GLP-1 Therapy: The Role of IL-6 Signaling and Adipose Tissue Remodeling in Metabolic Response

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Protocol Title:	**GLP-1 Therapy: The Role of IL-6 Signaling and Adipose Tissue Remodeling in Metabolic Response**
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Population:	This study will enroll 26 prediabetic (otherwise generally healthy) subjects, males and female, aged 18-50 years old, from the Houston, TX area.
Number of Sites:	Single site
Study Duration:	3 years
Subject Duration:	Up to 6 months

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I. Background and Significance

Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by increased blood glucose in the context of insulin resistance. Incretin mimetics, especially the analogs of glucagon-like peptide-1 (GLP-1), have shown great promise in T2DM therapy. Their physiological effects are complex and the mechanisms underlying their short-term and long-term glucose lowering effects are incompletely understood. Obesity, a condition of adipose tissue (AT) overgrowth, predisposes to type 2 diabetes. Various cells composing white AT (WAT) execute diverse functions implicated in the metabolic syndrome (1). It has been shown that dysfunctional visceral AT (VAT) in obese individuals contributes to metabolic disease (2). In contrast, subcutaneous AT (SAT) can develop anti-diabetic properties (3, 4) when it acquires mitochondria-rich adipocytes specialized to burn lipids through adaptive thermogenesis in response to sympathetic nervous system (SNS) stimuli (5). The organ specialized in executing this function is brown adipose tissue (BAT), which has fixed anatomic locations (6-8). The "inducible/recruitable" brown-like (beige aka brite) adipocytes, arising in SAT in response to catecholamines activating β3 adrenergic receptors, are functionally similar to adipocytes in the canonical (constitutive) BAT (9, 10). Brown and beige adipocytes enable thermogenic energy dissipation, which relies on the function of UCP1 leaking protons to uncouple substrate oxidation from ATP synthesis (4, 11). Both BAT and beige fat activate energy expenditure and can counteract metabolic consequences of obesity in mice (5, 12, 13). In adult humans, the presence of BAT in the supraclavicular area has been reported (14), the presence of beige adipocytes in various depots is unequivocal (5, 15), and activation of beige adipocytes by SNS has been demonstrated upon cold exposure (16). In humans, BAT and beige fat are traditionally visualized by ^{18}F-FDG PET-CT (17-19); however, these are also present in supraclavicular fat (via presence of UCP1) in absence of positive PET status (20). While it is currently believed that GLP-1 analogs lower glucose mainly by increasing insulin production by the pancreas (21), there is accumulating evidence that incretins also signal upon the immune system (22, 23). Importantly, while evidence in humans is not yet available, incretins induce browning of AT in mice (24-28), which could contribute to its efficacy. Our parallel human / mouse studies will elucidate important GLP-1 signaling mechanisms, which may ultimately guide development of new therapeutics for diabetes and obesity.

II. Background and Innovation

Obesity is a chronic low-grade inflammatory state, and WAT dysfunction is associated with leukocyte infiltration in AT and increased signaling via key inflammatory cytokines such is interleukin-6 (IL-6) (29). Indeed, AT and plasma IL-6 levels are higher in obese and insulin-resistant individuals, compared to healthy lean controls (30, 31). Interestingly, IL-6 also exhibits anti-inflammatory effects, leading to insulin sensitization and decreased atherogenesis (32). The evolving paradigm shift is that, while chronic inflammation underlies diabetes, transient low-level inflammation stimulates beige adipogenesis, and hence may be beneficial (33, 34). The nuances of chronic versus acute IL-6 signaling are not well understood. IL-6 is secreted by various cell types, mainly leukocytes, and the IL6 receptor (IL-6R) is expressed by numerous cell types, including adipocyte progenitors. In AT, IL-6 may act in autocrine/paracrine manner to influence insulin sensitivity (35, 36). IL-6 acts as a Th2 cytokine stimulating AT macrophage proliferation and (alternative) M2 macrophage activation (37, 38) and hyperplasia (39), which may indirectly affect AT physiology. In addition, IL-6 directly activates IL-6R in preadipocytes (40) and signaling of IL-6 family cytokines via STAT3 (41) can directly commit preadipocytes to beige adipogenesis (34). *The novelty of this study is the implication of IL-6 signaling potentially mediating the effects of GLP-1 analogs*. Our preliminary data indicate that incretin mimetics affect IL-6 secretion by leukocytes and IL-6 blood level, which has not been reported before. Interestingly, chronic GLP-1 analog therapy – administered in a weight-neutral manner – also displays increased circulating IL-6. Based on this, we hypothesize that transient IL-6 secretion, mediated by GLP-1R activation in leukocytes, such as AT macrophages, promotes AT browning. In SA 1, we will confirm that incretin treatment results in AT beiging. As a candidate of molecular pathway of IL-6 induction, we will test the known mediators of GLP-1R signaling, namely PI-3K, AKT, p38, and ERK, which are upstream of NFkB, a transcription factor regulating IL-6 expression (42-46). In SA 2, we will assess direct GLP-1R signaling and indirect incretin-induced IL-6 / IL-6R signaling via STAT3 activation (47) as mechanisms underlying beige adipogenesis. In vitro studies show that GLP-1 stimulates differentiation of adipocytes (48-51) and induces AT browning (24-28). We hypothesize that a direct GLP-1R activation leading to cAMP-mediated activation of the PKA pathway promotes brown adipogenesis (45, 48, 52-55). Our study will elucidate the role of GLP-1 / IL6 signaling on the immune system and AT in AT browning. The new insights on the anti-obesity mechanisms of incretin mimetic medications from our study will help re-evaluate the repercussions of IL-6-blocking antibodies currently considered as a therapy in diabetes / obesity clinical settings (56).

Preliminary data
Prediabetic, obese humans participated in two clinical studies.
Study #1: Randomized, crossover, placebo-controlled double-blinded prospective trial. Two study visits each utilized single doses of exenatide 10 mcg subcutaneous injection or saxagliptin 5 mg orally. For each visit, subjects presented to our Clinical Research Unit (CRU) after an overnight fast. Subjects received baseline blood draw, then study medication, and then a standardized meal. Blood draw was repeated in the postprandial period. 16 subjects completed this study.
Study #2: Nonrandomized, prospective extension study with exenatide ER (Extended-Release), a slow-release incretin. Subjects self-administered exenatide ER 2 mg weekly for six weeks and then presented to the CRU after an overnight fast. Baseline blood draw was followed by a standardized meal and postprandial blood draw. Exenatide ER was not re-administered due to its long duration of action (up to 10 weeks). 7 subjects completed this study.
Results showed a plasma IL-6 increases acutely after single treatments

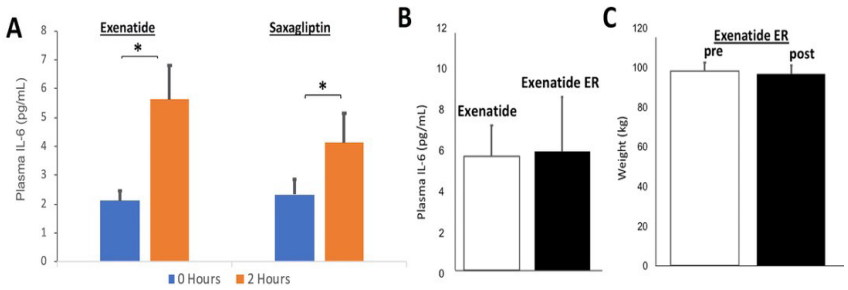

Figure 2. A) The effect of incretin mimetic therapy on plasma IL-6 levels. Prediabetic obese humans were challenged with single doses of exenatide or saxagliptin in a randomized blinded crossover trial. 2 hr after drug administration with standardized meal, plasma IL-6 levels increased in both exenatide and saxagliptin. n=16. *p<0.01 by paired t-test, B) Elevated IL-6 seen both after acute exenatide and after exenatide ER treatment. C) No weight change after exenatide ER therapy (6 weeks). n=7. Statistics was calculated using Wilcoxon signed-rank test.

per 2 types of incretin mimetics - exenatide (GLP-1 agonist) or DPP-IV inhibitor saxagliptin (Fig. 2). Elevated IL-6 levels were maintained after six weeks of treatment with exenatide ER, with no change in body weight (Fig. 2). Previous studies show that placebo does not significantly increase plasma IL-6 (57-59). Our results suggest the response to incretins is not due to AT loss but rather to GLP-1R signaling, potentially mediated by IL-6.

As our preliminary data showed that acute incretin therapy induces a transient increase in plasma IL-6, we tested if incretins induce IL-6 production by leukocytes. Treating adherent human PBMCs (mainly monocytes / macrophages) with GLP-1 analog in cell culture led to a robust increase in IL-6 secretion, comparable to that induced by LPS used as a positive control (Fig. 3).

