RESEARCH PROTOCOL

A phase 4, monocenter, randomized, double-blind, comparator-controlled, parallel-group, mechanistic intervention trial to assess the effect of 12-week treatment with the sodium-glucose linked transporters (SGLT)-2 inhibitor dapagliflozin versus the sulfonylurea (SU) derivative gliclazide on renal physiology and biomarkers in metformin-treated patients with type 2 diabetes mellitus (T2DM)

Short Title (Acronym):
RED: Renoprotective **E**ffects of **D**apagliflozin in Type 2 Diabetes

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CONFIDENTIAL

Investigators:

M.H.H. Kramer (PI)
D.H. van Raalte (PI)
E. van Bommel
M.H.A. Muskiet
L. Tonneijck

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Diabetes Center
PO Box 7057
1007 MB Amsterdam
The Netherlands
Telephone: +31-(0)20-44440651
Fax: +31-(0)20-4443349

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Contact information		
Principal Investigators	**Prof. M.H.H. Kramer, MD PhD** Department of Internal Medicine De Boelelaan 1117, Room 4A34 1081 HV Amsterdam Telephone: +31-(0)20-4443409 (assistant: Mrs. Annelies Kabalt–Zoet) Email: m.kramer@vumc.nl	**D.H. van Raalte, MD PhD** Department of Internal Medicine De Boelelaan 1117, Room ZH-4A58 1081 HV Amsterdam Telephone: +31-(0)20-4440534/40533 (secretary Mrs. Lilian van 't Hull) Email: d.vanraalte@vumc.nl
Sponsor	**Prof. M.H.H. Kramer, MD PhD** Department of Internal Medicine De Boelelaan 1117, Room 4A34 1081 HV Amsterdam Telephone: +31-(0)20-4443409 (assistant: Mrs. Annelies Kabalt–Zoet) Email: m.kramer@vumc.nl	
Independent physician	**E.J.G. Peters, MD PhD** Department of Internal Medicine De Boelelaan 1117, Room PK-1-Z108 1081 HV Amsterdam Telephone: +31-(0)20-4440596 Email: e.peters@vumc.nl	

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ABBREVIATIONS, DEFINITIONS AND TERMINOLOGY

8-OHdG	8-hydroxy-2' -deoxyguanosine
ACR	Albumin-to-Creatinine Ratio
ADA	American Diabetes Association
AE	Adverse Event
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANP	Atrial Natriuretic Peptide
ANS	Autonomic Nervous System
AR	Adverse Reaction
ARB	Angiotensin II Receptor Blocker
AST	Aspartate aminotransferase
BCM	Body Cell Mass (variable derived with BIA)
BIA	Bio-Impedance Analysis
BMI	Body Mass Index
BNP	Brain Natriuretic Peptide
CARTs	Cardiovascular autonomic reflex tests
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CKD	Chronic Kidney Disease
CRF	Case Report Form
eCRF	Electronic Case Report Form
CRU	Clinical Research Unit
CNS	Central Nervous System
DCCT	Diabetes Control and Complications Trial
DKD	Diabetic Kidney Disease
DNA	Deoxyrubonucleic acid
EASD	European Association for the Study of Diabetes
ECG	Electrocardiogram
ECW	Extracellular Water (variable derived with BIA)
EMA	European Medicines Agency
ERB	Ethical Review Board
ERBF	Effective Renal Blood Flow
ERPF	Effective Renal Plasma Flow
ERVR	Effective Renal Vascular Resistance
ESRD	End Stage Renal Disease
EudraCT	European Drug Regulatory Affairs Clinical Trials
FDA	Food and Drug Administration
FE	Fractional Excretion (measure of renal tubular function)
FPG	Fasting Plasma Glucose
FSH	Follicle-Stimulating Hormone
GCP	Good Clinical Practice
GFR	Glomerular Filtration Rate
GGT	Gamma-Glutamyl Transferase
GMP	Good Manufacturing Practice
HbA_{1c}	Glycated hemoglobin (A1c)
HDL-C	High Density Lipoprotein - Cholesterol
HF	High Frequency (component of HRV)
HRV	Heart Rate Variability
hsCRP	high sensitivity C-Reactive Protein
ICH	International Conference on Harmonisation
IFCC	International Federation of Clinical Chemistry
IIT	Investigator Initiated Trial
IL-6	Interleukin 6
IMP	Investigational Medicinal Product ('study drug')
IQR	Interquartile Range
KIM-1	Kidney Injury Molecule-1

LDL-C	Low Density Lipoprotein - Cholesterol
LF	Low Frequency (component of HRV)
Long-term tests	End-point assessments performed after 12-week study drug intervention
MCP-1	Monocyte Chemoattractant Protein-1
MedDRA	Medical Dictionary for Regulatory Activities
MSD	Merck Sharp & Dohme
NGAL	Neutrophil Gelatinase-Associated Lipocalin (a marker for renal tubular injury)
NT-proBNP	N-terminal pro-B-Type Natriuretic Peptide
NVDO	Dutch Association for the Study of Diabetes (in Dutch: Nederlandse Vereniging voor Diabetes Onderzoek)
NYHA	New York Heart Association
OGTT	Oral Glucose Tolerance Test
PAH	*Para*-aminohippuric acid
PROactive	PROspective pioglitAzone Clinical Trial In macroVascular Events
PWA	Pulse Wave Analysis
RAS	Renin-angiotensin-aldosterone system
RCT	Randomized Clinical Trial
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SGLT-2	Sodium glucose linked transporter-2
SMBG	Self-Monitoring Blood Glucose
SOP	Standard Operating Procedure
SPC	Summary of Product Characteristics
Sponsor	The sponsor is the party that commissions the organization or performance of the research, for example a pharmaceutical company, academic hospital, scientific organization or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidizing party
sRAGE	soluble receptor for advanced glycation end products
SU	Sulfonylurea derivative (antihyperglycemic agent)
SUSAR	Suspected Unexpected Serious Adverse Reaction
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TC	Total Cholesterol
TBW	Total Body Water (variable derived with BIA)
TG	Triglycerides
TGF- β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor alpha
TSH	Thyroid-Stimulating Hormone
UAE	Urinary Albumin Excretion
VUMC	VU University Medical Center
WFI	Water for injections
WBP	Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgegevens)
WMO	Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen)

ABSTRACT

Background: Worldwide, diabetic nephropathy or Diabetic Kidney Disease (DKD), is the most common cause of chronic and end-stage kidney disease. With the increasing rates of obesity and type 2 diabetes (T2DM), many more patients with DKD may be expected in the coming years. DKD is a multi-factorial condition, involving factors such as obesity, chronic hyperglycemia, advanced glycation end products, oxidative stress, pro-inflammatory cytokines, systemic- and glomerular hypertension. Large-sized prospective randomized clinical trials suggest that intensified glucose and blood pressure control, the latter especially by using agents that interfere with the renin-angiotensin-aldosterone system (RAS), may halt the progression of DKD, both in type 1 diabetes and T2DM. However, despite the wide use of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, a considerable amount of patients develop DKD during the course of diabetes, indicating an unmet need for renoprotective therapies. Sodium-glucose linked transporters (SGLT-2) inhibitors are novel glucose-lowering drugs for the treatment of T2DM. These agents seem to exert pleiotropic actions 'beyond glucose control', including reduction of blood pressure and body weight. In addition, SGLT-2 inhibitors decrease proximal sodium reabsorption and decrease glomerular pressure and albuminuria in rodents and type 1 diabetes patients. In rodents, SGLT-2 inhibitors also improved histopathological abnormalities associated with DKD. To date, the potential renoprotective effects and mechanisms of these agents have not been sufficiently detailed in human type 2 diabetes. The current study aims to explore the clinical effects and mechanistics of SGLT-2 inhibitors on renal physiology and biomarkers in metformin-treated T2DM patients with normal kidney function.

Hypothesis: Treatment with the SGLT-2 inhibitor dapagliflozin, as compared to the sulfonylurea (SU) derivative gliclazide, may confer renoprotection by improving renal hemodynamics, and decreasing blood pressure and body weight in type 2 diabetes.

We will test this hypothesis by addressing the following **research objectives**:
Primary objective: what are the long-term effects (i.e. after 12-week drug exposure) of the SGLT-2 inhibitor dapagliflozin versus the SU gliclazide on renal hemodynamics (glomerular filtration rate (GFR) / effective renal plasma flow (ERPF)) in metformin-treated T2DM patients?
Secondary objectives: renal tubular function, renal damage markers, blood pressure, heart rate, body anthropometrics, body fat, markers of inflammation, glycemic variables, lipid spectrum, microvascular function, arterial stiffness, systemic hemodynamics, cardiac autonomic nervous system function.
Exploratory objectives: additional markers of renal function/damage, inflammation and (cardiovascular)-biomarkers, deoxyribonucleic acid (DNA), gut microbiome, insulin sensitivity and measures of beta-cell function.

Methods:
Study Design: A phase 4, monocenter, randomized, double-blind, comparator-controlled, parallel-group, mechanistic intervention trial to assess the effect of 12-week treatment with the sodium-glucose linked transporters (SGLT)-2 inhibitor dapagliflozin versus the SU gliclazide on renal physiology and biomarkers in metformin-treated patients with type 2 diabetes mellitus (T2DM)
Patients: Patients with T2DM (male and female; HbA_{1c} 6.5-9%); aged 35-75 years; BMI ≥25 kg/m^2
Study Endpoints and methods: Renal hemodynamics, i.e. GFR and ERPF will be measured by the gold-standard inulin- or iohexol and para-aminohippurate clearance methods, respectively, during both euglycemic- and hyperglycemic clamp; renal tubular function will be measured by 24-hour urine sodium, potassium, chloride, calcium, magnesium, phosphate and urea and glucose; markers of renal damage will include urinary albumin excretion, neutrophil gelatinase-associated lipocalin and kidney injury molecule-1; blood pressure will be measured using an automated oscillometric blood pressure device (Dinamap®); body anthropometrics, including body weight, height, body-mass index and waist circumference, and body fat contents (by bio-impedance analysis) will be measured; blood samples will be obtained to determine glycemic variables, lipids and markers of inflammation; systemic hemodynamic variables (including stroke volume, cardiac output and total peripheral resistance) will be measured by continuous beat-to-beat hemodynamic monitor (NexFin®); heart rate will be determined by oscillometric blood pressure device; microvascular function will be measured

by capillary videomicroscopy and Laser Doppler; arterial stiffness will be assessed by applanation tonometry, (SphygmoCor®); additional urine, blood and feces will be collected for conditional determination of various markers of renal damage/function, inflammation, oxidative stress and (cardiovascular)-biomarkers and DNA; CANS will be measured by electrocardiography and NexFin®; insulin sensitivity will be measured by glucose infusion during the euglycemic clamp (M-value); beta-cell function will be measured by quantification of insulin secretion during the hyperglycemic clamp.

Expected results: This active-comparator controlled mechanistic intervention study, using state-of-the-art methodology will expand our knowledge regarding the potential renoprotective effects of SGLT-2 inhititors in human T2DM. We hypothesize that SGLT-2 inhibitors may confer renoprotection by improving renal hemodynamics, and decreasing blood pressure and body weight, all important factors in the development of DKD. Ultimately, we expect that this study will contribute to guiding clinicians in their decision-making process of individualizing therapies for T2DM patients at risk for, or suffering from, DKD.

1. INTRODUCTION

Diabetic Kidney Disease

Globally, 382 million adults are living with diabetes (primarily type 2 diabetes mellitus; T2DM), and this estimate is projected to rise to 592 million by 2035 (1). Among the various diabetes-related microvascular and macrovascular complications, approximately 20−40% of patients with T2DM will ultimately develop diabetic kidney disease (DKD) (2). DKD is defined as kidney disease attributable to diabetes and the clinical diagnosis is usually made by combined finding of decreased glomerular filtration rate (GFR) and albuminuria (3). In parallel with the diabetes pandemic, DKD has now become the leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) in developed countries (3). In the United States, the prevalence of DKD rose from 7.4% in 1988 to 9.6% in 2006, and this trend is forecast to continue (4). In addition, DKD results in high cardiovascular morbidity and mortality and decreased patients' health-related quality of life (5,6).

Current renoprotective treatment strategies

Chronic hyperglycemia and related glomerular hyperfiltration are the main driving pathogenic forces of DKD in type 1 diabetes (T1DM) patients. In contrast, in T2DM, the pathophysiology of DKD is more complex, since a cluster of cardiometabolic abnormalities (including obesity, systemic hypertension and dyslipidemia) may additionally contribute from early stages of the disease. In accordance with this complex etiology and based on landmark studies, clinical guidelines recommend a multifactorial treatment approach to improve renal outcome in T2DM, including lifestyle interventions (diet/exercise to achieve weight loss, smoking cessation) and pharmacological management of glucose, blood pressure and lipids are advocated (7–9). With respect to blood pressure control, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are especially recommended, since these inhibitors of the renin-angiotensin-aldosterone system (RAS) have demonstrated renoprotective effects beyond their ability to lower blood pressure. However, even targeting multiple risk factors simultaneously, residual risk for DKD development remains. Indeed, in the Steno-2 trial, although intensive multifactorial treatment of 160 T2DM patients with persistent microalbuminuria had sustained beneficial effects on renal complications, 25% of patients developed DKD nevertheless. Also, although rates of diabetes-related complications have declined substantially in the past two decades, the smallest decline was observed for ESRD (−28.3%; 95% CI: −34.6 to −21.6), leaving a tremendous burden of renal disease in this patient group (10). These numbers highlight the need for novel strategies or new therapeutic drugs to improve renal outcome in T2DM.

New management options in T2DM: SGLT-2 inhibitors

Sodium-glucose linked transporters (SGLT) are a main class of glucose transporters involved in glucose homeostasis. The SGLTs are located at the brush border membrane of the gut epithelium to enhance glucose absorption, and apical membrane of renal tubular cells to facilitate glucose reabsorption after glomerular filtration (11). Among the 12 SGLT family-members, the high-capacity low-affinity SGLT-2 is abundantly expressed in the S1 segment of the proximal renal tubule and accounts for the vast majority (90%) of filtered glucose reuptake, by coupling glucose transport to the electrochemical sodium gradient (12–14). The low-capacity high affinity SGLT-1 is mainly responsible for glucose absorption from the small intestine, while contributing to about 10% of renal glucose reabsorption in the straight S3 segment of the proximal renal tubule (13). Healthy human kidney's filter approximately 160-180 g of glucose daily, almost all of which is reabsorbed by SGLT-2 (90%) and SGLT-1 (10%) (14). The maximal capacity of the proximal tubule (Tm) for glucose-reabsorption varies among individuals and averages 375 mg/min for healthy subjects − reflecting a plasma glucose concentration of 10-11 mmol/l (14). In animal models of diabetes, the rate of renal glucose reabsorption in the proximal tubule is increased (15–18), whereas similar results were seen in humans with poorly controlled T1DM (19) and T2DM (20). On a molecular level, the increased renal glucose reabsorption may be explained by increased SGLT-2 and GLUT-2 expression and activity (15–17,21). Thus, in diabetes, a higher than normal amount of glucose is reabsorbed by the kidneys, which contributes to and sustains hyperglycemia (12).

Since renal glucose hyperreabsorption has been considered a maladaptive response to ambient hyperglycemia, SGLT inhibition was regarded a therapeutic target for glycemic control in diabetes (14). Rossetti *et al* were the first to show that phlorizin − a beta-D-glucoside isolated from the bark of the apple tree − could function as a non-selective SGLT-1/2 inhibitor (22). In a proof of concept study in partly pancreatectomised rats (a model for T2DM), subcutaneous injections of phlorizin induced glucosuria and normalized both fasting and postprandial glucose levels (22). Phlorizin has to be administered by injection and incites significant SGLT-1-mediated gastrointestinal side

effects, the compound has therefore not been developed as treatment for T2DM (12). However, results obtained with phlorizin have provided the basis for the development of specific SGLT-2 inhibitors. As a result, several SGLT-2 inhibitors have been successfully introduced, which have proven to be efficacious in reducing HbA1c levels (0.66% reduction versus placebo; similar efficacy compared to active comparators), providing a new insulin-independent approach for the treatment of T2DM (23), and potentially T1DM. Important side effects of SGLT-2 inhibitors comprise a modest increase in urinary and genital tract infections (23). Hypoglycemia rates for this novel drug class are similar to placebo. Currently, three SGLT-2 inhibitors −dapagliflozin, canagliflozine and empagliflozin− were granted marketing authorization by the European Medicines Agency.
Animal and human data indicate that SGLT-2 inhibitors may prevent the onset and progression of DKD. Treatment of db/db mice (a mouse model of obesity) with nonspecific (24) and specific SGLT-2 inhibitors (25), improved albuminuria and histological features of DKD. Similar findings were noted in streptozotocin induced diabetes (26–28) and Akita mice (29) (both models of T1DM). Also, in BTBR ob/ob mice (30) and T2DN rats (31) (models for T2DM), treatment with a SGLT-2 inhibitor resulted in decreased albuminuria and histopathological improvements, indicating decreased renal injury. In human registration phase-III trials, SGLT-2 inhibitors were associated with reduced albuminuria in T2DM (32,33). These data were confirmed and expanded in a pooled analysis of four phase-III trials of empagliflozin, which showed a significant reduction in microalbuminuria by 30% after 24 weeks of treatment (34). Larger renal outcome studies with SGLT-2 inhibitors are currently ongoing which focuses on albuminuria and estimated GFR.

Mechanisms of SGLT-2 inhibitors

The exact mechanism underlying the beneficial effects of SGLT-2 inhibitors on the renal system in T2DM remains to be elucidated. Phase-III trials demonstrate a reduction in body weight of about 1.80 kg −most likely due to osmotic diuresis and loss of calories− and systolic blood pressure (4.45 mmHg reduction versus active comparators) (23). In addition, preclinical studies (27–29,35) and human T1DM data indicate that SGLT-2 inhibitors reduce glomerular hyperfiltration (36). Hyperfiltration in diabetes may be explained by proximal sodium hyperreabsorption resulting in reduced sodium delivery at the distally located macula densa, leading to afferent vasodilation and increased glomerular pressure via tubuloglomerular feedback (37). Blockage of SGLT-2 may decrease proximal sodium reabsorption, thereby potentially restoring this maladaptive response (36,38). Interestingly, during the first weeks of SGLT-2 inhibitor treatment, the estimated GFR decreases in T2DM patients with moderate renal impairment, followed by long-term stabilization (32,38,39). Comparable changes in GFR have been observed for RAS inhibitors and were proven to be beneficial (38). Furthermore, since SGLT-2 inhibitors increase RAS activity comparable to other diuretic agents, it has been suggested that combined SGLT-2 and RAS inhibition yield additive renoprotective effects (36). SGLT-2 inhibitors may also diminish glucose influx of proximal renal tubular cells, thereby potentially reducing glucotoxicity and consequent inflammation and fibrosis in the diabetic kidney (38,40,41). Finally, SGLT-2 inhibitors increase the −urine measured− potential renoprotective enzyme ACE2 (42).

