PROTOCOL TITLE: Unacylated Ghrelin to Improve FuncTioning in PAD: the GIFT Trial

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1.0 Objectives

1.1 Purpose/ Objectives.

This pilot study will investigate the optimal subcutaneous dose and the therapeutic potential of unacylated ghrelin to improve peripheral artery disease (PAD)-related brachial artery flow mediated dilation and reduce inflammation.

Ghrelin is a peptide and hormone that is primarily produced by P/D1 cells of the gastric fundus and circulates in both acylated and unacylated forms. Acylated ghrelin acts on the Growth Hormone Secretagogue Receptor (GHSR), increasing growth hormone levels, appetite, and insulin resistance (1-4). Unacylated ghrelin was originally thought to be an inactive metabolite. However, recent evidence shows that unacylated ghrelin promotes skeletal muscle cell regeneration, improves mitochondrial function, and increases skeletal muscle capillary density (5-7). Unacylated ghrelin also improves brachial artery flow-mediated dilation in humans (8). Unacylated ghrelin has been studied in more than 70 healthy humans without adverse effects (4,8-14). Unacylated ghrelin does **not** act on GHSR and does not increase appetite, or cause insulin resistance (4,8-14). However, unacylated ghrelin has never been studied in people with PAD. In addition, unacylated ghrelin has never been administered in a subcutaneous form to humans.

The pilot GIFT Trial will obtain preliminary evidence to identify the optimal dose of subcutaneously administered unacylated ghrelin in people with PAD. The results of this pilot study may be used to design a randomized trial of unacylated ghrelin, in subsequent study, to improve functioning and prevent mobility loss in older people with PAD.

Specific Aim. Because subcutaneously administered unacylated ghrelin has not been studied in humans, we will establish the association of increasing doses of subcutaneous unacylated ghrelin with circulating levels of unacylated ghrelin and brachial artery flow-mediated dilation (FMD) and levels of circulating inflammatory biomarkers. To achieve this aim, six PAD participants age 55 and older will receive a single subcutaneous injection of unacylated ghrelin at doses of 10 ug/kg, 20 ug/kg, and 40 ug/kg, respectively, on three separate days at least one week apart. Unacylated ghrelin levels will be measured at baseline and at defined intervals after each subcutaneous injection (30 minutes, 60 minutes, 1.5 hours, 3 hours, 6 hours, 8 to 12 hours and 24 hours). Brachial artery FMD will be measured at baseline, before the unacylated ghrelin injection, approximately six to eight hours, and 24 hours after each unacylated ghrelin injection (8). Circulating levels of inflammatory biomarkers will be measured at baseline and at six hours, 8-12 hours, and 24 hours after the subcutaneous injection of unacylated ghrelin. The lowest dose of subcutaneously delivered unacylated ghrelin that maximizes brachial artery FMD, maximizes levels of unacylated ghrelin, and minimizes circulating levels of inflammatory biomarkers will be used in future study.

Specific Aim 2. We will assess the safety of increasing doses of unacylated ghrelin in six patients with PAD age 55 and older.

1.2 Hypotheses.

We hypothesize that unacylated ghrelin, administered subcutaneously, will increase brachial artery flow-mediated dilation (FMD) and reduce circulating levels of inflammatory biomarkers and that higher doses of subcutaneously administered unacylated ghrelin will be associated with greater increases in brachial artery FMD and lower circulating levels of inflammatory biomarkers.

2.0 Background

2.1 Relevant experience.

A. Lower extremity peripheral artery disease (PAD) affects 10-15% of community dwelling men and women age 65 and older (15-17) and will be increasingly prevalent as the U.S. population survives longer with chronic disease. Our prior work demonstrates that men and women with PAD have greater functional impairment and more rapid functional decline than those without PAD (15,18-24). The functional impairment documented in PAD is associated with loss of independence, increased mortality, and poor quality of life (22,25-27). Recent evidence shows that chronic disability, such as that associated with PAD, accounts for more than half of United States health burden (28). Furthermore, therapeutic advances have not kept pace with the growing burden of disability from chronic disease (28). Only two medications, pentoxifylline and cilostazol, are FDA-approved for treating PAD-associated walking impairment. Of these, pentoxifylline is usually ineffective and benefits from cilostazol are modest (29-32). New therapies are urgently needed for patients with PAD. This pilot study will test the therapeutic potential of subcutaneously administered unacylated ghrelin and the optimal dose of subcutaneously administered unacylated ghrelin in patients with PAD.

