Title Page

Role of Canagliflozin on gene expression and function of CD34+ endothelial progenitor cells and renal function in patients with type 2 diabetes

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PROTOCOL SYNOPSIS

Protocol Title:	Role of Canagliflozin on gene expression and function of CD34+ endothelial progenitor cells in patients with type 2 diabetes
Site Numbers & Names	GWU Medical Faculty Associates
Research Hypothesis:	Type 2 diabetes is associated with poor stem cell number and function. Poor viability and function of EPCs in diabetes affects the repair and regeneration of the endothelium. We hypothesize that use of Canagliflozin (Cana), along with certain anti-diabetic drugs may help reduce cardiovascular risk by improving EPC survival and function above and beyond adequate glucose metabolism control.
Study Schema: Drugs / Doses / Length of Treatment	We propose a 2-arm randomized, parallel group, longitudinal study of 16-week intervention duration. The 16-week time interval has been previously shown adequate to observe changes in biochemistry, EPCs and, importantly, pulse wave velocity (PWV) changes. Patients will be randomized to 2 groups: **Control (n=20) Concomitant Anti-Diabetic Therapy + Placebo.** **Treatment (n=20) Concomitant Anti-Diabetic Therapy + Cana 100mg.**

Study Objectives: • **Primary** • **Secondary**	The primary objective is to ascertain if the addition of Cana improves CD34+ cell function and gene expression in type 2 diabetes patients, which will be correlated to improvement in 24hr urinary protein estimation and creatinine clearance (obtained via micro-albumin/creatinine ratio from a spot urine sample). The secondary objective is to correlate the cellular outcome measures with other measures of endothelial function such as: 1. Arterial stiffness measures with Pulse wave analysis and pulse wave velocity measurements. 2. Serum biochemistry looking at surrogates of endothelial health, endothelial inflammation, appetite controlling hormone levels and fasting glucose, insulin and lipid profile. 3. Resting Metabolic Rate measurement **Study Outcome Measures:** **Primary:** To investigate the effect of Cana on Endothelial function in patients inadequately controlled (HbA1C ≥ 7.0 % to ≤10%) while being treated with a stable dose of certain anti-diabetic drugs. • **Cellular markers.** We will study pre and post Cana treatment changes in number, function and gene expression of patients' peripheral blood-derived CD34+ cells. • Renal Function Estimation: 24hr urinary **protein estimation,** creatinine clearance and urine exosome study. **Secondary:** To investigate the effect of Cana treatment on: • Serum endothelial inflammatory markers including high sensitivity C-reactive protein (hs-CRP), IL-6, TNF-alpha and fasting lipid profile. • Glycemic control will be evaluated by measuring fasting blood glucose, insulin, and HbA1c levels and assessing insulin resistance using HOMA-IR. • Adiposity, measured using a body composition analyzer scale, measured as percentage body fat. • Creatinine clearance estimation. • Vessel health will be assessed by systolic and diastolic blood pressure and Arterial stiffness assessed using Vascular Flow and wave measurement equipment, SphygmoCor CP system from ATCOR. • Central and aortic blood pressure estimated by the sphygmocor system. • Resting Metabolic Rate (RMR, similar to Resting Energy expenditure measurement) at baseline, midway point (week 8) and post therapy.

Study Design:	Prospective, double-masked, randomized placebo-controlled trial
Accrual Goal (Total number of subjects):	N = 40
Accrual Rate (Number of subjects expected per month):	Recruit 40 patients in approximately 36 months
Estimated Timeline:	FPFV: 10-06-2016 LPLV: 01-06-2019
Co-relative studies (PK/PD, etc.):	N/A
Inclusion Criteria:	1. Adults aged 30-70 years 2. Diagnosis of type 2 diabetes using criteria of the American Diabetes Association 3. Currently being treated with any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas as therapy for their Type 2 Diabetes 4. HbA1C between 7.0 to 10.0 % (both inclusive) 5. BMI 25 – 39.9 kg/m^2
Exclusion Criteria:	Patients with: 1. Type 1 DM 2. History of hyperosmolar non-ketotic coma 3. Abnormal CBC that is judged by physician to be unsafe to enroll or low hematocrit (<28 UNITS). 4. History of Pancreatitis 5. History of DKA in the past 3 months 6. History of active cancer or cancer treatment in the past 5 years (except basal cell carcinoma) 7. Heart attack or stroke within 6 months of screening 8. Clinically significant coronary and/or peripheral vascular disease that would be unsafe to enroll in the study. 9. Statin use started or dose change in the last 3 months, 10. CKD stages 3B-5 11. Use of oral anti-diabetic medication other than metformin, insulin, GLP-1 agonists, DPP-IV inhibitors, or sulfonylureas 12. Use of consistent long-term steroid medication in the last 3 months (oral, inhaled, injected). 13. Uncontrolled inflammatory disease, or current chronic use of anti-inflammatory drugs within the last 3 months. 14. Pre-existing liver disease and/or ALT and AST >2.5X's UNL 15. Serum creatinine levels ≥2.0 16. Estimated CrCl < 45 mL/min (measured by eGFR value) 17. Triglycerides >450 mg/dL 18. Implanted devices (e.g., pacemakers) that may interact with Body Composition scale 19. Untreated Systolic Blood Pressure > 150 mmHg and diastolic Blood Pressure > 90 mmHg

	20. Active wounds or recent surgery within 3 months. 21. Untreated hyper/hypothyroidism Additionally, patients who are active smokers, patients who are pregnant, nursing women, and post-menopausal women who are on estrogen hormone replacement therapy (not including oral contraceptives) will be excluded. Detailed Inclusion / exclusion criteria are listed on pages 12-15 of the protocol.
Criteria for Evaluation: (Efficacy, safety, stopping rules, etc.)	Subjects will be phone screened to determine initial eligibility. If the phone-screening indicates possible eligibility, then the subjects will come to an in-person screening visit at week -2, then evaluated for end points at weeks 0, 8 and 16. Follow up phone call will be done 30 days after last dose of study drug to assess for adverse effects. Study participation will be stopped for adverse effects at the discretion of the study investigator. Subjects may stop study participation at any time and for any reason.
Statistics:	The total sample size is requested, after accounting for attrition over the 16-week period, is 20 subjects per group or 40 subjects total. Sample size estimates were based on the effects of exercise on CD34+/KDR+ cells as described in the literature. The effect of a single session, as well as extended training, on healthy subjects or those with existing cardiovascular conditions appears to increase the CD34+/KDR+ cells. This is a pilot study; as a result appropriate power calculation is not feasible. We however did conduct power analysis, which provided us with 73% power (see statistics section). A p-value of less than 0.05 will be considered statistically significant.

INTRODUCTION

Diabetes affects more than 11% of adults in the United States and this is projected to nearly double by 2025.[1] Both diabetes and obesity are associated with endothelial dysfunction, oxidative stress, endothelial cell inflammation, cardiovascular pro-thrombotic states and are the most common causes of kidney disease[2,3,4]. Use of a sodium-glucose linked transporter (SGLT-2) inhibitor has shown promise in improving glycemic control, weight reduction, hypertension and even changes in circulating Renin-angiotensin-aldosterone system (RAAS) and nitric oxide (NO)[5,6] . However, whether these group of drugs have any effect on cardiovascular disease (CVD) risk modification or on endothelium or endothelial progenitor cells as a surrogate of cardiovascular and renal risk outcome measure, is unclear[5].

We have previously shown that CD34+ cells, derived from peripheral blood can act as a cellular biomarker that is more reliable than serum based markers[7] for CVD risk estimation. Serum based inflammatory markers are not useful until the endothelium is already damaged and inflamed[4]. Such serum based biomarkers takes several months to change and gives no preventive and predictable information as to whether a particular medication may affect future endothelium. This is why the study of endothelium progenitors is crucial. In our previous study of a prediabetes population with an aerobic exercise intervention, we have demonstrated that CD34+ cells are responsive to a change in therapy or intervention within 2-4 weeks and can be used as a reliable non serum based cellular bio-marker[7]. CD34+ cells or endothelial progenitor cells have been used clinically to improve collateral circulation and have been extensively studied as a robust cardiovascular biomarker [8,9,7]. Therefore studying CD34+ cells in patients, with or without Canagliflozin (Cana) can give vital information about the medication and its effect on endothelium. This is particularly important as another SGLT2 inhibitor Empagliflozin has shown unparalled positive cardiovascular effects with an oral hypoglycemic agent[10]. Of course, the question arises whether this clinical trial effect is secondary to glucose effect or direct effect of SGLT2 inhibitor on endothelium.

