DAPAGLIFLOZIN AND VASCULAR HEALTH IN PATIENTS WITH TYPE 2 DIABETES MELLITUS (T2DM).

ClinicalTrials.gov number: NCT05139914
Protocol Version Number: 1.6
Protocol Version Date: March 21, 2022
Funding Mechanism: American Heart Association 20SFRN35120118
Principal Investigator: Naomi M. Hamburg
Phone: 617-638-7260
E-mail: nhamburg@bu.edu

Statistical Analysis Plan- pages 18-19

CONFIDENTIAL

TABLE OF CONTENTS

1 List of Abbreviations

Abbreviation	Abbreviation definition
AE	Adverse Event
CRF	Case Report Form
CV	Cardiovascular
EC	Endothelial Cell
FMD	Flow-Mediated Dilation
HbA1c	Hemoglobin A1c
HIPAA	HealthCare Information Portability and Accountability Act
IPS	Investigational Pharmacy Service
IRB	Institutional Review Board
miRNA	microRNA
ncRNA	non-coding RNA
NO	Nitric Oxide
PAT	Peripheral Artery Tonometry
ROS	Reactive Oxygen Species
SGLT2 inhibitor	Sodium-Glucose Cotransporter-2 inhibitor
T2DM	Type 2 Diabetes Mellitus

2 Protocol Summary

Title:	DAPAGLIFLOZIN AND VASCULAR HEALTH IN PATIENTS WITH TYPE 2 DIABETES MELLITUS (T2DM)
Population:	Patients with T2DM (ages 30-75, all sexes). We will plan to consent 50 participants with the goal of randomizing 42 and having 35 subjects complete the study.
Intervention:	Dapagliflozin 10mg/day and Placebo each for 6 weeks in a cross-over design
Objectives:	1. To assess the impact of dapagliflozin on EC health in patients with T2DM 2. To assess the impact of dapagliflozin on vasodilator function and additional measures of vascular health including arterial stiffness and markers of inflammation
Design/Methodology:	The overall study design is a two-treatment, two-period cross-over study. Subjects will be randomized to treatment order in a 1:1 ratio to receive SGLT2 inhibitor (dapagliflozin) and then placebo or vice versa in a cross-over design. Total study period for each study subject is 14 weeks consisting of: two treatment periods (dapagliflozin and placebo) lasting 6 weeks each (12 weeks total) and a 2 week washout period between treatment periods. Each subject undergoes a washout period of 2 weeks after completing first 6 weeks of treatment with either placebo or dapagliflozin. This is followed by cross-over to the alternate treatment for a period of 6 weeks with dapagliflozin or placebo depending on their first

	treatment. Randomization will be done in block sizes of 2 or 4. Once assigned to treatment, participants will receive dapagliflozin 10 mg/day or placebo for 6 weeks. A detailed schedule of events is in the appendix.
Endpoints	Primary endpoint: EC health measured as response to insulin Secondary endpoint: Endothelial vasodilator function measured flow-mediated dilation of brachial artery Other Secondary Endpoints: vascular health measures, arterial stiffness, EC RNA, circulating biomarkers of vascular health
Total Study Duration:	We estimate that the study will last four years from IRB approval through data analysis.
Subject Participation Duration:	Total of 14 weeks after starting study intervention
Key Procedures:	1 screening visit, 4 study visits, 2 study phone calls.

3 Background/Rationale & Purpose

3.1 Background Information

Type 2 Diabetes Mellitus (T2DM) is a key public health problem worldwide with current trends predicting that 500 million people will have diabetes in 2035.[1] The escalating prevalence of T2DM worldwide presents a critical cardiovascular (CV) challenge.[2, 3] Patients with T2DM experience accelerated vascular aging, premature atherosclerotic CV disease, and increased rates of CV events.[4, 5] There is considerable interest in the clinical CV benefits of new agents to treat T2DM including SGLT2 inhibitors such as dapagliflozin.[6, 7] However, the mechanisms of that drive the cardiovascular health benefits of dapagliflozin remains incompletely understood.

Metabolic disturbances underlie the systemic vascular dysfunction in T2DM. Elevated glucose and lipids alter signal transduction thereby changing gene expression resulting disturbing endothelial homeostasis.[8] Alterations in endothelial function including inflammation and reduced nitric oxide (NO) production promote atherogenesis in T2DM.[9]

Several studies suggest activity of SGLT2 inhibitors on vascular function in animal models and humans.[10-12] Dapagliflozin has effects on endothelial inflammation in cultured cells and improved vasodilation in animal models[13]; however the effect on EC health in patients is not well-defined. Emerging experimental evidence in preclinical models also links SGLT2 inhibitors to reduction in mitochondrial oxidative stress[14]. Our study will provide novel evidence about EC effects of dapagliflozin that link to both animal models of T2DM and clinical trial data.

This study will be conducted in compliance with the protocol, applicable regulatory requirements, and Boston Medical Center/Boston University Medical Campus Human Research Protection policies and procedures.

3.2 Rationale and Purpose

There is considerable interest in the clinical CV benefits of new diabetes medications including dapagliflozin (SGLT2 inhibitor).[6, 7] However, the mechanisms that underlie the CV benefits remain incompletely defined. The robust clinical trial evidence with SGLT inhibitors has not yet been

accompanied by complete mechanistic evidence in the vasculature. Combining vascular functional assessment with targeted EC gene expression and NO characterization will expand critical evidence about the mechanisms of action. Prior clinical studies evaluating the effects of SGLT2 inhibitors have been limited to evaluation of vascular health without measures of EC phenotype that will provide additional mechanistic information. Dapagliflozin has effects on endothelial inflammation in cultured cells and improved vasodilation in animal models[13]; however, the effect on EC health in patients is not well-defined. Emerging experimental evidence in preclinical models also links SGLT2 inhibitors to reduction in mitochondrial oxidative stress[14].

