| Study Title | |
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| **Study Title:** | **Ertugliflozin: Cardioprotective Effects on Epicardial Fat** |
| **NCT Number:** | NCT04167761 |
| **Principal Investigator Contact Information** | |
| **Name:** | **Tracey McLaughlin** |
| **Title:** | **Professor of Medicine** |
| **Address 1** | **300 Pasteur Drive** |
| **Address 2** | **Rm S025** |
| **City, ST, Zip** | **Stanford, CA 94305-5103** |
| **E-mail:** | tmclaugh@stanford.edu **(preferred mode of contact)** |
| **Institution Contact Information** | |
| **Name:** | Sarah Daadi, CT Research Process Manager\| Institutional Official |
| **Address 1** | Research Management Group |
| **Address 2** | Stanford University School of Medicine \| 3172 Porter Drive, Palo Alto, CA 94304-1212 |
| **City, ST, Zip** | |
| **Phone/Fax:** | |
| **website** | |
| **Contracting Information (if applicable)** | |
| **Name:** | Dee Nguyen |
| **Phone/Fax:** | Phone\| 650-497-0753 Fax\| 650-721-2627 |
| **E-mail:** | nguyenmd@stanford.edu |
| **Study Information** | |
| **Last update:** | 9/19/2024 |
| **Number of Subjects:** | Goal: 36<br>61 enrolled (18 for cardiac tissue collection, 43 for bariatric tissue collection), 433 screened (293 for cardiac tissue collection, 140 for bariatric tissue collection) |

## Key Personnel

1. **Tracey McLaughlin, MD, MS.** Professor of Medicine, Department of Medicine, Division of Endocrinology, Stanford University School of Medicine. **Role: PI**. Expertise: Clinical/translational research related to diabetes, obesity, metabolism with focus on adipose cell/tissue contributions to metabolic disease. Performed over 1500 fat biopsies from subcutaneous, visceral and epicardial depots. Resources include full access to Clinical Translational Research Unit, offices and computing resources, private wet laboratory, and access to Stanford core laboratory facilities.

2. **Harold Sacks, MD.** Clinical Professor of Medicine, Department of Medicine, UCLA David Geffen School of Medicine. **Role: Scientific Collaborator**. Expertise: Epicardial fat biology in humans.

3. **Jack Boyd, MD.** Associate Professor of Surgery, Department of Cardiovascular Surgery. **Role: Cardiothoracic Surgeon.** Expertise: Cardiothoracic surgery.

4. **Frederick Kraemer, MD.** Professor of Medicine, Department of Medicine, Division of Endocrinology, Stanford University School of Medicine. **Role: Scientific Collaborator**. Expertise: Fatty acid metabolism/lipolysis.

5. **Samuel C Cushman, PhD.** Chief of Diabetes and Metabolism (emeritus), NIH/NIDDK. **Role: Scientific Collaborator**. Expertise: adipose cell size measurement and adipose cell biology including insulin-mediated glucose uptake via GLUT4 trafficking.

6. **Edgar Engleman, MD.** Professor of Medicine, Department of Medicine, Division of Immunology, Stanford University School of Medicine. **Role: Scientific Collaborator.** Expertise: Flow cytometry, immunology.

## Background and Rationale

- **Provide background on unanswered question(s) the study is attempting to answer (do not exceed one page)**

Sodium-glucose cotransporter 2-inhibitors (SGLT2i) decrease heart failure and cardiovascular death in patients with type 2 diabetes but the mechanism of risk reduction is unknown. Whereas SGLT2i lower glucose via binding receptors in the proximal tubule of the kidney, they may exert cardiac effects via direct or indirect mechanisms. Direct off-target effects of SGLT2i have been shown in cardiomyocytes, with reduced cytosolic and increased mitochondrial Ca2+, improved cell viability and ATP content following hypoxia in cardiomyocytes and endothelial cells, improve vasoreactivity in endothelial cells exposed to hyperglycemia, and attenuation of MCP-1 and IL-6 secretion by HUVEC[1]. Epicardial adipose tissue (EAT) is another potential target by which SGLT2i might decrease cardiovascular risk via as yet undefined mechanisms, either directly through small molecule properties or binding of the receptor, which has been demonstrated in epicardial fat[2], or indirectly, by altering systemic metabolism. Similar to very-low carbohydrate diets, SGLT2i lower not only glucose, but also insulin, thereby disinhibiting lipolysis and promoting ketogenesis. This is particularly We have previously shown that even moderately low-carbohydrate weight loss diets yield greater metabolic benefits than similarly hypocaloric high carbohydrate diets, with greater reductions in daylong triglyceride (despite higher circulating FFA) and ApoB, lower circulating markers of endothelial function[3], and greater reduction in adipose cell size[4]. SGLT2i may yield metabolic and cardiovascular benefits from similar systemic metabolic changes that promote release and utilization of stored FFA for energy, leading to reductions in EAT adipocyte size, fat mass, and inflammation.