Multiple glucose transporters have been identified in human cells these include GLUTs, SGLTs and even taste receptors (such as TLR2 and TLR3). We know SGLT transporters are present in tubular cells and clearly blocking of SGLT2 in these cells is beneficial. Information on glucose transporter in stem or progenitor cells is almost nil. In our lab we have shown presence of GLUT1, SGLTs and TLR3 on CD34+ cells. We have also demonstrated that hyperglycemia is toxic to CD34+ cells, more than CD31+ positive mature endothelial cells. We hypothesize that blocking SGLT2 in CD34+ cells will be beneficial rather than detrimental. As far as glucose uptake in CD34+ cells are concerned other glucose transporters should be sufficient, in fact

lesser amount of glucose entry in a hyperglycemic milieu (type 2 DM patients) may be less pro-inflammatory and less pro-apoptotic.

Our preliminary data indicates that mRNA gene expression of both SGLT1 and SGLT2 are noted on human CD34+ cells however only SGLT2 mRNA gene expression is up-regulated several fold in human CD34+ cells in presence of hyperglycemia (20mM glucose). However non primary commercially obtained human endothelium (HUVEC) do not show similar results. An explanation could be SGLT2 expression decreases as the cell transitions from progenitor to mature endothelium. From these results we believe SGLT2 inhibitor will be effective on progenitors and not mature endothelium. We therefore hypothesize that CD34+ cells will be an ideal biomarker to study the effect of the drug. It is possible that Cana, by blocking SGLT2 receptors, may influence other CD34+ cell surface receptors including other glucose transporters and influence its function (most importantly migration). If a particular medication positively influences stem/progenitor cell migration then that medication can positively influence endothelial dysfunction and vascular complications from diabetes. We are particularly interested to note effect of Canagliflozin, a SGLT2 inhibitor on other glucose transporters such as GLUT 1 and 4 while looking at SGLT 1 and 2 on CD34+ cells. It will be helpful to discern these effects particularly when choice of oral diabetic medication in a type 2 diabetes population is practically limited to metformin, DPP4 inhibitors and SGLT2 inhibitors[2]. We plan to investigate the effect of Cana on CD34+ cells, in a placebo matched study. We plan to recruit subjects with type 2 diabetes with the following characteristics: 1) overweight, mild and moderately obese (BMI=25.0-39.9); 2) individuals with type 2 diabetes with inadequate control, HbA1C= 7 to 10.0%, on any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas 3) with no current macrovascular complications. As per the ADA [2], Metformin, insulin, GLP-1 agonists, DPP-IV inhibitors, and sulfonylureas are all 1st line of care along with life-style modification. While Metformin on its own may affect inflammatory biomarkers, the effect is minimal at best, particularly in presence of endothelial dysfunction. For accurate comparison, both placebo and the Canagliflozin group will be on any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas We will recruit a total of 40 patients (20 individuals/per group) with approximately a 20% drop out rate over two years and we hope to retain 32 individuals (16/group). Individuals in each group will be matched by sex, age, and race. Participants will be assessed at baseline (week 0), and at 2 and 4 months of drug intake.

1 RESEARCH HYPOTHESIS

We hypothesize that Cana may be able to improve number and function of CD34+ endothelial progenitor cells. We also propose that this expected cardiovascular benefit is independent of HbA1C reduction[11,12].

In this proposed study we plan to recruit patients with type 2 diabetes, who are on any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas, but still remain inadequately controlled with HbA1C between 7.0% and 10% (both values inclusive), but not overtly out of control (> 10% of HbA1C).

Subjects will begin taking 100 mg of Cana or placebo after initial 4 weeks. Subjects will be withdrawn from the study if the medication or placebo is not tolerated.

2 STUDY OBJECTIVES

PRIMARY OBJECTIVE

To determine whether 4 months of Canagliflozin modifies CD34+ cell number, gene expression and migration function.

PROTOCOL FOR OBTAINING CD34+ CELLS:

We will obtain a total of approximately 95 mL of peripheral blood per visit. Of these 95 mL, 60-70 mL will be used to obtain CD34+ cells from mononuclear cell (MNC) population and 25-35 mL for biochemistry and plasma ELISA assays. MNC will be obtained from whole blood similar to protocols described before[13,14]. MNCs will be put through CD34 magnetic bead column to obtain CD34+ cells (Miltenyi Biotec). Purity of CD34+ cells, post sort, usually is above 90%, to be verified by FACS analysis.

A. From Mononuclear (MNC) population (pre CD34+ magnetic column sorting): We will assess the proportion of various cell types, progenitor stems and mature cells in MNCs to note if Cana influences stem vs non stem cell pool such as percentage of : CD34, CD309 (VEGFR2-KDR), CD184 (CXCR4), receptor substrate for SDF1 alpha, CD31 (PECAM-1, a mature circulating endothelial cell marker), CD133 (a progenitor marker), CD144 (Vascular Endothelial cadherin), Sytox Blue OR Propidium Iodide staining (to detect apoptotic cells).
B. MNC cells (prior to CD34 magnetic bead sorting) will be also used to assay CFU Hill Colony forming unit (Stem Cell Technologies- Cat#05900) media for Colony Forming unit (CFU) Assay. CFU will be counted on 5-7 days post plating (from initial plating).
C. From CD 34+ cell population (Post Sorting):
    a. Migration Assay using SDF1Alpha concentrations of 0, 10, 100ng/ml and VEGF-A concentrations of 20 and 50 ng/ml, using 150,000 cells in 300ul serum free cell suspension media per insert.
    b. Gene expression studies. Genes to be assessed on sorted CD34 positive cells:

i. Endothelial lineage cell surface markers: CD34, VEGFR2 (KDR), CD31, CD144
ii. For Anti-oxidant gene expression: Superoxide dismutase (SOD) 1, 2 and 3, catalase, glutathione- peroxidase.
iii. Apoptosis pathway: p53, p21, Bcl2, caspase-3
iv. Endothelial Function Assay Gene: endothelial Nitric Oxide Synthase, von-Willebrand's Factor
v. Genes associated with progenitor cell chemotaxis: VEGF-A, SDF1 alpha, CXCR4.

SECONDARY OBJECTIVE

To determine whether use of Canagliflozin alters other cardio-metabolic health indicator parameters such as: arterial stiffness measures, resting metabolic rate, body composition measures, hs-CRP, IL-6, TNF-alpha (inflammatory markers), fasting lipid profile, levels of insulin, glucose appetite controlling hormones and urine levels of microalubumin (Microalbumin/Creatinine ratio).

We expect to corroborate our findings of the Primary Objective (cellular outcome measures) with traditional markers of endothelial/ vascular function such as serum biochemistry and arterial stiffness.

Effect of Cana on Serum Biochemistry:
While we believe cell based biomarkers are superior to traditional serum biomarkers, we believe the outcome report will be stronger if one can show positive co-relation between the two outcome measures. We therefore will be looking at:

I. Inflammation, apoptosis and anti-oxidant protein levels: Highly selective C-reactive protein (hs-CRP), IL-6, TNF-alpha, fasting glucose, insulin and lipid profile.
   a. Glycemic control will be evaluated by measuring fasting blood glucose, insulin levels and HbA1c. Fasting blood glucose, insulin and lipid profile will be used to assess insulin resistance[15],[16] (32, 37)
II. Plasma SDF1 alpha (ELISA) and GLP-1 (ELISA) will be estimated to assess endothelial health and factors that may influence CD34+ cell chemotaxis
III. Estimation of Creatinine Clearance and proteinuria: 24hr urinary protein estimation and creatinine clearance (obtained via micro-albumin/creatinine ratio from a spot urine sample).
   a. *Subjects will have the option of opting into a sub-study that is being run by Dr. Eric Nylen at the Veterans Affairs (VA) Maryland Health Care System. 10-20 mL of the excess fresh spot urine that has been collected would be sent to the VA for exosome analysis. This is optional, and is not required of subjects. For details of urine-exosome study please see page 31.*
IV. The glomerular filtration rate (GFR) will be estimated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation. The CKD-EPI equation, expressed as a single equation, is:
   a. $GFR = 141 \times \min(Scr/\kappa,1)^{\alpha} \times \max(Scr/\kappa,1)^{-1.209} \times 0.993^{Age} \times 1.018$ [if female] $\times 1.159$ [if black]; where Scr is serum creatinine (mg/dL), κ is 0.7 for females and

0.9 for males, α is –0.329 for females and –0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1[17].

Effect of Cana on Body Habitus (Determination of body composition and visceral fat) AND Resting Metabolic Rate.

