Title:

Visceral Adiposity and Diabetes: Translating Form to Function Using Imaging

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The University of Texas Southwestern Medical Center at Dallas
Institutional Review Board
Protocol (Investigator Initiated Study)

1. Introduction and Purpose:

One out of three adults in the US is obese and forecasts predict that >50% of the US population will be obese by 2030.[1] Although obesity is associated with increased risk for diabetes, many obese individuals remain free of metabolic disease.[2] One factor that contributes to the heterogeneity of risk among obese individuals is visceral adipose tissue (VAT) distribution.[3] Excess VAT in obese adults is associated with insulin resistance, atherogenic dyslipidemia, and hepatic steatosis.[4] We have previously shown that higher VAT mass is associated with a significant increase in the risk of developing diabetes and that this effect is independent of body mass index (BMI).[5] One hypothesis linking excess VAT to the development of diabetes is that VAT alters glucose homeostasis by contributing excess glycerol from overactive lipolysis to stimulate hepatic gluconeogenesis.[6,7] However, this hypothesis has yet to be tested in human subjects. Furthermore, the effect of Empagliflozin (EMPA) on glucose homeostasis associated with visceral adiposity has not been explored previously.

My *long term goal* is to understand how VAT contributes to diabetes risk and to identify strategies to reduce the metabolic consequences of excess VAT.

My objectives here are to:

- Elucidate the *mechanistic relationship* between VAT and abnormal glucose homeostasis.
- Determine the *effect of EMPA*, a sodium glucose co-transporter 2 inhibitor shown to reduce surrogate markers and direct imaging-based assessments of VAT,[8,9] on glucose homeostasis in obese adults with differing VAT.

I will test the *central hypothesis* that visceral adiposity plays a key role in the pathogenesis of diabetes and that EMPA may positively impact glucose homeostasis in viscerally-obese individuals using functional studies of glycerol metabolism in hepatic gluconeogenesis using a well-validated nuclear magnetic resonance (NMR) spectroscopy platform. The *rationale* of this project is to utilize emerging technology to investigate potential mechanisms underlying diabetes risk related to excess VAT and explore the potential beneficial effects of EMPA on these pathways. I will achieve the scientific objectives of this application by pursuing the following *two specific aims*:

1. Elucidate the relationship between VAT mass and glucose homeostasis by measuring enrichment of glycerol in blood glucose by NMR spectroscopy. Here we will measure the differential enrichment of exogenous $^{13}C_3$-labeled glycerol vs. adipose-derived glycerol in blood glucose among non-diabetic obese adults with high vs. low VAT using a single venous blood sample.

Hypothesis 1: Participants with high VAT will have greater adipose contribution of glycerol and therefore lower $^{13}C_3$ glycerol enrichment compared with age, sex, and BMI-matched participants with low VAT.

2. Determine the effect of EMPA on glucose homeostasis in non-diabetic obese adults with differing VAT mass. In this aim we will measure changes in the enrichment of $^{13}C_3$ glycerol in response to treatment with EMPA or placebo among age, sex, and BMI-matched participants stratified by high vs. low VAT.

Hypothesis 2: $^{13}C_3$ glycerol enrichment will increase from baseline (signifying less contribution from adipose tissue) among EMPA-treated participants with high VAT, with no change in EMPA-treated low VAT or placebo-treated participants.

2. Background:

Diabetes mellitus type II is the consequence of insulin resistance and pancreatic beta cell failure resulting from a variety of metabolic insults, one of which is excess body adiposity/obesity. In the

diabetic individual, hepatic gluconeogenesis may go uninhibited due to failure of the body's normal feedback mechanisms to appropriately incorporate glucose into cells via insulin signaling, leading to excess gluconeogenesis and hyperglycemia. The substrate for this excess glucose derives from multiple sources in the liver including dietary glycerol, adipose-derived glycerol from lipolysis, and substrates from the citric acid cycle. In the normal state, lipolysis is maintained at a steady state in equilibrium between stored dietary triglycerides and free fatty acids. However, in situations of triglyceride excess (e.g. in the obese state), lipolysis may become overactive resulting in increased free fatty acids and adipose-derived glycerol. This excess glycerol drives hepatic gluconeogenesis and is incorporated into glucose and released into the blood, leading to hyperglycemia, and ultimately diabetes and its clinical sequelae.

A popular hypothesis linking visceral fat with excess gluconeogenesis is delivery of glycerol arising from mesenteric triglyceride turnover directly into the portal circulation and to the liver. Glycerol is a primary substrate for gluconeogenesis in the liver. Under normal conditions, hepatic gluconeogenesis begins from glycerol ingested in the diet which is converted to glycerol-3-phosphate and subsequently dihydroxyacetone phosphate (DHAP) in the liver. DHAP is converted to fructose-1,6-bisphosphate which undergoes a series of reactions to become a single 6-carbon glucose molecule. Adipocytes contribute glycerol to hepatic gluconeogenesis through lipolysis of triglyceride stores (Figure 1). Although glycerol-gluconeogenesis has been extensively studied in animals, the traditional reliance on radioactive tracers makes translation to humans difficult for many reasons. We aim to use new techniques to explore the mechanisms behind altered glucose metabolism related to excess visceral adiposity in obese adults by quantifying the relative contributions of varying substrates to liver-derived glucose. One such method uses $^{13}C_3$ labeled glycerol to trace the incorporation of glycerol from dietary sources to hepatic gluconeogenesis. This technology utilizes nuclear magnetic resonance (NMR) spectroscopy, a technique that does not require ionizing radiation and has been extensively validated, to analyze the NMR spectra of plasma glucose and quantify the "percent enrichment" of the circulating glucose molecules with labeled glycerol. In turn, differences in enrichment reflect variability in hepatic glucose metabolism as it relates to the contribution of glycerol from visceral adipose tissue to gluconeogenesis.