We propose to investigate treatment with novel medications (SGLT2 inhibitor) for T2DM will alter endothelial phenotype and ncRNA or biomarkers. We plan to test this hypothesis by conducting an interventional cross-over study with dapagliflozin (SGLT2 inhibitor). We anticipate that evaluating EC phenotype will provide key additional information regarding the ability of novel treatments for T2DM to restore vascular health.

4 Objectives

4.1 Study Objectives

The overarching objective of this mechanistic study is to learn how an SGT2 inhibitor, dapagliflozin, impacts vascular health in patients with T2DM. We aim to compare the changes in vascular health to changes in EC phenotype including ncRNA to develop evidence supporting the mechanism of cardiovascular benefit of SGLT2 inhibitors. This study will provide novel information regarding the mechanism of effects of novel treatments for endothelial function and vascular health in patients with T2DM to reduce CV risk.

1. To assess the effects of dapagliflozin on EC phenotype.
2. To assess the impact of dapagliflozin on vasodilator function and additional measures of vascular health including arterial stiffness and circulating markers of vascular health.

4.2 Study Outcome Measures.

4.2.1 Primary Outcome Measures

The primary outcome is <u>insulin-mediated eNOS phosphorylation</u> measured in ECs. The percentage change in the phosphorylation of eNOS is measured using quantitative immunofluorescence microscopy in EC collected before and after each treatment period.

4.2.2 Secondary Outcome Measures

<u>Key Secondary Outcome:</u> Flow-mediated dilation of the brachial artery measured by ultrasound. We measure the percentage change in the diameter of the brachial artery before and after a 5 minute cuff occlusion on the arm as a measure of endothelial function. We will measure before and after each treatment period.

<u>Other secondary Endpoints:</u>

- Arterial stiffness measured by tonometry
- Microvascular dilator function by EndoPAT
- Plasma measures of noncoding RNA
- EC measures of noncoding RNA
- EC measures of coding RNA
- Circulating biomarkers of vascular health including BNP and CRP
- Fasting glucose, insulin, and lipid profile
- Body weight and waist circumference

5 Study Design

5.1 Overall design:

The overall design is a single center randomized, double-blind, cross-over, placebo-controlled study of dapagliflozin (an SGLT2 inhibitor) compared to placebo in patients with T2DM. Each treatment period lasts 6 weeks with a 2 week washout period between study treatments. The study intervention is 10mg per day of dapagliflozin compared to placebo. Vascular health measures including EC, vasodilator function, and circulating biomarkers will be measured before and after each treatment period. An outline of the study visits and procedures is in the appendix. A cross-over design is being used in order to be able to be able to minimize variability by comparing within individual effects. Prior studies from our group and others have not shown carry-over effects with regards to the primary outcome measures.

Subjects will be randomized 1:1 ratio to receive SGLT2 inhibitor (dapagliflozin 10mg/day) and then placebo or vice versa. Treatment order will be block randomized in block sizes of 2 or 4. 50 subjects will be consented with the goal of randomizing 42 and having 35 study completers.

Participants will be adult men and women who are outpatients with established T2DM. All participants will receive both study interventions.

The study intervention is an FDA-approved medication used in the FDA-approved study population with the objective of identifying mechanisms of cardiovascular health effects thus the study does not fit into an FDA defined trial Phase.

The study is a single site study and all procedures will be conducted at Boston University using established methodology in Dr. Hamburg's laboratory. Clinical laboratory tests will be conducted in Boston Medical Center pathology. All data will be managed by BEDAC in a centralized manner using established quality control protocols.

6 Potential Risks and Benefits

6.1 Risks

Blood collection: will cause momentary discomfort and is associated with a very small risk of bruising or infection. There is a slight chance that a bruise or infection will develop at the puncture site.

Insertion of the J wire: there is the small possibility that a bruise or infection will develop at the puncture site. There is a very low chance that the J-wire could break during the procedure that could require a procedure to remove the part of the wire from your body. None of these are common occurrences. None of these have occurred in the more than 2500 times the procedure has been performed at BUMC. The procedure will be performed by a trained physician or nurse.

During inflation of the blood pressure cuff for 5 minutes, there will be tightness and numbness in the arm and hand that will pass within a few seconds after cuff release.

The nitroglycerin may cause a headache or hypotension lasting for up to 30 minutes. It will be given under the supervision of a physician while the subject is lying down. We measure blood pressure as part of the study, and the nitroglycerin portion of the study will be omitted if systolic blood pressure is less than 100 mmHg. Prior to nitroglycerin administration, we will ask the subject about history of migraine headaches, prior nitroglycerin use, and the use of Viagra®, Cialis®, or Levitra®. The nitroglycerin could cause a serious drop in blood pressure if taking any of these drugs. If the participant has taken any of these drugs within a week of any study visit they will still be eligible for the study, but the nitroglycerin portion of the vascular function testing will be omitted.

The study intervention: Potential Adverse Effects of dapagliflozin: (Please see attached package insert for additional information)[15].

- Hypoglycemia: There is a low risk of hypoglycemia even when dapagliflozin is combined with other common oral anti-diabetes medications. In the DECLARE-TIMI 58 study, there was a lower rate of a major hypoglycemic event (0.7%) in the dapagliflozin compared to the placebo group (1.0%)[16]. A meta-analysis that included 14 RCTs comparing the effects of dapagliflozin 10mg against placebo did not find a difference in the risk of hypoglycemic events (HR 0.97, CI 0.82-1.15)[17]. Another meta-analysis that included 15 RCTs compared dapagliflozin in conjunction with other diabetes medications metformin, insulin, or GLP1-agonist exenatide versus placebo plus these medications also showed no increased risk of hypoglycemia (RR 1.04, CI 0.93-1.17)[18]. The drug manufacturer recommends cautious use in patients taking insulin secretagogues (e.g. sulfonylureas) and insulin, which themselves carry a risk of hypoglycemia due to their mechanism of action[15, 19]. Taken together, these data suggest that the risk of hypoglycemia from dapagliflozin is only present when taken in conjunction with insulin or sulfonylureas, but not other commonly prescribed anti-diabetes medications metformin or GLP1-agonists. We will exclude participants with history of major hypoglycemic events in the past 6 months and/or HbA1c<6.5%. For patients on either insulin or sulfonylurea therapy we will consult with the patient's diabetologist to titrate diabetes medications appropriately to minimize risk of hypoglycemia.
- Volume depletion: Dapagliflozin has a mild diuretic effect. The rate of symptoms of volume depletion in DECLARE-TIMI 58 was similar in the dapagliflozin compared to the placebo group. We will exclude individuals with systolic blood pressure less than 100mmHg