As we plan to study cardio-metabolic effect of Cana we believe the RMR and body composition data is useful.

I. Using body composition scale:
   a. Height and weight will be measured and the body mass index (BMI=kg•m-2) used as an indicator of relative weight.
   b. The body composition scale calculates body fat%, total body water%, fat free mass, etc. in addition to BMI.

II. Resting Metabolic Rate (RMR): at weeks 0, 8 and 16 of the protocol using ReeVue (trademark) machine, with or without SGLT2 inhibitor therapy to ascertain if Cana has any effect on RMR. Other related trials have shown weight loss but effect on metabolic rate has not been studied[18].

Effect of Cana on Assays of Arterial Stiffness:

I. We will acquire Pulse Wave Analysis and Vascular Flow using SphygmoCor CP system from ATCOR as a measure of central arterial pressure and arterial stiffness. Again SGLT2 inhibitors have been shown to reduce systolic blood pressure but effect on stiffness has not been looked at, except with Empagliflozin[17,18]

II. Vessel health will be assessed by degree of arterial stiffness, using arterial tonometry.

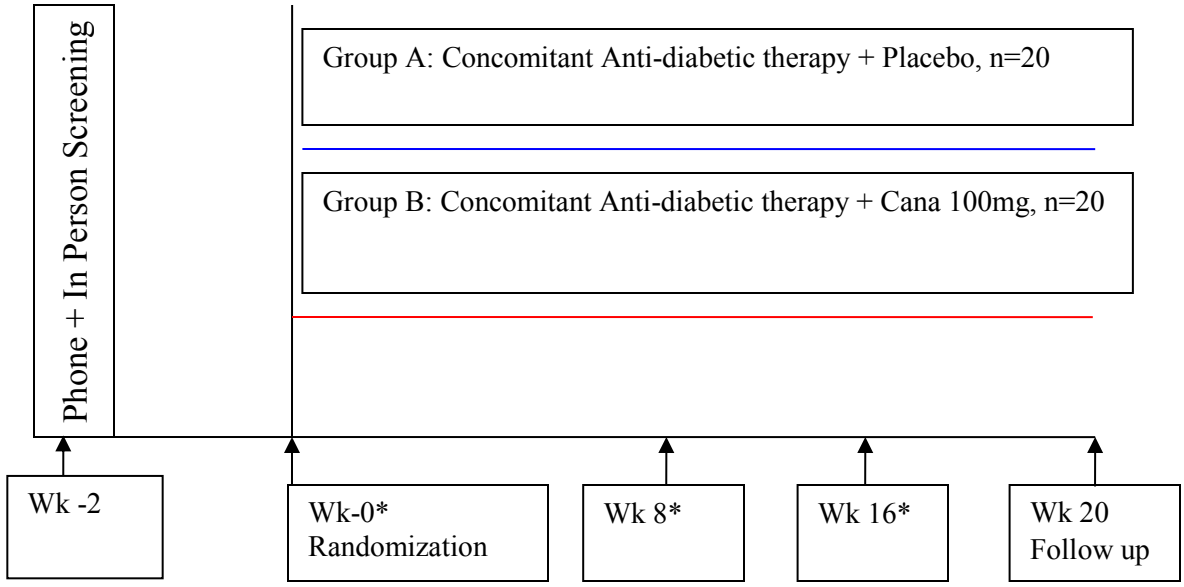
III. The central and the aortic pressure is assessed by pulse wave analysis (PWA) and pulse wave velocity (PWV).

IV. Arterial stiffness will be assessed using Vascular Flow and wave form analysis equipment, SphygmoCor CP system from ATCOR[19].

3 INVESTIGATIONAL PLAN

STUDY DESIGN AND DURATION

40 Type 2 diabetic Subjects Aged 30-70 on stable dose of certain anti-diabetic therapies



+/- **6 day window for visits**
*Assessed at week 0, 8 and 16: Biochemical and cellular markers of endothelial function and Pulse wave analysis and velocity (PWA and PWV), REE and Adiposity.

Week 20 - A telephone call to subjects will be made 30 days after last dose of study medication to determine if there have been any adverse events.

AVAILABLE RELATED STUDIES AND EXPECTED RESULTS:
Studies measuring changes to EPCs in type 2 diabetes patients are very limited.

Use of CD34 positive cells as a cardiovascular risk surrogate has been reported in the past. Various studies[20,21], have shown that various disease states, including those seen in diabetes, can deplete and damage EPC, thereby diminishing their regenerative potential. The inability to maintain or repair damaged endothelial tissue leads to cumulative vascular dysfunction and cardiovascular disease.

We hypothesize that monitoring number, function and gene expression of endothelial progenitors will allow us to quantify cardiovascular disease risk at the onset and regenerative potential post intervention at a cellular level. It will also help us to identify and correlate the best endothelial function bio-chemical inflammatory marker as an early indicator of cardiovascular disease progression in early type 2 diabetes and identify patients that are responders.

Cana can influence the glucose transportation and modify intercellular glucose and cell energetics, and so it can influence the migration of the cell. SDF-1alpha, being a chemotactic agent, will also influence migration depending on its level.

Cana 100 mg or placebo will be added to patients who have type 2 diabetes with HbA1C of 7.0 to 10 % (both inclusive) while being on any combination of the following anti-diabetic therapies:

metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas. As per ADA[22], Metformin is the 1st line of care along with life-style modification. While Metformin on its own may affect inflammatory biomarkers, the effect is minimal at best, particularly in presence of endothelial dysfunction. Also both the placebo and Canagliflozin group will be on any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas. Our study population will include equal numbers of adult male and female Type 2 diabetes (T2DM) patients aged 30-70 years, enrolled in both arms of the study.

Patients will be treated with 100 mg of Cana or placebo for 16 weeks. This time interval has been previously shown to be adequate to observe changes to Endothelial Progenitor Cells (EPCs)[13,23].

STUDY POPULATION

Inclusion Criteria:

1) Signed written informed consent
    - Before any study procedures are performed, subjects will have the details of the study described to them, and they will be given a written informed consent document to read. Then, if subjects consent to participate in the study, they will indicate that consent by signing and dating the informed consent document in the presence of study personnel.
2) Target Population
    - Subjects with a diagnosis of Type 2 diabetes mellitus using criteria of the American Diabetes Association.
    - Currently treated with any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas
    - HbA1C between 7.0% and 10% (both inclusive)
    - BMI 25-39.9 kg/m^2 both inclusive.

One of the prime outcome measure is estimation of renal function and accurate eGFR staging:Estimated Glomerular Filtration Rate Serum Cr should be used to estimate glomerular filtration rate (GFR). Estimated GFR (eGFR) is commonly reported by laboratories or can be estimated using formulae such as the Modification of Diet in Renal Disease (MDRD) study equation or the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. The latter is the preferred GFR estimating equation. GFR calculators are available at http:// www.nkdep.nih.gov.

Abnormal urinary albumin excretion and eGFR may be used to stage chronic kidney disease (CKD). The National Kidney Foundation classification (Table below) is based on **both kidney damage (UACR at or above 30 mg/g Cr) and eGFR.**

Normal UACR is defined as less than 30 mg/g Cr, and increased urinary albumin excretion is defined as at or above 30 mg/g Cr. Because of variability in urinary albumin excretion, ideally two (at least one) specimens of UACR should have been collected within a 3- to 6-month period before considering a patient to have albuminuria.

Stage -- Description -- GFR (mL/min/1.73 m2)

Stage 1 Kidney damage* with normal or increased GFR >=90
2 Kidney damage* with mildly decreased eGFR 60–89
3A Moderately decreased eGFR 45–59
3B Moderately decreased eGFR 30–45
4 Severely decreased eGFR 15–29
5 Kidney failure ,15 or dialysis

*Kidney damage is defined as abnormalities on pathological, urine, blood, or imaging tests.[24]

3) Age and Reproductive Status
   - Men and women, 30 to 70 years of age.
   - Enrolled women must not be pregnant, should not be breast-feeding and should not be taking estrogen hormone replacement therapy (HRT) during the study.

The following is a definition of Women of Child Bearing Potential (WOCBP):

- Women using the following methods to prevent pregnancy: Oral contraceptives, other hormonal contraceptives (vaginal products, skin patches, or implanted or injectable products), or mechanical products such as intrauterine devices or barrier methods (diaphragm, condoms, spermicides).
- Women who are practicing abstinence.
- Women who have a partner who is sterile (e.g., due to vasectomy).WOCBP must be using an acceptable method of contraception to avoid pregnancy throughout the study and for up to 4 weeks after the last dose of study drug in such a manner that the risk of pregnancy is minimized.
- WOCBP must have a negative serum or urine pregnancy test result within 0 to 72 hours before the first dose of study drug.
- Women must not be breast-feeding.