B. Calf skeletal muscle fibers in PAD are damaged. Electron microscopy demonstrates pathologic changes in myofibrils, mitochondria, nuclei, and sarcolemma of calf muscle myofibers in patients with PAD (33). In 34 participants with PAD and 21 without PAD who underwent calf muscle biopsy, PAD participants had 30% higher protein carbonyl content ($P<0.001$) and 40% higher 4-hydroxy-2-nonenal (HNE) levels ($P<.001$), indicating higher calf muscle oxidative stress levels in the PAD participants (33). More severe calf muscle oxidative stress was associated with more severe myofiber damage and fewer myofibers (33). Unacylated ghrelin has the potential to promote healthy calf muscle fibers in patients with PAD.

C. Calf muscle mitochondria are damaged in PAD. Skeletal muscle mitochondria from PAD patients also demonstrate a quantitative mitochondria dysfunction, with reduced energy production (34-36). One study used magnetic resonance spectroscopy to compare the efficiency of mitochondrial oxidative phosphorylation in 12 men with PAD and 14 without PAD who engaged in submaximal plantarflexion exercise. After submaximal exercise, and controlling for blood flow, the PAD participants had poorer phosphocreatine recovery (137 seconds $\pm$ 41 vs. 44 seconds $\pm$ 3, P=0.02) and poorer adenosine triphosphate (ATP) recovery (60 seconds $\pm$ 10 vs. 29 seconds $\pm$ 2, P=0.02) than patients without PAD (36). These and other results (34,35) demonstrate an intrinsic defect in calf muscle mitochondrial function in patients with PAD. Unacylated ghrelin has the potential to promote improved calf muscle mitochondrial function in patients with PAD.

2.2 Significance of the research.

A. **Overview of ghrelin**. Ghrelin is a peptide and hormone that circulates in acylated and unacylated forms. Unlike acylated ghrelin (1,3,), unacylated ghrelin does not bind to GHSR1a and does not increase appetite or cause insulin resistance (4,9,11). In studies of humans and animals, unacylated ghrelin improves endothelial function, regenerates skeletal muscle cells, improves mitochondrial function, and increases capillary density (37-42). In summary, unacylated ghrelin has therapeutic effects on skeletal muscle and the vasculature without promoting insulin resistance or weight gain.

B. **In a mouse model of hind-limb ischemia, unacylated ghrelin increased skeletal muscle regeneration, angiogenesis, and limb strength**. Togliatto and colleagues induced hindlimb ischemia in 81 mice and randomized them to intra-peritoneal injections of unacylated ghrelin, acylated ghrelin, or saline for 21 days (37). Mice who received unacylated ghrelin had significantly more skeletal muscle satellite cells (measured by Pax7/MyoD markers), significantly more regenerating skeletal muscle fibers, and significantly better plantarflexion function in the ischemic limb than mice who received acylated ghrelin or saline. Mice who received unacylated ghrelin had greater increases in capillary density in the skeletal muscle of their ischemic limb, compared to the other two groups. Greater increases in satellite cell number in mice who received unacylated ghrelin were mediated by P38/MAPK activation (37), consistent with previous reports that satellite cell proliferation depends on P38/MAPK protein activation. If these actions occur in humans with PAD, results will have major therapeutic implications for PAD patients. The purpose of this pilot study is to preliminarily test whether subcutaneously administered unacylated ghrelin improves brachial artery FMD in patients with PAD and to identify the most optimal dose of subcutaneously administered unacylated ghrelin in patients with PAD.