- Genital mycotic infections: there is a small risk of genital mycotic infections (0.9%). We will exclude participants with a history of more than one genital mycotic infection in the past two years.
- Hypersensitivity reaction: There is a low rate (0.4%) of hypersensitivity including rash or angioedema. Patients with history of allergy to SGLT2 inhibitors will be excluded.
- Bladder cancer: there is a very low rate of association with bladder cancer. Patients with a history of bladder cancer or a history of radiation therapy to the lower abdomen or pelvis will be excluded.
- Urinary tract infection: there is a small increase in the rate of urinary tract infection. We will exclude participants with a history of chronic cystitis and/or recurrent urinary tract infections (3 or more in the last year).
- Nausea (1-10%)
- Low rate of serious infections in the penis or vagina including necrotizing fasciitis of the peroneum.
- May increase LDL cholesterol level
- Dizziness or headache (1-10%)
- Low rate of ketoacidosis.

Protection of confidentiality: All samples and data files will be de-identified and coded. All paper files will be stored in locked cabinets and electronic files will be password protected. The studies will be opened to all participants regardless of sex or race/ethnicity. The participant voluntary recruited will be asked to provide consent before being included in the study.

Pregnancy during the study: If patients get pregnant while in this study, it could be bad for the fetus/baby. As described in the package insert, there is animal data suggesting adverse renal effects in the second and third trimesters of pregnancy. Limited data with dapagliflozin in pregnancy women are not sufficient to determine drug-associated risk for major birth defects or miscarriage. In order to mitigate this risk, we will complete a blood pregnancy test prior to the first visit to mitigate for this risk and collect information regarding use of birth control. If the subject is of child-bearing potential, she must use birth control if she is a woman having sex with men while in this study. She should also keep using birth control for three months after the study ends. Only some birth control methods work well enough to be safe while enrolled in this study. These methods are oral contraceptives (the pill), intrauterine devices (IUDs), contraceptive implants under the skin, contraceptive rings or patches or injections, diaphragms with spermicide, and condoms with foam. Subject should not be in this study if she is a woman who has sex with men and cannot use one of these birth control methods.

The investigators will continuously monitor subjects for adverse events by asking about such events at follow up phone call and study visit and by instructing the subjects to contact the investigators immediately if they experience unexpected problems. Investigators will call the participants by phone call two times (once during each treatment period) and will discuss adverse effects or change in medical status, and assess subject compliance. In addition, the principal investigator will monitor the literature for reports and studies of the clinical use SGLT2 inhibitors for new information on additional risks.

If there is any study related unexpected event, problems with recruiting, or new information derived from the literature, the principal investigator will review the situation, consult with co-investigators and decide about modifying the protocol or stopping the study.

There may be unknown risks or discomforts involved.

6.2 Potential Benefits

The study will provide novel information regarding vascular function in patients with T2DM who undergo treatment with SGLT2 inhibitors. CV disease is the leading cause of death and disability in this country and costs billions of dollars each year. The findings may be relevant to the management of CV disease. The results of these studies have the potential to be the foundation of novel therapeutic development for patients with T2DM to reduce CV risk.

6.3 Analysis of Risks in Relation to Benefits

In the opinion of the investigators, the potential benefits (understanding the mechanism of benefit of SGLT2 inhibitor treatment for T2DM for CV disease that could lead to improved outcomes) far outweigh the risks associated with this study (non-invasive testing of vascular function, blood collection, measuring endothelial function).

7 Study Subject Selection

7.1 Subject Inclusion Criteria

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Male and Female subjects.
2. Age 30-75 years old
3. Diagnosis of T2DM for minimum of 3 months
4. BMI>25
5. Willing to give informed consent and able to understand, to participate in and to comply with the study requirements.

7.2 Subject Exclusion Criteria

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Treatment with anticoagulation within last 30 days
2. Treatment with SGLT2 inhibitor within the last 60 days
3. HbA1c>9.5% within last 3 months timeframe
4. Systolic blood pressure less than 100mmHg
5. History of genital mycotic infections: more than one genital mycotic infection in the past two years.

6. History of recurrent urinary tract infections: history of chronic cystitis and/or recurrent urinary tract infections (3 or more in the last year).
7. History of allergy to SGLT2 inhibitor
8. History of bladder cancer or prior pelvic radiation
9. We will exclude participants with more than one hypoglycemic events in the past 6 months and/or HbA1c<6.5%. For patients on either insulin or sulfonylurea therapy we will consult with the patient's diabetologist to titrate diabetes medications appropriately to minimize risk of hypoglycemia.
10. Women who are lactating or pregnant. All women with childbearing potential will undergo a blood pregnancy test at baseline visit to exclude pregnancy and be required to use birth control throughout the study period.
11. Treatment with an investigational product within the last 30 days.
12. Clinically evident major illness of other organ systems, including clinically evident cancer, end-stage renal failure or other conditions that in the opinion of the principal investigator make this clinical study inappropriate.

8 Study Intervention

The study intervention will involve the use of an SGLT2 inhibitor dapagliflozin

8.1 Study Drug

Dapagliflozin, an SGLT2 inhibitor, is available as 5mg and 10mg tablets. Each bottle containing 50 of the 10 mg tablets will be purchased using funding from American Heart Association.