The rationale of this project is to utilize existing technology to investigate the impact of excess visceral adiposity on glycerol metabolism in hepatic gluconeogenesis in obese adults without diabetes and to explore the effects of treatment with EMPA on visceral adiposity related glucose homeostasis. Our hypothesis is that among individuals with excess visceral adipose tissue, high lipolytic turnover results in excess adipose-derived glycerol shunting to the liver and hyperactive gluconeogenesis. This phenomenon will be reflected in a relatively low enrichment of dietary (labeled) glycerol when analyzed by NMR spectroscopy when compared with obese individuals with low visceral adiposity. We also hypothesize that $^{13}C_3$ glycerol enrichment will increase from baseline (signifying less contribution from adipose tissue) among EMPA-treated participants with high VAT, with no change in EMPA-treated low VAT or placebo-treated participants.

3. Concise Summary of Project:

Study Population:

Obese (BMI ≥ 30 kg/m^2) participants who are referred, or enrolled in the DHS, without prevalent type 2 diabetes who have previously undergone VAT imaging by MRI[33] or DXA[34] or will have an MRI as part of this study will be recruited. Participants with high VAT will be selected and matched (age, sex, BMI) with an equal number of low VAT participants, defined by the highest and lowest age-and-sex stratified decile of VAT in the study population. Individuals with prevalent dyslipidemia or liver disease that could influence $^{13}C_3$ glycerol enrichment will be excluded. Recruitment is highly likely to be successful given that >450 DHS participants have been recruited to participate in additional mechanistic studies to date.

Exposure variables:

For the DHS subjects visceral adipose tissue (intraperitoneal + retroperitoneal) was measured by a 1.5-T MRI system (Intera, Philips Medical Systems, Best, The Netherlands) between 2000 and 2002 using a prospectively designed and validated method of fat mass prediction from a single MRI slice at the L2-L3 inter-vertebral level (Figure 2). Single slice measurement of visceral adipose tissue mass at this inter-vertebral level has been shown to be highly concordant with total abdominal fat mass measured at all inter-vertebral levels (R2=85-96%). Multiple studies have been published demonstrating the validity of this method of visceral fat quantification. Body composition was measured using dual x-ray absorptiometry (DXA, Delphi W scanner, Hologic Inc, Bedford, MA and Discovery software [version 12.2]) and proprietary software will be used to calculate visceral adipose tissue mass from the DXA data. For referral subjects, an MRI prior to glycerol administration will measure abdominal visceral adipose tissue (VAT). These participants will undergo a neck-to-knee scan on a 3T Philips Ingenia MRI scanner lasting approximately 6 minutes. No intravenous contrast will be administered during the study. The scan will be performed in the AIRC by expert technologists trained in the conduct of clinical research. The de-identified, HIPAA-compliant images will then be interpreted offline by expert readers blinded to the participant's study assignment and a report will be generated for use in the data analysis.

Body composition will be measured using height, weight, waist circumference, hip circumference; body fat percentage will be calculated using both skinfold thickness at 7 different anatomical sites and bio-impedence analysis.

Plasma blood glucose (mg/dL) measured by YSI glucose analyzer to determine glycemic status prior to study intervention.

Blood samples will also be used to measure hemaglobin A1C, adipocytokines, markers of inflammation, plasma insulin, alanine, catecholamines, and nonesterified free fatty acids.

Intervention:
[U-$^{13}C_3$] glycerol 50 mg/kg (obtained from Cambridge Isotopes, Andover, MA) will be given to participants to drink. It is non-radioactive and non-toxic. There is no known risk to drinking glycerol. Ingestion of this item is similar to drinking a commericial sugar sweetened beverage. If a participant cannot tolerate the drink initially, they will be given a second attempt at ingestion. If after two attempts they cannot ingest the entire item, they will be removed from the study and a replacement participant will be recruited.

Aim 2 will be an extension of Aim 1 in which all participants from Aim 1 will receive empagloflozin study drug or placebo for 12 weeks and then return for repeat testing. Participants stratified by VAT (high vs. low) will be randomized to either treatment or placebo using a random number generator (0-100, http://www.random.org) followed by the creation of numbered envelopes; ≤median will be assigned to treatment and >median will be assigned to placebo. The minimum approved dose of empagliflozin (10 mg tablet daily by mouth taken in the morning with or without food) will be used in Aim 2 of this study; this dose was chosen to minimize the chances of adverse events (specifically hypoglycemia, dehydration, and urinary tract infections) while still maintaining efficacy in clinical trials and preliminary data. We expect that this dose will be well tolerated, with the most common side effects being urinary tract infection or yeast infection (in women).

Outcome variables:

[U-$^{13}C_3$] glycerol enrichment in plasma blood glucose over time will be measured by nuclear magnetic resonance spectroscopy.

4. Study Procedures:

Study procedures are outlined below. Study procedures will be performed at baseline and then repeated after 3 months of study drug administration. There will also be two (2) interval visits during which study participants will meet with the study coordinator to review study drug adherence, obtain the next month's supply of study drug, and perform surveillance for any adverse effects.