EAT is of particular relevance to SGLT2i cardiovascular effects because 1) EAT thickness is a strong marker of clinical risk for CVD, including insulin resistance, myocardial lipid content, cardiomyocyte dysfunction[5,6], and also fatal and nonfatal coronary events[7], and 2) SGLT2 treatment in humans decreases EAT mass relative to total body fat, pointing to a potential cardioprotective mechanism[2,8,9]. Epicardial fat cells are derived from brown adipose tissue during embryogenesis, similar to mesenteric and omental fat cells. As compared with other fat depots, EAT demonstrates a high degree of metabolic flexibility, with increased capacity for FFA uptake, FFA synthesis, lipogenesis, and FFA release in order to meet the changing energy needs of the myocardium[10]. Indeed, lipolysis rates of EAT are twice that of visceral fat, and FFA oxidation accounts for 70% of myocardial energy production. Furthermore, because there is no physical barrier between EAT and the vessels that pass through or below the fat, secreted proteins and FFA can diffuse directly into the coronary arteries. EAT secretes a large number of proteins that regulate endothelial function, coagulation, and inflammation: these can be protective or harmful, as evidenced by increased expression of multiple inflammatory cytokines in EAT from individuals with CAD vs controls[11] and in depots overlying plaque vs clean vessel[11] as well as in those with type 2 diabetes[12].

There is remarkably scant data on the effect of SGLT2i on EAT. SGLT2 receptors have been demonstrated in human EAT[2], and EAT preadipocytes cultured with dapagliflozin demonstrated enhanced differentiation, decreased chemokine secretion, and increased glucose uptake[2]. In vivo, treatment with dapagliflozin for six months significantly decreased EAT mass compared to controls, along with a significant reduction in TNF-α[4], but subjects also lost total body weight which might have accounted for the observations. Two compelling studies in mice demonstrated that 1) empagliflozin promoted fat utilization and browning, and attenuated inflammation and insulin resistance[13], and 2) ipragliflozin promoted expansion of healthy EAT characterized by decreased inflammation including M1-polarized macrophages[14]. Comparable studies have not been done in humans, however, and given the clinical observations of reduced cardiac risk in SGLT2i-treated patients, mechanistic research is warranted.

Our laboratory has vast experience in working with human adipose tissue/cell metabolism, inflammation, morphology, and differentiation. We have established a working relationship with the cardiac surgery team and recently completed a study in 24 subjects with myocardial bridge, in whom we sampled five fat depots, including three from the epicardium, over vessels with and without atherosclerotic plaque. Gene expression (60 genes via Nanostring) and cytokine secretion analyses are in process. We also have spent over a decade performing collagenase digestion of subcutaneous and visceral human fat to isolate mature adipocytes for measurement of insulin-mediated glucose uptake (IMGU) and lipolysis, and stromal-vascular cells for flow cytometry and preadipocyte differentiation (methods published). We thus have the infrastructure, interdisciplinary relationships, wet lab space and methods, and scientific expertise in adipocyte biology and metabolism to investigate the effects of ertugliflozin on epicardial fat. We hypothesize that ertugliflozin will enhance lipolysis, improve insulin sensitivity, decrease adipose cell size, and reverse inflammatory phenotype in EAT.

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**Hypothesis**

- **List the clinical Hypotheses in order of priority:**

We hypothesize that SGLT2i treatment reduces cardiovascular risk via changes in epicardial adipose tissue (EAT) that yield smaller adipocytes with increased insulin sensitivity, capacity for lipid metabolism (metabolic flexibility) including FFA release via isoproterenol-stimulated lipolysis, which provides fuel for the myocardium during times of increased need, and decreased inflammation, which reduces atherosclerotic plaque and plaque instability.

