Contribution of Neuropeptide Y (NPY) to Vasoconstriction and Sympathetic Activation in the Setting of Dipeptidyl Peptidase IV (DPP4) Inhibition

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PI: Nancy J. Brown, M.D.

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1.0 Background

According to the 2005-2006 National Health and Nutrition Examination Survey (NHANES), approximately 65 million people in the United States have hypertension.[1] Hypertension is the most important modifiable risk factor for kidney disease, cardiovascular disease and stroke.[2-5] Comorbidities such as dyslipidemia, obesity, and insulin resistance account for additional cardiovascular risk. These cardiovascular risk factors comprise the metabolic syndrome, which in turn increases the risk of type 2 diabetes (T2DM). Diabetes has also been associated with a dramatically increased risk of heart attack, stroke, and renal failure.[6] It is estimated that more than 24 million people in the United States have diabetes. In these individuals, the prevalence of hypertension is 1.5 to 3 times greater than in sex-aged-matched controls.[7,8]

Given the additive cardiovascular risk of diabetes and hypertension, blood pressure goals in diabetic subjects are ≤ 130/80 mm Hg, as defined by the American Diabetes Association (ADA) [9] and the Seventh Joint National Committee for the Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7).[10] The evidence that supports the lower blood pressure goals in diabetic patients comes from large controlled studies, such as The United Kingdom Prospective Diabetes Study (UKPDS), which demonstrated a 45% reduction in relative risk of fatal and non-fatal stroke with tight BP control, with a BP 10/5 mm Hg lower than the group randomized to less controlled.[11] Similarly, the Hypertension Optimal Treatment study showed a 50% reduction in cardiovascular disease in the group randomized to achieve a diastolic blood pressure below 80 mm Hg.[12]

In order to achieve blood pressure goals, lifestyle modifications and pharmacologic therapy are the mainstay therapies. Lifestyle modifications have proven to be effective, however only few patients are able to achieve blood pressure control with those interventions.[13] The JNC 7 guidelines recommend starting both antihypertensive medication and lifestyle modifications in patients with diabetes when hypertension is diagnosed.[10] Among the antihypertensive drugs available, angiotensin converting enzyme (ACE) inhibitors are the first-line therapy in patients with hypertension and diabetes, according to guidelines from the ADA, the NKF, the World Health Organization, and the JNC 7. [9,10,14,15]

To reduce the risk of cardiovascular, cerebrovascular disease and renal failure in patients with both diabetes and hypertension, it is also important to achieve a good glycemic control. Among the anti-diabetic agents, dipeptidyl-peptidase-4 (DPP-4) inhibitors are novel anti-diabetic agents. These drugs inhibit the DPP4 enzyme that degrades the incretin hormones GLP-1 and GIP, which stimulate insulin release in response to an enteric glucose load.[16] The first DPP-4 inhibitor approved by the FDA was sitagliptin. Since its approval in 2006 this drug has become one of the leading branded oral anti-diabetic agents in the United States.[17] DPP-4 inhibitors offer advantages over commonly used anti-diabetic agents, such as a glucose-dependent mechanism of action and lack of weight gain.[18,19,20] However, there is information that remains to be known regarding safety, efficacy and drug interactions of DPP4 inhibitors.

2.0 Rationale and Specific Aims

Hypertension and diabetes are important modifiable morbidity and mortality risk factors. The prevalence of hypertension is nearly 1 billion worldwide.[21] On the other hand; approximately 246 million people have diabetes worldwide.[22] Among the latter the incidence of hypertension is 1.5 to 3 times higher than in sex and age matched control.[7] The first line therapy in patients with diabetes and hypertension are angiotensin converting enzyme (ACE) inhibitors because of its renoprotective and antithrombotic properties. Despite the wide variety of anti-diabetic drugs available, it remains difficult to achieve adequate glycemic control. DPP4 inhibitors are a promising new anti-diabetic drug class. By inhibiting the degradation of the DPP4 enzyme, they prevent the degradation of incretins GLP-1 and GIP, which increase the release of insulin, suppress glucagon release, and delay gastric emptying.[16]

Notably, DPP4 enzyme intervenes in the degradation of several vasoactive peptides such as brain natriuretic peptide (BNP), Neuropeptide Y (1-36), Peptide YY (1-36), and substance P (SP).[23] The ubiquitous nature of this enzyme would translate into diverse hemodynamic effects, and could potentially result in diverse drug interactions, as it was ascertained by Jackson et al. They found that the DPP-4 inhibitor, P32/98, significantly increased blood pressure in pre-treated (captopril 30 mg/kg or hydralazine 5mg/kg) adult spontaneous hypertensive rats (SHR).[24] Interestingly this effect was abolished by effective ganglionic blockade with chlorisondamine, suggesting that activation of the sympathetic nervous system was determinant for the increased blood pressure seen in the SHR model.[24] The hypertensive effect induced by the DPP-4 inhibitor in that study was attributed to neuropeptide Y; nevertheless a hypertensive effect mediated through other vasoactive peptides, such as substance P could not be ruled out.

Substance P is primarily inactivated by angiotensin-converting enzyme (ACE); under ACE inhibition however, DPP-4 becomes the major degradation pathway of this peptide.[25] Therefore, the interactive inhibition of DPP-4 and ACE will theoretically result in a significantly impaired degradation of substance P. Furthermore, ACE inhibition decreases the degradation of bradykinin which in turns increases the release of substance P by sensory nerves.[26] This has been associated with an increased risk of ACE inhibitor-associated angioedema in patients treated with the DPP-4 inhibitor, vildagliptin.[27] It is important to note that although SP induces vasodilation by acting on NK1 receptor,[26] it does not appear to decrease peripheral vascular tone, or systemic blood pressure.[28]

We have previously assessed the hemodynamic effects of the concomitant inhibition of the DPP-4 enzyme and the ACE in subjects with metabolic syndrome. The main finding of the study was that sitagliptin prevented the decrease in mean arterial blood pressure (MAP) when ACE was fully inhibited by enalapril, but not during sub-maximal ACE inhibition. Furthermore, the heart rate (HR) significantly increased in response to 10 mg enalapril plus sitagliptin. This was also associated with an increase in plasma catecholamine levels, which suggested an increased sympathetic outflow.[29] These effects on blood pressure, heart rate, and sympathetic activity could be due to an unrestrained amount of substance P, and may be enhanced by downstream effects of neuropeptide Y via Y1 receptor activation.[29]

