measured by MR spectrometry), cardiac function or cardiac metabolism compared to sitagliptin 50 mg daily [71].

In conclusion, existing studies in humans have shown divergent results regarding changes in cardiac diastolic function with little changes in cardiac metabolism. However, most studies have compared cardiac effects of SGLT2i to placebo, thus not accounting for the circumstances that characterised the EMPA-REG trial, where anti glycaemic treatment was intensified in the placebo group concurrently [26]. Thus, the CVD risk benefits of the study may have arisen from unfavourable metabolic consequences of the treatment in the placebo arm. In the one study with an active comparator empagliflozin was compared to sitagliptin, which not only affects the incretin system but also has less specific metabolic effect [72]. Therefore, to date our study, is the one to most directly pursue the coupling between metabolism and cardiac function, by choosing insulin as the comparator, and by including the effects of acute lowering of free fatty acid concentrations in plasma on cardiac function.

Disclosure summary

All authors have completed the ICMJE uniform disclosure form and declare: no support from any organisations for the submitted work; SM and NBJ have received research grants from Boehringer Ingelheim and JJH serves on advisory boards for Novo Nordisk, no other relationships or activities that could appear to have influenced the submitted work.

Funding

The study is funded by an investigator-initiated study grant from Boehringer Ingelheim.
Additional funding comes from Grosserer L. F. Foghts Fond, Charlottenlund, Denmark.
Grant numbers not applicable.

Contributions

Author contributions

Roopameera Thirumathyam (RT) will be conducting the study, collect all the data, perform data analyses and co-submit the study.

Erik A. Richter has contributed to the study design, designed the keton body- and biopsy analysis scheme and is going to perform these analyses.
Jens P. Goetze designed the pro-ANP and pro-BNP analysis scheme and is going to perform these analyses.
Mogens Fenger designed multiple metabolic analysis schemes (Triglycerides, insulin, free fatty acids, uric acid, urea and nitrogen) and is going to perform these analyses.
Gerrit van Hall designed the three-tracer measuring analysis schemes we are using in this study, and is going to perform these analyses.
Ulrik Dixen provided the study with analysis tools (holter-monitors and blood pressure monitors). He will perform the analyses of these data.
Jens Juul Holst participated in the protocol design, designed multiple metabolic analyses (GLP-1, glucagon and PYY) and will perform the analyses of these data.
Sten Madsbad co-planned the study and contributed with several analysing tools.
Niels Vejlstrup co-designed the cardiac MRI guideline for the study and contributed with analysing tool (MRI scanner).
Per Lav Madsen co-planned the study, co-designed the cardiac MRI guideline for the study and will analyse the cardiac MRI data.
Nils Bruun Jørgensen co-planned the study, will perform all data analyses with RT and co-submit the study.
All of the above mentioned authors contributed with writing and revising this manuscript.

Box 1: Eligibility criteria
Inclusion criteria
Age ≥18 years BMI ≥ 28 kg/m^2 HbA1c ≤ 9% (≤10% in diet or metformin treated only) Fasting C-peptide ≥ 500 pmol/L Unchanged glycaemic treatment for 3 months prior to inclusion High cardiovascular risk as one of the following: • Previous myocardial infarction, stroke or peripheral arterial disease more than 2 months prior to informed consent • Evidence of multi-vessel coronary arterial disease (CAD) but without prior myocardial infarction, if more than 50% stenosis is present, if revascularised (CABG or PCI) more than 2 months prior or if 1 vessel is vascularised and the other with 50% stenosis. • Single vessel CAD without prior myocardial infarction if more than 50% stenosis is present, not revascularised and positive stress test for ischemia.
Exclusion criteria
Insulin treatment within 3 months from informed consent Type 1 diabetes Psychiatric disorder or mental retardation

Drug or alcohol abuse within 3 months from informed consent Poor compliance Anaemia (Hb < 6.4 mmol/L) or other blood dyscrasias causing haemolysis or unstable erythrocytes. Indication of liver disease (ALT or Alkaline phosphatase elevation above 3x upper normal limit) Impaired renal function (eGFR<45 ml/min/1.73 m^2) Anti-obesity medication within 3 months from informed consent Systemic steroid treatment within 6 weeks from informed consent. Any uncontrolled endocrine disorder except T2D Bariatric surgery or other gastrointestinal conditions that may compromise gastrointestinal absorption Peptic ulcer – verified endoscopically Any form of surgery within 3 months of informed consent Acute myocardial infarction, stroke or peripheral arterial disease within 2 months of informed consent. Persistent or permanent atrial fibrillation Inability to undergo experimental procedures including exclusion criteria for CMR 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 Contraindications to adenosine: 2nd or 3rd degree atrioventricular block Severe hypotension (BP ≤ 90/50 mmHg) Long QT syndrome Unstable angina pectoris Decompensated heart failure Sinus node dysfunction Chronic obstructive pulmonary disease or asthma bronchiale (FEV1 ≤ 50% of expected) Allergy towards any of the drugs or diagnostics used in the protocol (insulin, empagliflozin, acipimox, glycopyrrolate, adenosine, gadolinium contrast enhancer). Any condition which in the opinion of the investigator may jeopardize subject safety or compliance with the protocol.
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 glycaemic dysregulation during washout periods.

Box 2: Endpoints	
Primary end point	Change in left ventricular peak filling rate (ΔLVPFR)
Secondary endpoints	Change in left atrial passive emptying fraction (ΔLAPEF) Change in left ventricular ejection fraction (ΔLVEF)
Explorative endpoints include	Cardiovascular: Change in VO2max and exercise tolerance test variables Change in central blood volume and haematocrite Change in heart rate variability Change in left ventricular volume Metabolic: Basal and postprandial AUC Free Fatty Acids and glycerol turnover Endogenous glucose production and tissue glucose disposal (metabolic clearance of glucose) Fasting and postprandial energy expenditure and respiratory quotient Glucagon-Insulin ratio Insulin sensitivity (AUC glucose metabolic clearance / AUC insulin concentration) Beta-cell function (prehepatic insulin secretion rate, correlated to ambient glucose)

Box 3: Screening procedures	
Blood samples	Haematology (haemoglobin, thrombocytes, haematocrit, leukocytes), liver and renal function tests (creatinine, eGFR (Cockroft-Gault formula), alkaline phosphatases, alanine aminotransferases, lactate dehydrogenase,

	bilirubin, amylase, sodium, potassium), fasting P-glucose, C-peptide, HbA1c, TSH, Urinary Albumin/creatinine mass ratio, and in fertile women, U-hCG.
Echocardiography	Parasternal long axis view, parasternal short axis view at aortic, mitral and apex levels, apical 4-chamber view, LVEF, E/E', E', LVEDV/BSA.
Estimation of VO2max	Maximum oxygen uptake is estimated using Åstrøm's two-point test performed on a cycle ergometer during indirect calorimetry. From measurements of VO2 at two sub-maximal pulse rates VO2max is estimated by linear extrapolation to the theoretical maximal pulse rate (220-age) [73].