**Study Design/Clinical Plan**

- **Provide a concise overview stating the type of experimental design**

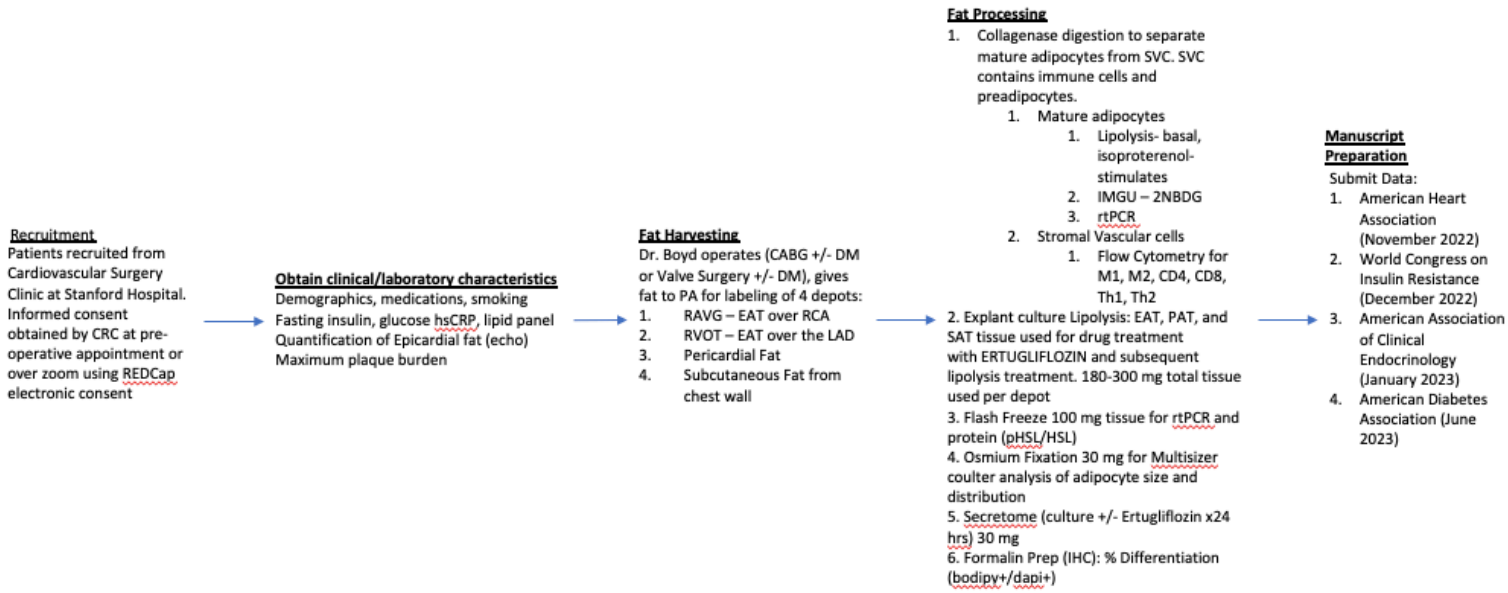
1. Test indirect effects of ertugliflozin on EAT: Because ertugliflozin lowers glucose renally, and thus does not invoke insulin secretion, ambient insulin concentrations are lower and FFA higher than other glucose-lowering therapies such as sulfonylureas. Insulin represents a metabolic switch from fasting (low insulin, catabolic, energy releasing) metabolism to fed (high insulin, anabolic, energy storing) metabolism, and the cardiovascular benefits of SGLT2i may be related to a switch in systemic metabolism from high to low insulin state, thereby promoting FFA release from EAT, thereby increasing energy substrate for myocardium, shrinking epicardial adipocytes, enhancing glucose uptake and insulin sensitivity, and decreasing inflammation. Because these patients were unable to take

the drug due to insufficient lead time prior to surgery, we decided to use these patients to test direct effects of ertugliflozin on EAT.

2. Test direct effects of ertugliflozin on EAT: Harvest fat from epicardial, pericardial, and subcutaneous (chest wall) depots from patients (with or without CAD and with or without type 2 diabetes) undergoing cardiothoracic surgery. Explants of whole tissue and/or collagenase digested mature fat cells will be used to measure lipolysis (basal/isopreteronol-stimualted/insulin-suppressed conditions), secretome (basal conditions only), and gene expression under mock vs drug-treated culture conditions for 2-24 hours[17]. In addition, mature adipocytes freshly isolated from collagenase digestion will be obtained from bariatric surgery patients from both SAT and VAT. This is necessary because bariatric samples yield greater quantities of fat for collagenase digestion whereas epicardial fat sample quantities are smaller which necessitates conducting an explant experiment on the tissue. Mature cells from bariatric samples will be tested for lipolysis, insulin-mediated glucose uptake, gene expression, and inflammatory cytokine secretion under mock vs drug-treated culture conditions for 2-24 hours. VAT has many similar qualities to EAT since they are both visceral depots, and thus will augment any results obtained from EAT and provide a mechanism for isolated mature fat cells which otherwise is not possible. In the case of minimal EAT availability from surgical collection, an explant culture will be performed to differentiate stromal vascular cells into the necessary amount of mature fat cells for a lipolysis assay. This experiment addresses the direct effect of ertugliflozin on EAT in the absence of systemic changes such as lower glucose, insulin, and higher FFA concentrations. Due to small amounts of EAT, we will also conduct lipolysis assays on whole tissue explants from all three depots.

    i Methodologic Details: The tissue collected during biopsy will be snap-frozen for rtPCR and phosphoproteomics, and osmium-fixed to quantify adipose cell size. Tissue will be placed in culture for 24 hours for collection of supernatant to measure secretome. Mature adipocytes isolated via collagenase digestion will undergo measurement of lipolysis, basal and isoproterenol-stimulated, and insulin-mediated glucose uptake. The remaining stromal vascular pellet will undergo flow cytometry for quantification of immune cell subsets per our usual gating protocols[15,16] (monocytes, macrophages, M1, M2, Th1, Th2, CD8, along with specific surface markers of activation (eg CD44, CD163) on macrophage and T-cell subsets.