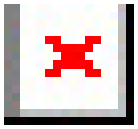

Figure 3. GLP-1 analog induces IL-6 secretion by human leukocytes. Human PBMC cells from a healthy donor were seeded at 2×10^6 cells/0.2 mL/well of RPMI 1640 containing 10% FBS onto a 48-well plate. After 24 h, medium was removed and the cells were washed with RPMI 1640 medium. Adherent cells were treated with 200ul RPMI 1640 medium containing 100ng/ml of LPS or 100nM of Liraglutide for 12 hours. The supernatants were collected and IL-6 was measured using R&D ELISA kit (Cat. #501030). **, p<0.001%, Student's t-test.

Combined, our preliminary data indicate that GLP-1R signaling induces IL-6 production by human leukocytes.

III. Objectives

Specific Aim 1: Investigate IL-6 induction and AT browning upon incretin treatment.
Hypothesis: GLP-1 analog signaling via GLP-1R induces IL-6 secretion by leukocytes.
Specific Aim 1A: Investigate IL-6 induction and AT browning upon incretin therapy in prediabetic humans.
Expected outcomes: Insulin resistance will decrease after long-term GLP-1 analog treatment. Long-term subcutaneous AT (SAT) browning will be detected by Oroboros and UCP1 expression measurement in biopsies as well as ^{18}F-FDG uptake activity with PET-CT. AT leukocytes and circulating peripheral blood mononuclear cells (PBMCs) will be established as the source of GLP-1-induced IL-6 secretion.
Specific Aim 1B: Validate mice as a model to study incretin-induced IL-6 as a mediator of AT browning.
Expected outcomes: Insulin resistance will decrease after long-term GLP-1 analog treatment in an insulin-resistant mouse model (WT DIO - wild type diet-induced obesity). AT browning will be observed long-term. AT leukocytes and circulating PBMCs will be established as the sources of GLP-1-induced transient IL-6 secretion.

Specific Aim 2: Investigate the mechanisms of incretin-induced adipose tissue browning.
Hypotheses: GLP-1 analog therapy induces adipose tissue browning via both direct GLP-1 / GLP-1R signaling and indirect incretin-induced IL-6 / IL-6R / STAT3 signaling.
Specific Aim 2A: Investigate if GLP-1 analog effects on human beige adipogenesis depend on IL-6 signaling. Human WT, IL-6R KO and GLP-1R KO preadipocytes will be subjected to treatment with GLP-1 analog, IL-6, or blocking IL-6 antibody, in cell culture.
Expected outcomes: IL-6 and GLP-1 signaling activation will synergize in inducing brown adipocyte differentiation. Brown adipogenesis will be less pronounced in IL-6R KO and GLP-1R KO than in WT cells. AT browning and incretin metabolic benefits will be reduced by a blocking IL-6 antibody co-administration during incretin treatment.
Specific Aim 2B: Investigate if GLP-1 analog effects on beige adipogenesis depend on IL-6 signaling in mice. Activation of STAT3 prior to AT beiging in SAT of GLP-1 analog-treated mice will be analyzed. A blocking IL-6 antibody injection will be used as an experimental intervention.
Expected outcomes: GLP-1 analog treatment will result in transient activation of IL-6-dependent STAT3 in SAT, which will be reduced by a blocking IL-6 antibody co-administered during incretin treatment.

These studies will delineate the novel link between GLP-1 and IL-6 signaling and its implications for AT browning. The mechanisms will serve as the basis for developing more targeted therapies for diabetes and obesity. Furthermore, revealing the effects of transient and chronic IL-6 signaling - a target of IL-6-blocking antibodies currently tested as therapeutics – may merit further clinical investigation.

IV. Study Design

Clinical studies: Our preliminary data and publications demonstrate our expertise (60-64).
Design: Single center, crossover, inactive comparator-controlled prospective trial (Fig. 4). Each subject will participate in both study arms: Cyanocobalamin (vitamin B12) 1000 mcg subcutaneous weekly x 6 weeks ("Drug A") followed by dulaglutide (titrated to 1.5 mg subcutaneous (sc) weekly) x 6 weeks ("Drug B"). There will be a three-week washout period between study arms.
Subjects: Twenty subjects (10 males and 10 females), 18-50 years old, with a BMI of ≤ 35 mg/kg^2 and prediabetes (via expanded version of ADA criteria) will participate. Individuals receiving beta blockers, corticosteroids, monoamine oxidase inhibitors, diabetes medications (including incretin mimetics and thiazolidinediones), hormonal therapy, and immunosuppressive therapy over the last 2 months will be excluded (17). If subject takes thyroid medications, these must be dosed to control hypo- or hyperthyroidism. NSAIDS and/or antioxidant vitamins will be discontinued one week prior to study initiation. Subjects using antihypertensive medications (other than beta blockers) and lipid-lowering medications will remain on stable doses for the duration of the study. Other exclusions are significant cardiac, hepatic, or renal disease, pregnancy, lactation, current tobacco use, active malignancy, history of medullary thyroid cancer, history of pancreatitis, and history of diabetes. Women of childbearing age must agree to use a method of pregnancy prevention.
Screening Visit (*Visit #1*): After subject consents to participation, we will perform a history, physical exam, and baseline lab tests to determine if subject qualifies for the study. A verbal consent will be done prior to this visit to see if subject consents to fasting (prior to screening visit) or if subject prefers to have fasting labs done before this visit. If subject qualifies, then subject will begin with the first study arm (cyanocobalamin; "Drug A").
Study Procedures: *Visit #2*: At 1-10 days after last dose of Drug A, subject will begin fasting at midnight of that day. Visit 2 must take place before Visit 3, and may be done in the morning or afternoon. Visit 2 may also be done on the same day as Visit 3 if Visit 2 is done in the morning. Subject will present to Radiology department at 8 am or 12 pm. At 9 am or 1pm, subject and study nurse will enter a room set to the temperature of 23˚ C (72˚ F) (65). Vital signs will be taken and an IV will be inserted. At 10 am or 2 pm, blood will be drawn for isolation of plasma and peripheral blood mononuclear cells (PBMCs), and isotope tracer will be administered. Lab personnel will pick up the blood, which will be immediately transported to the laboratory for processing. At 11 am or 3 pm, subject will be taken to separate room to receive PET-CT (65). After completion of PET-CT, IV will be removed and subject will be discharged. As PET-CT suite is located in a separate building from Interventional Radiology (IR), the studies will be either on separate days, or on the same day if Visit #2 is done in the morning.
Visit #3: One week after completing last dose of Drug A (±3 days, and after completing Visit #2), subject will undergo the same fasting as Visit #2. Subject will present to the Radiology department at 12 pm. At 1pm, subject will enter a room set to the temperature of 23˚ C (72˚ F). Vital signs will be taken and an IV will be inserted. The IV access is placed just in case patient experiences any adverse events requiring IV medication. At 3 pm, patient will go to IR receive CT-guided biopsy of supraclavicular adipose tissue under local anesthesia, as per previously described methods (66). Guidance from PET-CT ^{18}F-FDG uptake (from prior visit) will be used to direct the procedure. About 100-200 mg of AT collected will be split 50/50 for immediate Oroboros analysis / subsequent fixation and for -80˚C storage. A small adhesive bandage will be placed over the biopsy incision, which subject can remove at home the following morning. A three-week washout period will occur after completion of Visit #3. Then subject begin next study medication (dulaglutide; "Drug B") for another total duration of 6 weeks.
Visit #4: Identical procedures to Visit #2 after Drug B
Visit #5: Identical procedures to Visit #3 after Drug B.
The only exception is that patient will take no more study medication, and will complete the study.

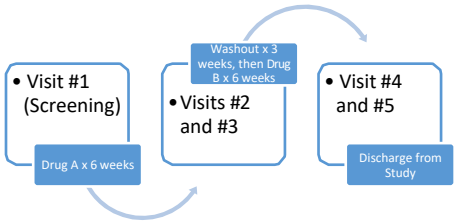

Figure 4. Study flow chart

Recruitment and Screening: A successful telephone prescreen will be followed by a verbal consent to see if (potential) subject consents to fasting prior to outpatient screening visit, or if subject prefers to have fasting screening labs done before the screening visit. We will cover the costs of all screening labs. If labs done before screening visit show disqualification, then subject will be informed (and results will be mailed to subject) and outpatient screening visit will be cancelled.

The prescreen process is followed by an outpatient screening visit (with the exception of prior disqualifying labs, as previously described). Completed consent procedures will be followed by a complete history and physical exam and blood draw while fasting for qualifying labs (complete metabolic panel, lipid panel, hemoglobin A1C, complete blood count, thyroid stimulating hormone, and urine pregnancy test (if applicable)). Subjects meeting inclusion criteria will be invited to participate in the trial. Participating subjects will be counseled on a weight-maintaining diet and instructed on study medication use. The PI will observe the subject self-inject first dose of study medication. Remainder of study medications will be administered at home by subject.

