Protocol Title

Effects of Liraglutide on Epicardial Fat Pro-Inflammatory Genes in Type 2 Diabetes and Coronary Artery Disease

INVESTIGATOR-SPONSORED STUDY PROPOSAL

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Abstract

Epicardial adipose tissue (EAT) is the visceral fat of the heart. EAT could locally affect the coronary arteries through local secretion of pro-inflammatory cytokines. EAT plays a role in the development of the coronary artery disease (CAD). EAT is a highly enriched with genes involved in inflammation. Given its rapid metabolism and simple measurability, as first developed by Iacobellis, EAT serves as target for medications targeting the fat. Glucagon-like peptide-1 agonists (GLP-1A) are anti-diabetic medications with recently suggested cardio-protective properties. Liraglutide, a GLP-1A, has recently shown to reduce the cardiovascular risk. Iacobellis'group found that EAT thickness decreased by an unprecedented 36% after 12 weeks of treatment with liraglutide. Remarkably, Iacobellis'group found for the first time that human EAT express GLP-1 Receptor (GLP-1R). GLP-1A effects may be therefore visceral fat specific and target EAT. Based on these preliminary data, we hypothesize that treatment with liraglutide will significantly and rapidly reduce EAT inflammation. Decreased EAT inflammation can reduce the burden of the coronary plaques. We will test our hypothesis in a 12-week randomized, double-blind, placebo-controlled, interventional study in 40 patients with type 2 diabetes mellitus (T2DM), and CAD, with an acceptable glycemic control on their current diabetes regimen who require elective CABG regardless of their participation in the study. A minimum time frame of 3-week treatment will be considered to detect significant changes in the study endpoints. Inclusion criteria for body fat markers will rule out the confounding effect of different body fast distribution at baseline. Study subjects will be randomized in two groups of 20 patients to receive additional liraglutide or to remain on current treatment/ placebo prior to cardiac surgery. EAT samples will be collected during cardiac surgery and processed for analysis of mRNA and protein expression of EAT inflammatory genes such as Tumor Necrosis Factor-alpha (TNF-α) and Interleukin 6 (IL-6), and GLP-1R.

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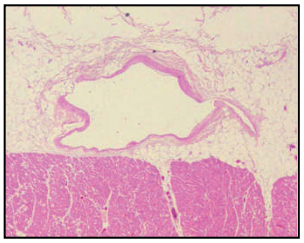
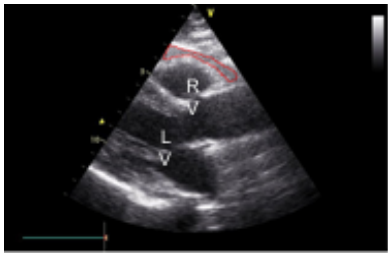
Background

EAT Anatomy and Physiology: EAT is the visceral fat depot of the heart. [1-3] EAT and intra-abdominal fat have the same embryogenesis and both evolve from brown fat (BAT). EAT is supplied by branches of the coronary arteries and no muscle fascia separates the fat depot and the myocardium (*as depicted in the microscopic figure of human EAT*). Hence, as the two tissues share the same microcirculation, a direct cross-talk between the EAT and the myocardium has been highly suggested. Under physiological conditions EAT could serve as a buffer, absorbing fatty acids and protecting the heart against high fatty acids levels, as a lipid storage and local energy source channelling fatty acids to the myocardium and as BAT to defend the myocardium against hypothermia. Under pathological conditions EAT releases factors that promote harmful coronary artery and myocardial changes. EAT is an extremely active organ that produces both pro-inflammatory, such as TNF-α, IL-6, and anti-inflammatory adipokines. [1-3] Given its anatomical proximity to the heart, EAT may interact locally and modulate the myocardium and coronary arteries through paracrine or vasocrine secretion of bioactive molecules. EAT transcriptome is also unique when compared to subcutaneous fat. EAT is a highly inflammatory tissue enriched with genes involved in inflammation, endothelial function, coagulation, immune signalling and apoptosis.[4]



EAT and Coronary Artery Disease: EAT plays a significant role in the development and progression of CAD.[5-6] EAT could alter the coronary arteries via multiple pathways including macrophage activation, inflammatory response, oxidative stress and plaque destabilization. Epicardial adipocytes display an intrinsic pro inflammatory and atherogenic profile. A dense inflammatory infiltrate, mainly represented by macrophages, is commonly detected in EAT of subjects with CAD. Regardless of the pathway, inflammatory cells secreted by the EAT surrounding the adventitia may stimulate the proliferation of vasa vasorum and ultimately cause intramural changes. EAT has been largely associated with the severity of CAD and its association with CAD was confirmed in several large population studies. The relationship of EAT thickness and coronary artery disease is driven by local mechanisms and partially independent of coronary calcification.

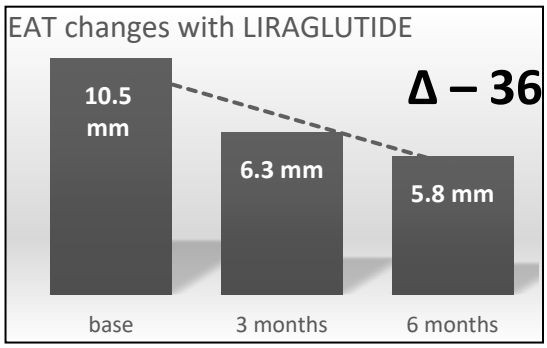
EAT as therapeutic target: EAT thickness can be visualized and measured with standard echocardiography, as first developed and validated by Iacobellis. [7-8] *(echo-free space within the red dot from the parasternal view*). In addition to the easy accessibility and excellent reproducibility, as reported by the majority of the studies using this technique, echocardiographic EAT independently reflects the intra-abdominal visceral fat, measured with Magnetic resonance imaging (MRI) and the intra-myocardial lipid content, calculated with MR-spectroscopy.[9] Given its rapid



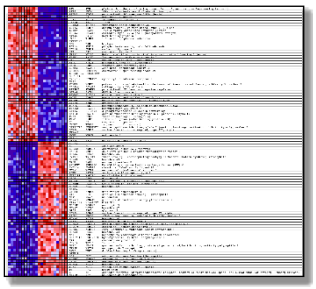
metabolism, organ fat specificity and simple objective measurability, EAT can serve as target for medications targeting the adipose tissue.[10-15]

Cardiovascular effects of Liraglutide: liraglutide, an analogue of glucagon-like peptide-1 (GLP-1), is indicated for the treatment of type 2 diabetes mellitus. In addition to its well established glucose-lowering effect, liraglutide causes a modest weight loss. Liraglutide has also been shown to provide cardio-protective effects beyond the glycemic control, although the mechanisms are still unclear. Very recently, the Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results (LEADER) trial showed that patients treated with liraglutide had a lower risk of first occurrence of cardiovascular death, nonfatal myocardial infarction, nonfatal stroke and lower risks of death from cardiovascular causes in comparison to placebo.[16]