**Study Workflow**:



**Preliminary Data:**
We hypothesize that fat metabolism in individuals taking ertugliflozin will resemble that seen in individuals randomized to a very low carbohydrate, non-insulinogenic diet: Figure 1 demonstrates increased lipolysis and Figure 2 demonstrates increased activity of hormone-sensitive lipase (normally inhibited by insulin, but more active during low insulin conditions)

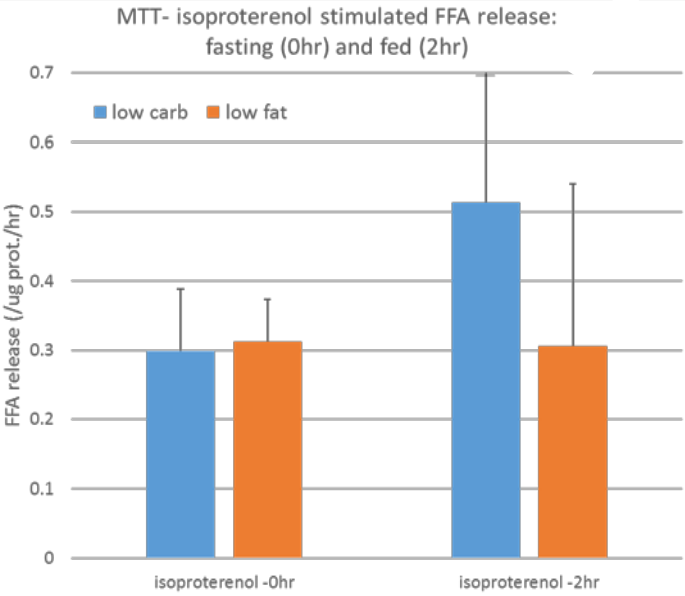



**Figure 1.** Demonstration of increased 2-hr isoproterenol-stimulated lipolysis in individuals assigned to very low carbohydrate diet (similar to hypothesized effect of ertugliflozin via decreased ambient insulin concentrations and enhanced FFA

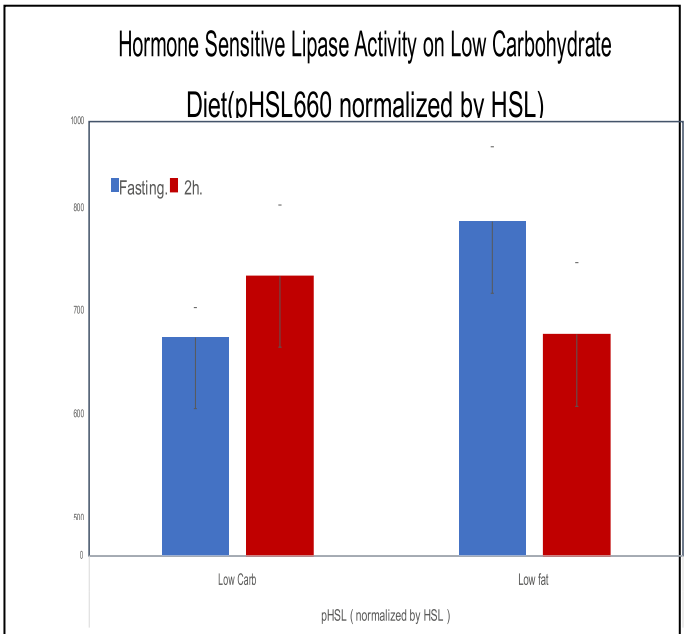



**Figure 2**: Increased HSL activity (as indicated by HSL phorphorylation) 2 hours after eating a low carbohydrate diet (non-insulinogenic, comparable to hypothesized effect of ertugliflozin) leading to FFA release & adipocyte shrinkage

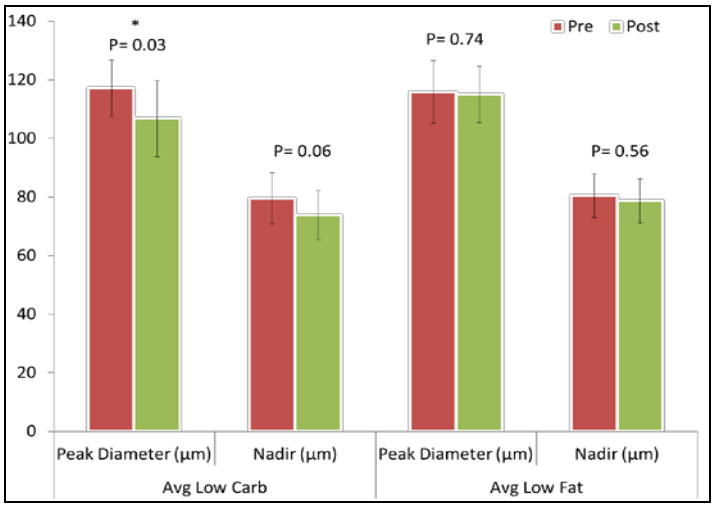



**Figure 3.** Mean changes in peak diameter and nadir in low carbohydrate (left, n=19) and low fat (right, n=19) groups: individuals on low carbohydrate diet demonstrated significant decrease in adipose cell size (p=0.03).