Box 4: Visit overview		
Metabolic study day	Cardiac MR	Cardiac MR, Acipimox
- 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.	- Fasting blood samples, before and after CMR. - Echocardiography - CMR Rest -- Without enhancement -- With enhancement and adenosine infusion - CMR Stress -- Unenhanced repeated during pharmacological chronotropic stress with glycopyrrolate infusion. - 24h ambulant blood pressure	Same protocol as Cardiac MR day, but during pharmacological suppression of hormone sensitive lipase activity and depletion of plasma free fatty acids. - 48h Holter monitoring.

Box 5: Blood samples on metabolic and CMR study days include	
Metabolic study day	Blood samples: glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, total amino acids and ketone bodies (betahydroxybuturate), tracers/tracees, gut hormones. HbA1c, urate, blood urea nitrogen, cortisol, is sampled at baseline.
Cardiac MRI days	Markers of cardiac function, including pro-ANP and pro-BNP, glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, ketone bodies, haematocrit are drawn before and after CMR.

Figure legends

Figure 1. Study outline. Included patients undergo a 7-week program of washout of pre-existing antiglycemic treatment (except metformin) and run-in of empagliflozin. Hereafter they are randomised to treatment for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over to 5±1 weeks treatment with the remaining study drug. Tests performed at each visit are summarised in Box 4 and 5.

Figure 2. Metabolic profile of the two study drugs. Schematic representation of the metabolic changes expected with the two study drug treatments in a patient randomized to insulin first. Insulin treatment is characterized by low glucose, low FFAs and high insulin concentrations; empagliflozin treatment by low glucose, high FFAs and low insulin.

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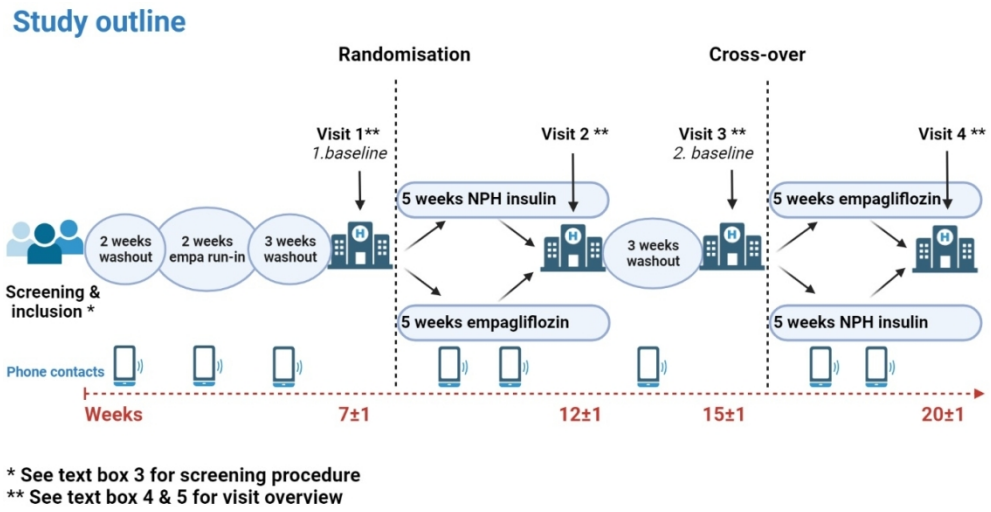

Figure 1. Study outline. Included patients undergo a 7-week program of washout of pre-existing antiglycemic treatment (except metformin) and run-in of empagliflozin. Hereafter they are randomised to treatment for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over to 5±1 weeks treatment with the remaining study drug. Tests performed at each visit are summarised in Box 4 and 5.

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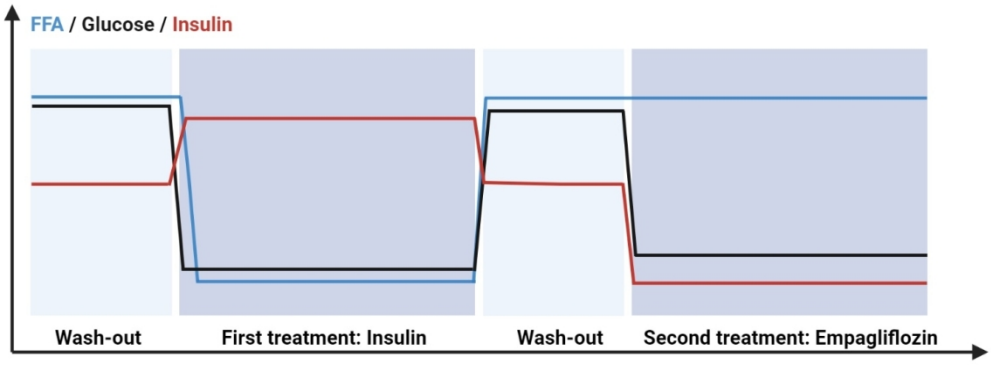

Figure 2. Metabolic profile of the two study drugs. Schematic representation of the metabolic changes expected with the two study drug treatments in a patient randomized to insulin first. Insulin treatment is characterized by low glucose, low FFAs and high insulin concentrations; empagliflozin treatment by low glucose, high FFAs and low insulin.

