

# Role of Canagliflozin on function of CD34+ endothelial progenitor cells (EPC) in patients with type 2 diabetes

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## Original investigation

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## Abstract

**Background:** Endothelial Progenitor cells (EPCs) has been shown to be dysfunctional in both type 2 diabetes mellitus (T2DM) and chronic kidney disease (CKD) leading to poor regeneration of endothelium and renal perfusion. EPCs have been shown to be a robust cardiovascular disease (CVD) risk indicator. Effect of sodium glucose channel inhibitors (SGLT2 inhibitors) such as Canagliflozin (CG) on a cellular CVD risk indicator such as CD34+ve cells, in patients with T2DM with established CKD has not been explored,

**Methods:** 29 subjects taking metformin and/or Insulin were enrolled in a 16 weeks, double blind, randomized placebo matched trial, with a low dose 100 mg CG compared to placebo. Type 2 diabetes subjects (30–70 years old), HbA1c of 7–10%, CD34+ cell number, migratory function, gene expression along with vascular parameters such as arterial stiffness, biochemistry, resting energy expenditure and body composition were measured. Data were collected at week 0, 8 and 16. A mixed model regression analysis was done with p value less than 0.05 were considered significant.

**Results:** Gene expression analysis of CD34+ve cells showed increase in antioxidants (superoxide dismutase 2 or SOD2, Catalase and Glutathione Peroxidase or GPX). A significant increase in gene expression of endothelial markers PECAM1 ( $p=0.002$ ), VEGF-A ( $p=0.04$ ) and CXCR4 receptor ( $p=0.06$ ) followed by an increase in migratory function of CD34+ve cells is observed in Canagliflozin treated group as compared to placebo group. A significant reduction in glucose ( $p=0.01$ ) levels was observed along with improved systolic and diastolic blood pressure in the CG group. Significant increase in adiponectin ( $p=0.02$ ) was also noted in treatment group. Urinary exosomal protein examining podocyte health (podocalyxin, Wilm's tumor and nephrin) showed improvement.

**Conclusion:** We conclude that Canagliflozin on addition to metformin and/or Insulin, in subjects with type 2 diabetes demonstrates a functional improvement of CD34+ Endothelial Progenitor Cell function and gene expression and improves endothelial function

## Background

Diabetes mellitus is a major public health concern worldwide as number of patients with type 2 diabetes mellitus (T2DM) is increasing rapidly and it is gradually reaching the proportions of a world epidemic. Currently, 463 million adults are living with diabetes and by 2045 estimated number of adult living with diabetes will reach to 700 million(1,2). CDC reported approximately, 9.4% of American adults have diabetes (1,2). Diabetes is closely associated with oxidative stress, inflammation, and as a result endothelial dysfunction and cardiovascular complications [1–3]. On the other hand, 15% of US adults are estimated to have chronic kidney disease and 38% of end stage renal disease developed CKD as a consequence of diabetes [4]. Use of a sodium-glucose linked transporter inhibitor (SGLT-2i) 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. It is extremely important to evaluate improvement, especially cardiovascular health of the patient after administration of diabetic drugs.

Available therapeutics are focused on glycemic control to delay the diabetes related complications[7, 8]. Widely used drugs to control hyperglycemia in patient with diabetes are metformin, DPP4 inhibitor, and SGLT2 inhibitor. Several studies showed SGLT2 inhibitor can be beneficial compare to DPP4 inhibitor, especially on subjects with type 2 diabetes and mild chronic kidney disease. A use of a sodium-glucose linked transporter (SGLT-2) inhibitor has a promise to improve glycemic control, weight reduction and hypertension has been reported by several groups[9–13]. Meta analysis studies have shown less risk of heart failure with SGLT2 inhibitors in comparison with DPP4 inhibitor and GLP1 agonist [14]. However, recently several studies have shown canagliflozin specifically lower the risk of cardiovascular disease in pre-clinical and clinical studies in type 2 diabetes [15–21]. Recent studies suggested canagliflozin may inhibit endothelial pro-inflammatory chemokine/cytokine secretion through AMPK dependent and independent mechanisms [22] and canagliflozin helps to improve myocardial ischemia-reperfusion injury [23].

Serum based biomarkers are being used for long time to identify cardiovascular improvement. However, use of serum based inflammatory markers are inefficient and does not change until the endothelium is already damaged and inflamed and generate pro-inflammatory molecules [24]. Serum based biomarkers are unable to detect effect on endothelium at an early stage. Several studies recently showed endothelial progenitor cells (defined as CD34 + ve cell) can serve as an important biomarker to monitor cardiovascular function [25–29].

Endothelial progenitor cells (EPC) defined as CD34 + progenitor cells are obtained from the hematopoietic stem cell pool by magnetic bead column separation. EPCs contribute to vascular repair angiogenesis and also vasculogenesis.

It is well established that EPCs respond to a chemo-attractant such as stromal derived factor 1 alpha (SDF1 $\alpha$ ) by migrating towards a chemoattractant released by wounded/damaged/ apoptosis prone tissue[30, 31]. The recruitment of EPCs help to repair the damaged tissue. It is also known that EPCs release or secrete growth factors and cytokines that promote repair and regeneration as part of their paracrine properties. [32, 33]. We reported earlier that CD34 + ve cells help to detect effect of therapy or intervention in an early onset [24]. A few preclinical and clinical studies showed, improvement of cardiovascular health by using a combination of DPP-4 inhibitor and metformin in type 2 diabetic patient by improving endothelial dysfunction and vascular complications from diabetes by increasing number of CD34 + EPCs and migratory capacity[34]. There are some previous studies indicate that SGLT2 inhibitors may help to improve endothelial dysfunction both in patients and animal studies [35–38]. However, such studies donot give us much information at a cellular level.

Previous studies from our laboratory have shown that CD34 + cells, derived from peripheral blood can act as a cellular biomarker that is more reliable than serum based markers for CVD risk estimation. In our previous studies using two dipeptylpeptidase inhibitors (DPP4i) such as saxagliptin and linagliptin in type 2 diabetes mellitus (T2DM) patients 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 [44]. The same is true for of a prediabetes population with an aerobic exercise intervention, where changes in gene expression are quite fast. CD34 + cells or endothelial progenitor cells have been used clinically to improve collateral circulation and have been extensively studied as a robust cardiovascular biomarker. Therefore, studying CD34 + cells in T2DM subjects with or without Canagliflozin can give vital information about the medication and its effect on hematopoetic stem cell pool and also endothelium. Our non cellular outcome measures were focused on parameters that are agreed to be indicators of endothelial function. In this 16-week intervention study we investigated the synergistic effect of canagliflozin, a SGLT2 inhibitor and metformin, on number and function of CD34 + EPCs while adjusting for variables that may affect stem cell number and function such as HbA1C, BMI, gender and age.