The direct effects of neuropeptide Y, particularly in the setting of DPP4 inhibition have not

been fully characterized in humans. Neuropeptide Y is co-stored with norepinephrine in large vesicles of the perivascular sympathetic nerves. Neuropeptide Y is degraded to neuropeptide Y (3-36) by DPP4. Whereas neuropeptide Y causes vasoconstriction via the Y1 receptor and amplifies adrenergic (α1)-mediated vasoconstriction, neuropeptide Y (3-36) has no effect at the Y1 receptor and suppresses release of norepinephrine and neuropeptide Y via presynaptic Y2 receptors. In support of a contribution of neuropeptide Y to the hypertensive effects of DPP4 inhibition, Jackson et al have reported that DPP4 inhibition elevates BP in spontaneously hypertensive rats pre-treated with an ACE inhibitor, but not those pre-treated with a ganglionic blocker; moreover, the hypertensive effect of DPP4 inhibition was blocked by treatment with a selective Y1 antagonist.[24]

In addition to releasing catecholamines such as norepinephrine, substance P stimulates the release of neuropeptide Y. Unlike substance P, however, neuropeptide Y is only degraded by DPP4 and not degraded by both DPP4 and ACE. Therefore, although vascular effects of combined DPP4 and ACE inhibition may be most prominent, DPP4 inhibition alone may have notable effects on catecholamine release and vasoconstriction via neuropeptide Y Y1 receptor activation irrespective of anti-hypertensive use. (**Figure 1**) Understanding these mechanisms of neuropeptide Y will provide insight to DPP4 inhibition effects and future potential therapeutic targets for the treatment of hypertension.

Specific Aim 1:
To test the hypothesis that DPP4 inhibition decreases degradation of neuropeptide Y to neuropeptide Y (3-36), resulting in increased vasoconstriction (decreased forearm blood flow) and increased sympathetic activation in individuals with T2DM.

Hypotheses:
1a) We hypothesize that DPP4 inhibition, due to decreased degradation of neuropeptide Y to neuropeptide (3-36), will lead to increased vasoconstriction, evidenced by decreased forearm blood flow (FBF), and will increase norepinephrine and NPY (1-36) concentrations compared to placebo.
1b) We hypothesize that Power Spectral Analysis will demonstrate increased sympathetic activation (i.e. higher LF/HF_{RRI} ratio) after higher neuropeptide Y concentrations are administered.
1c) We hypothesize that with combined ACE and DPP4 inhibition, there will be increased substance P dependent release of neuropeptide Y (rather than an interactive effect of ACE and DPP4 inhibition directly on neuropeptide Y degradation).

3.0 Animal Studies and Previous Human Studies

Substance P is a member of the tachykinin family peptides, which interact with neurokinin-1 (NK_1), NK_2, NK_3 receptors.[34] Its effects however, are mainly mediated by NK_1 receptor. This peptide is widely distributed in enteric, peripheral, and central nervous systems. SP is involved in a wide variety of mechanisms, such as pain perception, nociception, emesis, and inflammatory responses.[35,36] The evidence suggests that SP is also involved in cardiovascular regulation.

Numerous animal studies have described the effect of substance P in the nucleus of the tractus solitarious (NTS) and its effect in the modulation of cardiovascular function.[37,38,39,40]

Furthermore, Comet et al analyzed the effect of intra-NTS administration of the selective NK_1 receptor antagonist, GR205171, on baroreflex bradycardia inhibition observed during the defense reaction triggered by electrical stimulation of the dorsal periaqueductal grey matter in anesthetized rats. The bilateral microinjection of SP in NTS produced an immediate and brief (6-7 min) increase in MBP (+25±2 mm Hg from a baseline of 84±1 mm Hg, $P<0.05$) and a concomitant decrease in HR (-45±7 bpm from a baseline of 334±5 bpm, $P<0.05$). Moreover, bilateral microinjections of SP into the NTS produced a dose-dependent inhibition of phenylephrine evoked-cardiac response and aortic cardiac response which was reversed by the selective NK1 receptors antagonist, GR205171.[38]

Scheneider et al demonstrated that the intraventricular injection of substance P in Wistar rats induced a hypertensive effect.[41] SP increased MAP and HR +31.4±2.5 mm Hg and +190±13.7 bpm from baseline, respectively. An increase in plasma cathecolamines was also apparent. Interestingly, these effects were significantly attenuated in a dose-dependent manner by the administration the GABA agonist, muscinol.[41] Similarly, the microinjection of substance P in the nucleus of the amigdala centralis also elicits pressor responses, whereas pre-injection with [D-Pro^2, D-Phe^7, D-Trp^9]-SP, a substance P antagonist, in the same area attenuated the pressor response to glutamate.[42]

The mechanism underlying the pressor actions and heart rate responses are attributed to sympato-adrenal stimulations, as demonstrated by pharmacologic testing and sympathetic nerve recording.[30,31,32] Unger et al reported increased BP, HR, and sympathetic nerve activity (splenic, renal, and adrenal nerves) in conscious rats. They found that the acute administration of 1 μg of substance P increased the sympathetic nerve activity by + 85%, + 147%, and + 63%, respectively ($P < 0.001$).[32] In a similar fashion, Dzurik et al demonstrated that substance P increases sympathetic outflow in humans, as evidenced by a decrease muscle sympathetic nerve activity (MSNA) with the administration of the NK-1 receptor antagonist, aprepitant.[33]

We have previously reported that the DPP4 inhibitor, sitagliptin, attenuates the hypotensive effect of enalapril (10 mg) in patients with metabolic syndrome. Mean arterial blood pressure changes were -7.9±2.4 mm Hg with placebo plus enalapril, whereas MAP was -0.9±0.9±2.3 mm Hg with sitagliptin plus enalapril. Interestingly, this effect was only evidenced with 10 mg of enalapril, but not with 5 mg. Moreover, the HR significantly increased in response to 10 mg enalapril plus sitagliptin. This was also associated with increased in plasma catecholamine levels, which suggests an increased sympathetic outflow.[29] We proposed that the effects on blood pressure, heart rate, and sympathetic activity could be due to an unrestrained amount of substance P, secondary to the inhibition of the two main enzymes involved in its degradation.[29]