Study drug will be either empagliflozin (10 mg once daily by mouth) or matching placebo (once daily by mouth). Both active drug and placebo will be obtained from the manufacturer (Boehringer-Ingelheim) and will be administered by study staff (blinded to treatment allocation) in oral tablet form. Participants will randomized to either active drug or placebo using a random number generator (0-100, http://www.random.org); ≤median will be assigned to emagliflozin and >median will be assigned to placebo.

Study Procedures Visits 1 and 4

1. Arrive at AIRC
2. Review study procedures
3. Sign written informed consent
4. Urine pregnancy test to verify non-pregnant status (female only)
5. Measurement of height, weight, body composition, heart rate, blood pressure, and temperature.
6. Possible neck-to-knee MRI
7. IV insertion by AIRC nurse for blood draws
8. Baseline blood draw.
9. Ingestion of [U-13C3] glycerol (50 mg/kg body weight)
10. Blood draws: +30, +60, +90, +120, +150, +180 min. Each blood draw will be 41 ml for a total of 312 ml (includes 25 ml waste)
11. IV discontinued
12. Lunch and discharge from AIRC. Study participation completed.

Each study visit 1 and 4 will be approximately 5 hours.

Study Procedures Visits 2 and 3

1. Arrive at AIRC
2. Return study drug pill bottle and review study drug adherence
3. Assess for any interval adverse effects and document as necessary
4. Dispense study drug to be used in the next study visit interval (1 month)
5. Discharge from the AIRC.

Study participants do not need to be fasting for Visits 2 or 3 and no blood will be drawn at these interval visits. They are for study drug adherence/monitoring and drug dispensing only. Each study visit 2 and 3 will be approximately 30-60 minutes.

After the ingestion of the glycerol and during the blood draws, volunteers will be made comfortable in the AIRC procedure room. Conditions that would result in the subject exiting the study prior to the expected completion date include withdrawl of informed consent, intolerance of the glycerol drink, intolerance of study procedures (IV insertion, blood draws), or meeting exclusion criteria that were not previously known through telephone screening. Research subjects will not be responsible for any costs of the study.

5. Sub-Study Procedures: N/A

6. Criteria for Inclusion of Subjects:

- Obese, defined as BMI ≥ 30 kg/m^2, at both time of abdominal fat imaging and at study entry.
- Ages 30-65
- No prevalent diagnosis of type 2 diabetes mellitus, either at the time of abdominal fat imaging or at study entry.
- Previous abdominal fat quantification by magnetic resonance imaging in the Dallas Heart Study or possible neck-to-knee MRI for VAT measurement may be performed.

7. Criteria for Exclusion of Subjects:

- Pregnant or breastfeeding
- Incarcerated
- Chronic kidney or liver disease
- History of frequent (>2/year) urinary tract infections
- Non-obese either at time of abdominal fat imaging or at present.
- Greater than 10% change in body weight (kg) between time of abdominal fat imaging and present.
- Has donated blood within last 6 weeks
- Cannot give informed consent, understand the protocol, or tolerate any aspect of the protocol
- If undergoing MRI, persons with metal implants contraindicated for 3Tesla MRI exams will be excluded. Severe claustrophobia will also be assessed prior to an MRI exam.

<u>The inclusion/exclusion criteria above were chosen because:</u>

- Subjects must be obese and non-diabetic to meet the study objectives and test the study hypothesis.
- Pregnant or breastfeeding women, incarcerated individuals, and children/minors are excluded because these are special populations with higher risk and exclusion of these participants minimizes risk of the study.
- Chronic kidney or liver disease can possibly affect the outcome measurement in the study and confound the results. Additionally, for Aim 3, empagliflozin should be avoided in persons with chronic kidney or liver disease, so participants with these disorders will be excluded to minimize risks to human subjects.

- Since empagliflozin can increase risk for urinary tract infections, participants with a history of frequent urinary tract infections will be excluded to minimize risk to human subjects.

8. Sources of Research Material:

- The research materials that will be used for this proposal include demographic records and data (age, sex, weight, height, body mass index, abdominal MRI and dual x-ray absorptiometry imaging data currently collected and maintained in the Dallas Heart Study database), blood samples, and urine samples (females capable of pregnancy only). The blood and urine specimens will be taken at the time of study procedures and processed immediately. No samples will be stored. The database records that will be utilized include age, sex, race, cardiometabolic risk factor status, and data generated from blood tests and imaging phenotyping as part of the DHS study protocol.
- Prospective data to be obtained include current age, weight, height, waist circumference, hip circumference, calculated body fat percentage using both skinfold thickness at 7 different anatomical sites and bio-impedance analysis, a brief medical history, blood samples, and a urine pregnancy test (females only).
- For Aim 2, the FDA-approved drug, empagliflozin 10 mg by mouth daily, will be administered to those participants randomized to drug treatment. A matching placebo will be administered to participants not randomized to empagliflozin treatment. Both active drug and placebo will be obtained from the manufacturer (Boehringer-Ingelheim).
- No member of the study team will have access to individually identifiable private information about human subjects in the DHS. The DHS study coordinators have access to this information but are not part of the study team.
- Every study participant was assigned a randomly generated subject ID that is not linked to identifiable information. All data generated from a study participant is labeled with only their unique subject ID, including blood specimens and database records. The database records are kept on password protected servers in the DHS research center and managed by the study coordinators and statistical team. Investigators must have approved and authorized proposal to gain access to these data and all data transfers are managed by the database and statistical core. All analyses for this project will be conducted on site by the DHS statistical core. Measurements made during this study will be linked to participants in the database by the subject ID and merged into the database by the DHS statistical core.