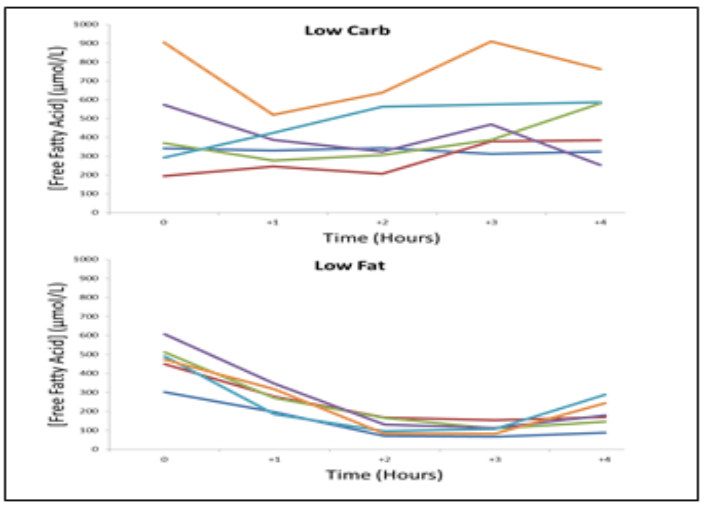



**Figure 4.** FFA concentrations in individuals given a low carbohydrate (non-insulinogenic) diet do not suppress during meal tolerance test as do those on low fat (high carbohydrate, insulinogenic)

## Laboratory Methods:

1) **Tissue harvesting and processing**

Tissues (SAT, VAT, EAT) are collected from surgical dissection. Flash freezing (50-100 mg) is performed in the laboratory for later RNA/miRNA isolation. For IHC staining, 30 mg tissue is fixed in 10% formalin in PBS at room temperature; for osmium fixation (for cell size distribution), 30 mg tissue is fixed in 2% osmium per protocol; for EM staining, 30 mg tissues are fixed in paraformaldehyde and glutaraldehyde (both E.M. grade). For secretome analysis, 30 mg tissue is placed in media for 48 hours after which supernatant is collected and frozen for cytokine analysis.

**2) Collagenase digestion**

Isolation of mature adipocytes and stromal-vascular cells (SVCs) from freshly collected adipose tissues (VAT and SAT) is performed using a modification of the original protocol of Rodbell[18]. Sterile harvested tissue is minced and placed in Krebs Ringer Bicarbonate Hepes Buffer with 1mg/ml of collagenase. The digestion is performed at 37°C in a shaking water bath for 3045 mins until the digestion mix becomes uniformed cell suspension. The cell suspension is passed through 500um nylon filters to remove the undigested tissue pieces. The flow-through contains the mature cell suspension on the top and preadipocytes mixture in the digested isolation. Subsequently, the serum is added to neutralize the enzymes, the top layer of mature adipocytes is collected and washed with Krebs Ringer Bicarbonate Hepes Buffer for three times. 100ul of packed cell volume (PCV) is flash frozen into tubes for later RNA processing. The freshly isolated mature cells suspension can be used for lipolysis assay and IMGU assay to measure fat metabolism reflecting the ambient conditions under which tissue was harvested (unlike culture studies in which preadipocytes are grown to maturity under controlled experimental conditions). The stromal vascular portion containing the immune cells and stromal vascular cells (Pre-adipocytes) is passed through the 100um cell strainer, and pelleted. Pelleted cells are washed with PBS and re-dissolved into the buffer that is suitable for flow cytometry and cell culture.

**3) rtPCR**

RNA is isolated from flash frozen tissues or from terminally differentiated cells on day 14 after confluence. Briefly, RNA isolation and purification were performed with the RNeasy Mini Kit (Qiagen Sciences, Inc., Germantown, MD.). After DNase treatment, reverse transcription of 0.5-1g of RNA is performed with Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif.). Quantitative real-time polymerase chain reaction is carried out using the Applied Biosystems QuantStudio Sequence

Detection System. Primerbank is used to design the specific primers for the genes (as markers of inflammation, TNF-alpha, IL-6, IL-8, IL1-B, MCP-1, CD68, CD45 and CD14 gene expression. To evaluate differentiation, we measure CREBP, SREBP-1, PPARG, pref-1, AP2, adiponectin, FABP, CD36, GLUT-4, PEPCK-c, perilipin, and leptin. To evaluate hypoxia, we evaluate HIF-1alpha-regulated genes that are both angiogenic (VEGF, Angio-1, apelin) and not angiogenic (leptin, GLUT-1, MMP3, COL6). The levels of gene expression are determined by normalizing to the values of GAPDH/18S. A common reference CDNA samples which is pooled from all the cDNA from the tissues is setup as control to obtain a normalized value for each tissue. Each reaction has three replicates (N=3).