Post-menopause is defined as:

- Women who have had amenorrhea for ≥ 12 consecutive months and/or who have a documented serum follicle-stimulating hormone (FSH) level > 35 mIU/mL.
- Women who have irregular menstrual periods and a documented serum FSH level > 35 mIU/mL.
- Women who are taking hormone replacement therapy (HRT).

Exclusion Criteria:

- Type 1 diabetes mellitus
- History of hyperosmolar non-ketotic coma
- Abnormal CBC that is judged by physician to be unsafe to enroll or low hematocrit (<28 UNITS).
- History of pancreatitis
- History of diabetic ketoacidosis in the last 3 months
- History of cancer (except basal cell carcinoma and cancer that is cured or not active or being treated in the past 5 years)
- Heart attack or stroke within 6 months of screening

- Clinically significant coronary and/or peripheral vascular disease that would be unsafe to enroll in the study. *
- Statin use started or dose change in the last 3 months. *
- CKD Stages 3B,4 and 5 (see definition above)
- Use of oral or injectable anti-diabetic medication other than any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas currently, or in the past 1 month. *
- Use of consistent long-term steroid medication (oral, inhaled, injected) within the last 3 months. *
- Uncontrolled inflammatory disease, or current chronic use of anti-inflammatory drugs within the last 3 months. *
- Implanted devices (e.g., pacemakers) that may interact with Body Composition scale
- Untreated Systolic Blood Pressure > 150 mmHg and diastolic Blood Pressure > 90 mmHg
- Active wounds or recent surgery within 3 months. *
- Untreated hyper/hypothyroidism

**These criteria may be allowed, judged on a case by case basis by the PI, with appropriate rationale.*

Physical and Laboratory Test Findings

- Pre-existing liver disease and/or ALT and AST >2.5X's UNL
- Serum creatinine levels ≥2.0
- Estimated CrCl < 45 mL/min (measured by eGFR value)
- Triglycerides >450 mg/dL

Allergies and Adverse Drug Reactions

- Subjects with a history of any serious hypersensitivity reaction to Cana or another SGLT2 inhibitor.

Sex and Reproductive Status

- Women in reproductive age group will be included in the study but encouraged to use contraceptive method to avoid pregnancy within 16 weeks of study duration.
- Women who are pregnant or breast-feeding will be excluded.

Other Exclusion Criteria

- Prisoners or subjects who are involuntarily incarcerated.
- Subjects who are compulsorily detained for treatment of either a psychiatric or physical (e.g., infectious disease) illness.
- Patients who are active smokers
- Patients who are pregnant
- Nursing women
- Post-menopausal women who are on estrogen hormone replacement therapy will be excluded.
- Patients on low dose oral contraceptives will be allowed to participate as these formulations contain very low amounts of estrogens.

- Eligibility criteria for this study have been carefully considered to ensure the safety of the study subjects and to ensure that the results of the study can be used. It is imperative that subjects fully meet all eligibility criteria.

DISCONTINUATION OF SUBJECTS FROM TREATMENT

Subjects **MUST** discontinue from the investigational product (and non-investigational product at the discretion of the investigator) for any of the following reasons:

- Withdrawal of informed consent (subject's decision to withdraw for any reason)
- Any clinical adverse event, laboratory abnormality, or inter-current illness which, in the opinion of the investigator, indicates that continued participation in the study is not in the best interest of the subject.
- Patients who progress to stages 3 and beyond, post screening will be excluded
- Pregnancy
    - Pregnant patients will be advised to contact the investigator or study staff immediately if they suspect they might be pregnant (e.g. missed or late menstrual period) at any time during study participation.
- Loss of ability to freely provide consent through imprisonment or involuntary incarceration for treatment of either a psychiatric or physical illness (e.g. infectious disease).
- If hyperglycemia and ketoacidosis is suspected, promptly discontinue treatment with Canagliflozin
- **All subjects who discontinue should comply with protocol-specified follow-up procedures outlined before.** The only exception to this requirement is when a subject withdraws consent for all study procedures or loses the ability to consent freely (i.e., is imprisoned or involuntarily incarcerated for the treatment of either a psychiatric or physical illness). If a subject withdraws before completing the study, the reason for withdrawal must be documented appropriately.

4 TREATMENTS

STUDY TREATMENT: CANAGLIFLOZIN

Definition of Investigational Product: A pharmaceutical form of an active substance or placebo being tested or used as a reference in a clinical study, including products already with a marketing authorization but used or assembled (formulated or packaged) in a way different from the authorized form, or used for an unauthorized indication, or when used to gain further information about the authorized form. In this protocol, the investigational product is Canagliflozin.

Definition of Non-Investigational Product: Other medications used in the study as support or escape medication for preventative, diagnostic, or therapeutic reasons as components of a given standard of care. In this protocol, the non-investigational products are Metformin, Insulin, GLP-1 agonists, sulfonylureas, and DPP-IV inhibitors. Patients will continue on any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas, as per their study entry dose added to Cana or placebo. As mentioned

before, patients on oral hypoglycemic other than any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas will not be invited to join the study.

This study is being conducted under FDA mandated guideline for use in type 2 diabetes. Though in this particular study Cana is referred to as the investigational drug, it is not a novel drug and we should not need a separate IND for execution of this study.

METHOD OF ASSIGNING SUBJECTS TO A TREATMENT

Subjects, as per selection criteria below will be randomized to treatments using a permuted block design, developed by the Epidemiology & Biostatistics Research Core. This approach ensures groups will be approximately balanced at any time during the study and at study completion. Randomization will be performed by the MFA IDS Pharmacy staff who never come in contact with subjects.

SELECTION AND TIMING OF DOSE FOR EACH SUBJECT

The recommended dose of Cana is 100 mg PO will be given once daily. Cana can be taken with or without food. Cana would preferably be taken in the morning prior to the first meal.

DOSE MODIFICATIONS

No dose modification is necessary prior to CKD stage 3B (eGFR less than 45)

BLINDING/ UNBLINDING

Blinding is critical to the integrity of this clinical study. However, in the event of a medical emergency or pregnancy in a subject, **in which knowledge of the investigational product is critical to the subject's management**, the blind **for that subject** may be broken.

Before breaking the blind of an individual subject's treatment, the investigator should have determined that the information is necessary, i.e., that it will alter the subject's immediate management. In many cases, particularly when the emergency is not investigational product-related, the problem may be properly managed by assuming that the subject is receiving active product without the need for un-blinding.

END OF STUDY UNBLINDING PROTOCOL

Upon completion of the research study, we will undergo the following protocol in order to unblind the research data.

Unblinding is the process by which the allocation code is broken so that the investigator, clinical staff and the trial statistician becomes aware of which intervention each subject enrolled in the research study was taking.
Unblinding at the end of the study is required in order to make unmasked analysis in accordance with the study analysis plan. It is also conducted in order to inform the participants of which investigational product they were assigned to.

Time to unblind:
Unblinding shall be conducted when all subjects enrolled in the research study have finished treatment, and all follow up visits. There must be no plan to recruit any more subjects in the research study. Additionally, all data points and outcome measures for each research subject

must have been collected, and ideally compiled. Prior to unblinding there will be a data lock on clinical outcome measures and basic side outcome measures and associated research data collected for the study.

Procedure to unblind

Once data (from both the clinical and the basic science side) and has been compiled and is data locked, the investigator can choose to unblind. The Principal Investigator must contact the study sponsor, and receive permission to un-blind. If, for unforeseen reasons, at the pre-determined date for full study unblinding the data analysis on the cellular or basic aspects of the study is lagging behind the clinical data outcome measures (though the data has been acquired) the Principal Investigator in consultation with the study sponsor may choose to un-blind the clinical outcome measures before the basic side data has been analyzed but compiled. Upon confirmation from the study sponsor, the principle investigator must make a written request to the designated party to unblind, hereto referred at the "*unblinder*". The unblinder is the bio-statistician of the MFA, **Dr. Richard Amdur**. Upon receipt of an instruction to unblind, the unblinder will sign the request form, indicating their agreement to unblind. This form will then be taken to the pharmacy, **MFA's IDS**, where a member of the IDS staff will take the form, and will give the unblinding study binder to the unblinder. The pharmacy will sign to indicate their release of the binder, and the unblinder will sign to indicate receipt of the binder. At this point in time the chain of custody of the pharmacy unblended binder has been transferred to the designated party to unblind. The form with all of the signatures will be provided to the study coordinator to be kept in the regulatory binder.

