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Cardiovascular Effects of GLP-1 Receptor Activation

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1.0 Background

Obesity leads to CVD. More than a third of people in the United States are obese.[1] Rates of obesity are higher among Black and Hispanic Americans than among non-Hispanic Whites.[1] Obesity leads to decreased life expectancy.[2, 3] Obesity is associated with an increased prevalence of cardiovascular risk factors such as hypertension, T2DM, and hyperlipidemia, but obesity is an independent risk factor for CVD, including atherosclerosis, heart failure, and atrial fibrillation.[4, 5] Whereas higher BMI has been associated with improved survival in CVD ("obesity paradox"), visceral obesity is associated with increased cardiovascular mortality.[6] In 2008 the medical cost of obesity in the U.S. was estimated to be $147 bil.[7]

In obesity, inflammation, oxidative stress, endothelial dysfunction and impaired fibrinolytic function all promote atherosclerosis. In obesity, activated macrophages infiltrate adipose tissue and lead to the release of inflammatory cytokines such as tissue necrosis factor-α (TNF-α) and interleukin-6 (IL-6).[8, 9] IL-6 increases expression of plasminogen activator inhibitor-1 (PAI-1), the primary inhibitor of tissue plasminogen activator, and increased PAI-1 concentrations predict incident myocardial infarction.[10] Circulating markers of oxidative stress such as F_2-isoprostanes are increased in obesity and correlate with BMI and visceral adiposity.[11] In the setting of increased oxidative stress and inflammation, endothelial function is decreased.[12, 13] Decreased endothelial function predicts adverse cardiovascular events and an atherogenic state.[14, 15] Inflammation and defective endothelial nitric oxide generation also contribute to platelet activation in obesity.[16]

Weight loss reduces cardiovascular risk. In the Finnish Diabetes Prevention Program weight loss (3.5-4.2 kg in the intervention group versus 0.8 kg in controls) reduced the incidence of T2DM, systolic blood pressure (SBP, by five versus one mmHg, P=0.007), diastolic blood pressure (DBP, by five versus three mmHg, P=0.02), and triglycerides (by 18 versus one mg/dL, P=0.01) in patients with impaired glucose tolerance and average BMI of 31 kg/M^2.[17] In the Diabetes Prevention Program, an intensive lifestyle intervention that included a low-calorie diet and a structured exercise program also reduced incident T2DM.[18] SBP and DBP were significantly reduced by three and four mmHg, and triglycerides by 25 mg/dL.[19 21]

Weight loss improves endothelium-dependent vasodilation.[22 24] Improvements in brachial artery flow mediated-vasodilation (FMD) are proportionate to decreases in weight. Weight loss also decreases circulating markers of inflammation and thrombosis, including TNF-α, IL-6, CRP, PAI-1.[25 27] Although some trials show no beneficial effect of weight loss on death due to CVD,[28] in a metaanalysis of 15 trials a mean reduction of 5.5 kg was associated with a 15% decrease in all-cause mortality.[29]

Administration of the stable GLP-1 analogues liraglutide and semaglutide reduce weight, BP and cardiovascular risk. Recent studies have focused on the cardiovascular effects of incretin-based therapies: long-acting GLP-1

analogues and drugs that inhibit the degradation of endogenous GLP-1, DPP4 inhibitors. Incretin-based therapies offer advantages over older oral anti-diabetic agents. They increase insulin secretion in a glucose-dependent manner, decreasing the risk of fasting hypoglycemia, and they suppress glucose-dependent glucagon secretion.[30] GLP-1 agonists also cause weight loss by increasing satiety.[30]

Liraglutide has been approved by the FDA for weight loss.[31] In a 56-week trial in obese patients (BMI ≥30 kg/M^2 or BMI ≥27 kg/M^2 and untreated hyperlipidemia and hypertension), liraglutide 3.0 mg/d and counseling on lifestyle modification resulted in a significantly greater reduction in weight (-6.4 kg, P<0.001) compared to counseling and lifestyle modification alone. Associated with this weight loss, liraglutide decreased SBP, DBP, fasting lipids, CRP, PAI-1 and adiponectin to a greater extent than placebo.[31] A dose of 1.8 mg/d reduced weight 5.0 kg and was associated with similar improvements in cardiovascular risk.

In the Liraglutide Effect and Action in Diabetes of Cardiovascular Outcomes Results (LEADER) trial, liraglutide 1.8 mg/d reduced the primary composite outcome of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke in patients with T2DM and high cardiovascular risk compared to placebo (from 14.9% to 13%, HR 0.87, 95% CI 0.78-0.97, P=0.01).[32] The mean BMI of the patients in the LEADER trial was 32.5 kg/M^2 and patients in the liraglutide group lost 2.3 kg more weight than the placebo treatment group. SBP was also significantly lower in the liraglutide group.

Semaglutide (0.5 or 1 mg/week) reduced the primary composite endpoint of first occurrence of death from cardiovascular causes, nonfatal myocardial infarction or nonfatal stroke in patients with T2DM and a hemoglobin A1c (HbA1c) 7% or more compared to placebo (HR 0.74, 95% CI 0.58 to 0.94, P<0.001).[33] Body weight decreased -3.6 kg and -4.9 kg in the 0.5 and 1.0 mg placebo group versus -0.7 and -0.5 in the placebo groups.

Understanding whether GLP-1 analogues reduce cardiovascular risk primarily by causing weight loss or by GLP1R-dependent effects on vascular endothelial function, inflammation and thrombosis will impact the treatment of obese patients, as well as the development of future GLP1R modulating drugs (Project 1). GLP-1 analogues are expensive and require injection. GLP-1 analogues increase serum lipase and amylase;[32, 33] liraglutide has been associated with gallstone disease.[31, 32] In addition, semaglutide use is associated with increased complications of retinopathy (HR 1.76, 95% CI 1.11 to 2.7, P=0.02).[33] *In Aim 1, we will compare the effect liraglutide and similar weight loss (hypocaloric diet) on endothelial vasodilator and fibrinolytic function, inflammation and other cardiovascular risk factors.*

Unlike the stable GLP-1 analogues, DPP4 inhibitors have a neutral effect on the risk of atherosclerotic events and may increase the risk of hospitalization for heart failure.[34 36] DPP4 inhibitors do not cause weight loss. DPP4 inhibitors prevent the degradation of vasoactive peptides in addition to the incretins and share many substrates with commonly used cardiovascular drugs the ACE inhibitors.[37] Our group has found an interactive effect of DPP4 inhibition with

ACE inhibition (see **Preliminary Studies**).[38] In a *post hoc* analysis of the EXAMINE trial, White et al. reported that alogliptin reduced BP compared to placebo in patients who were not taking an ACE inhibitor but not in those taking an ACE inhibitor.[39, 40]

Incretin-based therapies may alter cardiovascular risk through GLP1R-dependent or -independent mechanisms. In addition to increasing insulin secretion, GLP-1 causes vasorelaxation and enhances endothelial function in rodents.[36] In mice, GLP-1 causes vasodilation through both GLP1R-dependent and -independent mechanisms.[36] The latter requires degradation of GLP-1 to GLP-1 (9-36) by DPP4 and involves nitric oxide. Human studies provide conflicting information about the effect of GLP-1 or stable analogues on endothelial function.[41 46] Intravenous GLP-1 has been reported to improve cardiac function and decrease inotrope use after ischemia/reperfusion.[47] We found no effect of acute intra-arterial GLP-1 (**Preliminary Studies**) on forearm blood flow (FBF) in the presence or absence of a DPP4 inhibitor,[48] but are finding that the GLP1R antagonist Exendin (9-39) affects FBF after four-day treatment with sitagliptin, suggesting that GLP1R activation may improve endothelial function during DPP4 inhibition. Interestingly, GLP-1 and stable GLP-1 agonists have no effect on or increase BP when given acutely,[49] but reduce BP when given chronically;[32, 33, 50] whether this results from chronic GLP-1 activation or the effect of weight loss on BP is not known.

Although GLP-1 causes peripheral vascular relaxation in rodents, stimulation of GLP1Rs in brain increases BP and heart rate (HR) in mice by activating autonomic regulatory neurons.[51] GLP-1 agonists increase HR by one-two beats per minute in clinical studies.[32, 49, 50]

The contribution of GLP1R activation to effects of DPP4 inhibitors is more complex.[52] In addition to blocking the degradation of GLP-1, DPP4 inhibitors inhibit degradation of other vasoactive peptides such as brain natriuretic peptide (BNP), substance P, neuropeptide Y and peptide YY (PYY).[37] *In Aim 2, we will assess the contribution of GLP1R activation to the cardiovascular effects of liraglutide and sitagliptin using the GLP1R antagonist Exendin (9-39).*

Variation in the genes encoding the GLP1R or DPP4 could alter the effect of treatment on cardiovascular risk in obesity. Consistent with the beneficial effect of GLP-1 analogues on cardiovascular mortality, a low frequency missense variant (Ala316Thr, rs10305492) has been associated with lower fasting glucose and risk of T2DM and with protection against cardiovascular disease.[53] It is not known whether this variant affects response to therapy and the minor allele frequency (MAF) is just 0.015.

Rs6923761 is a *common* missense variant that results in substitution of a serine for glycine at position 168 (Gly168Ser).[54] In one study, circulating GLP-1 was increased in carriers of the A (Ser) allele.[55] Other studies have reported decreased untreated BMI, weight, fat mass, weight circumference, LDL or triglycerides in carriers of the A allele.[56] This variant has been associated with enhanced weight loss and decrease in SBP following 14-week treatment with liraglutide in patients with T2DM.[57] In contrast, most studies have not shown an association between the rs6923761 variant and weight loss in response to caloric

restriction.[58, 59]

In Aim 3, we will test the hypothesis that genetic and other individual factors predict the effect of liraglutide not only on metabolism and weight loss but also on vascular homeostasis.