8.2 Dosing

Subjects randomized to the dapagliflozin-then-placebo arm, will be instructed to administer dapagliflozin 10mg once daily for 6 weeks. Week 7 & 8 will be provided as a washout period. Afterwards, subjects will receive the placebo 0mg once daily for the remainder 6 weeks. The dose has been selected based on prior studies demonstrating the impact on cardiovascular health at the dose of 10mg and the safety profile is well-established with starting at the 10mg dose from multiple studies both including individuals with and without Type 2 diabetes with an acceptable safety profile.

Subjects randomized to the placebo-then-dapagliflozin arm, will be instructed to administer placebo 0mg once daily for 6 weeks. Week 7 & 8 will be provided as a washout period. Afterwards, subjects will receive the dapagliflozin 10mg once daily for the remainder 6 weeks.

8.3 Administration of Dapagliflozin

Subjects will receive Dapagliflozin 10mg capsules, to fulfill the 6 weeks treatment plus 3 tablets to account for lost doses. Subjects will be instructed to take 1 capsule (10mg) by mouth once daily preferably in the morning with or without food.

8.4 Preparation

Under USP 795 pharmaceutical compounding, BMC Investigational Pharmacy Service (IPS) will be responsible in maintaining compliance to good compounding practice in preparation of encapsulating dapagliflozin prior to dispensing.

Dapagliflozin (10mg tablets) will be over-encapsulated with **microcellulose,** which will serve as a filler to maintain the study blind. Matching placebo will also be packaged using **microcellulose**. Capsule size required will be determined by IPS with the goal of a minimum capsule size needed for over-encapsulation.

8.5 Medication distribution

Dapagliflozin will be stored in IPS at room temperature (15°C to 30°C [59°F to 86°F]) in a secured area until the time that medication is needed for study subjects. Stability and expiration dates will be monitored by IPS according to the labelling of the medication from the manufacturer and beyond use date in compounding.

IPS will be responsible for manual re-supply to ensure appropriate inventory of dapagliflozin are available upon enrollment and visits.

- How the study medications will be acquired: dapagliflozin will be purchased using funding from American Heart Association for the study.
- Medication distribution, storage and stability: The medication according to labeling as above.
- Dosage, preparation, and administration: subjects randomized to dapagliflozin will receive 10 mg/day during the treatment period.
- Instructions for modification of dose due to toxicity or other reason. If the participant shows any toxicity related to dapagliflozin he/she will be immediately withdrawn from the study.
- Accountability procedures and compliance assessment: Participant will be asked to keep a log of taking medication and to bring their package to each visit for pill count. The investigator will perform a pill count to assess subject compliance. Participants will be withdrawn from the study if their compliance is equal or lower than 80%.

8.6 Emergency Unblinding

There may be circumstances where it is necessary to unblind the subject's treatment assignment due to clinical care or ingestion of study medication by household member (ie child). The treating clinician will contact with study principal investigator to request emergency unblinding and provide the reason for unblinding. The emergency unblinding request will be sent to the IPS service by telephone during business hours and by email to IPS@bmc.org as well as paging the investigational pharmacist on call (2809) and the investigational pharmacist will provide the treatment assignment to the clinician.

9 Study Procedures

Recruitment

We will use two methods for recruiting participants in this study: 1) Recruitment within the internal medicine, endocrinology and cardiovascular clinics by study investigators who are also the care providers for potential participants as well as by care providers who are not also investigators on this study; 2) Recruitment using advertisements in the form of flyers in the endocrinology and cardiovascular clinics or on social media.

1. Recruitment in outpatient clinical practices

The study coordinator will check the EMR and clinic schedule for subjects who meet inclusion/exclusion criteria (if subjects do not have a recent HbA1C result or pregnancy status is not clear, subjects will still be considered as these assays can be done as part of a screening visit following full consent). The study coordinator will alert providers that a potential subject is to be seen in the clinic. If the provider agrees that the subject may suitable for the study, they will inform the subject about the study and if there is interest, they will notify the study coordinator.
The study coordinator will contact the potential subject and tell them more about the study. If they continue to be interested, the coordinator will move onto the telephone screening process. If they are not interested then only their name/EMR# and "not interested" will be kept in a separate log to prevent them from being contacted a second time.

2. Advertising in Clinics and Social Media

Advertisements will be posted within endocrinology and cardiovascular and internal medicine and family medicine clinics and flyers available for patients to view and on social media. If interested based on their review of the information on the flyer they can call the study team via the phone number on the flyer. These materials will be approved by BU Communications before being posted.
For those individuals who call in after seeing a flyer or advertisement about the study, the study coordinator will contact the individual and briefly review pertinent details about the study. If they continue to be interested, the coordinator will move onto the telephone screening process. If they are not interested then only their name/EMR# and "not interested" will be kept in a separate log to prevent them from being contacted a second time.

See "Recruitment Script" and other recruitment materials

Screening (prior to full consent)

EMR screening will be done by the study coordinator to determine initial eligibility. If subjects are interested in the study following the recruitment phone call, the coordinator will read them the full screening consent with HIPAA language. The procedures that will then be done are additional EMR review and screening questions. The EMR has to be accessed to establish whether or not the subject will need a screening visit for bloodwork or whether they can start the study without a screening visit. After this has been done, the coordinator will contact the subject to tell them whether they are eligible

For those subjects who do not continue to full consent, the only information that will be kept will be names/EMR and whether the potential subject was eligible or not, and interested or not. The primary reason this information will be kept will be to avoid recontacting individuals who decline or who are not eligible so as not to disrupt clinic flow unnecessarily. For subjects who go on to participate in the study, the screening data will become part of their study record.

For all subjects who agree to be screened, we will use the full screening consent with HIPAA language. We will request a partial HIPAA waiver to request that the signature be waived. Given the fact that we will be screening by phone as it is not possible to screen in person at the clinic due to clinic flow, and the fact that our BMC population by and large do not have the technological capacity to sign authorization in REDCap, we feel that not obtaining a signature is reasonable. If we do have to obtain a signature using REDCap, we may not be able to enroll many of our BMC patients.