## Methods

### 2.1 Trial Design and Oversight

This is a phase 4 (post-marketing), two arm, single site, parallel group, double blind, placebo controlled randomized clinical trial comparing low dose Canagliflozin 100 mg tablets, taken orally, once daily, with matching placebo. The study was conducted in accordance with Good Clinical Practice guidelines set forth by the International Conference of harmonization and any local regulatory guidelines with the approval and oversight of the George Washington University Institutional Review Board. The trial was funded by Janssen Scientific Affairs, LLC and conducted by the Investigator-Sponsor Dr Sabyasachi Sen at the George Washington University.

Subjects were initially pre-screened to assess eligibility. Once determined preliminary eligibility, they were brought in for a screening visit to confirm eligibility via interview, medical record check and laboratory workup once the subject signed the informed consent. The subjects were then enrolled into one of two arms of the study: 100 mg Canagliflozin or matching placebo. 15 subjects were enrolled into the active group and 14 subjects were enrolled into the placebo group.

There were 3 study visits total, first at week 0, second at week 8 (mid-point) and third and last visit at week 16. All three of the visits had same assessments. The assessments that were done were: vital measurements, adverse event (AE) check and a

peripheral blood draw. Approximately 80 ml of blood was drawn for CD34 + endothelial progenitor cell harvesting and routine blood work.

Other parameters tested were resting metabolic rate (RMR, energy expenditure), measurement of waist to hip ratio, urine sample collection, Tanita body composition scale, pulse wave analysis and pulse wave velocity to determine arterial stiffness. Subjects were advised to adhere to 150 minutes of weekly aerobic exercise and their activity levels were monitored using ACTi graph activity monitor.

A follow up phone call visit was done 30 days from the last in-person visit to assess for any residual adverse events (AE).

## **2.2 Participants**

Subjects were included if they were between 30 to 70 years old inclusive, with a diagnosis of Type 2 Diabetes for 15 years or less. Glycated hemoglobin level (HbA1c) inclusions were between 7.0 to 10.0% Inclusive. Their baseline medications were stable dose of Insulin (either short acting or long acting) and/or Metformin (1–2 grams per day). A stable dose was considered to be at least the maximum labeled dose or dose not associated with unacceptable side effects. Patients with BMI between 25 and 39.9 kg/m<sup>2</sup> were included, thereby excluding severe obesity. Only, patients with impaired renal function were included, with Chronic Kidney Disease stage 1 to 3, with lowest eGFR cut-off of 30 ml/min/1.73 (GFR, as calculated by MDRD formula). During the duration of the study alterations in baseline medications were done to keep HbA1C between 7–8% across all subjects.

Any patients with Type I diabetes, history of Diabetic ketoacidosis, low hematocrit (less than 28 units), history of recent pancreatitis or cancer, recent coronary or cerebrovascular event within 6 months, use of consistent steroid medications, untreated thyroid disease were excluded.

Additional Inclusion and Exclusion criteria can be found in **Appendix 1**.

## **2.3 Outcome Objectives**

The primary objective is to ascertain if addition of Canagliflozin improves CD34 + cell number, (CD34 + number, %CD34 + of total Mononuclear Cell population) function (cell migration function in response to SDF1 $\alpha$ ) and gene expression, in Type 2 Diabetes Mellitus, which will be correlated to improvement in 24 hour urinary protein estimation and serum Creatinine Clearance.

The secondary objective is to correlate the cellular outcome measures with other measures of endothelial function such as Arterial Stiffness (measured by Pulse Wave Analysis [Augmentation Index] and Pulse Wave Velocity [m/s]), Serum Biochemistry (CMP, IL6, hsCRP, Leptin, Serum insulin, TNF $\alpha$ ), Adiposity ( as % body fat), Resting Energy Expenditure (in kcal) and Glycemic control (through HbA1c).

### **Body Composition Measurement**

Body composition was measuring using Tanita™ BF-350 Body Composition Scale and manually. Manual measurement included height, waist circumference, hip circumference. Tanita scale uses a bio-impedance electrical impulse to measure body fat percent, fat mass (kg), fat free mass (kg), percent body water, water mass (kg) alongside weight. It then calculates the BMI and estimated basal metabolic rate.

### **Basal Metabolic Rate Measurement**

Resting Emergency Expenditure (REE) was measured using KORR REEVUE. Test was conducted with the subject sitting and well rested. Subject was instructed to keep a tight seal around the mouthpiece and use the nose clip to avoid breathing in from the nose. The test ran for about 10 mins. It calculated estimated REE, predicted REE, estimated TEE (Total Energy Expenditure), VO<sub>2</sub> Max and estimated calorie intake per day.

### **Arterial Stiffness**

This parameter was measured using AtCor SphygmoCor CP system. We obtained two outcomes such as: Pulse Wave Velocity and Pulse Wave Analysis. The patient was supine on the examination table, 3 leads were attached on right forearm, left forearm and left shin.

Pulse Wave Analysis (PWA) was measured on the left Radial Artery with the subject supine. At least three readings were taken with Operator Index  $\geq 80$ . Measurement includes Augmentation Index (AI), Augmentation Index adjusted for Heart Rate of 75 (AI-75), Augmentation Pressure (AP), Aortic and Radial reading of systolic, diastolic, pulse pressure and mean pressure.

Pulse Wave Velocity (PWV) was measured with the subject supine. This measurement requires a distal and proximal artery. Distal was used as right femoral artery with proximal being the left carotid. Index and ring fingers were used to manually localize the pulse, sometimes an arterial Doppler was used to localize the femoral pulse on patient with challenging body habitus. Once a stable pulse waveform was observed, the probe position was kept stable for 20 more pulses before the reading was finalized. Three readings were taken with standard deviation of less than 10%. The result reported a velocity in m/s, alongside the standard deviation with error.

### **Biological Sample and Vital Collection**

A venous blood sample was collected from the Antecubital fossa. About 80 ml of blood was collected. 60 ml for EPC analysis and 20 ml for standard of care blood works which included Basic Metabolic Panel, Lipid Panel, HbA1c, hsCRP, IL6, Adiponectin and Insulin. ELISA was performed to analyze serum GLP1 and SDF1 $\alpha$  using ELISA Immunoassay kit (Raybiotech, Norcross, GA) for GLP1 and Sandwich ELISA (EHCXCL12A, Thermo Scientific) for SDF1 $\alpha$ . Urine sample was collected for urine Microalbumin and Creatinine ratio. Vitals were gathered on the left arm, Systolic Pressure, Diastolic Pressure and heart Rate, along with sublingual temperature. Serum NAD/NADH were measured by using NAD/NADH assay kit from Abcam, MA, USA (Catalog No. ab65348) and Ketone bodies were from serum were measured by using Ketone Body Assay Kit from Millipore Sigma, MO, USA (Catalog No. MAK134).