2.0 Rationale and Specific Aims

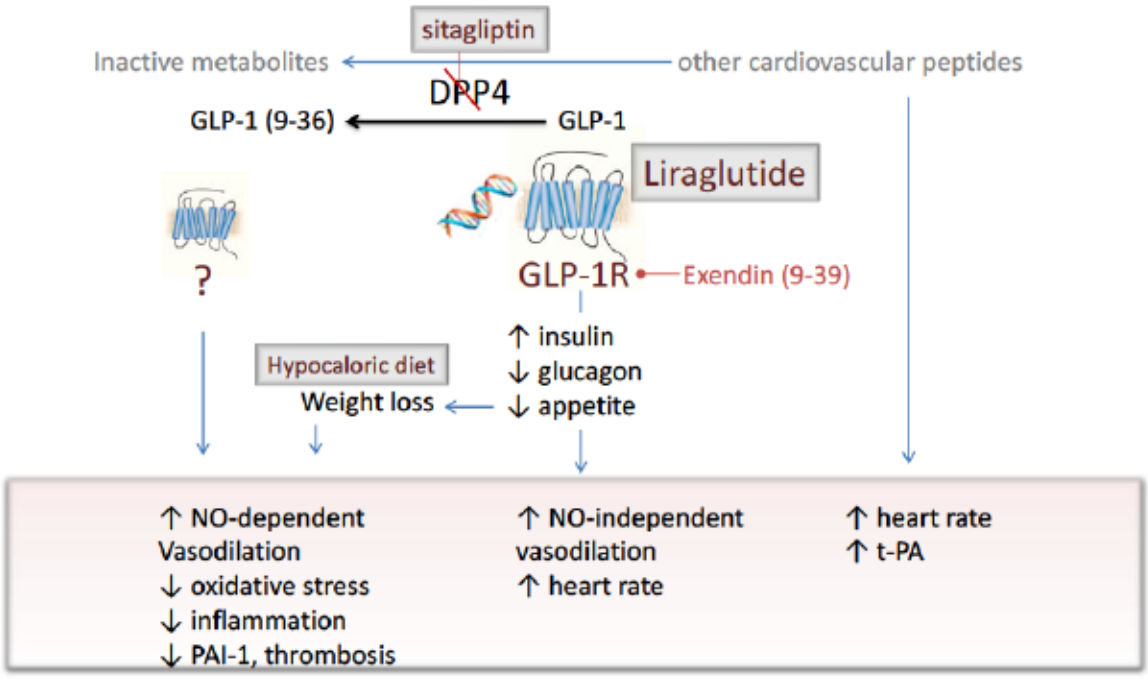
SPECIFIC AIMS

More than a third of people living in the United States are obese,[1] and obesity is associated with inflammation, endothelial dysfunction, fibrinolytic dysfunction and increased risk of cardiovascular disease (CVD).[12, 60 63] Recently the long acting glucagon-like peptide (GLP)-1 agonist liraglutide was approved by the FDA for the treatment of obesity.[31] Significantly, treatment with liraglutide, as well as the longer acting GLP-1 agonist semaglutide, reduces cardiovascular morbidity and mortality in patients with type 2 diabetes (T2DM).[32, 33] Whether this results from GLP-1 receptor (GLP1R) activation or from indirect effects of weight loss on cardiovascular risk factors such as hypertension, hyperlipidemia, and diabetes is not known.

Endogenous GLP-1 exerts beneficial endothelial and anti-inflammatory effects through GLP1R-dependent and -independent mechanism(s).[64, 65] The latter may require degradation of GLP-1 by dipeptidyl peptidase 4 (DPP4),[64] and thus may not occur during treatment with a stable analogue or when the degradation of endogenous GLP-1 is blocked by a DPP4 inhibitor. In addition, activation of the GLP1R by endogenous GLP-1 and stable analogues may couple with different downstream signaling pathways (see Project 1). Unlike the GLP-1 analogues, DPP4 inhibitors do not reduce the risk of acute coronary events and have been reported to increase the risk of hospitalization for heart failure in some trials in diabetic patients.[34 36] DPP4 inhibitors prevent the degradation of cardiovascular peptides in addition to GLP-1.[37] Also in contrast to GLP-1 analogues, DPP4 inhibitors do not cause weight loss.

Understanding the mechanism(s) through which GLP-1 analogues reduce cardiovascular risk in obesity has major implications for public health. Chronic treatment with a long-acting GLP-1 agonist is expensive compared to weight loss. GLP-1 analogues have neutral or detrimental effects on diabetic retinopathy.[32, 33] The effect of GLP-1 analogues compared to weight loss or DPP4 inhibition on cardiovascular risk may depend on individual factors. For example, functional genetic polymorphisms in the gene encoding the GLP1R can affect cardiovascular risk and the weight loss response to GLP-1 analogues (Project 3).[53, 54, 57, 66]

Figure 1: GLP-1 acts through a transmembrane receptor GLP1R, but may have GLP1R-independent effects that involve nitric oxide. Liraglutide is a stable GLP-1 analogue that is not degraded by dipeptidyl peptidase 4 (DPP4) and causes weight loss. Sitagliptin inhibits degradation of endogenous GLP-1 by DPP4 but affects the degradation of other peptides.



This project tests ***the principle hypothesis that stable GLP-1 analogues have specific GLP1R-dependent beneficial effects on vascular endothelial function, fibrinolysis and inflammation in obesity that exceed the benefits of weight loss, and that genetic or other individual factors that modulate GLP1R sensitivity can modify the effect of these analogues on cardiovascular risk (Figure 1).*** To do this, we will:

Aim 1: Test the hypothesis that a GLP-1 agonist (liraglutide) exerts beneficial effects on endothelial vasodilator and fibrinolytic function, inflammation and cardiovascular risk that exceed beneficial effects of equivalent weight loss (hypocaloric diet) alone.

Aim 2: Test the hypothesis that the beneficial effects of liraglutide on endothelial vasodilator and fibrinolytic function, inflammation and cardiovascular risk are GLP1R-dependent.

Aim 3: Test the hypothesis that functional genetic variants in the gene encoding for the GLP1R influence the beneficial effects of liraglutide on endothelial vasodilator and fibrinolytic function, inflammation and cardiovascular risk.

Understanding the mechanism(s) underlying the cardiovascular effects

GLP-1 analogues, DPP4 inhibitors and weight loss will lead to personalized, cost-effective approaches to reducing cardiovascular risk in obese patients.

3.0 Animal Studies and Previous Human Studies

Project investigators have extensive experience studying effects of obesity, metabolic syndrome, prediabetes and pharmacologic interventions on oxidative stress, inflammation, endothelial vasodilation, and fibrinolytic function. For example, the investigators have:

- Demonstrated that interruption of the renin-angiotensin-aldosterone system reduces inflammation and improves fibrinolytic balance in patients with hypertension, obesity or insulin resistance.[67 71]
- Elucidated strategies to improve endothelial vasodilator function in patients with hyperglycemia and diabetes.[13, 72, 73]
- Developed strategies including weight loss to improve endothelial fibrinolytic function in patients with hypertension, obesity, and insulin resistance.[25, 74 76]
- Demonstrated that increasing cGMP, either by increasing its endothelial-dependent production or by preventing its degradation, improves glucose homeostasis and reduces biomarkers of cardiovascular risk in patients with insulin resistance and pre-diabetes.[70, 75 77]

To optimize space utilization we highlight preliminary studies most relevant to the proposal.

DPP4 inhibitors increase BP, HR and sympathetic activity during ACE inhibition. Our group has identified interactive effects of DPP4 inhibitors and ACE inhibitors in obese hypertensive patients.[38] We initially compared the effect of sitagliptin (100 mg/d for five days) and matching placebo on the BP and HR response to acute enalapril (0, 5 or 10 mg) in obese patients with metabolic syndrome. Pre-treatment with DPP4 inhibitor attenuated the BP response to the highest enalapril dose and increased HR (**Figure 2**) and circulating norepinephrine. We subsequently reported that substance P increases sympathetic activity during combined DPP4 and ACE inhibition.[78] We are now studying cardiovascular effects of DPP4 inhibition during long-term treatment with an ACE inhibitor, an angiotensin receptor blocker (ARB) or a calcium channel blocker (CCB). This study is providing a wealth of information about the cardiovascular effects of modifying the incretin system in obesity.