Screening Visit (up to week -4) (30 minutes):
There are two possible pathways for obtaining the screening information that includes both medical history and laboratory values (HgA1c and pregnancy test for people of child-bearing potential). If participants have an available HgA1c within the past 3 months and are not of child-bearing potential, we will offer the subject the option of completing the screening visit on the same day as Study Visit 1. In this case, the subject will be instructed to come to the laboratory fasting and if applicable to avoid smoking on the morning of the study.

All other potential subjects will have a screening visit that is separate from study visit 1 and will not have to modify eating or smoking on the day of the screening visit.

For all potential subjects: After obtaining informed consent from the subject, investigators will review again the subject's medical history to confirm eligibility. Patients will undergo HbA1c testing unless they already have testing done in the last 3 months. For people of child-bearing potential, a blood based pregnancy test will be sent to the clinical laboratory at BMC.

After eligibility is confirmed, the first study visit will be scheduled either at a future date or on the same day as the screening visit.
For all 4 study visit: subjects will be instructed to fast after midnight on the day of the study, and if applicable, to avoid smoking on the morning of the study. Each study visit lasts about 2 hours.

Study Visit 1: patients will arrive at vascular research unit at Boston University School of Medicine (Evans Building Room E-748) in the morning. Vital signs will be measured and blood will be collected. We will then assess vascular function as follows:

1. **Endothelial vasodilator function (30minutes):** Brachial artery endothelium-dependent flow-mediated dilation and extent of reactive hyperemia will be measured using vascular ultrasound and fingertip tonometry as previously described [20]. Briefly, we will record 2-dimensional images and Doppler signals from the brachial artery and pulse signals from the fingertip at baseline and after 5-minute cuff occlusion of the upper arm and small arteries in the finger. After 10 min of

cuff release we will re-acquire the same image of the brachial artery scan before nitroglycerin administration. The patient will be given 0.4 mg of sublingual nitroglycerin, and 2 minutes 15 seconds after its administration images of the brachial artery will be acquired for 60 seconds.

2. **Arterial stiffness measurement (15 minutes):** Compliance of the central aorta and upper extremity will be assessed by measuring carotid-femoral and carotid-radial pulse wave velocity (PWV). A small probe is used to record signals from the carotid, radial, and femoral arteries.
3. **Endothelial cell collection (one hour):** We will place a 20-gauge intravenous catheter in an arm vein in each arm. Endothelial cells will be collected utilizing a J-wire inserted into the intravenous catheter [21, 22]. The J-wire is passed into the intravenous catheter and gently makes contact with the endothelial surface to collect cells which are then washed from the wire into physiological buffer. The typical yield is 200-400 cells per wire. Depending on the availability and quality of the arm veins, the investigator may use local anesthesia with 0.5 to 1.0 ml of 1% lidocaine injected subcutaneously. We will collect cells using four wires on each arm for total of eight wires.
4. **Biochemical analysis of plasma and serum:** The blood samples will be processed in the Clinical Chemistry Laboratory at Boston Medical Center to measure fasting glucose, insulin, and lipids. Serum, plasma, and white blood cell samples will also be collected and stored at -80º C for additional analyses of biomarkers relevant to cardiovascular health and for miRNA analysis.
5. **Biorepository:** Serum, plasma, and white blood cell samples will also be saved for future analyses. These samples will be stored in a -80º C freezer in the principal investigator's laboratory (Evans Building Room E-727) with key card only access.

Randomization: The study statistician at BEDAC will supervise computer generation of the randomization scheme. Randomization scheme will be provided to the IPS team in a blinded manner. Investigators and other team members will not have access to the randomization table. Un-Blinding will occur when all subjects have completed treatment and evaluation of the primary outcome data measures is complete. Un-blinding must be requested by the PI.

Treatment Period: Subjects will be randomized (1:1) to receive dapagliflozin and then placebo or vice versa. Total treatment period when participants are taking medication (either placebo or dapagliflozin) is 12 weeks consisting of 6 weeks of treatment for each dapagliflozin and placebo with 2 weeks of washout period in between. Subjects randomized to dapagliflozin will receive 10 mg/day (in capsule form). Subjects randomized to placebo will receive matching placebo capsule. Treatment order will be block randomized in block sizes of 2 or 4. We will call patients to ascertain compliance at week 3 into each treatment.

Treatment Plan:
The Boston Medical Center Investigational Drug Service will dispense 6 weeks supply of either dapagliflozin or placebo depending on their randomized group. Subjects will be instructed to contact the investigators if they experience adverse events or a change in healthy status.

Study Telephone Call 1: Subjects will be contacted in the 3rd week of the treatment period to evaluate self-reported compliance, assess for any relevant side effects, adverse events and change in clinical status.

Study Visit 2:
After arrival in the research unit, the investigators will perform a pill count to assess subject compliance, and complete an assessment of side effects and any change in clinical status. We will then perform all the study procedures to evaluate vascular health as described from Study Visit 1.

Study Visit 3:
After a washout period of 2 weeks (participants instructed not to take research medications during this time), patients will return to start treatment with either dapagliflozin or placebo for 6 weeks. We will perform all the study procedures from Study Visit 1. When assigned to dapagliflozin, participants will receive 10 mg/day (in capsule form) for the period of treatment. Subjects assigned to placebo will receive matching placebo capsules.

Telephone Call 2: We will call patients at week 3 as described for telephone Call 1.

Study Visit 4:
After arrival in the research unit, the investigators will perform a pill count to assess subject compliance, and complete an assessment of side effects and any change in clinical status. We will then perform all the study procedures to evaluate vascular health as described from Study Visit 1.

Earlier termination of the study:
There are no procedures in case of earlier termination of the study. All participants can voluntary withdraw from the study without suffering any penalty.
Participants will be withdrawn from the study if their compliance is equal or lower than 80% after performing a pill count on each visit, or if they develop an intervention illness that would impact the interpretation of the study.

