The Effect of SGLT2 Inhibition on Adipose Inflammation and Endothelial Function

05/25/2021

NCT # NCT04907214

The Effect of SGLT2 Inhibition on Adipose Inflammation and Endothelial Function

Mona Mashayekhi, MD, PhD
Division of Diabetes, Endocrinology, and Metabolism
Vanderbilt University Medical Center
Nashville, TN 37221
615-208-5037
mona.mashayekhi@vumc.org

Co-Investigators:

Curtis L. Gabriel, MD, PhD
Division of Gastroenterology, Hepatology and Nutrition
Department of Medicine
Vanderbilt University Medical Center
Nashville, TN 37221
curtis.l.gabriel@vumc.org

Wayne J. English, MD
Division of General Surgery
Department of Surgery
Vanderbilt University Medical Center
Nashville, TN 37221
wayne.english@vumc.org

Matthew D. Spann, MD
Division of General Surgery
Department of Surgery
Vanderbilt University Medical Center
Nashville, TN 37221
matthew.d.spann@vumc.org

Table of Contents:

1.0 Background

Obesity is on the rise globally and is linked to cardiometabolic disease and inflammation. Obesity is a major risk factor for cardiovascular disease (CVD) and type 2 diabetes, with odds ratio for a pooled cardiometabolic outcome of 4.5 for class I obesity (body mass index (BMI) 30.0-34.9 kg/m^2) and 14.5 for class II/III obesity (BMI ≥35.0 kg/m^2).[1] Obesity is also associated with chronic systemic and adipose tissue (AT) inflammation.[2-4] Inflammation has been linked with atherosclerosis and CVD development in a multi-factorial process involving endothelial cell activation, expression of adhesion molecules and recruitment of monocytes into the vasculature.[5] Differentiation of these cells into macrophages and foam cells propagates the local inflammatory response and leads to development and progression of atherosclerotic plaques.[6] Thus, low-grade inflammation is thought to be a critical link between obesity and CVD development.[7]

Macrophages and T cells are important in maintaining AT homeostasis and are dysregulated in obesity, contributing to systemic inflammation. A change from lean to obese AT is accompanied by a change in macrophage profile from homeostatic interleukin (IL)-10-producing to inflammatory IL-6 and tumor necrosis factor (TNF) α-producing phenotype.[2] Furthermore, lean AT is characterized by anti-inflammatory IL-10-producing invariant natural killer T cells (iNKTs), IL-5-and IL-13-producing type 2 innate lymphoid cells (ILC2s), gamma-delta T cells (γδT), and regulatory T cells (Tregs). With obesity, there is hypoxia associated with adipocyte hypertrophy and decreased blood supply leading to infiltration of pro-inflammatory CD8$^+$ T cells, a decrease in iNKT, ILC2, and Tregs, and an increase in pro-inflammatory interferon (IFN)γ- and IL-17-producing T-helper (Th) 1 and Th17 cells.[2,8] These changes not only affect local adipose inflammation, but contribute to adipocyte dysfunction, impaired lipid storage and handling, and systemic inflammation and metabolic disorders,[9] representing a rich potential target for interventions.

Importantly, while AT immune populations have been extensively characterized in obese animals, there are critical differences in AT immune cells in humans that require further investigation. For example, while AT macrophages in mice can be broadly classified into pro-inflammatory M1 and homeostatic M2 phenotypes based on markers such as CD11c and CD206, in humans there are mixed inflammatory phenotypes with markers of both M1 and M2 that require further investigation.[10] Furthermore, cryopreserved samples from human clinical studies cannot be assessed for macrophage phenotypes by flow cytometry in the way that T cells can, as macrophages do not survive the process. Thus, microscopy on tissues with intact architecture is needed to evaluate human AT macrophages as proposed in this study (**Aim 1b**).

Adipose depots contribute differentially to cardiometabolic disease in obesity. Visceral adipose tissue (VAT) that lines abdominal organs is approximately 10% of the fat in the human body, and VAT accumulation is a major risk factor for CVD and type 2 diabetes.[11] Subcutaneous adipose tissue (SAT) that is beneath the skin is approximately 85% of the fat in the body. While some studies have demonstrated independent contributions of both VAT and SAT to insulin resistance,[12] others have shown more significant contribution of VAT.[13-15] In addition, there is indirect evidence that preferential accumulation of SAT during weight gain is associated with improved metabolic parameters, acting as a “metabolically safe” depot for excess fat deposition.[9,16] While both VAT and SAT likely contribute to chronic inflammation of obesity, many of the cellular inflammatory features of obesity are more pronounced in VAT, including accumulation of pro-inflammatory macrophages,[17-19] Th1 and Th17 cells.[20] Studying VAT poses a special challenge in humans, as it is only accessible during abdominal surgery. SAT is more easily accessible either by biopsy or liposuction. Our study allows investigation of the inflammatory milieu in both VAT and SAT in relation to endothelial function, enabling us to better assess the contribution of each adipose depot to surrogate markers of CVD.

Current therapies to modulate inflammation in CVD leave a significant residual risk. Previous attempts to target inflammation in CVD have had mixed success.[21] A randomized controlled trial (RCT) of colchicine in patients with stable coronary disease found a 67% reduction of cardiovascular (CV) events.[22] Positive results were also reported with hydroxychloroquine in patients with rheumatoid arthritis,[23] while methotrexate had mixed results.[24,25] Finally, IL-1β antibody treatment in those with a prior myocardial infarction and high-sensitivity C-reactive protein (hsCRP) above 2mg/L resulted in 15% reduction in CV events.[26] However, treatment with these drugs is associated with increased adverse events including infections and liver injury, and none are currently standard-of-care in the management of obese individuals at risk for or with established CVD. Thus, there remains a significant need for specific, targeted drugs that will reverse obesity-associated inflammation and prevent adverse cardiometabolic outcomes.

SGLT2 inhibitors lower blood sugar and reduce major adverse CV outcomes. Sodium glucose co-transporter 2 (SGLT2) is a membrane protein found largely in the kidneys that is responsible for re-absorption of 90-97% of filtered glucose. SGLT2 inhibitors cause glucose excretion in the urine, reduce hemoglobin A1c (HbA1c) and induce weight loss of 2-3 kilograms (kg).[27] In the EMPA-REG OUTCOME study, the SGLT2 inhibitor empagliflozin significantly decreased major adverse cardiovascular events (MACE) in diabetic patients with established CVD.[28] Subsequent large CV outcome trials (CANVAS, DECLARE-TIMI 58, and CREDENCE) have now established a class effect for SGLT2 inhibitors in secondary prevention for MACE and primary and secondary prevention for heart failure and chronic kidney disease.[29-33] Both the American Diabetes Association and American Heart Association (AHA) guidelines now recommend consideration of an SGLT2 inhibitor in those at risk for CVD.[34,35]

SGLT2 inhibitors' mechanism(s) of CV benefit are unclear. Trials testing strict glucose control or similar degrees of weight loss have failed to show equivalent CV benefits, suggesting SGLT2 inhibition has independent effects on CV health. In addition, in the DAPA-HF study, patients with heart failure benefited from SGLT2 inhibition regardless of the presence or absence of diabetes, with decreased worsening heart failure or death from CV causes.[32] Thus, given that weight loss or glucose control has not demonstrated the same CV benefit as the SGLT2 inhibitors, and that SGLT2 inhibition has demonstrated benefits in non-diabetics, one can hypothesize that the improved CV outcomes with these drugs is not due to decreasing blood glucose or weight loss alone. It has been proposed that SGLT2 inhibitors may improve CV outcomes by having an anti-inflammatory function as discussed below.[36]

SGLT2 inhibitors have been shown to improve indices of NAFLD. Non-alcoholic fatty liver disease (NAFLD) is a spectrum of diseases that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), an inflammatory condition characterized by elevation of liver enzymes and the development of hepatic fibrosis. Obesity and insulin resistance are major risk factors for development of NAFLD, and those with NAFLD have increased prevalence of comorbid CVD.[37,38] There are currently no FDA-approved, NAFLD-specific treatments available. SGLT2 inhibitor treatment has been shown to be associated with improvement in liver function in diabetic patients through an unknown mechanism.[39,40] It is possible that this effect is mediated by the inflammatory milieu of the subcutaneous adipose tissue.