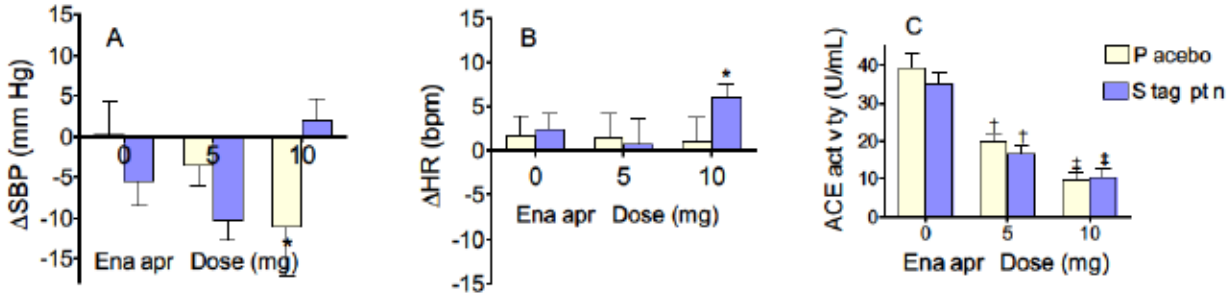


Figure 2: (A) Sitagliptin alters the dose-response to enalapril such that the anti-

hypertensive effect of enalapril 10 mg is attenuated.[38] **(B)** HR increases during enalapril 10 mg following sitagliptin pre-treatment. **(C)** Sitagliptin does not affect ACE inhibition. *p<0.05 vs placebo, †p<0.05, ‡p<0.01 vs 0 mg

Acute GLP-1 administration does not cause arterial vasodilation in the human forearm. Stable GLP-1 analogues may reduce cardiovascular risk through GLP1R-dependent effects or indirectly through beneficial effects on visceral obesity, BP and lipids. We studied the direct effect of GLP-1 on FBF.[48] We found no vasodilator effect of acute intra-arterial GLP-1 given alone or with sitagliptin to prevent its degradation (**Figure 3**). In addition, we have not found that increasing endogenous GLP-1 acutely with a DPP4 inhibitor alters the vasodilator response to endothelium-dependent vasodilators such as bradykinin or substance P.[78] As observed below, these data do not preclude a beneficial GLP1R-dependent effect of longer treatment with a GLP-1 analogue or DPP4 inhibitor on endothelial function, but highlight the importance of probing the GLP1R-dependent cardiovascular effects of these drugs.

The GLP1R antagonist Exendin (9-39) affects vasodilation in response to an arginine stimulation test during DPP4 inhibition. Co-investigator Devin is investigating the impact of DPP4 inhibition on the cardiovascular effects of growth hormone in collaboration with Dr. Brown. DPP4 degrades growth hormone releasing hormone (GHRH). We have found that four-day treatment with sitagliptin increases FBF following an arginine stimulation test and are investigating the molecular pathways involved. To determine whether effects of sitagliptin are mediated through the GLP1R we are measuring the effects of the GLP1R antagonist Exendin (9-39) versus placebo on vasodilation following arginine stimulation test during sitagliptin treatment. [redacted] *In Aim 2, we will use Exendin (9-39) to explore the role of the GLP1R in mediating the short- and long-term effects of the GLP-1 agonist liraglutide and the DPP4 inhibitor sitagliptin.*

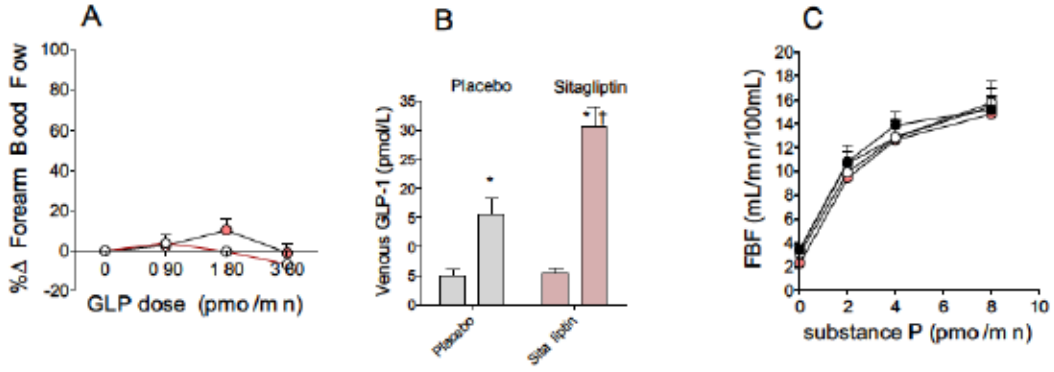
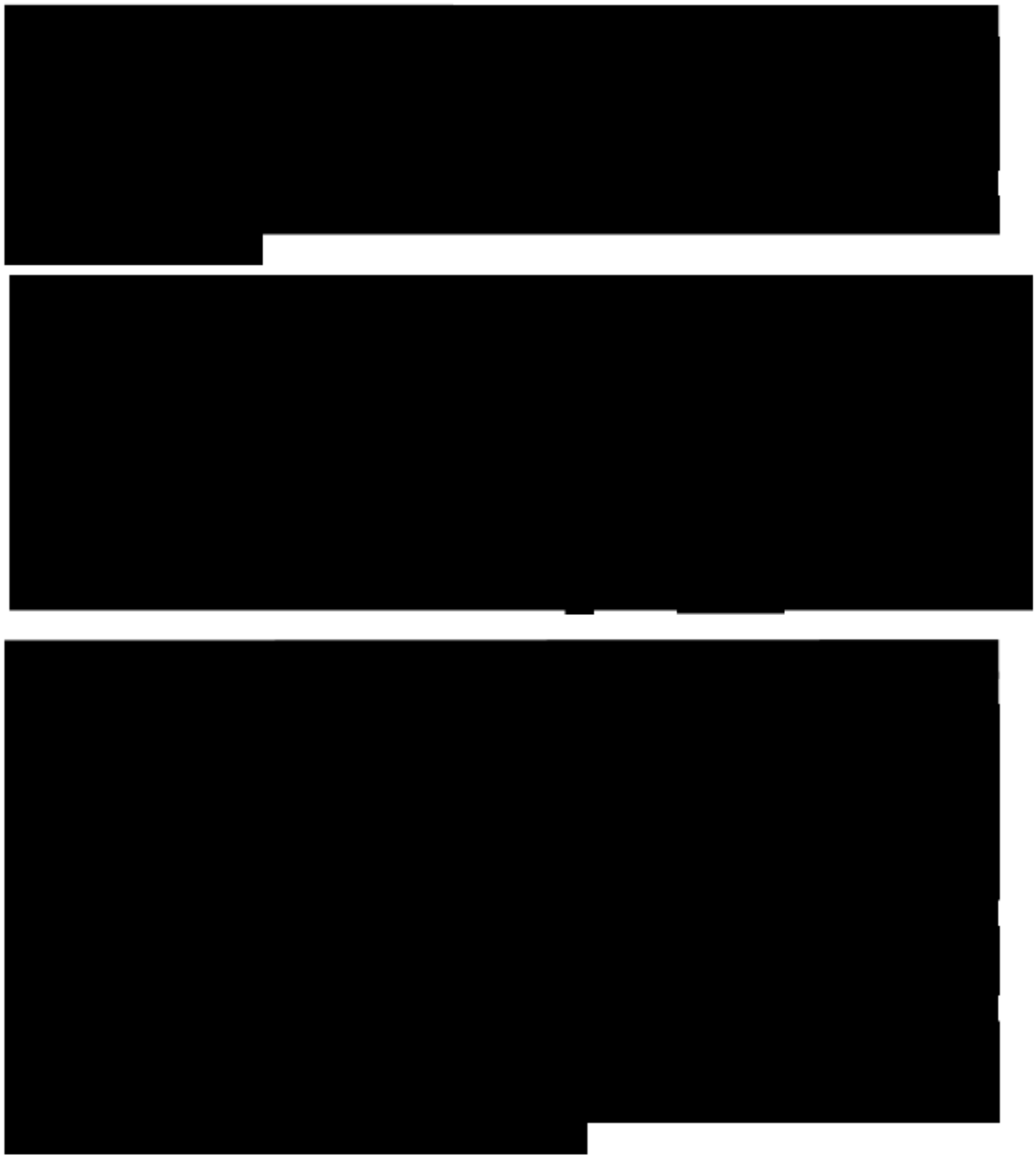


Figure 3: (A) Intra-arterial infusion of glucagon-like peptide-1 (GLP-1) after placebo (red) or sitagliptin (white) did not increase forearm blood flow (FBF), even though GLP-1 concentrations were significantly increased **(B)**.[78] **(C)**

Sitagliptin, alone (white) or in combination with an ACE inhibitor (black), did not change the FBF response to the endothelium-dependent vasodilator substance P.[48] In contrast, norepinephrine release (not shown) was increased by substance P during ACE and DPP4 inhibition. *p<0.05 versus baseline placebo, †p<0.05 versus GLP-1 infusion during placebo



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4.0 Inclusion/Exclusion Criteria

Inclusion

1. Men and women,
2. Age 18 to 65 years, and
3. FPG (100-125 mg/dL) or,
   IGT (two-hour plasma glucose 140-199 mg/dL) or,
   HbA1C 5.7-6.4%
4. BMI≥30 kg/M^2
5. The ability to provide informed consent before any trial-related activities.