9. Recruitment Methods and Consenting Process:

Obese (BMI ≥ 30 kg/m2) participants who are referred, or enrolled in the DHS, without prevalent type 2 diabetes who have previously undergone VAT imaging by MRI or DXA will be recruited. Participants with high VAT will be selected and matched (age, sex, BMI) with an equal number of low VAT participants, defined by the highest and lowest age-and-sex stratified decile of VAT in the study population. Individuals with prevalent dyslipidemia or liver disease that could influence 13-C3 glycerol enrichment will be excluded. Recruitment is highly likely to be successful given that >450 DHS participants have been recruited to participate in additional mechanistic studies to date. All participants will be volunteers and not recruited from a clinical practice. Both genders as well as minorities will be actively recruited for participation in this study. Participants will be recruited for a visit via telephone by the primary investigator (IN). A confidential telephone translation service will be used if the participant is a non-English speaker. All attempts to ascertain inclusion/exclusion criteria will be made prior to the visit. Subjects will participate after having the purpose and benefits of the study explained in detail by a research nurse. In addition, a detailed list of potential adverse reactions will be reviewed as part of informed consent. These participants will be assured that the lack of participation in the study will in no way affect their ability to obtain medical care at UTSW.

Documentation of permission to participate in this study will be obtained using a UTSW IRB approved written informed consent form. No waiver of informed consent will be requested. Participants will be paid an incentive to participate in the research. Parking tickets from the Clements Imaging Building valet service will be validated.

A partial waiver of HIPPA authorization for screening purposes will be requested. This is a waiver used only for collection of initial screening data to determine eligibility and/or recruit potential research subjects. Authorization by the subject will be obtained at the time of consent. This is justified because it is not practical to obtain a signed HIPPA Authorization form prior to review of the records of potential participants because review of records is required to screen for a participant's eligibility for study participation. Over 3000 HIPPA Authorization forms (all participants in phase 3 of the Dallas Heart Study) would need to be completed prior to screening of records to determine the eligibility of study participants if records cannot be reviewed for eligibility purposes beforehand. This is not practical and the study could not be effectively conducted in this manner. Inclusion and exclusion criteria could not be determined without access to the PHI. This would also not be possible with the use of de-identified information.

10. Potential Risks:

- *Adverse Reaction to Empagliflozin*

Known adverse effects of empagliflozin include hypoglycemia (0.4%), dehydration (0.3%), impaired renal function (change in eGFR -1.0 mL/min/1.73 m^2 compared placebo), and an increased risk of urinary tract infection (9.3%) or vaginal yeast infection (5.4%). The most common adverse reactions associated with empagliflozin were urinary tract infections and vaginal yeast infections.

- *Risks of IV Insertion and Blood Drawing*

Participants may experience a small amount of pain in the arm when the needle is inserted into the vein. Risks associated with drawing blood from the arm include minimal discomfort and/or bruising. Infection, excess bleeding, clotting, and/or fainting also are possible, although unlikely. Participants will have less than 1½ cups of blood collected because they are in this research study.

- *13C Labeled Glycerol*

Labeled glycerol is non-radioactive and non-toxic and there is no known risk to drinking labeled glycerol. The glycerol will taste sweet and will be mixed with a glass of drinking water.

- *MRI*

The subject's experience during this procedure is identical to what they would experience during a clinical MRI (magnetic resonance imaging). MRI involves the use of a magnet and radio frequency energy. Therefore, patients who have implanted metal devices, such as pacemakers, certain aneurysm clips, or metal in the eye will be excluded. If subjects have any potential for metal in the body, the technologist or investigators should be informed before entering the magnet room. Because of the strong magnetic field associated with the scanner, it is rare, but possible, that a metallic object could fly through the air toward the scanner and hit the subject. To reduce this risk, everyone near the magnet will remove all metal from his or her clothing or pockets when in the scanning environment. There are no known risks or adverse effects resulting directly from exposure to magnetic fields and radio frequency energy used in this study. Some people feel claustrophobic in the MR scanner. If the subject is unable to tolerate being in the scanner, the scan can be stopped at any time. In addition, the MR scanner produces tapping sounds during operation, which may reach very loud levels. To minimize any discomfort from this noise, the subject will be provided with disposable earplugs and/or headphones that suppress external noise levels but do not eliminate voice communication with the scanner operator. In some cases, it is possible that the subject might experience neurostimulation effects, such as muscle twitches and tingling sensations, due to the rapid switching of magnetic field gradients used in these examinations. There are no known risks associated with these effects.

All subjects will be carefully evaluated by trained personnel prior to entering the MR scanner to exclude metal objects in or on their body. Any female of childbearing age will be screened and subjected to a pregnancy test, if indicated, prior to any MR procedure.

The MR scans in this study are designed for research, not for medical purposes. They are not useful for finding problems or diseases. In the event that a researcher discovers a possible abnormality, the subject will be informed. The subject will then consult with their primary care physician to discuss further tests and/or follow-up and treatment.

- *Risks to an Embryo, Fetus or Breast-fed Infant*

Females**:** If females are part of this study while pregnant or breast-feeding an infant, it is possible that they may expose the unborn child or infant to risks. For that reason, pregnant and breast-feeding females cannot participate in the study. If participants can become pregnant, a pregnancy test will be done from a urine sample, and it must be negative before they can be a part of this study. If they do become pregnant during this study, they must tell the researchers immediately, and they will be removed from the study.

- *Loss of Confidentiality*

There is a potential risk of loss of confidentiality any time information is collected. Every effort will be made to keep participant information confidential; however, this cannot be guaranteed.