2.0 Rationale and Specific Aims

Obesity is associated with increased prevalence of type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease (CVD). Adipose tissue (AT) contains a complex immune environment and is a central contributor to heightened chronic inflammation in obese persons. Increased inflammation in obesity contributes to metabolic disease by increasing insulin resistance, to fatty liver disease by recruiting and activating Kupffer cells, and to CVD by increasing endothelial dysfunction. There are no currently approved medications to prevent or treat NAFLD, and while a number of anti-inflammatory drugs have shown promise in reducing cardiovascular (CV) events in at-risk populations, there remains a *residual risk* of adverse events and a need to specifically target inflammatory pathways activated in obesity to improve CV outcomes. This proposal will fill a critical research gap by defining the mechanisms linking AT inflammation to NAFLD and CVD in obesity.

Sodium glucose co-transporter 2 (SGLT2) inhibitors are anti-diabetic drugs that improve hepatic steatosis and decrease major adverse CV outcomes through unknown mechanism(s) and beyond what is expected from their anti-hyperglycemic and weight loss benefits. SGLT2 inhibitors reduce AT inflammation in animals, making these drugs an attractive candidate to probe linkages between inflammation, NAFLD and CVD. It is unknown if SGLT2 inhibitors decrease AT inflammation in humans, and access to human tissues is a significant barrier in clinical trials.

We will test the *central hypothesis* that SGLT2 inhibitors have anti-inflammatory effects systemically, and in AT specifically, which are accompanied by improvements in endothelial function as a surrogate measure of CVD and in liver inflammation, steatosis and fibrosis as a surrogate measure of NAFLD. In *Phase 1* of this study, we will complete an initial unblinded pilot to optimize planned study procedures and ensure quality of obtained samples. We will enroll six obese individuals with pre-diabetes and treat them with the SGLT2 inhibitor empagliflozin for 12 weeks. This population has known baseline inflammation, fatty liver, and endothelial dysfunction. We will subcutaneous AT (SAT), blood, and endothelial cells and measure endothelial function and liver steatosis at multiple time points (**Figure 1**).

[redacted]

In this study, we will:

Aim 1: Test the hypothesis that empagliflozin reduces systemic and AT inflammation. We will determine the balance of pro-inflammatory and anti-inflammatory cells in blood and AT before and after treatment with empagliflozin.

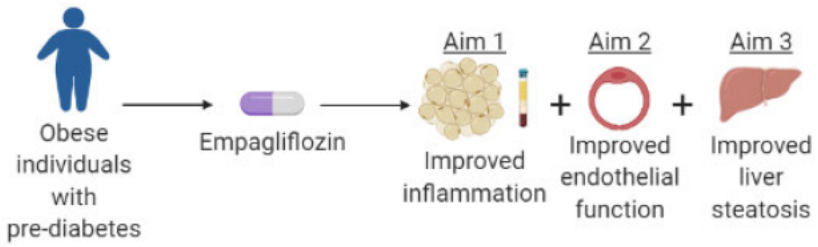
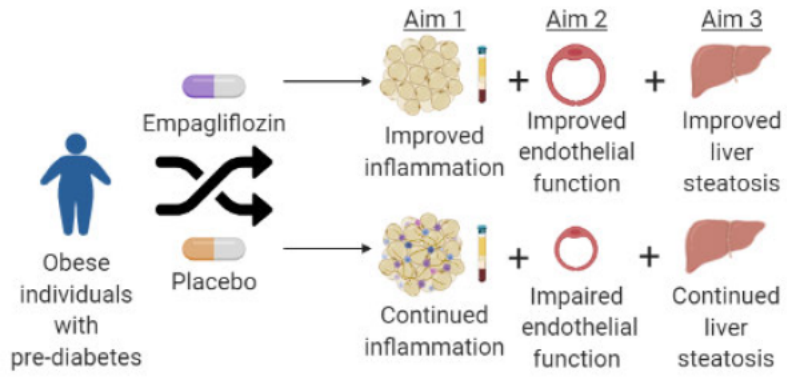


Figure 1: Schematic of Phase 1. Measurements at baseline, 2 weeks, and 12 weeks. N=6.

In **Aim 1a**, we will quantify pro-inflammatory cells including T helper (Th) 1, Th17 and $CD8^+$ T versus anti-inflammatory cells including regulatory T cells in blood and AT by flow cytometry. In **Aim 1b**, we will use microscopy to determine the inflammatory profile of macrophages in AT. In **Aim 1c**, we will correlate these findings with systemic measures of inflammation including interleukin (IL)-6 and high-sensitivity C-reactive protein (hsCRP).

Aim 2: Test the hypothesis that empagliflozin improves endothelial function. We will measure and compare conduit artery flow-mediated dilation (FMD) as a marker of endothelial function before and after treatment with empagliflozin. We will also collect endothelial cells and analyze markers of endothelial dysfunction and inflammation using flow cytometry. We will assess whether changes in endothelial function in response to the intervention are associated with changes in systemic and AT inflammation.



[redacted]

Aim 3: Test the hypothesis that empagliflozin improves liver steatosis. We will measure hepatic inflammation, steatosis and fibrosis using transient elastography-controlled attenuation parameter (TE-CAP) imaging and assess changes in liver function with NAFLD indices. We will assess whether changes in liver imaging and NAFLD indices in response to empagliflozin are associated with changes in systemic and AT inflammation.

SGLT2 inhibitors are recommended for diabetic patients with established or high risk of CVD. While these recommendations are based on benefits from large CV outcomes trials, the mechanism(s) for CV risk reduction are unknown. In addition, SGLT2 inhibitors improve markers of NAFLD beyond what is expected from their weight loss benefit. One important contributor to these findings may be the anti-inflammatory effect of SGLT2 inhibitors, which has been shown in animals. Dissecting this mechanism in humans may reveal potential future therapeutic targets for other conditions with dysregulated immune responses. For example, obesity is known to be a major risk factor in SARS-CoV-2-infected individuals for respiratory failure, and there is currently a trial of SGLT2 inhibitors in patients hospitalized with SARS-CoV-2 (NCT04350593). The proposed study will define the effect of SGLT2 inhibitors on systemic and AT inflammation and assess the association with endothelial function and liver steatosis. This will improve our understanding of the mechanisms by which SGLT2 inhibitors benefit our patients.