Conclusion

In conclusion, the global prevalence of DKD is increasing. Current pharmacological management to prevent renal disease in T2DM consists of antihyperglycemic agents, RAS-interfering agents and statins. However, these interventions do not prevent the development and progression of DKD in many patients, indicating an unmet need for additional renoprotective therapies. Based on preclinical and emerging clinical studies in patients with diabetes, SGLT-2 inhibitors are regarded a promising option to reduce the DKD burden. Conversely, to date, the potential renoprotective mechanisms underlying these beneficial effects have not been detailed in T2DM.

2. STUDY OBJECTIVES

Hypothesis

Treatment with the SGLT-2 inhibitor dapagliflozin, as compared to the sulfonylurea (SU) derivative gliclazide, may confer renoprotection by improving renal hemodynamics, and decreasing blood pressure and body weight in type 2 diabetes.

Primary Objective

To investigate the effects of 12-week treatment with the SGLT-2 inhibitor dapagliflozin (10 mg QD) versus the SU gliclazide (30 mg QD) on **renal hemodynamics** in metformin-treated T2DM patients, measured as:

- GFR (measured by the inulin or iohexol-clearance technique)
- Effective renal plasma flow (ERPF; measured by the para-aminohippurate acid (PAH) clearance technique)

Secondary Objectives

To investigate the effects of 12-week treatment with the SGLT-2 inhibitor dapagliflozin (10 mg QD) versus the SU gliclazide (30 mg QD) on

- Renal tubular function, measured as:
  - 24-h urine sodium-, potassium-, chloride-, calcium-, magnesium-, phosphate-, urea and glucose
- Renal damage, measured by urine biomarkers as:
  - UAE (Glomerular)
  - Neutrophil gelatinase-associated lipocalin (NGAL) and Kidney injury molecule-1 (KIM-1) (Tubular)
- Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) (measured by oscillometric blood pressure device)
- Heart rate (measured by oscillometric blood pressure device)
- Body anthropometrics: waist circumference, height, body weight and body mass index (BMI) (using the formula: weight (in kg) / height^2 (in m)), measured by a tape measure and calibrated weighing scale respectively
- Body fat content, measured by bio-impedance analysis (BIA)
- Marker of inflammation: high sensitivity C-reactive protein (hs-CRP)
- Glycemic variables: Glycated hemoglobin (HbA_{1c}) and fasting plasma glucose (FPG)
- Lipid spectrum (triglycerides (TG), total-cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) and free fatty acids (FFA))
- Microvascular function, measured by capillary videomicroscopy and Laser Doppler techniques
- Arterial stiffness (Pulse Wave Analysis), measured by applanation tonometry
- Systemic hemodynamic variables (SBP, DBP, MAP, heart rate (HR), stroke volume (SV), cardiac output (CO)/-index (CI), and total systemic vascular resistance (TPR)) derived from non-invasive beat-to-beat finger blood pressure measurements
- Cardiac autonomic nervous system (CANS) function, as derived from electrocardiographic (ECG) and non-invasive beat-to-beat finger blood pressure measurements (NexFin®) measured as:
  - Heart rate variability (HRV); Cardiovascular autonomic reflex tests (CARTs)

Exploratory Objectives

To investigate the effects of 12-week treatment with the SGLT-2 inhibitor dapagliflozin (10 mg QD) versus the SU gliclazide (30 mg QD) on:

- Complementary markers of renal function/damage (plasma cystatin C, fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH), soluble Klotho, urinary transforming growth factor-β1 (TGF-β1), collagen type IV, nephrin, podocin, microparticles, uric acid, 8-hydroxy-2' -deoxyguanosine (8-OHdG), Calcitriol, Monocyte Chemoattractant Protein-1 (MCP-1), tumor necrosis factor alpha (TNF-α)
- Additional markers of inflammation (interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1)
- (Cardiovascular)-biomarkers (N-terminal pro-B-Type Natriuretic Peptide (NT-proBNP), Brain Natriuretic Peptide (BNP), Atrial Natriuretic Peptide (ANP), urinary angiotensinogen, plasma renin

activity (PRA), angiotensin II, aldosterone, catecholamines, insulin, C-peptide, soluble receptor for advanced glycation end products (sRAGE)

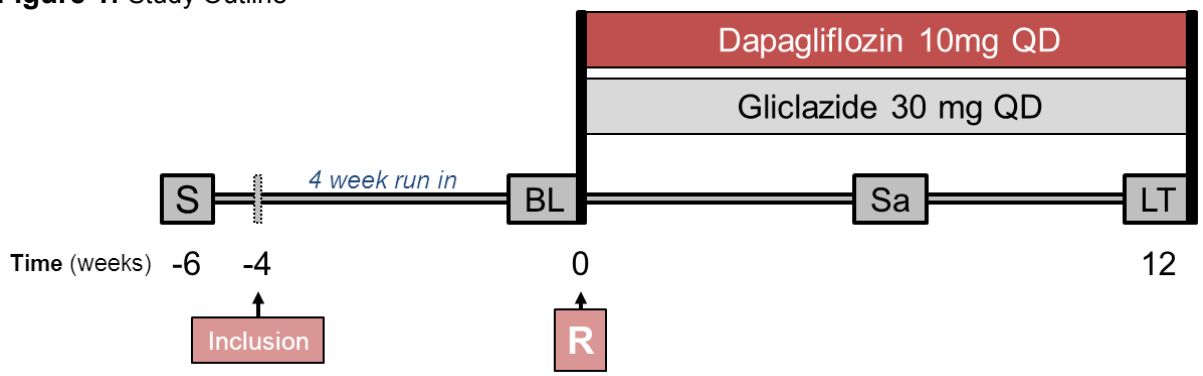
- Deoxyribonucleic acid (DNA) (to study the influence of genetic factors on the parameters measured and the response to SGLT-2 inhibitor)
- Gut microbiome composition
- Insulin sensitivity as derived from the euglycemic clamp
- Beta-cell function measures as derived from the hyperglycemic clamp

The extent of these complementary measurements is conditional to available budget.

3. STUDY DESIGN

A phase 4, monocenter, randomized, double-blind, comparator-controlled, parallel-group, mechanistic intervention trial to assess the effect of 12-week treatment with the sodium-glucose linked transporters (SGLT)-2 inhibitor dapagliflozin versus the sulfonylurea (SU) derivative gliclazide on renal physiology and biomarkers in metformin-treated patients with type 2 diabetes mellitus (T2DM)

Figure 1: Study Outline



BL = Baseline tests performed to assess study objectives at baseline
LT = Long-term follow-up tests performed to assess study objective at end-point (i.e. after 8 weeks of intervention)
R = Randomization
S = Screening visit and eligibility assessment
Sa = Safety visit and measurements

4. STUDY POPULATION

Subjects

Patients with previously diagnosed T2DM according to American Diabetes Association (ADA) criteria (43) .

Inclusion criteria

- Caucasian*
- Both genders (females must be post-menopausal; no menses >1 year; in case of doubt, Follicle-Stimulating Hormone (FSH) will be determined with cut-off defined as >31 U/L)
- Age: 35 - 75 years
- BMI: >25 kg/m^2
- HbA_{1c}: 6.5 – 9.0% Diabetes Control and Complications Trial (DCCT) or 48 - 86 mmol/mol International Federation of Clinical Chemistry (IFCC)
- Treatment with a stable dose of oral antihyperglycemic agents for at least 3 months prior to inclusion
- Metformin monotherapy
- Combination of metformin and low dose SU derivative**
- Hypertension should be controlled, i.e. ≤140/90 mmHg, and treated with an ACE-I or ARB (unless prevented by side effect) for at least 3 months.
- Albuminuria should be treated with a RAS-interfering agent (ACE-I or ARB) for at least 3 months.
- Written informed consent

* In order to increase homogeneity

** In order to accelerate inclusion, patients using combined metformin/SU derivative will be considered. In these patients, a 12 week wash-out period of the SU derivative will be observed, only when combined use has led to a HbA_{1c} <8% at screening. Subsequently, patients will be eligible to enter the study, now using metformin monotherapy, provided that HbA_{1c} still meets inclusion criteria.

Exclusion criteria

- History of unstable or rapidly progressing renal disease
- Macroalbuminuria; defined as ACR of 300mg/g.
- Estimated GFR <60 mL/min/1.73m^2 (determined by the Modification of Diet in Renal Disease (CKD-EPI) study equation)
- Current/chronic use of the following medication: TZD, SU derivative, GLP-1RA, DPP-4I, SGLT-2 inhibitors, glucocorticoids, immune suppressants, antimicrobial agents, chemotherapeutics, antipsychotics, tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs). Subjects on diuretics will only be excluded when these drugs cannot be stopped for the duration of the study.
- Volume depleted patients. Patients at risk for volume depletion due to co-existing conditions or concomitant medications, such as loop diuretics should have careful monitoring of their volume status.
- Chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) will not be allowed, unless used as incidental medication (1-2 tablets) for non-chronic indications (i.e. sports injury, head-ache or back ache). However, no such drugs can be taken within a time-frame of 2 weeks prior to renal-testing
- History of diabetic ketoacidosis (DKA) requiring medical intervention (eg, emergency room visit and/or hospitalization) within 1 month prior to the Screening visit.
- Current urinary tract infection and active nephritis
- Recent (<6 months) history of cardiovascular disease, including:
    - Acute coronary syndrome
    - Chronic heart failure (New York Heart Association grade II-IV)
    - Stroke or transient ischemic neurologic disorder
- Complaints compatible with neurogenic bladder and/or incomplete bladder emptying (as determined by ultrasonic bladder scan)
- Severe hepatic insufficiency and/or significant abnormal liver function defined as aspartate aminotransferase (AST) >3x upper limit of normal (ULN) and/or alanine aminotransferase (ALT) >3x ULN
- (Unstable) thyroid disease; defined as fT4 outside of laboratory reference values or change in treatment within 3 months prior to screening visit
- History of or actual malignancy (except basal cell carcinoma)

- History of or actual severe mental disease
- Substance abuse (alcohol: defined as >4 units/day)
- Allergy to any of the agents used in the study
- Individuals who are investigator site personnel, directly affiliated with the study, or are immediate (spouse, parent, child, or sibling, whether biological or legally adopted) family of investigator site personnel directly affiliated with the study
- Inability to understand the study protocol or give informed consent

5. STUDY DRUGS

This paragraph will briefly address the different agents. For more information we refer to the included Summary of Product Characteristics (SPCs).

Study Medication

In addition to their ongoing oral antihyperglycemic medication (metformin only) and drugs for cardiovascular risk management (e.g. RAS-interfering agent and/or statin), subjects will be assigned to oral treatment with either encapsulated dapagliflozin or gliclazide (see Table 2).

Table 2: Overview treatment allocation

Group	Treatment	Number of subjects
I	Dapagliflozin 10 mg QD	22
II	Gliclazide 30 mg QD	22

QD, once daily

Dapagliflozin *(Forxiga® by AstraZeneca)*
The SGLT-2 inhibitor Forxiga (dapagliflozin tablet à 10 mg) will be used, i.e. a yellow, biconvex, approximately 1.1 x 0.8 cm diagonally diamond-shaped, film-coated tablets with “10” engraved on one side and “1428” engraved on the other side (44). The agent will be purchased commercially and masked by encapsulation (see below), such that this drug will be visually undistinguishable from encapsulated gliclazide.. Following randomization, participants will start using dapagliflozin 10 mg once daily (QD), titration is not necessary. Dapagliflozin will be taken orally, with or without food, preferably in the evening.

Gliclazide (*Diamicron® by Servier)*
A generic SU derivative of gliclazide (tablet à 30 mg) will be used, i.e. a white oblong tablet of 10 mm x 5 mm x 2,5 mm, with "DIA 30" engraved on one side and on the other side (45). The agent will be purchased commercially and masked by encapsulation (see below), such that this drug will be visually undistinguishable from encapsulated dapagliflozin. Following randomization, participants will start using gliclazide 30 mg QD, titration is not necessary. Gliclazide will be taken orally, with or without food, preferably in the evening.

Test agents

Following the meal and during the renal-test procedures, three medicinal products will be used.

- Renal test = inulin or iohexol/PAH-clearance. Inulin is approved by the Austrian drug authority, iohexol is produced by GE Healthcare and marketed under the brand name Omnipaque, and PAH is produced by Bachem in GMP-grade and the product will undergo quality assurance prior to administration (46). These agents are widely used for the determination of renal hemodynamics.
- Short-acting insulin (Novorapid®, obtained from Novo Nordisk) will be infused during the euglycemic clamp. The drug is widely used in patients with diabetes and is FDA approved.
- Arginine 10% HCl will be infused during the hyperglycemic clamp after which insulin response will be measured. It is produced by our in house pharmacy and widely used in trials to determine beta cell function.

Detailed information regarding these study agents can be found in Appendix B and in corresponding SPCs.

Randomization

The blinded treatment assignment will be performed by the trial pharmacists of VU University Medical Center (who have ample experience with this process), in order to maintain blinding for investigator, site personnel and patients:

- Block randomization will be performed with
  - Allocation ratio of 1:1
  - Block-size of 4
  - Stratification for ACE-I or ARB (yes/no) therapy

It must be noted that the ‘Apotheek A15’ will provide randomization lists for encapsulated dapagliflozin and gliclazide for this trial.

Labeling, Drug dispensation and Accountability

All trial drugs and the Total Dispensing Unit lists will be received and handled by the trial pharmacist. Patients will receive a capsule containing either dapagliflozin or gliclazide . To be undistinguishable and packed blinded, the Total Dispensing Unit list is needed for dispensing trial products. Dapagliflozin and gliclazide will be encapsulated by an external GMP (Good Manufacturing Practice)-certified organization (A15 pharmacy). The trial products will be provided in labeled and specific boxes packages with participant identifying information to our institutional trial pharmacist who, subsequently, will store the trial drugs until they are needed. The trial pharmacist will deliver the study medication to the research personnel (who remain blinded). These will then dispense the study medication to the participants at the designated visit (visit 2, see Appendix A), combined with extensive instructions on use and administration of the study drugs. The number of daily dosages dispensed will be sufficient to cover the complete expenditure of the study. No trial products will be dispensed to any person not enrolled in the trial.
On the designated day (visit 3; see Appendix A), the participants will bring their trial medication. In order to perform proper drug accountability, the investigator will keep track of all received, used and unused trial products and -if possible- all empty packaging.

Drug storage

Both dapagliflozin (44) and gliclazide (45) do not require any special storage conditions. Nevertheless, when not distributed to participants, drugs will be stored at the institutional trial pharmacy. Keeping a temperature log document, proper storage conditions will be ensured and records and evaluations will be kept as required by International Conference on Harmonisation – Good Clinical Practice (ICH-GCP). Storage facilities will be checked frequently (temperature at least every working day).

Blinding and Unblinding

Throughout the study duration, all study personnel will remain blinded with regard to the medication used. The randomization –in order to achieve blinding for study personnel- will be performed by the trial pharmacist of the VU Medical Center. The investigator will receive the subjects blinding information concerning the study medication in the form of a sealed envelope; this envelope will be stored on a secure location in the Diabetes Center VU University Medical Center (VUMC). The blind shall not be broken by the investigator unless information concerning the study medication is necessary for the medical treatment of the subjects. Procedures regarding emergency unblinding are described in Chapter 10. If the investigator is unblinded, study medication will be stopped immediately in addition to the withdrawal of the study subject from the study.
At the end of the study, following the last patients last visit and data-base lock (which will only occur after medical/scientific review), the blind will be broken by the trial pharmacist.

6. STUDY OUTLINE

Recruitment

Subjects will be recruited by researcher physicians involved in this trial using methods that are established practice for all human studies in the Diabetes Center VUMC:

1) Via advertisements in (local) newspapers
2) Use of a Diabetes Center database to approach volunteers from previous studies, who have given written informed consent to be available for future studies
3) Through the Diabetes Center website (www.diabetescentrum.nl)
4) Via general practitioner offices with whom we have collaborations, we will identify potential participants who will be approached by the general practitioner
5) Where possible, subjects will be recruited from the out-patient clinic of the Diabetes Center / Department of Internal Medicine VUMC or affiliated hospitals / pharmacies

In case of a positive response, the information letter and informed consent forms will be sent to these individuals. They will then be contacted by telephone by the research physician to answer any remaining questions and make an appointment for a screening visit if the individual wants to participate.

Screening and eligibility (visit 1 and T1)

After giving extensive oral and written information, a written informed consent form will be obtained from the subjects before screening. Once the participant signs the informed consent form he/she will be assigned a unique study number which will be used for identification.

The screening procedure will consist of obtaining an extensive medical history (including disease history, current medical conditions and use of medication, substance use, employment, education, family history, self-reported birth weight), complete physical examination, drawing blood for hematologic and biochemistry testing (Full blood count, glucose, HbA_{1c}, creatinine, AST, ALT, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) and thyroid-stimulating hormone (TSH)), urine screening, urine creatinine and albumin, and a 12-lead electrocardiogram (ECG).

Table 3: Timetable of end-point assessments performed at baseline and 12-week drug intervention

	Visit 2	Visit 3	Visit 4
0700	Renal Protocol and Cardiovascular Tests		Renal Protocol and Cardiovascular Tests
0800		Safety evaluation + measurements	
0900			
1000			
1100			
1200			
1300			
1400			
1500			
1600			
1700	Randomization + Instructions		

Postvoid residual urine volume will be determined by ultrasonic bladder scan. In females, the menopausal status will be verified by history taking (no menses > 1 year) and in case of doubt measurement of FSH-level (cut-off defined as >31 U/L). If subjects are eligible, the investigator will inform them within two weeks after screening (T1, see Appendix A). After inclusion, participants will enroll in a 4-week run-in period to allow for study effects.

Endpoint visits: Visit 2 (baseline) and Visit 4 (long-term follow-up)

During the baseline measurements (visit 2; Table 3 and Appendix A), various tests will be performed to obtain baseline values for the predefined endpoints. The baseline measurements of the renal system, as well as the cardiovascular assessments, will be performed during one test visit and serve as reference values. The endpoint assessments will be repeated during visit 4 (Table 3 and Appendix A).

Visit 2 (baseline assessments)

Seven days prior to visit 2, subjects will adhere to a `normal-salt' (9 - 12 grams or 150 - 200 mmol per day) and protein (1.5 - 2.0 g/kg per day) diets, in order to minimize variation in renal physiology due to salt and protein intake (47,48). Participants will abstain from alcohol (24 hours), caffeine (12 hours) and nicotine (12 hours) and heavy exercise prior to and during visits 2 (49). One day prior to the testing day, subjects will collect 24-hour urine.