Laboratory Methods: Subcutaneous AT (SAT) will be collected via needle biopsy and stored as described (67). PBMCs will be isolated immediately upon collection, via previously described methods (68) and then stored at -80˚ C until further studies. Analysis of AT biopsies by Oroboros and measure UCP1 expression, by immunofluorescence (IF) will be performed as we have reported (14). Both SAT and PBMCs, we will be subjected to IL-6 qRT-PCR and Western blotting. Plasma IL-6 will be measured via ELISA (R&D Systems). Other cytokines, including TNF-α, IL-4, IL-10, IL-11, and IL-13, will be measured by Luminex. Plasma insulin and GLP-1 will be measured via radioimmunoassay and plasma glucose will be measured with an automated glucose analyzer. Free fatty acids (FFAs) will be measured by using a quantification kit (Wako).

Animal studies: The same methods will be used for studies SA 1B and 2B. Numerous publications demonstrate our expertise in methodology (69-75). We will analyze metabolism and AT composition in the experimental groups of mice placed on HFD at weaning and developing diet-induced obesity (DIO) mice after 4 months on HFD. The main variable in considering metabolic change will be the glucose tolerance test (GTT) and insulin tolerance test (ITT) performed as described in our previous studies (69, 72, 74, 75). Body composition will be measured by Echo MRI; blood levels of glucose, insulin, and other key metabolites will be measured as described (69, 72, 74, 75). To analyze energy expenditure, indirect calorimetry will be performed with Oxymax CLAMS (Columbus Instruments) metabolic chambers as described (69, 72, 74). Food intake will be quantified over a 3-day time course at the same time. Core body temperature and spontaneous locomotor activity will be measured upon sc sensor implantation on the upper flank by using PDT-4000 G2 E-mitter sensors (MiniMitter, Responics). We will also measure cold tolerance**/** adaptive thermogenesis by placing mice into environmental chamber as described (75). To assess AT composition, upon necropsy at the end of experiment, tissues will be recovered for IF, gene expression, and protein expression analyses. We routinely use all of these methods as reported (69, 72, 74, 75).

As evident from our published (70-76) and preliminary data, we have a set of complementary methods to analyze AT composition and function. For mouse AT analysis, VAT (mesenteric, retroperitoneal and gonadal) and SAT (interscapular and inguinal) depots will be analyzed separately to establish possible site-specificity. For each sample, we will prepare whole mounts and 10% formalin-fixed paraffin-embedded sections for IF analyses. H/E histology and IF will be used to identify UCP1+ multilocular beige from unilocular UCP1- white adipocytes (Perilipin1+). Isolectin B4 staining will be used to identify EC. For IF, cells/tissues will be permeabilized with 0.3%

Triton X-100 as needed, blocked, and then incubated with primary antibodies followed by fluorophore-labeled (Cy3 and Alexa-488) secondary antibodies. Nuclei will be stained with DAPI. Confocal microscopy will be performed using a Leica TCS SP5 microscope equipped with LAS AF software. Quantification will be performed by processing multiple microscopy fields with NIH IMAGE J software. Another portion of AT will be digested with collagenase-I / dispase and adipocytes will be separated by centrifugation. The remaining stromal/vascular cell suspension will be used to separate cell populations by fluorescence-activated cell sorting (FACS) as described with FACS Aria II / FlowJo software. Leukocytes will be isolated as $CD45^{bright}$ cells; EC as $CD34^{dim}CD31^{bright}$CD45- cells and adipocyte progenitors as $CD34^{bright}$CD31-CD45- cells as described (70, 75). IgG clones we use are anti-CD34 MEC14.7, anti-CD31 MEC 13.3 and anti CD45 30-F11.

Plasma IL-6 will be measured via ELISA. STAT3, NFkB, AKT, and ERK1/2 phosphorylation and activation will be measured as we previously described (77). Immunoblotting with Phospho-Stat3 (Ser727) antibody 9134 as well as previously described Phospho-NFkB, Phospho-ERK1/2 and Phospho-AKT antibodies from Cell Signaling. Analysis of fixed mouse tissue or cultured cells will be performed as we previously described with optimized antibodies (70, 71, 73-76). LipidTox will be used to stain lipid droplets. For IF analysis, tissues will be permeabilized with 0.3% Triton X-100, blocked in serum-free Protein Block, incubated with primary antibodies, washed, and then incubated with fluorophore-labeled (Cy3 and Alexa-488) secondary antibodies. Nuclei will be visualized by DAPI staining. Confocal microscopy will be performed using a Leica TCS SP5 microscope equipped with LAS AF software. Quantification will be performed by processing multiple microscopy fields with NIH IMAGE J software. To further quantify the extent of AT beiging, protein extracts will be made from the banked snap-frozen tissue and analyzed by Western blotting. In addition, mRNA will be isolated and RT-qPCR analysis will be performed to measure the expression of beige AT markers *Ucp1, Cidea, Pgc1α*, and *Tbx1* as routinely done in our laboratory (72, 75).

V. Statistics/Outcomes

Patients: SAT IL-6 mRNA (compared between dulaglutide and placebo treatments) will serve as the primary outcome variable. Assuming skewed distribution, we consider log-transformed IL6 mRNA (i.e., log(IL6 mRNA)) data for sample size calculation. With a correlation coefficient of 0.92 for log(IL6 mRNA) and common coefficient of variation of 0.9, power calculation given a sample size of n=20 shows that paired t-test with a 0.05 two-sided significance level will have 80% power to detect 1.2-fold difference (i.e., 20% increase) in IL-6 mRNA between two study arms. For all biochemical and molecular endpoints, we will conduct univariable and multivariable mixed effect regression models that account for within-subject correlations. All analyses will be performed primarily using widely available tools in SAS® version 9.4 (SAS Institute, Cary, NC), at a significance level of 0.05.

Animals: Based on power calculations (nQuery 5.0), 10 mice will be used for each genotype/gender/treatment/control group to see a >25% difference with a power of 80 and $p<0.05$. Quantitative biochemical and molecular endpoints, such as gene and protein expression data, will be analyzed as continuous variables using the two-tailed Student's t test or a Mann-Whitney U test for pairwise comparison with $P<0.05$ significance threshold. As needed, assistance will be received from the UTHealth Biostatistics / Epidemiology/ Research Design (BERD) Core.

Specific Aim 1: Investigate IL-6 induction and AT browning upon incretin treatment.
Hypothesis: GLP-1 analog signaling via GLP-1R induces IL-6 secretion by leukocytes.

Specific Aim 1A: Investigate IL-6 induction and AT browning upon incretin therapy in prediabetic humans. Subjects will be treated with both dulaglutide and cyanocobalamin as described in Clinical studies. To test if GLP-1 analogs signal via GLP-1R to induce IL-6 secretion by leukocytes in humans, SAT and PBMCs will be collected after treatment, as described above for Clinical studies. To assess AT browning, we will measure oxygen consumption by Oroboros and UCP1 expression as we have reported (14) as shown in Figure 5. qRT-PCR will be used to measure IL-6 expression in SAT and PBMCs. ELISA will measure IL-6 levels in plasma. qRT-PCR will measure SAT browning via expression of UCP-1, PRDM16, NDUFS3, ARDRB1, ADRB2, and ADRB3. STAT3 in SAT and NFkB phosphorylation / activation in PBMCs will be measured by Western blotting. We will also perform IF on formalin-fixed paraffin-embedded AT sections to assess UCP-1 expression and IL-6 expression in M1 and M2 macrophages. To measure insulin /glucose tolerance, plasma will be measured for IL-6, free fatty acids, insulin, glucose, HOMA-IR, GLP-1, TNF-α, IL-4, IL-10, IL-11, and IL-13. In vivo quantification of fat browning will be measured as standardized uptake value (SUV) from PET-CT

To test if GLP-1 analog treatment results in transient IL-6R / STAT3 signaling activation and brown adipocyte differentiation, we will analyze biopsies of SAT collected from humans before and after treatment, as described in Clinical studies above. STAT3 phosphorylation will be assessed by qRT-PCR and Western blotting. H/E histology and IF will be used to identify UCP1+ multilocular adipocytes as beige and unilocular UCP1-negative adipocytes as white. Perilipin-1 IF will be used to distinguish beige adipocytes from fibrotic areas (72). An example of analysis from our recent study (14) is shown in Figure 5.

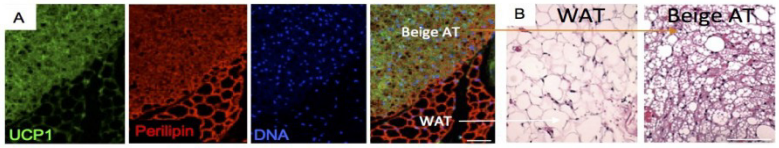

Figure 5. A: Human UCP1+ brown adipocytes identified by IF next to perilipin+UCP1- supraclavicular WAT. B: H&E staining showing that human beige adipocytes have multilocular lipid droplets. From Porter et al. 2016, Kolonin et al. 2016.