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BMJ Open

Investigating the roles of hyperglycemia, hyperinsulinemia, and elevated free fatty acids in cardiac function in patients with type 2 diabetes via treatment with insulin compared with empagliflozin: protocol for the HyperCarD2 randomised, crossover trial

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-054100.R2
Article Type:	Protocol
Date Submitted by the Author:	27-Jun-2022
Complete List of Authors:	Thirumathyam, Roopameera; Hvidovre Hospital, Department of Endocrinology Richter, Erik; University of Copenhagen, Department of Nutrition, Exercise and Sports, Section of Molecular Physiology, Faculty of Science Goetze, Jens; University of Copenhagen, Department of Clinical Biochemistry, Rigshospitalet Fenger, Mogens; Hvidovre Hospital, Department of Clinical Biochemistry Van Hall, Gerrit; Rigshospitalet, Clinical Metabolomics Core Facility, Clinical Biochemistr; University of Copenhagen, Department of Biomedical Sciences, Faculty of Health Sciences Dixen, Ulrik; Hvidovre University Hospital, Department of cardiology Holst, Jens; University of Copenhagen, NovoNordisk Center for Basic Metabolic Research; University of Copenhagen, Department of Biomedical Sciences, Faculty of Health Sciences Madsbad, Sten; Hvidovre Hospital, Department of Endocrinology Vejlstrup, Niels; Rigshospitalet, Department of Cardiology Madsen, Per; Herlev Hospital, Department of Cardiology Jørgensen, Nils Bruun; Hvidovre Hospital, Department of Endocrinology
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Diabetes and endocrinology
Keywords:	Coronary heart disease < CARDIOLOGY, Ischaemic heart disease < CARDIOLOGY, Myocardial infarction < CARDIOLOGY, Diabetic nephropathy & vascular disease < DIABETES & ENDOCRINOLOGY, DIABETES & ENDOCRINOLOGY

TITLE

"Investigating the roles of hyperglycemia, hyperinsulinemia, and elevated free fatty acids in cardiac function in patients with type 2 diabetes via treatment with insulin compared with empagliflozin: protocol for the HyperCarD2 randomised, crossover trial"

Short title: The HyperCarD2 study

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WORD COUNT: Max 4000 words

ABSTRACT

Introduction: Type 2 diabetes (T2D) is characterised by elevated plasma glucose, free fatty acid (FFA) and insulin concentrations, and this metabolic profile is linked to diabetic cardiomyopathy, a diastolic dysfunction at first, and increased cardiovascular disease (CVD) risk. Shifting cardiac metabolism towards glucose utilisation has been suggested to improve cardiovascular function and CVD risk, but insulin treatment increases overall glucose oxidation and lowers lipid oxidation, without reducing CVD risk, whereas SGLT-2 inhibitors (SGLT-2i) increase FFA, ketone body concentrations and lipid oxidation while decreasing insulin concentrations and CVD risk. The aim of the present study is to elucidate the importance of different metabolic profiles obtained during treatment with a SGLT2i versus insulin for myocardial function in patients with T2D.

Methods and analyses: Randomised, cross-over study, where 20 patients with T2D and BMI >28 kg/m2 receive 25 mg empagliflozin qd or NPH insulin bid first for 5 weeks followed by a 3-week washout before crossing over to the remaining treatment. Insulin treatment is titrated to achieve similar glycaemic control as with empagliflozin. In those randomised to insulin first, glycaemia during an initial empagliflozin run-in period prior to randomisation serves as target glucose. Metabolic and cardiac evaluation is performed before and at the end of each treatment period. The primary endpoint is change (treatment – washout) in left ventricular peak filling rate, as assessed by cardiac MR (CMR) with and without acute lowering of plasma FFAs with acipimox. Secondary and explorative endpoints are changes in left atrial passive emptying fraction, left ventricular ejection fraction, central blood volume and metabolic parameters.

Ethics and dissemination: This study is approved by the Danish Medicines Agency (ref. nr.: 2017061587), the Danish Data Protection Agency (ref. nr.: AHH-2017-093) and the Capital Region Ethics Committee (ref. nr.: H-17018846). The trial will be conducted in accordance with ICH-GCP guidelines and the Helsinki Declaration and all participants will provide oral and written informed consent (see supplementary appendix 1). Our results, regardless of outcome, will be published in relevant scientific journals and we also will seek to disseminate results through presentations at scientific meetings.
Trial registration number: EudraCT: 2017-002101.

STRENGTHS AND LIMITATIONS

- Comparison with NPH Insulin, which has opposite metabolic effects to empagliflozin, provides a strong basis for detecting metabolic effects on cardiac function.
- Repeated cardiac MR, during depletion of plasma FFAs with acipimox during treatments and washouts allows for dissection of the individual roles of hyperglycaemia, hyperinsulinemia and elevated free fatty acids on cardiac function in T2D.
- A cross-over design is vulnerable to dropout, but provides greater statistical power

- Effects of metabolic changes on cardiac function are limited to the 5-week intervention period, which excludes effects arising from longer-term treatment.

INTRODUCTION

Type 2 diabetes (T2D) is characterised by hyperglycaemia, hyperinsulinemia, increased free fatty acids and impaired tissue glucose uptake and oxidation [1]. T2D is associated with an increased cardiovascular morbidity, and the more dysregulated the metabolic state, the greater the cardiovascular risk [2–5]. T2D develops when insulin secretion can no longer compensate for the ambient insulin resistance, and therefore previous treatments has focused on increasing insulin signalling by either exogenous insulin administration, stimulation of endogenous insulin secretion or enhancing insulin sensitivity [1].

Diabetic cardiomyopathy (DCM), is an early "silent" complication to T2D, independent of hypertension and/or coronary heart disease. It is characterised by left ventricular (LV) hypertrophy and diastolic dysfunction [6,7] and has been linked to the increased cardiovascular risk in T2D [8] . DCM may be accurately described by measuring left ventricular peak filling rate (LVPFR) and left ventricular ejection fraction (LVEF) using cardiac magnetic resonance imaging (CMR) [9,10]. Both diastole and systole are energy requiring processes and sensitive to changes in energy availability [11,12]. Interestingly, cardiac metabolism in patients with T2D is altered and depends more on lipid oxidation and less on glucose oxidation compared to non-diabetic controls [13,14]. It has been argued that glucose oxidation is a better source of energy for the heart than lipid oxidation, especially during stress such as myocardial ischemia, because this yields more ATP pr. unit oxygen [15]. However, manipulating cardiac metabolism towards glucose oxidation, by administering glucose-insulin (-potassium) infusions in patients with hyperglycaemia and myocardial infarction has been attempted, but did not improve survival in neither diabetic nor non-diabetic patients [16–18]. In intensive care unit patients, strict glycaemic control using insulin has been associated with increased mortality [19], and in patients with T2D and increased CVD risk, intensive glycaemic control has not reduced CVD risk compared to conventional glycaemic control [20–23] and in the ACCORD study, which involved aggressive insulin treatment resulted in excess mortality [24]. Thus, insulin treatment does not prevent cardiovascular events in patients with T2D nor improve prognosis when such occur [25].