In addition, neuropeptide Y (NPY) has previously been administered intra-arterially in humans. Nilsson et al infused 0.1, 0.3, 1.0, and 3.0 nmol/min of NPY (total concentration for each 1mL/min) to the brachial artery. They reported a dose dependent reduction in forearm blood flow. During the highest dose infusion of NPY, forearm blood flow was reduced from 4.3±2.0 to 2.3±1.2

mL/min/100mL. These findings did not differ significantly between subjects with or without hypertension.[49]

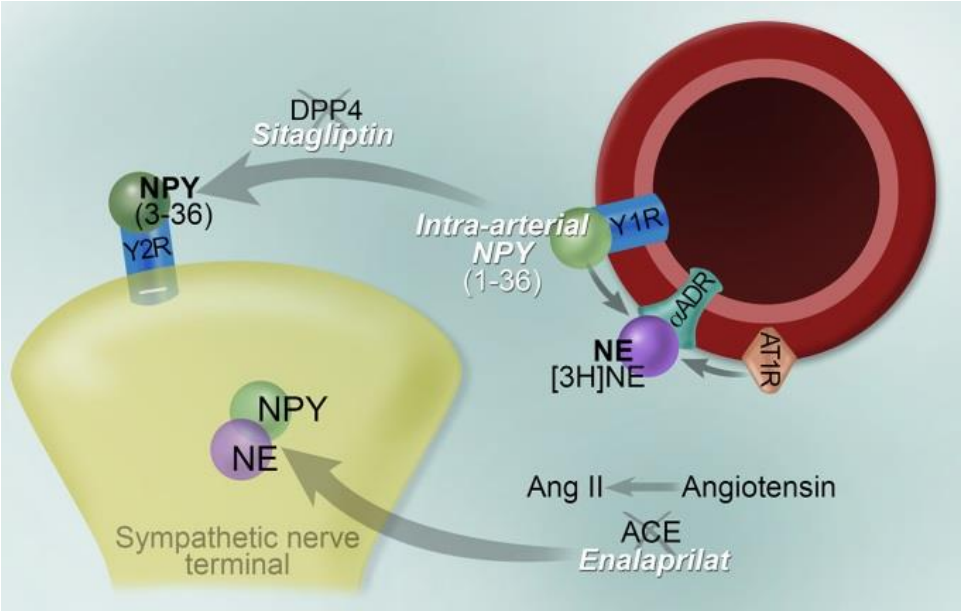
Similarly, Clarke and colleagues studied intra-arterial NPY administration. They also found that among their study population (normotensive subjects) NPY decreased forearm blood flow in a dose dependent manner. At maximal dose of 1,000 pmol/min administered for 6 minutes, the mean decrease in forearm blood flow was from 3.8±0.6 to 1.8±0.2 ml/100ml/min.[50]

NPY has also been administered intravenously in humans. Ullman et al studied intravenous doses of NPY: 3, 10, 30 pmol/min/kg infused at a rate of 1ml/min over 20 min and bolus infusions of 90, 200, or 900 pmol/kg in healthy subjects. Forearm blood flow was not measured in this study, however, arterial blood pressure increased with increasing doses of NPY, and this effect was attributed to vasoconstriction (baseline MAP 94±2 mmHg compared to 104±6 mmHg with highest dose NPY).[51]

Finally, Schuerch et al studied 12 healthy subjects who received intravenous phenylephrine with or without one of two doses of NPY: 1.4 and 14.3 pmol/kg/min for a total of 90min. Subjects who received higher doses of NPY were noted to have lower forearm blood flow and increased forearm vascular resistance, and these effects were more prominent with increased doses of co-administered phenylephrine.[52]

In agreement with this, Linder and colleagues evaluated healthy subjects who received phenylephrine administration with and without combined NPY at concentrations of 1 or 30 pmol/min. They found that the effects were synergistic, meaning that even at relatively small doses of NPY, NPY can potentiate α-adrenergic effects.[53]

Figure 1. Schematic Representation of Mechanisms Studied



4.0 Inclusion/Exclusion Criteria

Inclusion criteria

- Age 18 to 55 years old.
- For female subjects the following conditions must be met:
  - Postmenopausal status for at least 1 year, or
  - Status post-surgical sterilization, or
  - If of childbearing potential, utilization of some form of birth control and willingness to undergo β-HCG testing prior to drug treatment and on every study day
- T2DM, as defined by one or more of the following OR healthy controls
  - Hgb A1C ≥6.5%, or
  - Fasting plasma glucose ≥126mg/dL, or
  - 2-hour plasma glucose ≥200 mg/dL following 75gr oral glucose load

Exclusion criteria

- Type 1 diabetes.
- Poorly controlled T2DM, defined as Hgb A1C>8.7%.
- Use of anti-diabetic medications other than metformin.
- Hypertension.
- Subjects who have participated in a weight-reduction program during the last 6 months and whose weight has increased or decreased more than 5 kg over the preceding 6 months.
- Pregnancy. Breast-feeding.
- Treatment with any of the following drugs: cisapride, pimozide, terfenadine, astemizol
- Clinically significant gastrointestinal impairment that could interfere with drug absorption
- Cardiovascular disease that would pose risk for the subject to participate in the study, such as: myocardial infarction within 6 months prior to enrollment, presence of angina pectoris, significant arrhythmia, congestive heart failure (LV hypertrophy acceptable), deep vein thrombosis, pulmonary embolism, second- or third-degree AV block, mitral valve stenosis, or hypertrophic cardiomyopathy.
- Impaired hepatic function (aspartate amino transaminase [AST] and/or alanine amino transaminase [ALT] >2 x upper limit of normal range)
- Impaired renal function (eGFR< 60mL/min/1.73m^2 as determined by the MDRD equation).
- History or presence of immunological or hematological disorders.
- History of pancreatitis or known pancreatic lesions.
- History of angioedema or cough while taking an ACE inhibitor.
- Hematocrit <35%.
- Treatment with anticoagulants.
- Growth hormone deficiency.
- Diagnosis of asthma requiring use of an inhaled β-2 agonist more than 1 time per week.