Exclusion

1. Diabetes type 1 or type 2, as defined by a FPG of 126 mg/dL or greater, a two-hour plasma glucose of 200 mg/dL or greater, or the use of anti-diabetic medication
2. Resistant hypertension, defined as hypertension requiring the administration of more than three anti-hypertensive agents including a diuretic to achieve control
3. Known or suspected allergy to trial medications, excipients, or related products.
4. Family or personal history of multiple endocrine neoplasia type 2 (MEN2) or familial medullary thyroid carcinoma
5. Personal history of non-familial medullary thyroid carcinoma
6. History of pancreatitis
7. Contraindications to study medications, worded specifically as stated in the product's prescribing information
8. Pregnancy or breast-feeding. Women of child-bearing potential will be required to have undergone tubal ligation or to be using an oral contraceptive or barrier methods of birth control
9. Subjects who have participated in a weight-reduction program during the last three months or whose weight has increased or decreased more than two kg over the preceding three months
10. Cardiovascular disease such as myocardial infarction within six months prior to enrollment, presence of angina pectoris, significant arrhythmia, congestive heart failure (left ventricular hypertrophy acceptable), deep vein thrombosis, pulmonary embolism, second or third degree heart block, mitral valve stenosis, aortic stenosis or hypertrophic cardiomyopathy
11. Treatment with anticoagulants
12. History of serious neurologic disease such as cerebral hemorrhage, stroke, or transient ischemic attack
13. History or presence of immunological or hematological disorders
14. Diagnosis of asthma requiring regular inhaler use
15. Clinically significant gastrointestinal impairment that could interfere with drug absorption
16. Impaired hepatic function (aspartate amino transaminase [AST] and/or alanine amino transaminase [ALT] >3.0 x upper limit of normal range)
17. Individuals with an eGFR<30 mL/min/1.73 m2 or with a UACR >1000µg/mg, where eGFR is determined by the four-variable Modification of Diet in Renal Disease (MDRD) equation, where serum creatinine is expressed in mg/dL and age in years: eGFR (mL/min/1.73m2)=186 • Scr-1.154 • age-0.203 • (1.212 if black) • (0.742 if female)
18. Hematocrit <35%
19. Any underlying or acute disease requiring regular medication which could possibly pose a threat to the subject or make implementation of the protocol or interpretation of the study results difficult

20. Treatment with chronic systemic glucocorticoid therapy (more than 7 consecutive days in 1 month)
21. Treatment with lithium salts
22. History of alcohol or drug abuse
23. Treatment with any investigational drug in the one month preceding the study
24. Previous randomization in this trial
25. Mental conditions rendering a subject unable to understand the nature, scope and possible consequences of the study
26. Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study
27. Inability to undergo a magnetic resonance imaging (MRI), e.g. cardiac pacemaker, artificial heart valve, non-compatible metallic implant, or any retained foreign metallic bodies. This will be an optional procedure, so the subjects will not be excluded from the study because of this criterion.

5.0 Enrollment/Randomization

We will study 160 subjects in total. Exendin infusion for aim 2 will be stopped after 60 patients, based on our power to detect an effect of the antagonist. Dropouts will be replaced. If a replaced subject also drops out, the subject will be replaced. From our experience, we expect a 10-15% dropout rate during the study, thus the double-dropout rate is expected to 1-2%. Subjects will be randomized to liraglutide, sitagliptin, or hypocaloric diet study group in a 2:1:1 ratio. This ratio will be modified to 30:3:15 due to the SARS-CoV2 pandemic to enrich our ability to compare liraglutide versus hypocaloric diet with an anticipated drop in total subjects studied, sacrificing numbers in sitagliptin arm while maintaining integrity of blinded study design. Within each study arm the order of placebo and Exendin (9-39) will also be randomized. Dr. Chang Yu, study biostatistician will prepare the allocation schedule. Vanderbilt Investigational Drug Services will be responsible for storage, preparation, and labeling of all agents and for maintaining accurate drug storage and dispensing logs. If Novo Nordisk provides liraglutide (6 mg/mL) and its corresponding placebo, the products will be labelled by Novo Nordisk.

6.0 Study Procedures

The protocol will be registered at http://www.clinicaltrials.gov before any subject is enrolled.

Written advertisements which have been approved by the Vanderbilt Institutional Review Board (IRB) and which give the name and phone number of a contact research nurse will also be placed on Vanderbilt clinic bulletin boards. A description of the study will also be placed on the Vanderbilt patient portal. Potential participants who call for information will be given a brief description of

the study protocol and, if interested, will be invited to the Vanderbilt CRC for more information. During meetings with potential subjects, the research nurse or investigator will describe the study protocol in detail. Interested participants will be invited to read and sign an IRB-approved consent form and will be given a copy of the consent form to take with them.

Protocols will be carried out according to the principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, as well as ICH GCP guidelines. The investigators will comply with all regulatory and legal requirements, ICH GCP guidelines and the Declaration of Helsinki in obtaining and documenting the informed consent.

Screening

Once informed consent has been obtained, subjects will report to the Clinical Research Center (CRC) after an overnight fast, and we will obtain a medical history and complete a physical exam. We will measure height, weight, waist (horizontal umbilicus) and hip (at the largest horizontal pan) circumferences to 0.5 cm in triplicate, using a spring-loaded tape measure (Gulick II, Country Technology, Gay Mills, WI). We will draw screening laboratory including fasting glucose and lipids, electrolytes and creatinine, complete blood count, HbA_{1C}, liver enzymes, urinalysis, and electrocardiogram (ECG). Approximately two days later subjects will return to the CRC fasting to undergo OGTT. Prior to the OGTT, we will collect a spot urine for UACR. (Subjects will refrain from heavy exercise for 24 hours before providing any sample for UACR.) We will obtain a baseline DEXA scan for measurement of body composition and measure resting energy expenditure. We will also invite subjects to undergo magnetic resonance imaging (MRI) of the thigh (see **Standard Techniques**) to measure intermuscular adipose tissue (IMAT) and intramyocellular (IMCL) content as an optional procedure.

Protocol

Figure 6 illustrates the overall study protocol for this single-center, randomized, placebo-controlled study with three treatment arms. Following

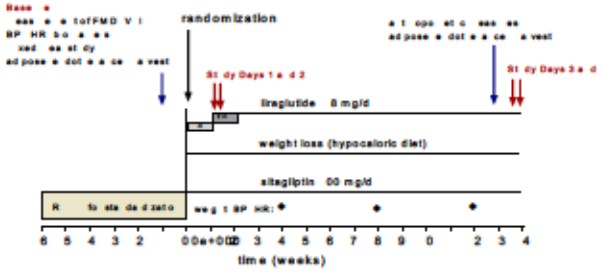

Figure 6: Overall study protocol for Aims 1 through 3. The study day protocols for days 1 through 4 appears in **Figure 7**. BP, blood pressure; HR, heart rate; FMD, flow-mediated vasodilation; VTI, velocity time interval

screening, eligible subjects will undergo a six-week run-in phase during which their medical management will be optimized. Lipids will be managed according to current American Heart Association (AHA) guidelines. Any subject who smokes will be advised regarding

smoking cessation. Participants will be given a target BP goal of 140/90 mmHg (or a revised goal from anticipated AHA recommendations). If a subject is hypertensive and not taking an ACE inhibitor or ARB, these drugs will be recommended for initial treatment of hypertension unless contraindicated. For subjects already taking an ACE inhibitor or ARB, the dose will be optimized. Amlodipine will be recommended as a second agent, starting at a dose of 2.5 mg/d and increasing to 10 mg/d. Chlorthalidone 12.5 mg/d will be recommended as a third agent. Participants will be advised on the use of low-dose (81 mg) aspirin based on US Preventive Services Task Force guidelines.

After the six-week run-in period, subjects will undergo a **baseline study day**, during which they report to the CRC in the fasting state. We will obtain a second spot urine sample for UACR. We will weigh subjects and place an IV catheter for blood sampling. After the subject has been supine for 45 min, we will measure FMD and hyperemic velocity time interval (VTI), BP and HR in triplicate (see **Standard Techniques**). We will draw blood for measurement of fasting glucose and insulin, PAI-1, t-PA, von Willebrand factor (vWF), P-selectin, 11-dehydro-thromboxane B_2, platelet function assay, F2-isoprostanes, Il-6, TNF-α, adipokines and peripheral blood mononuclear cell (PBMC) analysis. We will obtain adipose tissue for measurement of resident immune cell populations and gene expression profiles related to local inflammatory mediators, and adipocyte metabolism and regulation, and endothelial cells (ECs) for measurement of inflammatory phenotype and endothelial function. Subjects will then ingest a standardized mixed meal plus acetaminophen mixture over ten min (see **Standard Techniques**). We will collect blood at 0, 15, 30, 45, and 60 min, and then every half-hour for a total of four hours for measurement of insulin, glucose, C-peptide, GLP-1, PYY, catecholamines, VDL, glycerol and free fatty acids (FFA). We will collect blood once for PBMC analysis at the same time as adipose tissue harvest. We will measure BP and HR prior to each blood draw. We will ask subjects to rank their satiety using a previously validated visual analog scale[79, 80] prior to and at one and three hours after the meal. If DEXA scan or REE was not previously completed, it will be done at this baseline visit.

Approximately one week after the baseline study day subjects will be randomized to liraglutide (6 mg/mL, Novo Nordisk), sitagliptin (Merck and Co, Inc), or hypocaloric diet study group in a 2:1:1 ratio (modified to 30:3:15 with SARS-CoV2 pandemic as above). Randomization will be stratified by race. Based on the distribution of the GLP1R rs6923761 genotypes among Americans of European (GG:GA:AA=40.1%:45.1%:14.2%) and African (GG:GA:AA=85.5%14.5%:0) ancestry, we expect that 47% of subjects in the liraglutide group will carry the rs6923761 A allele. Assignment to liraglutide or sitagliptin treatment groups will be double-blind with a double dummy setup. Subjects in the liraglutide group will receive s.c. liraglutide (0.6 mg/d for one week, 1.2 mg/d for one week, and then 1.8 mg/d for 13 weeks) and oral placebo. We have chosen this dose of liraglutide because it reduced risk of cardiovascular disease in the LEADER trial; the dose escalation follows FDA-approved recommendations to reduce gastrointestinal side effects. The sitagliptin group will

receive s.c. placebo/d and sitagliptin p.o. 100 mg/d for 14 weeks. This is the FDA-approved dose of sitagliptin. The same dosing regimen including doses, timing, and period of placebo treatment will be administered exactly in parallel to its corresponding active treatment to ensure blinding. Treatment with hypocaloric diet will not be blinded. Subjects in the hypocaloric diet group will begin their dietary instruction (see **Standard Techniques**). Subjects will be instructed to take their subcutaneous medication between 2100 and 2200 each day and their oral medication at 0700 each day. If the adipose tissue biopsy, DEXA scan, or REE was not previously completed, it will be done at this randomization visit.