3.0 Animal Studies and Previous Human Studies

SGLT2 inhibitors decrease adipose and vascular inflammation in animals and systemic inflammatory markers in humans. The SGLT2 inhibitor canagliflozin decreased production of pro-inflammatory IL-1β, IL-6, and TNFα both in vitro in mouse and human cell lines, and in vivo in a mouse lung injury model after four hours of lipopolysaccharide treatment.[41] In diabetic *ApoE* knockout mice prone to atherosclerosis, SGLT2 inhibitor treatment for seven days decreased aortic messenger RNA levels of the pro-inflammatory genes F4/80, TNFα, IL-1β, and IL-6 independently of weight loss, and suppressed atherosclerosis at six months.[42] Further, empagliflozin skewed AT macrophages from pro-inflammatory M1 (F4/80+, CD11c+) to anti-inflammatory M2 (CD206+, CD163+) in a mouse model of diet-induced obesity after 16 weeks.[43]

In human studies, empagliflozin reversed TNFα-mediated vasoconstriction in an in vitro culture system using human cardiac endothelial cells and cardiomyocytes,[44] demonstrating an important anti-inflammatory mechanism with endothelial benefit. Add-on empagliflozin in a trial

of diabetics decreased hsCRP, which correlated with improvements in insulin resistance.[45] Of note, while some of the anti-inflammatory effects of SGLT2 may be modulated by weight loss especially in longer-term studies, it is unlikely that the small degree of weight loss of 2-3kg is sufficient to explain all the anti-inflammatory findings. In a study by Magkos et al., a careful evaluation of systemic and AT inflammation was performed after increasing degrees of weight loss in obese participants. This group found no improvement in inflammation after 5-11% weight loss, with trends in improvement starting to appear at 16% weight loss although not statistically significant.[46] To date, no human studies have evaluated the effect of SGLT2 inhibition on the AT immune environment.

SGLT2 inhibitors improve endothelial function and decrease progression of atherosclerosis in animals and may improve endothelial function in humans. SGLT2 inhibitors decrease plaque burden and improve insulin resistance in *ApoE* knockout mice.[47-49] These animals also have improved inflammation as demonstrated by decreased pro-inflammatory M1 macrophages in AT, and decreased circulating hsCRP, TNFα, IL-6 and monocyte chemoattractant protein (MCP)-1.[47] In addition, endothelial function is improved in diabetic *ApoE* knockout mice after eight weeks of empagliflozin as measured by vascular response to acetylcholine.[50]

The data in humans is less clear and complicated by study design and population differences. Several trials without comparator arms have shown an improvement in flow-mediated dilation (FMD) after 6-12 months of SGLT2 inhibitor therapy.[51,52] FMD is a measure of endothelial function and surrogate marker of CVD. In a RCT of empagliflozin in type 2 diabetes, improvement in arterial stiffness was correlated with change in hsCRP after 6 weeks,[53] suggesting a link to inflammation. In contrast, a randomized open-label trial of patients with type 2 diabetes and hypertension treated for four weeks with the SGLT2 inhibitor dapagliflozin or hydrochlorothiazide failed to find an improvement in FMD.[54] In a blinded RCT, 12 weeks of treatment with dapagliflozin did not improve FMD as compared to placebo in patients with type 2 diabetes and ischemic heart disease.[55] Finally, Solini et al. evaluated the effect of acute treatment with dapagliflozin and showed that FMD improved after 2 days of treatment as compared with hydrochlorothiazide, with the caveat that this was not a randomized study design.[56] Thus, while animal and human studies suggest an improvement in endothelial function with SGLT2 inhibitor therapy is likely, a randomized, double-blinded, controlled trial as proposed here will clarify the controversies in the literature, and simultaneous measurement of inflammation will assess the association between these factors during SGLT2 inhibition.

SGLT2 inhibition is associated with improvements in NAFLD in humans and animals through unknown mechanisms. Several small clinical trials have shown that SGLT2 inhibitors improve indices of NAFLD, including reductions in hepatic steatosis and inflammation. An RCT of 50 diabetic patients showed that empagliflozin reduced liver fat and alanine transaminase (ALT) compared to untreated controls.[57] Another RCT of 32 diabetic patients showed that luseogliflozin reduced hepatic fat relative to metformin, and despite higher visceral fat area.[58] Equal improvements in hepatic steatosis and ALT were observed in both arms of an RCT comparing ipragliflozin to pioglitazone.[59] Similar results were shown in other single-arm clinical trials of SGLT2 inhibitors.[60,61] A study in a mouse model of NAFLD showed reduced liver fat and liver inflammation in mice treated with canafligozin, and these improvements were associated with AT inflammation.[62] The mechanisms by which SGLT2 inhibitors improve NAFLD have not been adequately explored in humans.

4.0 Inclusion/Exclusion Criteria

Phase 1:

Inclusion criteria:

1. Age 18 to 70 years old
2. Impaired glucose tolerance (two-hour plasma glucose 140-199 mg/dL) *or* impaired fasting glucose (100-125mg/dL) *or* HbA1c 5.7-6.4%
3. BMI ≥ 30 kg/M^2
4. The ability to provide informed consent

Exclusion criteria:

Criteria Related to Medical Diagnoses/Conditions/Treatments:

1. Diabetes type 1 or type 2, as defined by a fasting plasma glucose of 126 mg/dL or greater, a two-hour plasma glucose of 200 mg/dL or greater, HbA1c ≥6.5%, or the use of anti-diabetic medication
2. Pregnancy or breast-feeding. Women of child-bearing potential will be required to have undergone tubal ligation or to be using an oral contraceptive or barrier methods of birth control
3. Cardiovascular disease such as myocardial infarction within six months prior to enrollment, presence of angina pectoris, significant arrhythmia, congestive heart failure (left ventricular hypertrophy acceptable), deep vein thrombosis, pulmonary embolism, second or third degree heart block, mitral valve stenosis, aortic stenosis or hypertrophic cardiomyopathy
4. Presence of implanted cardiac defibrillator or pacemaker
5. History of serious neurologic disease such as cerebral hemorrhage, stroke, or transient ischemic attack
6. History of pancreatitis or pancreatic surgery
7. History or presence of immunological or hematological disorders
8. Clinically significant gastrointestinal impairment that could interfere with drug absorption
9. History of advanced liver disease with cirrhosis
10. Individuals with an eGFR<45 mL/min/1.73 m2, where eGFR is determined by the four-variable Modification of Diet in Renal Disease (MDRD) equation, where serum creatinine is expressed in mg/dL and age in years: eGFR (mL/min/1.73m2)=186 • Scr-1.154 • age-0.203 • (0.742 if female)
11. Treatment with chronic systemic glucocorticoid therapy (more than 7 consecutive days in 1 month)
12. Treatment with anticoagulants
13. Any underlying or acute disease requiring regular medication which could possibly pose a threat to the subject or make implementation of the protocol or interpretation of the study results difficult
14. History of alcohol abuse (>14 per week for men and >7 per week for women) or illicit drug use
15. Treatment with any investigational drug in the one month preceding the study
16. Previous randomization in this trial
17. Mental conditions rendering a subject unable to understand the nature, scope and possible consequences of the study
18. Inability to comply with the protocol in the opinion of the principal investigator, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study

Criteria Related to Known Adverse Effects of Drug:

19. Uncircumcised men or men with history of balanitis
20. History of urinary incontinence
21. History of recurrent (>3) episodes of vulvovaginitis per year, or severe symptoms
22. History of Fournier's gangrene
23. History of recurrent (≥3) UTIs per year or pyelonephritis
24. History of symptomatic hypotension or conditions predisposing to volume depletion
25. Known peripheral vascular disease, neuropathy, history of foot ulcers or lower limb amputations
26. Treatment with loop diuretics furosemide, torsemide, bumetanide, ethacrynic acid
27. Known or suspected allergy to trial medications, excipients, or related products
28. Contraindications to study medications, worded specifically as stated in the product's prescribing information

[redacted]

5.0 Enrollment/Randomization

Phase 1: We will study six participants in Phase 1, recruited from prior studies of this population within our research group in which permission was granted for the patient to be contacted again regarding future studies. Patients will be contacted by phone or e-mail and asked if they would agree to hear about a new study. The recruiter will provide a brief description of the study. Interested participants will be invited to read and sign an IRB-approved e-consent form. There will be a separate section for consent for genetic research that is optional. We will conduct a verbal discussion by phone to review the document and address questions.