- 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
- Treatment with systemic glucocorticoids within the last 6 months.
- Treatment with lithium salts
- Ongoing tobacco use or recreational drug use.
- Treatment with any investigational drug in the 1 month preceding the study
- Mental conditions rendering the subject unable to understand the nature, scope, or possible consequences of the study
- Inability to comply with the protocol, e.g., uncooperative attitude, inability to return for follow-up visits, and unlikelihood of completing the study

5.0 Methods and Protocol

Recruitment of subjects

Subjects will be recruited from the Vanderbilt outpatient clinics identified through Subject Locator (Starbrite/StarPanel). A member of the subject's health care team will request permission from the patient to introduce a member of the study team. We will also recruit from a pool of study volunteers (ResearchMatch) who have identified themselves to the Vanderbilt University Clinical Research Center (CRC) as interested in studies involving T2DM and/or hypertension, as well as using MyResearch@Vanderbilt and the ResearchDerivative. Written advertisements approved by the Vanderbilt Institutional Review Board (IRB) will be placed on Vanderbilt bulletin boards. Contact information for the study will be placed on the advertisements. Subjects 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 this meeting the research nurse and/or investigator will describe the study protocol in detail and answer all questions. Interested subjects will be invited to read and sign an IRB-approved consent form and will be given a copy of that consent form to take home.

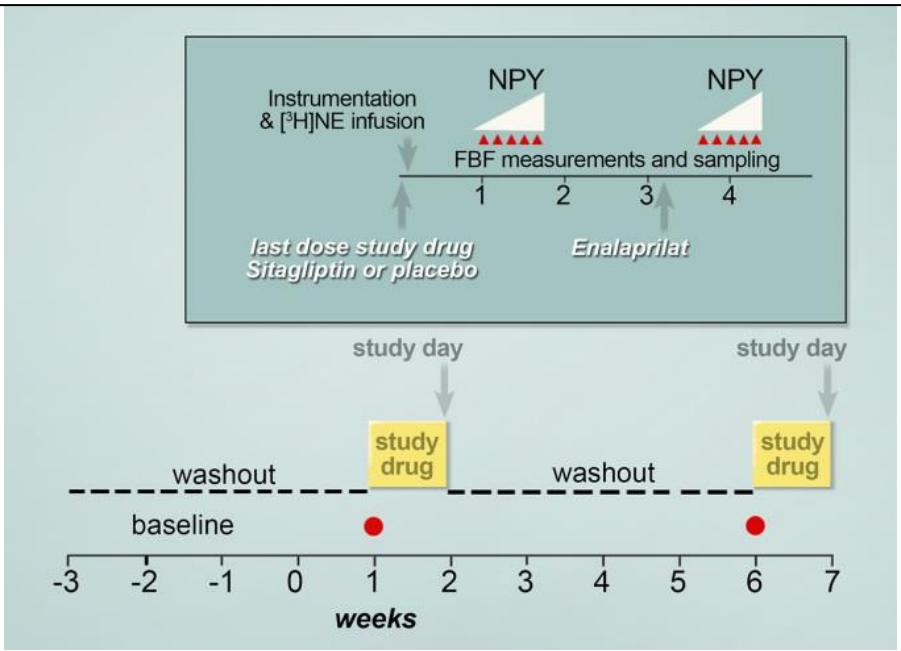
Enrollment and Randomization

Figure 2 illustrates the randomized, double-blind, placebo-controlled study protocol. Subjects who meet inclusion and exclusion criteria will be have a 4-week medication washout period before starting the study. This will be from medications that are known to have the potential to affect vascular blood flow, such as: non-steroidal anti-inflammatory drugs, oral estrogens, ACE inhibitors if used for microalbuminuria (not for purposes of hypertension, as this is an exclusion criteria), beta blockers, or medications that may affect sympathetic tone including stimulants.

Following washout, we will randomize subjects in a 1:1 ratio to receive one of two treatments. Each subject will undergo two treatment periods separated by a 4 week washout. Subjects will receive either sitagliptin 100mg/d for one week or matching placebo in random order. On the last day of each treatment, subjects will report to the CRC after an overnight fast for the first study day (**Study Day Procedures**).

After 4-week washout, subjects will undergo a second treatment period using the opposite drug (sitagliptin or placebo) and repeat the study day.

Figure 2. Study Protocol.



The Vanderbilt Investigational Drug Service will be responsible for the storage, preparation, and labeling of all investigational agents and for maintaining accurate drug storage and dispensing logs. At the time of randomization the research nurse will fax a copy of the consent form and a prescription containing check boxes for inclusion and exclusion criteria. The pharmacist will assign the subject a randomization number and provide the investigator a 1 week supply of treatment drug along with supply of study day medications (neuropeptide Y, enalaprilat). On study days 1 and 2 the pharmacy will provide the investigator with a 1-week supply with the remaining two interventions. The tablets will have an identical appearing form. An extra label containing the randomization number will be attached to each bottle of study drug. The investigator will affix this extra label to the subject's records. The Investigational Drug Service will retain a secure set of sealed envelopes containing the treatment assignment. Those envelopes will be opened in the event of a clinical scenario that compromises the safety of the subject, in which unblinding will alter the clinical outcome, as determined by the PI and the data safety monitoring committee.

Blinding

This study will be double–blinded. Neither the investigator nor the patient will be aware of the treatment assignment or the order of randomization.

6.0 Endpoints

Primary Endpoints

The primary endpoint is vasoconstriction measured by forearm blood flow (FBF) (**Standard Techniques**), and the primary analysis will focus on comparing this endpoint in the setting of placebo administration or sitagliptin, and enalaprilat vs enalaprilat plus sitagliptin.

Secondary Endpoints

Secondary endpoints include comparisons of catecholamine concentrations, neuropeptide Y and neuropeptide Y (3-36) during each intervention. Secondary mechanistic analyses include comparisons of additional measurements during sitagliptin versus placebo and during sitagliptin+enalaprilat versus sitagliptin alone to determine the effects of sitagliptin and concurrent DPP4 and local ACE inhibition on sympathetic activation and hemodynamic parameters: Power spectral analysis (as a measure of sympathetic/ vagal tone), MAP, BP, and HR. Additional laboratory analysis will include: ACE activity, DPP4 activity, tPA, and glucose homeostasis biomarkers (plasma glucose, insulin, and GLP-1).