Before completion of aim 2, on the 1st and 3rd days of the second week each subject will undergo a study day to assess the GLP1R-dependent effects of short-term therapy (**Figure 7**). On each day they will report to the CRC in the morning in the fasting state. We will call them at 2100 the night before to confirm that they have taken their s.c. medication. That morning we will weigh subjects and place an IV catheter for sampling. After the subject has been supine for 45 min, we will measure BP and HR in triplicate (**Standard Techniques**). We will draw blood for measurement of fasting glucose and insulin, PYY, catecholamines, PAI-1, t-PA, vWF, P-selectin, 11-dehydro-thromboxane B_2, platelet function assay, F2-isoprostanes, Il-6, TNF-α, adipokines. Subjects will then be given either the GLP1R antagonist Exendin-(9-39) (Clinalfa®, Bachem Distribution Services; Weil am Rhein, Germany; IND #122,217, IV bolus of 7500 pmol/kg over one min at time 0 followed by continuous infusion of 350 pmol/kg/min for the remainder of the study) or matching placebo in double-blind, randomized fashion. Sixty min after that, we will measure FMD and VTI and repeat BP and HR and blood sampling. Subjects will then be asked to ingest a standardized mixed meal over ten min and we will measure BP and HR and collect blood and assess satiety as during the baseline study. After completion of aim 2, exendin infusion will be discontinued and only one study day will be done on the second week after randomization. Subjects will receive placebo (carrier) infusion on this study day to preserve the protocol.

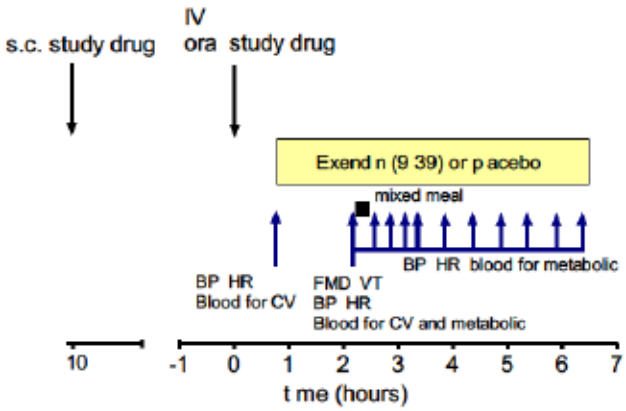

Figure 7: Protocol for study days 1 through 4. The study protocol for the baseline study day is similar without the administration of the GLP1R antagonist Exendin (9-39) or placebo. BP, blood pressure; HR, heart rate; CV, cardiovascular biomarkers:

Following completion of the second study day, subjects will continue their double-blind therapy (liraglutide and sitagliptin groups) or hypocaloric diet. They will report to the CRC monthly for measurement of weight, BP, and

HR.

At the end of the 13th week of therapy, subjects will report to the CRC to provide a spot urine sample for UACR, and to undergo repeat DEXA scanning, REE, adipose tissue, PBMC and endothelial cell harvesting. We will also obtain an MRI of the thigh during this visit if the subjects agree to this procedure. Prior to completion of aim 2, on the 5th and 7th days of the 14th week of treatment, they will repeat the study days in which we measure hemodynamics, endothelial function, biomarkers of fibrinolysis, markers of platelet function, markers of oxidative stress and inflammation and metabolic response to a mixed meal after they have received either the GLP1R antagonist Exendin (9-39) or placebo on each day in random order. The mixed meal on these study days will include acetaminophen (see **Standard Techniques**). After completion of aim 2, exendin infusion will be discontinued and only one study day will be done on the 14th week of treatment. Subjects will receive placebo (carrier) infusion on this study day to preserve the protocol.

Anticipated Results

Aim 1: We have designed the study to achieve equivalent weight loss in the hypocaloric diet and liraglutide arms. We do not expect sitagliptin to cause weight loss. We expect that the effects of liraglutide on conventional risk factors such as BP and lipids will be similar to weight loss. HR may be increased during liraglutide. If activation of the GLP1R contributes to beneficial effect of liraglutide on endothelial vasodilator and fibrinolytic function, inflammation, or thrombosis we expect to see a greater effect of liraglutide than weight loss alone. If liraglutide does not have greater effects on biomarkers of cardiovascular risk than weight loss alone, this would affect interpretation and implementation of recent trials.

Aim 2: We expect that acute endothelial effects of liraglutide, as well as metabolic effects, will be mediated via the GLP1R, and therefore decreased by the GLP1R antagonist Exendin (9-39). Probing the GLP-1-dependent cardiovascular effects of this stable analogue will provide important information for the future design of allosteric modulators of the GLP1R (see also Project 1). We expect that sitagliptin will have GLP1R-dependent and -independent cardiovascular effects. The latter may be mediated by other DPP4 peptide substrates with vascular effects such as substance P and PYY.

Aim 3: Previous studies suggest that the rs6923761 genotype affects the weight loss response to liraglutide. We expect to find this, but also hypothesize that vascular endothelial function and markers of cardiovascular risk will be improved to a greater extent by liraglutide in carriers of the rs6923761 A allele than in individuals who are homozygous (GG). We predict that rs6923761 genotype will also affect GLP1R-dependent cardiovascular responses to sitagliptin. Project 1 will explore the effects of this genotype on receptor coupling to downstream signaling. We do not expect that the polymorphism will affect response to weight loss alone. We will explore the effect of and control for race and gender in these analyses.

Limitations and Future Directions

It is possible we would not achieve equivalent weight loss in the liraglutide and hypocaloric arm. Dr. Silver and her team have a successful track-record of creating diets to enable research subjects to achieve targeted weight loss in collaboration with Dr. Niswender (Project 1), however.[25, 81, 82] If necessary we will include weight loss as a covariate.

We will study equal numbers of women and men in this study. The study population will be enriched with subjects of African descent compared to the general population – 20 in each study arm or 50% in the weight loss and sitagliptin arms and 25% in the liraglutide arm. Clinical trials of GLP-1 analogues and cardiovascular disease have studied a majority of men and ~6.5% were African American in the semaglutide study.[32, 33] Because the rs6923761 A allele is less common in individuals of African descent, it is possible that responses to GLP-1 will differ.

We are studying the effect of rs6923761 genotype on cardiovascular biomarkers during treatment with liraglutide because this genotype has been associated with weight loss response and the variant is common. We are not planning to study the rs10305492 variant. While we could prescreen for this genotype, with an MAF of 1.5% we would have to screen more than 2500 individuals to enroll enough subjects with the minor allele to have adequate statistical power. It is likely that studies in Project 3 will reveal other individual subphenotypes or genotypes and we will consider these in *post hoc* analyses.

In exploratory studies of adipose tissue and ECs we will study subcutaneous rather than visceral adipose (due to feasibility) and venous ECs rather than arterial (in order to avoid arterial injury). Studies suggest that effects of obesity and other environmental factors have directionally similar effect on subcutaneous and visceral fat and venous and arterial ECs.[83 85]

Standard Techniques

BP Measurements: During screening, washout, and active treatment outpatient BP will be measured with an aneroid sphygmomanometer (Welch Allyn, Skaneateles Falls, NY), using the appearance and complete disappearance of the Korotkoff sounds (K1 and K5) as SBP and DBP. The mean of three seated measurements will be used. During study days, BP will be measured using an automated oscillometric recording device (Dinamap, Critikon, Carlsbad, CA) and will be completed with subjects supine. The mean of three measurements one min apart will be used.

Oral Glucose Tolerance Test: After overnight fast, subjects will be given 75-g glucose by mouth. We will draw blood at 0, 30, 60, 90, and 120 min after administration of glucose for measurement of plasma glucose, insulin, and C-peptide. Although the primary outcome will be two-hour glucose, we will calculate insulin sensitivity as both 1/fasting insulin and as the homeostasis model assessment (HOMA) of insulin sensitivity (HOMA-S) using the Web-based HOMA calculator for nonspecific insulin (hggp://www.dru/ox.ac.uk). We will also calculate the early insulin response as the ratio of change in insulin to the change in glucose from 0 to 30 min (ΔI0-30/ΔG0-30). We will calculate the oral DI as

(ΔI0-30/ΔG0-30) x 1/fasting insulin.

Caloric Restriction (Weight Loss Treatment Arm): Subjects in the weight-loss arm will be given a caloric goal designed to achieve a weight loss similar to that expected in the liraglutide treatment arm; based on prior studies of 1.8 mg/d liraglutide this is predicted to be ~0.27kg/week.[31, 87 89] Each subject's caloric goal will be determined by measuring resting energy expenditure (REE) at baseline and calculated to reduce total energy balance by 390 kcal/d.[90] Subjects will be provided counseling and written instructions on how to achieve their daily caloric goal, including use of their own mobile phone applications to monitor caloric intake. To assure compliance with the prescribed caloric goal, subjects will meet with the study dietitian every other week for problem solving and review of diet intake logs.

REE will be measured in the supine position using a Parvo TrueOne 2400 portable metabolic system (ParvoMedics, Sandy, Utah) in a dimly lit, temperature-controlled room. A ventilated plexiglass hood will be placed over the subject's head and connected to the metabolic cart by a single expired-gas hose. Indirect calorimetry will proceed for 25-30 min, with the first 5-10 min eliminated from analysis. Before each use, the system will be calibrated using room air and a single gas tank (~16% O_2, 1% CO_2). Whole-body rates of O_2 consumption and CO_2 production will be determined from measurements of expired volume, and the differences in O_2 and CO_2 concentration between inspired and expired air. Ventilation is measured by a mass flow meter, oxygen concentration by a paramagnetic O_2 analyzer, and CO_2 by an infrared analyzer. REE will be calculated from the Weir equation.[91]

Dietary recalls will be performed by trained registered dietitians using the validated U.S.D.A. multi-pass recall method, a standardized script and software generated prompts. Intakes will be analyzed for energy and nutrient content via Nutrition Data System (NDSR version 2016, U Minn) which automatically calculates average energy, macronutrients, micronutrients, nutrient ratios, food components and indices. Our standardized protocols and portion estimation methods minimize underreporting even among people with higher BMI. In comparing data from our prior cohorts to NHANES data, we show <5% variation for expected average energy and macronutrient intakes. To reduce subject burden, our trained dietitians and technicians are able to complete recalls in ~20 min. Weight cycling history will be obtained via a questionnaire which will be administered by the study dieticians.