4) **NanoString**

Nanostring gene expression analysis will be performed using a customized panel of 60 genes related to IR and inflammation in adipose tissue and PBMC as previously described[19]. RNA is isolated by the Hybrid-R RNA extraction kit (Geneall BioTechnologies, Palo Alto, CA) according to the manufacturer's instructions. RNA amount and quality are checked by Bioanalyzer analysis. RNA samples will be analyzed in the Stanford Genomic Core Facility. Inter- and intra-cartridge replicates are included in the assay. Normalization of nCounter results are carried out using nSolver Analysis Software Version 2.5 (NanoString Technologies), according to the manufacturer's guidelines.

5) **Preadipocyte differentiation protocol**

SVCs are pelleted by centrifugation, erythrocytes lysed, and the isolated cells are seeded onto 12-well plates at a density of 10,000 cells per well for assessment of adipogenesis. Adipogenic differentiation medium purchased from Zenbio (Cat#DM2 for subcutaneous depot cells and #OMDM for visceral depot cells) are used to induce differentiation. ERTUGLIFLOZIN and vehicle are supplemented to the adipogenic differentiation medium. At 3 days, medium is changed to maintenance medium from ZenBio (Cat#AM-1) based on the manufacturer's protocol and ertugliflozin is supplemented. Differentiation is carried out for 14 days to achieve terminal differentiation as described[17,18].

6) **Optimization of ertugliflozin concentration for pre-adipocyte culture studies**

SVC are placed in wells with vehicle control and three different concentrations of ERTUGLIFLOZIN (provided by the company). Planned concentrations to test include 10, 100 and 300nmol/L. Media (with or with drug at the listed concentrations)

will be changed every other day with media replenishment. BrdU can be used to determine the proliferation effect at day 3 and day 7. Optimal concentration will be defined as the concentration that yields 50% of growth inhibition.

7) **IMGU using 2NDGB method for cultured adipocytes**

Assay of Insulin Mediated Glucose Uptake (IMGU) is carried out after 14 days of adipogenic differentiation. On day 14 terminally-differentiated cells are washed with glucose-free PBS three times to remove the residual glucose in the media. Glucose-free PBS is used to fast the cells for additional one hour at 37C. After one hour incubation, 10ug/ml of insulin is added to the cells to stimulate the glucose release, after 10 mins, 100ug/ml of 2-NDBG solution is added to the cells for florescence labeling. 30 mins later, cells are washed with PBS twice to remove the residual 2NDBG in the buffer and cells are lysed in the protein lysis buffer (Sigma, Inc). Subsequently, 100ul of lysis are read by excitation/emission=485/535nm to detect the florescence incorporation to the cells. The protein lysis concentration are measured by Coomassie Plus™ Protein Assay. Each samples florescence reading is normalized by its protein concentration as the final reading. Each assay is set to have three replicates, mean and SD are calculated compared to the control.

**8) Total fat uptake using Oil Red O on day 14 of culture**

Oil red O staining and quantification are performed after two weeks of differentiation. Briefly, differentiated cells are fixed with 10% formalin in PBS, Oil Red O solution (Sigma. Inc) is used to stain the cells. After washing, stained cells will be dried at room temperature and photos will be taken by light microscope. To extract and quantify the cell staining, we use 100% isopropanol and subtract the background signal. Readings of absorbance at 492 nm are calculated compared to control[17].

**9) % Differentiation using immunofluorescence on day 14 culture**

Briefly, terminal differentiated cells after two weeks are fixed in 4% glucose/paraformaldehyde in PBS solation (made for freshly use) for one hour and stain with DAPI (blue) and Bodipy FL (ER-Tracker Green) in 37C for two hours. Fluorescence microscope is used to capture the lipid droplets of green staining with internal control of DAPI (nuclei staining). Photos are counted using NIH imagine J software. The total Green stained cells or percent of green cells will be counted normalized by the blue staining of nuclei numbers as the differentiated cells[17,18].

**10) Basal and isoproterenol-stimulated lipolysis on mature adipocytes isolated via collagenase digestion**

Lipolysis assay is carried out by using Lipolysis Colorimetric Assay Kit (Biovision, Inc.), Cat# K577-100 as described[4]. Briefly, the washed mature cells (in PCV) are diluted into 1:2 in Lipolysis Assay Buffer based on manufacturer's protocol. A glycerol standard is built with supplies offered in the kit and a colorimetric assay is performed with human mature cells mixture and glycerol standard at the same time in 96-well reaction. Afterwards, cells are incubated with 100nM of isoproterenol (set to induce the glycerol release) for 3 hours at room temperature. Glycerol standard and mature cells suspension are measured at OD of 570nm. Based on the standards, the mature cell glycerol amount can be calculated.