Preliminary Data

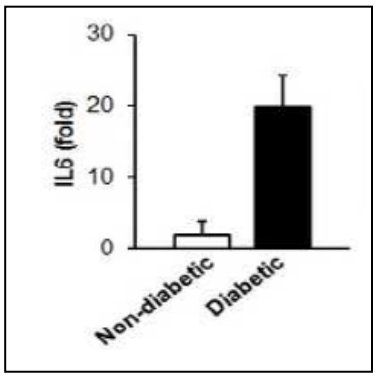


Liraglutide targeting EAT: GLP-1 activation targeting EAT has been recently evaluated.[11-13] Our group recently evaluated the effect of liraglutide on EAT in a 24-week interventional case-controlled study in overweight/obese subjects with T2DM on metformin monotherapy.[11] Individuals were randomized in two groups to receive additional liraglutide up to 1.8 mg sc once daily or to remain on Metformin. Our results showed that ultrasound measured EAT thickness decreased from 10.2±2 to 6.9±1.9 and 5.8±1.9 mm ($p< 0.001$) after 12 and 24 weeks, respectively, accounting for approximately 40% of reduction at 12 and 24 weeks (*graphic on the right*), whereas there was no significant EAT reduction in the Metformin group. EAT shrunk independently of overall weight loss and improved glucose control. A milder, as compared to our results, yet noticeable (-13%), reduction of EAT thickness was recently observed after 12 weeks of treatment either with liraglutide or exenatide in a smaller group of patients with type 2 diabetes. [12] However, EAT results in this study were pooled together making difficult to discriminate differences between the two agents.



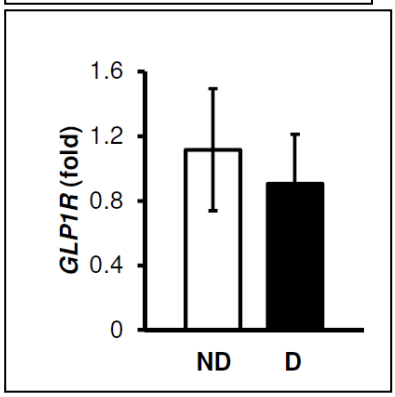
EAT as pro-inflammatory organ: EAT of CAD subjects is enriched with pro-inflammatory genes, as our recent microarray analysis (*microarray table on the left*) showed. [4] Pro-inflammatory cytokines are secreted and transported from EAT into the coronary lumen through vasocrine and paracrine pathways. [1-3]

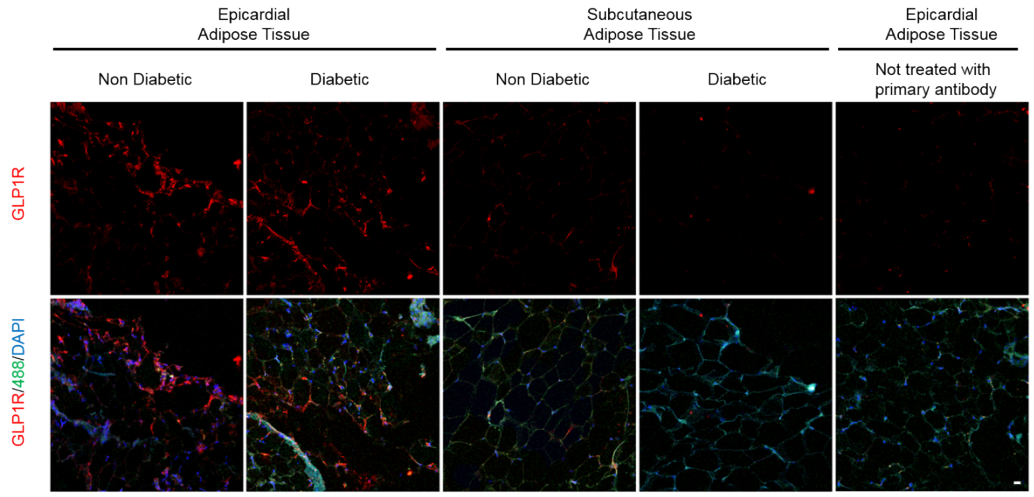
We recently showed that diabetic EAT was mainly enriched in inflammatory genes, such Interleukin-6 (IL-6) (*graphic on the right*) and Tumor Necrosis Factor-alpha (TNF-α).[17] Our study suggests a unique and novel atherogenic pathway in diabetes mediated by EAT inflammatory profile.



EAT expresses GLP-1 receptor (GLP-1R)

Whether EAT expressed GLP- 1 receptor (GLP-1R) was unknown. We recently performed a RNA-sequencing (RNA-seq analysis) and Quantitative real-time RT-PCR (qRT-PCR) to evaluate the presence of GLP-1R in EAT obtained from 8 subjects with coronary artery disease and type 2 diabetes mellitus undergoing elective cardiac surgery. Immunofluorescence was also performed on EAT and SAT samples using Mab 3f52 against GLP-1R. RNA-seq analysis showed that EAT expresses *GLP-1R* gene. qRT-PCR analysis confirmed GLP-1R expression by using two different sets of intron-spanning primers (*graphic on the right*). Immunofluorescence showed that GLP-1R is present and more abundant in EAT than SAT (*immunofluorescence images*). [18]





In the upper quadrants (A), the immunofluorescence images (starting from the left) show definite higher signal in GLP-1R antibody-treated EAT, irrespective of diabetes, when compared to GLP-1R antibody-treated SAT and to not treated with primary antibody EAT (last image on the right). Lower quanrants (B) images show the amplification of the GLP-1R signal using biotinylated anti mouse IgG, horseradish peroxidase-streptavidin and alexa fluor 647 tyramide. DAPI was used to label the nucleus and autofluorescence of the tissue was collected at 488nm excitation.

So, for the first time, we found that human EAT express GLP-1R. GLP-1A effects may be therefore visceral fat specific and target EAT. However, the mechanisms mediating the actions of GLP-1 analogues on adipose tissue are still unclear. Liraglutide may improve EAT insulin sensitivity and microcirculation, stimulate EAT thermogenesis and adipocyte browning.[19-22]

Short term effects of Liraglutide on inflammation

The experimental study by Shiraki et al showed a rapid effects of liraglutide in reducing TNF-α-induced oxidative stress and inflammation in endothelial cells (25). A rapid anti-inflammatory effects of liraglutide on endothelial cells was also reported by Krasner et al Liraglutide reduced the inflammatory responses to TNFα and LPS stimulation, with a significant reduction of protein expression of the adhesion molecules VCAM-1 and E-Selectin, and THP-1 monocyte adhesion in cultured human aortic endothelial cells (26).