Mixed meal study: Following baseline sampling, participants will be given a test meal (712 kcal, 33% carbohydrate, 45% fat, and 22% protein) composed on two 8-ounce cans of Glucerna®1.5 cal. They will be asked to consume the meal within ten min and we will sample at the time points noted in the study day protocol. We used this protocol in the studies presented under preliminary data.

Acetaminophen ingestion and measurement: On baseline study day 1 and study days 3 and 4, the liquid mixed meal will be mixed with 1,500mg liquid acetaminophen, compounded at the IDS pharmacy in 22.5mL. Remainder of procedure on these days remains unchanged as compared with typical mixed

meal study described above, including timing and amount of blood samples after ingestion.

Vascular endothelial function: Measurement of endothelium-dependent and endothelium-independent vasodilation will be performed in the CRC in a quiet, temperature-controlled (23°C) room. Patients will be asked to refrain from alcohol and caffeine for at least 12 hours prior to vascular function measurements. In addition, subjects will be asked to abstain from phosphodiesterase type 5 (PDE5) inhibitors for at least one week as these drugs may confound the measurement of vascular function or cause precipitous hypotension after nitroglycerin. Any premenopausal women will be studied during the follicular phase of the menstrual cycle to avoid variability of vascular function measures.

Vascular function will be measured as reported previously by the applicants.[92 95] Brachial artery diameter is measured using B-mode ultrasonography equipped with a high resolution linear array transducer (7.5 mHz). A longitudinal image (parallel to the artery) is acquired just proximal to the antecubital fossa with the transducer positioned to optimize images of the near and far wall interfaces. A simultaneous electrocardiographic signal is recorded and images are digitally acquired at end-diastole, synchronized to the R wave on the electrocardiogram. To assess endothelium-dependent vasodilation, brachial artery diameter is measured under basal conditions and during reactive hyperemia. Reactive hyperemia results after five min of ischemia produced by inflating a BP cuff on the upper arm to suprasystolic pressures. We have found that following cuff deflation, the maximal increase in brachial artery diameter occurs at approximately one min of reactive hyperemia, a response mediated by endothelium-derived nitric oxide. [93] After a rest period, endothelium-independent vasodilation will be assessed by imaging the brachial artery under basal conditions and following the administration of sublingual nitroglycerin (0.4 mg). Maximal brachial artery dilation occurs three to four min after sublingual nitroglycerin. The video output and electrocardiographic signal of the ultrasound machine will be connected to a computer equipped with a Data Translation frame grabber videocard. The R wave on the electrocardiogram will be used as a trigger to acquire frames. Digitized images will be stored on the hard drive and backed up on removable media. Acquisition and analysis of the stored images will be performed using software designed for this purpose by Medical Imaging Applications. The vessel wall lumen interface will be determined by derivative-based edge detection following identification of the region of the anterior and posterior walls by the investigator. The maximum diameter of the vessel will be determined and the percent change in diameter calculated. We have found that this technique yields an inter-observer variability of 0.05±0.16% and intra-observer variability of 0±0.15%.

Adipose harvest: Adipose tissue will be obtained from the periumbilical area using a Tulip CellFriendly™ GEMS closed syringe system for lipoaspiration. Under aseptic conditions and local lidocaine anesthesia, a small incision is made

in the skin. The GEMS Johnnie Snap lock is placed into a 60-cc syringe, the Tulip liposuction cannula with 60-cc syringe attached is inserted at an angle through the incision to below Scarpa's fascia, and suction is applied until the syringe activates the clicker lock. The needle is moved in and out at a rate of approximately 1 Hz without breaking suction with a twisting motion. The sampling continues until approximately 5-10 g of tissue is removed (~5.5-11 cc at a specific gravity of 0.918 for human fat). The syringe containing the sample will be taken on ice to the lab where it will be separated into pieces for DNA and RNA isolation, adipocyte size and number, cytokines, and adipokines, and extraction of T cells. Immune cell extraction will be performed using a gentleMACS™ Dissociator from Miltenyi Biotec followed by incubation with collagenase, mononuclear cell separation with Ficoll-Paque and cryopreservation for flow cytometry/sorting.

Endothelial cell harvest: Endothelial cell harvest will be performed by passing a 0.018" sterile J- wire (Arrow International, PA) back and forth two to six cm beyond the tip of a venous catheter placed in a cubital vein. Approximately 1000-1500 ECs can be isolated after four passes of the wire. Cell viability is >90% with >90% staining positive for expected EC markers (nitric oxide synthase, vWF) without contamination by leukocytes or smooth muscle cells.

PBMC harvest: PBMCs will be obtained through the peripheral IV into 3 EDTA tubes, and transported at room temperature to the lab for processing. Cells will then be passed through a Ficoll-Paque barrier, washed with PBS, and stored in DMSO.

Magnetic Resonance Imaging (MRI): Consecutive images of the thigh will be obtained using a 3.0 tesla MRI unit (Philips Medical Systems), Coronal slides of the thigh will be used to estimate the IMAT volume and infiltration. The later will be obtained by calculating the ratio of IMAT to total muscle volume. IMAT is defined as the fat beneath the deep fascia of the thigh. The calculations will be performed using a custom written Matlab (Mathworks, Natick, MA, USA) program. IMCL will be measured using ^{1}H-magnetic resonance spectroscopy. Spectra will be obtained from 20 x 20 x 20 mm voxels localized in the quadriceps muscle of the thigh using a PRESS sequence. Analysis will be performed using the AMARES algorithm in the MRUI software package.

Assessment of platelet activation: Blood for assessment of platelet activation will be collected after at least 5 ml of whole blood has been collected for another purpose. Blood will be collected at the baseline visit before the mixed meal and study days 1, 2, 3, and 4 after exposure to exendin-9-39 or placebo infusion. Whole blood will be removed from the sodium citrate tubes for antibody staining to detect platelet-leukocyte aggregates via flowcytometry. From whole blood, platelets will be counted and platelet-rich plasma generated. Platelet-rich plasma will be stimulated with various platelet agonists and antagonists and platelet aggregation assessed using a luminoaggregometer. In those samples which respond to platelet agonists, glucagon-like peptide-1 receptor agonists and antagonists will be coincubated with agonists to assess platelet aggregation.

Stimulated-platelet rich plasma will be separated into platelets and supernatant for assessment of platelet protein, cytokine, and RNA function and concentration.

Laboratory Analyses

Clinical assays will be run in Vanderbilt CLIA-approved laboratories. Blood drawn for research assays will be centrifuged immediately at 0ºC for 20 min, and plasma or serum will be divided into at least two aliquots, labeled, logged and stored separately at -80˚C until sampling.

Genotyping: DNA will be extracted from whole blood using the AutoPure LS extraction system (Qiagen, Valencia, CA, USA). *GLP1R* rs6923761 will be genotyped using a TaqMan assay (Applied Biosystems, Foster City, CA). SDS v2.4 (Applied Biosystems) will be used to create cluster plots and identify sample-associated fluorescent markers for genotype call.

Albumin and creatinine: Creatinine will be measured using the Jaffe method. Urine albumin will be measured by turbidimetric immunoassay with endpoint determination.

Glucose, C-peptide, Insulin, HbA_{1C} and metabolic parameters: Plasma glucose will be measured by glucose oxidase method with a YSI glucose analyzer (YSI Life Sciences, Yellow Springs, OH). Plasma insulin will be measured by radioimmunoassay (RIA; Millipore, St. Charles, MO). This assay cross-reacts with intact human pro-insulin (38%) but not with C-peptide (≤0.01%). Samples for C-peptide will be drawn in heparinized tubes containing 250 KIU/mL aprotinin, and C-peptide will be measured using RIA (Millipore). HbA_{1C} will be measured using high pressure liquid chromatography (HPLC). Blood for GLP-1 will be collected in tubes containing EDTA and aprotinin. GLP-1 will be determined using a multiplex magnetic bead assay (Milliplex MAP Human Metabolic Hormone Magnetic Bead Panel, EMD Millipore) that detects active GLP-1(7-36) from 4 to 3033 pmol/L with no cross-reactivity for GLP-1(9-36). We will measure total PYY and PYY (3-36) by RIAs (Millipore). FFA will be measured via gas chromatography in the Vanderbilt Diabetes and Research Center Lipid Core. Catecholamines and acetaminophen will be measured by HPLC with electrochemical detection.

Markers of inflammation, oxidative stress, and fibrinolysis: Inflammatory cytokines such as IL-6 and TNF-α will be measured by BD™ Cytometric Bead Array in the Vanderbilt Immunology Core. P-selectin will be measured using a commercially available ELISA kit (R&D, Minneapolis, MN). We will measure 11-dehydro-thromboxane B_2 and F_2-isoprostanes using mass spectrometric methods developed at Vanderbilt.[96, 97] Blood for PAI-1, t-PA, and vWF will be collected in 0.105 M acidified sodium citrate and samples will be analyzed using commercially available two-site ELISA using chromogenic substrates (TriniLIZE, Trinity Biotech, Berkeley Heights, NJ and American Diagnostica Inc, Stamford, CT).