- *Other Risks*

There may be other side effects that are unknown at this time. If participants are concerned about other, unknown side effects, they will discuss this with the researchers.

11. Subject Safety and Data Monitoring:

Type of Research Data or Events to be Monitored:

All research activities described in the project summary will be monitored in addition to study data accrual, protocol deviations, protocol violations, unanticipated problems, and adverse events.

Methods and Frequency of Analysis:

All research staff will have appropriate training to ensure the safety of participants, the appropriate conduct of research, and the integrity of safety and/or efficacy of data. This training includes completion of all University-required training in the policies and procedures to protect the rights and welfare of people who participate in research.

Subjects will be fully informed about the study requirements throughout the conduct of the trial by the study personnel. At the beginning of the study, it will be noted in the research record that the subject received complete information about the study requirements. Study personnel will give participants information relevant to continued participation.

The PI will perform monitoring activities. Monitoring will be performed weekly by the PI. The conclusions of the monitoring will be reported to the IRB in writing if there is any change in the risk/benefit ratio for subjects or if there is any new information which may affect a subject's willingness to continue participation. Monitoring will include consideration for (1) progress of the trial, (2) data quality, (3) timeliness of data collection, (4) recruitment, (5) accrual and retention, (6) risk vs. benefit for participants, (7) protocol violations, and (8) other factors that could affect the outcome of the study. Monitoring will be documented in the research record.

Person(s) Responsible for Data Monitoring:

The persons responsible for data monitoring in this research are Ian Neeland, MD and Bienka Milton, MPH BS. Dr. Neeland will oversee study operations on a day-to-day basis. Ms. Milton is a certified study coordinator. Ms. Milton will be responsible for aiding in subject recruitment and retention, protocol execution, and general study coordination.

Reporting Unanticipated Problems, Adverse Events, Protocol Deviations and Protocol Violations:
The principal investigator and research coordinator will monitor subject involvement in the study on a daily basis. The PI will report all classes of unexpected adverse events and any serious adverse events within 48 hours of recognition to the Federal agencies and University and hospital offices as specified by the IRB. The PI will submit a summary of adverse events in continuing review reports to the IRB at intervals specified by the IRB. Subject identifiers will be excluded from all reports.

Stopping Rules:
Subjects will be informed of the voluntary nature of the research and their option to stop study procedures for any reason. They will also be advised that the PI may stop their participation if it is deemed to be in the subject's best interest, if new information becomes available during the research, or if a subject cannot follow study instructions or keep appointments.

Procedures and Time Frames for Communicating Outcomes:
Medical literature for scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the study will be monitored by the PI.

Investigational New Drug (IND) Exemption:
Empagliflozin has been FDA approved as an adjunct to diet and exercise to improve glycemic control in adults with type 2 diabetes The empagliflozin intervention component of the proposed study should be IND exempt based on meeting IND exemption criteria. Specifically,

IND exemption criteria:

(1) it is not intended to be reported to FDA in support of a new indication for use or to support any other significant change in the labeling for the drug;	As a small pilot clinical study looking at an intermediate phenotype (glycerol enrichment in blood glucose), this will not support a label change or new indication.
(2) it is not intended to support a significant change in the advertising for the product;	See response to (1).
(3) it does not involve a route of administration or dosage level, use in a subject population, or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the use of the drug product;	The route of administration and dosage level are FDA approved for use in patients with type 2 diabetes. The lowest approved dosage will be used for a short period of time to minimize risks. The subject population will be obese volunteers who are otherwise healthy; it is expected that use of the drug product will not significantly increase risks in this population.
(4) it is conducted in compliance with the requirements for IRB review and informed consent [21 *CFR* parts 56 and 50, respectively];	IRB review is being obtained for the intervention/clinical trial aim of this study.
(5) it is conducted in compliance with the requirements concerning the promotion and sale of drugs [21 *CFR* 312.7];	Use of empagliflozin in this capacity will be investigational; promotional claims of safety and

	effectiveness for obesity or diabetes will not be made.
(6) it does not intend to invoke 21 *CFR* 50.24.	We will obtain written informed consent.

12. Procedures to Maintain Confidentiality:

Subjects will be assigned an identification code. A subject's name and code will be contained within a small notebook and will be kept in a locked cabinet in the research coordinator's locked office. The research chart will contain personal health information and will be kept in locked file cabinet in the research coordinator's locked office. All other data on a computer will be de-identified with the subject's identification code. Blood samples waiting for analysis will not be labeled with the subject's name, but with the assigned identification code.

The study database will be password protected. No data will be sent over the internet unless it is encrypted. All email with subject-identifiable information will be password protected. Only key personnel will have access to the information in the study database on an as-needed basis. Key personnel may not alter the data in the database or directly view all of it without specific cause and approval of the PI. Subjects will be advised that representatives of the Sponsor and the UT Southwestern Medical Center IRB may review their medical and research records to assure the quality of information used in this research. A HIPAA "Authorization for Disclosure" will be obtained from each study participant. A Certificate of Confidentiality is not needed for this study. The research coordinator will keep the original signature copy of the informed consent document in the research records, which will be kept in a locked file cabinet in a locked office. Each subject will receive a photocopy of consent documents.

Neither the PI nor any other key personnel have any real or perceived conflict of interest in the research outcome. All have submitted a report of financial interest to the Conflict of Interest Office at UT Southwestern within the past twelve months.