[redacted]

[redacted]

6.0 Study Procedures

The protocol will be registered at http://www.clinicaltrials.gov before any subject is enrolled.

Protocols will be carried out according to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, as well as ICH GCP guidelines. The investigators will comply with all regulatory and legal requirements, ICH GCP guidelines, and the Declaration of Helsinki in obtaining and documenting the informed consent.

Intervention: Empagliflozin is an FDA-approved SGLT2 inhibitor used for the treatment of type 2 diabetes, with off-label use for diabetic kidney disease and for heart failure with reduced ejection fraction even in those without diabetes. Treatment doses used clinically are 10mg or 25mg per day. Participants will be treated with a dose of 25mg per day. Adverse events include acute kidney injury, volume depletion, genitourinary infections, and rare cases of ketoacidosis.

[redacted]

Protocol: Table 1, **Figures 3** and **4** illustrate the protocol and study timeline. After obtaining consent, participants will be asked to fast for their screening visit. At the screening visit, the research nurse or investigator will again review the study protocol in detail and answer any questions. We will complete a medical history and physical exam, measure height and weight, and record blood pressure and heart rate. Participants will undergo an oral glucose tolerance test (OGTT), and we will collect additional screening laboratory tests including basic metabolic panel, complete blood count, liver enzymes, hemoglobin A1c, urinalysis, and electrocardiogram (ECG).

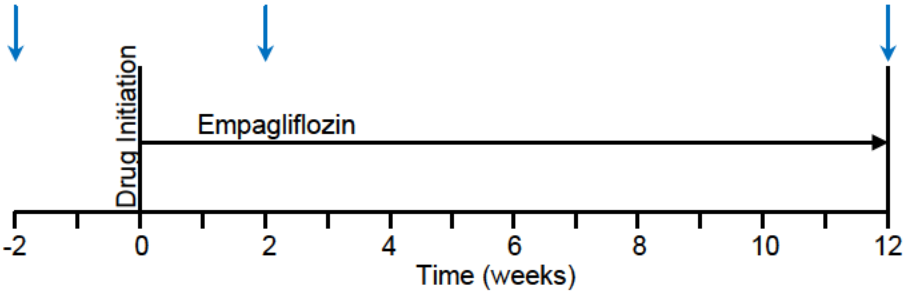
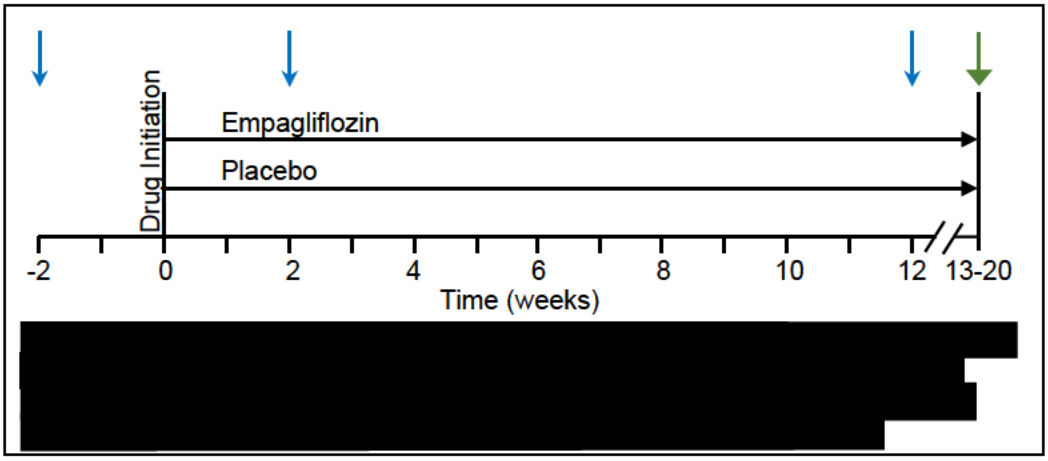


Figure 3: Study timeline Phase 1. All participants will undergo the same treatment at study days 1, 2 and 3 (**blue arrows**).

All participants will complete three study days, one at baseline prior to drug initiation, one after approximately two weeks of drug, and one after approximately 12 weeks of drug. To optimize vascular studies, alcohol and caffeine will be withheld for 12 hours prior to the study days, and PDE5 inhibitors will be withheld for one weeks prior to the first study day. In addition, all vascular studies will be made at roughly four-week intervals to approximate the same phase of menstruation in pre-menopausal women, given the known role of cyclical changes in estradiol



and FMD.[63]

Study drug will be provided at the baseline visit, to be started approximately 2 weeks later. On study day 1, a weight cycling history will be obtained via a questionnaire which will be administered by study personnel, and we will collect blood for DNA if consented. On study days 1 and 3, we will quantify VAT and SAT with a dual-energy X-ray absorptiometry (DXA) scan, and liver steatosis and fibrosis with TE-CAP (**Aim 3**). On all study days, we will collect blood for plasma, PBMCs and laboratory measures, and SAT via liposuction (**Aim 1**); measure endothelial function by FMD and endothelial cell harvest via wire collection from a vein (**Aim 2**); take anthropometric and hemodynamic measurements including weight, waist and hip circumference, blood pressure, and heart rate, collect urine, administer a urine pregnancy test if required, and administer an alcohol-use questionnaire.

Table 1. Schedule of Events

	Screening Visit	Study Day 1	Drug Initiation	Study Day 2	Study Day 3	[redacted]
Visit Window	<30 days and ≥24 hours prior to Study day 1	-2 weeks ±14 days	Day 0	+2 weeks -2/+5 days	+12 weeks -7/+14 days	[redacted]
Medical History	X					
Medication History	X					
Weight Cycling Questionnaire		X				
Height Measurement	X	X (if not completed)				
Weight Measurement	X	X		X	X	
Hip Circumference Measurement		X		X	X	
Waist Circumference Measurement		X		X	X	
Complete Physical Exam	X	X (if not completed)				
Concomitant Medications Review		X		X	X	
Alcohol-Use Questionnaire		X		X	X	
CBC	X					
Platelet				X	X	
BMP	X (with fasting glucose)					
Liver Function	X			X	X	
Hemoglobin A1c	X	X (if not completed)				
Spot Urine	X	X (if not completed)		X	X	
Pregnancy Testing		X		X	X	
DNA Collection		X				
OGTT	X					
BP and HR Measurement	X	X		X	X	
ECG	X					
Drug Dispense		X				
Drug Initiation			X			
DXA		X			X	

TE-CAP		X			X	
FMD		X		X	X	
Endothelial Cell Collection		X		X	X	
Blood Collection and Storage		X		X	X	
Urine Collection and Storage		X		X	X	
SAT Collection and Storage		X		X	X	X
[redacted]						[redacted]

In **Aim 1a**, we will investigate the number and function of T cell subsets in PBMCs and AT using flow cytometry, including identification of pro-inflammatory Th1 and Th17 cells, and characterization of anti-inflammatory cells including γδT, iNKT, ILC2, Th2, and Tregs. We will compare PBMCs and SAT liposuction samples both within individuals at the three time points, as well as between individuals randomized to placebo or empagliflozin. [redacted]

In **Aim 1b**, we will perform *in situ* characterization of AT immune cells in surgical SAT and VAT samples using immunofluorescence microscopy and compare individuals on placebo versus empagliflozin. *We will focus on macrophages, which cannot be characterized in cryopreserved samples by flow cytometry.* We will look for expression of markers associated with activated macrophages such as CD11c, CD16, CD40 and CD9, as contrasted with markers of M2-like homeostatic macrophages including CD206 and CD163.