Following an overnight fast (10 hours prior to the start of investigation), participants will be instructed to drink 500 mL of water, but to delay all morning-time medication (apart from metformin) until conclusion of the examination day. Subjects will arrive at the Clinical Research Unit (CRU) at 07.30 AM. After taking subjects history, current weight and blood pressure, intravenous catheters will be placed in both forearms after which blood and urine will be collected (see Figure 2). Then, subjects will assume a semi-recumbent position throughout the duration of the visit and after a resting period, an automatic blood pressure measurement is performed. Thereupon the combined renal and clamp protocol will commence (Chapter 7, sections A and B). During the first equilibration period, body fat content assessment and the cardiovascular tests are carried out.

Figure 2: Schematic representation of measurements during visits 2 and 4

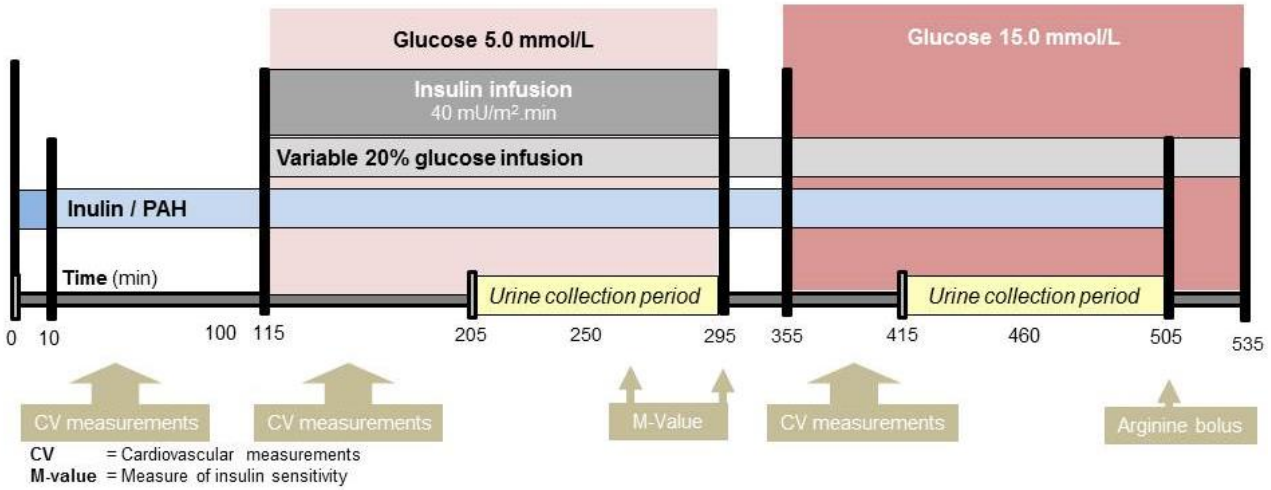


Figure 2. During the renal testing days, renal hemodynamics will be evaluated in fasting state, during an euglycemic-hyperinsulinemic clamp and a hyperglycemic clamp. Plasma glucose levels will be kept at 5.0 mmol/L and 15.0 mmol/L, respectively. Total duration of the visits: approximately 10 hours.

Safety and tolerability: Telephone consultation 2 and Visit 3 (follow-up)

The 12-week intervention study includes a telephone consultation (T2 at week 2) and a follow-up visit (Visit 3 at week 6) (see Table 3 and Appendix A). During T2 and visit 3, participants will be asked about the occurrence of adverse events and the intake of study drugs will be checked. Furthermore, recent history will be taken, physical examination/anthropometric measurements performed and blood and urine samples collected.

Early-term assessments

At any time during the course of the study, all subjects have the right to withdraw without providing a reason, as required by ICH-GCP and Dutch laws. When participants decide to withdraw their participation, they will be asked to participate in 'early-term assessments'. During these assessments, some end-point parameters will be measured, which can possibly be used for final data analyses (see Appendix A).

Withdrawal from study

After subjects have entered the study, participation will be stopped in case of occurrence of any of the following:

- Participants own decision
- The occurrence of an AE or clinically significant laboratory change or abnormality that, in the judgment of the investigator, warrants withdrawal of the study
- Subjects need medical procedures that are not allowed in the protocol
- Subjects cannot undergo the procedures to investigate the research questions as outlined in this protocol
- Non-compliance
- In addition to these requirements for study drug discontinuation, the investigator should discontinue study participation for a given subject if, on balance, he thinks that continuation would be detrimental to the subjects well-being

The study physician can be guided by the criteria above, but may discontinue participation at any time based on his/her clinical judgment.

Study Completion

The study will be considered completed for an individual participant when he/she completes the final tests (visit 4, i.e. the last day of the 12-week end-point assessments). The study as a whole will be considered completed when all planned randomized subjects have completed the treatment phase, followed by the final measurements.

7. STUDY PROCEDURES

This paragraph provides short definitions of the study procedures used in this study. Detailed information or 'standard operation procedures' (SOPs) for most of the procedures can be found in the respective appendices.

A. Renal protocol (visit 2 and 4):
During this protocol a combined inulin or iohexol/PAH-clearance technique will be performed to examine the GFR and ERPF respectively (36,50–52). In order to examine tubular function, sodium, potassium, chloride, calcium, magnesium, phosphate, urea and glucose will be measured in urine. In addition, urine osmolality will be determined. Moreover, biomarkers of renal damage (UAE, NGAL and KIM-1) will be measured in urine (see Appendix C1).

B. Clamp protocol
A combined hyperinsulinemic-euglycemic and hyperglycemic clamp will be performed. During the euglycemic clamp insulin will be infused at 40 mU/m^2.min and glucose will be kept at 5 mmol/l by variable glucose 20% infusion. This technique allows for renal measurements during normoglycemia in all participants. After 3 hours of insulin infusion and an one hour rest period for exogenous insulin to be cleared, glucose will be infused in order to reach a plasma concentration of 15 mmol/l. In this matter, renal tests can be performed under stable hyperglycemia (see Appendix C2). From the euglycemic clamp, insulin sensitivity will be determined from the amount of glucose infused (M-value). Blood will be stored during hyperglycemia in order to be able to obtain measures of beta-cell function, including 1st phase insulin secretion, 2nd phase insulin secretion and arginine-induced insulin secretion.

C. Blood pressure (visit 1, 2, 3 and 4):
While the auscultatory method (sphygmomanometry) might be considered the gold standard, it is hampered by intra- and inter-observer variability. Because blood pressure measurements will be performed on multiple occasions, we will use an automatic oscillometric device (Dinamap®, GE Healthcare) during the entire study to reduce possible bias (53,54). After an acclimatization period of >5 minutes, blood pressure will be measured three times at the non-dominant arm, with the subject in a semi-recumbent position, using an appropriate cuff-size. The mean of the last 2 measurements will be used as final value (53,54).
Should the automated technique not be possible or yields erroneous results, the auscultatory technique will be used, using a sphygmomanometer according to methods of Riva-Rocci. SBP is defined as the appearance of vascular tones (Korotkoff sound 1), DBP as the disappearance (Korotkoff sound 5) (54). To assess the differences in automatic versus auscultatory techniques, we will perform the latter in the fasted state during the baseline- and long-term follow-up assessments.

D. Body composition (visit 1, 2, 3 and 4):
Waist circumference will be assessed by a constant tension tape with the patient in a standing position with the arms relaxed at the sides and without clothing, that is, directly over the skin. Measurement will be performed at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest at the end of a normal expiration (55).
A height measuring board will be used to assess height. Patients will be asked to remove their footwear and headgear and to stand on the board with their feet together, heels against the back board and knees straight. The patient should be looking straight ahead, without tilting the head up, with eyes and ears at the same level. The measure arm will gently be moved down onto the head of the participant and height will be read in centimeters at the exact point.
Body weight will be recorded by a digital calibrated weighing scale on a firm, flat surface. Patient will be asked to remove their footwear, extra layers of clothing, jewelry and any items in his/her pockets before standing still on the weighing scale with arms placed on the side. Measurement will be recorded on the Case Report Form (CRF) to the nearest kilogram. No deduction for clothing will be permitted.
BMI will be derived from height and body weight by using the mathematical formula: BMI = [kg] / [m]^2.

E. Body fat content: (visit 2 and 4):
To measure body fat content we will use the tetrapolar Soft Tissue Analyzer® (STA, Akern, Florence, Italy), which can perform BIA. This technique measures the resistance in the body tissues when a 50

kHz AC electrical current is given between four electrodes. Resistance can be measured by placing two electrodes on the dorsal hand and two on the foot at the unilateral side of the body (56). Extracellular water (ECW) as a percentage of total body water (TBW) and body cell mass (BCM) will be calculated independent of weight and height.

F. Systemic hemodynamics (visit 2 and 4):
To test drug-related changes on the cardiovascular system we will measure hemodynamic variables (including stroke volume, cardiac output, total peripheral resistance) non-invasively. For this, an automated, beat-to-beat blood pressure and ECG recording monitor (Nexfin®, BM Eye, Amsterdam, The Netherlands (57) will be used.

G. Heart rate (visit 1, 2, 3 and 4):
Heart rate will be measured using the automated oscillometric blood pressure device. Should the automated technique not be possible or yield erroneous results, heart rate will be assessed by counting the radial pulse rate during 30 seconds and multiplying the outcome by two.

H. Microvascular function (visit 2 and 4):
Microvascular function will be measured by nail fold skin capillary density on the middle finger, before and after 4 minutes of arterial occlusion with a digital cuff, yielding data on baseline capillary density and post-occlusive reactive hyperemia, respectively. Moreover, vasomotion analyses will be performed, in which skin blood flow will be measured using a Laser Doppler probe (58,59) (see Appendix D).

I. Pulse Wave Analysis (PWA) (visit 2 and 4):
Applanation tonometry using the SphygmoCor® system (Atcor Medical, West Ryde, Australia) will be performed to measure variables of arterial stiffness. For PWA, the tonometer will be applied to the radial artery of the non-dominant arm. The SphygmoCor® system will calculate the Augmentation index (AIx) from the pulse waves (see Appendix E) (60–62).

J. CANS function (visit 2 and 4):
To test drug-related changes in the autonomic nervous function and their impact on the cardiovascular system we will measure resting heart rate variability (HRV) (63) and perform CARTs, a modified battery of the Ewing tests (64). Using an automated, beat-to-beat blood pressure and ECG recording monitor (Nexfin®, BM Eye, Amsterdam, The Netherlands (57) heart rate and blood pressure will be recorded during: resting, taking deep breaths, exhalation into a mouth-piece with a measurable resistance (Valsalva maneuver) and standing up from a supine position. Information regarding changes in parasympathetic and sympathetic nervous system function can be derived from the measurements using dedicated software (65–67) (see Appendix F).

K. ECG (visit 1):
A 12-leads ECG will be made in the fasting resting state during screening in order to assess cardiac conduction parameters. Heart rate on the ECG will be defined as the mean rate of 10 R-R intervals. PQ-time, QRS-time and QT-time will be calculated, QT-time will be corrected for heart rate by using the Bazett formula (68). The cardiac vector and any changes in ECG morphology will be assessed.

L. Other (potential) analytes in blood/urine:
In addition to the determination from blood samples as outlined for the above-mentioned procedures, additional blood and plasma samples will be collected and stored to determine relevant (emerging) biomarkers of renal / cardiovascular damage. The extent of these additional measurements are conditional to available budget:

Renal damage	Conditional: Plasma cystatin C, FGF23, PTH, calcitriol, soluble Klotho, urinary TGF-β1, collagen type IV, nephrin, podocin, microparticles, urinary 8-OHdG, urinary TNF-α

(Cardiovascular) biomarker	Conditional: NT-proBNP, BNP, ANP, PRA, angiotensin II, angiotensin 1-7, aldosterone, endothelin, catecholamines, insulin, C-peptide, GLP-1, glucagon, GIP, sRAGE, urinary angiotensinogen, uric acid
Inflammation	hsCRP, Conditional: interleukin 6 (IL-6), PAI-1, urinary MCP-1
Genetic sampling	Conditional: DNA

M. Fecal examinations:

Each participant will collect a stool sample before the baseline tests and once before the long-term follow-up tests. At home, one feces sample must be stored at room temperature and one in the freezer and when arrived at the CRU, the feces will be stored at -20^{0}C and -80^{0}C, respectively. Stool samples will be stored for possible analysis of biomarkers, including calprotectin (69). Since it is becoming increasingly clear that the gut microbiome might be involved in the development of obesity and T2DM (70) and CKD disease (71,72), we will also store and analyze feces for microbial content (94). (see Appendix G).

8. LABORATORY ASSESSMENTS

Sample collection and testing

During the study blood and urine will be collected at various time points (see Appendix A). Blood sampling will be performed by drawing blood from intravenous catheters during the test days, or, when no catheter is placed (e.g. during the screening (V1) and safety/follow-up visit (V3), by venipuncture. 24-hour urine and spot urine will be collected using designated non-sterile containers.
All procedures regarding biosample collection as well as the necessary administration will be in line with ICH-Good Clinical Practice (GCP)/ Good Laboratory Practice (GLP).

Screening and Safety Measurements

During the screening visit (V1) hematology (hemoglobin (Hb), hematocrit (Ht), leucocytes and thrombocytes) and chemistry (glucose, HbA_{1c}, AST, ALT, GGT, ALP, creatinine, TSH and FSH (in females only, in case of doubt)). In addition, urine will be screened using a urine test strip. These parameters will be used to assess eligibility.
At visit 3, i.e. after 6 weeks of treatment blood samples will be obtained to assess hematology (Hb, Ht, leucocytes and thrombocytes) and chemistry (sodium, potassium, creatinine, BUN, AST, ALT, GGT, ALP) markers.

Endpoint Measurements

Throughout the study blood will be drawn for endpoint assessments at various time points. Many biochemical and hematological analyses will be performed directly. Blood for biomarkers will be centrifuged and plasma/serum will be stored at -80°C. An overview of the different endpoint laboratory measurements is merged with the efficacy measurements in Chapter 9.

Blood volume

The total amount of drawn blood is detailed in Table 4.

Table 4: Description of estimated drawn blood volume

Test	Screening -4 wk	Baseline 0 wk	Safety 6 wk	Long-term 12 wk	Total
Screening	11 mL				**11** mL
Control visit			7 mL		**7** mL
Chemistry/Hematology		25 mL		25 mL	**50** mL
Renal protocol		52 mL		52 mL	**104** mL
Clamp protocol		90 mL		90 mL	**180** mL
Storage samples*		71 mL		71 mL	**142** mL
Genetic sampling		6 mL			**6** mL
Total	**11** mL	**244** mL	**7** mL	**238** mL	**500** mL

***Storage samples** for determination of additional (cardiovascular)biomarkers connected to the study objectives (depending on study budget)

9. EFFICACY MEASUREMENTS

Primary Efficacy Measures

Renal hemodynamics

Inulin-, iohexol- and PAH- measurements will be performed in a fasting state and during euglycemic- and hyperglycemic clamps in a semi-recumbent position at visits 2 (baseline) and 4 (week 12). Both plasma and urine samples will be collected and stored for determination of inulin or iohexol/PAH levels, which will be used for the calculation of the GFR and ERPF. This can be corrected for hematocrit to calculate renal blood flow (RBF). Filtration fraction (FF) can be calculated by dividing GFR by ERPF. In addition, effective renal vascular resistance (ERVR) can be calculated (for specifics, see Appendix C1).

Secondary Efficacy Measures

Renal Tubular Function

Prior to visits 2 (baseline) and 4 (week 12), subject will collect 24-hour urine for measurements of sodium, potassium, chloride, calcium, magnesium, phosphate, urea and glucose. Furthermore, urine osmolality is measured. These measurements are all markers for renal tubular function.

Renal damage

Prior to visits 2 (baseline) and 4 (week 12), subject will collect 24-hour urine for measurement of microalbuminuria. In addition, urine will be stored for subsequent measurements of NGAL and KIM-1 (tubular damage).

Blood Pressure

Oscillometric measurements for assessment of blood pressure will be performed at visits 2 (baseline), 3 and 4 (week 12). The mean of the last 2 out of 3 measurements will be used as final outcome parameter.

Heart rate

Oscillometric measurements for assessment of heart rate will be performed at visits 2 (baseline), 3 (week 6) and 4 (week 12). The mean of the last 2 out of 3 measurements will be used as outcome parameter.

Body anthropometrics and Body fat content

Measurements for anthropometrics and body composition will be performed at visits 2 (baseline), 3 (week 6) and 4 (week 12). Weight will be recorded using a weighing scale, waist circumference will be measured using a tape measurement and body fat content will be assessed by BIA. The latter will produce different variables independent of weight or height (ECW, TBW and BCM).

Glycemic variables, Lipid spectrum, Inflammation, Biochemistry and Hematology

Blood will be drawn for direct determination of fasting glucose, HbA_{1c}, FPG, TG, TC, HDL-C,LDL-C, FFA and hs-CRP at visits 2 (baseline), 3 (week 6) and 4 (week 12). Furthermore, different end-point assessments at visits 2 (baseline) and 4 (long-term; week 12) will serve as safety measurement at visit 3 (week 6). To this end, blood chemistry (sodium, potassium, creatinine, ALT, AST, GGT, ALP) and hematology (Hb, Ht, leukocytes, thrombocytes) will be determined.

Microvascular function

Measurements for assessing microvascular function will be performed at visits 2 (baseline) and 4 (week 12). Capillary videomicroscopy will be used to assess capillary density and capillary recruitment (after arterial occlusion). Laser Doppler will be applied to measure skin blood flow to define microvascular vasomotion (for specifics, see Appendix D).

Arterial stiffness (PWA)

Applanation tonometry will be performed at visits 2 (baseline) and 4 (week 12) in order to measure PWA (a collection of variables: augmentation pressure, augmentation index, ejection duration and subendocardial viability ratio; for specifics, see Appendix E).

Systemic hemodynamics
Non-invasive beat-to-beat finger blood pressure measurements will be performed at visits 2 (baseline) and 4 (week 12) for measurement of systemic hemodynamic variables (systolic and diastolic blood pressure, mean arterial pressure, heart rate, stroke volume, cardiac output/-index, systemic total peripheral resistance).

CANS function
ECG and non-invasive beat-to-beat finger blood pressure measurements will be performed at visit 2 (baseline) and 4 (week 12) during a time-period of 7 minutes following rest of at least 10 minutes in a semi-recumbent position, for determination of resting heart rate variability (HRV) defined by the High Frequency (HF) component, Low Frequency (LF) component and their ratio, as attained from Fourier analysis, in order to assess changes in the balance of the parasympathetic and sympathetic nervous system on resting heart rate due to study drugs. Identical measurements will be performed whilst performing the deep breathing, valsalva maneuver, and orthostasis in order to yield information on the cardiovascular autonomic reflexes (for specifics on cardiac ANS function, see Appendix F).