Specific Aim 1B: Validate mice as a model to study incretin-induced IL-6 as a mediator of AT browning.

Anti-diabetic efficacy of incretin mimetics has been reported in the mouse model and previously established for dulaglutide and liraglutide (28, 78, 80). Our preliminary data demonstrate that liraglutide injection results in rapid and transient increase in circulating IL-6 (Fig. 6). These data match clinical observations (Fig. 2) and indicate mice as a relevant model to study incretin effects.

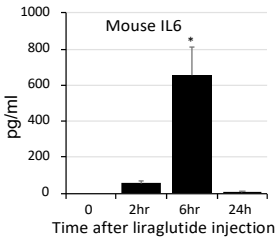

Figure 6. The effect of acute incretin mimetic therapy on plasma IL-6 levels in mice. Prediabetic overweight C57/B6 females (n=3) were challenged with a single subcutaneous dose of Liraglutide (400mg/kg BW). Plasma was collected indicated time points after drug administration and IL-6 levels were measured by ELISA. Note a dramatic increase in circulating IL-6 after 6 hrs (P<0.0001).

To test if incretin mimetics signal via GLP-1R induce IL6 secretion in mice, diet-induced obesity (DIO) and pre-diabetic conditions will be induced by 4 months of feeding with high-fat-diet (HFD) 58 kcal % (fat) diet (Research Diets, D12331). Upon DIO induction, the following cohorts will be analyzed:

Untreated (n=20, 10M / 10F) Liraglutide-treated (n=20, 10M / 10F)
Liraglutide will be subcutaneously administered to mice for 6 weeks at 400 mcg/kg daily (28). Metabolic profiles of males and females will be separately compared before and after incretin mimetic therapy as described in Research Design and Methods. IL-6 levels in plasma will be measured by ELISA as shown in preliminary data (Fig. 6). At endpoint, we will sacrifice mice and isolate various cell populations (leukocytes, EC and ASC) by FACS from PBMC and WAT (SAT and VAT). We will measure GLP-1R activity and IL-6 expression in them separately by qRT-PCR and Western blotting. Induction of pAKT, pNFkB, and pERK1/2 will serve as a readout of GLP-1R activation. IF will be used to detect pNFkB and IL-6 in M1 and M2 macrophages (76), as well as in EC, and other cells ofWAT.

We will analyze AT from necropsies of mice described in SA1B. H/E histology and IF will be used to identify UCP1+ multilocular adipocytes as beige and unilocular UCP1-negative adipocytes as white (Fig. 5). Perilipin-1 IF will be used to distinguish beige adipocytes from fibrotic areas (72). Mitotracker staining (identifying active mitochondria) will be used to confirm beige adipocyte identity (75). To test if incretin treatment results in signaling activation coupled with brown adipocyte differentiation, we will use immunoblotting as shown (Fig. 7). We will also analyze various cell populations (leukocytes, EC and ASC) isolated by FACS from AT. IL-6R signaling in them will be assessed in them based on STAT3 phosphorylation by qRT-PCR and Western blotting. IF will also be used to measure pSTAT3 in ASC (adipocyte progenitors) and other WAT cells.

Expected outcomes: Insulin resistance will decrease after long-term GLP-1 analog treatment in both prediabetic humans and insulin-resistant mice. AT leukocytes and PBMCs will be established as the sources of GLP-1-induced transient IL-6 secretion. AT browning (UCP1 expression) will be observed in subcutaneous AT (SAT) long-term. In vivo AT browning will be observed long-term via increased ^{18}F-FDG uptake activity on PET-CT.
Alternative Approaches: We expect to determine M1, M2 (or both) macrophages are the main source of IL-6. However, we will also explore other cell types as potential IL-6 sources. Regardless of whether IL-6 is confirmed as a mediator of incretin signaling, we will also consider a screen to identify other potential molecular players by also measuring plasma levels of IL-11, as well as IL-4, IL-10, TNFα and other cytokines, involved in inflammation.

Specific Aim 2: Investigate the mechanisms of incretin-induced adipose tissue browning.
Hypotheses: GLP-1 analog therapy induces adipose tissue browning via both direct GLP-1 / GLP-1R signaling and indirect incretin-induced IL-6 / IL-6R / STAT3 signaling.

Specific Aim 2A: Investigate if GLP-1 analog effects on human beige adipogenesis depend on IL-6 signaling.

We have derived adipocyte progenitors from both SAT and VAT of both lean and obese patients and have used them in adipogenesis studies (75). We will test if IL-6 / IL-6R and GLP-1 / GLP-1R signaling in adipocyte progenitors can directly induce brown adipogenesis. Our pilot data indicate that IL-6 does induce STAT3 phosphorylation (Fig. 7-8). This signaling is predicted to favor brown adipogenesis, which will be tested at long-term time points during the clinical trial.

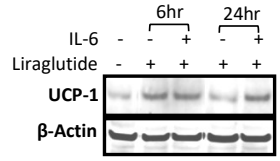

Figure 7. Mouse immortalized brown pre-adipocytes (IBP), cultured in 50 nM insulin / 0.5 mM IBMX, 1 µM dexamethasone, 1 nM 3,5,3'-Triiodothyronine (T3) and 5µM rosiglitazone for 24 hr, were washed and cultured in DMEM containing 10% FBS supplemented with 500pg/ml of mIL-6 (R&D, Cat.# 406-ML/CF) and/or 100nM of Liraglutide for 6 and 24 hr. UCP-1 expression was measured by Western blotting.

We will co-culture human preadipocytes with monocytes from PBMC to provide a physiological source of IL-6 signaling in a paracrine fashion. Upon co-culture setup, cells will be treated with liraglutide concomitantly with adipogenesis induction. Using protocols we described previously (75), we will induce brown adipogenesis with 50nM insulin / 0.5 mM IBMX, 1 µM dexamethasone, 1 nM 3,5,3'-Triiodothyronine and 5 µM rosiglitazone. Cell will be grown in 1.7 µM insulin post-induction (71, 72, 75). To test the dependency of brown adipogenesis on IL-6 signaling, we will perform cell culture experiments with and without IL-6 added to the culture medium. We will also knock-out IL-6R in human ASC used for adipocyte differentiation in these studies. For this, we will use the CRISPR/Cas9 technology as in our previous studies (79). sgRNAs will be selected and Cas9n vectors will be used as described (75). Expression of brown adipogenesis markers will be assessed by IF and immunoblotting for UCP1 as described above (Fig. 5, 7).

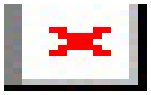

Figure 8. Human subcutaneous pre-adipocytes were seeded at 5×10 ^{5} cells/well of 10% FBS DMEM medium onto a 12-well plate. After 24 h, medium was removed and the cells were washed with 1XPBS. The cells were maintained in 1% FBS DMEM overnight. The cells were treated with 20ng/ml of human IL-6 at the indicated time points, then were lysed in RIPA buffer. Western blot analyses were performed by standard methods using antibodies against pStat3 and beta-Actin.

Specific Aim 2B: Investigate if GLP-1 analog effects on beige adipogenesis depend on IL-6 signaling in mice.
To test if the AT browning and anti-diabetic effects of incretin mimetics are mediated by IL6 in mice, DIO and pre-diabetic conditions will be induced by 4 months of feeding with high-fat-diet (HFD) 58 kcal % (fat) diet (Research Diets, D12331). Upon DIO induction and liraglutide / IL-6 antibody treatment, the following cohorts will be analyzed: IL-6 antibody-treated (n=20, 10M / 10F) Liraglutide / IL-6 antibody-treated (n=20, 10M / 10F)

Metabolic profiles of males and females will be separately compared in DIO mice before and after incretin mimetic therapy. Liraglutide will be subcutaneously administered for 6 weeks at 400 mcg/kg daily (28)
A blocking IL-6 antibody (Tocilizumab) will be concurrently administered at 5 mg/kg intraperitoneally weekly for six doses (56, 80) concomitantly with liraglutide. Activation of STAT3 in SAT of GLP-1 analog-treated mice will be analyzed as described above.

Expected outcomes: IL-6 and GLP-1 signaling activation will synergize in inducing brown adipocyte differentiation. Brown adipogenesis will be less pronounced in IL-6R KO and GLP-1R KO than in WT cells. AT browning and incretin metabolic benefits will be reduced by a blocking IL-6 antibody co-administration during incretin treatment. In mice, GLP-1 analog treatment will result in transient activation of IL-6-dependent STAT3 in SAT, which will be reduced by a blocking IL-6 antibody co-administered during incretin treatment.