### **ACTi graph Activity Monitor**

Subjects level of activity was measured using Actigraph wGT3x-BT activity monitors. Subjects were advised on diet and exercise instructed to wear the meter during all waking hours and was advised to adhere to 150 min of moderate intensity aerobic exercise per week. Actigraph served as a measure of this exercise compliance, and to verify for exercise as a confounding variable.

### **Polyethylene Glycol (PEG) enrichment of Extracellular vesicles**

The cells debris and large apoptotic bodies were removed from the urine samples by centrifugation at 500 g for 5 minutes followed by 3000 g for 30 minutes at 4<sup>0</sup> C. Transfer supernatant into ultracentrifugation tubes and centrifuged at 100,000 g at 4<sup>0</sup> C for 75 min (Optimal XPN-100 centrifuge, Beckmann Coulter Inc, US). After ultracentrifugation the pellet was dissolved in RIPA buffer with protease inhibitor cocktail and stored the sample at -80<sup>0</sup> C for further analysis.

Western blotting: Extracellular vesicle extracts were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min. The membrane was washed once with TBST and incubated with antibodies against CD9 (1:1000), CD81 (1:1000), CD63 (1:1000), HSP70 (1:1000), anti-podocalyxin (PODXL, 1:1000), anti-wilms tumor protein (1:1000) and anti-Nephrin antibody (1:1000) at 4 °C for 12 h. Membranes were washed three times for 10 min and incubated with a 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody for 90 min at room temperature. Blots were washed with TBST three times and developed with Pierce ECL kit (ThermoFisher Scientific, USA).

### **Cellular and Clinical Assessments**

#### **CD34+ Endothelial Progenitor Cell Analysis**

Peripheral blood samples (approximately 60 ml) were drawn from patients and phosphate buffered saline (1:1) was added. Identification and quantification of circulating cell phenotypes was performed on fresh blood samples, within 3 hours after collection, using flow cytometry. Briefly, mononuclear cells (MNCs) were then isolated from whole blood using a Ficoll density centrifuge method. MNCs were counted and aliquot was used for CFU-Hill colony formation assay following the manufacturer's instruction (Stem Cell Technologies, Vancouver, BC, Canada). Colony forming unit was counted at day 14. A fraction of the MNC were stained with fluorescein isothiocyanate (FITC)-conjugated antihuman CD34, Allophycocyanin (APC) conjugated antihuman CD184 (CXCR4) and FITC conjugated antihuman CD31 antibodies (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) in order to analyze specific progenitor cell surface marker (CD34) and mature endothelial cell surface markers (CD31) or receptor for SDF1a ligand, CXCR4) by flow cytometry. After gating mononuclear cells in the side scatter (SSC)-A vs forward scatter (FSC)-A plot, CD34/CD184 single- and double-positive cells were identified. Cells were acquired on a fluorescence-activated cell sorter (FACS) Canto instrument (Becton Dickinson) and scored with the Flo-Jo software.

To isolate EPCs (CD34+), MNCs were magnetically sorted through a column after cells were stained with CD34+microbeads antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). An aliquot of CD34+ cells were then stained with trypan blue and counted using an Auto Cellometer Mini (Nexcelom Bioscience, Lawrence, MA) to assess viability.

CD34+ gene expression analysis was performed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as previously described (33). CD34 +ve cell total mRNA was extracted and purified using the RNeasy Minikit (Qiagen, Germany). mRNA was then converted into cDNA by using the high capacity cDNA reverse transcriptase kit (Thermo Fisher Scientific, MA). Possible gene expression changes promoted by Canagliflozin was assessed by a CFX96 real-time PCR systems (Bio-Rad, CA.) using Taqman Universal masters Mix II (Thermo Fisher Scientific, MA) and inventoried probes. The gene expression analysis included antioxidants, apoptosis, endothelial functions, chemotaxis, inflammation and endothelial lineage cell surface markers. The expression of each individual gene was normalized to either housekeeping 18S or GAPDH and calculated using C-ddct method considering the difference in cycle threshold between visit 2 and 3 and baseline (Visit 1). mRNA gene expression of CD34- cell population (from MNC population) was also analyzed along with CD34+ cells.

The migratory capacity of CD34+ was evaluated using the CytoSelect 24-well Cell Migration Assay kit (Cell Biolabs, Inc., San Diego, CA). Cells were suspended in Serum free media and seeded at 100,000 cells per insert. Migration of the cells through a 3 um polycarbonate membrane to the wells containing a serum-free media (control) and chemoattractant SDF-1 $\alpha$  (10 or 100 ng/mL) was assessed after cells were kept overnight in incubator. Migratory cells were dissociated from the membrane and subsequently lysed and quantified by fluorescence (480 nm/530 nm) using CyQuant GR dye (Cells Biolabs, Inc, San Diego, CA). The fluorescence ratios between cells exposed to the chemotactic factor and cells exposed to chemoattractant-free media (control) along the visits were used to analyze the migratory capacity of the cells.

As is the case with diabetic patients, number of isolated CD34+ cells is usually not as high as anticipated due to established endothelial damage and because the progenitor cells are very susceptible to apoptotic death in hyperglycemia. Hence in order to understand some of the effect of protein upregulation we have done the analysis on all remaining CD34- cells as they are the remaining 99% of Polymorphonuclear cells. Idea is that any gene expression upregulation on CD34+ cells should also be prevalent in CD34- cells and should by and large reflect the mRNA analysis of unsorted MNC population.

## 2.5 Statistical Analysis

### Statistical Analysis

Continuous variable distributions were examined for skewness or outliers using histograms. When these were present, we log-transformed the variables. Outliers > 5sd from the mean were capped at 5sd from the mean. We used 2-tailed between-groups t-tests to examine differences between treatment groups at baseline on continuous variables, and either chi-square or the Fishers Exact test for categorical variables. To examine differences between treatment groups across all time points, as well as time effects, and whether the slope of change over time differed between treatments, we used random effects mixed model regression, examining the main effects of treatment (Canagliflozin vs placebo), and time (v1, v2, v3), and the treatment by time interaction. This method allows us to use all non-missing subject data and adjusts for within-subject auto-correlation. For

variables with significant effects in the mixed models, we examined the means graphically. SAS (version 9.4, Cary, NC) was used for data analysis with  $p < .05$  considered significant.

Since subjects were randomized to treatment, chance of baseline subject characterizations acting as confounders are minimized. Therefore, randomized control trials do not usually adjust for baseline differences. However, in small studies, imbalances may exist as a result of random group assignment, and may thus function as mediators of an association between treatment and outcome. In this situation, one would need to adjust for the mediators in order to obtain the direct effect of treatment on outcome, which is the effect of interest.