**11) IMGU using 2NDBG for mature adipocytes isolated via collagenase digestion**

IMGU assay of mature adipocyte is carried by using the isolated PCV of mature cells after digestion. 2-NDBG (dilutions need to be standardized) will be added in for measuring the glucose uptake after insulin stimulation (with 10ng/ul insulin and without). Briefly, PCV of mature adipocyte is washed three times after digestion. Krebs Ringer Bicarbonate Hepes Buffer (Ph 7.4) is used to dilute the cells into 5% -10% to use for the assay. 10ng/ul of insulin or vehicle control are added to the fixed volume of cell suspension and incubated for 30 mins. Cell reaction (2NDBG incorporation) will be counted at excitation/emission=485/535nm to detect the florescence in the mature cells.

**12) Measurement of secretome from preadipocytes in culture**

During the 2-weeks of adipogenic differentiation, we will collect the media of the preadipocytes on day 3, 7, and 14 of differentiation. Media will be harvested and spun, then frozen at -80C for future cytokine assays. The attached preadipocyte cell number in each well at the time of seeding will be used for cytokine normalization (expressed per number of cells) as described[17].

**13) Measurement of secretome from adipose tissue**

Briefly, 30 mg of adipose tissue is incubated in buffer (Krebs-Ringr Solution) plus albumin (3ml/g of tissue) for approximately 5–30min to reduce contamination of the tissue with blood cells and soluble factors. At the conclusion of this brief incubation, the tissue explants are flash centrifuged for 30s at 400g in order to remove blood cells. The explants (~100mg/ml ratio) are then incubated at 37C for 48 hrs in suspension culture under aseptic conditions. After 48 hrs incubation, tissues are spun down, and the

supernatant is collected and flash frozen for future cytokine assays. We use the Human Adipocyte (cell culture samples, Cat# HADCYMAG-61K) panel to assay 11 cytokines by luminex (Millipore). Cytokines are expressed per mg tissue.

**14) Adipose cell size distribution**

Two 30 mg samples of osmium-fixed tissue are incubated in a water bath at 37ºC for 48 h and then washed as previously described[18], after which adipose cell size was determined via a Beckman Coulter (Miami, FL, USA) Multisizer III with a 400-um aperture. The effective cell-size range using this aperture is 20 to 240 μm, excluding preadipocytes and cells of mononuclear origin. The instrument is set to count 6,000 particles and the fixed-cell suspension is diluted so that coincident counting is less than 10%. After collection of pulse sizes, the data is expressed as particle diameters and displayed as histograms of counts against diameter using linear bins and a linear scale for the x-axis. Cell size distribution is described via a mathematical model (non-linear least squares function nls, R 1.9(http://r-project.org])), as previously described[18], in which a single formula using 7 cell-size parameters describes the cell distribution, in which the small cells are represented by a double exponential, and the large cells by a Gaussian curve, as follows: $y = h1 \exp(-(x - x0)/w1) + h2 \exp(-(x – x0)/w2 + hp \exp(-(x - cp)2/wp)$ where x = cell diameter, and x0 = the smallest diameter; h1 and w1 = height and width of the first exponential; h2 and w2 = height and width of the second exponential; and hp, cp, and wp = height, center, and width-squared of the Gaussian curve. From these fitted curves the following estimates are possible: 1) Peak diameter – middle of the Gaussian: reflects mean size of mature adipose cells; 2) AUC Gaussian: represents fat storage capacity; 3) Width of the Gaussian - this represents the variability in size of mature cells. This is evident from mouse models utilizing curve fitting from multisizer curves in which weight gain is associated first with increase in peak diameter, followed by widening of the Gaussian and an increase in the number/fraction of small cells, consistent with studies from other groups in humans, and rodents, showing "hypertrophy" followed by "hyperplasia". When the curve widens and flattens, it is associated with increased number of cells and likely represents movement of small cells into the large cell subfraction rather than shrinking size due to lipolysis; 4) % small cells –represents the proportion of all adipose cells that reside in the small cell subfraction, defined as diameter less than the nadir. An increased number of cells in this fraction indicates either increased adipogenesis, with movement from preadipocytes to small adipocyte population now apparent on multisizer, or an inability of small cells to move into the large cell population with resultant accumulation.