Additionally, we provided evidence that epicardial fat expresses the GLP-1R protein and mRNA, such as the heart. Hence, liraglutide is more likely to have a direct effect on epicardial fat transcriptome than mediated by weight loss or glycemic control.

The LEAD trials showed a decrease in mean weight ranging from 1 to 3.2 kg (2.2 to 7.04 pounds) over the course of 26 or 52 weeks with liraglutide.

Significance We believe that this study proposal may have a great relevance and impact in the research and clinical management of CAD. From a pathophysiological perspective, this study proposal can provide data that will contribute the understanding of the complex mechanisms that lead to the development and progression of CAD. Understanding whether EAT plays a metabolic role in CAD is key. This study can provide evidences of the recently suggested cardioprotective mechanisms of Liraglutide. Treatment with Liraglutide and other GLP1-A may reduce EAT inflammation and revert EAT to its physiological function and therefore to a cardioprotective role. From a clinical perspective, this study proposal can provide data that will open new avenues and have an important impact in the management of CAD. Liraglutide may become an effective treatment of patients with CAD and diabetic cardiovascular complications. EAT can be easily measured, as we first developed, and then targeted by Liraglutide, other GLP-1A or other anti-inflammatory drugs in patients with CAD.

Innovation This proposal contains a number of novel concepts with important clinical applications. For the first time this project will evaluate the idea that GLP-1 activation may reduce EAT inflammation in subjects with T2DM and CAD. This is will be the first time that liraglutide effects will be evaluated on EAT samples obtained from subjects with T2DM and CAD. These important points will be addressed in well-studied population of subjects, as was never done before. Based on pre-existing data, the expected liraglutide effects on the epicardial adipose tissue inflammatory genes, primary endpoint, may not necessarily be time dependent. The shorter term of treatment that may occur in some patients may actually rule out the potential confounding effect of the liraglutide-associated weight loss on the study outcomes

Study Rationale Liraglutide, may have properties that go beyond the glucose-lowering effects, as the recent LEADER trial showed that Liraglutide reduces the risk of major cardiovascular events. Hence, our timely proposal will help the understanding of the

mechanisms of the cardio-protective effects of Liraglutide. As our preliminary data are showing that a) EAT is highly inflammatory fat depot affecting the coronary arteries, b) Liraglutide causes a massive and rapid reduction of EAT thickness, c) EAT expresses GLP-1R our hypotheses can be likely proven in this study. Our proposal would provide stronger evidences for using GLP-1As in the treatment of CAD. In addition, as EAT is an easily measurable and modifiable risk factor, as we first developed, it can be targeted by Liraglutide. The randomized, double-blind, placebo-controlled study design will allow for an independent and unbiased effect of Liraglutide on study results. In addition, actions to equalize weight loss and maintain similar glucose control between study arms will rule out the confounding effects of these factors on the study outcomes.

Specific Objectives

Primary Objective:

- to test the hypothesis that liraglutide can reduce the pro-inflammatory transcriptome profile of EAT of subjects with T2DM and CAD.

Secondary Objectives:

- to assess the hypothesis that liraglutide will reduce the ultrasound measured EAT thickness in subjects with T2DM and CAD.
- to evaluate the hypothesis that liraglutide can produce more anti-inflammatory effects in the EAT as comparted to SAT of subjects with T2DM and CAD.
- to test the hypothesis that EAT mRNA and protein expression of GLP-1R will be greater in subjects receiving liraglutide as compared to those continuing their standard therapy

Research Design and Methods

Study Hypotheses

Hypothesis for Primary Objective: We hypothesize that EAT inflammatory gene mRNA and protein expression will be lower in subjects receiving additional therapy with liraglutide as compared to those who will continue with their current treatment.

Hypothesis for Second Objectives: based on our biomolecular and clinical preliminary data, we hypothesize that a) EAT thickness will respond and decrease more on liraglutide than on standard therapy, b) inflammatory genes and protein expression will be different in EAT *vs* SAT c) EAT GLP-1R expression will be greater in patients on liraglutide *vs* those on standard therapy.

Endpoints

Primary Endpoint:

- Change from baseline in EAT inflammation (measured as mRNA and protein expression of TNF-α and IL-6) within a minimum of 3 and up to 12 weeks of treatment

Secondary Endpoints: Change from baseline within a minimum of 3 and up to 12 weeks of treatment for the following parameters:

- Ultrasound measured EAT thickness
- SAT inflammation (measured as mRNA and protein expression of TNF-α and IL-6)
- EAT GLP-1R (measured as mRNA and protein expression of GLP-1R)

Study Type

This is an Investigator Sponsored Study (ISS).

This will be a 12-week randomized, double-blind, placebo-controlled interventional study in 40 patients with T2DM and CAD with an acceptable glycemic control on their current diabetes regimen who require elective coronary artery bypass grafting (CABG) regardless of their participation in the study.

A minimum time frame of 3-week treatment will be considered to detect significant changes in the study endpoints. If not all subjects would be able to complete the 12 weeks, study outcomes will be evaluated at the 3 week time point.

Study Design Study subjects will be randomized in two groups of 20 patients to receive additional liraglutide, (L-group) or to remain on current treatment or placebo (D-group).

- L-group will be started on liraglutide. Liraglutide will be started and administered for 12 weeks prior to CABG with a starting dose of 0.6 mg (after a least one week) and subsequent increments to 1.2 mg (after a least one week) and to 1.8 mg (after at least a week on 1.2 mg). The dose of 1.8 mg daily will be maintained until the end of the 12-week study. Other and current diabetes treatment will be continued. In those study participants who may be impacted by CABG scheduling and COVID-19, and cannot complete the 12-week treatment, we will consider a minimum time frame of 3 weeks to detect significant changes in the study primary endpoints.
- D-group: placebo will be administered in addition to current treatment prior to the CABG with a starting dose of 0.6 mg (after a least one week) and subsequent increments to 1.2 mg (after a least one week) and to 1.8 mg (after at least a week on 1.2 mg). Patients will be started on a supervised diet to achieve approximately 5% of weight loss between 3 and 12 weeks. In those study participants who may be impacted by CABG scheduling and COVID-19, and cannot complete the 12-week treatment, we will consider a minimum time frame of 3 weeks to detect significant changes in the study primary endpoints.

  Ultrasonographic measurement of EAT thickness will be performed at baseline and after 12 weeks in all patients, regardless of the study arm.

  All groups will receive lifestyle and diabetes education, and supervised diet as part of the standard care, to insure similar glycemic control between arms and weight loss. Study patient will be also required to check and log their finger stick

blood glucose twice daily (fasting before breakfast and at bedtime) and report the readings to the study coordinator on a bi-weekly base.

Rationale for study Design

The randomized, double-blind, placebo-controlled study design will allow for an independent and unbiased effect of liraglutide on study results.

Rationale for minimum study time line of 3 weeks.