## Results

### Primary Outcome

The study population was representative of subjects with uncontrolled type 2 diabetes, but with no preexisting macro-vascular complications. All adverse effects that occurred throughout the duration of the study were either not related to the study medication and design or fell within the expected side effects profile for Canagliflozin. Table 1 shows blood biochemistry measures across the three visits. No statistical significance was observed between the groups for body composition measures. Mean percent body fat is low and mean percent body water is high in canagliflozin group and showed no significance difference throughout the study (table 2). This implies that the changes that we note in cellular and serum based outcome measures are independent of body composition changes.

### Venous Blood Biochemistries

Venous blood biochemistries were gathered, both through Labcorp of America and through serum ELISA and both standard of care and research labs were collected.

Detailed lab values of selected significant parameter are on table 1. We found statistically significant decrease in the Glucose levels in Canagliflozin group. ( $p = 0.01$ ) whereas an opposite effect is seen in placebo group. The HbA1c levels went down substantially in the Canagliflozin group from visit1 to 2 ( $p = 0.0919$ ) and overall it shows a trend in decrease in Canagliflozin group where as in placebo group it increased from visit 1 to 3 (**Figure. 1**). The inflammatory marker IL-6 levels in serum decreased significantly. As shown in the Fig. 1, in Canagliflozin treated subjects from visit1 to visit 3 IL-6 levels reduced ( $P = 0.05$ ). Adiponectin and endocrine factor synthesized and released from adipose tissue. We have observed a significant increase in adiponectin levels ( $p = 0.02$ ) in Canagliflozin group, where as in placebo group adiponectin levels decreased from visit 1 to 3 (Fig. 1).

**Table.1: Blood Biochemistry before and after Canagliflozin treatment.**

Parameter	Placebo			Canagliflozin			Interaction P value
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	
<b>Glucose</b>	132.59 ± 13.60	176.12 ± 14.82	146.73 ± 14.91	183.34 ± 13.16	154.03 ± 13.52	149.61 ± 13.04	0.01
<b>HbA1c</b>	7.77 ± 0.39	7.81 ± 0.40	8.06 ± 0.40	8.27 ± 0.37	7.88 ± 0.38	7.92 ± 0.38	0.11
<b>eGFR</b>	85.22 ± 4.43	79.85 ± 4.22	88.33 ± 4.52	80.24 ± 4.27	81.13 ± 4.56	79.90 ± 4.21	0.31
<b>Creatinine</b>	0.84 ± 0.05	0.94 ± 0.05	0.80 ± 0.06	0.96 ± 0.05	0.88 ± 0.06	0.95 ± 0.05	0.37
<b>HDL</b>	51.56 ± 3.60	52.23 ± 3.66	51.67 ± 3.69	42.96 ± 3.47	46.97 ± 3.48	46.17 ± 3.48	0.28
<b>Cholesterol</b>	163.40 ± 10.09	166.23 ± 10.53	147.76 ± 10.61	167.78 ± 9.74	176.26 ± 9.84	171.57 ± 9.84	0.20
<b>LDL/HDL</b>	1.68 ± 0.15	2.26 ± 0.15	1.60 ± 0.16	2.19 ± 0.15	1.43 ± 0.16	2.08 ± 0.15	0.92
<b>C-reactive protein</b>	1.03 ± 0.2	0.40 ± 0.2	0.92 ± 0.2	0.54 ± 0.2	0.96 ± 0.2	0.63 ± 0.2	0.44
<b>IL-6</b>	2.81 ± 0.37	2.76 ± 0.4	2.59 ± 0.4	2.55 ± 0.3	2.78 ± 0.3	1.69 ± 0.4	0.40
<b>Adiponectin</b>	1.53 ± 0.1  4	1.65 ± 0.1	1.33 ± 0.1	1.13 ± 0.1	1.24 ± 0.1	1.27 ± 0.1	0.02
<b>Insulin</b>	2.49 ± 0.21	2.63 ± 0.20	2.71 ± 0.22	2.50 ± 0.20	2.46 ± 0.22	2.56 ± 0.20	0.42
<b>Leptin</b>	39.84 ± 3.34	42.42 ± 3.52	40.28 ± 3.65	28.36 ± 3.30	29.92 ± 3.36	27.79 ± 3.29	0.96
<b>SDF-1α</b>	6.34 ± 0.47	5.74 ± 0.45	5.85 ± 0.47	5.49 ± 0.46	6.41 ± 0.48	5.68 ± 0.44	0.51
<b>3Hydroxy- butyric Acid</b>	0.03 ± 0.01	0.0	0.03 ± 0.01	0.01 ± 0.01	0.001 ± 0.01	0.02 ± 0.01	0.75
<b>Acetoacetic Acid</b>	0.72 ± 0.25	0.0	0.30 ± 0.27	1.08 ± 0.24	0.05 ± 0.25	0.69 ± 0.24	0.86

To study the effect of Canagliflozin on **mitochondrial function** serum NAD and NADH levels in placebo and Canagliflozin groups, we measured serum NAD and NADH levels from visit 3. We did not see any significant difference between the groups (For Placebo 0.8454 ± 0.3239 and for Canagliflozin 0.7335 ± 0.2730). Serum ketone bodies, 3hydroxybutyric acid and

acetoacetic acid were measured. As shown in the table 1, there is no significant difference between the groups between either of the two ketone bodies.

### **Adiposity**

Body composition measurement showed no statistically significant change amongst the group throughout the visits. As expected, given short duration of the treatment the subjects were asked to maintain activity level as advised by ADA for healthy living. There was no statistically significant change in hip to waist ratio, body weight and body fat percent amongst the treatment and control group. As we know from our prior study that physical activity, even for a short duration, can improve endothelial function and CD34 + circulating progenitor cells in patients with improvement in endothelial dysfunction [43].

### **Cellular Outcome Measures:**

#### **Characterization of endothelial progenitor cells (CD34 + ve)**

To find out the effect of SGLT2 inhibitor, Canagliflozin, on the endothelial progenitor cell number we counted the total CD34 + cells by using Cellometer Mini (Nexcelom Biosciences, Lawrence, MA), after microbead column separation (BD Biosciences, San Jose, CA). As shown in Table 2, there is no statistical significance in difference in CD34 + ve cell numbers between placebo and Canagliflozin group.