10 Assessment of Safety and Data Safety Monitoring Plan (DSMP)

10.1 Definitions

The following definitions will be used in the assessment of safety:

Adverse Event (AE) is any untoward or unfavorable medical occurrence in a human subject, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research.

Serious Adverse Event (SAE) is any adverse event that

(1) results in death;
(2) is life-threatening;
(3) results in inpatient hospitalization or prolongation of existing hospitalization;
(4) results in a persistent or significant disability/incapacity;
(5) results in a congenital anomaly/birth defect; or
(6) based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition (examples of such events include allergic bronchospasm requiring intensive treatment in the emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse).

Life-threatening means that the event places the subject at immediate risk of death from the event as it occurred.

Unanticipated Problem is defined as an event, experience or outcome that meets **all three** of the following criteria:

- is unexpected; AND
- is related or possibly related to participation in the research; AND
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

Possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research

Unexpected means the nature, severity, or frequency of the event is not consistent with either:

- the known or foreseeable risk of adverse events associated with the procedures involved in the research that are described in (a) the protocol–related documents, such as the IRB-approved research protocol, any applicable investigator brochure, and the current IRB-approved informed consent document, and (b) other relevant sources of information, such as product labeling and package inserts; or
- the expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the adverse event and the subject's predisposing risk factor profile for the adverse event.

10.2 Safety Review

Risks will be monitored as follows:

The study staff will ask participants about potential adverse events at each study visit and at the telephone calls at 3 weeks into each treatment period. Participants will be given contact information to report any changes in their medical status or concerns related to study procedures. All AE's will be recorded in REDcap and reviewed by the investigators. If the study team finds out about any SAE, they will document the date that the information was obtained and inform the PI within 24 hours. The PI will review any potential SAE and classify as described above in 10.1 and report as described in 10.3 and will determine follow- up with the subject including potential withdrawal from the study.
Adverse Events (AE) definition are described in section 10.1. The principal investigator in this study, who is a physician, would evaluate rather the AE are expected given the participant illness or they are due to study intervention. All AE will be reported as described below.
The study team and investigators will review all AE on a 4 month basis to determine whether the pattern of adverse events, in total, does not suggest that the research places subjects or others at a greater risk of harm than was previously known. The quarterly reviews will be documented.

10.3 Reporting Plans

The principal investigator at Boston Medical Center/Boston University Medical Campus will report Unanticipated Problems, and Adverse Events to the Boston Medical Center/Boston University Medical Campus IRB in accordance with IRB policies:

- Unanticipated Problems occurring at Boston Medical Center/Boston University Medical Campus will be reported to the IRB within 7 days of the investigator learning of the event.
- Adverse Events (including Serious Adverse Events) will be reported in summary at the time of continuing review, along with a statement that the pattern of adverse events, in total, does not suggest that the research places subjects or others at a greater risk of harm than was previously known.

10.4 Stopping Rules

The study has no stopping rules.

11 Data Handling and Record Keeping

11.1 Source Documents

Source data in the study will consist of vascular images, laboratory results and microscope images.
We have standard operating protocols in our Research Unit that meets the "ALCOA" standards: Attributable, Legible, Contemporaneous, Original and Accurate.

Data will be entered directly into REDCap in CRFs designed by BEDAC. Laboratory values will be entered into the master data set by the data management team at BEDAC. BEDAC has uniform policies describing source documents, how to make corrections, and who can access them.

11.2 Case Report Forms

We have contracted with BEDAC to create case report forms using REDCap using established data validation methodology. Primary data entry will be directly into the REDCap instruments. For selected instruments that are used during the vascular health testing, there will be paper forms available to the research assistant performing the study that will be entered into REDCap at a later time. BEDAC has uniform policies for quality assurance at the data entry level and for data quality monitoring.

11.3 Case Report Forms

We have contracted with BEDAC to create case report forms using REDCap using established data validation methodology. Primary data entry will be directly into the REDCap instruments. For selected instruments that are used during the vascular health testing, there will be paper forms available to the research assistant performing the study that will be entered into REDCap at a later time. BEDAC has uniform policies for quality assurance at the data entry level and for data quality monitoring.

11.4 Study Records Retention

Study records will be retained for at least seven years after completion of the study. Documentation of informed consent of subjects will be retained for at least seven years after the study is closed. Such records will be preserved in hardcopy, electronic or other media form and will be accessible for inspection and copying by authorized individuals.

12 Statistical Plan

The overall study design is an AB/BA two-treatment, two-period cross-over trial.

12.1 Study Hypotheses

We hypothesize that treatment with the SGLT2 inhibitor dapagliflozin will have favorable effects on EC function and vascular health in patients with T2DM.

Primary outcome: Treatment with dapagliflozin (SGLT2 inhibitor) will improve EC insulin-signaling in patients with T2DM.

- *Null hypothesis:* There is no effect of dapagliflozin on EC insulin-signaling.
- *Alternative hypothesis:* EC insulin-signaling is changed by dapagliflozin.

Secondary outcome: Treatment with dapagliflozin (SGLT2 inhibitor) will improve endothelial function as measured by flow-mediated dilation in patients with T2DM.

- *Null hypothesis:* There is no effect of dapagliflozin on flow-mediated dilation.
- *Alternative hypothesis:* Flow-mediated dilation is changed by dapagliflozin.

Additional endpoints will include additional measures of additional measures of vascular function and endothelial cell health and circulating biomarkers of endothelial health.

12.2 Sample Size Determination

The primary analysis for this randomized cross-over study will be comparison of the primary endpoint, EC insulin-signaling, and the secondary outcome, flow-mediated dilation, during the dapagliflozin treatment period compared to the placebo treatment period. These continuous outcomes were used to power the study.