The pre-operative clinic schedule may not always allow to wait the full 12 weeks between the patient's enrollment and the CABG, standard of care procedure. Dr Lamelas is the only cardiac surgeon at UM performing elective CABGs. Once the patient is randomized and randomly allocated to one of the study arms, the length of the treatment is set by the date of standard of care CABGs. During the current COVID-19 pandemic, the number of elective CABGs has been reduced and we cannot reschedule or delay the surgery

Study Population: Study group will be formed by 40 patients with T2DM and with clinically and angiographically stable CAD who will undergo CABG surgery, as part of their standard medical care. Cardiac surgery will be elective procedure in hemodynamically stable patients taking their standard cardiac treatments and under the care of the cardiologist.

Study team and site: Dr Iacobellis is Professor of Medicine, Division of Endocrinology, Diabetes and Metabolism. Dr Iacobellis pioneered EAT echocardiographic measurement and he is considered the leading expert in the epicardial fat. Dr Iacobellis will lead and supervise liraglutide therapy, EAT measurement, analysis and interpretation of the data. Dr Frasca, Research Assistant Professor, Dept. Microbiology and Immunology, will lead and supervise adipose tissue analysis. Dr Claudia Martinez is associate professor of Cardiology and contributes to patient recruitment. Dr Joseph Lamelas, professor of cardiothoracic surgery will be the cardiac surgeons for this study. Low calorie diet (LCD) and other nutritional aspects will be managed and supervised by the Division of Endocrinology registered dietitian (RD), as part of the standard care. University of Miami has been the site of several National Health Institute (NIH) studies and many peer-reviewed publications. The Division of Endocrinology Diabetes and Metabolism is fully equipped and trained to visit, follow up and manage patients with diabetes. The Division of Endocrinology provides multiple outpatient diabetes clinics at the Diabetes Research Institute (DRI). Division of Endocrinology clinical laboratory is fully accredited and equipped to perform state-of- the-art diagnostic tests assisting in the diagnosis and management of diabetes.

Inclusion Criteria

- T2DM as defined by American Diabetes Association (ADA) criteria
- Adult patients with T2DM who are indicated to receive liraglutide, not as first-line therapy, in addition to diet and exercise to improve glycemic control
- Hemoglobin A1c (HbA1c) ≤ 9%
- Age ≥ 18 years old
- Body mass index (BMI) ≥ 27 Kg/m^2 and/or waist circumference ≥ 102 cm (40 inches) in men and 88 cm (35 inches) in women, respectively.
- Clinically and angiographically stable CAD who requires CABG as part of the standard medical care, as CAD does not represent a contraindication for using liraglutide. The stability of the CAD further warranties that study patients will not be exposed to higher risk by using liraglutide.

Exclusion Criteria

- Patients with a personal or family history of medullary thyroid carcinoma or patients with Multiple Endocrine Neoplasia syndrome type 2
- Patients with a prior serious hypersensitivity reaction to liraglutide
- Other contra-indications to liraglutide in accordance with risks and safety information included in the latest updated prescribing information
- Type 1 diabetes, as defined by ADA criteria
- Current use of other GLP-1A, dipeptidyl peptidase 4 (DPP4) or Sodium Glucose transportes 2 (SGLT2) inhibitors, thiazolidinediones (TZDs), pramlintide and fixed prandial insulin.
- Patients with unstable CAD, assessed by the Cardiology team and defined as new onset angina, rest angina, rapidly increasing or crescendo angina
- History of diabetic ketoacidosis, pancreas or beta-cell transplantation, or diabetes secondary to pancreatitis or pancreatectomy; acute or chronic infective diseases, cancer or chemotherapy, history of pulmonary, renal or liver diseases, and drug abuse
- Patients with chronic and acute inflammatory conditions such as sepsis, rheumatoid arthritis, ectopic dermatitis, asthma, ulcerative colitis.
- Current use of systemic corticosteroids in the 3 months prior this study.
- Pregnant women
- Women of childbearing potential who are not using adequate contraceptive methods (as required by local law or practice)

Investinational drug

Liraglutide [rDNA origin] injection, solution for subcutaneous use. Liraglutide will be prescribed according to the same, current indications of the marketed Victoza.

Withdrawal Criteria

1. The subject may withdraw at will at any time.
2. The subject may be withdrawn from the trial at the discretion of the investigator due to a safety concern or if judged non-compliant with trial procedures or included in contravention to the inclusion and/or exclusion criteria.
3. Subject diagnosed with acute pancreatitis by clinical and/or radiographic criteria.
4. Pregnancy, positive pregnancy test prior to the enrollment or intention to become pregnant.
5.
6. Subjects who will not tolerate liraglutide dose increase to 1.2 or 1.8 mg will remain in the study at the highest tolerated dose

Subject Replacement

There will be no replacement of subjects in this trial.

Rationale for Study Population

We anticipate that approximately 400 patients will be screened in a 2-year period. We will recruit patients with T2DM and clinically and angiographically stable CAD who will undergo CABG surgery, as part of their standard medical care. Cardiac surgery will be elective procedure in hemodynamically stable patients taking their standard cardiac treatments and under the care of the cardiologist.

Recruitment strategy: Participants will be recruited among the outpatient population who routinely refer to the Division of Cardiothoracic Surgery (Dr Lamelas and collaborators), Division of Cardiology (Dr Martinez) and Division of Endocrinology, Diabetes and Metabolism outpatient clinics (Dr Iacobellis), at University of Miami.

Visit Procedures

Study Timetable: This study will consist of two clinical visits prior the cardiac surgery and adipose tissue collection during the surgical intervention. Consecutive subjects with T2DM and CAD who are candidate for elective cardiac surgery regardless of their participation in the study will be selected, informed and consented. Patients suitable to wait 12 weeks for cardiac surgery will be identified by the cardiology team and accordingly scheduled, as part of the standard care. Anthropometrics, blood draw for laboratory measures and echocardiographic measurements of EAT thickness will be performed at baseline and before cardiac surgery that will be scheduled 12 weeks after study enrollment and treatment. Adipose tissue collection will be collected during the cardiac surgery.
Study outcomes will be collected at 3 weeks in both groups in those patient who may not be able to complete the 12- week treatment due to CABG logistics and COVID-19 impact on scheduling

Assessments for Efficacy

Adipose tissue collection: EAT biopsy samples (average 0.5-1.0 g) will be taken, before heparin administration, near the proximal tract of the right coronary artery. Each tissue sample will be snap frozen in liquid nitrogen and stored at −80 °C until analysis in a freezer. The subcutaneous fat tissue (SAT) will be harvested at the site of the thoracic wound and preserved in the same manner.

Gene expression: in freshly isolated mature adipocytes (from EAT and SAT), extraction of total RNA, quantification of mRNA, c-DNA generation qRT-PCR will be performed for measuring expression of the inflammatory genes (TNF-α, IL-6) and GLP-1R.