**Table.2: Cellular Parameters before and after Canagliflozin treatment.**

Parameter	Placebo			Canagliflozin			Interaction P value
	Visit 1	Visit 2	Visit 3	Visit 1	Visit 2	Visit 3	
Mean MNC	167.00± 16.08	153.79± 16.08	155.09± 16.08	147.75± 15.54	145.93± 15.54	155.09± 15.54	0.71
Mean CD 34+	2527.64± 508.69	3128.79± 508.69	3157.36± 508.69	2452.07± 491.44	2031.40± 491.44	2682.93±491.44	0.32
% of CD34+	1.01 ± 0.19	1.04 ± 0.17	1.23 ± 0.21	0.94 ± 0.19	0.85 ± 0.22	0.93 ± 0.17	0.75
% of CD31+	2.85± 0.33	2.47± 0.44	2.16± 0.43	2.75± 0.33	2.34± 0.38	2.58± 0.35	0.59
CD34 + CD184	0.57± 0.09	0.45± 0.10	0.61± 0.11	0.43± 0.09	0.33± 0.10	0.37± 0.09	0.62
CFU	10.30± 2.03	13.62± 2.10	13.61± 2.19	6.08± 1.95	7.62± 1.95	7.53± 1.89	0.82
SDF 10 NG	0.20± 0.031	0.20± 0.031	0.23± 0.03	0.15± 0.03	0.15± 0.03	0.26± 0.03	0.26
SOD2	0.97± 0.23	1.45± 0.23	0.92± 0.24	1.00± 0.22	1.05± 0.24	1.36± 0.24	0.22
Catalase	-0.03± 0.25	-0.09± 0.25	-0.61± 0.26	-0.55± 0.23	-0.55± 0.26	0.20± 0.26	0.04
GPX35	2.00± 0.54	2.00± 0.54	1.71± 0.54	2.00± 0.47	1.64± 0.50	2.25 ±0.54	0.68
CXCL12	1.00± 0.16	0.53± 0.17	0.33± 0.17	1.00± 0.15	0.60± 0.16	0.63± 0.18	0.59
CXCR4	1.06± 0.17	1.03± 0.17	0.90± 0.18	1.00± 0.16	1.03± 0.18	1.58± 0.18	0.06
EDN1	1.22± 0.27	1.05± 0.29	1.03± 0.29	1.00± 0.24	0.79± 0.27	2.10± 0.27	0.02
VEGEF-A	0.95± 0.17	1.46± 0.18	0.83± 0.19	1.00± 0.17	0.86± 0.18	1.12± 0.19	0.04
PECAM	0.94± 0.11	1.43± 0.11	0.89± 0.12	1.00± 0.11	0.87± 0.12	1.28± 0.12	0.002
KDR	1.00± 0.18	0.53± 0.18	0.50± 0.20	1.00± 0.18	0.55± 0.20	1.16± 0.20	0.13
NOS3	1.11± 0.21	0.06± 0.21	-0.25± 0.21	0± 0.18	-0.23± 0.19	0.34± 0.21	0.08
Wilm'S Tumor	6.52 ± 0.65	6.13 ± 0.72	6.36 ± 0.75	6.27 ± 0.60	6.68 ± 0.62	5.75 ± 0.59	0.45
Nephrin	6.15 ± 0.55	5.10 ± 0.62	5.87 ± 0.65	5.39 ± 0.51	5.70 ± 0.53	5.24 ± 0.50	0.25
PODXL	6.72 ± 0.33	6.08 ± 0.38	6.68 ± 0.40	7.05 ± 0.31	6.92 ± 0.32	6.62 ± 0.30	0.34

To discern the direct effect of Canagliflozin on CD34 + ve cells percentage the values were adjusted for HbA1c. HbA1c adjusted value for CD34 + ve cell number is statically significant (  $p = 0.0047$ ). Consistent with this HbA1C adjusted values for CD34 + ve/ CD 184 + ve cells (dual positive) were also statistically significant (  $p = 0.0039$ ) between the groups. A dual positive cell would indicate a CD34 + ve cell that can respond to chemotactic factor SDF1a for stem cell migration.

### **CFU-Hill's colonies**

The CFU-Hill's colony formation was improved in the Canagliflozin treatment group. CFU numbers increased sharply from visit1 to 2 and visit1 to 3(data not shown). Even though there is no significant difference between the visits, HbA1c adjusted value for CFU is statically significant (  $p = 0.042$ ) with higher CFU count. In placebo group despite an increase in CFU number from visit1 to 2 but overall decrease in CFU number from visit1 to 3.

### **Migration Response**

Next, we wanted to study the effect of Canagliflozin on migration of CD34 + ve cells. The migratory response of CD34 + cells to the chemotactic factor SDF1 $\alpha$  (concentration of 10 ng/ml ) increased in canagliflozin group as compared to placebo group. However from visit 2 to 3 there is a significance increase in migration of CD34 + ve cells in canagliflozin group as compared to placebo group (Fig. 2).

### **Gene Expression Analysis**

#### **The effect of Canagliflozin on the gene expression of CD34 + ve cells**

We wanted to study the gene expression on CD34 + ve cells for antioxidants SOD2 (superoxide dismutase 2), GPX3 (glutathione peroxidase 3), CAT (catalase). The mean fold change in gene expression of all 3 antioxidants that we studied were increased in canagliflozin group where as in placebo group it is decreased from visit 1 to visit 3 ( Fig. 3A,B,C). The mean gene expression for endothelial markers VEGF-A (  $p = 0.04$ ), KDR(  $p = 0.13$ ) and PECAM1(  $p = 0.002$ ) had increased significantly in Canagliflozin group from visit 1 to visit 3 (Fig,4A,B,C) as compared to placebo group. Since the prominent endothelial gene expression (VEGF, KDR) is increased significantly, we were interested to see the expression of endothelial functional genes NOS3(endothelial nitric oxide synthase). Over all there is a trend in increase in NOS3 expression (  $P = 0.08$ ) in Canagliflozin treated group as compared to placebo group but no statistical significant difference was observed between the groups (Fig. 4D). We also checked the inflammatory markers and we did not see any differences in the inflammatory markers IL6 and TNF- $\alpha$  expression between the groups (data not shown), however the serum IL-6 levels decreased significantly in Canagliflozin treated group as compared to placebo group (Fig. 1F). This is difference is statistically significant from visit1 to 3 (  $p = 0.0649$  ) and from visit2 to vist3 (  $p = 0.0259$ ).

### **Urine Podocyte Function Marker**

#### **Quantification of Exosomal proteins in urine samples by western blot**

Our recent study using Linagliptin showed protection from renal injury with patients taking Linagliptin for a duration of 12 weeks [44]. We were interested to study the effect of Canagliflozin on podocyte health by quantifying the exosome expression for Nephryn, renal Wilm's Tumor (WT-1) and podocalaxyn like protein 1( PODXL) in urine samples from placebo and Canagliflozin group. As described in the methods after isolating exosomes from urine samples of both placebo and canagliflozin group exosomes were identified as CD9 positive. As shown in the **Fig. 5**, the mean band intensities for the podocyte specific exosomal protein leak of PODXL and Nephryn and WT-1 were decreased from visit 1 to 3 in Canagliflozin group as compared to placebo group. Even though the difference in band intensities between the groups is not significant (taking all visits together) a trend in decrease intensities is observed in Canagliflozin treated group, particularly in visit 2 to visit 3, indicating a late onset protection effect of Canagliflozin. Interestingly, the HbA1c adjusted value for Nephryn in Canagliflozin treated group is statically significant (  $p = 0.0133$ ).