5 ADVERSE EVENTS: DEFINITIONS, REPORTING, GUIDANCE

I. Management of Safety Data

This Study has been designated as an interventional study. Janssen requirements for IIS interventional studies are all adverse events regardless of causality and special situations excluding those from subjects not exposed to a Janssen Medicinal Product and product quality complaints with or without an adverse event will be reported, once the subject has signed and dated an Informed Consent Form is obtained until the subject has completed participation in the study and for 30 days after the last dose of study drug.

II. Definitions

Adverse Event (AE)

An adverse event is any untoward medical occurrence in a clinical study subject administered a medicinal (investigational or non-investigational) product. An adverse event does not necessarily have a causal relationship with the treatment. An adverse event can therefore be any unfavorable and unintended sign (including an abnormal finding), symptom, or disease temporally associated with the use of a medicinal (investigational or non- investigational) product, whether or not related to that medicinal (investigational or non-investigational) product. (Definition per International Conference on Harmonisation [ICH]).

This includes any occurrence that is new in onset or aggravated in severity or frequency from the baseline condition, or abnormal results of diagnostic procedures, including laboratory test abnormalities.

Adverse Events of Special Interest

Events that Janssen Scientific Affairs is actively monitoring as a result of a previously identified signal (even if non-serious). These adverse events are:

- Diabetic ketoacidosis (DKA)
- Genital mycotic infection
- Urinary tract infection
- Osmotic diuresis-related AEs
- Volume related AEs

Individual Case Safety Report (ICSR)

A valid ICSR must contain the four minimum criteria required to meet regulatory reporting requirements.

- an identifiable subject (not disclosing the subject's name and address)
- an identifiable reporter (investigational site)
- a Janssen medicinal product
- an adverse event, outcome, or certain special situation

The minimum information required is:

- suspected Janssen product (doses, indication)
- date of therapy (start and end date, if available)
- batch or lot number, if available
- subject details (subject ID and country)
- gender
- age at AE onset
- reporter ID
- adverse event detail (AE verbatim in English), onset date, relatedness, causality, action taken, outcome, (if available)
- Janssen protocol ID

Product Quality Complaint (PQC)

A product quality compliant is related to a potential quality issue during manufacturing, packaging, release testing, stability monitoring, dose preparation, storage or distribution of the product, or delivery system. Not all PQCs involve a subject. Lot and batch numbers are of high significance and need to be collected whenever available.

Examples of PQC include but not limited to:

- Functional Problem: e.g., altered delivery rate in a controlled release product
- Physical Defect: e.g. abnormal odor, broken or crushed tablets/capsules
- Potential Dosing Device Malfunction: e.g., autoinjector button not working, needle detaching from syringe
- Suspected Contamination
- Suspected Counterfeit.

Serious Adverse Event (SAE)

A serious adverse event based on ICH and EU Guidelines on Pharmacovigilance for Medicinal Products for Human Use is any untoward medical occurrence that at any dose:

- Results in death
- Is life-threatening
- (The subject was at risk of death at the time of the event. It does not refer to an event that hypothetically might have caused death if it were more severe.)
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect
- Is a suspected transmission of any infectious agent via a medicinal product
- Is medically important*

*Medical and scientific judgment should be exercised in deciding whether expedited reporting is also appropriate in other situations, such as important medical events that may not be immediately life threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed in the definition above. These should usually be considered serious.

NOTE: Death for ANY reason should be reported as a SERIOUS ADVERSE EVENT.

For reports of hospitalization, it is the sign, symptom or diagnosis which led to the hospitalization that is the serious event for which details must be provided.

Any event requiring in-patient hospital admission (or the prolongation of hospitalization) must be reported as an SAE. Events that do not meet the criteria for SAE reporting are:

- Reasons described in the Protocol, e.g. drug administration, Protocol-required testing
- Social reasons, e.g. overnight stay because of distance between home and hospital
- Surgery or procedure planned and documented prior to entry into the Study.

Any event requiring hospitalization or prolongation of hospitalization that occurs during the study must be reported as a serious adverse event, except hospitalizations for the following:

- Hospitalizations not intended to treat an acute illness or adverse event (e.g., social reasons such as pending placement in long-term care facility)
- Surgery or procedure planned before entry into the study. [Note: Hospitalizations that were planned before the start of data collection and where the underlying condition for which the hospitalization was planned has not worsened will not be considered serious adverse events. Any adverse event that results in a prolongation of the originally planned hospitalization is to be reported as a new serious adverse event.]

The cause of death of a subject in a study within 30 days of the last dose of study drug, whether or not the event is expected or associated with the study drug, is considered a serious adverse event.

Unlisted (Unexpected) Adverse Event/Reference Safety Information

An adverse event is considered unlisted if the nature or severity is not consistent with the applicable product reference safety information. For a medicinal product(s) with a marketing authorization, the expectedness of an adverse event will be determined by whether or not it is listed in the applicable product information.

III. Special Situations

Safety events of interest for a Janssen medicinal product that require expediting reporting and/or safety evaluation include, but are not limited to:

- Drug exposure during pregnancy (maternal and paternal)
- Overdose of a Janssen medicinal product
- Exposure to a Janssen medicinal product from breastfeeding
- Suspected abuse/misuse of a Janssen medicinal product
- Inadvertent or accidental exposure to a Janssen medicinal product
- Any failure of expected pharmacological action (i.e., lack of effect) of a Janssen medicinal product
- Medication error involving a Janssen medicinal product (with or without patient exposure to the Janssen medicinal product, e.g., name confusion)
- Suspected transmission of any infectious agent via a medicinal
- Unexpected therapeutic or clinical benefit from use of a Janssen medicinal product

These safety events may not meet the definition of an adverse event; however, they are treated in the same manner as adverse events. Special situations should be recorded on the Adverse Event page of the CRF.

Any special situation that meets the criteria of a serious adverse event should be recorded on a Serious Adverse Event Report Form and be reported to Janssen Scientific Affairs **within 24 hours of becoming aware of the event.**

IV. Pregnancy

All initial reports of pregnancy must be reported to Janssen Scientific Affairs by the Sponsor Investigator **within 24 hours of their awareness of the event** using the Serious Adverse Event Form. Abnormal pregnancy outcomes (e.g. spontaneous abortion, stillbirth, and congenital anomaly) are considered serious adverse events and must be reported using the Serious Adverse Event Form.

Any subject who becomes pregnant during the study must be promptly withdrawn from the study and discontinue further study treatment.

Because the effect of the Janssen medicinal product on sperm is unknown, pregnancies in partners of male subjects exposed to a Janssen medicinal product will be reported by the Sponsor Investigator **within 24 hours of their awareness of the event** using the Serious Adverse Event Form. Depending on local legislation this may require prior consent of the partner.

Follow-up information regarding the outcome of the pregnancy and any postnatal sequelae in the infant will be required.

V. Reporting Procedures for Adverse Events and Pregnancies [and/or Pregnancies in Partners]

All adverse events, whether serious or non-serious, related or not related, special situations, pregnancy exposures and/or pregnancies in partners following exposure to a Janssen medicinal product are to be documented by the investigator and recorded in the CRF and in the subject's source records. Investigators must record in the CRF their opinion concerning the relationship of the adverse event to a Janssen medicinal product.
All (serious and non-serious) adverse events reported for a Janssen medicinal product should be followed-up in accordance with clinical practice.

All serious adverse events that have not resolved by the end of the study, or that have not resolved upon discontinuation of the subject's participation in the study, must be followed until any of the following occurs:

- The event resolves
- The event stabilizes
- The event returns to baseline, if a baseline value/status is available
- The event can be attributed to agents other than the study drug or to factors unrelated to study conduct
- It becomes unlikely that any additional information can be obtained (subject or health care practitioner refusal to provide additional information, lost to follow-up after demonstration of due diligence with follow-up efforts)

All serious adverse events, pregnancy exposures and/or pregnancies in partners for Janssen medicinal products under study should be reported directly by the Sponsor Investigator, **within 24 hours of becoming aware,** to Janssen Scientific Affairs using the Janssen Scientific Affairs Serious Adverse Event Report Form. In the event the study is blinded, the Sponsor Investigator will submit an unblinded SAE or pregnancy exposure report to Janssen Scientific Affairs.

All follow-up information for serious adverse events that are not resolved at the end of the study or by the time of patient withdrawal must be reported directly by the Sponsor Investigator, **within 24 hours becoming aware**, to Janssen Scientific Affairs using the Janssen Scientific Affair's Serious Adverse Event Report Form.