7.0 Study Procedures

Screening Visit 1: After informed consent is obtained, subjects will undergo a medical history and physical examination. During this and all visits to the CRC we will measure blood pressure three times, every two minutes as described under **Standard Techniques**. We will measure weight, height, and hips and waist (horizontal umbilicus) circumference to 0.5 cm precision in triplicate. We will use a spring-loaded tape measure (Gulick II by Country Technology, Gay Mills,WI) for these measurements to ensure the tightness of the tape is consistent. We will obtain screening laboratory tests including: electrolytes, serum creatinine, fasting glucose level, complete blood count, liver enzymes, lipid profile, hemoglobin A1C, prothrombin time/ international normalized ratio (PT/INR), and urinalysis. Data will be transferred using the REDCap Dynamic Data Pull (DDP) to enable transfer of relevant study related data directly from the Vanderbilt Research Derivative, if available.

Screening Visit 2: subjects whose screening labs are adequate will return to the CRC to undergo an oral glucose tolerance test (OGTT).

Medication withdrawal: Subjects taking medications that may alter forearm blood flow (for example non-steroidal anti-inflammatory drugs, beta blockers, ACE-inhibitors, stimulant medications, etc) will be asked to withhold this medication during the washout period and

throughout the remainder of the study. Female subjects taking oral contraceptives will be asked to continue the same regimen throughout the duration of the study.

Medication Visit: Subjects will be asked to come to the CRC at least 14 days before each study day to pick up the study medications. At each visit, we will request female subject of childbearing potential undergo β-HCG testing. Subjects will be instructed on how to take the medication.

Study days: During each study day subjects will be asked to report to the CRC in the morning in a fasting state to undergo serial blood pressure and heart rate measurements. Subjects will be asked not to exercise for at least 48 hours prior to each study day and to refrain from alcohol and caffeine intake for 1 week prior to each study day.

Subjects will be studied in the supine position in a temperature-controlled room. Upon arrival, female subjects of childbearing potential will undergo pregnancy testing. Baseline hemodynamic monitoring (BP, HR, MAP, repeated three times), will be obtained, followed by administration of the last dose of the study drug. An indwelling IV catheter will be placed in the non-dominant arm along with one blood pressure cuff. On the non-dominant arm, an intra-arterial line (**Standard Techniques**) will be placed by a trained study physician. Forearm blood flow (FBF) cuffs will be placed on the same arm as the intra-arterial line.

Fifty minutes after study drug administration, subjects will undergo Power Spectral Analysis (**Standard Techniques**) followed by baseline laboratory collection: baseline catecholamines (venous and arterial), tPA (venous and arterial), insulin, GLP-1, BG, substance P, neuropeptide Y including total and (3-36)), DPP4 and ACE activity levels.

Starting at time point 1 hour, we will give NPY (U-1230, Clinalfa, Bachem AG) in doses of 0.1, 0.3, and 1.0 nmol/min intra-arterially via the brachial artery catheter. Each dose will be infused for 5 min. We will measure FBF and collect simultaneous venous samples for substance P, NPY and its fragments, and arterial and venous catecholamines (norepinephrine). After the first series of NPY infusions is complete, Power Spectral Analysis will be repeated. The finger blood pressure cuff for Power Spectral Analysis will be placed on the contralateral arm as the arterial line. In addition, venous samples will be collected to measure insulin and blood glucose and venous and arterial samples will be collected to measure tPA after completion of the first set of NPY infusions.

Ninety min after the last dose of NPY, enalaprilat will be infused via the brachial artery catheter at 0.33 μg/min/100mL. Twenty minutes later, the Power Spectral Analysis will be repeated. After this is completed over ten minutes (thus time point 3 hours 40 minutes, also two hours after the first NPY infusion), we will repeat a second NPY infusion at the same rates as previously described. Again, we will collect venous samples prior to and at the end of each dose of NPY to measure NPY and its fragments, substance P, and venous and arterial samples to measure catecholamines (norepinephrine). In addition, venous samples for GLP-1, insulin, blood glucose, DPP4 and ACE activity levels as well as venous and arterial tPA will be collected just prior to the initiation of the second set of NPY infusions. We will measure FBF at the end of each dose of NPY, similar to during the first infusion. At the end of the NPY infusion, Power Spectral Analysis will be repeated and venous insulin and blood glucose as well as venous and arterial tPA will be collected.

Hemodynamic monitoring will include MAP, BP, HR via blood pressure cuff, measured at baseline, at the start of both NPY infusion periods, and at the end of each dose administered.

After 4 week washout, subjects will undergo a second treatment period using the opposite drug (sitagliptin or placebo) and repeat the study day.

DNA samples: A blood sample for DNA analysis will be drawn once during the study and will be stored for analysis of genetic markers related to subject's susceptibility to the development of hypertension, cardiovascular disease, metabolic alterations, and/or response to treatment. This sample is not required for participation in the study. Genetic information will be linked with data collected during the conduct of this trial. As the relationships between genes and diseases become less equivocal, it might be possible to predict an individual's susceptibility to a disease, its prognosis, and the response to treatment. Blood specimens will be coded. Codes will not include any identification information; such as name, date of birth, or medical record number. Only study key personnel will have access to those codes. The identity of each code will be kept in a file in an encrypted computer.