## **Discussion**

## Primary Outcome: Cellular

Reduction in CD34 + ve cells is a consistent observation in people with T2D and this defect may contribute to the development of chronic complications. Previous studies demonstrated that SGLT2 inhibitors reduce the risk of cardiovascular events in patients with T2D and high cardiovascular risk [45]. Despite several attempts to explain such striking cardiovascular protection exerted by SGLT2 inhibitors we still have a poor understanding of the potential mechanisms at work. Therefore, in this study we attempted to test whether SGLT2 inhibitor Canagliflozin exert beneficial effects on endothelial progenitor cells which are considered as biomarkers and have role in the pathogenesis of chronic diabetic complications. We investigated the effect of SGLT2 inhibitor Canagliflozin in addition to metformin and/or Insulin on CD34 + EPCs and CD34 + CD184 + cells as a marker for vascular endothelial function. Our data shows canagliflozin did not increase the endothelial progenitor cells in this short time 16 week study. CD34 cells decreased from visit 1 to 2 and then increased to slightly higher by the end of Visit 3 in canagliflozin group. The decrease in EPCs is in parallel with the recent study on effect of SGLT2 inhibitors on EPCs [36]. Even though, CD34 positive cells are not increased, the expression of CXCR4 and SDF1 (CD184+) expression is increased in the CD34 + ve cells correlated with the increased migratory response to SDF1- $\alpha$  of CD34 + cells in canagliflozin group (Fig. 2B)..It is likely that long term treatment with canagliflozin will increase the CD34 + ve cells with good glycemic control [46].

Next to understand the effect of Canagliflozin on gene expression of CD34 + ve cells we performed qPCR analysis. We looked at pathways involving inflammation, oxidative stress, EPC chemotaxis pathways and endothelial function markers, in CD34 + ve cells obtained post magnetic column separation of MNCs. We found the expression of SDF1- $\alpha$  receptor CXCR4 expression is very close to statistical significance in Canagliflozin treatment. This was an unexpected result as we did not expect an SGLT2i to have effect of stem cell migration, unlike DPP4i treatment where migration can be expected to increase secondary to increased SDF1a increased bio-availability. Antioxidants and endothelial markers are upregulated in Canagliflozin treatment group, indicating improved vascular health. The upregulation of endothelial markers, VEGF-A and its receptor KDR indicates VEGF signaling most likely plays an important role in vasculogenesis and tissue repair upon Canagliflozin treatment. Interestingly eNOS expression increased significantly from visit 2 to visit 3 in Canagliflozin group. Another interesting finding were increase in serum adiponectin levels by ELISA and reduction in serum IL-6 values particularly between visit 2 to visit 3. These two results concurrently indicate improved endothelial health following canagliflozin treatment. (Fig. 1E and F).

As nephropathy is an important progressive complication of T2DM we specifically looked at markers beyond urine microalbumin: creatinine ratio which did not show a difference between the groups. However, when we examined a more sensitive marker such as urine exosomes that specifically look at podocyte derived protein such as podocalyxin, nephrin and Wilm's tumor we noted that canagliflozin appears to reduce these podocyte proteins particularly between visit 2 to 3.

Lastly, we wanted to examine any possible differences in ketone bodies between placebo and low dose canagliflozin as the latter has been shown to cause euglycemic ketosis [47], however at this dose we did not note any significant difference in serum ketone body levels. There are reports of canagliflozin influencing mitochondrial function via AMPK-Sirt1-Pgc-1 $\alpha$  signalling pathway [48] however we did not note any significant differences in NAD: NADH ratio, however it will be worthwhile to investigate into these effects at higher doses.

## Limitations Of Our Study

Limitations of our pilot study may include the relatively short duration of 16-week Canagliflozin therapy, which may have been inadequate to see significant changes in certain clinical and cellular parameters. This may have been also because of the small sample size, However, we were happy to note statistical significance in various cellular outcomes and even in some serum based markers. Further studies with a larger population and longer duration may be helpful to further define the mechanisms behind our findings.

## Conclusion

It could be concluded that when Canagliflozin was added to subjects with Type 2 Diabetes along with metformin and/or Insulin, demonstrates a functional improvement of CD34+ Endothelial Progenitor Cell migratory function through increased

CD34/CXCR4 positivity, and improved endothelial function

We believe CD34+ cells can act as a valuable biomarker for assessment of endothelial function, in a setting of diabetes and can help provide valuable clinical information leading to appropriate therapeutic intervention choices. It may also allow an individual approach to medication response rather than standard serum based assays with set normal ranges

## List Of Abbreviations

AI - Augmentation Index

AI-75 - Augmentation Index adjusted for a heart rate of 75

AP - Augmentation Pressure

AS - Arterial stiffness

BCI-2 - B-cell lymphoma 2

BMI – Body mass index

BUN – Blood urea nitrogen

CAT -- Catalase

CDKN1A - cyclin-dependent kinase inhibitor 1A

CD34 – Progenitor marker

CFU - Colony forming units

CVD - Cardiovascular disease

CXCR4 - C-X-C Motif Chemokine Receptor 4

DBP - Diastolic blood pressure

DPP-4 Inhibitors - Dipeptidyl peptidase-4 inhibitors

EDN-1 - Endothelin 1

eGFR – Estimated glomerular filtration rate

ELISA – Enzyme-Linked Immunosorbent Assay

eNOS - Nitric Oxide Synthase 3 (Endothelial Cell)

EPCs - Endothelial Progenitor Cells

FFM - Fat free mass

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

GLP1 – Glucagon Like Peptide 1

GPX1 - Glutathione Peroxidase 1

HbA1C – Hemoglobin A1C, glycated hemoglobin test

IGF1 - Insulin-like growth factor 1

IL-6 – Interleukin 6

kg – Kilograms (weight)

lbs – Pounds (weight)

MNCs - Mononuclear cells

PECAM1 - Platelet And Endothelial Cell Adhesion Molecule 1

PWA - Pulse Wave Analysis

PWV - Pulse Wave Velocity

qRT-PCR - Quantitative reverse transcriptase polymerase chain reaction

REE - resting energy expenditure

SBP - Systolic blood pressure

SDF1 $\alpha$  - Stromal cell-derived factor-1 $\alpha$

SOD1, SOD2 - Super oxide dismutase 1 & 2

TBW - Total body water

TNF $\alpha$  – Tumor Necrosis Factor  $\alpha$

TP53 – Tumor protein p53

VEGF - vascular endothelial growth factor

VEGFA - Vascular Endothelial Growth Factor A

VEGFR2 - Vascular endothelial growth factor receptor 2

VO<sub>2</sub> - Maximal oxygen consumption

18S - 18S ribosomal RNA

## Declarations

**Ethics approval and consent to participate:** Our study was approved by the George Washington University Institutional Review Board and was conducted in accordance with Good Clinical Practices of the National Institutes of Health. Signed informed consent was obtained by all subjects prior to engaging in any research.