[redacted]

[redacted]

Measurement of adipose-resident immune cell populations and gene expression profiles related to local inflammatory mediators, adipocyte metabolism and regulation (exploratory). Based on experience we expect to collect ~40-80,000 CD3+ T cells and 50-100,000 CD68+ macrophages per 5-10 g adipose. We will use flow cytometry to assess activated, memory, exhausted, and senescent CD4 and CD8 populations using surface markers CD38, HLA-DR, CD69, CD57, PD1, and CD45RO (as examples), as well as M1 (CD14+CD16+ with either CD86 or CD36) and M2 (CD163, CD301, and CD206) macrophage polarization. T cell and macrophage subsets will be FACS sorted and cryopreserved for later analyses. Adipose can also be used to assess quantitative mRNA expression of major inflammatory and adipocyte regulatory genes using the Nanostring nCounter Plex2 Assay Kit in the Vanderbilt Technologies for Advanced Genomics core. Genes of interest include peroxisome proliferator-activated receptor-γ (PPAR-γ, regulator of adipocyte function), glucose transporter type 4 (GLUT4), lipoprotein lipase, adiponectin, and CCAAT enhancer–binding protein-α (C/EBPα), in addition to pro-inflammatory mediators including TNF-α, IL-6, macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein 1 (MCP-1), and PAI-1, as well macrophage activation markers (e.g., MIP-1α/β, MCP-1) and cytokines to differentiate M1 vs. M2 macrophage polarization (e.g., IL-12, IL-23 for M1, and IL-10, TGF-β for M2, and others).

Evaluation of PBMC immune cell populations: We will use flow cytometry to assess activated, memory, exhausted, and senescent CD4 and CD8 populations using surface markers CD38, HLA-DR, CD69, CD57, PD1, and CD45RO (as examples), iNKT cells using CD1d tetramer, as well as M1

(CD14+CD16+ with either CD86 or CD36) and M2 (CD163, CD301, and CD206) macrophage polarization.

7.0 Risks

1. Insertion of venous catheters may cause bleeding, bruising, or infection.
2. Frequent blood draws can cause anemia.
3. During measurement of flow-mediated vasodilation, the administration of sublingual nitroglycerin can cause low blood pressure. Nitroglycerin may also cause severe headache. Patients will be monitored for this and the effects of nitroglycerin are transient. Patients will be asked to refrain from taking a PDE5 inhibitor at least one-week before the study day.
4. Spending study days at the CRC can be inconvenient for subjects.
5. Harvesting adipose tissue could cause pain, bleeding or infection. We will use local lidocaine injection to numb the area and sterile technique.
6. Harvesting endothelial cells from veins could cause pain, bleeding or infection, primarily from intravenous placement, as well as damage to the vein. We will use local lidocaine injection to number the area and sterile technique. We utilize a wire that has a soft rounded end to reduce any risk. We will utilize superficial veins. We will allow at least four weeks before repeating the procedure in the same vein.
7. Liraglutide causes dose- and duration-dependent thyroid C-Cell tumors in rats and mice. There have been a handful of cases of thyroid C-cell hyperplasia among liraglutide-treated patients. At present monitoring calcitonin concentrations is not recommended. We will exclude patients with risk factors for medullary carcinoma of the thyroid. We will check patients for thyroid nodules at the screening physical examination and at the end of the study. In the unexpected event that someone were to develop a nodule, we would refer them for evaluation. The duration of exposure to liraglutide in this study is only 14 weeks.
8. Liraglutide and sitagliptin use have been associated with an increased risk of pancreatitis, but patients often had other risk factors for pancreatitis. We will exclude patients with a history of pancreatitis and monitor them for symptoms.
9. Antidiabetic agents such as liraglutide and sitagliptin can cause hypoglycemia.
10. Liraglutide can cause adverse gastrointestinal symptoms including nausea (28.4%), diarrhea (17.1%), vomiting (10.9%), and constipation (9.9%). We are starting with a lower dose for one week to reduce these symptoms.
11. DPP-4 inhibitors such as sitagliptin can increase the risk of angioedema in patients who are taking ACE inhibitors, but this is a rare event. We will advise patients of this potential risk and instruct them to stop their ACE

inhibitor and study medication should this occur. We will provide contact numbers.

12. Exendin-(9-39) (**IND #122,217**) is an investigational agent (Clinalfa®, Bachem Distribution Services; Weil am Rhein, Germany) that will be infused intravenously following dissolution in 0.25% human serum albumin. Salehi et. al. have not experienced any untoward effects with the use of this antagonist in healthy participants, those with T2DM, or history of gastric bypass.(40;42;48) Deane et. al. reported that blockade of GLP-1 action was associated with accelerated gastric emptying and larger glucose excursions in the hour following meal intake(49). We have not observed any adverse events in nine normal subjects we have studied to date under another protocol.
13. During the mixed meal study, patients can develop hyperglycemia. Because we are not studying individuals with diabetes, this should not be significant.
14. Acetaminophen given at higher than total daily recommended doses or to individuals with liver disease can cause acute liver failure. The dose used in this study of 1.5 gram is significantly less than the 4 gram daily limit, and we exclude participants with liver disease based on abnormal liver function tests. We will also ask participants to refrain from the use of acetaminophen for 4 days before and 4 days after the study days.

8.0 Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

A Data and Safety Monitoring Committee (DSMC) will provide objective review of human safety and data quality. Committee members will be Marie R. Griffin, MD, Professor of Health Policy; Alvin C. Powers, MD, Director of the Vanderbilt Diabetes Center and Joe C. Davis Professor of Medicine and Biomedical Sciences; Dr. Naji N. Abumrad, John L. Sawyers Professor of Surgery and Chairman Emeritus, Department of Surgery. Dr. Abumrad will chair the committee. All members hold a primary appointment outside the Department of Medicine (Griffin and Abumrad) or lead a Research Center that reports directly to the Dean (Powers).

The DSMC will also receive quarterly reports of enrollment, protocol adherence, data quality, and adverse events (AE)s. The DSMC will review all serious AEs (SAEs), suspected unexpected serious adverse reactions (SUSARS), serious adverse drug reactions (SADRs). Any SAE, SUSAR, or SADR will be reported to the DSMC, IRB, NIH, Novo Nordisk (if supplying liraglutide) and FDA if appropriate [regarding EXENDIN (9-39)] as soon as possible, but not more than 10 days from the investigators' awareness of the event. Any pregnancy occurring during the trial would be reported similarly.

Subjects will be questioned about AEs at each study visit. Any untoward medical event will be classified as an AE, regardless of its causal relationship with the study. Relationship to a study medication will be assessed as probably,

possible or unlikely based on the United States Package Insert for the drug and in the case of Exendin (9-39) based on temporal association. An AE will be classified as serious if it a) results in death, b) is life-threatening, c) requires inpatient hospitalization or prolongation of existing hospitalization, d) results in persistent or significant disability or incapacity, e) is a congenital anomaly or birth defect. Suspicion of transmission of infectious agents will also be considered an SAE. The DSMC may choose to become unblinded; however, it is expected that such unblinding would not occur without reasonable concern related either to patient safety or to data validity.

9.0 Study Withdrawal/Discontinuation

Subjects who develop an adverse event that is not transient (such as persistent nausea, more than one hypoglycemic event, etc.) will have any study drug discontinued and will be withdrawn from the study. Subjects who do not tolerate the 1.8 mg/d dose of liraglutide will be discontinued. Subjects who are withdrawn will be treated and/or followed as appropriate until any symptoms are resolved. If it is determined by Vanderbilt and the PI that an adverse event occurred as a direct result of the tests or treatments that are done for research, then neither the subject nor his or her insurance will have to pay for the cost of immediate medical care provided at Vanderbilt to treat the adverse event. This includes clinically significant laboratory values related to the study.

If in the opinion of the investigator a subject is non-compliant, the subject will be withdrawn from the study.

10.0 Statistical Considerations

Sample Size Calculation and Statistical Analysis

The primary endpoints are endothelial vascular function (FMD, UACR), and markers of endothelial fibrinolytic function (e.g. PAI-1). Secondary endpoints will include BP, HR, lipids, fasting insulin and glucose, insulin and glucose in response to a mixed meal, F_2-isoprostanes, circulating markers of inflammation (IL-6 and TNF-α), and markers of platelet function (P-selectin and 11-dehydro-thromboxane B_2). Exploratory endpoints will include adipose-resident immune cell numbers and gene expression, and endothelial cell inflammatory phenotype and eNOS and phosphorylated eNOS expression.

In Aim 1, the primary analyses will focus on liraglutide versus weight loss, and on liraglutide versus sitagliptin, on primary, secondary and exploratory endpoints.

In Aim 2, primary analyses will focus on liraglutide + placebo versus liraglutide + the GLP-1 antagonist Exendin 9-39, and on sitagliptin + placebo versus sitagliptin + Exendin 9-39 comparisons on primary, secondary and exploratory endpoints.

In Aim 3, the primary analyses will focus on the effect of GLP1R rs6923761 genotype on the primary and secondary endpoint responses to liraglutide.