Exploratory Efficacy Measures

Biomarkers and gut microbiome
During visits 2 (baseline assessment), 3 (safety follow-up; week 6) and 4 (long-term assessment; week 12), blood will be drawn in appropriate test tubes and serum/plasma will be stored to measure (emerging) biomarkers (conditional to available budget). Also urine is obtained and stored to measure (emerging) biomarkers (conditional to available budget). Possible biomarkers include: plasma NT-proBNP, BNP, ANP, plasma cystatin C, FGF23, PTH, soluble Klotho, PRA, angiotensin II, aldosterone, endothelin, catecholamines, plasma insulin, C-peptide, lipids, GLP-1, glucagon, GIP, sRAGE, hs-CRP, IL-6, PAI-1, urinary TGF- β1, angiotensinogen, endothelin, nephrin, podocin, microparticles. Feces will be stored for potential gut microbiome analysis and biomarkers, such as calprotectin.

DNA
During visit 2 (baseline), blood will be collected and stored for possible DNA-analyses. This will only be performed after specific and separate consent by the participant. DNA can be used to study the influence of genetic factors on the parameters and the response measured to the SGLT-2 inhibitor.

Insulin sensitivity
During visit 2 and 4, insulin sensitivity will be measured by gold-standard euglycemic clamp. The M-value (metabolic clearance of glucose) will be calculated and expressed as mg/kg.min.

Beta-cell function
During hyperglycemia, insulin secretion will be quantified as measure for beta-cell function. The following parameters are obtained: 1st phase insulin secretion (early secretion), 2nd phase insulin secretion and arginine-induced insulin secretion (the latter as measured of total secretory capacity).

10. SAFETY (REPORTING)

In accordance with the guidelines of ICH-GCP, the European Clinical Trials Directive (2001/20/EC) and the local regulations, the investigator will inform the subjects and the reviewing Ethical Review Board (ERB) if anything occurs, on the basis of which it appears that the disadvantages of participation may be significantly greater than was foreseen in the research proposal.

Safety Evaluations

The following safety measures will be collected during the study (as outlined in Appendix A):

- Occurrence of adverse events (as reported by the subject)
- Vital signs (pulse rate, blood pressure, temperature)
- Different end-point assessments that also serve as safety measurements: blood chemistry and hematology.

Collection of adverse event information will begin at the signing of the informed consent and continues through 30 days after administration of the last dose of study medication.

Adverse Event

AEs are defined as any undesirable experience occurring in a clinical investigation subject during the study and which does not necessarily have a causal relationship with the treatment. All AEs reported spontaneously by the subject or observed by the investigator or study site staff will be recorded on special CRF. The AE term will be recorded using standard medical terminology (Medical Dictionary for Regulatory Activities; MedDRA™). Every attempt will be made to describe the AE in terms of a diagnosis. Whenever the investigator is confident in making a unifying diagnosis, all related signs, symptoms and abnormal test results will be grouped together and recorded as a single AE. All adverse events will be evaluated for intensity and causal relationship with use of the study medication (using the definitions in Table 5).

Table 5: Safety Definitions

Severity	
Mild	The event is transient and easily tolerated by the subject
Moderate	The event causes the subject discomfort and interrupts the subject's usual activities
Severe	The event causes considerable interference with the subject's usual activities
Causality	
Definite	An AE that follows a reasonable temporal sequence form administration of the drug (including the course after treatment withdrawal of the drug) AND that satisfies any of the following: • Reappearance of similar reaction upon readministration (rechallenge) • Positive results in a drug sensitivity test • Toxic level of the drug revealed by measurement of drug concentrations
Probable	An AE that follows a reasonable temporal sequence form administration of the drug (including the course after treatment withdrawal of the drug) AND for which involvement of factors other than the drug – such as underlying diseases, complications, concomitant drugs, and concurrent treatment – can reasonably be excluded.
Possible	An AE that follows a reasonable temporal sequence form administration of the drug (including the course after treatment withdrawal of the drug) AND for which involvement of factors other than the drug – such as underlying diseases, complications, concomitant drugs, and concurrent treatment – may also be responsible
Not related	An AE that follows a reasonable temporal sequence form administration of the drug or that can be reasonably explained by other factors, such as underlying diseases, complications, concomitant drugs, and concurrent treatment.

Serious Adverse Event (SAE)

A SAE is any untoward medical occurrence or effect that:

- Results in death
- Is life threatening (Note: the term "life-threatening" refers to an event/reaction in which the patient was at risk of death at the time of the event/reaction; it does not refer to an event/reaction which hypothetically might have caused death if it were more severe).
- Requires inpatient hospitalization or results in prolongation of existing hospitalization
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly or birth defect
- Is a medically important event or reaction. Medical and scientific and scientific judgment should be exercised in deciding whether other situations should be considered serious, such as important medical events that might not be immediately life-threatening or result in death or hospitalization, but might jeopardize the patient or might require intervention to prevent one of the other outcomes listed in the definition above.

It must be noted that the term "severe" is often used to describe the intensity (severity) of a specific event; the event itself, however, may be of relatively minor medical significance. By contrast, the term "serious" is used to describe an event based on a (possible) event outcome or actions usually associated with events that pose a threat to a patient's life or functioning.

Adverse Drug Reaction (ADR)

There is a reasonable possibility according to the Sponsor-Investigator that the product may have caused the event.

Suspected unexpected serious adverse reaction (SUSAR)

An ADR, the nature or severity of which is not consistent with the applicable product information (e.g. SPC). An expected ADR with fatal outcome should be considered unexpected.

AE of special interest (AESI)

An AE (serious or non-serious) of scientific and medical concern, specific to the product or program of study drug supplier (AstraZeneca), for which ongoing monitoring and rapid communication by the investigator may be appropriate. Please refer to the site master file for the current list of topics.

Reporting

Each SAE will be investigated thoroughly and an assessment will be made whether the SAE is a SUSAR (i.e. unexpected and drug-related, as indicated in the most recent SPC provided by the drug supplier). Because this study is an investigator sponsored trial (IST), the investigator also acts as sponsor. Therefore, the investigator will be responsible for reporting the SAEs and SUSARs to the accredited ERB. This will be done within 15 days after first knowledge, using the specified web portal ('ToetsingOnline'), which automatically leads to informing the EMA and the respective national health authorities. For fatal or life threatening SAEs and SUSARs this term will be 7 days for a preliminary report ('expedited') with another 8 days for completion of the report. The expedited reporting through the web portal 'ToetsingOnline' is sufficient as notification to the competent authority. Since this study is a monocenter study, no other sites have to be informed.

The involved pharmaceutical company (AstraZeneca) will be notified of all SAEs, pregnancy, clinical relevant overdosing and AESIs, if any. These events must be transmitted within 1 working day of the Investigator's awareness or identification of the event, using standardized forms. Reports will consist of patient identification (subject number, initials, sex, age), event (diagnosis), drug reporter identification (name, initials), causality and outcome and should be written in English. Results of any relevant complementary exams performed to obtain the final diagnosis of any SAE (e.g., hospital discharge summary, autopsy, consultation) will be made available to AstraZeneca entity upon request.

Contact details for safety reporting pharmaceutical company:
AEMailboxClinicalTrialTCS@AstraZeneca.com

Annual safety report

In addition to the expedited reporting of SAEs, the investigator will include a safety section containing an overview of all study SAEs in the periodic report, which will be submitted to the accredited ERB and the competent authorities once a year throughout the clinical trial.

Follow-up of AEs

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist. The follow-up information should describe whether the event has resolved or continues, if and how it was treated, and whether the patient continued or discontinued study participation.

11. DATA MANAGEMENT AND QUALITY ASSURANCE

Handling and storage of data and documents

In accordance with the Dutch law 'Wet Bescherming Persoonsgegevens' (WBP; Personal Data Protection Act) and GCP all patient data will be handled confidentially and anonymously. Therefore, all participants will get a study code ('RED-xxx'). The code list, with both identifier data and study number, will be stored securely on the server of the VUMC, protected by passwords only known by the responsible research physicians and principal investigator.

All subject data will be recorded on printed CRF by the authorized investigator (i.e. research physician) and/or personnel that is instructed in performing these administrative tasks and study procedures. In the latter case, data will always be reviewed by the investigator. All subject related data will be stored for 15 years. Subject data, both anonymous (e.g. CRFs) or not (e.g. source documents), will always be stored securely, in a locked cabinet in the Diabetes Center VUMC or on password secured computers.

Data Management

To ensure correct entrance of data from the CRF to the database which will be used for statistical analyses, a special database program called OpenClinica© (Community Edition, v3.1.2, OpenClinica©, LLC, Waltham, MA, USA) will be used. A study environment will be build, containing the specific visits and necessary data entry fields. Electronic-CRFs (eCRF) will be made, similar to the hardcopy CRFs. These eCRFs will be enhanced by safeguards and queries to warrant correct entry of data. The investigator, or his/her designated representative, will enter all data from the hardcopy printed CRF on the eCRFs, which automatically stores the data in the database. All changes made to the database will be logged ('audit trail'), which is required by GCP. On a regular basis all data from the OpenClinica©-database will be extracted to an Excel-file for backup purposes.

Monitoring

As required by GCP, a monitoring procedure will be performed in order to oversee the progress of the clinical trial and ensure that it is conducted, recorded and reported in accordance with the protocol, SOPs, GCP and the applicable regulatory requirements. Because the risk classification of the current study is estimated to be "moderate risk" (see Chapter 13), 2-3 monitor visits is required per year (73).

Monitoring will be performed by a GCP-certified assistant of the Clinical Research Bureau VUMC. The monitor will verify inclusion of patients, the use of the investigational medicinal products (e.g. drug accountability), source documents and reporting of adverse events according to a pre-defined format (for specifics, see Appendix H). The findings will be reported to the investigator.

12. STATISTICS

Sample Size Calculations

The primary end-point is change in GFR/ERPF after 12 weeks of treatment with dapagliflozin or gliclazide, as derived from the inulin or iohexol/PAH clearance test. Based on literature we used the following calculation to define our sample size: to detect a change in GFR from 89 mL/min to 75 mL/min (16%), SD 13.0 mL/min, assuming α = 0.05 (2-sided testing), a power (1-β) of 90% (36,39,74,75), we need 19 subjects per group. However, taking into account a maximum dropout percentage of 15%, we have chosen to increase the amount of subjects per group to N=22. This sample size was calculated using Stata-software (v.11.2, College Station, TX, USA).

Statistical Analysis Plan

General considerations

- All tests of differences between treatments will be conducted at a two-sided significance level of <0.05. In general, the null hypothesis (*H_0: no difference exists between the treatment groups*) will be tested against the hypothesis *H_1: a difference exists between treatment groups, with respect to the underlying parameter of interest.*
- All variables will be expressed as mean ± SEM when normally distributed, or -in case of non-normal distribution- be expressed as median and interquartile range (IQR). Log transformation will be applied to obtain normal distribution as appropriate for the statistical tests.
- In case of missing data, a multiple imputation technique will be applied and compared with complete case analysis.

Analysis population

The efficacy analysis population will be based on the per protocol (PP) principle – i.e. all subjects who completed the entire treatment period using the treatment as originally allocated. Safety analysis will be performed in all patients who received one or more doses of the study medication.

Statistical analysis of study endpoints

In order to compare the effects between the two study arms we will perform t-tests and/or multivariable linear regression models. The latter technique allows us to use continuous outcome variables and both continuous and dichotomous independent variables. The efficacy endpoint of interest will be added to the model as dependent variable. Treatment group will be added as independent categorical variable. Appropriate covariates may be added to the model. Baseline measurements of specific variables (e.g. age, gender) will be added to the regression models as covariates should differences in the baseline values of these variables occur between treatment groups. Moreover, corrections will be performed when relevant for the specific endpoint. The possible covariates will be elaborated prior to conducting the statistics. For possible confounders, we will test whether there is an association with both the dependent and independent variable (p <0.1). If this is the case, this confounder will be added to the model as covariate. Since all outcome variables – primary, secondary and exploratory – are continuous, this statistical technique can be applied.
Our primary outcomes will be the most important outcomes for testing the hypothesis that SGLT-2 inhibitor dapagliflozin versus the SU gliclazide causes an improvement in renal hemodynamics (GFR and ERPF). The large amount of secondary/exploratory endpoints potentially justifies the use of corrections for multiple tests. However, there is an increasing debate on whether this should be done, because of the increased risk of type 2 errors which could lead to prematurely discarding of potential useful observations (76). As recently pointed out (77), in a hypothesis-generating study – such as the current study – correction for multiple outcomes can be detrimental. A final decision will be made in the detailed Statistical Analysis Plan (SAP) prior to the conduction of the analysis.

13. ETHICS

The trial will be conducted in accordance with the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013) for biomedical research involving human subjects and in accordance with ICH-GCP. The investigator will ensure that all aspects of the institutional ERB review are conducted in accordance with current institutional, local and national regulations.

Ethical review

Any member of the ERB who is directly affiliated with this study as an investigator or as site personnel must abstain from the ERB vote on the approval of the protocol.

Recruitment

Subjects will be recruited by researcher physicians involved in this trial using methods that are established practice for all human studies in the Diabetes Center VUMC:

1) Via advertisements in (local) newspapers
2) Use of a Diabetes Center database to approach volunteers from previous studies, who have given written informed consent to be available for future studies
3) Through the Diabetes Center website (www.diabetescentrum.nl)
4) Via general practitioner offices with whom we have collaborations, we will identify potential participants who will be approached by the general practitioner
5) Where possible, subjects will be recruited from the out-patient clinic of the Diabetes Center / Department of Internal Medicine VUMC or affiliated hospitals / pharmacies

In case of a positive response, the information letter and informed consent forms will be sent to these individuals. They will then be contacted by telephone by the research physician to answer any remaining questions and make an appointment for a screening visit if the individual wants to participate.

Informed consent form for trial subjects

Prior to any trial related activity, the investigator will give oral and written information about the trial in a form that the subject can read and understand. It is the investigators responsibility to ensure the trial subject is fully informed about the aims of the trial, procedures, potential risks, any discomforts and expected benefits. The investigator must ensure the subject is informed and agrees that VUMC personnel, their representatives and possibly health authority (national or other) personnel can require access to the subject's data. It must be emphasized that participation is voluntary and that the subject has the right to withdraw from the trial at any time without prejudice.

A voluntary, signed and dated 'Informed Consent' will be obtained from the subject prior to any trial related procedure. The written informed consent must be signed by the person who conducted the informed consent. If information becomes available that may be relevant to the subject's willingness to continue participating in the trial, the investigator must inform the subject in a timely manner and a revised written informed consent must be obtained.

Unexpected findings will be reported to the participant and to his general practitioner. If the participant does not want to be informed, he/she cannot participate in the trial.

A physician who is not involved nor has any interest in the performance of the trial will be available; this person has knowledge about the protocol and can be consulted by subjects potentially interested in participation.

Financial Compensation

All subjects will receive a financial compensation for their participation (based on the minimum wages in The Netherlands). We have determined this to be €300 when the participant completes the entire study (as defined in Chapter 6). Moreover, reimbursement for travelling expenses will be given based on current prices of public transportation.

Insurance

In case of possible damage as a result of participating in this research project, the loss is, in agreement with legal demands, covered by insurance. This is in accordance with article 7, subsection 6 of the Medical

Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen (WMO).

The sponsor (also) has an insurance which is in accordance with the legal requirements in The Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. € 650.000,-- (i.e. six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 5.000.000,-- (i.e. five million Euro) for death or injury for all subjects who participate in the Research;
3. € 7.500.000,-- (i.e. seven million and five hundred thousand Euro) for the total damage incurred by the organization for all damage disclosed by scientific research for the Sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study. The insurance is covered by:

Onderlinge Waarborgmaatschappij Centramed B.A.
Postbus 191
2270 AD Voorburg
The Netherlands

Benefits and risk assessment

Study Medication

Participants will be randomized for a 12 week treatment with two different active study agents. Consequently, independent of treatment allocation, beneficial effects can be expected, as both SGLT-2 inhibitors and SU derivatives improve glycemic control. Both study medications have been approved for blood-glucose lowering treatment in T2DM patients and, based on currently available data, are considered to be safe. Furthermore, SGLT-2 inhibitors in general may decrease blood pressure and body weight compared to gliclazide.
The most common side effects for dapagliflozine are hypoglycemia (especially when used in combination with a SU derivative or insulin) and genitcal mycotic- and urinary tract infections. These side effects are usually mild and transient. In addition dyslipidemia and increased hematocrit have been described. Long-term (side) effects of SGLT-2 inhibitors are currently being evaluated in large-scaled outcome trials. For gliclazide, hypoglycemia and blurred vision in the initiation phase of treatment (due to changes in blood glucose levels) are the most common side effects. Also gastrointestinal side-effects (nausea, vomiting, diarrhea or constipation) have been reported. Of note, the gliclazide in this study will be used at a relatively low dose (i.e. 30 mg per day) for a relatively short treatment duration (12 weeks). Therefore, also given the inclusion- and exclusion criteria of the study participants, the overall risk of hypoglycemia is believed to be low. In the unlikely case of hypoglycemia symptoms, patients will be provided with a self-monitoring blood glucose (SMBG) device and instructed to perform ambulatory blood glucose checks in case of symptoms, during the course of the study. Also, they will receive standardized diabetes education, including information regarding carbohydrate use and self-management / resolution of hypoglycemia. As in all drug intervention trials, in this study, we will closely monitor patients for adverse drug and study events during the follow-up visit and by telephone consultation according to GCP (see Appendix A). Participants can contact the research staff 24 hours a day.

Possible inconvenience for participants

Over the last 10 years, we have gained ample experience with similarly demanding mechanistic drug intervention studies in T2DM patients. Based on the positive feedback from our participants, the low drop-out rate (max 5-10%) and the large proportion of participants that returns to participate in yet another (similarly demanding) study, we are confident that the burden on participants is perceived as not being too high. Indeed, we have built in different ways to alleviate the burden for participants, including clear, repeated communication, frequent contacting, intensified (diabetes) care, 24-hour availability of research staff, study and travel reimbursement, enabling participants to receive the newest study medication (that for most of them would not

be reimbursed in daily practice) and offering follow-up care in our out-patient clinic. During test-days, we provide meals and allow participants to read or watch TV/DVDs when possible. Finally, it should be noted that several tests are similar to currently or previously performed in patient care for diagnostic purposes (e.g. inulin or iohexol/PAH-clearance).
We are aware of the fact that in the current study participants will undergo multiple tests that demand a considerable time investment from their end. The total duration of visits is 1-2 (screening- and control visit) and 9 hours (visits 2 and 4). In addition, the renal/cardiovascular test-days during euglycemic- and hyperglycemic clamp may be perceived as demanding that amongst others involves frequent blood and urine collection, infusions, blood pressure, heart rate and microvascular measurements. During the cardiac autonomous nervous system function tests participants may experience transient dizziness or lightheadedness. As mentioned above, all possible measures will be taken to minimize the discomfort for the participants during the tests (e.g. comfortable beds are available which allow a semi-recumbent position).