**Consent for publications:** Not applicable

**Availability of data and material:** All associated data will be available to the public, as requested. Demography of subjects including detailed description of baseline characteristics and parameters have been included in the Appendices

**Competing interests:** The authors declare that they have no competing interests

**Funding:** Janssen Scientific Affairs, LLC

## Author's contributions:

SRN performed the experiments and were responsible for collection and compilation of data, analysis of data, and in writing the manuscript.

NK contributed to cellular outcome measures, compilation of the data and helped write the manuscript.

HA responsible for collection of clinical data and contributed to clinical assessments.

BB conducted the urine exosome-based assay, including related data compilation and analysis

MF has contributed in collection and complication of the clinical and cellular data sets.

NA and AE contributed in performing cellular experiments particularly the gene expression studies.

RA performed the statistical analyses of the data set, and

SS designed and supervised the study and revised the manuscript.

All authors read and approved the final manuscript.

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## Appendix 1

### APPENDIX I

#### Inclusion Criteria

1. Adults aged 30-70 years.
2. Diagnosis of type 2 diabetes within the previous 15 years using criteria of the American Diabetes Association
3. Currently treated with a stable dose of Insulin, Metformin (1-2 grams/day), or a stable combination of the two as therapy.
4. HbA1C between 7 to 10% (both inclusive)
5. BMI 25 to 39.9 kg/m<sup>2</sup> (both inclusive)

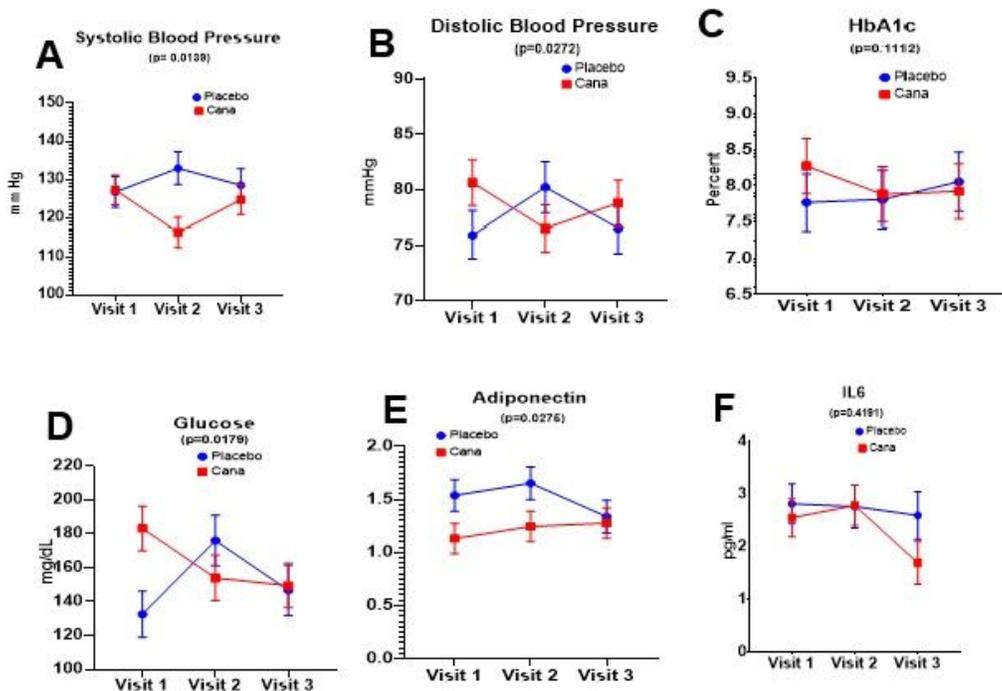
## Exclusion Criteria

1. Implanted devices (e.g., pacemakers) that may interact with Tanita scale
2. Previous coronary or cerebrovascular event within 6 months of screening or active or clinically significant coronary and/or peripheral vascular disease
3. Low hematocrit (<28 UNITS).
4. Pre-existing liver disease and/or ALT and AST >2.5X's UNL
5. CKD stage 4 and
6. History of pancreatitis, or cancer (except basal cell carcinoma and cancer that is cured or not active or being treated in the past five years)
7. Statin use started or dose change in the last 3 months
8. Use of oral anti-diabetic medication other than Metformin, or Insulin.
9. Use of consistent long-term steroid medication in the last 3 months (oral, inhaled, injected).
10. Systolic BP> 140 mmHg and Diastolic BP> 90 mmHg
11. Active wounds or recent surgery within 3 months.
12. Inflammatory disease, or chronic current use of anti-inflammatory drugs within the last 3 months
13. triglycerides >450 mg/dL
14. untreated hyper/hypothyroidism
15. Auto antibody confirmed type 1 diabetes

Additionally, patients who are active smokers, patients who are pregnant, nursing women, and post-menopausal women who are on hormone replacement therapy will be excluded.

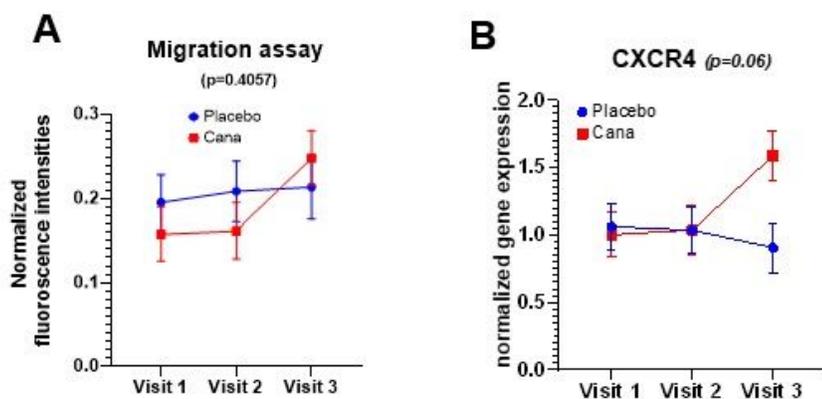
Patients on low dose oral contraceptives will be allowed to participate as these formulations contain lesser amount of estrogens.