Alternative Approaches: In addition to treating human preadipocytes with liraglutide or IL-6 separately, we will also use their combination treatment to determine a possible synergy. Because STAT3 is activated by many signaling pathways, we will also analyze other signaling mediators (STAT1 and MAPK) to confirm that IL-6R

signaling is indeed activated. For the mouse study, we will also analyze signaling in skeletal muscle and other organs (pancreas, liver, brain) to assess signaling changes.

VI. Study Population

Subjects: Twenty subjects (ten males and ten females), 18-50 years old, with a BMI ≤ 35 kg/m^2 and prediabetes (via expanded version of American Diabetes Association criteria) will participate in this study. Anticipating a 25% drop out rate, we will recruit 26 subjects. Each subject will participate in both study arms.

Inclusion criteria:

1. Men and women, ages 18-50 years
2. Diagnosis of Prediabetes - defined as either impaired fasting glucose (fasting glucose of 100-125 mg/dL), impaired glucose tolerance (2-hour postprandial blood glucose of 140-199 mg/dL after 75-gram oral glucose challenge), and/or a hemoglobin A1C ranging from 5.5% to 6.4%.
3. BMI ≤ 35 kg/m^2
4. Women of childbearing age must agree to use an acceptable method of pregnancy prevention (barrier methods, abstinence, oral contraception, vaginal rings, long-acting reversible contraceptives, or surgical sterilization) for the duration of the study
5. Patients must have the following laboratory values: Hematocrit ≥ 33 vol%, estimated glomerular filtration rate ≥ 60 mL/min per 1.73 m^2, AST (SGOT) < 2.5 times ULN, ALT (SGPT) < 2.5 times ULN, alkaline phosphatase < 2.5 times ULN
6. If patients are receiving antihypertensive medications (other than beta blockers) and/or lipid-lowering medications, they must remain on stable doses for the duration of the study.
7. If patients are receiving NSAIDs or antioxidant vitamins, these must be discontinued one week prior to study initiation and cannot be restarted during the study.
8. If patient takes thyroid medications, these must be dosed to control hypo- or hyperthyroidism.

Exclusion Criteria:

1. History of Type 1 or Type 2 diabetes mellitus
2. Pregnant or breastfeeding women
3. Medications: Beta blockers, corticosteroids, monoamine oxidase inhibitors, diabetes medications (including incretin mimetics and thiazolidinediones), and/or immunosuppressive therapy over the last 2 months.
4. Uncontrolled hypo- or hyperthyroidism
5. Current tobacco use
6. Active malignancy
7. History of clinically significant cardiac, hepatic, or renal disease.
8. History of any serious hypersensitivity reaction to study medications, any other incretin mimetic, any other formulation of supplemental vitamin B12, and/or cobalt
9. Personal or family history of Leber hereditary optic nerve atrophy
10. Prisoners or subjects who are involuntarily incarcerated
11. Compulsorily detention for treatment of either a psychiatric or physical (e.g., infectious disease) illness
12. Prior history of pancreatitis, medullary thyroid cancer, or multiple endocrine neoplasia type 2 (MEN 2)
13. Serum vitamin B12 level above the upper limit of assay detection

VII. Study Procedures

Table 1: Time and Event Schedule for Protocol Procedures

Procedure	Visit 1 (Screening)	Visit 2	Visit 3	Visit 4	Visit 5
Obtain Informed Consent	X				
Confirm Eligibility	X				
Medical History	X				
Concomitant Medications	X	X	X	X	X
Physical Examination	X				
Vital Signs	X	X	X	X	X
Pregnancy Test (if applicable)	X	X	X	X	X
applicable)	X				
CMP – including FPG, LFTs, serum creatinine	X				
Lipid panel	X				
HbA1C	X				
CBC	X				
TSH (thyroid stimulating hormone)	X				
Vitamin B12	X				
Plasma IL-6 (Interleukin-6)		X		X	
Plasma FFA (free fatty acids)		X		X	
Other plasma cytokines (TNF-α, IL-4, IL-10, IL-11, and IL-13)		X		X	
Plasma Glucose		X		X	
Plasma Insulin		X		X	
Peripheral blood mononuclear cell (PBMC) isolation		X		X	
^{18}F-FDG-PET/CT		X			
Subcutaneous Adipose Tissue Biopsy			X		X
Targeted Noncontrast CT			X		X
Assess for Adverse Events		X	X	X	X
Supply Study Medication to Patient (for home use)	X		X		

Subject recruitment: Participants will be recruited via IRB-approved advertisements in the press and in our clinics, as well as IRB-approved UT Physicians All Scripts search. Prior to beginning the study, all potential subjects will be prescreened for the above criteria via a telephone interview. If a subject meets prescreening criteria, he or she will be invited to participate in an hour-long outpatient screening visit.

After prescreening and prior to screening visit, subjects will be asked 1) whether or not they verbally consent to fasting laboratories to be performed before the screening visit, or 2) whether or not they verbally consent to fasting laboratories to be done during the screening visit. Verbal response will be documented on a limited verbal

consent form. We will follow FDA guidance during the consent process (82). It preferred that fasting laboratories be collected before anticipated screening visit, as many subjects will screen out of the study with these alone. We will cover the costs of all screening labs. If labs done before screening visit show disqualification, then subject will be informed (and results will be mailed to subject) and outpatient screening visit will be cancelled. If subject prefers to wait until screening, fasting is preferred but not required for day of screening visit.

Study Design: Single center, crossover, inactive comparator-controlled prospective trial (Fig. 4). Each subject will participate in both study arms: Cyanocobalamin (vitamin B12) 1000 mcg subcutaneous weekly x 6 weeks ("Drug A") followed by dulaglutide (titrated to 1.5 mg subcutaneous (sc) weekly) x 6 weeks ("Drug B"). There will be a three-week washout period between study arms.

Study Procedures:

Visit #1 (Screening Visit): The nature and the risks of the procedures, and the amount of financial remuneration for participating in the study will be discussed with each subject prior to obtaining written informed consent. All subjects who consent to participate will undergo a complete history and physical exam and receive a blood draw (for laboratory analysis) in the fasting state (i.e. blood draw can be done at the outpatient visit, or at a later time) to verify inclusion and exclusion criteria. If subject already obtained any of the required screening blood labs at an accredited laboratory during the 2 months prior to the screening visit, then it is not necessary to repeat those labs. Alternatively, subject may also obtain labs after the screening visit via another physician's order, if using an accredited laboratory (e.g., labs may be obtained by subject's primary care physician at another accredited laboratory). Qualified subjects will be invited to participate in the clinical trial.

Only subjects who give their written informed consent will take part in these studies. This will be obtained by the investigators in conjunction with research staff associated with the project. A subject may withdraw at any time without prejudice.

Clinical trial participants (determined after successful screening visit) will begin with cyanocobalamin 1000 mcg ("Drug A") subcutaneous weekly x 6 weeks , followed by minimum of 3 week washout, followed by 2) dulaglutide ("Drug B") – beginning at 0.75 mg sc weekly x 2 weeks, then 1.5 mg weekly x 4 weeks (for a combined total 6 weeks of treatment).

Qualified subjects will then have a brief appointment at the Endocrinology Clinic at UT Physicians Professional Building (6th floor) to pick up the first set of study medications. Parking validation will be given for this appointment.

Visit #2: At 1-10 days after last dose of Drug A, subject will begin fasting at midnight of that day. Visit 2 must take place before Visit 3, and may be done in the morning or afternoon. Visit 2 may also be done on the same day as Visit 3 if Visit 2 is done in the morning. Subject will present to Radiology department at 8 am or 12 pm. At 9 am or 1pm, subject and study nurse will enter a room set to the temperature of 23˚ C (72˚ F) (65). Vital signs will be taken and an IV will be inserted. At 10 am or 2 pm, blood will be drawn for isolation of plasma and peripheral blood mononuclear cells (PBMCs), and isotope tracer will be administered. Lab personnel will pick up the blood, which will be immediately transported to the laboratory for processing. At 11 am or 3 pm, subject will be taken to separate room to receive PET-CT (65). After completion of PET-CT, IV will be removed and subject will be discharged.

Visit #3: One week after completing last dose of Drug A (±3 days, and after completing Visit #2), subject will undergo the same fasting as Visit #2. At 12 pm, subject will present to our Department of Radiology, where

he/she will check in and change into a hospital gown. At 1pm, subject will enter a room set to the temperature of 23˚ C (72˚ F). If applicable, a urine pregnancy test will be collected and analyzed. Vital signs will be taken and an IV will be inserted. The IV access is placed just in case patient experiences any adverse events requiring IV medication. Subject will stay in room for 2 hours. At 3 pm, patient will go to IR receive CT-guided biopsy of supraclavicular adipose tissue under local anesthesia, as per methods described below. Guidance from PET-CT (from prior visit) will be used to guide the procedure. About 100-200 mg of tissue will be collected, which will be immediately stored at -80˚C by our lab personnel. A small adhesive bandage will be placed over biopsy site (which subject can remove the next day).