Western Blot: Total proteins will be extracted from frozen EAT and SAT adipocytes and blotted for TNF-α, IL6, as previously described.

In addition to the mRNA expression of GLP1R, we will also measure protein expression of GLP-1R by western blotting. Total cell lysates from the EAT will be obtained as follows. Cells will be resuspended in M-PER (Mammalian Protein Extraction Reagent, Thermo Scientific), incubated on ice for 20 min, sonicated for a few seconds and then centrifuged (14,000 rpm, 15 min, 4°C) to obtain protein lysates which will be stored at -80°C until use. Protein content will be determined by Bradford assay before western blotting runs. Antibodies will be anti-GLP1R Mab 3F52 (Novo Nordisk) and anti-GAPDH (GeneTex) as loading control.

Immunofluorescence

To confirm GLP-1R protein expression, immunofluorescence will be performed on EAT and SAT samples using Mab 3f52 against GLP-1R, as previously described by Pyke et al [23-24]. This mouse monoclonal antibody has been extensively validated to show its specificity. Due to the relatively low abundance of GLP-1R the immunofluorescence will be performed using a tyramide signal amplification reagent (TSA kit#26 from Molecular probes) according to the manufacturer instructions. In summary, cryosection of the samples will be performed at -35 °C with 12 μm thickness using a Leica CM1850 UV cryostat (Wetzlar, Germany). The sections will be fixed for 20 min with 4% PFA, washed with PBS and dried at room temperature overnight. Sections will be washed 3 x with PBS and incubated with PBS with 3% BSA and 0.4% triton X for 1 hour. Sections will then incubated with Mab 3f52 (1:50 dilution) antibody overnight in PBS with 3% BSA and 0.4% triton X. Sections will be then washed 4 times with PBS and incubated with biotinylated anti mouse IgG for 1 hour at room temperature, washed 4 times with PBS, then incubated at room temperature with horseradish peroxidase-streptavidin for another hour, washed 4 times with PBS and then incubated at room temperature with alexa fluor 647 tyramide for 10 min. Sections will be further washed 3 times and incubated with DAPI (Thermo fisher, Waltham, MA) for 30 min. The samples will be washed 3 times with PBS and mounted on Mowiol 4-88 (Sigma-Aldrich, St Louis, MO). The tyramide signal can amplify from 10 to 200 times the immunofluorescence signal from low expressed proteins (Molecular probes, Eugene, OR). The fluorescence images will be acquired using a Zeiss LSM 710 confocal microscope (Oberkochen, Germany).

Echocardiographic EAT Thickness:

Echocardiogram for EAT thickness measurement will be performed at baseline and after 12 weeks in all patients, regardless of the study arm. EAT thickness will be measured according to the method firstly described and validated by Iacobellis [7-8]. Briefly, EAT will be identified as the echo-free space between the outer wall of the myocardium and the visceral layer of pericardium. EAT thickness will be measured perpendicularly on the free wall of the right ventricle at end-systole in three cardiac cycles. Parasternal long views allow the most accurate measurement of EAT on the right ventricle, with optimal cursor beam orientation. Maximum epicardial fat thickness will be measured at the point on the free wall of the right ventricle along the midline of the ultrasound beam, perpendicular to the aortic annulus, used as anatomical landmark for this view. The average value of three cardiac cycles will be considered.

Reliability of echocardiographic measurement of EAT

Reliability of echocardiographic measurement of EAT will be assessed by the intra-class correlation coefficient. Inter- and intra-observer reproducibility will be evaluated by the intra- class correlation coefficient in all subjects. Echocardiograms will be read by Dr Iacobellis and an experienced cardiac imaging technician. Previously published studies have shown that intra- and inter-observer reproducibility of epicardial fat measurement was excellent. Intra-class correlation coefficients varied from 0.90 to 0.98 and from 0.93 to 0.98, respectively indicating good reproducibility and reliability. Both readers will be blinded to the subjects' clinical data.

Anthropometrics and Clinical measures: As standard of care, height (in cm) and weight (in kg) will be measured, and BMI will be calculated. Waist circumference (in cm or inches) will be measured as the minimum circumference between the lower rib margin and the iliac crest. Blood pressure will be also measured.

Blood Measurements: Lipid panel, lipase, and amylase are not study end-points and will be collected if made available through a standard of care procedure at baseline and after 4 or 12 weeks.

Low calorie Diet (LCD) A low calorie diet program will be prescribed to study patients and controls. A RD who will reach out to patients to insure adherence to the dietary program will supervise the LCD. This will help patients to achieve approximately 5% of weight loss between 3 and 12 weeks.

Assessments for Safety

Potential Risks to the Subjects

The risks of this study are minimal are mainly related to risks associated with the investigational drug liraglutide, echocardiogram, blood draw, and a potential breach of confidentiality.

Drug therapy: liraglutide, (Victoza®), is a GLP−1A indicated as an adjunct to diet and exercise to improve glycemic control in adults with T2DM. Liraglutide is taken once daily at any time of day, without regard to the timing of meals. Liraglutide is injected

subcutaneously in the abdomen, thigh, or upper arm. Liraglutide may cause mild and usually well-tolerable gastro-intestinal side effects (5%), such as nausea, constipation. Liraglutide is contraindicated in patients with a personal or family history of medullary thyroid cancer and in patients with Multiple Endocrine Neoplasia (MEN). Based on spontaneous postmarketing reports, acute pancreatitis has been observed in patients treated with liraglutide. Subjects will be instructed to contact the research staff if they develop new symptoms on the drugs. In case that an acute pancreatitis will be suspected, lab and imaging tests will be ordered. Marked or severe side effects will necessitate discontinuation of study drug.

Echocardiography: Diagnostic ultrasound imaging has been used in routine medical care in various forms for over 45 years. Over this period of time, no harmful effects have been identified from its use. Mild discomfort when the probe is pressed against the chest may be experienced, but otherwise there are no known risks.

Adipose tissue collection: Intra-operative collection of a very small sample of adipose tissue do not represent a clinical relevant risk to the study subjects

Blood draw: Bruising at the intravenous puncture site may occur which will clear up in a few days. Rarely the skin or vein at the site can become infected. A total of approximately 12 cc of blood will be drawn during the course of each of the patient visits which poses no significant risk to a subject who is not severely anemic.

Confidentiality: Patient confidentiality is an important issue in any clinical research study. All stored blood will be deidentified using established protocols. Subject’s name will not be used in publications or data analyses nor will they be available for discussion by any investigators other than the treating physicians.