8.0 Risks of Investigational Agents/Devices (side effects)

Risks/Inconvenience

- Insertion of venous catheters may cause bleeding, bruising, or infection.
- Frequent blood draws can cause anemia.
- Spending study days at the CRC can be inconvenient for subjects.
- It has been reported that vildagliptin, a DPP-4 inhibitor, increases the risk of angioedema in patients who are taking ACE inhibitors. In clinical trials the mean exposure to vildagliptin prior to the development of angioedema was 5.5 months. During the study the subjects will receive sitagliptin during two 1-week interventions, separated by at least 4 weeks. The half-life of sitagliptin is 12 hours, therefore it is unlikely that subjects develop angioedema.
- Sitagliptin can cause hypoglycemia, peripheral edema, or nausea. In addition, sitagliptin can rarely increase the risk of pancreatitis; however, this is not likely to occur given the short duration of the study and the exclusion of subjects with past medical history of pancreatitis.
- The FDA has warned that dipeptidyl peptidase-4 (DPP-4) inhibitors such as sitagliptin, saxagliptin, linagliptin, and alogliptin may cause joint pain that can be severe and disabling. After the patients discontinued the DPP-4 inhibitor medicine, their symptoms were relieved, usually in less than a month. Some patients developed severe joint pain again when they restarted the same medicine or another DPP-4 inhibitor.
- Enalaprilat theoretically increases the risk of hypotension; although, generally this does not lower blood pressure significantly in individuals with normal blood pressure.
- Insertion of brachial artery and venous catheters may cause bleeding, bruising, or infection. Insertion of a brachial artery catheter may cause damage to the artery or thrombosis. This is potentially serious and could result in loss of blood supply to the arm, requiring intra-arterial administration of fibrinolytic agents and/or surgery. Complications arising from arterial line insertion are usually seen in the setting of low flow and prolonged arterial line placement, neither of which occur in these protocols. We have conducted studies involving the insertion of brachial artery catheters safely in over 400 subjects. We will limit subjects to two arterial line study days.

- We will use lidocaine to numb the skin prior to the insertion of the brachial artery catheter. Lidocaine can cause numbness and burning and/or local rash or irritation in subjects who are allergic to it.
- Not exercising or taking caffeine, anti-inflammatories for 48hours

Protection against risk

- A nurse or physician will be present at all times during the study days.
- Subjects with a hematocrit less than 35% will be excluded from study.
- We will exclude any subject with a history of angioedema.
- We will monitor BP and HR and rhythm throughout each study day.
- We will exclude individuals with a history of pancreatitis
- We will ask subjects to monitor their home glucoses for hypoglycemia.
- We are limiting each subject to participation in two arterial line study days to minimize risk of thrombosis.
- A trained study physician will insert the arterial line.
- We are giving NPY locally so as to avoid systemic effects.

Study Withdrawal/Discontinuation

If at any time the blood glucose is determined to be at an unsafe level (sustained > 300mg/dL or < 70mg/dL), the subject will be withdrawn from the study.

If the subject experiences significant side effects, allergic reaction, or other concerning signs/ symptoms related to study day procedures or medications as determined by the study physician, the subject will be withdrawn from the study.

The subject may withdraw from the study at any time.

Arm 2

In five additional healthy subjects, we would like to study the effect of angiotensin receptor blockade on forearm blood flow and sympathetic activation in the setting of DPP4 inhibition. This will test the hypothesis that combined DPP4 inhibition and angiotensin receptor blockade does not result in increased substance P dependent release of NPY when compared to combined DPP4 and ACE inhibition.

As before, healthy subjects will be enrolled in this randomized, double-blind, and placebo-controlled study protocol. Subjects who meet inclusion and exclusion criteria will have a 4 week medication washout period before starting the study. This will be from medications that are known to have the potential to affect vascular blood flow, such as: non-steroidal anti-inflammatory drugs, oral estrogens, ACE inhibitors if used for microalbuminuria (not for purposes of hypertension, as this is an exclusion criteria), beta blockers, or medications that may affect sympathetic tone including stimulants. Following washout and seven days before the first study day, subjects will receive the angiotensin receptor blocker valsartan 160mg/d.

Additionally, each subject will be randomized in a 1:1 ratio to receive one of two treatments. Each subject will undergo two treatment periods separated by a 4 week washout. Subjects will receive either sitagliptin 100mg/d for one week or matching placebo in random order. On the last day of each treatment, subjects will report to the CRC after an overnight fast for the first study day. After 4 week washout, subjects will undergo a second treatment period using the opposite drug (sitagliptin or placebo) and repeat the study day.

The primary and secondary endpoints will be the same for this sub-study as they are in the previous study protocol. Also, subjects will undergo the same procedures for Screening Visit 1, Screening Visit 2, and the Medication Visits.

On the study day, subjects will be studied in the supine position in a temperature-controlled room. Upon arrival, female subjects of childbearing potential will undergo pregnancy testing. Baseline hemodynamic monitoring (BP, HR, MAP, repeated three times) will be obtained, followed by administration of the last dose of the study drug and the angiotensin receptor blocker. An indwelling IV catheter will be placed in the non-dominant arm and a blood pressure cuff will be placed on the dominant arm. On the non-dominant arm, an intra-arterial line will be placed by a trained study physician. Forearm blood flow (FBF) cuffs will be placed on the same arm as the intra-arterial line.

Fifty minutes after study drug administration, subjects will undergo Power Spectral Analysis followed by baseline laboratory collection: baseline catecholamines (venous and arterial), tPA (venous and arterial), insulin, GLP-1, BG, substance P, neuropeptide Y including total and (3-36)), DPP4 and ACE activity levels.

Starting at time point 1 hour, we will give NPY (U-1230, Clinalfa, Bachem AG) in doses of 0.1, 0.3, and 1.0 nmol/min intra-arterially via the brachial artery catheter. Each dose will be infused for 5 min. We will measure FBF and collect simultaneous venous samples for substance P, NPY and its fragments, and arterial and venous catecholamines (norepinephrine). After the NPY infusion is complete, Power Spectral Analysis will be repeated. The finger blood pressure cuff for Power Spectral Analysis will be placed on the contralateral arm as the arterial line. In addition, venous samples will be collected to measure insulin and blood glucose and venous and arterial samples will be collected to measure tPA after completion of the NPY infusion. Hemodynamic monitoring will include MAP, BP, HR via blood pressure cuff, measured at baseline, at the start of the NPY infusion period, and at the end of each dose administered.

After 4 week washout, subjects will undergo a second treatment period using the opposite drug (sitagliptin or placebo) and repeat the study day.

Valsartan Risks

Common risks of valsartan include diarrhea, high blood potassium, and low blood pressure. Uncommon risks include headache, dizziness, and excessive tiredness. Rarely, valsartan can cause fainting.

(Of note, enalaprilat will not be infused for this specific study protocol)

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

The Principal Investigator will closely oversee the protocol in conjunction with the assigned research nurse. Any adverse events or toxicities that occur while subject are enrolled in the study will be reported to the IRB as per IRB guidelines. Any untoward medical event will be classified as an adverse event, regardless of its causal relationship with the study. An adverse event it is considered to be serious if it results in: death, life-threatening condition, requires inpatient hospitalization or prolongation of existing hospitalization, results in persistent or significant disability or incapacity, or results in a congenital anomaly or birth defect. Serious adverse events will be reported to the Data and Safety Monitoring Committee (DSMC) and the IRB within 10 days of the PI's notification of the event. Non-serious adverse events will be reported at the time of continuing review.