In **Aim 1c**, we will quantify systemic markers of inflammation including hsCRP, TNFα, IFNγ, IL-1β and IL-6.

In **Aim 2**, we will perform FMD and endothelial cell collection at baseline, after 2 weeks, and after 12 weeks of treatment. We will compare results within individuals at the three time points.

In **Aim 3**, we will assess liver function with a liver function panel and platelet count to calculate NAFLD indices, and perform imaging using TE-CAP. We will compare results within individuals at multiple time points.

Anticipated Results:

SGLT2 inhibition in rodent models decreases acute pro-inflammatory responses to lipopolysaccharide in vitro, prevents lung inflammation in vivo,[41] and skews AT macrophages from pro-inflammatory M1 to anti-inflammatory M2.[43,64] Human trials have noted decreased serum markers of inflammation,[65] and in vitro studies have demonstrated anti-inflammatory effects.[41,44,66] We expect that SGLT2 inhibition with empagliflozin will be associated with decreased systemic and AT inflammation in **Aim 1**, as measured by decreased inflammatory Th1, Th17, and $CD8^+$ T cells, and increased homeostatic ILC2, iNKT, Treg, Th2, and γδT cells. We also expect that empagliflozin treatment will result in decreased systemic pro-inflammatory cytokines and mediators, including IL-6, hsCRP, IL-8, TNFα, IL-1β, IFNγ, IL-17 and MCP-1.

SGLT2 inhibition in rodent models improves atherosclerosis and endothelial function.[47,50] Human trials have demonstrated improvement in FMD as early as 2 days and up to a year after treatment with SGLT2 inhibitors,[51,52,56] but these results have not been consistent across

studies.[54,55] We expect that SGLT2 inhibition will improve FMD as measured in **Aim 2**, and that the relative change in AT inflammation will be associated with improvements in endothelial function.

SGLT2 inhibition attenuates the development of hepatic steatosis and inflammation in a mouse model of NAFLD in spite of the accumulation of epididymal fat mass.[62] These changes were associated with reduced expression of inflammatory genes in the epididymal fat pad. Multiple small clinical trials have also shown that SGLT2 inhibitors improve hepatic steatosis and markers of liver inflammation.[39,40] We hypothesize that this is partly mediated by SGLT2-specific effects on adipose tissue, but this has not been adequately tested in humans. We expect that SGLT2 inhibition will improve NAFLD indices and TE-CAP imaging as measured in **Aim 3**, and that the relative change in AT inflammation will be associated with improvements in hepatic steatosis.

Methods:

Oral Glucose Tolerance Test: After an overnight fast, subjects will be given 75-g glucose by mouth. We will draw blood at 0, 30, 60, 90, and 120 min after administration of glucose for measurement of plasma glucose, insulin, and C-peptide.

Vascular endothelial function (FMD): Measurement of endothelium-dependent and endothelium-independent vasodilation will be performed using methods employed in multiple prior studies by our research team.[67-70] Brachial artery diameter is measured using B-mode ultrasonography, and a simultaneous electrocardiographic signal is recorded and images are digitally acquired at end-diastole, synchronized to the R wave on the electrocardiogram. To assess endothelium-dependent vasodilation, brachial artery diameter is measured under basal conditions and during reactive hyperemia. After a rest period, endothelium-independent vasodilation will be assessed by imaging the brachial artery under basal conditions and following the administration of sublingual nitroglycerin (0.4 mg). Acquisition and analysis of the stored images will be performed using software designed for this purpose by Medical Imaging Applications. The vessel wall lumen interface will be determined by derivative-based edge detection following identification of the region of the anterior and posterior walls by the investigator. The maximum diameter of the vessel will be determined and the percent change in diameter calculated. We have found that this technique yields an inter-observer variability of 0.05±0.16% and intra-observer variability of 0±0.15%.

Subcutaneous adipose harvest by liposuction: SAT will be obtained from the periumbilical area using a Tulip CellFriendly™ GEMS closed syringe system for lipoaspiration. Approximately 5-10 g of tissue is removed (~5.5-11 cc at a specific gravity of 0.918 for human fat). Immune cell extraction from the stromal vascular fraction will be performed using a gentleMACS™ Dissociator from Miltenyi Biotec followed by incubation with collagenase, mononuclear cell separation with Ficoll-Paque and cryopreservation for flow cytometry/sorting.

PBMC harvest: PBMCs will be obtained through the peripheral IV into 3 EDTA tubes for total of 60 milliliters, and transported at room temperature to the lab for processing. Cells will then be passed through a Ficoll-Paque barrier, washed with PBS, and stored in DMSO in a liquid nitrogen freezer for later analysis.

Endothelial cell harvest: Endothelial cell harvest will be performed by passing a 0.018" sterile J- wire (Arrow International, PA) back and forth two to six cm beyond the tip of a venous catheter placed in a cubital vein. Approximately 1000-1500 ECs can be isolated after four passes of the wire. Cell viability is >90% with >90% staining positive for expected EC markers (nitric oxide synthase, vWF) without contamination by leukocytes or smooth muscle cells.

TE-CAP: Liver elastography will be assessed using the FibroScan system by Echosens. All

scans will be performed at the Vanderbilt Digestive Diseases Clinic or the Clinical Research Center by one experienced, certified FibroScan technician. Participants will need to fast at least 4 hours before the procedure. The participant's abdomen will be exposed and will be placed supine with his or her right arm resting above his or her head during the procedure. A water-soluble gel will be applied to the participant's abdomen and a FibroScan probe will be placed over the intercostal space over the liver. Final results will be expressed as the median of 10 successful measurements. Success will be defined as 10 captures with an interquartile range lower than 30% of the median kPa (fibrosis) and dB/m (steatosis). Patients with significant fibrosis defined as >8.0 kPa will be offered referral to a hepatologist for further evaluation.

Laboratory Analyses:

Clinical assays will be run in Vanderbilt CLIA-approved laboratories. Blood drawn for research assays will be centrifuged immediately at 4°C for 20 min, and plasma or serum will be divided into at least two aliquots, labeled, logged and stored separately at -80°C until sampling.

Genotyping: DNA will be extracted from whole blood using the AutoPure LS extraction system (Qiagen, Valencia, CA, USA). Samples will be stored for future exploratory analyses.