PROTECTION AGAINST RISKS

The inclusion and exclusion criteria were designed to limit the possibility of adverse events. Any adverse event will be reported to the sponsor and Institutional Review Board (IRB). Subjects will be informed that they are free to withdraw at any point during the study. We will follow safeguards to minimize the risk to our subjects: a) we will carefully monitor response to medical treatment every 2 weeks by telephone contact, b) as standard clinical procedure women of reproductive age who are sexually active will undergo a urine pregnancy tests prior to participation in the study, c) female subjects whom are pregnant, breast-feeding, or not willing to use appropriate contraception at time of enrollment will not be included in the study. Participation in a research study introduces some inherent loss of privacy. However, full measure will be taken to protect the integrity of patient identifying information. All data used in the analysis and reporting of this evaluation will be without identifiable reference to the patient. Data used for reports or publications will never include identifiable information such as name, date of birth, social security number, address, or medical record number and in most cases will not include any identifiers other than sex, age, race, and diagnosis.

Scheduling CABG timing

In patients with stable CAD found to have CAD anatomy that requires CABG, the timing and schedule for revascularization will be determined by Dr Lamelas. Patients suitable to wait between 3 and 12 week- period for revascularization will be identified and managed with optimal medical therapy, and scheduled for complete revascularization with CABG.

Managing patients becoming unstable or developing acute events during the study

The inclusion and exclusion criteria will limit the possibility of recruiting patients with unstable CAD/angina, as defined before. Study patients showing unstable angina or developing acute cardiac events will be transferred promptly to the local emergency department for evaluation and treatment. If patient status becomes acute and emergent, the adverse event will be reported to the IRB.

Unblinding

The investigator will follow the study's randomization procedures. The blind should ordinarily be broken for serious and unexpected adverse experiences that are associated with the use of a drug to determine if there is a reasonable possibility that the experience may have been caused by the drug. Certain adverse events such as ***known consequences of the underlying disease under investigation*** or events common in the study population generally should ***not*** require unblinding.

We feel that in the not likely case (given the inclusion criteria) that a study patient will become unstable or develop acute events during the study, this event would more likely fall in the ***known consequences of the underlying disease under investigation*** rather than caused by the study drug, and therefore should ***not*** require premature unblinding. Nevertheless, it will be upon the principal investigator's best judgement and discretion to

break the blind. In this case, the principal investigator will promptly document and report to the IRB and Novo Nordisk any premature unblinding.

Subject Compliance

We estimate that the majority of the participants who will be considered eligible will complete the study. Attrition can be estimated as lower than 10%. In the event that participants are lost to follow-up, the research coordinator will contact with the study participant by telephone. The research coordinator will be trained in effective telephone technique to maximize recruitment success. All groups will receive lifestyle and diabetes education, as part of the standard care, to insure similar glycemic control between arms. Study patients will be advised to monitor their capillary glucose twice daily, fasting in the morning and post-prandially. Glucose profile will be tracked bi-weekly by the research coordinator by email or telephone.

Statistical Considerations

POWER ANALYSIS

The statistical power (two-sided, α=0.05) of the study was calculated to detect statistically and clinically meaningful differences in the primary endpoint between the two study arms. Our preliminary data showed that EAT inflammatory genes, including IL-6 and TNF-α are upregulated in subjects with T2DM and CAD and higher in EAT as compared to SAT [4-17]. From our recently published study, we calculated the average coefficient of variance (CV) for differential inflammatory genes to be 42%, but for only 5 T2DM patients. [17] As there are no data on the effects of liraglutide on EAT inflammation, the power analysis was based on our previous data and on the assumption that the difference in EAT TNF-α and IL-6 expression between liraglutide treated and placebo treated patients would be clinically meaningful at 33% (approx. 1.3X fold). Based on these data, we propose to sample 40 individuals. There will be 20 on liraglutide plus standard therapy versus 20 subjects on liraglutide-matching placebo plus standard therapy contributing EAT and SAT samples collected during cardiac surgery. Using the power set at 90% and α=0.05, and given the CV of 42%, this sample size will provide adequate power to detect the clinically significant expected difference (33%) in EAT TNF-α and IL-6 between the two study arms.

Based on pre-existing data, this sample size will provide adequate power to detect statistically significant differences in EAT TNF-α and IL-6 between the two study arms in those patients who will receive treatment only for 3 weeks. The difference in EAT TNF-α and IL-6 between liraglutide treated and placebo treated patients is expected to be approximately 1X fold after 3 weeks of treatment.

The larger sample proposed in this current project would likely reduce the variability. To further assure consistency of results, mRNA and protein expression values will be log-

transformed prior to statistical analyses unless they will be normally distributed. Housekeeping genes will be also used for both RT-PCR and western blot analyses.
In those patients who will not complete the 12-week treatment, the analysis of EAT genes from the samples will be time adjusted

Statistical power was also calculated to detect statistically significant differences in the ultrasound measured EAT thickness, secondary endpoint, between the two study arms Our group very recently showed that additional therapy with liraglutide for 12 weeks caused a reduction in ultrasound measured EAT from 9.6±2 to 6.2±1.5 mm ($p< 0.001$) in 54 patients with T2DM [11]. Given the reference value and the expected difference in EAT, the statistical power of the study (two-sided, $\alpha=0.05$) was calculated.

Outcome	**Reference value±SD**	**Expected Difference**	**Detectable difference with 80% power α=0.05**	**Detectable difference with >99% power α=0.05**
EAT (mm)	9.6±2	-20-or -30%	-20% (absolute difference = 1.9)	-30% (absolute difference = 2.88)

Assuming a difference of approximately 2 mm (20%) in EAT to be clinically significant (based on our recent and previously published data [10-14]), a convenience sample of 20 individuals for each group will therefore provide at least the statistical power (80%) to detect an expected difference of at least -20% in the EAT before and after treatment with liragutide. Baseline standard Deviation (SDs) of 2 is expected to lower to approximately 1.5 for the expected change. The expected change of SD will further assure significant statistical power of the comparative test.

In those study patients who will not be able to complete the 12-week treatment, we anticipate to see a 15% of EAT reduction after 3 weeks in patients who will be allocated to liraglutide. This anticipated 15% of EAT reduction will remain clinically significant. For example, a EAT thickness of 10 mm may likely decrease to 8.5 mm after 3 weeks of treatment with liraglutide, a clinically meaningful change for the patient cardio-metabolic profile.

This sample size also considers the difficulties with recruitment in the real world clinics. Based on our previous studies in humans, some participants may be lost to follow-up.

Student's t-test or non-parametric (Mann–Whitney) tests, depending on whether the genes mRNA expression and protein present or not a normal distribution, will be used to compare the effects of treatment (liraglutide versus placebo) on the variables measured. Student's t-test will be also used to compare ultrasound measured EAT thickness before and after treatments. Continuous variables will be considered as age-adjusted, sex-adjusted and weight-adjusted means with their SDs or median, if values are skewed.

Univariate regression models will be performed to assess the correlation between clinical variables, including echocardiographic EAT thickness and blood tests, and EAT gene and protein expression. Two-tailed $p < 0.05$ indicates statistical significance.