VI. Product Quality Complaints for Janssen Medicinal Products

A PQC may have an impact on the safety and efficacy of the product. Timely, accurate, and complete reporting and analysis of PQC information from studies are crucial for the protection of patients, investigators, and Janssen Scientific Affairs, and are mandated by regulatory agencies worldwide. Janssen Scientific Affairs has established procedures in conformity with regulatory requirements worldwide to ensure appropriate reporting of PQC information. Lot and/or Batch #s shall be collected or any reports of failure of expected pharmacological action (i.e., lack of effect).

All initial PQCs involving a Janssen product under study must be reported to Janssen Scientific Affairs by the Sponsor Investigator **within 24 hours after being made aware of the event.**

If the defect for a Janssen product under study is combined with either a serious adverse event or non-serious adverse event, the Sponsor Investigator must report the PQC to Janssen Scientific Affairs according to the serious adverse event reporting timelines. A sample of the suspected product should be maintained for further investigation if requested by Janssen Scientific Affairs.

VII. Maintenance of Safety Information

All safety data should be maintained in a clinical database in a retrievable format. The Institution and Sponsor Investigator shall provide all adverse events, both serious and non-serious, in report format. However, in certain circumstances more frequent provision of safety data may be necessary, e.g. to fulfill a regulatory request, and as such the data shall be made available within a reasonable timeframe at Janssen Scientific Affair's request.

VIII. Transmission Methods:

The following methods are acceptable for transmission of safety information to Janssen Scientific Affairs:

- Electronically via Janssen SECURE Email service (preferred)
- For business continuity purposes, if SECURE Email is non-functional:
  - Facsimile (fax), receipt of which is evidenced in a successful fax transmission report
- Telephone (if fax is non-functional).

Please use the contact information and process information provided by Janssen Scientific Affairs.

IX. Procedures for Reporting Adverse Events (AE), Serious Adverse Events (SAE), Pregnancy, and Product Quality Complaints (PQC) to Janssen Scientific Affairs

A. AEs, SAEs, Special Situations and Pregnancy Reporting.

The Institution and the Sponsor Investigator will transmit SAEs and Special Situations in a form provided by Janssen Scientific Affairs in accordance with Section VIII Transmission methods, in English **within 24-hours** of becoming aware of the event(s).

All available clinical information relevant to the evaluation of a related SAE or Special Situation is required.

- The Institution and/or Sponsor Investigator are responsible for ensuring that these cases are complete and if not are promptly followed-up. A safety report is not considered complete until all clinical details needed to interpret the case are received. Reporting of follow-up information should follow the same timeline as initial reports.
- Copies of any and all relevant correspondences with regulatory authorities and ethics committees regarding any and all serious adverse events, irrespective of association with the Janssen Product under study, are to be provided to Janssen Scientific Affairs using a transmission method in Section VIII **within 24 hours of such report or correspondence being sent to applicable health authorities.**

B. PQC Reporting

The Institution and the Sponsor Investigator will report any suspected PQC to the Janssen contact within 24 hours of becoming aware of the complaint. The product should be quarantined immediately and if possible, take a picture.

X. Reconciliation of SAEs

At a minimum, on a quarterly basis and at the end of the Study, Janssen Scientific Affairs will provide to the Institution and/or Sponsor Investigator, a listing of all SAEs reported to Janssen Scientific Affairs. The Sponsor Investigator will review this listing and provide any discrepancies to Janssen Scientific Affairs.

Upon request, Institution and/or Sponsor Investigator shall provide Janssen Scientific Affairs with a summary list of all SAEs, and AEs of Special Interest and Special Reporting Situation reports to date, for reconciliation purposes.

XI. Dissemination of Safety Information from Janssen Scientific Affairs to Institution/Sponsor Investigator

Janssen Scientific Affairs agrees to provide to the Sponsor Investigator SUSAR reports for the Study Product as they become available until all subjects in the Protocol have completed their last Study visit according to the Protocol (i.e. Last Subject Last Visit has occurred).

6 STUDY SCHEMATIC: TIME AND EVENTS SCHEDULE

Procedure	Phone Screening (Before week -2)	Screening Visit (Week -2)	Baseline/ Randomization Visit 1 (Week 0)	During Treatment Visit 2 (Week 8)	End-of-Treatment Visit 3 (Week 16)	Follow up phone call (Week 20)
Eligibility Assessments						
Informed Consent		X				
Inclusion / Exclusion Criteria	X	X				
Medical History		X				
Safety Assessments						
Physical Examination		X			X	
Targeted Physical Examination (as needed)			X	X	X	
Vital Signs		X	X	X	X	
Assessment of Signs and Symptoms		X	X	X	X	
Adverse Events Assessment			X	X	X	X
Laboratory Tests (Biochemical)		Screening	X	X	X	
Urine Pregnancy Test (As needed)		X	X	X	X	
Spot Urine Sample			X	X	X	
Waist/Hip Measurements			X	X	X	
Body Composition Scale			X	X	X	
Efficacy Assessments						
Peripheral blood draw for CD34+cell harvest			X	X	X	
Pulse wave analysis and Pulse wave velocity assessment			X	X	X	
RMR			X	X	X	
Clinical Drug Supplies						
Randomize			X			
Dispense Study Treatment			X	X		

RESEARCH STUDY DESIGN AND METHODS

Patient Definition and Selection:

Adults aged 30-70 years will be recruited mainly from the GWU MFA research patient database provided by MFA Information Technology, and then contacted via email and phone, from the endocrinology and other provider clinics at GWU and through referrals from the Veterans Affairs (VA) clinics. Suitable advertisements for the study patient enrollment will be posted. Patients will be included in the study if they have been diagnosed with type 2 diabetes within the previous 15 years using criteria of the American Diabetes Association[22], and are currently treated with any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas. We wanted to capture a population where the subjects will be classified as having early stage of diabetes where endothelial dysfunction can be reversed. Late stages of diabetes may be associated with irreversible vascular damage. We plan to enroll subjects with HbA1C between 7.0% and 10% (both inclusive) and with BMI 25-39.9 kg/m^2 (both inclusive). Post enrollment, patients will be randomized to Cana 100 mg or placebo as detailed in Study Design for direct comparison between the two regimens.

Before any study procedures are performed, subjects will have the details of the study described to them, and they will be given a written informed consent document to read. Then, if subjects consent to participate in the study, they will indicate that consent by signing and dating the informed consent document in the presence of study personnel.

Recruitment:

Subjects will be recruited from the physician clinics at GWU, Medical Faculty Associates, where Dr. Sen is a clinician, as well as through referral from the VA. All patients will receive counseling regarding the study aims and methods.

Study Design Overview:

We propose a 2-arm randomized, double-masked, placebo-controlled, parallel group, longitudinal study of 16-weeks duration. (See study design below), recruited over 2 years. The 16 week time interval has been previously shown adequate to observe changes in endothelial progenitor stem cells, biochemistry and importantly, PWV changes[25,26]. Patients will be randomized to 2 groups:

Control, (n=20), metformin, insulin or Combo + Placebo
Treatment, (n=20) metformin, insulin or Combo + Cana 100mg

This proposed study is based on patients with type 2 diabetes, who are on any combination of the following anti-diabetic therapies: metformin (1-2 grams), insulin, GLP-1 agonists, a DPP-IV inhibitor, or sulfonylureas but still remain poorly controlled with HbA1C between 7.0% and 10% (both inclusive), but not overtly out of control (> 10% of HbA1C).

Though our study is over 16 weeks we do not anticipate significant drop in HbA1C in between the two groups in this relatively short time period to act as a confounding factor on CD34+ cell number, function and gene expression.

Subjects will be taking 100 mg of Cana or placebo. Dose titration of metformin, will not be allowed. Dose titration of insulin will be allowed to avoid hypoglycemia while maintaining HbA1C under 10%.

Most clinical studies looking at HbA1C reduction were of a much longer duration of 6 months or above and most DPP-4 inhibitor studies are with animal EPCs where comparison with human CD34+ population is difficult to make. In one study[27] where saxagliptin was used for 12 weeks

in drug-naïve type 2 diabetes patients there was placebo-subtracted HbA1c reduction noted of 0.45-0.63%. It showed mean placebo-subtracted reductions in fasting serum glucose of 20 mg/dL. From our laboratory studies of in vitro human EPC studies we do not think glucose level change of 20 mg/dL is significant enough to account for changes in EPC number, function and gene expression. However we will take the possibility of glycemia change/ reduction in experimental arm into account during statistical analysis of the results between the Canagliflozin and placebo groups.