7.0 Risks

1. Insertion of venous catheters may cause bleeding, bruising, or infection.
2. Frequent blood draws can cause anemia.
3. During measurement of flow-mediated vasodilation, the administration of sublingual nitroglycerin can cause low blood pressure. Nitroglycerin may also cause severe headache. Patients will be monitored for this and the effects of nitroglycerin are transient. Patients will be asked to refrain from taking a PDE5 inhibitor at least one-week before the study day.
4. Spending study days at the CRC can be inconvenient for subjects.
5. Harvesting adipose tissue could cause pain, bleeding, or infection. We will use local lidocaine injection to numb the area and sterile technique.
6. Harvesting endothelial cells from veins could cause pain, bleeding or infection, primarily from intravenous placement, as well as damage to the vein. We will use local lidocaine injection to numb the area and use sterile technique. We utilize a wire that has a soft rounded end to reduce any risk. We will utilize superficial veins. We will allow at least four weeks before repeating the procedure in the same vein.
7. Pruritus or skin irritation may result from the vibration of the TE-CAP probe or the water-soluble ultrasound gel needed for the procedure.
8. Rare cases of ketoacidosis have been reported in postmarketing surveillance but only in those with type 1 or type 2 diabetes. We are studying an obese population with pre-diabetes that is unlikely to develop this condition. We will advise patients to contact us immediately if they develop symptoms of nausea, vomiting, abdominal pain, malaise or shortness of breath so they can be evaluated. We will exclude those at increased risk for ketoacidosis including pancreatic insulin deficiency from any cause, caloric restriction and alcohol abuse.
9. Empagliflozin causes intravascular volume contraction. We will screen patients for risk of symptomatic hypotension, which is more likely in presence of renal impairment, elderly, patients with low systolic blood pressure, and those on diuretics. Adverse reactions related to volume depletion occur in 0.3% of those on placebo and 0.3% of those on empagliflozin in pooled studies.

10. Empagliflozin can cause renal impairment from volume contraction, and there are postmarketing reports of acute kidney injury.
11. Empagliflozin can increase incidence of urinary tract infections (17% on empagliflozin versus 16.6% on placebo in females, and 4.1% on empagliflozin versus 3.2% on placebo in males). We will exclude those with history of recurrent UTIs.
12. Rare cases of urosepsis and pyelonephritis have been reported in post-marketing analysis.
13. Empagliflozin increases the risk for genital mycotic infections, especially in those with history of chronic or recurrent infections. In females the rate is 6.4% in empagliflozin as compared with 1.5% in placebo, and in males 1.6% with empagliflozin as compared with 0.4% in placebo. We will exclude uncircumcised men or men with history of balanitis, and women with history of severe vulvovaginitis or recurrent vulvovaginitis (>3) per year.
14. Empagliflozin causes an increase in urination (0.8% in empagliflozin versus 0.4% in placebo) and increase in thirst.
15. Necrotizing fasciitis of the perineum has been identified in post-marketing surveillance in patient with diabetes.
16. Hypoglycemia can be observed with concomitant use of insulin and insulin secretagogues. We will study non-diabetic patients who are not on any medications.
17. Empagliflozin can increase LDL-C.
18. There may be unknown or unanticipated adverse effects.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

A Data and Safety Monitoring Committee (DSMC) will provide objective review of human safety and data quality. Committee members will be Dr. C. Michael Stein, Adriana Hung, and Tebeb Gebretsadik. Dr. Stein will chair the committee. All members hold a primary appointment outside the Division of Diabetes, Endocrinology and Metabolism.

The DSMC will also receive quarterly reports of enrollment, protocol adherence, data quality, and adverse events (AE)s. The DSMC will review all serious AEs (SAEs), suspected unexpected serious adverse reactions (SUSARS), and serious adverse drug reactions (SADRs). Any SAE, SUSAR, or SADR will be reported to the DSMC, IRB, and FDA, if appropriate, as soon as possible, but not more than 7 days from the investigator's notification of the event. Any pregnancy occurring during the trial would be reported similarly.

Subjects will be questioned about AEs at each study visit. Any untoward medical event will be classified as an AE, regardless of its causal relationship with the study. Relationship to a study medication will be assessed as probably, possible or unlikely based on the United States Package Insert for the drug. An AE will be classified as serious if it a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, e) is a congenital anomaly or birth defect. Suspicion of transmission of infectious agents will also be considered an SAE. The DSMC may choose to become unblinded; however, it is expected that such unblinding would not occur without reasonable concern related either to patient safety or to data validity.

Non-serious AEs and instances of noncompliance with the protocol will be reported at the time of annual review.

9.0 Study Withdrawal/Discontinuation

Subjects who develop an adverse event that is not transient (such as persistent nausea, severe mycotic genital infection or recurrent infection, recurrent or severe UTI, etc.) will have any study drug discontinued and will be withdrawn from the study. Subjects who do not tolerate empagliflozin at 25mg per day will be discontinued. Subjects who are withdrawn will be treated and/or followed as appropriate until any symptoms are resolved. If it is determined by Vanderbilt and the PI that an adverse event occurred as a direct result of the tests or treatments that are done for research, then neither the subject nor his or her insurance will have to pay for the cost of immediate medical care provided at Vanderbilt to treat the adverse event. This includes clinically significant laboratory values related to the study.

If in the opinion of the investigator a subject is non-compliant, the subject will be withdrawn from the study. Subjects may request to stop the study at any time.

10.0 Statistical Considerations

Sample Size and Power Calculation:

The primary endpoints are macrophage activation and a pro-inflammatory T cell profile in PBMCs and AT in **Aim 1**, endothelial function in **Aim 2**, and liver steatosis in **Aim 3** at 12 weeks post-treatment comparing placebo to empagliflozin. Secondary endpoints include pro-inflammatory T cell profile in PBMCs and AT, systemic cytokine profile, FMD, liver steatosis and weight at baseline, two weeks post-treatment, and 12 weeks post-treatment. [redacted]

[redacted]

Data Analysis Plan: We will use standard graphing and screening techniques to detect outliers and ensure data accuracy. [redacted]

[redacted]

11.0 Privacy/Confidentiality Issues

We will use the web-based Vanderbilt Research Electronic Data Capture (REDCap) system to design electronic data-collection forms in all Aims. These forms will be pilot tested before use. Data will be input into a protected, web-based case report form (which can be readily downloaded into SAS, STATA, R, or SPSS). The form allows for direct data entry by investigators and is designed to minimize errors and erroneous values. Results from the Vanderbilt Clinical Laboratory can also be directly imported to REDCap, which further reduces typographical data-entry errors. Expected ranges are pre-specified to prevent errors such as the shifting of decimal points. The program includes a computerized audit trail so that the identity of individuals entering or changing data and, in the case of changes, both original and revised data are saved. Data are backed up daily. Clinical and research data will be entered by the research nurse, fellow, or research technician in the laboratory.

A unique identification case number will be used to protect the confidentiality of the study participants. Only case numbers will be included in spreadsheets used for the statistical analysis.

12.0 Follow-up and Record Retention

The study duration is expected to be three years. All research records will be accessible for inspection and copying by authorized representatives of the IRB, federal regulatory agency representatives, and the department or agency supporting the research. All study documents will be retained for at least six years after closure of the study with the IRB.