Safety issues

The study examinations/tests are considered to be safe. No invasive procedures (besides intravenous peripheral catheters) are involved. During the study tests, two ‘diagnostic agents’ (i.e. inulin or iohexol and PAH) need to be administered; both agents are inert and have no side effects. As defined in Chapter 8, the total amount of drawn blood will be 500 mL during a total period of 16 weeks. Side effects are not expected because the blood volume taken per visit is relatively small, especially in comparison with regular blood donation, which amounts 500 mL per donation (and is allowed 5 times a year for men and 3 times for women).

Risk classification

The Dutch Federation of University Medical Centers (in Dutch: ‘Nederlandse Federatie van Universitair Medische Centra’) proposed a guideline for determination of the risk classification of a clinical trial (73). As stated above, the study medication and examinations/test are generally considered to be safe, with a small increased risk for hypoglycemia. For the latter, appropriate measures will be taken to minimize risk. Within the T2DM population, our study population can be considered as relatively low risk. However, sporadic serious side effects have been described (dehydration, hypervolemia, severe hypoglycemia), although most patients recover quickly. Therefore, while there is a low risk, the possible consequences might be ‘moderate’ to ‘serious’. Therefore, we feel that the current study has a classification of ‘moderate risk’.

14. ADMINISTRATIVE ASPECTS AND PUBLICATION

Amendments

Amendments are changes made to the research after a favorable opinion by the accredited ERB has been given. A 'substantial amendment' is defined as an amendment to the terms of the ERB application or to the protocol or any other supporting documentation that is likely to affect to a significant degree:

- The safety or physical or mental integrity of the subjects of the trial
- The scientific value of the trial
- The conduct or management of the trial
- The quality or safety of any intervention used in the trial

All substantial amendments will be notified to the ERB, the competent authority and subsidizing party/study (AstraZeneca). Non-substantial amendments will not be notified to ERB and competent authority, but will be recorded and filed by the sponsor/investigator and reported to AstraZeneca.

Study time lines

(Please note that the time lines stated below are estimations as they may vary according to, among others, study drug availability, approval by the ERB and inclusion rates).

Date	Description
01-06-2015	Study initiation: familiarizing with literature; attending courses for GCP / methodology; writing study full-protocol/SOPs; consulting statisticians/ methodologists; writing deliverables for pharma-companies for drug requests; exploring and arranging logistics/infrastructural collaborations; familiarizing with and validating of methods / training personnel; preparing data base/CRF
01-09-2015	Submission research committee
01-10-2015	Submission ERB
01-01-2016	Start Study (First patient in)
01-08-2017	Last drug dispensation (Last patient in)
01-11-2017	Collecting data (Last patient out)
01-01-2018	Database Lock
01-01-2018 – 01-10-2018	Data analysis
01-06-2018 – 01-12-2018	Manuscript submissions
01-05-2018	Final study report (ERB / Competent Authority)

Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited ERB once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems and amendments.

End of study report

The sponsor will notify the accredited ERB and the competent authority of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.
In case the study is ended prematurely, the sponsor will notify the accredited ERB and the competent authority within 15 days, including the reasons for the premature termination.
Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited ERB and the Competent Authority.

Publication policy

AstraZeneca will be provided will all intended publication(s) and abstract(s), preceding submission at least 30 days and 7 days, respectively, AstraZeneca can make suggestions to change the wording of any

planned publication or abstract in relation to the study and/or the results. The sponsor-investigator is under no obligation to implement such changes, unless local regulatory requirements specify otherwise.
The study will be part of a PhD project for one MD PhD student. A PhD-project has to fulfill the requirements of the Medical Schools at VU University, Amsterdam, the Netherlands. In general, a PhD-thesis has to contain at least 5 chapters, consisting of manuscripts published in international peer-reviewed journals. We anticipate that this study will yield sufficient data to meet these criteria. Papers will be written at the end of the study, i.e. second half of 2018, and will be submitted to different relevant international peer-reviewed journals (Diabetes, Diabetes Care, etc.). Authorship of the papers will be determined using the Vancouver criteria for authorship.

Data will be presented at relevant national and international scientific meetings, including those from the Dutch Association for the Study of Diabetes (NVDO), European Association for the Study of Diabetes (EASD), American Diabetes Association (ADA), etc. Since the competition in this field is considerable, the usual caution should be entertained when presenting abstracts, which usually contain preliminary results.

REFERENCES

1. International Diabetes Federation. IDF Diabetes Atlas, 6th edn. Brussels, Belgium: International Diabetes Federation, 2013. Available from http://www.idf.org/diabetesatlas, accessed 14 December 2014.
2. Dronavalli S, Duka I, Bakris GL. The pathogenesis of diabetic nephropathy. Nat Clin Pract Endocrinol Metab. 2008 Aug;4(8):444–52.
3. Tuttle KR, Bakris GL, Bilous RW, Chiang JL, de Boer IH, Goldstein-Fuchs J, et al. Diabetic Kidney Disease: A Report From an ADA Consensus Conference. Diabetes Care. 2014 Oct;37(10):2864–83.
4. De Boer IH, Rue TC, Hall YN, Heagerty PJ, Weiss NS, Himmelfarb J. Temporal trends in the prevalence of diabetic kidney disease in the United States. JAMA. 2011 Jun 22;305(24):2532–9.
5. Pálsson R, Patel UD. Cardiovascular complications of diabetic kidney disease. Adv Chronic Kidney Dis. Elsevier; 2014 May 1;21(3):273–80.
6. Atkins RC, Zimmet P. Diabetic kidney disease: act now or pay later. Kidney Int. Nature Publishing Group; 2010 Mar;77(5):375–7.
7. Kidney Disease: Improving Global Outcomes (KDIGO) Work Group. KDIGO clinical practice guideline for evaluation and management of chronic kidney disease. Kidney Int Suppl 2013; 3: 1–163. Sep;
8. Standards of medical care in diabetes--2014. Diabetes Care. 2014 Jan;37 Suppl 1(October 2013):S14–80.
9. KDIGO Blood Pressure Work Group. KDIGO clinical practice guideline for the management of blood pressure in chronic kidney disease. Kidney Int 2012; 2(Suppl): 337–414.
10. Gregg EW, Li Y, Wang J, Burrows NR, Ali MK, Rolka D, et al. Changes in diabetes-related complications in the United States, 1990-2010. N Engl J Med [Internet]. 2014 Apr 17 [cited 2014 Oct 1];370(16):1514–23. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24738668
11. Wright EM, Loo DDF, Hirayama BA. Biology of human sodium glucose transporters. Physiol Rev. 2011 Apr;91(2):733–94.
12. DeFronzo R a, Davidson J a, Del Prato S. The role of the kidneys in glucose homeostasis: a new path towards normalizing glycaemia. Diabetes Obes Metab. 2012 Jan;14(1):5–14.
13. Ferrannini E, Solini A. SGLT2 inhibition in diabetes mellitus: rationale and clinical prospects. Nat Rev Endocrinol. Nature Publishing Group; 2012 Aug;8(8):495–502.
14. Abdul-Ghani MA, DeFronzo RA. Inhibition of renal glucose reabsorption: a novel strategy for achieving glucose control in type 2 diabetes mellitus. Endocr Pract. 2008 Sep;14(6):782–90.
15. Kamran M, Peterson RG, Dominguez JH. Overexpression of GLUT2 gene in renal proximal tubules of diabetic Zucker rats. J Am Soc Nephrol. 1997 Jun;8(6):943–8.
16. Dominguez JH, Song B, Maianu L, Garvey WT, Qulali M. Gene expression of epithelial glucose transporters: the role of diabetes mellitus. J Am Soc Nephrol. 1994 Nov;5(5 Suppl 1):S29–36.
17. Dominguez JH, Camp K, Maianu L, Feister H, Garvey WT. Molecular adaptations of GLUT1 and GLUT2 in renal proximal tubules of diabetic rats. Am J Physiol. 1994 Feb;266(2 Pt 2):F283–90.
18. Freitas HS, Anhê GF, Melo KFS, Okamoto MM, Oliveira-Souza M, Bordin S, et al. Na(+) -glucose transporter-2 messenger ribonucleic acid expression in kidney of diabetic rats correlates with glycemic levels: involvement of hepatocyte nuclear factor-1alpha expression and activity. Endocrinology. 2008 Feb;149(2):717–24.
19. Mogensen CE. Maximum tubular reabsorption capacity for glucose and renal hemodynamcis during rapid hypertonic glucose infusion in normal and diabetic subjects. Scand J Clin Lab Invest. Informa UK Ltd UK; 1971 Sep 8;28(1):101–9.
20. FARBER SJ, BERGER EY, EARLE DP. Effect of diabetes and insulin of the maximum capacity of the renal tubules to reabsorb glucose. J Clin Invest. 1951 Feb;30(2):125–9.
21. Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J. Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. Diabetes. 2005 Dec;54(12):3427–34.
22. Rossetti L, Smith D, Shulman GI, Papachristou D, DeFronzo RA. Correction of hyperglycemia with phlorizin normalizes tissue sensitivity to insulin in diabetic rats. J Clin Invest. 1987 May;79(5):1510–5.
23. Vasilakou D, Karagiannis T, Athanasiadou E, Mainou M, Liakos A, Bekiari E, et al. Sodium-glucose cotransporter 2 inhibitors for type 2 diabetes: a systematic review and meta-analysis. Ann Intern Med. 2013 Aug;159(4):262–74.
24. Arakawa K, Ishihara T, Oku A, Nawano M, Ueta K, Kitamura K, et al. Improved diabetic syndrome in C57BL/KsJ-db/db mice by oral administration of the Na(+)-glucose cotransporter inhibitor T-1095. Br J Pharmacol. 2001 Jan;132(2):578–86.
25. Terami N, Ogawa D, Tachibana H, Hatanaka T, Wada J, Nakatsuka A, et al. Long-Term Treatment with the Sodium Glucose Cotransporter 2 Inhibitor, Dapagliflozin, Ameliorates Glucose Homeostasis and Diabetic Nephropathy in db/db Mice. PLoS One. 2014 Jan;9(6):e100777.

26. Adachi T, Yasuda K, Okamoto Y, Shihara N, Oku A, Ueta K, et al. T-1095, a renal Na+-glucose transporter inhibitor, improves hyperglycemia in streptozotocin-induced diabetic rats. Metabolism. 2000 Aug;49(8):990–5.
27. Malatiali S, Francis I, Barac-Nieto M. Phlorizin prevents glomerular hyperfiltration but not hypertrophy in diabetic rats. Exp Diabetes Res. 2008 Jan;2008:305403.
28. Tahara A, Kurosaki E, Yokono M, Yamajuku D, Kihara R, Hayashizaki Y, et al. Effects of sodium-glucose cotransporter 2 selective inhibitor ipragliflozin on hyperglycaemia, oxidative stress, inflammation and liver injury in streptozotocin-induced type 1 diabetic rats. J Pharm Pharmacol. 2014 Jul;66(7):975–87.
29. Vallon V, Gerasimova M, Rose M, Masuda T, Satriano J, Mayoux E, et al. SGLT2 Inhibitor Empagliflozin Reduces Renal Growth and Albuminuria in Proportion to Hyperglycemia and Prevents Glomerular Hyperfiltration in Diabetic Akita Mice. Am J Physiol Renal Physiol. 2013 Nov 13;(9151).
30. Gembardt F, Bartaun C, Jarzebska N, Mayoux E, Todorov VT, Hohenstein B, et al. The SGLT2 inhibitor empagliflozin ameliorates early features of diabetic nephropathy in BTBR ob/ob type 2 diabetic mice with and without hypertension. Am J Physiol Renal Physiol. 2014 Jun 18;
31. Kojima N, Williams JM, Takahashi T, Miyata N, Roman RJ. Effects of a new SGLT2 inhibitor, luseogliflozin, on diabetic nephropathy in T2DN rats. J Pharmacol Exp Ther. 2013 Jun;345(3):464–72.
32. Kohan DE, Fioretto P, Tang W, List JF. Long-term study of patients with type 2 diabetes and moderate renal impairment shows that dapagliflozin reduces weight and blood pressure but does not improve glycemic control. Kidney Int. Nature Publishing Group; 2013 Oct 25;85(4):962–71.
33. Cefalu WT, Leiter LA, Yoon K-H, Arias P, Niskanen L, Xie J, et al. Efficacy and safety of canagliflozin versus glimepiride in patients with type 2 diabetes inadequately controlled with metformin (CANTATA-SU): 52 week results from a randomised, double-blind, phase 3 non-inferiority trial. Lancet. 2013 Sep 14;382(9896):941–50.
34. Cherney D. 1125-P. American Diabetes Association (ADA), 74th Scientific Session, June 2014.
35. Thomson SC, Rieg T, Miracle C, Mansoury H, Whaley J, Vallon V, et al. Acute and chronic effects of SGLT2 blockade on glomerular and tubular function in the early diabetic rat. Am J Physiol Regul Integr Comp Physiol. 2012 Jan 1;302(1):R75–83.
36. Cherney DZI, Perkins B a, Soleymanlou N, Maione M, Lai V, Lee A, et al. Renal hemodynamic effect of sodium-glucose cotransporter 2 inhibition in patients with type 1 diabetes mellitus. Circulation. 2014 Feb 4;129(5):587–97.
37. Vallon V, Komers R. Pathophysiology of the diabetic kidney. Compr Physiol. 2011 Jul;1(3):1175–232.
38. Gilbert RE. Sodium-glucose linked transporter-2 inhibitors: potential for renoprotection beyond blood glucose lowering? Kidney Int. Nature Publishing Group; 2013 Nov 20;1–8.
39. Yale J-F, Bakris G, Cariou B, Yue D, David-Neto E, Xi L, et al. Efficacy and safety of canagliflozin in subjects with type 2 diabetes and chronic kidney disease. Diabetes Obes Metab. 2013 May;15(5):463–73.
40. De Nicola L, Gabbai FB, Liberti ME, Sagliocca A, Conte G, Minutolo R. Sodium/Glucose Cotransporter 2 Inhibitors and Prevention of Diabetic Nephropathy: Targeting the Renal Tubule in Diabetes. Am J Kidney Dis. Elsevier Inc; 2014 Mar 24;1–9.
41. Panchapakesan U, Pegg K, Gross S, Komala MG, Mudaliar H, Forbes J, et al. Effects of SGLT2 inhibition in human kidney proximal tubular cells--renoprotection in diabetic nephropathy? PLoS One. 2013 Jan;8(2):e54442.
42. Burns KD. Poster 534-P, American Diabetes Association (ADA) 74th Scientific Session, June 2014.
43. ADA. Standards of medical care in diabetes--2014. Diabetes Care. 2014 Jan;37 Suppl 1(October 2013):S14–80.
44. Sanofi. Summary of Product Characteristics - Lyxumia. 28-11-2013. Available from: URL: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/002445/WC500140401.pdf.
45. Sanofi. Summary of Product Characteristics - Apidra. 29-11-2013. Available from: URL: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000557/WC500025250.pdf. :1–163.
46. Fresenius Kabi. Summary of Product Characteristics - Inutest. 2011. Available from: URL: http://fresenius-kabi.at/en/files/4.2.3.Inutest_SPC_2011.pdf. 0.
47. Miller JA. Renal responses to sodium restriction in patients with early diabetes mellitus. J Am Soc Nephrol. 1997 May;8(5):749–55.
48. Frank H, Graf J, Graf J, Amann-Gassner U, Bratke R, Daniel H, et al. Effect of short-term high-protein compared with normal-protein diets on renal hemodynamics and associated variables in healthy young men. Am J Clin Nutr. 2009 Dec;90(6):1509–16.
49. Sargent WQ, Simpson JR, Beard JD. The effect of acute and chronic alcohol administration on renal hemodynamics and monovalent ion excretion. J Pharmacol Exp Ther. 1974 Feb;188(2):461–71.

50. Donnelly SM, Miller JA. Losartan may modulate erythropoietin production. J Renin Angiotensin Aldosterone Syst. 2001 Dec;2(4):255–60.
51. Goddard J, Johnston NR, Hand MF, Cumming AD, Rabelink TJ, Rankin AJ, et al. Endothelin-A receptor antagonism reduces blood pressure and increases renal blood flow in hypertensive patients with chronic renal failure: a comparison of selective and combined endothelin receptor blockade. Circulation. 2004 Mar 9;109(9):1186–93.
52. Van der Zander K, Houben AJHM, Hofstra L, Kroon AA, de Leeuw PW. Hemodynamic and renal effects of low-dose brain natriuretic peptide infusion in humans: a randomized, placebo-controlled crossover study. Am J Physiol Heart Circ Physiol. 2003 Sep;285(3):H1206–12.
53. Mengden T, Asmar R, Kandra A, Di Giovanni R, Brudi P, Parati G. Use of automated blood pressure measurements in clinical trials and registration studies: data from the VALTOP Study. Blood Press Monit. 2010 Aug;15(4):188–94.
54. Pickering TG, Hall JE, Appel LJ, Falkner BE, Graves J, Hill MN, et al. Recommendations for blood pressure measurement in humans and experimental animals: part 1: blood pressure measurement in humans: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Cou. Circulation. 2005 Feb 8;111(5):697–716.
55. R A, S H, Ruma D. The waist circumference measurement: a simple method for assessing the abdominal obesity. J Clin Diagn Res. 2012 Nov;6(9):1510–3.
56. Lukaski HC, Bolonchuk WW. Estimation of body fluid volumes using tetrapolar bioelectrical impedance measurements. Aviat Space Environ Med [Internet]. 1988 Dec [cited 2013 Dec 16];59(12):1163–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3240217
57. Eeftinck Schattenkerk DW, van Lieshout JJ, van den Meiracker AH, Wesseling KR, Blanc S, Wieling W, et al. Nexfin noninvasive continuous blood pressure validated against Riva-Rocci/Korotkoff. Am J Hypertens. 2009 Apr;22(4):378–83.
58. Serne EH, Gans ROB, ter Maaten JC, Tangelder G-J, Donker a. JM, Stehouwer CD a. Impaired Skin Capillary Recruitment in Essential Hypertension Is Caused by Both Functional and Structural Capillary Rarefaction. Hypertension [Internet]. 2001 Aug 1 [cited 2013 Sep 4];38(2):238–42. Available from: http://hyper.ahajournals.org/cgi/doi/10.1161/01.HYP.38.2.238
59. Serne EH, Stehouwer CD a., ter Maaten JC, ter Wee PM, Rauwerda J a., Donker a. JM, et al. Microvascular Function Relates to Insulin Sensitivity and Blood Pressure in Normal Subjects. Circulation [Internet]. 1999 Feb 23 [cited 2013 Sep 4];99(7):896–902. Available from: http://circ.ahajournals.org/cgi/doi/10.1161/01.CIR.99.7.896
60. Wilkinson IB, Fuchs SA, Jansen IM, Spratt JC, Murray GD, Cockcroft JR, et al. Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. J Hypertens. 1998 Dec;16(12 Pt 2):2079–84.
61. Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J. 2006 Nov;27(21):2588–605.
62. Davies JI, Struthers AD. Pulse wave analysis and pulse wave velocity: a critical review of their strengths and weaknesses. J Hypertens. 2003 Mar;21(3):463–72.
63. Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ. Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. Science. 1981 Jul 10;213(4504):220–2.
64. Ewing DJ, Martyn CN, Young RJ, Clarke BF. The value of cardiovascular autonomic function tests: 10 years experience in diabetes. Diabetes Care. 1985;8(5):491–8.
65. Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. Diabetes Care. 2003 May;26(5):1553–79.
66. Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Circulation. 1996 Mar 1;93(5):1043–65.
67. Kahn R. Proceedings of a consensus development conference on standardized measures in diabetic neuropathy. Autonomic nervous system testing. Diabetes Care. 1992 Aug;15(8):1095–103.
68. Bazett H. An analysis of the time relations of electrocardiograms. Ann Noninvasive Electrocardiol. 1997;
69. Judd TA, Day AS, Lemberg DA, Turner D, Leach ST. Update of fecal markers of inflammation in inflammatory bowel disease. J Gastroenterol Hepatol. 2011 Oct;26(10):1493–9.
70. Burcelin R, Serino M, Chabo C, Blasco-Baque V, Amar J. Gut microbiota and diabetes: from pathogenesis to therapeutic perspective. Acta Diabetol. 2011 Dec;48(4):257–73.
71. Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. J Am Soc Nephrol. 2014 Apr;25(4):657–70.
72. Sabatino A, Regolisti G, Brusasco I, Cabassi A, Morabito S, Fiaccadori E. Alterations of intestinal barrier and microbiota in chronic kidney disease. Nephrol Dial Transplant. 2014 Sep 4;