Sample Size and Power Calculation:

FMD: In studies of weight loss, improvement in endothelial function is proportionate to weight loss and the degree of weight loss achieved in LEADER has been associated with a 1.3%±2.1% increase in brachial artery FMD.[22] With 80 subjects in the liraglutide group and 40 subjects in each of the hypocaloric diet and sitagliptin groups, we will have 80% power to detect an improvement in FMD of 2.45%±2.1% in the liraglutide versus 1.3%±2.1% in the hypocaloric diet or sitagliptin group. In Aim 2, for the within-subject comparison, we will have 80% power to detect a 24.5% reduction in ΔFMD (from 2.45% to 1.85%) by Exendin 9-39 in the liraglutide group, and a 65% decrease (from 1.3% to 0.45%) in ΔFMD in either the weight loss or the sitagliptin group. The latter is of the order of magnitude of the difference in FBF following arginine stimulation during sitagliptin in our preliminary study with Exendin (9-39).

In Aim 3, we expect the distribution of rs6923761 to be 42 GG subjects: 38 A allele carriers (GA + AA), based on our prior observed genotype frequencies in blacks and whites. In a prior study of the relationship between rs6923761 genotype on weight loss in response to 14-week treatment with liraglutide 1.8 mg/d, those in the GG group lost 50% less fat mass than the mean, and A carriers lost 37% more fat mass than the mean for all subjects.[57] If the effect of genotype on FMD is similar we would expect to find a 1.68% increase in the GG group versus a 3.3% increase in A carriers in response to liraglutide. With the expected genotype frequencies (38 A carriers) we should have 92% power to detect this difference.

UACR: von Scholten reported a 30% (95% CI -44% to -12%) decrease in 24-hour urine albumin excretion from 32.7 (10.4-61.4) mg to 23.0 (8.5-43.4) mg after seven weeks of treatment with liraglutide 1.8 mg/d.[98] Intensive lifestyle modification had no effect on UACR in the Diabetes Prevention Program study.[99] Sitagliptin has been reported to have a minimal effect (-0.18, -0.35 to 0.20, µg/mg Cr) on UACR in well-controlled studies.[100] In a recent study by the investigators of obese patients with pre-diabetes UACR was 12.67±14.67 µg/mg Cr at baseline.[76] With 80 subjects in the liraglutide group and 40 subjects in each of the hypocaloric diet and sitagliptin groups, we will have 80% power to detect the difference between a 30% decrease in the liraglutide group versus a 5% decrease in UACR in either of the other two groups using an SD of 45.5. In Aim 2, for the within-subject effect of Exendin 9-39, we have 80% power to detect a 43% reduction (from 30% to 17%) in the decrease in UACR in the liraglutide group. For Aim 3, if the magnitude of the influence of rs6923761 genotype on UACR is similar to the effect on loss of body fat, we would expect to observe a 17% versus 44% decrease in UACR in the GG versus A carriers, respectively. With 38 A carriers in the liraglutide group, we will have 85% power to detect this difference using SD of 40.

PAI-1: Courrèges reported that 14-week treatment with liraglutide 1.2

mg/d reduced PAI-1 29% (6.38 ng/dL) from a baseline of 22.0±13.0 ng/mL in T2DM.[101] This is similar to the magnitude of change we have observed in studies of the effect of ACE inhibitors on PAI-1.[102, 103] Surgical and dietary weight loss are also associated with decreases in PAI-1.[25] (We have not observed an effect of sitagliptin on PAI-1.) With 80 in the liraglutide and 40 in the sitagliptin group, we have 80% power to detect the difference between a 6.38±11.6 ng/mL (11.6 is the SD of the decrease assuming a correlation of 0.6 between baseline and post-treatment measures) decrease in the liraglutide versus sitagliptin group. For the within-group comparison in Aim 2, we have 80% power to detect a 58% reduction in the PAI-1 response to liraglutide by Exendin 9-39 (from 6.38 to 2.7 ng/mL). For Aim 3, if the magnitude of the influence of rs6923761 genotype on PAI-1 is similar to the effect on loss of body fat, we would expect to observe a 14.5% versus 39.7% decrease in PAI-1 in the GG versus A carriers, respectively. We are not adequately powered to detect this difference (56% power), but we will have perfect (99.5%) power to detect a 39.7% or 8.73±11.6 ng/mL decrease in PAI-1 within A carriers.

Data Analysis Plan: We will use standard graphing and screening techniques to detect outliers and to ensure data accuracy. We will assess continuous outcomes for normality. If normality is violated, we will apply data transformation or consider non-parametric analysis methods. We will provide summary statistics for both numerical and categorical variables by study arms. We will assess comparability among randomization groups.

We will use a 2x2 crossover of Exendin (9-39)/placebo in the three arms. Although we have designed the study to avoid carryover effect, we will evaluate for potential carryover using two approaches. First, we will test for carryover effect using the T-test approach described in Section 2.3 (page 21) of Jones and Kenward (2003).[104] Second, we plan to take baseline measurement of study endpoints right before study subjects are given study medication [Exendin (9-39) or placebo] in both treatment periods. This will allow us to estimate any residual carryover effect using baseline measurements obtained in the Exendin (9-39) followed by placebo sequence. This estimate would enable us to evaluate still the Exendin (9-39) vs placebo difference using data collected in both crossover periods versus the usual approach in which one would have to discard period two data in the presence of carryover.

We will use mixed-effects models with a random subject effect and with treatment factor 1 (liraglutide versus weight loss versus sitagliptin), treatment factor 2 [Exendin (9-39) versus placebo], and GLP1R rs6923761 genotype groups (GG versus A allele carriers) as fixed effects. We will use an autoregressive model of order 1 [AR(1)] or other plausible covariance structures for the error covariance. If it appears to be present, residual carryover effect can be included in the model as a fixed effect. We will evaluate treatment effects using properly set-up contrasts in the mixed-effects models. Week 2 and week 14 data will be analyzed separately.

We will also utilize mixed-effects models to evaluate treatment factor 2 (Exendin (9-39) versus placebo) within each treatment arm. We will conduct

additional sub-analyses such as general linear models (GLM) to evaluate the effect of treatment factor 1 (liraglutide versus weight loss versus sitagliptin) or GLP1R rs6923761 genotype groups (GG versus A carriers) using data collected during the placebo or Exendin (9-39) period of treatment factor 2. Both GLM and mixed-effects models provide the flexibility of controlling for and of evaluating covariates, such as gender, race, and concurrent medication.

In addition to evaluating treatment effects using the above regression models, we will estimate direct between-group difference in means with its 95% confidence interval (CI) in Aims 1 and 3. These differences will be tested using either two-sample t-test or Wilcoxon Rank Sum test. We will calculate within-subject mean difference and its 95% CI for Exendin (9-39) vs placebo comparison within one of the three treatment arms in Aim 2. These differences will be tested using paired t-test or sign rank test.

Subjects who drop out early in the study will be replaced to minimize the problem of missing data. Nevertheless, if data are missing for a particular time point, mixed-effects models are robust in that subjects with missing data at some time points can be included to estimate effects of interest. In addition, we will conservatively impute missing data to perform corroborative analyses with and without missing data.

Analyses of secondary endpoints and other exploratory analyses will be conducted similarly. We will test all hypothesis at the level of α=0.05. We will use SPSS for Windows (Version 24.0, SPSS, Chicago) and the open source statistical package R (version 3.1.0, R Core Team, 2014) for analyses.

The principal investigator will strive to publish findings in a timely manner, will acknowledge research support, and ensure that it is accessible. It is also expected that the investigator or her collaborators or trainees will present data at one or two national meetings per year such as the American Diabetes Association or the Scientific Sessions of the American Heart Association.

11.0 Privacy/Confidentiality Issues

We will use the web-based Vanderbilt Research Electronic Data Capture (REDCap) system to design electronic data-collection forms in all Aims. These forms will be pilot tested before use. Data will be input into a protected, web-based case report form (which can be readily downloaded into SAS, STATA, R, or SPSS). The form allows for direct data entry by investigators and is designed to minimize errors and erroneous values. Results from the Vanderbilt Clinical Laboratory can also be directly imported to REDCap, which further reduces typographical data-entry errors. Expected ranges are pre-specified to prevent errors such as the shifting of decimal points. The program includes a computerized audit trail so that the identity of individuals entering or changing data and, in the case of changes, both original and revised data are saved. Data are backed up daily. Clinical data, including clinical laboratory, will be entered by the research nurse. Research laboratory data will be entered by a fellow or research technician in the laboratory.

A unique identification case number will be used to protect the confidentiality of the study participants. Only case numbers will be included in spreadsheets used for the statistical analysis.

12.0 Follow-up and Record Retention

All research records will be accessible for inspection and copying by authorized representatives of the IRB, federal regulatory agency representatives, and the department or agency supporting the research. All study documents will be retained for at least six years after closure of the study with the IRB.

Appendix A
***Informed consent will be obtained prior to Screening Visit 1**

	Screening 1	Screening 2		Start of Run-in	Baseline phenotyping	Randomization	Study Day 1	Study Day 2				Re-phenotype	Study Day 3	Study Day 4
Time (weeks)	-7	-7	-6		-1	0	1	1	4	8	12	13	14	14
History	X													
Physical Exam	X													
Height	X													
Weight	X				X		X	X	X	X	X	X	X	X
Waist and hip circumference	X													
Screening laboratory	X													
ECG	X													
OGTT		X												
UACR		X			X							X	X	
Optimization of medications			X	X										
Pregnancy test		X			X	X	X	X				X	X	X
Randomization						X								
Study Medication refill									X	X	X			
FMD					X		X	X					X	X
BP HR					X		X	X		X	X	X	X	X
Blood sampling					X		X	X				X	X	X
Adipose harvest					X							X		
Endothelial cell harvest					X							X		
Mixed-Meal Study					X		X	X					X	X
DEXA		X										X		
MRI (optional)		X										X		
Resting energy expenditure		X										X		
Exendin or placebo infusion							X	X					X	X
AE questionnaire	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Concomitant meds			X	X	X	X	X	X	X	X	X	X	X	X

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