13.0 REFERENCES:

1. Kivimaki M, Kuosma E, Ferrie JE, et al. Overweight, obesity, and risk of cardiometabolic multimorbidity: pooled analysis of individual-level data for 120 813 adults from 16 cohort studies from the USA and Europe. *Lancet Public Health.* 2017;2(6):e277-e285.
2. Exley MA, Hand L, O'Shea D, Lynch L. Interplay between the immune system and adipose tissue in obesity. *J Endocrinol.* 2014;223(2):R41-48.
3. Lontchi-Yimagou E, Sobngwi E, Matsha TE, Kengne AP. Diabetes mellitus and inflammation. *Curr Diab Rep.* 2013;13(3):435-444.
4. Berg AH, Scherer PE. Adipose tissue, inflammation, and cardiovascular disease. *Circ Res.* 2005;96(9):939-949.
5. Nosalski R, Guzik TJ. Perivascular adipose tissue inflammation in vascular disease. *Br J Pharmacol.* 2017;174(20):3496-3513.
6. Golia E, Limongelli G, Natale F, et al. Inflammation and cardiovascular disease: from pathogenesis to therapeutic target. *Curr Atheroscler Rep.* 2014;16(9):435.
7. Wang Z, Nakayama T. Inflammation, a link between obesity and cardiovascular disease. *Mediators Inflamm.* 2010;2010:535918.
8. Kohlgruber A, Lynch L. Adipose tissue inflammation in the pathogenesis of type 2 diabetes. *Curr Diab Rep.* 2015;15(11):92.
9. Vegiopoulos A, Rohm M, Herzig S. Adipose tissue: between the extremes. *EMBO J.* 2017;36(14):1999-2017.
10. Russo L, Lumeng CN. Properties and functions of adipose tissue macrophages in obesity. *Immunology.* 2018;155(4):407-417.
11. Patel PS, Buras ED, Balasubramanyam A. The role of the immune system in obesity and insulin resistance. *J Obes.* 2013;2013:616193.
12. Kusters YH, Schalkwijk CG, Houben AJ, et al. Independent tissue contributors to obesity-associated insulin resistance. *JCI Insight.* 2017;2(13).
13. Preis SR, Massaro JM, Robins SJ, et al. Abdominal subcutaneous and visceral adipose tissue and insulin resistance in the Framingham heart study. *Obesity (Silver Spring).* 2010;18(11):2191-2198.
14. Ross R, Aru J, Freeman J, Hudson R, Janssen I. Abdominal adiposity and insulin resistance in obese men. *Am J Physiol Endocrinol Metab.* 2002;282(3):E657-663.
15. Gastaldelli A, Miyazaki Y, Pettiti M, et al. Metabolic effects of visceral fat accumulation in type 2 diabetes. *J Clin Endocrinol Metab.* 2002;87(11):5098-5103.
16. Stefan N, Haring HU, Hu FB, Schulze MB. Metabolically healthy obesity: epidemiology, mechanisms, and clinical implications. *Lancet Diabetes Endocrinol.* 2013;1(2):152-162.
17. Wouters K, Gaens K, Bijnen M, et al. Circulating classical monocytes are associated with CD11c+ macrophages in human visceral adipose tissue. *Sci Rep.* 2017;7:42665.
18. Aron-Wisnewsky J, Tordjman J, Poitou C, et al. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. *J Clin Endocrinol Metab.* 2009;94(11):4619-4623.
19. Cancello R, Tordjman J, Poitou C, et al. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. *Diabetes.* 2006;55(6):1554-1561.
20. McLaughlin T, Liu LF, Lamendola C, et al. T-cell profile in adipose tissue is associated with insulin resistance and systemic inflammation in humans. *Arterioscler Thromb Vasc Biol.* 2014;34(12):2637-2643.
21. Kosmas CE, Silverio D, Sourlas A, Montan PD, Guzman E, Garcia MJ. Anti-inflammatory therapy for cardiovascular disease. *Ann Transl Med.* 2019;7(7):147.

22. Nidorf SM, Eikelboom JW, Budgeon CA, Thompson PL. Low-dose colchicine for secondary prevention of cardiovascular disease. *J Am Coll Cardiol.* 2013;61(4):404-410.
23. Shapiro M, Levy Y. The association between hydroxychloroquine treatment and cardiovascular morbidity among rheumatoid arthritis patients. *Oncotarget.* 2018;9(5):6615-6622.
24. Mangoni AA, Zinellu A, Sotgia S, Carru C, Piga M, Erre GL. Protective Effects of Methotrexate against Proatherosclerotic Cytokines: A Review of the Evidence. *Mediators Inflamm.* 2017;2017:9632846.
25. Ridker PM, Everett BM, Pradhan A, et al. Low-Dose Methotrexate for the Prevention of Atherosclerotic Events. *N Engl J Med.* 2019;380(8):752-762.
26. Ridker PM, Everett BM, Thuren T, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. *N Engl J Med.* 2017;377(12):1119-1131.
27. Brown E, Wilding JPH, Barber TM, Alam U, Cuthbertson DJ. Weight loss variability with SGLT2 inhibitors and GLP-1 receptor agonists in type 2 diabetes mellitus and obesity: Mechanistic possibilities. *Obes Rev.* 2019;20(6):816-828.
28. Zinman B, Wanner C, Lachin JM, et al. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *N Engl J Med.* 2015;373(22):2117-2128.
29. Neal B, Perkovic V, Mahaffey KW, et al. Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes. *N Engl J Med.* 2017;377(7):644-657.
30. Wiviott SD, Raz I, Bonaca MP, et al. Dapagliflozin and Cardiovascular Outcomes in Type 2 Diabetes. *N Engl J Med.* 2019;380(4):347-357.
31. Perkovic V, Jardine MJ, Neal B, et al. Canagliflozin and Renal Outcomes in Type 2 Diabetes and Nephropathy. *N Engl J Med.* 2019;380(24):2295-2306.
32. McMurray JJV, Solomon SD, Inzucchi SE, et al. Dapagliflozin in Patients with Heart Failure and Reduced Ejection Fraction. *N Engl J Med.* 2019;381(21):1995-2008.
33. Zelniker TA, Wiviott SD, Raz I, et al. SGLT2 inhibitors for primary and secondary prevention of cardiovascular and renal outcomes in type 2 diabetes: a systematic review and meta-analysis of cardiovascular outcome trials. *Lancet.* 2019;393(10166):31-39.
34. Arnett DK, Blumenthal RS, Albert MA, et al. 2019 ACC/AHA Guideline on the Primary Prevention of Cardiovascular Disease: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation.* 2019;140(11):e596-e646.
35. American Diabetes A. 9. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes-2020. *Diabetes Care.* 2020;43(Suppl 1):S98-S110.
36. North EJ, Newman JD. Review of cardiovascular outcomes trials of sodium-glucose cotransporter-2 inhibitors and glucagon-like peptide-1 receptor agonists. *Curr Opin Cardiol.* 2019;34(6):687-692.
37. Targher G, Bertolini L, Padovani R, et al. Prevalence of nonalcoholic fatty liver disease and its association with cardiovascular disease among type 2 diabetic patients. *Diabetes Care.* 2007;30(5):1212-1218.
38. Younossi Z, Anstee QM, Marietti M, et al. Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol.* 2018;15(1):11-20.
39. Shao SC, Kuo LT, Chien RN, Hung MJ, Lai EC. SGLT2 inhibitors in patients with type 2 diabetes with non-alcoholic fatty liver diseases: an umbrella review of systematic reviews. *BMJ Open Diabetes Res Care.* 2020;8(2).
40. Sumida Y, Yoneda M, Tokushige K, et al. Antidiabetic Therapy in the Treatment of Nonalcoholic Steatohepatitis. *Int J Mol Sci.* 2020;21(6).