SGLT2-inhibition (SGLT2i), on the other hand, is a newer treatment principle in T2D, which has proven effective in attenuating the risk of myocardial infarctions, worsening of heart failure, cardiovascular mortality and all-cause mortality in patients with T2D [26–28]

SGLT2i increases renal glucose excretion thereby lowering plasma glucose and insulin levels and increasing glucagon release, lipolysis and ketogenesis [29,30]. Additionally, tissue glucose uptake and oxidation is reduced and hepatic glucose production increased [31]. The exact cardioprotective mechanisms of SGLT2i are not yet understood, but has been proposed to be linked to improved

haemodynamics [32], inhibition of myocardial Na^+/H^+ exchange [33,34] or reductions in inflammatory activity [35,36].

An early and interesting hypothesis proposed that changes in cardiac metabolism may be responsible for the cardioprotective effect of SGLT2i. The lowered glucose and insulin concentrations, persistent hyperketonaemia and elevated free fatty acids, caused by SGLT2i treatment, leads to reduced glucose uptake, increased ketone body uptake and oxidation and unchanged uptake of free fatty acids in the heart while overall lipid oxidation is increased [37,38]. This altered energy metabolism may rapidly improve myocardial function, especially during myocardial stress [39–42]. The SGLT2i induced myocardial fuel switch from glucose to fatty acids and ketone bodies, has been suggested to ameliorate adverse cardiac remodelling and heart failure in nondiabetic porcine models [43], and it is noteworthy that eliminating the availability of free fatty acids to insulin resistant hearts can lead to cardiac dysfunction in rodents and in humans, suggesting an important role for lipid metabolism in cardiac function [44–47]. Cardiovascular endpoint trials with SGLT2 inhibitors have shown effects within weeks after initiation of treatment, coinciding with the metabolic effects of the treatment [26,38]

Altogether, SGLT2 inhibitors "amplify" some components of the dysmetabolic profile of T2D and works opposite the metabolic effects of insulin. This raises the question of how cardiac function in patients with T2D depends on lipid and glucose oxidation in the resting state and during stress, and how increasing or lowering blood glucose, free fatty acids, ketone bodies and insulin concentrations influence cardiac function.

Objective

The primary objective of the present study is to evaluate myocardial function in patients with T2D and high risk of CV events using advanced cardiac magnetic resonance imaging (CMR) scans during rest, chronotropic stress and under depletion of plasma free fatty acids before and after 5 weeks of empagliflozin treatment (high free fatty acid and ketone body concentrations, high lipid oxidation and low insulin concentrations) and before and after 5 weeks of human insulin treatment titrated to yield glycaemic control similar to the empagliflozin treatment period (low free fatty acid and ketone body concentrations, high insulin concentrations and glucose oxidation).

Hypothesis

We hypothesise that hyperinsulinemia and hyperglycaemia are conditions that negatively affect cardiac function in T2D, while the availability of free fatty acids and ketone bodies and switching metabolism towards lipid oxidation improves cardiac diastolic and systolic function. Thus, we expect that lowering plasma glucose insulin-independently, and increasing fatty acid concentrations, lipid oxidation and ketone body availability with empagliflozin treatment, improves myocardial function in patients with T2D, and that depleting plasma of free fatty acids during empagliflozin treatment will impair cardiac function.

METHODS AND ANALYSES

Design

This is a 20-week prospective, investigator-initiated, comparator controlled, open label, 2-arm cross-over, human study where subjects are randomised in blocks of 3-5 to NPH insulin or Empagliflozin treatment (25 mg once daily) for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over of treatment for 5±1 weeks (figure 1). For 7 weeks preceding randomisation, but after inclusion, patients undergo a program of 2 weeks of washout of pre-existing antiglycaemic treatment (except metformin), 2 weeks of empagliflozin run-in (used for glycaemic target and titration of treatment in participants randomised to insulin first, see below) followed by 3 weeks of wash-out. During run-in and treatment periods, participants measure blood glucose twice daily (fasting and before evening meal), and during washouts patients measure fasting blood glucose. After the screening visit (V0) there are four study visits (V1-4) – before and at the end of each treatment period. Each visit consists of three study days – a metabolic study day (MET) and two CMR study days. Randomisation is performed at V1 after the metabolic study day.

Participants

Twenty subjects older than 18 years diagnosed with T2D, a BMI ≥28 kg/m^2, HbA1c ≤ 9%, fasting C-peptide >500 pmol/L and unchanged antiglycaemic treatment for 12 weeks prior to screening, and who are at a risk of cardiovascular disease (CVD), are eligible for the study. High CVD risk is modified from the EMPA-REG protocol [48]. Inclusion, exclusion and withdrawal criteria are listed in Box 1.

Recruitment

Participants are recruited from the Department of Endocrinology and Cardiology at Hvidovre Hospital and are identified by reviewing laboratory results and patient files. Potential participants will be contacted by means of a recruitment letter, in which they are informed of the opportunity to participate in a scientific research project. We also will advertise for participant in local newspapers and on the internet as well as social media (e.g. www.forsøgsperson.dk; www.sundhed.dk and www.facebook.com).

Outcomes

The primary outcome is change in myocardial diastolic function. This was chosen because firstly, diastole is a highly energy requiring process [11,12], and secondly, because diastolic dysfunction (with or without LV hypertrophy) is the notable early manifestation of diabetic cardiomyopathy [49]. Thus, if changes in overall energy metabolism are to affect cardiac function in patients with T2D, it may well occur in diastole at the earliest. Diastolic cardiac function can be accurately assessed using CMR by measuring left ventricular peak filling rate (LVPFR) and left atrial passive emptying fraction (LAPEF) [50]. Our primary outcome measure is change ($LVPFR_{treatment} - LVPFR_{wash\text{-}out}$) in LVPFR (ΔLVPFR). All endpoints are listed in Box 2.