73. Nederlandse Federatie van Universitair Medische Centra. Kwaliteitsborging van mensgebonden onderzoek. 2010. Available from: URL: http://www.nfu.nl/img/pdf/NFU-12.6053_Kwaliteitsborging_mensgebonden_onderzoek_2.0.pdf.
74. Sochett EB, Cherney DZI, Curtis JR, Dekker MG, Scholey JW, Miller J a. Impact of renin angiotensin system modulation on the hyperfiltration state in type 1 diabetes. J Am Soc Nephrol. 2006 Jun;17(6):1703–9.
75. Cherney DZI, Miller JA, Scholey JW, Bradley TJ, Slorach C, Curtis JR, et al. The Effect of Cyclooxygenase-2 Inhibition on Renal Hemodynamic Function in Humans With Type 1 Diabetes. 2008;57(March).
76. Rothman KJ. No adjustments are needed for multiple comparisons. Epidemiology. 1990 Jan;1(1):43–6.
77. Streiner DL, Norman GR. Correction for multiple testing: is there a resolution? Chest. 2011 Jul;140(1):16–8.

APPENDIX A

TEST MEDICATIONS

Contact / Visit	**V1**	**T1**	**V2**	**T2**	**V3**	**V4**	**ET***
Weeks relative to 1 test visit	**-4**	**-4**	**0**	**2**	**6**	**12**	
Informed consent	X						
Screening (in-/exclusion)	X						
Inform about eligibility		X					
Medical history	X						
Recent history / Adverse event review	X		X	X	X	X	X
Physical examination / anthropometrics	X				(X)		
Vital Signs / Body weight	X		X		X	X	X
Bio-Impedance Analysis			X			X	X
Laboratory sampling	X[A]		X[B,C]		X[C,D]	X[B,C]	X[C,E]
Renal Protocol[F]			X			X	(X)
Clamp Protocol[G]			X			X	(X)
Cardiovascular tests[H]			X			X	(X)
ECG	X						
DNA collection			X				
Randomization			X				
Intervention period			start				
Study medication dispensed			X		X		
Study drug accountability					X	X	X
Time spent in CRU (hours)	1.5	phone	9	phone	1.0	9	1.5

CRU=Clinical research unit; ET=Early-Term assessments; T=Telephone consultation; V=Visit

[A] Measurements: Blood (Hematology (Hb, Ht, leucocytes and thrombocytes), glucose, HbA_{1c}, AST, ALT, GGT, ALP, sodium, potassium, creatinine, BUN, TG, TSH and FSH (in females only, in case of doub) and urine (screening, ACR)

[B] Measurements: Hematology (Hb, Ht, leukocytes and thrombocytes), fasting glucose, HbA_{1c}, TG, TC, HDL-, LDL-C, creatinine, BUN, AST, ALT, GGT, ALP.

[C] Blood/urine sampled and stored for various (cardiovascular- & inflammatory)-biomarker analyses (cystatin C, FGF23, , PTH, soluble Klotho, NT-proBNP, BNP, ANP, PRA, angiotensin II, aldosterone, catecholamines, insulin, C-peptide, lipids, GLP-1, glucagon, GIP, sRAGE, hsCRP, IL-6, PAI-1, TGF-β1, collagen type IV, nephrin, podocin, angiotensinogen, mRNA, microparticles)

[D] Safety (and efficacy) lab measurements: Hematology (Hb, Ht, leucocytes and thrombocytes) and chemistry (sodium, potassium, creatinine, BUN, AST, ALT, GGT, ALP)

[E] Measurements: Blood (Hematology, HbA_{1c}, TG, TC, HDL-, LDL-cholesterol, creatinine, Na, BUN, albumin, AST, ALT, GGT, ALP, lipase, amylase) and urine (sodium, creatinine, BUN)

[F] Renal protocol consists of: inulin or iohexol/PAH-clearance, tubular function tests and renal damage measurements

[G] Cardiovascular test-battery consists of oscillometric blood pressure measurements, applanation tonometry, capillary videomicroscopy, ECG and beat-to-beat finger blood pressure measurements

[H] Clamp protocol consists of: insulin and glucose infusion; measurements of insulin sensitivity and beta-cell function will be obtained.

* When a subject wants to discontinue participation prior to the end of the study protocol, all possible effort will be made to counsel the participant to undergo as much endpoint measurements as possible. The procedures mentioned in the ET-column should at least be performed.

APPENDIX B

TEST MEDICATIONS

Inulin ***(Inutest® by Fresenius Kabi Austria GmbH)*** **(1)**

Supplied as: 20 mL ampoules with a clear, colorless to slight yellow solution containing 25% sinistrin.

Approved agent: Approved by the Austrian drug authority (Bundesamt für Sicherheit im Gesundheitswesen).

Action: Sinistrin is a naturally occurring sugar polymer which, like inulin, belongs to the fructan group. While traditionally inulin has been used for determination of GFR (hence the so called 'inulin-clearance'), sinistrin shows a better solubility in water and improved alkaline stability, while the clearance ratios of inulin and sinistrin (inutest) are virtually identical. The laboratory determination of inulin and sinistrin is identical. Like inulin, sinistrin is inert and therefore has no effects on human physiology. While we will use sinistrin, for clarity and recognition, throughout the protocol the term inulin is used.

Side effects: Side effects are rare, but extremely large doses may cause osmotic diuresis. Allergic and anaphylactic reactions have been reported, but are very uncommon.

Dosing: For the steady-state inulin-clearance test, an initial bolus dose is given, followed by continuous infusion with a dose calculated to maintain the plasma concentration between 200mg/L and 250mg/L.

Storage: Inutest can be stored up to 3 years below 25°C.

Iohexol ***(Omnipaque® by GE Healthcare B.V.)***

Supplied as: 200 mL ampoules with a clear, colorless solution containing 302 mg iohexol per mL.

Approved agent: Approved by the Dutch drug authority (College ter Beoordeling van Geneesmiddelen; RVG 09821)

Action: Iohexol is a non-ionic, monomeric, triiodinated, water-soluble substance mostly used as X-ray contrast medium.In the concentration of 140 mg I/ml it is isotonic with blood and tissue fluid. Close to 100 per cent of the intravenously injected iohexol is excreted unchanged through the kidneys within 24 hours in patients with normal renal function. The maximum urinary concentration of iohexol appears within approximately 1 hour after injection. No metabolites have been detected. The protein binding of iohexol is very low (less than 2%).

Side effects: The risk of serious reactions in connection with use of Omnipaque is regarded as minor. However, iodinated contrast media may provoke serious, life-threatening, fatal anaphylactic/anaphylactoid reactions or other manifestations of hypersensitivity.

Dosing: For the steady-state iohexol-clearance test, an initial bolus dose is given, followed by continuous infusion with a dose calculated to maintain the plasma concentration between 100 mg/L and 150 mg/L.

Storage: Iohexol can be stored up to 3 years below 30°C.

PAH ***(4-aminohippuric acid as sodium salt by Bachem Distribution Services GmbH.)*** **(2)**

Supplied as: 10 mL vials containing sterile 20% (2 g/10 mL) of 4-Aminohippuric acid dissolved in sodium hydroxide solution and water for injections (WFI)

Approved agent: PAH by Bachem has no EMA or FDA approval. However, PAH is widely used in clinical trials for the assessment of ERPF. Bachem produces PAH in GMP-grade and the product will undergo quality assurance prior to administration (document verification, chemical/physical/ microbiological properties and stability testing). A QP-release (release by a qualified person) will be given prior to use of PAH.

Action: PAH is a derivative of hippuric acid, which is derived from the amino acid glycine. It is inert in normal doses.

Side effects: Extremely large doses may cause osmotic diuresis. Some patients may have a sensation of warmth, or the desire to defecate or urinate during or shortly following initiation of infusion. Allergic and anaphylactic reactions have been reported, but are very uncommon.

Dosing: During one PAH-clearance test, an initial bolus dose will be given, followed by a continuous infusion with a dose calculated to maintain the plasma concentration between 15mg/L and 20mg/L.

Storage: PAH will be stored at room temperature (excursions between 15 - 25°C are allowed) protected from light.

Insulin aspart ***(NovoRapid® by Novo Nordisk).*** **(3)**

Supplied as: 100 units/ml solution for injection, containing a clear, colorless and aqueous solution
Approved agent: EMA-approved
Action: Rapid-acting insulin analogue
Side effects: The most frequently reported adverse reaction during treatment is hypoglycemia. At the beginning of the insulin treatment, refraction anomalies and edema may occur. These reactions are usually of transitory nature. Fast improvement in blood glucose control may be associated with acute painful neuropathy, which is usually reversible.
Dosing: Each insulin infusion will be primed with an intravenous (IV) insulin bolus of 0.1 x kg body weight x desired elevation of plasma insulin level of 100 mU/L. The insulin infusion rate will be maintained at 40 mU/min/m^2 body surface area during the 3-hr euglycemic clamp.
Shelf life: Before opening: 30 months. During use or when carried as a spare: The product must be stored for a maximum of 4 weeks. Store below 30°C.
Storage: Before opening: Store in a refrigerator (2°C - 8°C). Do not freeze. During use or when carried as a spare: Store below 30°C. Do not refrigerate. Do not freeze.

Arginine 10% HCl (50g=500ml) ***(VUmc pharmacy).***
Supplied as: 500 ml infusion fluid
Approved agent: Arginine by the VUmc pharmacy has no EMA or FDA approval. However, it has been widely used in clinical trials (including at our department) to measure beta cell function during hyperglycemic clamp. The VUmc pharmacy has been producing arginine for many years and has ample experience with the process.
Action: beta cell stimulation
Side effects: side effects are rare. Dysgeusia, headaches, hot flashes have been reported.
Dosing: At the end of the hyperglycemic clamp, 5 mg arginine (50ml) will be administered.
Shelf life: *Arginine can be stored for 3 years*
Storage: *At room temperature (below 25 degrees) and in the dark*

References

(1) Fresenius Kabi. Summary of Product Characteristics - Inutest. 2011. Available from: URL: http://fresenius-kabi.at/en/files/4.2.3.Inutest_SPC_2011.pdf
(2) Bachem (Christoph Hörth, Pharmacist). Documentation on 4-aminohippuric acid solution 20% (2g/10ml). 6 May 2015
(3) Novo Nordisk A/S. Summary of Product Characteristics – NovoRapid. 30-04-2009. Available from: URL: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000258/WC500030372.pdf
(4) Arginine HCl 10% infusievloeistof, farmacotherapeutische plaatsbepaling apotheek VUmc
(5) SPC Arginine HCl 50 g= 500 ml, Apotheek haagse ziekenhuizen
(6) SPC Omnipaque

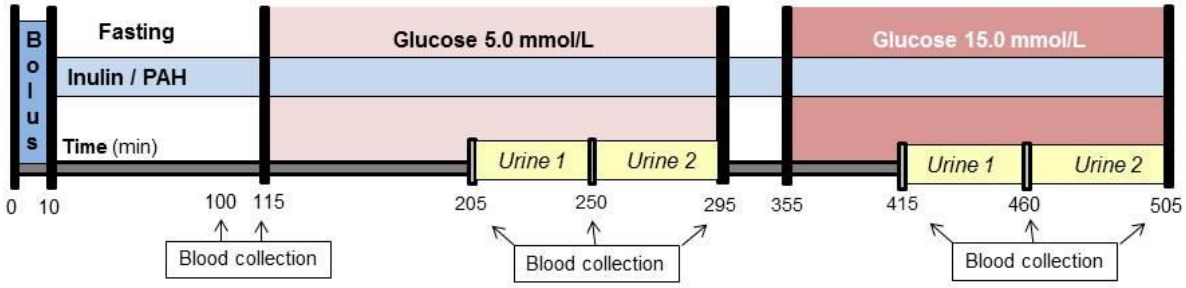
APPENDIX C1

RENAL PROTOCOL

The 'renal protocol' consists of the measurements of the GFR, ERPF, tubular function and renal damage parameters. The GFR and ERPF will be assessed using the gold standard inulin or iohexol/PAH-clearance (1-4).

Preparation

Seven days prior to the renal examination, the subject will adhere to a 'normal-salt' (9-12 grams or 150-200 mmol per day) and -protein (1.5-2 g/kg per day) diet, in order to minimize variation due to salt and protein intake (5;6). Moreover, participants will abstain from alcohol (24 hours), caffeine (12 hours) and nicotine (12 hours) and heavy exercise prior to and during the visits, as these have been shown to affect renal physiology (7-10). Participants will be instructed to abstain from taking any kind of medication on the morning after an overnight fast (at least 8 hr) of the renal tests, except for metformin. Prior to coming to the clinical research unit, the participant is asked to drink 500 mL of water.



The participant will assume a semi-recumbent position, in a temperature controlled room (22-23^{0}C). A peripheral 18 gauge venous cannula will be placed in both forearms. One of the cannulas will be used for infusion of the test substances, while the other will be used for venous blood sampling. The cannulas will be kept patent using 0.9% saline (no heparin will be used).

Procedure

Baseline measurements

Prior to the start of the infusion of inulin or iohexol/PAH, urine will be collected by spontaneous voiding and a blood sample will be taken. This blood and urine will be used to determine the reference levels of inulin or iohexol and PAH for the clearance tests. Moreover, tubular function and renal damage will be determined using these samples.

Start of Inulin or iohexol/PAH-protocol

In order to measure GFR and ERPF, respectively inulin (Inutest®, Fresenius Kabi, Austria) (11) or iohexol and para-aminohippurate (PAH sodium salt, Bachem, Germany) (12) need to be administered. In order to yield steady state concentrations quickly, a priming dose will be given. Then, a continuous infusion will be started to maintain the plasma concentration at a level sufficient for the determination of the GFR and ERPF. The specific dosages will be derived from the SPCs.

Because frequent voiding is necessary, a constant water intake is advocated to maintain an adequate urine output. During the equilibration period, the participant needs to drink 10 mL/kg body weight (with a maximum of 1000 mL). During the collection periods, subjects need to drink 200 mL/hour.

Collection periods

After an equilibration period of 90 minutes and and 15 minutes later blood samples will be drawn to measure PAH and inulin or iohexol to estimate GFR and ERPF. After 90 minutes during the euglycemic clamp (see figure), the participant is asked to void and blood will be drawn. This urine is of no value and will be discarded. From then on, urine will be collected by spontaneous voiding every 45 minutes for 2 periods of time. At the beginning and end of each collection period blood will be sampled from the venous cannula which is not used for the Inutest/PAH-infusion. Both urine and blood will be analyzed for inulin or iohexol and PAH.

Sample handling and measurements

All blood and urine will be collected and processed according to standard operating procedures. In short, urine will be collected at all times using a urine chair or urinal. The volume of urine will be recorded and the urine will be transferred to a plastic container, which will be stored at -20^{0}C or -80^{0}C until assay.
Blood will be drawn at set times. The different analyses will be performed as descripted in the table.

In order to measure tubular function, blood and urine will be analyzed for sodium, potassium, chloride, calcium, magnesium, phosphate and urea and fractional excretions will be calculated (see Table). Urine osmolality will also be tested and plasma osmolality calculated. In order to calculate the fractional excretion, the blood and urine level of creatinine need to be measured.

For renal damage the urine will be analyzed for 24-hour albumin, and NGAL and KIM-1 in spot urine.
Serum and urine samples will be stored for possible (emerging) analyses depending on budget and when deemed interesting (e.g. cystatin C, FGF23, PTH, soluble Klotho, TGF-β1, collagen type IV, nephrin, podocin, microparticles).

Table: schedule for renal protocol

Test Time (min)	Procedure	Collection + Analyses	Blood volume (mL)
		Inulin or iohexol / PAH	
0	Start bolus Inutest/PAH Fasting state	Blood + urine	20
10	Start Continuous infusion Inutest/PAH		
100	Measure fasting serum Inutest/PAH	Blood	15
115	Measure fasting serum Inutest/PAH Start euglycemic clamp	Blood	15
205	Voiding (measure urine volume + discard urine)	Blood	15
255	Measure urine volume	Blood + urine	15
295	Measure urine volume	Blood + urine	15
355	Start hyperglycemic clamp		
415	Voiding (measure urine volume + discard urine)	Blood	15
460	Measure urine volume	Blood + urine	15
505	Measure urine volume	Blood + urine	15

Calculations
Different calculations will be performed, using the formulas in the box. During fasting state GFR and ERPF will be calculated without collecting urine, assuming that a steady state has been reached and infusion rate equals excretion rate. During the clamps a mean will be calculated for plasma levels of inulin or iohexol and PAH, using the blood collected at the beginning and end of each collection period. This mean will be used in the formulas. The two different values (two collection periods) will be combined to calculate the mean GFR, ERPF, FF, ERBF and ERVR. The mean value will be the reference value (the baseline value for the rest of the study).