A minimum of a three-week washout period will occur after completion of Visit #2. Then subject subjects will then have a brief appointment at the Endocrinology Clinic at UT Physicians Professional Building (6th floor) to pick up Drug B, also to be taken for a total duration of 6 weeks. Parking validation will be given for this appointment.

Visit #4: Identical procedures to Visit #2 after Drug B.

Visit #5: Identical procedures to Visit #3 after Drug B. The only exception is that patient will take no more study medication, and will complete the study.

Compensation: We will compensate participants for their time in participating in screening and study visit. Participants will be compensated $40 for the screening visit (Visit #1), regardless of whether or not they qualify for the clinical trial. Visits #2 and #4 will be compensated at $75 per visit. Visits #3 and #5 will be compensated at $200 per visit. The maximum potential compensation for each subject is $590.

To maximize retention and monitor for adverse events, subjects will be contacted approximately every 3 weeks by research personnel to verify medication adherence, ask about potential adverse events, and answer any questions or concerns raised by the subject.

The PI will retain, in a confidential manner, all private identifiable information collected during these procedures. All information will be recorded in file folders and binders, which will be stored in the Clinical Research Unit in locked, secure cabinets which will only be accessible to the PI and his team.

Research Procedures in Detail:

Blood sample collection and processing

For the screening visit (or prior to screening visit if subject verbally consents), two tablespoons of blood will be drawn and analyzed immediately by our affiliated vendor (Quest Diagnostics). During Visit #2 and Visit #4 (see above), three tablespoons of blood will be drawn at each time point, making for a total of 12 tablespoons per Study Visit. For plasma markers, whole blood will be centrifuged to separate serum from plasma; then plasma will be separated and stored in labeled plastic screw-cap vials and stored as described below.

For peripheral blood mononuclear cells (PBMCs), there is a specific isolation technique. Blood samples will be collected in EDTA tubes and layered on a density gradient cell separation medium. Samples will be centrifuged serially to yield a pellet of mononuclear cells, and stored as described below.

Serum and plasma specimens, and PBMCs will be stored in a -80 degrees C freezer for no more than 6 years. Samples are stored while waiting processing. Any unused sample may be used for future studies if the subject

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consents to this. The freezer will be locked an accessible only to the PI and his team.

^{18}F-FDG-PET/CT

At 1 pm, subject and study nurse will enter a room set to the aforementioned temperature of 23˚ C (72˚ F). An intravenous line (IV) will be inserted. One hour later, a bolus injection of 185 MBq of 2-deoxy-2-[18F]fluoroglucose (18F-FDG) was will be administered to the study subject. One hour later, images will be obtained using a multidetector helical PET–CT scanner. Tissue is classified as brown adipose tissue (BAT) in each axial slice on a pixel-by-pixel basis as follows: when CT is in the range of -250 to -10 Hounsfield Units and when the Standard Uptake Value (activity in MBq per unit pixel volume of 64 µL within the region of interest (supraclavicular region) divided by the injected dose in MBq per body mass in g) was ≥ 1.5 (SUV*g/mL). Although a ratio of two activities, the denominators are different, so the SUV therefore has the units of "SUV*g/mL": [SUV *(mCi/mL)/(mCi/g) ⍰ SUV*g/mL]. BAT activity in each pixel is defined as the product of the volume of BAT in mL and its mean SUV (mL*SUVmean*g/mL). After completion of imaging study, the IV will be removed and patient will be discharged. This is similar to the protocols described by Cypess et al. 2015, and by Chondronikola et al. 2015.

Adipose tissue sample collection and processing

During each Visit #3 and Visit #5, we will take 100-200 mg of subcutaneous supraclavicular adipose tissue a 6-mm Bergström needle. Compared to other adipose tissue stores, supraclavicular adipose tissue contains the highest amount of brown adipose tissue (Leitner B et al. 2017).

Subjects will be taken to a CT procedure room to receive noncontrast scout CT images of the lower neck, for visualization of the supraclavicular area anatomy. Using major anatomical landmarks, the interventional radiologist will identify the location of the supraclavicular brown adipose tissue (BAT) depot in the CT images corresponding to the BAT in the PET/CT scan. If no BAT is visualized, then the interventional radiologist will plan for a white adipose tissue biopsy in the same area. The skin over the area of the biopsy will be cleaned using chlorhexidine and covered with a sterile drape. Approximately 5 mL of 2% lidocaine will be injected in the dermis of the biopsy site and in the subcutaneous adipose tissue depot. The injection will be advanced in the targeted adipose tissue area and an additional noncontrast CT scan will be performed to ensure the correct placement of the needle at the biopsy site, avoiding any adjacent superficial veins. The interventional radiologist will determine the depth of the adipose of the adipose tissue deposit and plan for the advancement of the biopsy needle.

Afterwards, an incision (<1 cm) will be made the skin using a scalpel and a 6-mm Bergström needle will be inserted through the incision and advanced into the supraclavicular adipose tissue depot. After proper positioning of the needle, the nurse will apply suction using a 60 ml syringe and a suction catheter. Bacitracin ointment will be applied to the top of the inner cannula to maintain suction and avoid leakage of air between the needle and the inner cannula. The needle will be rotated 180° and the target depot will be sampled. Up to three attempts will be made to obtain tissue. After procedure is completed, the radiologist or nurse will apply manual compression for 3–5 min to minimize bleeding. A small adhesive bandage will be placed over the incision, which you can remove at home the next morning. Participant can also shower with the bandage while at home. The duration of the procedure is approximately 30–40 minutes. This is similar to the protocol described by Chondronikola M et al. 2015.

Freshly obtained subcutaneous (sc) adipose tissue will be preserved in RNAlater or embedded in optimal cutting temperature (OCT) and stored at -80°C until further analyses. Some sc adipose tissue will be cut further into smaller pieces, rinsed in cold phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 24 hours and

then embedded in paraffin.

The subject will be instructed to inspect the wound daily, and inform the study team if you experience bleeding or worsening redness, pain, or discomfort.

Previously Collected Biospecimens: not applicable to this study.

Potential Risks

There are minimal risks to the study participants, as we will only add one medication to the treatment regimen. The invasive procedures proposed also pose minimal risks.

Blood draws and IV placement: For blood draws (related to screening visit), the principal risk is hematoma at the venipuncture site, which is not serious and disappears after several days. Phlebotomists and nurses drawing blood will be trained in venipuncture techniques to minimize extravasation.

The insertion of the peripheral intravenous (IV) catheter, during Visit #2 and Visit #4, may cause some discomfort, lightheadedness, fainting, bruising, clotting and/or bleeding from the site of the needle stick and, in rare cases, infection. A trained nurse will insert and remove the catheter at appropriate times.

Medication: Six weeks of dulaglutide and six weeks of cyanocobalamin – per dosing schedule described above – will be given to each subject as per study protocol.

Dulaglutide: Likely and not serious adverse reactions (10-20% of subjects) are nausea, vomiting, and diarrhea. Less likely, non-serious adverse reactions (1-10% of subjects) are dizziness, gastrointestinal upset, hypoglycemia, and injection site erythema and pruritus. A serious and rare adverse reaction (<1%) is acute pancreatitis. Subjects with prior history of pancreatitis, medullary thyroid carcinoma, and/or MEN2 are excluded from the study.

Cyanocobalamin: Water soluble vitamins, including B vitamins, can generally be tolerated at high doses, with toxicity occurring only at doses thousands of times the Recommended Dietary Allowance (RDA) (81). Likely and not serious adverse reactions (10-20% of subjects) are headaches and mild loss of strength. Less likely, non-serious adverse reactions (1-10% of subjects) are paresthesias, nausea, rhinitis, and injection site erythema. Subjects with the following characteristics will be excluded: Vitamin B12 levels beyond the upper limit of assay detection, hypersensitivity reactions to B12 supplementation and/or cobalt, and personal or family history of Leber hereditary optic neuropathy (which can be exacerbated by B12 supplementation).

If the subject experiences any adverse reaction from medication, he or she will contact the PI (who is a licensed and practicing clinical endocrinologist). The PI will direct the patient to the appropriate medical care and decide whether or not the patient may continue study participation. In the case of an emergency, the patient will be directed to a nearby Emergency Department.

^{18}F-FDG-PET/CT: During each Visit #1 and Visit #3, we will obtain PET-CT, as per protocol described above. An IV contrast load (185 MBq of 18F-FDG) will be given one hour prior to obtaining images, as described above. Because the dose of radiotracer administered is small, there is a relatively low radiation exposure to the patient. Nuclear medicine diagnostic procedures (including PET-CT) have been used for more than five decades, and there are no known long-term adverse effects from such low-dose exposure. Allergic reactions to radiopharmaceuticals may occur but are extremely rare and are usually mild. Injection of the radiotracer may

cause slight pain and redness which should rapidly resolve within a few days. As these procedures are not safe during pregnancy, a urine pregnancy test will be collected on the day of the exam (only in female subjects of childbearing potential), to ensure there is no pregnancy prior to participation.