Randomisation and intervention

Participants are randomised consecutively by lottery in blocks of 3-5 to treatment with either subcutaneous NPH insulin (Insulatard®) twice daily or oral empagliflozin (Jardiance®) 25 mg once daily first. All patient will receive both treatments during the trial. Randomisation is performed at V1. NPH insulin is initiated at a dose of 0.2 IU/kg body weight/day and is titrated daily over phone (phone contacts, figure 1) by 0.05 IU/kg body weight/day until average blood glucose over three consecutive days is within ±1 mmol/L of the individual glycaemic target. In participants randomised to insulin first, the glycaemic target is average fasting and evening glucose concentrations during the second week of empagliflozin run-in. In patients randomised to insulin second, the glycaemic target is average fasting and pre-prandial evening BG values of week 3 and 4 during the first (empagliflozin) treatment period.
As previously discussed, insulin and empagliflozin represents two metabolically opposing methods for lowering plasma glucose concentrations. By titrating insulin treatment to match the glycaemic control found with empagliflozin in the same participants, 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 glycaemic control (Figure 2). NPH Insulin has been chosen over more modern human insulin analogues, as it is not albumin bound and can be measured in an ordinary insulin assay.

Safety considerations
During washout periods blood glucose concentrations will increase – that is a separate point of the study, but severely dysregulated diabetes is an exclusion criterium to ensure participant safety. The risk of severe hyperglycaemia is reduced in several ways in the study:

- Existing metformin treatment is continued throughout the whole study as background antiglycaemic treatment.
- In case of fasting BG concentrations of more than 13 mmol/L, patients are instructed to contact 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 hyperglycaemic events or other adverse events.
- As soon as the final day (CMR with acipimox) of a washout visit (visit 1 or 3) is completed, antiglycaemic treatment according to study drug sequence is commenced to minimise time spent in hyperglycaemia.

In case of fasting 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 antihyperglycaemic treatment is initiated.

Screening visit (V0)
Once oral and written informed consent is obtained by the study investigator, the screening procedure follows. Medical history is recorded, screening blood samples drawn, and an ECG, recording of blood pressure, pulse rate and registration of anthropometric data are performed, and patients are screened according to in- and exclusion criteria. A standard transthoracic echocardiography is performed, and VO2max is estimated (Box 3).

Study visits

All study visits consist of three study days – a metabolic study day and two CMR study days (Box 4).

The metabolic study day

The metabolic study is conducted at the Department of Endocrinology, Hvidovre Hospital, to document the metabolic effects of each study drug.

Participants meet in the morning after an overnight fast. Anthropometric data, blood pressure, pulse rate and an ECG are recorded, and two catheters, one in each arm are inserted for infusion of tracers and for repeated drawing of arterialised blood samples respectively. Baseline and safety blood samples are taken (Box 5), the participant empties bladder and the investigational drug (V2, V4) and the participants usual medications are administered at 0800h. Body composition is determined by Dual energy x-ray absorptiometry scan (DXA).

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 at -30, -15 and -2 min to characterise glucose, lipid and amino acid metabolism. The patient empties bladder, urine is weighed, and samples are taken for determination of tracer concentrations and urinary nitrogen excretion, and the 5h-OGTT is initiated.

5h-OGTT

The patient ingests anhydrous glucose (72 g) with added [U-13C6]-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 characterisation of postprandial glucose, lipid and amino acid metabolism (Box 4 and 5). The patient empties bladder regularly during and at the end of the OGTT. Urine is sampled for nitrogen excretion and tracers/tracees.

Fat and muscle biopsies: Biopsies are obtained during the basal (T=-60 min) and the maximally insulin stimulated (T=60 min) state. Muscle biopsies are considered proxies for cardiomyocyte metabolic status. 30 min ventilated hood indirect calorimetry (Vyaire Vyntus® CPX) is performed during the basal period (prior to biopsies) t= -90 min and postprandially at t=60 min for determination of fasting and postprandial energy expenditure and respiratory quotient.

Exercise test (50% VO2max)

At T=300 min, the participant is exercised at 60 W for 4 minutes after which work load is increased until oxygen consumption is 50% of estimated VO2max. Pulse rate is recorded with a chest mounted pulse rate monitor and oxygen consumption with a mask mounted indirect calorimeter. Blood is sampled to characterise glucose, lipid and amino acid metabolism (Box 4 and 5). After 30 min, VO2max is estimated by increasing workload by 50W until a pulse rate increase of 30. When

pulse is steady for 2 min, oxygen consumption and pulse rate are recorded, and the test is stopped. VO2max is estimated by linear extrapolation to the theoretical maximum pulse rate (220-age).

Ad libitum meal test

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 [38]. Therefore, the metabolic study day is ended with an ad *libitum meal*, consisting of thoroughly mixed pasta bolognese (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 ad libitum meal intake defined as the difference. Throughout the day patients are asked to score their hunger, satiety and sensation of fullness on a visual analogue scale [51].

Cardiac evaluation

Two CMR days are performed during each visit (V1-V4). In addition, diurnal blood pressure and Holter monitoring are performed. CMR is conducted at the Department of Cardiology, Rigshospitalet, Copenhagen, whereas Holter monitoring and diurnal blood pressure monitoring are performed at the Department of Cardiology, Hvidovre Hospital.

Participants meet fasting and morning medication, including investigational medicinal product (V2 and V4), is administered. Anthropometric data are recorded, and two intravenous catheters are inserted into an antecubital and the contralateral dorsal hand vein for infusion of adenosine, gadolinium contrast and glycopyrrolate and for blood sampling respectively (Box 5). Prior to CMR a transthoracic echocardiography is performed (Box 3)

CMR is performed on a 1.5 Tesla scanner (Siemens Aera; Siemens; Erlangen; Germany) with the patient lying supine on the back, using an 18-channel cardiac coil with continuous ECG gating.

Cine 2-, 3- and 4-chamber images, complete transverse and short axis cine stacks covering the whole heart are acquired. All images are obtained during end-expiratory breath-holds. Myocardial perfusion images during rest and stress are obtained at the basal, mid-ventricular and apical cardiac short-axis level. Rest perfusion images of the myocardium is acquired using an intravenous bolus of gadolinium contrast (Gadovist®, Bayer AG, Germany) 0.075 mmol/kg bodyweight. The time of gadolinium contrast entry into the right and the left ventricle is accurately determined, and this transit time of gadovist multiplied by cardiac output is used to calculate the pulmonary and central (pulmonary + cardiac) blood volume.

Myocardial stress perfusion images are obtained with an i.v dose of 0.075 mmol/kg of gadovist during and 10 min after an intravenous adenosine (140 ug/min) administrated for maximum 4 minutes). This is followed by evaluation of cardiac function during chronotropic stress, where short axis cine stack will be reacquired 10 minutes after the administration of intravenous glycopyrrolate

(4 µg/kg, max. 400µg, given as a bolus). This approach has been shown to unmasque subclinical diastolic dysfunction as has been demonstrated in normal healthy elderly [50].