In our prior work, we have reported that patients with T2DM have an EC insulin response of -10±9% and a flow-mediated dilation response of 6.5±4.3%. These measures are expected to be similar in patients enrolled in the present study. An effect size of (how much) was used to calculate the expected sample size for the study.
The analysis will use a an analysis of repeated measures using Proc Mixed in SAS with conventional REML analysis to compare baseline and follow-up results for each treatment group with an overall significance level two-sided alpha=0.025 given the planned primary and secondary endpoints. In order to ensure 90% power to detect an effect size of 0.25 for each outcome, the study needs to

have at least 35 participants who complete the study (SD of differences, σ=5.8% for EC insulin-signaling and 3.5% for flow-mediated dilation). We estimate that 85% of consented participants (50) will be randomized (42). We estimate that 7% of participants will not complete and 83% will complete (35). The sample size has been calculated using NQuery Advisor [version 8.7.2.0, Statistical Solutions, San Diego, CA].

12.3 Statistical Methods

We will compare baseline and follow-up results for each treatment group using analysis of repeated measures with Proc Mixed in SAS. The measures of endothelial phenotype (EC insulin-signaling) and vascular function (flow-mediated dilation) will be our dependent variables and treatment, period, and sequence will be the main independent measures. That is the variables are denoted as y_{ikj} is the observation for the i^{th} sequence (i-1,2) j^{th} subject (j=1,2,…n) k^{th} period (k=1,2.

The model can be described by:

$$y_{ijkl} = \beta_0 + \beta_1 X_{treat} + \beta_2 X_{period} + \beta_{12} X_{treat} X_{period} + s_{ij} + \varepsilon_{ijk}$$

s_{ij} is a random subject effect term and is assumed to be independently and identically distributed with N (0, σ_s^2)

ε_{ijk} is an error term and is assumed to be independently and identically distributed with N (0, σ^2)

Two dummy variables are introduced as:

X_{treat} = 1 if the subject is in treatment B, and X_{treat} = 0 otherwise;

X_{period} = 1 if the subject is in period 2, and X_{period} = 0 otherwise;

Model fitting and interference will follow conventional REML analysis in the Proc Mixed procedure in SAS. The between-subject variation includes a carry-over treatment effect and between-subject residuals. The within-subject variation includes direct treatment effect, period effect, and within-subject residuals. A significant period effect reflects evidence of carry-over effect. A significant within-subject treatment effect is consistent with differences between the two treatments adjusting for carry-over and period effect. In exploratory analyses, we will evaluate for effect modification by sex and race. We will evaluate additional exploratory outcomes in a similar fashion.

Analysis Datasets: The intention-to-treat (ITT) analysis dataset will be the source of data for primary analyses. This will include all randomized participants regardless of actual receipt or compliance with therapy. The per protocol analysis will be conducted based on adherence to assigned treatment (defined as more than 80% based on pill returns). Use of Prox Mixed analyses allows all participants to be included if they contribute any data to the analysis dataset.

Baseline Descriptive Statistics: All variables will be summarized using mean, median, standard deviation, and range (for continuous variables) and frequency (for categorical variables). Treatment order groups will be compared with respect to baseline characteristics to verify randomization balance.

13 Ethics/Protection of Human Subjects

This study is to be conducted according to applicable US federal regulations and institutional policies (which are based in federal regulations, guidance, and ICH Good Clinical Practice guidelines).

This protocol and any amendments will be submitted to the Boston Medical Center and Boston University Medical Campus IRB, for formal approval of the study conduct. The decision of the IRB concerning the conduct of the study will be made in writing to the investigator. A copy of the initial IRB approval letter will be provided to the sponsor before commencement of this study.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. The consent form will be submitted with the protocol for review and approval by the IRB. The consent of a subject, using the IRB-approved consent form, must be obtained before that subject is submitted to any study procedure. Consent will be documented as required by the IRB.

14 Literature References

1. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Despres JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER, 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB, American Heart Association Statistics C, Stroke Statistics S. Heart disease and stroke statistics--2015 update: A report from the american heart association. *Circulation*. 2015;131:e29-322
2. Collaboration NCDRF. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. *Lancet*. 2016;387:1513-1530.
3. Rawshani A, Rawshani A, Franzen S, Eliasson B, Svensson AM, Miftaraj M, McGuire DK, Sattar N, Rosengren A and Gudbjornsdottir S. Mortality and Cardiovascular Disease in Type 1 and Type 2 Diabetes. *N Engl J Med*. 2017;376:1407-1418.
4. Niiranen TJ, Lyass A, Larson MG, Hamburg NM, Benjamin EJ, Mitchell GF and Vasan RS. Prevalence, Correlates, and Prognosis of Healthy Vascular Aging in a Western Community-Dwelling Cohort: The Framingham Heart Study. *Hypertension*. 2017;70:267-274.
5. Einarson TR, Acs A, Ludwig C and Panton UH. Prevalence of cardiovascular disease in type 2 diabetes: a systematic literature review of scientific evidence from across the world in 2007-2017. *Cardiovasc Diabetol*. 2018;17:83.
6. Wiviott SD, Raz I, Bonaca MP, Mosenzon O, Kato ET, Cahn A, Silverman MG, Zelniker TA, Kuder JF, Murphy SA, Bhatt DL, Leiter LA, McGuire DK, Wilding JPH, Ruff CT, Gause-Nilsson IAM, Fredriksson M, Johansson PA, Langkilde AM, Sabatine MS and Investigators D-T. Dapagliflozin and Cardiovascular Outcomes in Type 2 Diabetes. *N Engl J Med*. 2019;380:347-357.
7. Cefalu WT, Kaul S, Gerstein HC, Holman RR, Zinman B, Skyler JS, Green JB, Buse JB, Inzucchi SE, Leiter LA, Raz I, Rosenstock J and Riddle MC. Cardiovascular Outcomes Trials in Type 2 Diabetes:

Where Do We Go From Here? Reflections From a Diabetes Care Editors' Expert Forum. *Diabetes Care*. 2018;41:14-31.