Data Handling and Record Keeping

Each of the dedicated clinic exam rooms provides direct access to the clinical research study electronic data capture (EDC) eVelos system provided by the Clinical Research Informatics and Data Management Unit of the Center for Health Informatics and Bioinformatics at University of Miami (UM). The investigator and research coordinator will collect and manage the data.

Data collection records with personal identifiers will be stored in locked file cabinets, at Dominion Tower suite 805, 1400 NW 10th Ave, Miami, FL, 33136. Blood samples drawn and stored in conjunction with this study will not be labeled with information that could directly identify study subjects. Presentation of the study results at scientific meetings or in publications will not identify subjects. Access to research and confidential records will be limited to clinical investigators, research coordinators, and the IRB at UM.

Adipose tissue samples storing

Each adipose tissue sample will be snap frozen in liquid nitrogen and stored at −80 °C until analysis in a freezer, in Dr Frasca's lab, University of Miami, Dept of Microbiology, 1600 NW 10th Ave Miami FL 33136-1015. Once the sample is taken, it will be unlinked from patient's name. This will assure confidentiality and anonymity. Current or future research on adipose tissue samples will need to be approved by the local IRB.

Ethical Considerations

The study has been approved by the UM local institutional review board (IRB). The study will be conducted in accordance with the Declaration of Helsinki. The study will be conducted in accordance with the ICH GCP guidelines. The investigator will comply with all applicable regulatory and legal requirements, ICH GCP guidelines and the Declaration of Helsinki in obtaining and documenting the informed consent**.**

Informed Consent: Eligible and willing participant will be approached by the investigators or the research coordinator and consented. Written informed consent form will be obtained from the subjects before "baseline" activities begin. Each patient will sign and date the informed consent. As standard procedure, patients will be informed of the risks and complications related to cardiac surgery, regardless of their participation in the study.

Study Schedule

Expected milestones:

- start of study: 09/01/2017

- first patient first visit 09/01/2017
- last patient last visit 10/31/2023
- Planned completion of integrated final study report 12/2023

Study Drugs and Materials

Trial drugs

Trial drugs will be supplied as liraglutide and matching liraglutide-placebo pre-filled pens for subcutaneous injection, provided by Novo Nordisk. Pre-filled, multi-dose pen for subcutaneous injection delivers doses of 0.6 mg, 1.2 mg, or 1.8 mg (6 mg/mL, 3 mL).

Labelling of trial drugs: All the pre-filled pens will be labeled "liraglutide/placebo"

Trial drugs supply Trial drugs (liraglutide and matching placebo) will be supplied to each study subject by Novo Nordisk.

Clinical Supply in Novo Nordisk Headquarter will revert with timeline for delivery of trial drugs when protocol is approved, and Novo Nordisk Headquarter will receive a signed contract and other additional information. Standard delivery is 4 months.

Storage and Drug Accountability of Study Medication

The investigator will ensure the availability of proper storage conditions and record and evaluate the temperature. Prior to first use, trial drugs will be stored in a refrigerator between 36°F to 46°F (2°C to 8°C). After initial use of the trial drug pen, the pen can be stored for 30 days at controlled room temperature (59°F to 86°F; 15°C to 30°C) or in a refrigerator (36°F to 46°F; 2°C to 8°C). Trial drugs will be not dispensed to any person not enrolled in the study. Unused trial drugs will be stored separately from used trial medication.

Randomization and Blinding

This will be a double-blind, parallel group, placebo controlled study. The method of allocation generation will be a computerized random-number generator. The sequence will be generated by the process of restricted randomization. Computer-based randomization process will be managed by the UM Research pharmacy.

Concomitant Illnesses and Medications

Background medications:

Metformin. Metformin is considered background medication (non-investigational medicinal product) and will not be provided during the trial. Dose adjustments could occur during the trial at the investigator's discretion. As Iacobellis et al [12] recently showed no significant effects of metformin on EAT, we do not anticipate that metformin dose adjustment may influence the study outcomes.

Long acting insulins. Insulin is considered background medication (non-investigational medicinal product) and will not be provided during the trial. The total daily dose of insulin could be adjusted during the trial at the investigator's discretion. Iacobellis' group recently performed a 24-week interventional study to compare EAT changes in insulin-naïve

inadequately controlled patients with type 2 diabetes following basal insulin initiation with detemir vs. glargine. Not significant changes in EAT between detemir and glargine were observed. [21] Based on these findings we do not anticipate that insulin treatment may influence the study outcomes.

Sulfonylureas. Sulfonylurea treatment is considered background medication (non-investigational medicinal product) and will not be provided during the trial. During the study, no up-titration of sulfonylurea dosage will be allowed. Dose reduction of SU due to hypoglycemia may be allowed at the investigators discretion. In the event of hypoglycemia, the dose of sulfonylurea can be reduced or the drug can be stopped at the investigator's discretion.

Adverse Events

In the case of an adverse event (AE), the investigator will comply with all local legal, regulatory, and IRB requirements. The investigator will report to IRB all adverse events including not serious and serious adverse events (SAE), suspected unexpected serious adverse reactions (SUSARs), serious adverse drug reactions (SADRs). The investigator will report all SAEs, SUSARs, and SADRs at the same time such events are reported to regulatory authorities or within 15 days from the investigator becoming aware of such adverse events, whichever comes first.

The investigator will use the approved Updated Prescribing Information for Victoza®. The investigator will collect the following information at minimum for each of these events: 1. Study name2. Patient identification 3. Event (preferably a diagnosis) 4. Drug 5. Reporter identification. 6. Causality 7. Outcome

Definitions An AE is any undesirable medical event occurring to a subject in a clinical trial, whether or not related to the trial product(s). This includes events reported from the first trial related activity after the subject has signed the informed consent and until post treatment follow-up period as defined in the protocol. The following should not be recorded as AEs, if recorded as medical history/concomitant illness at screening:• Pre-planned procedure, unless the condition for which the procedure was planned has worsened from the first trial related activity after the subject has signed the informed consent • Pre-existing conditions found as a result of screening procedures

Clinical Laboratory Adverse Event: A clinical laboratory AE is any clinical laboratory abnormality regarded as clinically significant i.e. an abnormality that suggests a disease and/or organ toxicity and is of a severity, which requires active management, (i.e. change of dose, discontinuation of trial product, more frequent follow-up or diagnostic investigation).