Visits with outcome measurements will be scheduled for the morning while overnight fasting (except water) to reduce diurnal variability, and clinical parameters will be obtained at scheduled office visits as depicted in the study schema and will be measured at each outcome visit as follows:

Week -2: Subjects will be phone screened to determine initial eligibility. If the phone-screening indicates possible eligibility, then the subjects will come to an in-person screening visit that same week (If within two weeks that is acceptable).
In-person screening (Week -2): Subjects will be screened and eligibility will be verified based on physical and laboratory tests. Equal numbers of male and female patients will be recruited.

Week 0: At this stage patients will be randomized into the study. Subjects will have the first set of measurements. We will make sure that patients still meet the inclusion criteria. They will be started on medication or placebo.
Weeks 0, 8 and 16: Subjects will all have the same measurements and will continue on study medication or placebo. All primary and secondary measures as outlined before will be carried out.

PRIMARY OBJECTIVE

The cellular outcome measures are as follows:
To determine whether 4 months of Canagliflozin modifies CD34+ cell number, gene expression and function.

Protocol for obtaining CD34+ cells: We will obtain a total of approximately 95 mL of peripheral blood per visit. Of these 95 mL, 60-70 mL will be used to obtain CD34+ cells from mononuclear cell (MNC) population and 25-35 mL for biochemistry and plasma ELISA assays. No samples will be stored for future research. MNC will be obtained from whole blood similar to protocols described before[13,14]. MNCs will be put through CD34 magnetic bead column to obtain CD34+ cells (Miltenyi Biotec). Purity of CD34+ cells, post sort, usually is above 90%, to be verified by FACS analysis.

A. From Mononuclear (MNC) population (pre CD34+ magnetic column sorting): MNC cells (prior to CD34 magnetic bead sorting) will be also used to assay CFU Hill Colony forming unit (Stem Cell Technologies- Cat#05900) media for Colony Forming unit (CFU) Assay. CFU will be counted on 5-7 days post plating (from initial plating).
B. From CD 34+ cell population (Post Sorting):
    a. Migration Assay using SDF1Alpha concentrations of 0, 10, 100ng/ml and VEGF-A concentrations of 20 and 50 ng/ml, using 150,000 cells in 300ul serum free cell suspension media per insert.

b. Gene expression studies. Genes to be assessed on sorted CD34 positive cells:
    i. Endothelial lineage cell surface markers: CD34, VEGFR2 (KDR), CD31, CD144
    ii. For Anti-oxidant gene expression: Superoxide dismutase (SOD) 1, 2 and 3, catalase, glutathione- peroxidase.
    iii. Apoptosis pathway: p53, p21, Bcl2, caspase-3
    iv. Endothelial Function Assay Gene: endothelial Nitric Oxide Synthase, von-Willebrand's Factor
    v. Genes associated with progenitor cell chemotaxis: VEGF-A, SDF1 alpha, CXCR4.

SECONDARY OBJECTIVE

To determine whether use of Canagliflozin alters other cardio-metabolic health indicator parameters such as: arterial stiffness measures, resting metabolic rate, body composition measures, hs-CRP, IL-6, TNF-alpha (inflammatory markers), fasting lipid profile, levels of insulin, glucose appetite controlling hormones and urine levels of microalubumin (Microalbumin/Creatinine ratio)..

We expect to corroborate our findings of Aim 1 (cellular outcome measures) with traditional markers of endothelial/ vascular function such as serum biochemistry and arterial stiffness.

A. Effect of Cana on Serum Biochemistry & Urine:

a. Looking at inflammation, apoptosis and anti-oxidant protein levels: Highly selective C-reactive protein (hs-CRP), IL-1, IL-6, TNF-alpha, MCP-1 , fasting glucose, comprehensive metabolic panel, insulin and lipid profile.
b. Plasma SDF1 alpha (ELISA) and GLP-1 (ELISA) will be estimated to assess endothelial health and factors that may influence CD34+ cell chemotaxis
c. Estimation of Creatinine Clearance and proteinuria: 24hr urinary protein estimation and creatinine clearance (obtained via micro-albumin/creatinine ratio from a spot urine sample).
    i. *Optional sub-study to investigate urine exosomes. See page 31.*
d. The glomerular filtration rate (GFR) will be estimated using the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation. The CKD-EPI equation, expressed as a single equation, is:

GFR = 141 X min $(Scr/\kappa,1)^{\alpha}$ X max$(Scr/\kappa,1)^{-1.209}$ X 0.993^{Age} X 1.018 [if female] X 1.159 [if black]; where Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is –0.329 for females and –0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1[17].

B. Effect of Cana on Body Habitus (Determination of body composition and visceral fat) AND Resting Metabolic Rate.

a. Height and weight will be measured and the body mass index (BMI=kg•m-2) used as an indicator of relative weight. The body composition scale calculates body fat% and total body water% in addition to BMI.

b. We also intend to follow Resting Metabolic Rate (RMR): at weeks 0, 8 and 16. Indirect calometric measurements of basal metabolic rate will be obtained using machine obtained from Korrs Medical. This is a noninvasive procedure. We plan to measure the oxygen that the body consumes. It will help us calculate a patient's Resting Energy Expenditure (REE), commonly referred to as a Resting Metabolic Rate (RMR). As our protocol is directed to overweight and obese individuals using incretin therapy, it will be beneficial to note if the treatment modifies RMR. Description: The machine ReeVue by KORR directly measures the concentration of oxygen breathed out by each patient. The patient breathes through a simple mouthpiece as all the exhaled air is collected and analyzed. Because there is a direct correlation between oxygen consumed and calories burned (4.813 calories for every milliliter of oxygen consumed), an accurate measurement of oxygen consumption is an effective measurement of calorie consumption. The machine uses a one-way valve in the disposable (MetaBreather) mouthpiece. This draws in fresh room air with each inspiratory breath and eliminates concerns about cross contamination. The disposable mouthpiece is thrown away. It is un-necessary to clean the equipment or the tubings between each patient. This equipment is FDA approved and has been used in physician offices as an oxygen uptake test with associated CPT code #94690[27]. Other related trials have shown weight loss but effect on metabolic rate has not been studied[18].

C. Effect of Cana on Assays of Arterial Stiffness:

a. We will acquire Pulse wave analysis and Vascular Flow using SphygmoCor CP system from ATCOR as a measure of central arterial pressure and arterial stiffness. Again SGLT2 inhibitors have been shown to reduce systolic blood pressure but effect on stiffness has not been looked at, except with Empagliflozin[17,18].

b. We also intend to acquire Pulse wave analysis and Vascular Flow using SphygmoCor CP system from ATCOR to measure central arterial pressure and arterial stiffnesss[28,29,30,31,32]. The secondary measures are indirect measures of endothelial inflammation in early type 2 diabetes patients[11,12,19,33,34].

D. Other Measures:

a. Urine pregnancy testing for women of child bearing potential.

b. Creatinine Clearance and proteinuria estimation (obtained via micro-albumin/creatinine ratio from a spot urine sample).

STUDY MATERIALS

Janssen, manufacturers of Cana is expected to provide Cana at no cost for this study.

SAFETY ASSESSMENTS

Study drug toxicities will be assessed continuously. Adverse events will be evaluated on a continuous basis while the patient is on study and until 30 days after the last dose of study drug. Patients should be followed until all treatment-related adverse events have recovered to baseline or are deemed irreversible by the principal investigator.

7 STATISTICAL CONSIDERATIONS

STATISTICAL ANALYSIS

Sandri et al. (2005, Study A, patients with peripheral arterial occlusive disease) found that in the control group, $CD34^+$ cells increased from 372 cells per mL blood at baseline (SD 156) to 402 (SD 183) at 4 weeks. In their exercise training group, the increase was from 458 (SD 252) at baseline to 2977 (SD 852) at 4-weeks. We created a simulated data set with these parameters in order to calculate the percent of $CD34^+$ cell variation that was explained by the group x time interaction. We tested two random effects mixed models, one without and the other with, a group x time interaction (group is treatment vs control; time is pre vs. 4-weeks post). The percent variance explained = (Vno-int – Vint)/Vno-int, where Vno-int is the residual covariance parameter estimate without a group x time interaction term in the model, and Vint is the residual covariance parameter estimate with a group x time interaction term in the model. We found that based on the baseline and 4-week mean and standard deviation of CD34+ cell concentrations reported by Sandri et al. (2005), the interaction explained 84.5% of the variance in CD34+ cell concentration (a very large effect size). The correlation among repeated measures was r=.62. Using these parameters in G-Power3 (version 3.1.3), with 2 groups measured at 2 time points, in order to achieve power >.95 would require a total sample size of 6 subjects (3 per group) each measured at 2 time points. Assuming 25% subject loss, we would need to start with 4 per group. If the group x time interaction explained only 50% of the variance in CD34+ cell count (still a large effect), we would need total sample size of 8 (4 per group) in order to achieve power >.95. In order to account for possible 25% drop-out, 6-8 per group should be randomized.