## Figures



**Figure 1**

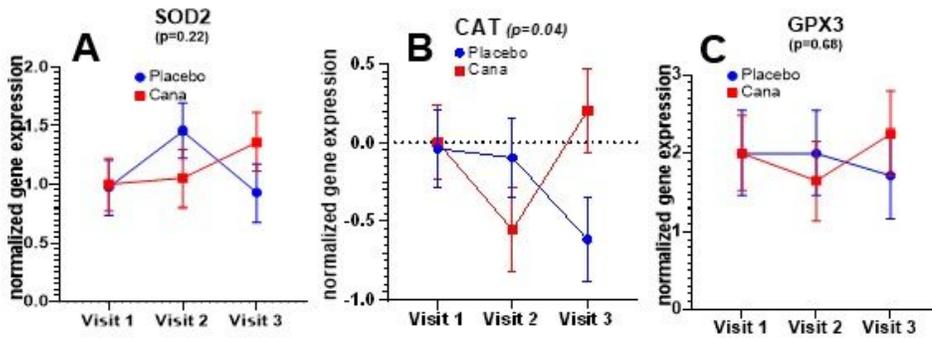
A significant difference in mean systolic blood pressure (A) and diastolic blood pressure (B) is observed between Canagliflozin and placebo groups. In Canagliflozin group both decreased from visit 1 to 2 and increased by visit 3. Whereas in placebo group both increased from visit 1 to 2 and decreased by visit 3. C). HbA1c went down substantially in Canagliflozin group whereas it increased in Placebo group. The sharp decrease in HbA1c is seen in visit 1 to visit 2 (p=0.0919) and is stabilized from visit 2 to visit 3 in Canagliflozin group. D). Glucose levels also followed the same trend as HbA1c in Canagliflozin group by sharp decrease from visit 1 to 2 and then stabilized till visit 3. Whereas, in placebo group glucose levels increased from visit 1 to 3. (E). A significant rise in adiponectin levels is noted in Canagliflozin group where as a sharp decrease is observed in placebo group from visit 2 to 3. (F) Inflammatory marker serum IL6 levels decreased. The difference was significant from visit 2 to 3 (p=0.0259) in Canagliflozin group as compared to placebo group.



**Figure 2**

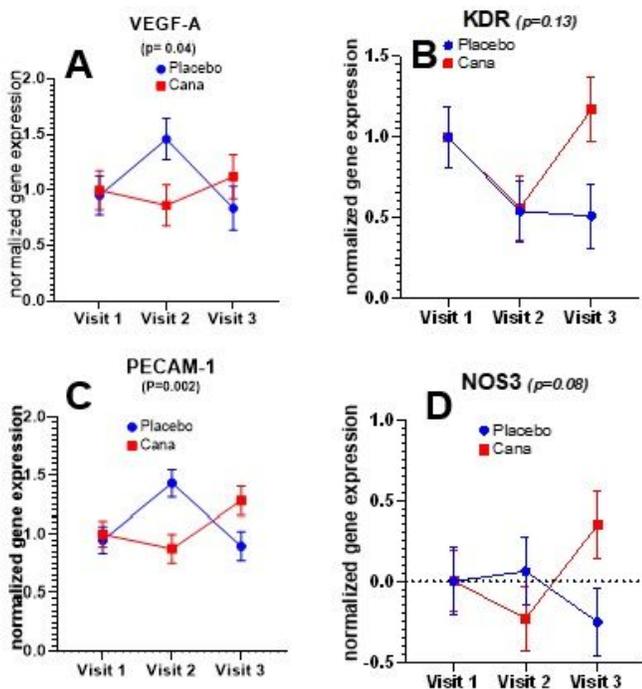
Migration Assay in response to SDF1alpha at 10ng/ml concentration and gene expression of SDF1a receptor, CXCR-4 on CD34+ve cells: A). Mean migration of CD34+ve cells towards SDF1-  $\alpha$  (at 10ng/ml) increased from visit 1 to 3 in Canagliflozin

group as compared to placebo group. (For visit1 to 3  $p=0.03$  and for Visit 2-3  $p=0.05$ ). B) CXCR4 gene expression on CD34 positive cells is increased statistically significant( $p=0.036$ ) in Canagliflozin group as compared to placebo group at Visit 3.



**Figure 3**

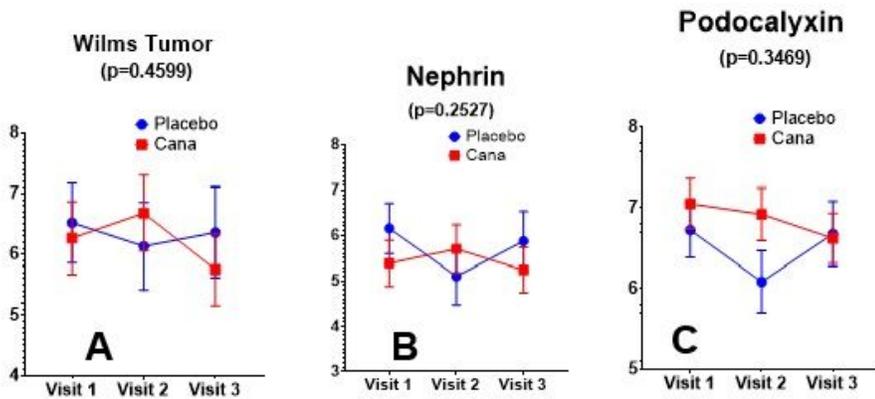
The effect of Canagliflozin on CD34+ve cell antioxidants gene expression. Fold change in gene expression of antioxidants SOD2 (A) in CD34+ve cells are increased in Canagliflozin group from Visit 1 to 3. However, the same for the placebo group is decreased. Whereas the antioxidants (B) Catalase(CAT) and (C) GPX3 gene expression increased in CD34+ve cells in Canagliflozin group from Visit 2 to 3. However, the same for the placebo group is decreased. In summary, all the antioxidant genes showed increased expression when visit 2 values were compared to visit 3, whereas in the placebo group, there was a downward trend. Expression of the catalase gene expression reached statistical difference between Cana and Placebo group, when all visits were considered together.



**Figure 4**

The effect of Canagliflozin on CD34+ve cell antioxidants gene expression. Fold change in gene expression of antioxidants SOD2 (A) in CD34+ve cells are increased in Canagliflozin group from Visit 1 to 3. However, the same for the placebo group is decreased. Whereas the antioxidants (B) Catalase(CAT) and (C) GPX3 gene expression increased in CD34+ve cells in

Canagliflozin group from Visit 2 to 3. However, the same for the placebo group is decreased. In summary, all the antioxidant genes showed increased expression when visit 2 values were compared to visit 3, whereas in the placebo group, there was a downward trend. Expression of the catalase gene expression reached statistical difference between Cana and Placebo group, when all visits were considered together.



**Figure 5**

Urinary exosome markers associated with damaged podocyte Wilm's tumor-1 (WT-1), Nephryn and Podocalyxin (PODXL) were identified by Western blot and followed by quantification. A trend in reduced mean band intensities for urinary exosome markers are observed in Canagliflozin group from visit 1 to visit 3 when compared to placebo group.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [StudyDesign.pdf](#)