GFR: $\frac{Urine\ concentration\ of\ inulin * urine\ flow}{Plasma\ concentration\ of\ inulin}$ or $\frac{Infusion\ rate\ of\ inulin}{Plasma\ concentration\ of\ inulin}$

ERPF: $\frac{Urine\ concentration\ of\ PAH * urine\ flow}{Plasma\ concentration\ of\ PAH}$ or $\frac{Infusion\ rate\ of\ PAH}{Plasma\ concentration\ of\ PAH}$

Filtration Fraction: $\frac{GFR}{ERPF}$

Effective Renal Blood Flow: $\frac{ERPF}{1-hematocrit}$

Effective Renal Vascular Resistance: $\frac{Mean\ Arterial\ Pressure}{ERBF}$

Urinary solute excretion: $urinary\ solute\ x\ urine\ flow$

FE-solutes: $\frac{Urinary\ solute}{Plasma\ solute}\ x\ \frac{Plasma\ inulin/creatinin}{Urinary\ inulin/creatinin}\ x\ 100$

References

(1) Donnelly SM, Miller JA. Losartan may modulate erythropoietin production. J Renin Angiotensin Aldosterone Syst 2001 Dec;2(4):255-60.
(2) Goddard J, Johnston NR, Hand MF, Cumming AD, Rabelink TJ, Rankin AJ, et al. Endothelin-A receptor antagonism reduces blood pressure and increases renal blood flow in hypertensive patients with chronic renal failure: a comparison of selective and combined endothelin receptor blockade. Circulation 2004 Mar 9;109(9):1186-93.
(3) van der Zander K, Houben AJ, Hofstra L, Kroon AA, de Leeuw PW. Hemodynamic and renal effects of low-dose brain natriuretic peptide infusion in humans: a randomized, placebo-controlled crossover study. Am J Physiol Heart Circ Physiol 2003 Sep;285(3):H1206-H1212.
(4) Cherney DZI, Perkins B a, Soleymanlou N, Maione M, Lai V, Lee A, et al. The Renal Hemodynamic Effect of SGLT2 Inhibition in Patients with Type 1 Diabetes. Circulation 2014 Feb;129:587-97
(5) Frank H, Graf J, Amann-Gassner U, Bratke R, Daniel H, Heemann U, et al. Effect of short-term high-protein compared with normal-protein diets on renal hemodynamics and associated variables in healthy young men. Am J Clin Nutr 2009 Dec;90(6):1509-16.
(6) Miller JA. Renal responses to sodium restriction in patients with early diabetes mellitus. J Am Soc Nephrol 1997 May;8(5):749-55.
(7) Pawlik WW, Jacobson ED, Banks RO. Actions of nicotine on renal function in dogs. Proc Soc Exp Biol Med 1985 Apr;178(4):585-90.
(8) Sargent WQ, Simpson JR, Beard JD. The effect of acute and chronic alcohol administration on renal hemodynamics and monovalent ion excretion. J Pharmacol Exp Ther 1974 Feb;188(2):461-71.
(9) Leonard TK, Watson RR, Mohs ME. The effects of caffeine on various body systems: a review. J Am Diet Assoc 1987 Aug;87(8):1048-53.
(10) Poortmans JR. Exercise and renal function. Sports Med 1984 Mar;1(2):125-53.
(11) Fresenius Kabi. Summary of Product Characteristics - Inutest. 2011. Available from: URL: http://fresenius-kabi.at/en/files/4.2.3.Inutest_SPC_2011.pdf
(12) Bachem (Christoph Hörth, Pharmacist). Documentation on 4-aminohippuric acid solution 20% (2g/10ml). 6 May 2015

APPENDIX C2

CLAMP PROTOCOL

Preparation of subjects. The subjects will arrive at the clinical research unit in the morning after an overnight fast (at least 8 hr). All subjects will remain fasted throughout the clamp. At the evening prior to the clamp procedure, subjects will be instructed to take their usual medication. They should refrain from taking any drugs in the morning of the clamp day, except for metformin.
Subjects will be placed in a semirecumbent position, and a cannula will be inserted into the non-dominant (if available) antecubital fossa for infusion of saline, glucose, and insulin. A contralateral dorsal hand vein will be cannulated in a retrograde manner and maintained at 55º C to permit sampling of arterialized venous blood at 5 min intervals throughout the clamp protocol. The cannulae will be kept patent using 0.9% saline. After both cannulae have been inserted, the veins will be allowed at least 15 min of rest time prior to starting blood draws for the clamp. Glucose (20%) will be infused at a variable rate to maintain glucose at the desired concentration. The glucose infusion rate will be adjusted according to bedside measurement of blood glucose (BG) every 5 min based on a predefined algorithm. Blood samples will be taken before and during the tests as described in the following table. In all subjects, a euglycemic insulin clamp will be performed during the first 3-hr followed by an interval ("rest period") of 1 hour during which insulin infusion will be discontinued and glucose concentration maintained by the clamp technique, and a subsequent hyperglycemic clamp procedure with a standard 80-min square-wave hyperglycemia of 15 mmol/L. During the last 30 min of the hyperglycemic clamp, an arginine bolus will be administered. The duration of the total procedure is about 450 min, during which approximately 90 mL blood will be collected.

The euglycemic clamp procedure. Insulin infusion will be be maintained at 40 mU/min/m^2 body surface area during the 3-hr euglycemic clamp. The blood glucose concentration will be maintained at 5.0 mmol/L by performing manual glucose clamp for the remaining duration of the euglycemic clamp procedure. The glucose infusion rates will be adjusted according to the equation originally described by DeFronzo et al. (1979) with supplementary glucose infusion when BG decreases by > 0.2 mmol/L in 5 min.
Resting period. During the subsequent resting period of 60 min the subjects will receive an infusion of saline or glucose to maintain euglycemic levels at around 5 mmol/L (or at the blood glucose concentration reached at 90 min of the euglycemic clamp). Insulin infusion will be discontinued during this period.
The hyperglycemic clamp procedure combined with arginine stimulation. The hyperglycemic clamp procedure will be started with a body weight-adjusted IV bolus of 20% glucose administered over 1 min followed by a continuous infusion to achieve target blood glucose concentration of approximately 15 mmol/L (27 x bodyweight [kg] x desired increase in blood glucose [mmol/L] = bolus dose [mg]). The glucose infusion will be adjusted to maintain BG at 15 mmol/L. After 150 min of hyperglycemia, an IV bolus of 5 g arginine (dissolved in 50 mL) will be injected over 45 s while the glucose level will be maintained at 15 mmol/L.
Blood collections and sample handling. Blood glucose concentrations will be determined by the glucose oxidase method with a Yellow Springs glucose analyzer (Yellow Springs, OH, US) at 5 min intervals, except for the first 10 min of the hyperglycemic clamp (at 3 min, 5 min, 8 min, and 10 min) and the first 5 min after the arginine bolus (at 2 min, 3 min, 4 min, and 5 min). Sampling for plasma insulin and C-peptide will be performed as in the following table. All samples will be processed according to standard operating procedures within 1 hr and stored at -80°C until assay. The amount of infused glucose and saline will be recorded.

Euglycemic and Hyperglycemic Clamp Procedures

Test time (min)	Real time (min)	Procedures	Period	Analytes		Blood Volume (mL)
				Insulin	C-Peptide	
-30	0			X	X	1.5
-15	15			X	X	1.5
0	30	**Begin insulin IV**	Euglycemic Clamp			
10	40					
60	90					
90	120	**Reach glucose clamp target** (5 mM)				
100	130	Start period 1		X	X	1.5
145	175	Start period 2		X	X	1.5
190	220	End period 2		X	X	1.5
190	220	End insulin IV	Rest Period			1.5
-10 (=50)	270			X	X	1.5
-5 (=55)	275			X	X	1.5
0 (=60)	280	**Begin glucose IV** (target 15 mM)	Hyperglycemic Clamp			
1				X	X	1.5
2				X	X	1.5
3				X	X	1.5
4				X	X	1.5
5				X	X	1.5
6				X	X	1.5
7				X	X	1.5
8				X	X	1.5
9				X	X	1.5
10	290			X	X	1.5
15	295			X	X	1.5
30	310			X	X	1.5
60	340	Start period 1		X	X	1.5
70	350			X	X	1.5
105	385	Start period 2		X	X	1.5
150	430	End period 2		X	X	1.5
150		**I.V. Arginine Bolus** (45s)	Arginine Challenge	X	X	1.5
151				X	X	1.5
152				X	X	1.5
153				X	X	1.5
154				X	X	1.5
155				X	X	1.5
156				X	X	1.5
157				X	X	1.5
158				X	X	1.5
159				X	X	1.5
160	440			X	X	1.5
170	450			X	X	1.5
180	460	**End of Clamp**	**Recovery Period**	X	X	1.5
190	470	**End Recovery**				
Subtotal Blood Volume (mL)						55.5
Blood Volume for Glucose Analysis						44.5
Total Blood Volume (mL)						90.0

BG, blood glucose; YS, Yellow Springs glucose analyzer

APPENDIX D

MICROVASCULAR FUNCTION ASSESSMENTS

In order to measure the microvascular function we will perform capillary videomicroscopy and Laser Doppler methods (1-3).

Preparation

Subjects will be in a fasting state (for at least 10 hours) and will be asked to refrain from alcohol, caffeine and nicotine for at least 12 hours. Participants will be instructed to abstain from taking any kind of medication on the morning of the tests, except for metformin.
The participant will assume a semi-recumbent position, with the non-dominant hand at heart level. Measurements will be conducted after 30 minutes of acclimatization in a quiet, temperature controlled room (22-23 ^{0}C).

Procedure

Capillary Videomicroscopy

Microvascular function will be measured by assessing skin capillary density and capillary recruitment after arterial occlusion by video-microscopy of the nail fold capillaries in the fasting state, according to a previously described protocol (2;3).
In short, perfused nail fold capillaries in the dorsal skin of the third finger of the non-dominant hand will be visualized by a capillary microscope (Carl Zeiss) linked to a television camera, a video recorder and a monitor. To visualize the capillaries, a 3.2X objective (Zeiss 3.2/0.07) is used, with a total system magnification of 99X. Two separate visual fields of 1 mm^2 will be recorded before and after 4 minutes of arterial occlusion with a digital cuff (300 mmHg). The images will be stored on videotape for off-line analysis. Capillary density is defined as the number of erythrocyte-perfused capillaries per mm^2 of nail fold skin. Percentage capillary recruitment during post-occlusive reactive hyperemia is assessed by dividing the capillary density during post-occlusive reactive hyperemia by the number of capillaries at baseline.
Although performed in the original protocol (as described in (2;3)), data by van Genugten *et al* (SPHINX-study) suggests that venous occlusion does not lead to additional information (4). Therefore, in this study, no venous occlusion will be performed.

Laser Doppler

To perform vasomotion analyses, skin blood flow will be measured with a Laser Doppler probe (PF 457; Perimed, Stockholm, Sweden) at the dorsal side of the third finger of the non-dominant arm. Signals will be recorded for 30 minutes in the fasting state. Fast-Fourier transform analysis will be performed by means of Perisoft dedicated software (PSW version 2.5, Perimed) to determine the contribution of the five frequency components of the variability of the Laser Doppler signal (i.e. endothelial: 0.01 – 0.02 Hz; neurogenic: 0.02 – 0.06 Hz; myogenic: 0.06 – 0.15 Hz; respiratory: 0.15 – 0.40 Hz; and heart beat: 0.40 – 1.60 Hz).

References

(1) Antonios TF, Rattray FE, Singer DR, Markandu ND, Mortimer PS, MacGregor GA. Maximization of skin capillaries during intravital video-microscopy in essential hypertension: comparison between venous congestion, reactive hyperaemia and core heat load tests. Clin Sci (Lond) 1999 Oct;97(4):523-8.
(2) Serne EH, Stehouwer CD, ter Maaten JC, ter Wee PM, Rauwerda JA, Donker AJ, et al. Microvascular function relates to insulin sensitivity and blood pressure in normal subjects. Circulation 1999 Feb 23;99(7):896-902.
(3) Serne EH, Gans RO, ter Maaten JC, Tangelder GJ, Donker AJ, Stehouwer CD. Impaired skin capillary recruitment in essential hypertension is caused by both functional and structural capillary rarefaction. Hypertension 2001 Aug;38(2):238-42.
(4) van Genugten RE, Serné EH, Heymans MW, van Raalte DH, Diamant M. Postprandial microvascular function deteriorates in parallel with gradual worsening of insulin sensitivity and glucose tolerance in men with the metabolic syndrome or type 2 diabetes. Diabetologia. 2013 Mar;56(3):583-7.

APPENDIX E
PULSE WAVE ANALYSIS

Pulse Wave Analysis (PWA) will be assessed using the SphygmoCor® System (Atcor Medical, West Ryde, Australia), a non-invasive system using applanation tonometry (1-3).

Preparation

The subject will assume a supine position in a temperature-controlled room (22-23^{0}C) for 10-30 minutes prior to taking any recordings. Participants will be in a fasting state, and will be asked to refrain from alcohol, caffeine and nicotine for at least 12 hours. Participants will be instructed to abstain from taking any kind of medication on the morning of the test, except for metformin.

Procedure

Pulse Wave Analysis

The SphygmoCor® PWA-setting will be used to derive a calibrated blood pressure waveform at the ascending aorta from a peripheral pressure waveform recorded at the radial artery of the non-dominant arm. To realize this, the radial artery will be sought by palpation. The tonometer will be placed in a 90^0 angle on top of the artery. Once a consistent pressure waveform has been found, a minimum of 12 seconds / 3 full waveforms should be recorded. The SphygmoCor® system will derive an Augmentation Pressure (AP), Augmentation Index (AIx), Augmentation Index corrected for heart rate (AIx@HR75), Ejection Duration (ED) and Subendocardial Viability Ratio (SEVR) (1).

Quality control

In order to use valid measurements, the 'Quality Control Section' of SphygmoCor® will be used. If any of the parameters for PWA (average pulse height, pulse height variation, diastolic variation or shape deviation) is outside of the predefined range, the measurement will be repeated.

References

(1) Davies JI, Struthers AD. Pulse wave analysis and pulse wave velocity: a critical review of their strengths and weaknesses. J Hypertens 2003 Mar;21(3):463-72.
(2) Laurent S, Cockcroft J, Van BL, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J 2006 Nov;27(21):2588-605.
(3) Wilkinson IB, Fuchs SA, Jansen IM, Spratt JC, Murray GD, Cockcroft JR, et al. Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. J Hypertens 1998 Dec;16(12 Pt 2):2079-84.

APPENDIX F

CARDIOVASCULAR AUTONOMIC NERVOUS SYSTEM FUNCTION TESTS

A non-invasive, automated, beat-to-beat blood pressure and ECG recording monitor (Nexfin®, BM Eye, Amsterdam, the Netherlands) (1) will be used to non-invasively measure heart rate and blood pressure.

Preparation

Participants will be in a fasting state and have restrained from drinking caffeine and smoking for at least 12 hours. Participants will be instructed to abstain from taking any kind of medication on the morning of the tests, except for metformin. Before starting the tests, subjects will assume a supine position for at least 10 minutes in a temperature controlled room (22-23 ^{0}C).

Procedures

Throughout the years many different tests have been developed and used in order to test cardiovascular ANS function (2;3), however the following tests were recommended by a joint panel (4):

Resting HRV (heart rate variability)

Using the Nexfin® monitor, the resting heart rate will be recorded during 7 minutes. With the use of dedicated software, different time domain and power spectral analyses of heart rate fluctuations can be performed in order to quantitatively evaluate beat-to-beat cardiovascular control (5;6). Different possible measures and their definition are explained in the table. Resting heart rate variability reflects the balance between the sympathetic nervous system and parasympathetic. Also, the baroreflex sensitivity can be assessed, reflecting the sensitivity of spontaneous baroreflex control of the heart rate (see table).

CARTs

Deep breathing: In a supine position, subjects will be asked to breathe standardized frequency during 1 minute. They will be asked to take 10 seconds for every in- and expiration (5 seconds in, and 5 seconds out), resulting in 6 completed breathings per minute. Heart rate is recorded throughout the period of deep breathing and a marker will be used to indicate the start of the in- and expiration. The Expiration-Inspiration difference (E-I-Difference) will be calculated (see table), reflecting cardiac parasympathetic activity.

Valsalva maneuver: In a supine position, subjects will be asked to blow into a mouth piece connected to an anaeroid manometer. They will be asked to hold it at a pressure of 40 mmHg during 15 seconds. During this performance, heart rate is recorded. This maneuver will be performed 3 times. From this maneuver the Valsalva ratio can be calculated (see table), reflecting both cardiac sympathetic and parasympathetic function.

Orthostasis: Subjects will be asked to stand up from a supine position. Prior to this action and up to 2 minutes afterwards, heart rate and blood pressure will be recorded. A characteristic immediate increase in heart rate is observed around the 15th beat after standing and a compensatory overshoot bradycardia is seen around the 30th beat. This physiologic response is used to calculate the “30:15-ratio” or R-R interval Max/Min (see table) and is a marker for cardiac parasympathetic function.
In response to standing, blood pressure will usually fall and slowly rise again. The fall in blood pressure will be reported as the difference between the positions in systolic blood pressure and reflects cardiac sympathetic function.

Each separate test will be started with a resting period of 1 minute between the tests, to prevent influences by previous test conditions. It must be emphasized that throughout all procedures the participant must remain silent.

Screening

During the screening visit, Ewing tests will be performed as stated above (deep breathing, Valsalva and orthostasis). For this, cut-off values for ‘normal’, ‘borderline’ and ‘abnormal’ will be used as described by Ewing (3).

Table: Frequently used measures of cardiovascular autonomous nervous system function

Measure	Unit	Definition
During spontaneous breathing in a supine position		
Mean NN	ms	The mean of all normal to normal, i.e. sinus rhythm, RR intervals
SDNN	ms	The standard deviation of all normal to normal, i.e. sinus rhythm, RR intervals
LF Power	ms^2	Low frequency power, in absolute units: energy in the power spectrum between 0.004 and 0.12 Hz
HF Power	ms^2	High frequency power, in absolute units: energy in the power spectrum between 0.12 and 0.40 Hz
LF/HF		The ratio of low frequency power to high frequency power
LF/(LF+HF)		The ratio of low frequency power to the sum of the low and high frequency power
HF/(LF+HF)		The ratio of high frequency power to the sum of the low and high frequency power
Baroreflex sensitivity (BRS)	ms $mmHg^{-1}$	A measure of baroreflex sensitivity, computed as gain, i.e. ratio of the energy in the cross-spectrum of systolic blood pressure and RR intervals and the energy in the power spectrum of the RR interval; all between 0.05 and 0.15 Hz and with a squared coherence (γ^2) of 0.5 or higher (*BRS can also be calculated during deep breathing*).
During Valsalva maneuvre		
Valsalva ratio		The longest RR interval divided by the shortest RR interval within 45 seconds of peak heart rate, averaged over the three consecutive maneuvers
During deep breathing		
E-I-Difference	ms	The difference in maximum and minimum RR interval duration during expiration and inspiration, averaged over the six consecutive breaths
During an active change in position from lying to standing		
RR Max	ms	The difference between the mean RR interval during 1 min of rest prior to standing up and the minimum RR interval within 15 s after standing up
RR Max/Min (or “30:15”)		Maximum RR interval between 15 and 30 s after standing up divided by minimum RR interval within 15 s after standing up
SBP difference	mmHg	Systolic blood pressure after standing up (mean of 1.5–2 min after standing) minus systolic blood pressure in supine position

Abbreviations: BRS: baroreflex sensitivity; HF: High Frequency; LF: Low Frequency; mmHg: millimeter mercury; ms: millisecond NN: normal-to-normal; RR: time span between two pulse beats; SBP: systolic blood pressure; SDNN: standard deviation of normal-to-normal.
It must be noted that this table summarizes the frequently used measures. However, for the analysis, measures not listed in this table can be used.