CT Imaging: Targeted noncontrast CT images will be obtained during the supraclavicular adipose tissue biopsies on Visit #3 and Visit #5, as described above. There is always a slight chance of cancer from excessive exposure to radiation. However, this is less likely given the small doses used in this protocol. No contrast dye will be used for these CT images. As these procedures are not safe during pregnancy, a urine pregnancy test will be collected on the day of the exam (only in female subjects of childbearing potential), to ensure there is no pregnancy prior to participation.

Supraclavicular Adipose Tissue Biopsies: During each Visit #3 and Visit #5, we will obtain 100-200 mg of subcutaneous supraclavicular adipose tissue, as per protocol described above. Risks and side effects related to these biopsies are as follows:

Less likely and not serious:

- Bleeding and/or bruising and/or mild swelling at site of biopsy. As the incision is small (less than 1 cm), participants will not require sutures. A small adhesive bandage will be placed over the incision, which can be removed the next morning. If there is any swelling or bruising, it usually goes away with rest after a few days, although sometimes it may take a week. The bruising is helped by using hot packs.

Rare and Serious:

- Rarely, bleeding from a subcutaneous biopsy may be severe enough to require hospitalization.
- Very rarely, some subjects may experience numbness or tingling at the biopsy site. This is usually temporary and goes away in a few days.
- There is a small risk of infection at the site of the muscle biopsy. Infections can be usually treated effectively with oral antibiotics.
- Allergic reactions to the local anesthetic we use for the muscle biopsy are extremely rare, but could include a skin sore, swelling, or hives.

Safety Measures:

- The subject will be instructed to inspect the wound daily, and inform the study team if he or she experiences bleeding or worsening redness, pain, or discomfort.

Screening measures: Subjects may experience discomfort or embarrassment about answering questions about their medical history. The probability of these events is generally low. We will monitor these situations and provide support if needed. Such events will be documented fully in the case records.
Confidentiality: There is potential risk to subjects for a breach in confidentiality of identifying information and/or private data. Confidentiality risks are low given our measures to protect confidentiality (see below).
Alternatives: The only alternative is to not take part in this study.

VIII. Data and Safety Monitoring for Adverse Events

During the course of the study, trained research staff supervised directly by the PI will monitor participants. The study coordinator and data analysts will meet weekly with the PI (and/or appropriate co-investigators), to review data and study issues including data analysis, recruitment, and confidentiality.

In the event of identification of a previously unknown adverse outcome, the research staff will immediately communicate to the PI, who will determine whether the patient should be referred to the emergency room or follow up with primary care physician. This information will also be communicated immediately to the IRB. If changes to the protocol become necessary to ensure continued confidentiality measures, or to ensure the level of risk associated with this study, the IRB will be notified immediately, and these modifications will be addressed for review and approval as quickly as possible. Except for adverse events or modifications, a summary of study progress will be forwarded to the IRB with the annual renewal application**.**

Monitoring Adverse Events

All adverse events, including those that are serious, will be graded by the investigator as follows:

- Mild (Grade 1): awareness of event but easily tolerated
- Moderate (Grade 2): discomfort enough to cause some interference with usual activity
- Severe (Grade 3): inability to carry out usual activity
- Very Severe (Grade 4): debilitating; significantly incapacitates subject despite symptomatic therapy.

The following categories and definitions of causal relationship to a study medication or procedure as determined by a physician will be used:

- Related: There is a reasonable causal relationship to study medication administration and the adverse event.
- Not Related: There is not a reasonable causal relationship to study medication administration and the adverse event.

The expression “reasonable causal relationship” is meant to convey in general that there are facts (e.g., evidence such as de-challenge/re-challenge) or other arguments to suggest a positive causal relationship.

Collection and Reporting

Adverse events can be spontaneously reported or elicited during open-ended questioning, examination, or evaluation of a subject. To prevent reporting bias, subjects should not be questioned regarding the specific occurrence of one or more adverse events.

If known, the diagnosis of the underlying illness or disorder should be recorded, rather than its individual symptoms. The following information will be captured for all AEs: onset, duration, intensity, seriousness, relationship to investigational product, action taken, and treatment required. If treatment for the event was administered, it will be recorded in the medical record. The investigator will supply the IRB with any additional information requested, notably for reported deaths of subjects.

Serious Adverse Events

Following the subject's written consent to participate in the study, all SAEs, whether related or not related to study drug and/or procedures, will be collected, including those thought to be associated with protocol-

specified procedures. All SAEs must be collected that occur during the screening period and within 30 days of discontinuation of medication dosing. If applicable, SAEs must be collected that relate to any later protocol-specified procedure. The investigator will report any SAE occurring after these time periods that is believed to be related to study drug or protocol-specified procedure.

An SAE report will be completed for any event where doubt exists regarding its status of seriousness. If the investigator believes that an SAE is not related to study drug and/or procedure but is potentially related to the conditions of the study (such as withdrawal of previous therapy, or a complication of a study procedure), the relationship will be specified in the narrative section of the SAE Report Form.

All SAEs, whether related or unrelated to study medications, and all pregnancies will be reported to the University of Texas (UT) Health Sciences Center at Houston IRB within 24 hours of study personnel becoming aware of the event. If only limited information is initially available, follow-up reports are required. The original SAE form must be kept on file at the study site.

All SAEs will be reported to the UT Health Sciences Center at Houston IRB.

Safety Monitoring

The PI and his team will monitor the study at each visit. All treatment-emergent AEs will be recorded on source documents (i.e. original documents, data, and records). AEs include those reported spontaneously by the subject and those noted incidentally or as observed by the investigator or study personnel. All clinically significant abnormalities noted upon physical examination, or other diagnostic test results will be reported as an AE, except for baseline measurements that may be considered part of the medical history. In addition, all clinically significant AEs that continue at Study Termination will be followed up by the investigator and evaluated with additional tests if necessary, until the underlying cause is diagnosed or resolution occurs. All AEs will be evaluated for intensity and causal relationship with use of the study medication and/or study procedures by the investigator and reported to the UT Health Sciences Center at Houston IRB. All SAEs will be reported to the UT Health Sciences Center at Houston IRB and the sponsor within 24 hours. In addition, a safety report will be submitted to the IRB annually. Any new information regarding the safety of dulaglutide will be submitted the IRB.

IX. Adequacy of Protection Against Risks

A. *Informed Consent and Assent*

At the initial screening visit, if the subjects choose to participate, informed consent forms specific to this project will be administered and signed as specified by the IRB. The informed consent form will specify the purpose of the study, the procedures, potential risks and benefits, and the ability to leave the study at any time without prejudice.

The investigators will ensure that subjects are clearly and fully informed about the purpose, potential risks, and other critical issues regarding clinical studies in which they volunteer to participate. Freely given, written informed consent must be obtained from every subject before clinical study participation, including informed consent for any screening procedures conducted to establish subject eligibility for the study. Persons who are not able to consent will not participate in this study.

Investigators must:

1) Provide a copy of the consent form and written information about the study in the language in which the subject is most proficient prior to clinical study participation. The language must be non-technical and easily understood.
2) Allow time necessary for the subject to inquire about the details of the study.
3) Obtain an informed consent signed and personally dated by the subject and by the person who conducted the informed consent discussion.
4) Obtain the IRB's written approval/favorable opinion of the written informed consent form and any other information to be provided to the subjects, prior to the beginning of the study, and after any revisions are completed for new information.
5) Revise the informed consent whenever important new information becomes available that is relevant to the subject's consent. The investigator, or a person designated by the investigator, should fully inform the subject of all pertinent aspects of the study and of any new information relevant to the subject's willingness to continue participation in the study. This communication should be documented.

The rights, safety, and well-being of the study subjects are the most important considerations and should prevail over interests of science and society.

B. *Protections Against Risk*

<u>Screening</u> - Staff are well trained in interviewing procedures and techniques with patient subjects to minimize embarrassment about questions. Strict informed consent procedures will allow the subjects to understand the procedures, potential risks and benefits, and the ability to leave the study at any time without prejudice.

<u>Study Procedures</u>

Blood draws: Phlebotomists and nurses drawing blood will be trained in venipuncture techniques to minimize extravasation. For the use of a peripheral intravenous line, a trained nurse will insert and remove the catheter at appropriate times. Any complications arising from procedures will be reported to the study PI, who is a licensed and practicing clinician. The PI will make the determination regarding the appropriate course of care, and whether or not the patient may continue study participation.

Medication: If the subject experiences any adverse reactions from the study medication, he or she will contact the PI, who is a licensed and practicing clinician. The PI will direct the patient to the appropriate medical care and decide whether or not the patient may continue study participation. In the case of an emergency, the

patient will be directed to a nearby Emergency Department.