Serious Adverse Event (SAE): A serious AE is an experience that at any dose results in any of the following:

• Death

• A life-threatening* experience

• In-patient hospitalization or prolongation of existing hospitalization

Suspicion of transmission of infectious agents must always be considered an SAE
• A persistent or significant disability/incapacity
• A congenital anomaly/birth defect
• Important medical events that may not result in death, be life-threatening*, or require hospitalisation may be considered an SAE when, based upon appropriate medical judgement, they may jeopardise the subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition
*The term life-threatening in the definition of SAE refers to an event in which the subject was at risk of death at the time of the event. It does not refer to an event which hypothetically might have caused death if it was more severe.
SADR**:** an adverse drug reaction (ADR) is an adverse event for which a causal relationship (Possible/Probable relation) between the study drug and the occurrence of the event is suspected. The ADR should be classified as serious if it meets one or more of the seriousness criteria.
Medical Events of Special Interest (MESI): A MESI is (1) a medication error (e.g. wrong drug administration or wrong route of administration) or (2) a suspected transmission of an infectious agent via the product
Non-Serious Adverse Event: A non-serious AE is any AE which does not fulfil the definition of an SAE. Severity Assessment Definitions:
• Mild: Transient symptoms, no interference with the subject's daily activities
• Moderate: Marked symptoms, moderate interference with the subject's daily activities
• Severe: Considerable interference with the subject's daily activities, unacceptable
Relationship to study medication Assessment Definitions:
• Probable: Good reasons and sufficient documentation to assume a causal relationship
• Possible: A causal relationship is conceivable and cannot be dismissed
• Unlikely: The event is most likely related to an etiology other than the trial product

Outcome Categories and Definitions: • Recovered: Fully recovered or by medical or surgical treatment the condition has returned to the level observed at the first trial related activity after the subject signed the informed consent• Recovering: The condition is improving and the subject is expected to recover from the event. This term should only be used when the subject has completed the trial • Recovered with sequelae: As a result of the AE, the subject suffered persistent and significant disability/incapacity (e.g. became blind, deaf, paralysed). Any AE recovered with sequelae should be rated as an SAE• Not recovered• Fatal• Unknown
Collection, Recording and Reporting of Adverse Events: All events meeting the definition of an adverse event will be collected and reported from the first trial related activity after the subject has signed the informed consent and until the end of the post-treatment follow-up period as stated in the protocol.
Follow-up of Adverse Events: During and following a subject's participation in a clinical trial, the investigator and institution will provide adequate medical care to the study subject

for any study-related adverse events, including clinically significant laboratory values related to the study. This medical care for study subjects will be provided regardless of their insurance status. All adverse events classified as serious or severe or possibly/probably related to the trial product will be followed until the subject has recovered and all queries have been resolved. For cases of chronic conditions follow-up until the outcome category is "recovered" is not required, as these cases can be closed with an outcome of "recovering" or "not recovered". All other adverse events will be followed until the outcome of the event is "recovering" (for chronic conditions), or "recovered" or until the end of the post-treatment follow-up stated in the protocol, whichever comes first, and until all queries related to these AEs have been resolved.

Pregnancy: Pregnancy test must be negative prior to be enrolled in the study, as per standard care. However, study subjects will be instructed to notify the investigator immediately if they become pregnant. The investigator will report to IRB any pregnancy occurring during the trial period. Reporting of pregnancy by investigator will occur within the same timelines described above for reporting of Adverse Events.

Precautions/Over-dosage: Precautions and procedures will be observed in the event of overdose of liraglutide provided during the study.

Liability and Subject Insurance

The investigator must state that during and following a subject's participation in trial, the investigator and his/her institution will provide adequate medical care to the study subject for any study-related adverse events, including clinically significant laboratory values related to the study. This medical care for study subjects will be provided regardless of their insurance status.

The investigator will be responsible for the conduct of the study and agrees to defend, indemnify, and hold harmless Novo Nordisk, any of its parent companies, affiliates, or subsidiaries, and their respective officers, directors, employees, agents, representatives, distributors, salespersons, customers, licensees, and end-users from and against any claim, suit, demand, loss, damage, expense or liability imposed by any third party arising from or related to: (a) any breach of investigator's obligations; or (b) investigator's negligent or grossly negligent use or willful misuse of the study drug, the results, or services derived there from. This indemnification shall not apply in the event and to the extent that a court of competent jurisdiction or a duly appointed arbiter determines that such losses or liability arose as a result of Novo Nordisk's gross negligence, intentional misconduct, or material breach of its responsibilities.

Publication Plan

Data generated from the study will be published in highly ranked peer reviewed scientific journals, such as Diabetes, Diabetes Care, Obesity or Circulation and presented at national and international meetings, such as ADA 2018-19, ENDO Society 2018-19 and American

Heart Association 2018-19. Novo Nordisk will have any manuscripts for publication for review with a right to comment.

The investigator will register the study with a publicly assessable database such as clinicaltrials.gov.

REGULATORY CONSIDERATIONS

We plan to conduct the trial under an IND exemption. After reviewing 21 CFR 312.2(b)(1) and FDA's "'Guidance for Clinical Investigators, Sponsors, and IRBs Investigational New Drug Applications (INDs) Determining whether human research studies can be conducted without an IND, September 2013", we believe an IND exemption is applicable to this study as it meets all the criteria, as reported in the table:

857 21CFR 312.2 (B) EXEMPTIONS

858 **(1) the clinical investigation of a drug product that is lawfully marketed in the**
859 **united states is exempt from the requirements of this part if all the following**
860 **apply:**
861

(i) The investigation is not intended to be reported to FDA as a well-controlled study in support of a new indication for use nor intended to be used to support any other significant change in the labeling for the drug;	**Meets criteria**. This clinical trial is not intended to be reported to the FDA in support of a new indication or change in Victoza labeling.
(ii) If the drug that is undergoing investigation is lawfully marketed as a prescription drug product, the investigation is not intended to support a significant change in the advertising for the product.	**Meets criteria**. This investigation is not intended to support a significant change in the Victoza advertising.
(iii) The investigation does not involve a route of administration or dosage level or use in a patient population or other factor that significantly increases the risks (or decreases the acceptability of the risks) associated with the product	**Meets criteria**. In this clinical trial liraglutide (and the matching placebo) will be administered according to the labeled route and dosage (Pre-filled, multi-dose pen for subcutaneous injection that delivers doses of 0.6 mg, 1.2 mg, or 1.8 mg (6 mg/mL, 3 mL). In this clinical trial liraglutide (and the matching placebo) will be used according to the indications. Liraglutide will be used in adults with type 2 diabetes (T2DM) in adjunct to diet and exercise advices and not as first-line therapy, as study patients will continue their standard diabetes therapy. Hence, liraglutide/placebo is prescribed under inclusion criteria that are the same as for marketed Victoza. Exclusion criteria for liraglutide are also the same as marketed Victoza. This investigation will not increase the risks in the study population.
(iv) The investigation is conducted in compliance with the requirements for institutional review set forth in part 56 and with the requirements for informed consent set forth in part 50;	**Meets criteria**. This study will be reviewed and approved by the University of Miami IRB Institutional Review board prior to study start and will meet with the requirements for informed consent.
(v) The investigation is conducted in compliance with the requirements of 21 CFR 312.7.	**Meets criteria**. Study Investigator, or any person acting on behalf of the investigator, will not represent in a promotional context that the study drug is safe or effective for the purposes for which it is under investigation or otherwise

	promote the study drug.

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