15) **Flow cytometry**

We will harvest mononuclear cells from fresh biopsy specimens for macrophage, dendritic cells and T cell activation marker analyses using flow cytometry as described[15]. Cells can be stained simultaneously with multiple antibodies for surface markers and intracellular cytokines. Single cell suspensions of mononuclear cells from adipose tissue will be analyzed by multicolor flow cytometry for the frequency and activation state of macrophages (CD14+), DCs (Lin-DCsign+ CD11c+) and CD4+ T cells and their subsets. The cells will be stained for activation markers, including CD80, CD86 and HLA-DR on DCs and macrophages, and CD25, CD69, CD71 and HLA-DR on CD4+ T cells. CD4+ T cells, macrophages and DCs from VAT and blood from obese insulin sensitive and insulin resistant subjects, will be cultured in the absence of exogenous stimuli, and the amount of intracellular and secreted IL6, IL10, IL12, IL17, IFNa, TGFβ, and TNFa in the cells and culture supernatant will be assayed after 24 and 48 hours by intracellular flow cytometry and ELISA, respectively. To measure intracellular cytokines, cells are fixed and permeabilized by Cytofix/Cytoperm for 30 minutes at 4°C, washed twice with Perm/Wash buffer, and stained with cytokinespecific, fluorochrome-conjugated mAbs anti-IFN-g APC and anti-IL-4 P or isotype-matched control mAbs (all reagents purchased from BD Biosciences).

**16) Immunohistochemistry**

Studies will be performed on paraformaldehyde-fixed tissue stored in isopropyl alcohol. Paraffin-embedded tissue will be sectioned and stained for leukocytes and macrophages using Hematoxylin and Eosin and anti-CD45 and anti-CD68 monoclonal antibodies, and for lymphocyte using CD4 and CD3 monoclonal antibodies. Inflammatory cells present per hpf (40x) will be quantified and averaged over each tissue section.

**17) Explant Culture for Drug Treatment and Lipolysis Treatment**

Surgical biopsy tissues (EAT, PAT, or SAT) are washed in PBS to remove excess blood, and then cut into small pieces (30-50 mg weighted for each test). Cut tissue biopsy is treated with ERTUGLIFLOZIN (-/+, 25 uM) for overnight in CO2 incubator (37C), with culture medium (CM) collected for cytokine release (-/+ Ertu). Tissue biopsy is washed with wash buffer (1x) and then treated with lipolysis condition (Basal, Isoproterenol (15nM), and Isoproterenol (15nM) + Insulin (3nM)), for 1 hr in in CO2 incubator (37C). Then culture medium (CM*) collected for free glycerol release assay (lipolysis) by a commercial kit (EGLY-200).

## Treatment

- **List the clinical dosage/dosage form, route, and dose regimen:**

Tissue will be collected during surgery for cell culture (in vitro, direct effects) work.

## Collateral Research

- **Include biomarkers, PK, etc.**

The planned study already has multiple endpoints. Optional pilot studies: miRNA from current EAT study, co-culture treated and untreated adipocytes with human cardiomyocytes and ipscendothelial cells (stem cell lab Joseph Wu, Stanford).

## Statistical Plans

- **Include justification for clinical sample size and primary hypothesis testing:**

**Primary end point: Isoproterenol-stimulated lipolysis:**
Based on prior low carbohydrate diet study, difference to detect between ertugliflozin and control vehicle (mature adipocytes) will be based on differences observed between low and high carbohydrate diets: 0.2 ug FFA release( FFA/ug protein/hour) with SD 0.2, which yields 5 subjects per group for 80% power and alpha 0.05 for unpaired student's t-test (two-tailed). Cell culture with ertugliflozin vs control vehicle: in order to attain statistical power for testing direct actions on EAT from a given subject (comparison of differentiated cells after culture with ertugliflozin vs control vehicle), 8 subjects are needed for similar statistical power using a paired student's t-test. Cell culture with ertugliflozin vs control vehicle: in order to attain statistical power for testing direct actions on VAT and SAT from a given subject (comparison of differentiated cells after culture with ertugliflozin vs control vehicle), 14 subjects are needed for similar statistical power using a paired student's t-test. Measurement of phosphorylated hormone-sensitive lipase (pHSL/HSL ratio reflects HSL activity) will comprise supportive data (in vivo study) for this endpoint and it is expected that ertugliflozin will increase HSL activity, consistent with increased lipolysis.

**Secondary endpoint: Inflammatory cytokine expression:**
In a prior study (McLaughlin & Engleman, Arteriosclerosis, Thrombosis, and Vascular Biology. 2014;34:2637–2643) in which we measured relative gene expression of inflammatory cytokines in subcutaneous vs visceral fat we found a difference of 1.4 (fold expression) with SD of 1.4, again yielding a number of 5 subjects per group for 80% power and alpha 0.05 for unpaired student's t-test (two-tailed). Cell culture with ertugliflozin vs control vehicle: in order to attain statistical power for testing direct actions on EAT from a given subject (comparison of differentiated cells after culture with ertugliflozin vs control vehicle), 8 subjects are needed for similar statistical power using a paired student's t-test. Cell culture with ertugliflozin vs control vehicle: in order to attain statistical power for testing direct actions on VAT and SAT from a given subject (comparison of differentiated cells after culture with ertugliflozin vs control vehicle), 14 subjects are needed for similar statistical power using a paired student's t-test. Secretome in the supernatant (cell culture and in vivo study) will comprise supportive data.

**Other endpoints including differentiation, FFA uptake and IMGU will be analyzed similarly using unpaired and paired student's t-tests.**