In Sen et al. (2015), in a sample of patients with pre-diabetes (n=11), CD34+ cell number increased from 0.8 (SEM 0.1) before exercise, to 1.4 (SEM 0.2) after exercise, a pre-post effect size (Cohen's d) of 1.81. In order to detect an effect of this size using a 2-tailed paired t-test, with alpha=.05, for power >.80, >.90, and >.95, the number of subjects required would be 5, 6, or 7, respectively.

However the two quoted studies quoted above are very different from our proposed study on patients' with established endothelial dysfunction entity such as diabetes. It is well known (Werner et al, Ref No. 11) that diabetes can affect CD34+ number and function, therefore in our proposed study, number of CD34+ cell availability may be a limiting factor. Therefore we propose to use double the number suggested by power calculation and use 8x2=16 patients per group so that CD34+ cell based end-point studies are not compromised.

DATA ANALYSIS

Digital data files will be stored in a protected limited-access folder on the MFA network. Only the Study Biostatistician and the PI will have direct access to the data once it is on this server. The data file will have random subject identifiers and will have no HIPAA identifiers.

The distributional assumptions of all measures will be examined. Means and standard deviations will be computed for continuous measures and proportions for categorical variables. Graphical representations of the mean group slopes and individual slopes will be generated and inspected.

Study groups will be compared to determine whether any imbalance between the groups on patient characteristics remain after randomization. If imbalance is evident, by inspection, all models will be adjusted for the unbalanced covariates. Change in outcome over time will be examined using a multilevel approach with linear mixed models (LMM)[35,36]. The longitudinal multilevel modeling approach will enable us to examine characteristics of within-person change, as well as between-group differences that may influence change. LMMs handle missing data more efficiently than traditional ANOVA designs. In the linear mixed models, the patient is considered a random effect and the outcome measured at specific time points is nested within patient. Time (week 0, 8, 16) will be modeled as a continuous variable in order to assess linear effects from 0 to 8 to 16 weeks. In order to determine whether there are non-linear time effects (e.g., rapid increase from 0 to 8 weeks followed by little change from 8 to 16 weeks; or initial decrease at 8 weeks followed by increase at 16 weeks, etc.) we will also need to include models in which time is defined as a class variable (with 0 as the reference group). Subsequent analyses will add study group and other patient characteristics as covariates. We are particularly interested in whether there is a significant interaction between study group and time, adjusting for other patient level covariates. A significant interaction would indicate that the slopes in outcome over time differ by study group. If the global test of interaction is significant, subsequent significance testing of the pair wise comparisons of group slopes group will be adjusted for multiple comparisons. The model also includes coefficients for the group-by-time interactions and random effects for patient and time.

$$Y_{ij} = \beta_0 + \beta_1 Time_{ij} + \beta_2 Group_i + \beta_3 (Time_{ij}\ x\ Group_i) + + \varepsilon_{ij}$$

The interaction between GROUP and TIME will be modeled in a similar fashion using two additional indicator variables. The above multivariable model enables us to adjust for time-varying covariates, such as glucose. Since glucose will be measured contemporaneously with the outcomes at each visit, however, causal interpretations of glucose and any outcome are suspect. As such, we will consider glucose as a nuisance covariate, and consider any relationship between glucose and an outcome as merely associative.

Privacy Protection

All data collected for the research study will be collected under a random subject ID.

- All samples being sent to LabCorp for standard of care labs will be labeled with the subject ID, DOB, Date of visit, time of collection, and Visit number. These samples will not be stored as they will be sent down to LabCorp immediately.
- The whole blood and plasma being used for ELISA and CD34+ analysis will be labeled with the subject ID, Date of visit, and Visit number. No additional data will be provided with the samples. These samples will be analyzed and stored at The George Washington University Ross Hall in Dr. Sen's Laboratory,
- The urine sample being used for the exosome sub-study will be labeled with the subject ID, Date of visit, time of collection, and Visit number. They will also be provided with subject gender, and age. These samples will be analyzed and stored (as per the protocol below) at the VA by Dr. Eric Nylen.

All other study data will be both electronically stored on a secure server, and physically stored as patients files locked in the Department of Medicine. The only individuals who will have access

to the study data will be the members of the research staff team, as indicated in the GW IRB Form "Research Team Personnel Form (HRP-201)".

8. APPENDIX A: OPTIONAL URINE EXOSOME STUDY, Veterans Affairs, Dr. Eric Nylen

A urine sample is already required as a part of Dr. Sen's Canagliflozin protocol. This urine will be used for urine pregnancy tests (if applicable), and labcorp tests for microalbumin/creatinine ratio. There will be excess urine.

Subjects will have the option to opt in or out of a research study being conducted by Dr. Eric Nylen of the VA, in which the urine will undergo a urine exosome analysis.

Purpose of this Study: **Exosomes** are cell-derived vesicles that are present in many and perhaps all biological fluids, including blood, urine, and cultured medium of cell cultures. The reported diameter of **exosomes** is between 30 and 100 nm, which is larger than LDL, but much smaller than for example, red blood cells. Exosomes are either released from the cell when multivesicular bodies fuse with the plasma membrane or they are released directly from the plasma membrane.[3] Evidence is accumulating that exosomes have specialized functions and play a key role in, for example, coagulation, intercellular signaling, and waste management.[1] Consequently, there is a growing interest in the clinical applications of exosomes. Exosomes can potentially be used for prognosis, therapy, and biomarkers for health and disease. In this study we are particularly interested in exosomes released from podocytes as a marker of podocyte function and podocyte inflammation. Podocytes are often considered a modified endothelium and one of its more commonly used parameter is microalbuminuria.
In this study we are using urine exosomes as an added parameter of kidney function other than microalbuminuria and GFR calculation.

The procedure for this study is as follows:
10-50 mL of the fresh spot urine is placed into a urine cup containing 1 tab of cOmplete Ultra - protease inhibitor cocktail (Roche) for exosome analysis performed at the Veterans Affairs (VA) Maryland Health Care System.
The specimen can be refrigerated at 4ºC for ~ 1 week, frozen at -80C, or processed immediately. Transfer to the VA will be done following appropriate procedures outlined in the MFA-VA Materials Transfer Agreement (MTA). The urine will be processed for exosomes as follows:

The supernatant may be subjected to ultracentrifugation, with final centrifugation at 200,000g for 2 hr at 4ºC to obtain the urinary exosome pellet. The pellet would then be tested for exosomes markers, namely CD63, CD9, CD81, Hsp70, and WT-1, nephrin and podocalyxin, using one of two methods. Preferably mRNA will be extracted and then RT-PCR will be run. Alternative analysis will be done using gel electrophoresis followed by Western blot techniques.

Method 1: **Urine exosome mRNA extraction**
mRNA extraction from exosomes will be accomplished by using commercial kits (Norgen Biotek Corp., Catalog # 47200). Thereafter, a pre-amplification step will take place, this is a

necessary step to measure mRNA in exosomes and will be followed by quantification of specific endothelium and podocyte proteins such as CD9, WT-1, nephrin and podocalyxin by RT-PCR.

Total urinary protein content, albumin and creatinine will be measured via Lapcorp for the same urine samples that have been collected.

Method 2: Gel electrophoresis and Western blot
Exosome pellet will be dissolved in Laemli buffer and proteins will be resolved by gel electrophoresis (4-12% polyacrylamide gel) under denaturing conditions. Subsequently, proteins will be transferred to a PVDF membrane by Western blotting using a dry blotting method (iBlot). The blot will be incubated with blocking solution for 1h, and subsequently exposed to a solution with the first antibody overnight, at 4ºC. After washing (5 x 5 min), the membrane will be exposed to a secondary antibody (horseradish peroxidase [HRP]-conjugated) for 1h at room temperature. Finally, the membrane will be incubated with an HRP substrate reagent to reveal the proteins bound to the antibodies tested. Quantitative analysis will be performed by densitometry readings of the positive signals corrected to creatinine concentration of the urine aliquot equivalent to each exosome sample.

End-point: Exploratory but in conjunction with urine albumin/creatinine ratio will help to establish impact of Linagliptin on renal function in diabetes related CKD population.

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