The PI will be responsible for submitting summary reports to the IRB annually. The report will include: the number of adverse events and an explanation of how each event was handled, the number of complaints and how each complaint was handled, the number of subject withdrawals with an explanation of why the subject withdrew or was withdrawn, and the number of instances of noncompliance with the protocol with explanation. Before submission, the report will be reviewed internally by the PI.

The DSMC will provide objective review of treatment results as they relate to human safety. The committee will be comprised of [redacted], MD, Professor of Health Policy; [redacted] MD, Director of the Vanderbilt Diabetes Center, Professor of Medicine and Joe C. Davis Professor of Biomedical Science; and [redacted], John L. Sawyers Professor of Surgery and Chairman, Vanderbilt Department of Surgery. [redacted] will chair the committee.

The DSMC will meet at least 3 times, once to review the protocol and twice to receive reports of the progress of the study, at the end of the first year and at the end of year two. These reports will provide information regarding safety. The committee will assess safety data including hypotension, common adverse events, hospitalizations, and other serious adverse events. During regularly scheduled meetings, the DSMC will also be provided with a list of non-serious adverse events. Interim data will be provided to the committee by Drs. Brown and [redacted]. The DSMC may

choose to become unblinded; however, it is expected that such unblinding would not occur without reasonable concern related to either patient safety or data validity.

10.0 Statistical Considerations

Sample Size and Power Calculation

We calculated the sample size based on previously published data for the effect of NPY on FBF.[49] Specifically, in normotensives NPY reduced FBF from 4.3 to ~3.55±2.0, ~2.91±1.5, and 2.3±1.2 mL/min/100mL at 0.3, 1, and 3 nmol/min NPY, respectively. With a correlation of 0.7 between two repeated measurements in the same subject, a sample size of 18 will provide a power of 93% to detect an increase in vasoconstriction at the 0.3 nmol/min NPY dose during sitagliptin from FBF 3.55 to 2.3 mL/min/100mL (i.e., comparable to the 3-nmol/min dose in the absence of sitagliptin or a 10-fold shift to the left in the dose-response curve) and a power of 61% to detect an increase in vasoconstriction at 1 nmol/min NPY from 2.91 to 2.3 mL/min/100mL (a 3-fold shift to the left in the dose-response curve). Based on prior studies of the effect of ACE inhibition on the vasodilator response to bradykinin we expect to see at least an 8-fold shift to the left of the dose-response curve. We will replace dropouts.

We will recruit a small number of healthy individuals (5) to inform us of the differences in vascular pathophysiology in persons with diabetes compared to non-diabetics.

Data Analysis Plan

We are using a 2x2 crossover design. Although we have designed the study with a prolonged 4 week washout to avoid carryover, we will evaluate potential residual 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 Kenwood.[118] Second, we will take additional baseline measurement of key study endpoints right before study subjects take study medication (placebo or sitagliptin) in both treatment periods. This will allow us to estimate any residual carryover effect using the following described mixed effects models with factor 1 effects of placebo or sitagliptin replaced with their carryover effect in period 2 and without treatment factor 2. This estimate would enable us to assess still the sitagliptin vs placebo difference using data collected in both crossover periods versus the normal approach in which one would have to discard period 2 data in the presence of carryover.

We will use mixed-effect models with a random subject effect and with treatment factor 1 (sitagliptin versus placebo) and treatment factor 2 (absence or presence of enalaprilat) as fixed effects. We will evaluate treatment effects and interactions using properly set up contrast in the mixed-effect models. We will include gender, race, and glucose as covariates. In addition to evaluating treatment effects and interactions using regression analyses, we will calculate within-subject mean differences and 95% confidence intervals for specific effects of interest and test for treatment effects using paired t-test or sign rank test as appropriate.

If normality of the data is in question, we will utilize corresponding nonparametric tests. We will test all hypothesis at the level of $\alpha=0.05$. We will use SPSS for Windows (Version 22.0, SPSS, Chicago) and the open source statistical package R (version 2.12, R Development Core Team, 2006) for analyses.

Because subjects who drop out will be replaced and based on prior experience, missing data will be unusual. 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.

Interim analysis

Due to the size of the project, with 18 subjects (9 male, 9 female), there is no plan for an interim analysis.

Data Management and Quality Control

We will use the Vanderbilt REDCap system to design an electronic data collection form. This form will be tested before its use. The form allows for direct data entry by study key personnel and will be designed to minimize the amount of erroneous values. Clinical data, including clinical laboratory values, will be entered by the research nurse. Research laboratory data will be entered by the research assistant. A unique identification case number will be used to protect the confidentiality of the study participants. The electronic data collection system will allow us to monitor the quality performance by tracking the missing data. Before analysis raw data will be reviewed to assess its accuracy and completeness.

11.0 Privacy/Confidentiality Issues

Clinical data, including clinical laboratory data, will be accessible only to key study personnel. A unique identification case number will be used to protect the confidentiality of the study participants. The case numbers and participants names will be included in the protected source Redcap database, accessible only to members of the research team. For statistical analysis purposes the information will be de-identified, and only case numbers will be included.

12.0 Follow-up and Record Retention

All records will be retained for 7 years following publication of the data. After that time, records may be archived for an additional 5 years and then shredded.

13.0 Standard Techniques

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

Instrumentation for intra-arterial infusions: An intravenous catheter will be placed in the antecubital vein of the non-dominant arm. After subdermal administration of 1% lidocaine, a 3-French catheter (Cook Inc., Bloomington, IN) will be inserted into the brachial artery of the non-dominant arm for direct intra-arterial infusion of peptides and enalaprilat. Following placement of the catheters, subjects will be allowed to rest at least 30 min before baseline measurements are made. Arterial catheter patency will be maintained by infusion of normal saline at a rate of 1 mL/min. After measurement of basal FBF and blood sampling, peptides will be infused at the doses and durations described. FBF will be measured during the last two min. Drug concentrations in the infusate will be adjusted to maintain infusion volumes at 1 mL/min.