8. Tabit CE, Chung WB, Hamburg NM and Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. *Rev Endocr Metab Disord*. 2010;11:61-74.
9. Schmidt AM. Diabetes Mellitus and Cardiovascular Disease. *Arterioscler Thromb Vasc Biol*. 2019;39:558-568.
10. Shigiyama F, Kumashiro N, Miyagi M, Ikehara K, Kanda E, Uchino H and Hirose T. Effectiveness of dapagliflozin on vascular endothelial function and glycemic control in patients with early-stage type 2 diabetes mellitus: DEFENCE study. *Cardiovasc Diabetol*. 2017;16:84.
11. Lee DM, Battson ML, Jarrell DK, Hou S, Ecton KE, Weir TL and Gentile CL. SGLT2 inhibition via dapagliflozin improves generalized vascular dysfunction and alters the gut microbiota in type 2 diabetic mice. *Cardiovasc Diabetol*. 2018;17:62.
12. Sugiyama S, Jinnouchi H, Kurinami N, Hieshima K, Yoshida A, Jinnouchi K, Nishimura H, Suzuki T, Miyamoto F, Kajiwara K and Jinnouchi T. The SGLT2 Inhibitor Dapagliflozin Significantly Improves the Peripheral Microvascular Endothelial Function in Patients with Uncontrolled Type 2 Diabetes Mellitus. *Intern Med*. 2018;57:2147-2156.
13. Gaspari T, Spizzo I, Liu H, Hu Y, Simpson RW, Widdop RE and Dear AE. Dapagliflozin attenuates human vascular endothelial cell activation and induces vasorelaxation: A potential mechanism for inhibition of atherogenesis. *Diab Vasc Dis Res*. 2018;15:64-73.
14. Tanajak P, Sa-Nguanmoo P, Sivasinprasasn S, Thummasorn S, Siri-Angkul N, Chattipakorn SC and Chattipakorn N. Cardioprotection of dapagliflozin and vildagliptin in rats with cardiac ischemia-reperfusion injury. *J Endocrinol*. 2018;236:69-84.
15. Bristol-Myers Squibb Company (2014) Farxiga [package insert]. Princeton, NJ: Bristol-Myers Squibb Company.
16. Wiviott SD, Raz I, Bonaca MP, Mosenzon O, Kato ET, Cahn A, Silverman MG, Zelniker TA, Kuder JF, Murphy SA, Bhatt DL, Leiter LA, McGuire DK, Wilding JPH, Ruff CT, Gause-Nilsson IAM, Fredriksson M, Johansson PA, Langkilde AM, Sabatine MS; DECLARE–TIMI 58 Investigators. Dapagliflozin and Cardiovascular Outcomes in Type 2 Diabetes. N Engl J Med. 2019 Jan 24;380(4):347-357. doi: 10.1056/NEJMoa1812389. Epub 2018 Nov 10. PMID: 30415602.
17. Zaccardi F, Webb DR, Htike ZZ, Youssef D, Khunti K, Davies MJ. Efficacy and safety of sodium-glucose co-transporter-2 inhibitors in type 2 diabetes mellitus: systematic review and network meta-analysis. Diabetes Obes Metab. 2016 Aug;18(8):783-94. doi: 10.1111/dom.12670. Epub 2016 May 13. PMID: 27059700.
18. Xu X, Xu W, Zhuo Q, Yan Y. The efficacy and safety of dapagliflozin combined with oral hypoglycemic agents in patients with type 2 diabetes: a systematic review and meta-analysis. Ann Palliat Med. 2022 Mar;11(3):1028-1037. doi: 10.21037/apm-22-121. PMID: 35365032.
19. Strojek K., Yoon K., Hruba V., Elze M., Langkilde A., Parikh S. (2011) Effect of dapagliflozin in patients with type 2 diabetes who have inadequate glycaemic control with glimepiride: a randomized, 24-week, double-blind, placebo-controlled trial. *Diabetes Obes Metab* 13: 928–938.
20. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R, International Brachial Artery Reactivity Task F. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: A report of the international brachial artery reactivity task force. *J Am Coll Cardiol*. 2002;39:257-265

21. Feng L, Stern DM, Pile-Spellman J. Human endothelium: Endovascular biopsy and molecular analysis. *Radiology*. 1999;212:655-664
22. Colombo PC, Ashton AW, Celaj S, Talreja A, Banchs JE, Dubois NB, Marinaccio M, Malla S, Lachmann J, Ware JA, Le Jemtel TH. Biopsy coupled to quantitative immunofluorescence: A new method to study the human vascular endothelium. *Journal of applied physiology*. 2002;92:1331-1338

15 Appendix

Schedule of Events

Visit number	Screening	Visit1	Phone call	Visit2		Visit3	Phone call	Visit4
Study week	Up to -4	0	3	6		8	11	14
Eligibility	x				2 weeks washout period			
Demographics & Medical History	x							
HbA1c with 3 months	x							
Assessment of Inclusion/Exclusion criteria	x							
Pregnancy test for women of childbearing potential	x							
In person consent	x							
Medication history	x	x	x	x		x	x	x
Weight, height, waist circumference	x	x		x		x		x
Randomization		x						
Vascular function, BP, HR, arterial stiffness		x		x		x		x
Lipids, Glucose		x		x		x		x
Plasma, biomarkers, miRNA		x		x		x		x
Endothelial cell phenotype		x		x		x		x
Endothelial Cell RNA		x		x		x		x
Dispense Study drug		x				x		
Assess Compliance			x	x			x	x
		Dapagliflozin 10mg/day or placebo	Dapagliflozin 10mg/day or placebo	Dapagliflozin 10mg/day or placebo		Dapagliflozin 10mg/day or placebo	Dapagliflozin 10mg/day or placebo	Dapagliflozin 10mg/day or placebo
Symptom Assessment		x	x	x		x	x	x

Schematic of Study Design

Figure 10. Study Overview.