13. Potential Benefits:

- There is a potential benefit of the proposed research to study participants in the form of weight loss. However, researcher cannot guarantee any direct benefit to the study participants. Knowledge gained from this project will likely lead to better understanding of diabetes in the general population and across ethnic minorities and women as well as potentially lead to improved targets of therapies aimed at reducing diabetes risk.
- The risks outlined in this proposal are reasonable for this societal benefit as it may lead to improved care to reduce diabetes risk, a leading cause of morbidity and mortality in the U.S.

14. Biostatistics – Statistical Analysis Plan:

Aim 1 Sample size calculation: Assuming 20% $^{13}C_3$ enrichment in subjects with low VAT (based on prior work), and an expected enrichment of between 15-18% among those with high VAT (SD 2%), for Aim 1 we expect to require 20 subjects per group to achieve 87% power to detect a 2% difference between groups at an alpha level of 0.05. Because we expect some patients may be deemed ineligible or withdraw consent in the interim between recruitment/informed consent is signed and undergoing study procedures, we will recruit up to a total of 50 subjects in order to ensure that 40 total subjects complete the study procedures (see sample size table). The study will be stopped after 40 subjects complete all study procedures and no further patients will be included in the study.

Variable	Mean Difference	Standard Deviation	Type 1 error (α)	Power (1-β)	Total N

[U-$^{13}C_3$] glycerol enrichment	2%	2%	0.05	0.87	40
	3%	2%	0.05	0.89	20
	5%	2%	0.05	0.97	12
	7%	2%	0.05	0.89	6

Aim 1 Statistical analysis: Data will be expressed as means ± standard deviations. The primary outcome of mean [U-$^{13}C_3$] enrichment of blood glucose at study end will be compared between high VAT and low VAT groups using the Student's t-test. Plots of [U-$^{13}C_3$] enrichment over time for each group will be constructed to compare the trajectory of glycerol enrichment over time between groups and compared using a mixed linear model. The association between VAT and change in plasma blood glucose with glycerol challenge will be analyzed within each subject using a paired t-test and between groups by mean change in plasma glucose using the Student's t-test. The relationship between VAT and glycerol enrichment will also be modeled using linear regression and adjusted for potential confounders of age, ethnicity, family history of diabetes, triglycerides, lean mass, liver fat, abdominal subcutaneous fat mass, and biomarkers.

Aim 2 Sample size calculation: Assuming a mean change of 2% (SD 1.5%) [U-$^{13}C_3$] enrichment after treatment with EMPA, for Aim 2 we expect to require 8 paired measurements in each arm to achieve 84% power to detect this difference at an alpha level of 0.05. Assuming an estimated 20% dropout rate consistent across groups we will still have sufficient power to detect this mean difference within each treatment arm using the planned enrollment strategy.

Aim 2 Statistical analysis: See Aim 1 above. The primary outcome of change in mean [U-$^{13}C_3$] enrichment of blood glucose within each treatment assignment will be tested using the paired t-test. Secondary outcomes comparing post-treatment glycerol enrichment across groups will be tested with ANOVA.

For all statistical testing, a 2-sided *p*-value <0.05 will be considered statistically significant. All statistical analyses will be performed using SAS version 9.2 software (SAS Corporation, Cary, NC).

15. References:

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Figure Legend

Figure 1.

Title: Schematic of Glycerol Metabolism in Gluconeogenesis in Liver and Adipose Tissues

Caption: Glucose as a product of hepatic gluconeogenesis is comprised of a 6-carbon molecule derived from glycerol via multiple different metabolic pathways including exogenous glycerol from dietary sources and adipose tissue-derived glycerol produced from lipolysis of triglyceride stores. In the liver, glycerol (Gly) is converted to glycerol-3-phosphate (G3P) and subsequently dihydroxyacetone phosphate (DHAP) which is in equilibrium with glyceraldehyde-3-phosphate (GA3P). Both DHAP and GA3P are converted to fructose-1,6-bisphosphate which is serially enzymatically converted to fructose-6-phosphate, glucose-6-phosphate, and ultimately glucose. In adipose tissue, triglycerides (TG) undergo lipolysis to form free fatty acids (FFA) and glycerol (Gly) which then shuttles to the liver to enter into the same gluconeogenesis pathway as that of exogenous glycerol. Therefore, free plasma glucose can be comprised of two glycerol molecules from either exogenous or lipolytic sources in any combination.

Figure 2.

Title: Representative examples of abdominal fat by MRI in two subjects with divergent metabolic phenotypes in the Dallas Heart Study.

Caption: A. Transverse abdominal MRI images of visceral (VAT) and abdominal subcutaneous (SAT) adipose tissue in a 21-year-old black female with BMI of 36 kg/m2 and total body fat of 4.2 kg (41%) demonstrates very low VAT (0.22 kg/m2) and high SAT (4.45 kg/m2). B. In contrast, images of VAT and in a 59-year-old white male with a BMI of 31 kg/m2 and total body fat of 4.0 kg (34%) demonstrate very high VAT (1.80 kg/m2) and low SAT (1.46 kg/m2).

Figure 1.