Forearm blood flow measurements: FBF will be measured using mercury-in-silastic strain-gauge plethysmography. The wrist will be supported to raise the forearm above the level of the atrium, and a strain gauge placed on the widest part of the forearm. The strain gauge will be connected to a plethysmograph (Model EC-4, D.E. Hokanson, Issaquah, WA), calibrated to measure percent change in volume and connected to a chart recorder to record flow measurements. For each measurement, a cuff around the upper arm will be inflated to 40 mmHg with a rapid cuff inflator (Model E-10, Hokanson) to occlude venous outflow from the extremity. The hand will be occluded from measurement of FBF by inflation of a pediatric sphygmomanometer cuff to 200 mmHg around the wrist. Flow measurements will be recorded for approximately 7 sec out of 15 sec, and the slope will be derived from the first 3-4 pulses; 7 readings will be obtained for each mean.

Assays: All blood samples will be centrifuged for 20 minutes immediately following blood drawing. Plasma will be storage at -80°C until analysis.

Laboratory Analyses: Catecholamines will be quantified after batch alumina extraction by high-performance liquid chromatography (HPLC) using electrochemical detection. We will measure ACE (Olympus AU400/AU600, Alpco Diagnostics, Salem, NH) and DPP4 activity by kinetic analysis.[54,55] Details regarding sample collection and assays appear in the cited publications.

Substance P and Neuropeptide Y (NPY total and NPY (3-36)): Plasma samples will be collected in a protease cocktail inhibitor containing an aminopeptidase P inhibitor (apstatin), DPP4 inhibitor (vildagliptin), serine protease inhibitor (phenylmethylsulfonyl fluoride), ETA, and phosphoramidon to avoid *ex vivo* degradation of the peptides. NPY will be concentrated from plasma by immunoextraction using an anti-NPY monoclonal antibody (NPY02) coupled to magnetic beads, followed by C18 micro-extraction (Ziptip, Millipore). Recovery (~70%) will be calculated using 13C, 15N-labeled internal standards for NPY and NPY (3-36). NPY and NPY (3-36) will be quantified using an IonKey-based system (Acquity M-Class + IonKey + Xevo TQ-S) using a modification of Dr. Grouzmann's previously published method that allows detection of

physiologic concentrations NPY and its metabolites in plasma. The assay can detect 0.001 pg NPY and fragments injected and is linear for NPY, NPY (3-36), NPY (3-35) from 0 to 8 pM (all r^2>0.99). We have previously used HPLC to separate substance P from substance P (3-11) prior to assay.[55,56] Since our prior submission, we have refined a UPLC-MS/MS method to detect substance P. We have markedly increased sensitivity of the assay by increasing the MS/MS working temperature from 270 to 325ºC to reduce background, utilizing a 0.1% formic acid (FA) in H_2O/1% FA in CHC3N gradient to optimize separation of substance P, and by purifying ethanol-extracted blood samples with Nexus and C_{18} Sep-Pak cartridges prior to analysis. $^{13}C_6{}^{15}N_4$-substance P serves as internal standard. The limit of detection of substance P is 0.1 pg injected. The standard curve is linear (r^2=0.995) from 0.1 pg to at least 50 pg. The coefficient of variation is 2.2%. The basal concentration of substance P in plasma is 9.2±7.8 pg/mL (mean±SD, N=11).

Glucose, Insulin, GLP-1: Plasma glucose will be measured by the glucose oxidase method with a YSI glucose analyzer (YSI Life Sciences, Yellow Springs, OH). Plasma insulin concentrations will be determined by RIA. The insulin assay cross-reacts with 38% intact human pro-insulin and with C-peptide, ≤0.01%. GLP-1 (glucagon like peptide 1) will be measured using an ELISA assay.

Power Spectral Analysis*:* Blood pressure fluctuates with a 10-second periodicity, and these fluctuations are commonly termed "Mayer" waves. The low frequency variability (LF_{SBP}) is thought to reflect sympathetic regulation of vasomotor tone. LF_{SBP} is increased by maneuvers that induce sympathetic activation, such as upright posture, lower body negative pressure, or infusion of depressor substances[47] and correlates with direct measurements of sympathetic traffic using microneurography. A derivative-threshold algorithm provided the continuous series of RR intervals from ECG signal, continuous non-invasive BP from finger arterial pressure (Finapress 2300, Ohmeda, Madison, WI), and respiratory rate will be recorded. All the data recorded will be used for determination of spectral analysis. Beat-to-beat data will be digitized using the WINDAQ data acquisition system (DI220, DATAQ, Akron, OH, 14 Bit, 1000Hz) and processed off-line using a custom-written software in PV-Wave language (PV-wave, Visual Numerics Inc., Houston, TX). Detected beat-to-beat values of R-R intervals and blood pressure values will be interpolated and low pass filtered (cutoff 2 Hz). Data segments of interest are used for spectral analysis. Linear trends will be removed, and power spectral density will be estimated with the FFT-based Welch algorithm. The power in the frequency range of low frequencies (LF: 0.04 to <0.15 Hz), and high frequencies (HF: 0.15 to < 0.40 Hz) will be calculated according to the Task Force recommendations.[48]

Appendix A: Study Procedure Calendar

	Screening Visit 1	Screening Visit 2	Medication Visit 1	Study Day 1	Medication Visit 2	Study Day 2
Complete History and Physical Examination	√					
Baseline Blood Pressure Measurements	√		√	√	√	√
Height, Weight	√			√		√
Hips and Waist Circumference	√					
CBC, CMP, lipid profile, urinalysis, 12-Lead ECG, HbA1C, PT/INR	√					
Oral Glucose Tolerance Test		√				
Human Chorionic Gonadotrophin (β-HCG)			√	√	√	√
Power Spectral Analysis				√		√
Arterial line insertion				√		√
Serial Blood Pressure and Heart Rate Measurements				√		√
Plasma NPY, NPY (3-36), substance P, DPP4 activity, tPA, GLP-1, insulin, glucose, catecholamines				√		√
DNA	√					
Pill Dispensing			√		√	
Pill Count and Structured Pill taking Interview				√		√
Concurrent Conditions Interview				√		√
Adverse Event Checklist				√		√

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