Cardiac MRI, Acipimox

CMR scans will follow the same procedure as described above, but participants are instructed to ingest 250 mg acipimox p.o. twice, 4 hours before and right before the scan, to determine myocardial function. This repeated administration of acipimox is required for adequate suppression of hormone sensitive lipase activity and depletion of plasma FFAs (28). This has been shown to gradually impair cardiac function [52], and is done to disclose any coupling between FFA availability and cardiac function.

CMR image analysis

Is performed using Circle42 (Circle Cardiovascular Imaging Inc., Calgary Canada, v5.5.1). LV volumes, LV mass, LV ejection fraction (LVEF) and LV peak filling rate are determined by tracing of the endo- and epicardial contours in end-diastolic and end-systolic phases. The papillary muscles are excluded from the myocardium. On native and post-contrast T1-mappings, endocardial and epicardial borders are traced, and the mean extra cellular volume (ECV) is calculated from areas outside late gadolinium enhancement (LGE) lesions. For determination of the ECV within an LGE lesion, myocardium without LGE in the segment is excluded. Myocardial perfusion scans are inspected for perfusion defects. Regions with infarctions, sub-endocardial perfusion defects or dark-rim artefacts will be excluded. Blinded to clinical data, the analyses will be reviewed and finalised by two CMR specialist.

Diurnal blood pressure and Holter monitoring

Between study days during each visit, diurnal blood pressure is recorded (ScottCare, ABP 320, Cleveland, OH)) for 24 hours with 15 minutes intervals between 6.00 to 22.00- and 60-min intervals during night-time. Cardiac rhythm is evaluated with Holter monitoring (SCOTTCARE, CHROMA, model RZ153C, Cleveland, OH) for 48 hours.

Patient and public involvement

No patient involved.

ANALYSES

Blood and tissue samples

Subcutaneous fat- and muscle biopsies: Local analgesia is applied before sampling with a Bergströms cannula. Samples are immediately frozen in liquid nitrogen and stored at -80°C. Blood and urine samples: Samples are spun, aliquoted and stored at –20°C (GLP-1, PYY, Glucagon) or –80°C for later analysis. Bedside plasma glucose measurements are performed using the glucose oxidase technique (YSI model 2300 STAT Plus; YSI,Yellow Springs, OH). Home blood glucose measurements are carried out on Contour XT (Ascensia Diabetes Care Holdings AG, Basel,

Switzerland). Safety blood- and urine samples are analysed on the same day at the Department of Clinical Biochemistry, Hvidovre Hospital.

Statistical methods

Sample size calculation

Measures of myocardial function are highly reproducible when assessed using CMR, and interstudy and cohort coefficients of variation are in the range of 3-5% [53–55]
Using the same CMR protocol as the present, 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) [55]. 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. In case of a 20% dropout rate, power would still be acceptable (83%, p=0.01).

Statistical analysis plan

The primary and secondary endpoints are analysed assuming no period effect or treatment-period interaction. This assumption is reasonable, given results from similar studies, where no such interactions or effects have been reported [56]. Normally distributed data are presented using standard descriptive statistics, and reported as mean (SD) for normally distributed and median (Q1;Q3) for non-normally distributed data. Likewise, comparisons of normally distributed data is done using the paired Student's t-test for all completers, whereas Wilcoxon's paired signed rank test will be used if data is non-normally distributed.

Timeframe

Screenings are performed from January 2018. Last patient, last visit is expected second half of 2021 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 by Winter 2022. No later than 12 months after unregistering of the study, will results be made available at www.clinicalregister.eu. Trial registration number: EudraCT: 2017-002101.

ETHICS AND DISSEMINATION

The study is conducted according to ICH GCP guidelines E6 (R2) and the Helsinki Declaration and all participants will provide oral and written informed consent. The study is approved by the Danish Medicines Agency (ref. id: 2017061587), The Capital Region Ethical Committee (H-17018846) and the Danish Data Protection Agency (AHH-2017-093). Our results, regardless of outcome, 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.

DISCUSSION

The profound and swift benefits of SGLT2i on cardiovascular risk in T2D have inspired the discussion of metabolism and its importance for cardiac function in patients with T2D [57–61]. Especially since, SGLT2 inhibitors have metabolic effects that by and large are opposite to those of insulin treatment. Thus, insulin treatment is associated with increased tissue glucose uptake and utilisation, but suppression of lipid mobilisation and oxidation as well as lowering of plasma concentrations of ketone bodies [62]. SGLT2 inhibitors increase lipid mobilisation and oxidation, increase plasma ketone body concentrations and reduce tissue glucose uptake [38,63]. Both treatments lower plasma glucose, but insulin treatment increases whereas SGLT2i treatment decreases plasma insulin concentrations. Whether such changes in metabolism affect cardiac function, is still unsettled, but forcing cardiac glucose uptake and utilisation through insulin treatment has been suggested by some to benefit and by others to impair cardiac function [47,64–67], while yet others have suggested increased lipid and ketone body oxidation to be important for proper cardiac function in T2D [46,58,67]. Studies on SGLT2i treatments and the effects on cardiac function are beginning to emerge. In a recent study, 42 patients with T2D were randomised to 12 weeks of empagliflozin 10 mg or placebo once daily. SGLT2 inhibition was shown to rapidly improve diastolic cardiac function as evaluated with echocardiography [68]. In a placebo controlled cross-over design, after 4 weeks of empagliflozin treatment in patients with T2D, myocardial glucose uptake was reduced and fatty acid oxidation unaltered, but this did not significantly change myocardial oxygen consumption or cardiac efficiency, nor any measure of cardiac function [69]. In a Swedish study, 6 weeks dapagliflozin treatment showed unchanged cardiac fatty acid uptake, a trend toward reduced left atrial maximal volume, and reduced LV oxygen consumption and external work compared to placebo [70], and in the only study found, where an active comparator was used, 10 mg empagliflozin once daily for 12 weeks did not change cardiac lipid accumulation (as measured by MR spectrometry), cardiac function or cardiac metabolism compared to sitagliptin 50 mg daily [71].