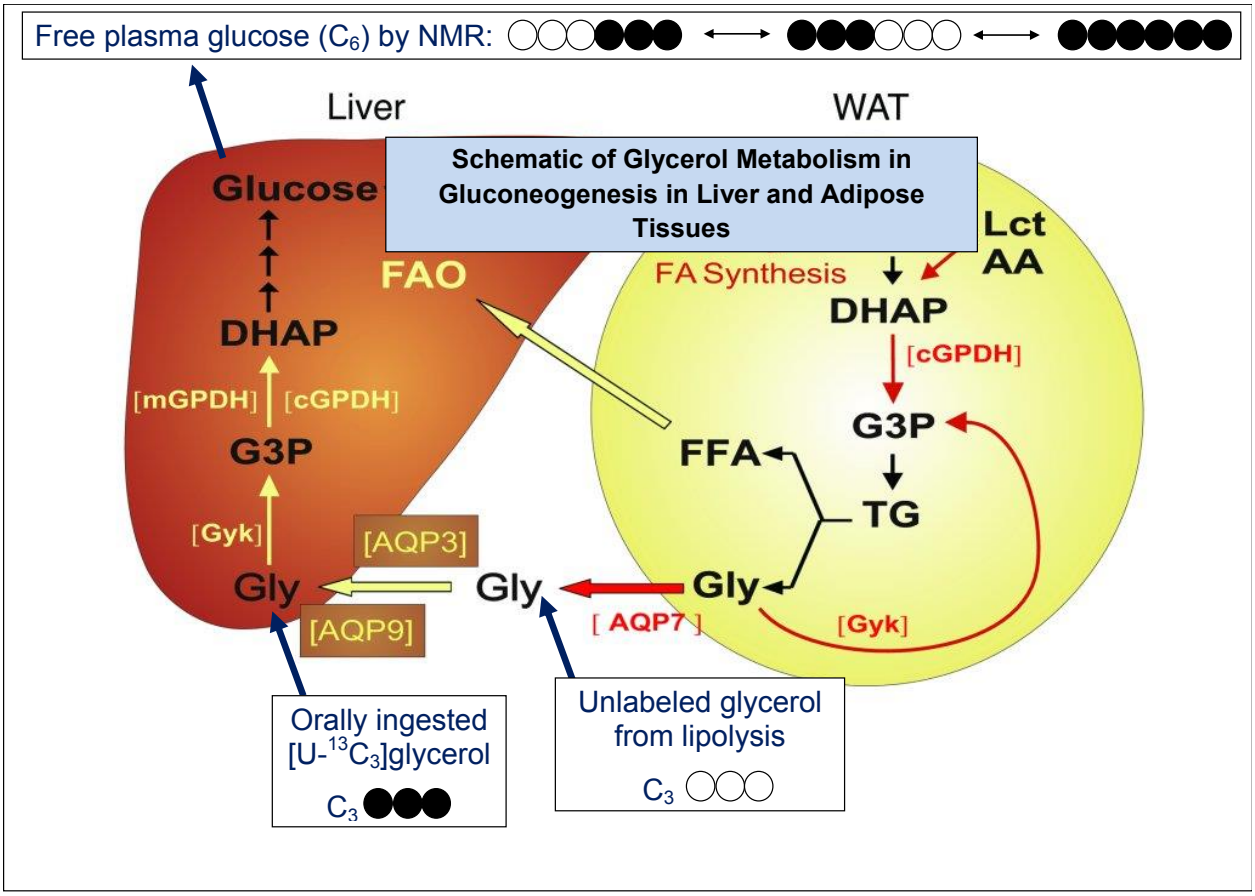
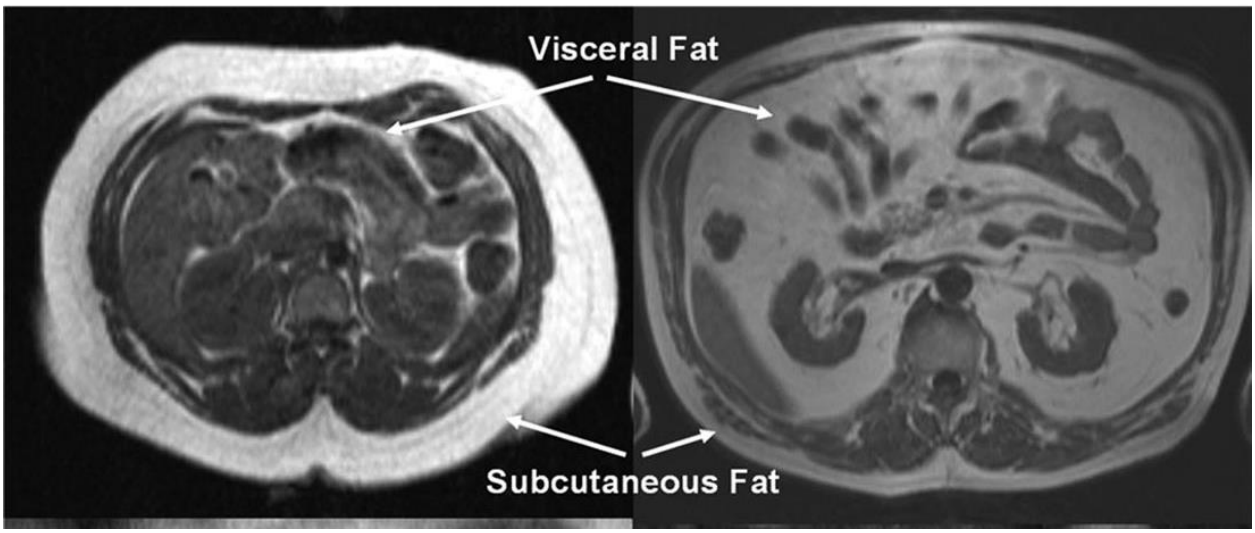

Free plasma glucose (C_6) by NMR:
Liver
WAT
Schematic of Glycerol Metabolism in Gluconeogenesis in Liver and Adipose Tissues
Glucose
DHAP
[mGPDH]
[cGPDH]
G3P
[Gyk]
Gly
[AQP9]
FAO
[AQP3]
Gly
Gly
[AQP7]
FA Synthesis
Lct
AA
DHAP
[cGPDH]
G3P
FFA
TG
[Gyk]
Orally ingested [U-$^{13}C_3$]glycerol
C_3
Unlabeled glycerol from lipolysis
C_3

Figure 2.