^{18}F-FDG-PET/CT: An experienced nuclear medicine physician (who is also a study Co-Investigator) and nurse will conduct these procedures (including administration of intravenous contrast load). For the use of a peripheral intravenous line, a trained nurse will insert and remove the catheter at appropriate times. Any complications arising from these procedures will be reported to the study PI and nuclear medicine Co-Investigator, who are both licensed and practicing clinicians. The PI will make the determination regarding the appropriate course of care, and whether or not the patient may continue study participation.

CT Imaging and Supraclavicular Adipose Tissue Biopsies: An experienced interventional radiologist (who is also a study Co-Investigator) and nurse will conduct these procedures. Any complications arising from these procedures will be reported to the study PI and interventional radiology Co-Investigator, who are both licensed and practicing clinicians. The PI will make the determination regarding the appropriate course of care, and whether or not the patient may continue study participation.

Incidental Findings: If any incidental findings – including those which impact a patient's health - are found from any of the above procedures, the PI will notify the patient immediately. The patient will be referred to primary care physician and/or appropriate specialty physician to address the incidental findings in a timely manner. The PI will make the determination whether or not the patient may continue study participation. The patient's safety will be prioritized above all other considerations.

Women of Childbearing Potential: Women of childbearing potential (WOCBP) must use an acceptable method of birth control (as in inclusion/exclusion criteria) during the course of the study, in such a manner that the risk of failure is minimized. Urine pregnancy testing will be done at screening at during all study visits.

All WOCBP MUST have a negative urine pregnancy test before receiving any study medications or study procedures. A urine pregnancy test will be done at the beginning of each study day. If the urine pregnancy test is positive, the subject must not receive the study medication or any study procedures that day. The subject must see her primary care physician or OB/GYN physician for further evaluation. If pregnancy is confirmed by this medical provider, the subject will be discontinued from the study. If the medical provider determines that the subject is not pregnant, the subject may be allowed to resume the study on a later date if there is minimal deviation from study protocol; otherwise subject will be discontinued from the study.

In addition, all WOCBP will be instructed to contact the investigator and/or other study personnel immediately if they suspect they might be pregnant (e.g., missed or late menstrual period) at any time during study participation.

If, following initiation of the study medication, it is subsequently discovered that a study subject is pregnant or may have been pregnant at the time of study medication exposure, including at least 6 half-lives after medication administration, the subject will be discontinued from the study. The subject must establish or continue care with a qualified primary care or OB/GYN physician. The protocol-specific duration of exposure to the study medication is not expected to have a significant impact on a pregnancy, but the pregnancy should be monitored by the above medical provider.

The PI must immediately notify the IRB in accordance with established reporting procedures.

Confidentiality: This potential risk will be minimized by careful medical evaluations and procedures, regular monitoring of subjects' clinical status, careful screening of subjects, and well-defined procedures for maintaining

confidentiality. The clinical information obtained from subjects will be part of their study records and

maintained at UT in a facility with adequate safeguards for the protection of confidentiality. The research data will be collected and recorded using only arbitrary code numbers for identification, in order to safeguard the confidentiality. All data will be kept in a secure area. Only the members of the research group will have access to the data files or to the master list for the codes. For publication purposes, the patients will be designated only by codes. Our research team is in compliance with all HIPPA privacy guidelines.

Strict confidentiality will be maintained with the use of the subjects' records and files (in accordance with federal, state, and UT Health policies and procedures). Following the required retention period, hard copy data or research records will be destroyed. Electronic, password-protected databases will be maintained. Data will be identified by study code and will not contain personal identifiers. UT Health policy specifies that all research records must be retained for a minimum of 7 years following study completion. Research interviews and related procedures will be conducted in private and research staffs are schooled in issues of confidentiality and sign a confidentiality statement. All personal information is kept in locked cabinets/offices separate from the data. Research data will be maintained in charts, identified by subject ID only. All data will be stored in locked files. Data being analyzed will be recorded and identified by subject code. Only members of the investigative group will have access to secured files or to master lists for subject code numbers and will be well educated regarding the protection of patients' rights to confidentiality. Only personnel involved in the study will have access to research records. Any items requiring names and subject number will be handled according to strict procedures to maintain privacy. The collection of sensitive information is limited to that needed to achieve the aims of the research and is not recorded in narrative format or saved in medical records. Research participants are not scheduled together at the same time. If it becomes apparent that study personnel know a participant from outside the project, the participant is informed and given the opportunity to make a choice about participation; if the participant then chose to participate, the staff member will have no access to this participant's identifiable information or data.

Computer-based files will only be made available to personnel involved in the study and limited to that purpose. Whenever feasible, identifiers will be removed from study-related information. Precautions are in place to ensure the data is secure by using passwords and encryption, etc. No identifiable data will be released to anyone without the signed consent of the participant, and no identification of the subject as a participant will be made to outside sources without signed consent. We keep no detailed written narratives with private information. Computerized files will be kept in a similar fashion to hard copies: personal information separate from actual data, both tied together only by subject number. The lists that associate the subject number and this information will also be separate. All members of the investigative group given access to these files are given this access by the data management group. Such access (the type of access and to what types of data) is recorded and monitored. Access is removed if it is no longer needed or as soon as staff leaves. All access is protected by logon and passwords. Again, all staff is educated regarding the protection of patients' rights to confidentiality.

C. *Vulnerable Subjects*: Not applicable. See above for Women of childbearing potential.

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X. Potential Benefits of the Proposed Research to Subjects and Others

Participants may learn more their prediabetes health status; if participant is overweight, he or she may learn more about that status as well. Information from laboratory studies and procedures will be available when the study ends. This information might guide the participant to more effective treatment from his or her physician. Studies may help the investigators find better alternatives for the treatment of prediabetes and overweight status, as well as the prevention of diabetes. However, participants may receive no benefits from participating. The specific aims of this research proposal may benefit society by furthering our understanding of the insulin-resistant states as well as the mechanisms the overweight state and obesity. More information will be gained about the mechanisms of human response to incretin treatment, and this information may be used to guide the development of new therapeutics.

XI. Importance of the Knowledge to be Gained

Knowledge to be gained from this research can be quite relevant for society. Our findings may improve the understanding of the role of GLP-1R signaling in adipose tissue IL-6 signaling and adipose tissue browning. Uncovering these molecular pathways will serve as a basis for developing more targeted therapy for obesity and insulin resistance. Given the importance of this knowledge to be gained, we believe the value of participating in the research outweighs the risks described above.

XII. References

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82. From Page 17 of FDA guidance - https://www.fda.gov/media/88915/download

XIII. Appendix I: Outcome Measures

1. Name: Interleukin-6 (IL-6) mRNA (from adipose tissue)

Type

Primary

Time Frame: after treatment with each intervention (i.e., after 6 weeks of exenatide and after 6 weeks of placebo)

Brief Description: cytokine

2. Name: Uncoupling protein 1 (from adipose tissue)

Type

Primary

Time Frame: after treatment with each intervention

Brief Description: marker of beige/brown fat

3. Name: STAT3 band intensity/Western blot (from adipose tissue)

Type

Primary

Time Frame: after treatment with each intervention

Brief Description: signaling intermediary with interleukin-6

4. Name: PRDM16 (from adipose tissue)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of beige/brown fat

5. Name: NDUFS3 (from adipose tissue)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of beige/brown fat

6. Name: ADRB1 (from adipose tissue)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of beige/brown fat

7. Name: ADRB2 (from adipose tissue)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of beige/brown fat

8. Name: ADRB3 (from adipose tissue)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of beige/brown fat

9. Name: NfKappaB p65 band intensity/Western blot (from peripheral blood mononuclear cells)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: signaling intermediary with interleukin-6

10. Name: IL-6 mRNA (from peripheral blood mononuclear cells)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

11. Name: IL-6 (from peripheral blood mononuclear cells)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

12. Name: Suppressor of cytokine signaling 3 band intensity/Western blot (from peripheral blood mononuclear cells)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: signaling intermediary with interleukin-6

13. Name: IL-6 (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

14. Name: free fatty acids (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: signaling intermediary with interleukin-6, marker of insulin resistance

15. Name: insulin (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of insulin resistance

16. Name: glucose (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of insulin resistance

17. Name: Tumor necrosis factor - alpha (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

18. Name: Interleukin-4 (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

19. Name: Interleukin-10 (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

20. Name: Interleukin-11 (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

21. Name: Interleukin-13 (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: cytokine

22. Name: Glucagon like peptide-1 (from plasma)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: incretin

23. Name: Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: marker of insulin resistance, calculated from fasting plasma glucose and fasting plasma insulin values

24. Name: Standard Uptake Value (from PET-CT reading)

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: radiologic marker of brown fat

25. Name: Oroboros oxygen consumption

Type

Secondary

Time Frame: after treatment with each intervention

Brief Description: measure of oxygen consumption