In conclusion, existing studies in humans have shown divergent results regarding changes in cardiac diastolic function with little changes in cardiac metabolism. However, most studies have compared cardiac effects of SGLT2i to placebo, thus not accounting for the circumstances that characterised the EMPA-REG trial, where anti glycaemic treatment was intensified in the placebo group concurrently [26]. Thus, the CVD risk benefits of the study may have arisen from unfavourable metabolic consequences of the treatment in the placebo arm. In the one study with an active comparator empagliflozin was compared to sitagliptin, which not only affects the incretin system but also has less specific metabolic effect [72]. Therefore, to date our study, is the one to most directly pursue the coupling between metabolism and cardiac function, by choosing insulin as the comparator, and by including the effects of acute lowering of free fatty acid concentrations in plasma on cardiac function.

Disclosure summary

All authors have completed the ICMJE uniform disclosure form and declare: no support from any organisations for the submitted work; SM and NBJ have received research grants from Boehringer Ingelheim and JJH serves on advisory boards for Novo Nordisk, no other relationships or activities that could appear to have influenced the submitted work.

Funding
The study is funded by an investigator-initiated study grant from Boehringer Ingelheim.
Additional funding comes from Grosserer L. F. Foghts Fond, Charlottenlund, Denmark.
Grant numbers not applicable.

Contributions
Author contributions
RT will be conducting the study, collect all the data, perform data analyses and co-submit the study. EAR has contributed to the study design, designed the keton body- and biopsy analysis scheme and is going to perform these analyses. JPG designed the pro-ANP and pro-BNP analysis scheme and is going to perform these analyses. MF designed multiple metabolic analysis schemes (Triglycerides, insulin, free fatty acids, uric acid, urea and nitrogen) and is going to perform these analyses.
GVH designed the three-tracer measuring analysis schemes we are using in this study and is going to perform these analyses. UD provided the study with analysis tools (holter-monitors and blood pressure monitors). He will perform the analyses of these data.
JJH participated in the protocol design, designed multiple metabolic analyses (GLP-1, glucagon and PYY) and will perform the analyses of these data.
SM is the principal investigator, co-planned the study and contributed with several analysing tools.
NV co-designed the cardiac MRI guideline for the study and contributed with analysing tool (MRI scanner).PLM co-planned the study, co-designed the cardiac MRI guideline for the study and will analyse the cardiac MRI data.
NBJ co-planned the study, will perform all data analyses with RT and co-submit the study. All of the authors contributed with writing, revising and approving this manuscript.

Box 1: Eligibility criteria
Inclusion criteria
Age ≥18 years BMI ≥ 28 kg/m^2 HbA1c ≤ 9% (≤10% in diet or metformin treated only) Fasting C-peptide ≥ 500 pmol/L Unchanged glycaemic treatment for 3 months prior to inclusion High cardiovascular risk as one of the following: • Previous myocardial infarction, stroke or peripheral arterial disease more than 2 months prior to informed consent • Evidence of multi-vessel coronary arterial disease (CAD) but without prior myocardial infarction, if more than 50% stenosis is present, if revascularised (CABG or PCI) more than 2 months prior or if 1 vessel is vascularised and the other with 50% stenosis. • Single vessel CAD without prior myocardial infarction if more than 50% stenosis is present, not revascularised and positive stress test for ischemia.

Exclusion criteria
Insulin treatment within 3 months from informed consent Type 1 diabetes Psychiatric disorder or mental retardation Drug or alcohol abuse within 3 months from informed consent Poor compliance Anaemia (Hb < 6.4 mmol/L) or other blood dyscrasias causing haemolysis or unstable erythrocytes. Indication of liver disease (ALT or Alkaline phosphatase elevation above 3x upper normal limit) Impaired renal function (eGFR<45 ml/min/1.73 m^2) Anti-obesity medication within 3 months from informed consent Systemic steroid treatment within 6 weeks from informed consent. Any uncontrolled endocrine disorder except T2D Bariatric surgery or other gastrointestinal conditions that may compromise gastrointestinal absorption Peptic ulcer – verified endoscopically Any form of surgery within 3 months of informed consent Acute myocardial infarction, stroke or peripheral arterial disease within 2 months of informed consent. Persistent or permanent atrial fibrillation Inability to undergo experimental procedures including exclusion criteria for CMR 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 Contraindications to adenosine: 2nd or 3rd degree atrioventricular block Severe hypotension (BP ≤ 90/50 mmHg) Long QT syndrome Unstable angina pectoris Decompensated heart failure Sinus node dysfunction Chronic obstructive pulmonary disease or asthma bronchiale (FEV1 ≤ 50% of expected)

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

Box 2: Endpoints	
Primary end point	Change in left ventricular peak filling rate (ΔLVPFR)
Secondary endpoints	Change in left atrial passive emptying fraction (ΔLAPEF) Change in left ventricular ejection fraction (ΔLVEF)
Explorative endpoints include	Cardiovascular: Change in VO2max and exercise tolerance test variables Change in central blood volume and haematocrite Change in heart rate variability Change in left ventricular volume Metabolic: Basal and postprandial AUC Free Fatty Acids and glycerol turnover Endogenous glucose production and tissue glucose disposal (metabolic clearance of glucose) Fasting and postprandial energy expenditure and respiratory quotient Glucagon-Insulin ratio Insulin sensitivity (AUC glucose metabolic clearance / AUC insulin concentration) Beta-cell function (prehepatic insulin secretion rate, correlated to ambient glucose)

Box 3: Screening procedures	
Blood samples	Haematology (haemoglobin, thrombocytes, haematocrit, leukocytes), liver and renal function tests (creatinine, eGFR (Cockroft-Gault formula), alkaline phosphatases, alanine aminotransferases, lactate dehydrogenase, bilirubin, amylase, sodium, potassium), fasting P-glucose, C-peptide, HbA1c, TSH, Urinary Albumin/creatinine mass ratio, and in fertile women, U-hCG.
Echocardiography	Parasternal long axis view, parasternal short axis view at aortic, mitral and apex levels, apical 4-chamber view, LVEF, E/E', E', LVEDV/BSA.
Estimation of VO2max	Maximum oxygen uptake is estimated using Åstrøm's two-point test performed on a cycle ergometer during indirect calorimetry. From measurements of VO2 at two sub-maximal pulse rates VO2max is estimated by linear extrapolation to the theoretical maximal pulse rate (220-age) [73].

Box 4: Visit overview		
Metabolic study day	Cardiac MR	Cardiac MR, Acipimox
- 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.	- Fasting blood samples, before and after CMR. - Echocardiography - CMR Rest -- Without enhancement -- With enhancement and adenosine infusion - CMR Stress -- Unenhanced repeated during pharmacological chronotropic stress with glycopyrrolate infusion. - 24h ambulant blood pressure	Same protocol as Cardiac MR day, but during pharmacological suppression of hormone sensitive lipase activity and depletion of plasma free fatty acids. - 48h Holter monitoring.