References

(1) Eeftinck Schattenkerk DW, van Lieshout JJ, van den Meiracker AH, Wesseling KR, Blanc S, Wieling W, et al. Nexfin noninvasive continuous blood pressure validated against Riva-Rocci/Korotkoff. Am J Hypertens 2009 Apr;22(4):378-83.
(2) Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. Diabetes Care 2003 May;26(5):1553-79.
(3) Ewing DJ, Martyn CN, Young RJ, Clarke BF. The value of cardiovascular autonomic function tests: 10 years experience in diabetes. Diabetes Care 1985 Sep;8(5):491-8.
(4) Kahn R. Proceedings of a consensus development conference on standardized measures in diabetic neuropathy. Autonomic nervous system testing. Diabetes Care 1992 Aug;15(8):1095-103.
(5) Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Circulation 1996 Mar 1;93(5):1043-65.
(6) Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ. Power spectrum analysis of heart rate fluctuation: a quantitative probe of beat-to-beat cardiovascular control. Science 1981 Jul 10;213(4504):220-2.

APPENDIX G
FECAL TESTS

During this study, participants will collect multiple feces samples. The gut microbiome will be sequenced in order to gain insight in the association of the measured renal parameters with the intestinal flora (1-3). Also, the possible effect of GLP-1 receptor agonist therapy on the gut microbiome will be examined (1-3). Additional feces samples will be used to measure other explorative markers, i.e. calprotectin (marker for gut inflammation) (4).

Collection
Participants will receive a collection kit containing sample tubes, gloves and tools to transfer feces into the tubes. One tubes need to be frozen and stored immediately (at -20 ºC) in order to reduce bacterial growth. One tube can be stored in a dark and cool place. In order to prevent thawing of the frozen tubes, a container with ice packs will be provided.

Sample handling
After arrival at the clinical research unit, the research personnel will immediately store the frozen samples (meant for analysis of the gut microbiome) at -80 ºC. The non-frozen sample will be homogenized and transferred to multiple smaller cups, which will also be stored at -20 ºC.

Analyzes
Microbiota analyses: using a multi-level system of increasing sophisticated measurements:

1) All samples will be characterized using LCR/PCR analysis, a fast 'screening' technique, providing an insight in the 25 major phylogenetic bacterial groups and methanogens.
2) A selection of 60 samples (n=10 in each treatment arm, at baseline and after long-term treatment) will be analyzed in detail using the 'HITChip' (Human Intestinal Tract phylogenetic microarray). The HITChip recognizes over 4800 specific 16S rRNA probes of intestinal microbes, identifying 1200 phylotypes (5;6).
3) A subselection of 30 samples will be made based on the above analysis that will be targeted for 16S rRNA metagenomic sequencing (using 454 Titanium or other sequencing systems) as previously reported (6). These techniques allow deep sequencing, thereby able to map the entire microbiome.
4) In order to provide a first hypothesis between diversity and function we will target 30 samples selected for metaproteomics analysis using advanced Fourier Transformation MS/MS analysis. We will analyze the generated sequences for specific diagnostic signatures that will be analyzed further by advanced functional search analysis. The selection of these subjects will take place at the end of the project when all clinical parameters and microbiota analysis have been completed.

Calprotectin: this inflammatory markers will be assessed using an EliA Phadia-method.

References
(1) Burcelin R, Serino M, Chabo C, Blasco-Baque V, Amar J. Gut microbiota and diabetes: from pathogenesis to therapeutic perspective. Acta Diabetol. 2011 Dec;48(4):257–73.
(2) Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. J Am Soc Nephrol. 2014 Apr;25(4):657–70.
(3) Sabatino A, Regolisti G, Brusasco I, Cabassi A, Morabito S, Fiaccadori E. Alterations of intestinal barrier and microbiota in chronic kidney disease. Nephrol Dial Transplant. 2014 Sep 4;
(4) Judd TA, Day AS, Lemberg DA, Turner D, Leach ST. Update of fecal markers of inflammation in inflammatory bowel disease. J Gastroenterol Hepatol 2011 Oct;26(10):1493-9.
(5) Rajilic-Stojanovic M, Heilig HG, Molenaar D, Kajander K, Surakka A, Smidt H, et al. Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. Environ Microbiol 2009 Jul;11(7):1736-51.
(6) Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, et al. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One 2009;4(8):e6669.

APPENDIX H
MONITORING PROCEDURE

Monitor
The monitoring procedure will be performed by an independent monitor from the Clinical Research Bureau VUMC.

Frequency
Because of the anticipated moderate risk of this study, monitoring has to take place 2-3 times a year.

Procedure
General
- Report of the amount of screened patients, inclusion rate, screen-failures
- Verification of GLP-certification for laboratories

Source Data Verification
- ***100%*** verification for presence and completeness (names, dates, signatures) of the informed consent forms. Informed consent must be obtained prior to participation in the trial.
- ***100%*** verification for in- and exclusion criteria for the first 5 participants, followed by ***10%*** of the remaining subjects. *When participants are wrongly included all dossiers may be verified.*
- ***10%*** verification of CRFs with source documentation
- ***100%*** verification of reported SAEs and SUSARs. Report appropriately? Timely? Correct parties?
- ***10%*** of participants will be checked for possible missed SAEs and SUSARs

Investigational Medicinal Products (IMP)
- Verification of GMP-certification for the involved pharmacies
- Verification of storage times, conditions, sufficient supplies and whether IMPs are supplied only to those who are eligible to receive it
- Verification that subjects are provided with instructions on the proper use of the IMPs
- ***100%*** verification of receipt, use and return of the IMPs for Drug Accountability
- Ensure that the most recent version of the SPC is present (for each IMP)

Protocol
- Verification that investigator(s) follow the approved protocol and amendment(s)
- Verification of the presence of Standardized Operating Procedures for all study tests and whether study site personnel is adequately trained.
- Determination of the maintenance of the essential documents (GCP, Chapter 8)

Report
The monitor will summarize his/her findings in an official report which will be handed to the PI. The 'monitor visiting report' will include the date, name of the monitor and the name of the investigator/individual(s) contacted. It should contain a summary of what the monitor reviewed and the monitor's statements concerning the significant findings/facts, deviations and deficiencies, conclusions, actions taken or to be taken and/or actions recommended to secure compliance.

APPENDIX I

RED substudy - RECOLAR

Title: Renoprotective Effects of Combined dapagliflOzin and LosARtan (RED- RECOLAR)

Background: This substudy elaborates on the primary results of the RED study, which seriously challenge our current understanding of the effects of SGLT-2 inhibitors on renal hemodynamics. From our results it seems that, in contrast to the current consensus based on results from animal studies and type 1 diabetes studies (1-3), the hypothesized protective hemodynamic mechanism in type 2 diabetes – a drop in glomerular pressure, indivated by an acute drop in eGFR and subsequent stabilization over time – is not due to afferent vasocontriction, but due to efferent vasodilatation. As is known, RAS inhibitors also act on this efferent arteriole, causing a protective vasodilative effect (1). The population of the RED study exists of participants treated with a RAS inhibitor (participants with hypertension were only included if treated with a RAS inhibitor) and non-hypertensive participants without RAS inhibition. Moreover, RAS inhibition using angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) was both allowed. This hetergenity in RAS inhibition use hampers clinical interpretation of the results. This substudy will assess and untangle the acute hemodynamic effects of RAS inhibition and SGLT-2 inhibition using a simplified version of the renal protocol used in the RED study both in combination- and mono therapy in a T2DM population with identical in- and exclusion criteria used in the RED study.

Hypothesis: Mono- and combination therapy with SGLT-2 inhibitor dapagliflozin and RAS inhibitor losartan may confer renoprotection by improving renal hemodynamics in T2DM.

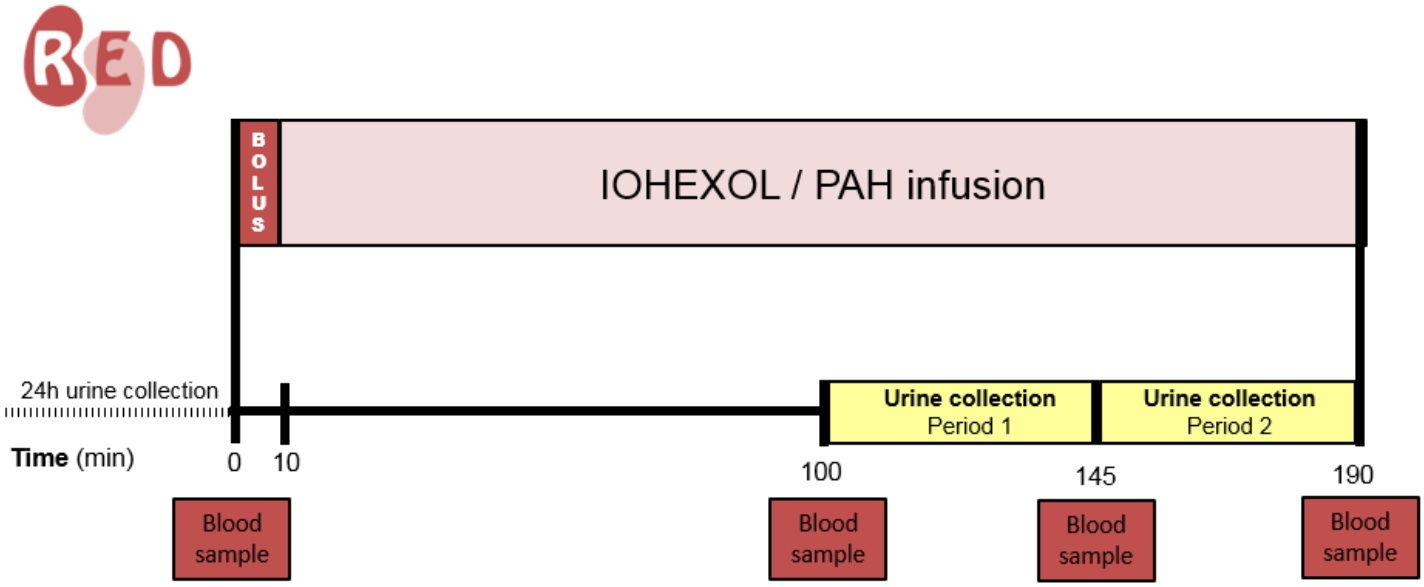
We will test this hypothesis by addressing the following **research objectives**:
Primary objective: what are the acute effects (i.e. after 3 days of drug exposure) of mono- and combination therapy with the SGLT-2 inhibitor dapagliflozin and RAS inhibitor losartan on renal hemodynamics (glomerular filtration rate (GFR) / effective renal plasma flow (ERPF)) in metformin-treated T2DM patients?
Secondary objectives: renal tubular function, renal damage markers, blood pressure, heart rate, body anthropometrics, markers of inflammation and (cardiovascular-)biomarkers.

Methods:
Design: An randomized, cross-over, mechanistic substudy to assess the effect of 3 days of mono- and combination therapy with the SGLT-2 inhibitor dapagliflozin 10 mg QD and RAS inhibitor losartan 50 mg QD on renal hemodynamics (glomerular filtration rate (GFR) / effective renal plasma flow (ERPF)) in metformin-treated T2DM patients.
Patients: 12 patients with T2DM (male and female; HbA_{1c} 6.5-9%); aged 35-75 years; BMI ≥25 kg/m^2. Same in- and exclusion criteria as the RED study except for antihypertensive agent use. Only use of alpha blockers is allowed as background therapy. Patients using an antihypertensive agent will be considered if this agent can be stopped (i.e. blood pressure adequate to stop at screening) or replaced by an alpha blocker. In these patients, a 4 week wash-out/run-in period will be observed prior to visit 2.
Study Endpoints and methods: Renal hemodynamics, i.e. GFR and ERPF will be measured by the gold-standard fasting iohexol and para-aminohippurate continue infusion methods, respectively; renal tubular function will be measured by 24-hour urine sodium, potassium, chloride, calcium, magnesium, phosphate and urea and glucose; markers of renal damage will include 24-hour urinary creatinine and albumin excretion, neutrophil gelatinase-associated lipocalin and kidney injury molecule-1; blood pressure and heart rate will be measured using an automated oscillometric blood pressure device (Dinamap®); body anthropometrics, including body weight, height, body-mass index and body fluid contents (by bio-impedance spectroscopy) will be measured; additional urine and blood samples will be obtained to determine markers of inflammation and (cardiovascular)-biomarkers.

Expected results: This cross-over mechanistic substudy will expand our knowledge regarding the potential renoprotective effects of mono- and combination therapy with SGLT-2 inhititors and RAS in human T2DM. We hypothesize that SGLT-2 inhibitors and ARBs may confer renoprotection by improving renal hemodynamics. Moreover, combined use might lead to a additive or even synergistic effect, since we know from the RED study

that both agents act on the efferent arteriole. Ultimately, we expect that this study will contribute to guiding clinicians in their decision-making process of individualizing therapies for T2DM patients at risk for, or suffering from, DKD.

Figure. Test vistit with simplified renal protocol.



(1) van Bommel EJ, Muskiet MH, Tonneijck L, Kramer MH, Nieuwdorp M, van Raalte DH. SGLT2 Inhibition in the Diabetic Kidney-From Mechanisms to Clinical Outcome. Clin J Am Soc Nephrol. 2017 Apr 3;12(4):700-710
(2) Cherney DZ, Perkins BA, Soleymanlou N, Maione M, Lai V, Lee A, Fagan NM, Woerle HJ, Johansen OE, Broedl UC, von Eynatten M. Renal Hemodynamic Effect of Sodium-Glucose Cotransporter 2 Inhibition in Patients With Type 1 Diabetes Mellitus. Circulation. 2014 Feb 4;129(5):587-97.
(3) Kidokoro K1, Cherney DZI2, Bozovic A3, Nagasu H1, Satoh M4, Kanda E5, Sasaki T1, Kashihara N4. Evaluation of Glomerular Hemodynamic Function by Empagliflozin in Diabetic Mice using In Vivo Imaging. Circulation. 2019 Feb 18 (4) Judd TA, Day AS, Lemberg DA, Turner D, Leach ST. Update of fecal markers of inflammation in inflammatory bowel disease. J Gastroenterol Hepatol 2011 Oct;26(10):1493-9.

APPENDIX J

RED substudy - REGROUP

Title: Renoprotective Effects of dapaGliflozin in vaRisOUs Populations (RED-REGROUP)

Background: This substudy elaborates on the primary results of the RED study, which led us to the research question whether the effects that we observe are dependent of hyperglycemia and whether they are preserved in patients with diabetic kidney disease. From our results it is clear that dapagliflozin affects renal hemodynamics in type 2 diabetes patients with adequate kidney function. However, if these effects remain the same in other populations, such as T2DM patients with impaired renal function, non-diabetic patients with impaired renal function and non-diabetic patients without renal impairment is currently unknown. This substudy will assess the acute hemodynamic effects of dapaglifozin in four different populations using a simplified version of the renal protocol used in the RED study.

Hypothesis: Treatment with SGLT-2 inhibitor dapagliflozin may confer renoprotection by improving renal hemodynamics regardless of renal function or the presence of diabetes.

We will test this hypothesis by addressing the following **research objectives**:
Primary objective: what are the acute effects (i.e. after 3 days of drug exposure) of treatment with SGLT-2 inhibitor dapagliflozin on renal hemodynamics (glomerular filtration rate (GFR) / effective renal plasma flow (ERPF)) in either metformin-treated T2DM patients with normal kidney function, or metformine-treated T2DM patients with impaired kidney function, or non-diabetic individuals with impaired kidney function, or non-diabetic patients without impaired renal function?

Secondary objectives: renal tubular function, renal damage markers, blood pressure, heart rate, body anthropometrics, markers of inflammation and (cardiovascular-)biomarkers.

Methods:
Design: An open-label, parallel-group, mechanistic substudy to assess the effect of 3 days of treatment with SGLT-2 inhibitor dapagliflozin on renal hemodynamics (glomerular filtration rate (GFR) / effective renal plasma flow (ERPF)) in T2DM patients with normal kidney function, T2DM patients with impaired renal function, non-diabetic patients with impaired renal function and non-diabetic patients without renal impairment but with hypertension.
Patients: Hypertensive patients (male and female); aged 35-75 years; BMI ≥25 kg/m^2; using RAS inhibition

- T2DM with an eGFR (CKD-EPI) above 75 mL/min/1.73m^2 at the Screening Visit or
- T2DM with an eGFR (CKD-EPI) between 25 and 50 mL/min/1.73m^2 at the Screening Visit or
- Non-diabetic with an eGFR (CKD-EPI) between 25 and 50 mL/min/1.73m^2 at the Screening Visit or
- Non-diabetic with an eGFR (CKD-EPI) above 75 mL/min/1.73m^2 at the Screening Visit.

Diagnosis of T2DM according to RED study protocol. Other in- and exclusion criteria are the same as in the RED study.

Study Endpoints and methods: Renal hemodynamics, i.e. GFR and ERPF will be measured by the gold-standard fasting iohexol and para-aminohippurate continue infusion methods, respectively; renal tubular function will be measured by 24-hour urine sodium, potassium, chloride, calcium, magnesium, phosphate and urea and glucose; markers of renal damage will include 24-hour urinary creatinine and albumin excretion, neutrophil gelatinase-associated lipocalin and kidney injury molecule-1; blood pressure and heart rate will be measured using an automated oscillometric blood pressure device (Dinamap®); body anthropometrics, including body weight, height, body-mass index and body fluid contents (by bio-impedance spectroscopy) will

be measured; additional urine and blood samples will be obtained to determine markers of inflammation and (cardiovascular)-biomarkers;

Expected results: This parallel-group, mechanistic substudy will expand our knowledge regarding the potential renoprotective effects of treatment with SGLT-2 inhititor dapagliflozin in various populations. We hypothesize that SGLT-2 inhibitors may confer renoprotection by improving renal hemodynamics not only in the diabetic population with normal kidney function, but also in the population without diabetes or with impaired kidney function. We expect that this study will contribute to guiding clinicians in their decision-making process of individualizing therapies for T2DM patients at risk for, or suffering from, DKD.

Figure. Test visit with simplified renal protocol.