A. **B.**



Glycerol Study Protocol Flowsheet

Advanced Imaging Research Center
eIRB File #: 012015-064
Visceral Adiposity and Diabetes: Translating Form to Function Using Imaging

Subject Name: **Visit Date:**

Scheduled Time	Actual Time	Initials	AIRC Outpatient Procedure
0800			Meet patient in lobby of Rogers MRI Center and verify name & DOB.
0810			Explain study procedures and obtain informed consent. Verify NPO except water since midnight.
0825			Obtain urine sample for pregnancy test (female only)
0830			Obtain vital signs and anthropometric measurements. T_____ P_____BP_____/_____ R_____ Body Fat % _____ Ht_____Wt_____BMI____ Waist Circ____ Hip Circ____
0845			Insert peripheral IV with 150ml bag of normal saline to KVO. Verify good blood return and flushes easily.
0855			Collect 40ml blood sample in purple top and 1ml blood sample for blood sugar. YSI Result: _____/_____mg/dl
0915 -0930			Prepare for subjects receiving [U-13C3]glycerol in ½ cup filtered water: _____kg x 0.05 gm/kg x 1 ml/1.302 gm = **_____ml** Lot#: _____ Exp: n/a
0930			Have subject drink Glycerol. Follow with 1 cup filtered water to rinse bottle.
1000 +30			Collect 1ml for blood sugar and 40ml blood sample in purple top tubes. YSI Result: _____/_____mg/dl
1030 +60			Collect 1ml for blood sugar and 40ml blood sample in purple top tubes. YSI Result: _____/_____mg/dl
1100 +90			Collect 1ml for blood sugar and 40ml blood sample in purple top tubes. YSI Result: _____/_____mg/dl
1130 +120			Collect 1ml for blood sugar and 40ml blood sample in purple top tubes. YSI Result: _____/_____mg/dl
1200 +150			Collect 1ml for blood sugar and 40ml blood sample in purple top tubes. YSI Result: _____/_____mg/dl

Advanced Imaging Research Center
eIRB File #: 012015-064
Visceral Adiposity and Diabetes: Translating Form to Function Using Imaging

Subject Name: **Visit Date:**

Scheduled Time	Actual Time	Initials	AIRC Outpatient Procedure
1230 +180			Collect 1ml for blood sugar and 40ml blood sample in purple top tubes. YSI Result: _____/_____mg/dl
			Discontinue peripheral IV line after last blood sample obtained.
			Obtain vital signs. BP: _____/_____ P: _____ T: _____°F R: _____
			Provide lunch.
			Perform MRI scan if indicated
			On Study Day 1 Provide Emagliflozin/Placebo and Instructions.
			Discharge subject from AIRC.

Advanced Imaging Research Center eIRB File #: 012015-064 Visceral Adiposity and Diabetes: Translating Form to Function Using Imaging				
Study Time	**Plasma (Pre) (40ml)**	**Glucose (1ml)**	**NMR (40ml)**	**Comments**
				Arrive @ AIRC
				Start IV x 1
-60 (Pre)	X	X		
0				Glycerol consumed
+15		X	X	
+30		X	X	
+60		X	X	
+90		X	X	
+120		X	X	
+150		X	X	
+180		X	X	
				D/C IV & provide lunch
Tubes	10ml purple top tube x 1	1ml microfuge tube	10ml purple top tube x 4	
Processing	On ice → spin → plasma in small vial → REFRIGERATOR for Dr. Jin/Neeland	IMMEDIATELY spin → run → record result → plasma in cryo vial for Dr. Jin/Neeland	On ice → spin → plasma divide into 3 10ml plastic vials → REFRIGERATOR for Dr. Jin/Neeland	

Glycerol Study Procedure Grid

VISIT 2 and VISIT 3 STUDY MEDICATION COMPLIANCE FORM

DID SUBJECT RETURN UNUSED AND/OR EMPTY MEDICATION CONTAINERS?
☐ **YES** ☐ **NO**
If no, comment below:
_____
_____

Date of Last Dose Dispensed: _____/_____/_____
Day Mon Year

Total Number of Tablets Dispensed at last visit: _____

Total Number of Tablets Returned Today: _____

Last Dose Kit #: _____ Last Dose Lot #:_____

$$\text{COMPLIANCE \%} = \frac{\text{TOTAL NUMBER OF TABLETS USED}}{\text{TOTAL NUMBER OF TABLETS REQUIRED PER PROTOCOL}} \times 100$$

_____ /_____ X 100=_____

Is Subject compliant with Medication? ☐ **YES** ☐ **NO**
If no, comment below and counsel/educate subject again:
_____
_____

If medication compliance percentage is <80%, please re-educate subjects. The subject can be withdrawn from study after 2 visits of medication noncompliance. The principal investigator should be notified for each subject's visit of not meeting medication compliance. It is up to the discretion of the principal investigator to allow the subject to remain in the study.

MEDICATION RE-DISPENSED ☐ **YES** ☐ **NO**
If no, comment below:
_____
_____

Kit #: _____ Lot#: _____ Expiration Date: _____

NUMBER OF TABLETS DISPENSED? _____

MEDICATION DOSAGE GIVEN TO AND REVIEWED WITH SUBJECT ☐ **YES** ☐ **NO**