41. Xu C, Wang W, Zhong J, et al. Canagliflozin exerts anti-inflammatory effects by inhibiting intracellular glucose metabolism and promoting autophagy in immune cells. *Biochem Pharmacol.* 2018;152:45-59.
42. Nakatsu Y, Kokubo H, Bumdelger B, et al. The SGLT2 Inhibitor Luseogliflozin Rapidly Normalizes Aortic mRNA Levels of Inflammation-Related but Not Lipid-Metabolism-Related Genes and Suppresses Atherosclerosis in Diabetic ApoE KO Mice. *Int J Mol Sci.* 2017;18(8).
43. Xu L, Nagata N, Nagashimada M, et al. SGLT2 Inhibition by Empagliflozin Promotes Fat Utilization and Browning and Attenuates Inflammation and Insulin Resistance by Polarizing M2 Macrophages in Diet-induced Obese Mice. *EBioMedicine.* 2017;20:137-149.
44. Juni RP, Kuster DWD, Goebel M, et al. Cardiac Microvascular Endothelial Enhancement of Cardiomyocyte Function Is Impaired by Inflammation and Restored by Empagliflozin. *JACC Basic Transl Sci.* 2019;4(5):575-591.
45. Hattori S. Anti-inflammatory effects of empagliflozin in patients with type 2 diabetes and insulin resistance. *Diabetol Metab Syndr.* 2018;10:93.
46. Magkos F, Fraterrigo G, Yoshino J, et al. Effects of Moderate and Subsequent Progressive Weight Loss on Metabolic Function and Adipose Tissue Biology in Humans with Obesity. *Cell Metab.* 2016;23(4):591-601.
47. Han JH, Oh TJ, Lee G, et al. The beneficial effects of empagliflozin, an SGLT2 inhibitor, on atherosclerosis in ApoE (-/-) mice fed a western diet. *Diabetologia.* 2017;60(2):364-376.
48. Terasaki M, Hiromura M, Mori Y, et al. Amelioration of Hyperglycemia with a Sodium-Glucose Cotransporter 2 Inhibitor Prevents Macrophage-Driven Atherosclerosis through Macrophage Foam Cell Formation Suppression in Type 1 and Type 2 Diabetic Mice. *PLoS One.* 2015;10(11):e0143396.
49. Nakajima K, Mita T, Osonoi Y, et al. Effect of Repetitive Glucose Spike and Hypoglycaemia on Atherosclerosis and Death Rate in Apo E-Deficient Mice. *Int J Endocrinol.* 2015;2015:406394.
50. Ganbaatar B, Fukuda D, Shinohara M, et al. Empagliflozin ameliorates endothelial dysfunction and suppresses atherogenesis in diabetic apolipoprotein E-deficient mice. *Eur J Pharmacol.* 2020;875:173040.
51. Sezai A, Sekino H, Unosawa S, Taoka M, Osaka S, Tanaka M. Canagliflozin for Japanese patients with chronic heart failure and type II diabetes. *Cardiovasc Diabetol.* 2019;18(1):76.
52. Sawada T, Uzu K, Hashimoto N, et al. Empagliflozin's Ameliorating Effect on Plasma Triglycerides: Association with Endothelial Function Recovery in Diabetic Patients with Coronary Artery Disease. *J Atheroscler Thromb.* 2019.
53. Bosch A, Ott C, Jung S, et al. How does empagliflozin improve arterial stiffness in patients with type 2 diabetes mellitus? Sub analysis of a clinical trial. *Cardiovasc Diabetol.* 2019;18(1):44.
54. Solini A, Seghieri M, Giannini L, et al. The Effects of Dapagliflozin on Systemic and Renal Vascular Function Display an Epigenetic Signature. *J Clin Endocrinol Metab.* 2019;104(10):4253-4263.
55. Zainordin NA, Hatta S, Mohamed Shah FZ, et al. Effects of Dapagliflozin on Endothelial Dysfunction in Type 2 Diabetes With Established Ischemic Heart Disease (EDIFIED). *J Endocr Soc.* 2020;4(1):bvz017.

56. Solini A, Giannini L, Seghieri M, et al. Dapagliflozin acutely improves endothelial dysfunction, reduces aortic stiffness and renal resistive index in type 2 diabetic patients: a pilot study. *Cardiovasc Diabetol.* 2017;16(1):138.
57. Kuchay MS, Krishan S, Mishra SK, et al. Effect of Empagliflozin on Liver Fat in Patients With Type 2 Diabetes and Nonalcoholic Fatty Liver Disease: A Randomized Controlled Trial (E-LIFT Trial). *Diabetes Care.* 2018;41(8):1801-1808.
58. Shibuya T, Fushimi N, Kawai M, et al. Luseogliflozin improves liver fat deposition compared to metformin in type 2 diabetes patients with non-alcoholic fatty liver disease: A prospective randomized controlled pilot study. *Diabetes Obes Metab.* 2018;20(2):438-442.
59. Ito D, Shimizu S, Inoue K, et al. Comparison of Ipragliflozin and Pioglitazone Effects on Nonalcoholic Fatty Liver Disease in Patients With Type 2 Diabetes: A Randomized, 24-Week, Open-Label, Active-Controlled Trial. *Diabetes Care.* 2017;40(10):1364-1372.
60. Seko Y, Nishikawa T, Umemura A, et al. Efficacy and safety of canagliflozin in type 2 diabetes mellitus patients with biopsy-proven nonalcoholic steatohepatitis classified as stage 1-3 fibrosis. *Diabetes Metab Syndr Obes.* 2018;11:835-843.
61. Sumida Y, Murotani K, Saito M, et al. Effect of luseogliflozin on hepatic fat content in type 2 diabetes patients with non-alcoholic fatty liver disease: A prospective, single-arm trial (LEAD trial). *Hepatol Res.* 2019;49(1):64-71.
62. Shiba K, Tsuchiya K, Komiya C, et al. Canagliflozin, an SGLT2 inhibitor, attenuates the development of hepatocellular carcinoma in a mouse model of human NASH. *Sci Rep.* 2018;8(1):2362.
63. Luca MC, Liuni A, Harvey P, Mak S, Parker JD. Effects of estradiol on measurements of conduit artery endothelial function after ischemia and reperfusion in premenopausal women. *Can J Physiol Pharmacol.* 2016;94(12):1304-1308.
64. Miyachi Y, Tsuchiya K, Shiba K, et al. A reduced M1-like/M2-like ratio of macrophages in healthy adipose tissue expansion during SGLT2 inhibition. *Sci Rep.* 2018;8(1):16113.
65. Bonnet F, Scheen AJ. Effects of SGLT2 inhibitors on systemic and tissue low-grade inflammation: The potential contribution to diabetes complications and cardiovascular disease. *Diabetes Metab.* 2018;44(6):457-464.
66. Panchapakesan U, Pegg K, Gross S, et al. Effects of SGLT2 inhibition in human kidney proximal tubular cells--renoprotection in diabetic nephropathy? *PLoS One.* 2013;8(2):e54442.
67. Beckman JA, Goldfine AB, Dunaif A, Gerhard-Herman M, Creager MA. Endothelial function varies according to insulin resistance disease type. *Diabetes Care.* 2007;30(5):1226-1232.
68. Owens CD, Wake N, Conte MS, Gerhard-Herman M, Beckman JA. In vivo human lower extremity saphenous vein bypass grafts manifest flow mediated vasodilation. *J Vasc Surg.* 2009;50(5):1063-1070.
69. Nohria A, Kinlay S, Buck JS, et al. The effect of salsalate therapy on endothelial function in a broad range of subjects. *J Am Heart Assoc.* 2014;3(1):e000609.
70. Nguyen PL, Jarolim P, Basaria S, et al. Androgen deprivation therapy reversibly increases endothelium-dependent vasodilation in men with prostate cancer. *J Am Heart Assoc.* 2015;4(4).
71. Shigiyama F, Kumashiro N, Miyagi M, et al. Effectiveness of dapagliflozin on vascular endothelial function and glycemic control in patients with early-stage type 2 diabetes mellitus: DEFENCE study. *Cardiovasc Diabetol.* 2017;16(1):84.

72. Benjamin Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological).* 1995;57(1):289-300.