Box 5: Blood samples on metabolic and CMR study days include	
Metabolic study day	Blood samples: glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, total amino acids and ketone bodies (betahydroxybuturate), tracers/tracees, gut hormones. HbA1c, urate, blood urea nitrogen, cortisol, is sampled at baseline.
Cardiac MRI days	Markers of cardiac function, including pro-ANP and pro-BNP, glucose, insulin, C-peptide, glucagon, FFAs, triglycerides, ketone bodies, haematocrit are drawn before and after CMR.

Figure legends

Figure 1. Study outline. Included patients undergo a 7-week program of washout of pre-existing antiglycemic treatment (except metformin) and run-in of empagliflozin. Hereafter they are randomised to treatment for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over to 5±1 weeks treatment with the remaining study drug. Tests performed at each visit are summarised in Box 4 and 5.

Figure 2. Metabolic profile of the two study drugs. Schematic representation of the metabolic changes expected with the two study drug treatments in a patient randomized to insulin first. Insulin treatment is characterized by low glucose, low FFAs and high insulin concentrations; empagliflozin treatment by low glucose, high FFAs and low insulin.

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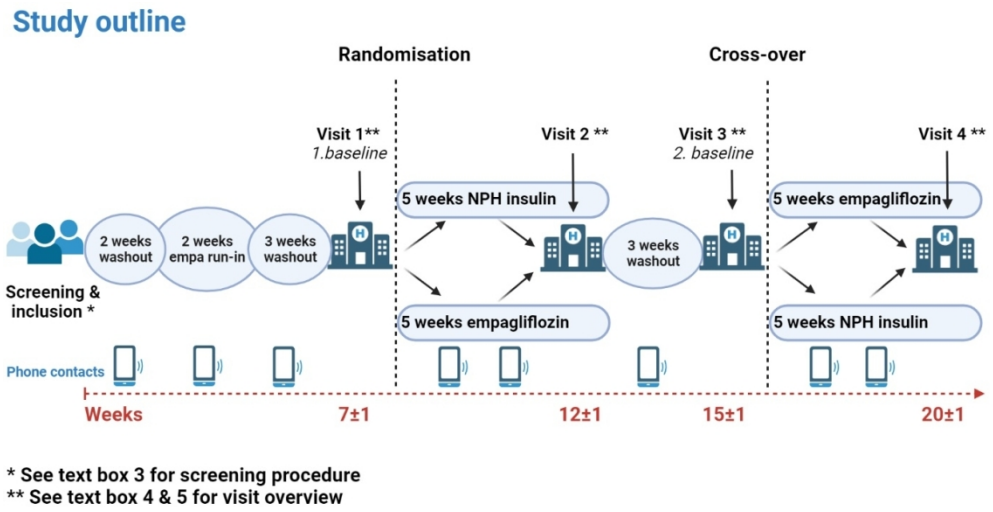

Figure 1. Study outline. Included patients undergo a 7-week program of washout of pre-existing antiglycemic treatment (except metformin) and run-in of empagliflozin. Hereafter they are randomised to treatment for 5±1 weeks, followed by 3±1 weeks wash-out and cross-over to 5±1 weeks treatment with the remaining study drug. Tests performed at each visit are summarised in Box 4 and 5.

369x187mm (96 x 96 DPI)

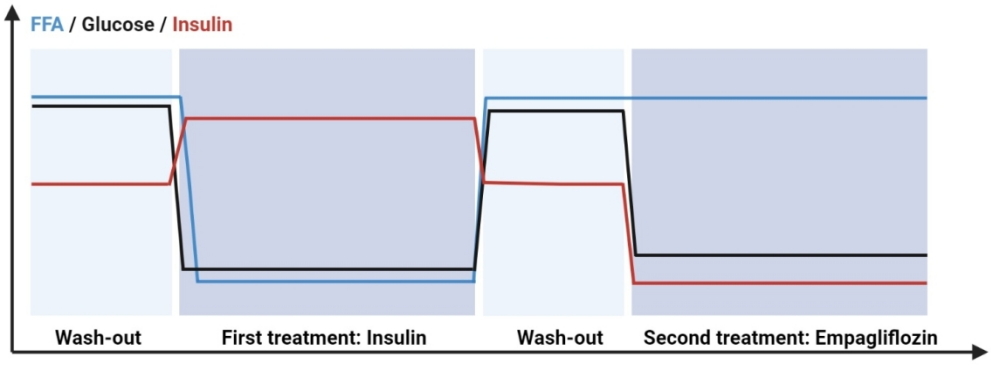

Figure 2. Metabolic profile of the two study drugs. Schematic representation of the metabolic changes expected with the two study drug treatments in a patient randomized to insulin first. Insulin treatment is characterized by low glucose, low FFAs and high insulin concentrations; empagliflozin treatment by low glucose, high FFAs and low insulin.

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NB: THIS CONSENT FORM IS TRANSLATEDE FROM THE ORIGINAL DANISH FORM

(S4)

Informed consent to participate in a health science research study.

Title of the research project:" The role of hyperglycemia, hyperinsulinemia and elevated free fatty acids for cardiac function in patients with type 2 diabetes – the HyperCarD2 study".

Declaration from the patient:

I have received written and oral information and I know enough about the purpose, method, benefits and

disadvantages of saying yes to participating.

I know that participating is voluntary and that I can always withdraw my consent without losing my current or future rights to treatment.

I give my consent to participate in the research study and to have my biological material collected and stored in a research biobank. I have received a copy of this consent form as well as a copy of the written information about the study for my own use.

Name of the patient: _____

Date: _____ Signature: _____

If new essential health information about you appears in the research study, you will be informed. If you would like to **decline** receiving any new information about your health that appears in the research project, please mark here: _____ (insert an x)

Do you want to be informed about the result of the research study and any possible consequences for you?

Yes _____ (insert an x) No _____ (insert an x)

Declaration from the person providing the information:

I declare that the patient has received oral and written information about the research study.

In my opinion, sufficient information has been provided to enable a decision to be taken on participation in the study.

The name of the person who provided the information: Roopameera Thirumathyam

Date: _____ Signature: _____

Studytidentification: (E.g comiteé ID, EudraCT no., version no./date or similar.)

EudraCT 2017-002101-35