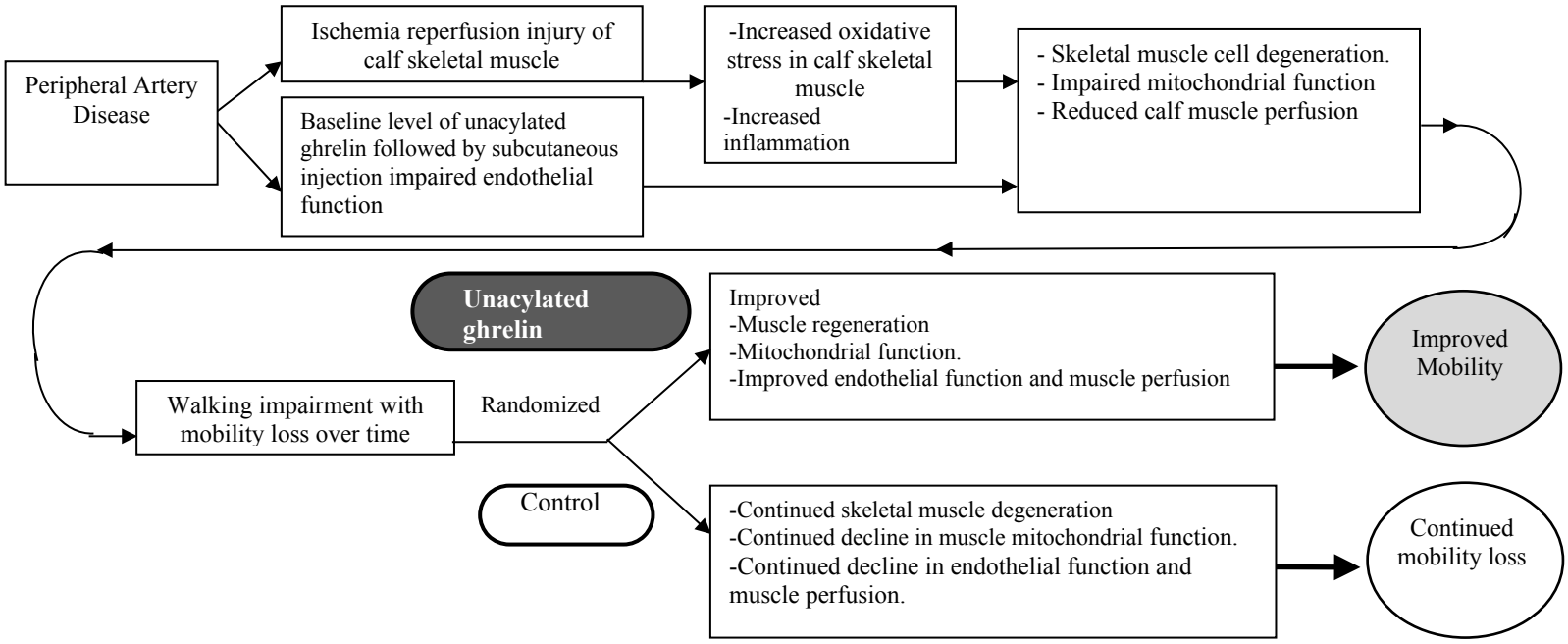
C. **Unacylated ghrelin promotes myocyte regeneration via the p38 pathway**. In a separate *in vitro* study, unacylated ghrelin induced differentiation of proliferating skeletal myoblasts and promoted their fusion into multi-nucleated syncytia (7). A high affinity binding site for deacylated ghrelin was identified on C2C12 myoblasts that is distinct from the (GHSR)-1a receptor that causes insulin resistance and increased appetite (7). In summary, preliminary evidence from *in vitro* and animal studies supports our hypothesis that unacylated ghrelin may improve functioning by promoting myocyte regeneration and differentiation in calf skeletal muscle damaged by PAD-related ischemia-reperfusion injury.

D. **Unacylated ghrelin and mitochondrial function**. In the hind limb ischemia mouse model by Togliatto et al (37), unacylated ghrelin reduced oxidative stress in ischemic muscle, by increasing the mitochondrial specific enzyme, superoxide dismutase-2 (SOD-2), suggesting improved mitochondrial function. In a rat model of chronic kidney disease, Barazzoni and colleagues administered acylated ghrelin or saline subcutaneously to healthy mice for four days. As compared to saline, acylated ghrelin significantly improved oxidative metabolism and increased activity of the mitochondrial enzymes citrate synthase and COX enzyme (38). These favorable changes in mitochondrial function were not related to increases in appetite, suggesting that this beneficial effect may be distinct from the GHSR-1a receptor. Thus, we hypothesize that unacylated ghrelin will improve mitochondrial oxidative metabolism in PAD participants.

E. **Unacylated ghrelin improves endothelial function**. *In vitro* study shows that unacylated ghrelin inhibits apoptosis of endothelial cells (41). In rats, unacylated ghrelin causes endothelium-dependent vasodilation of the mesenteric vascular bed (42). In 12 humans with metabolic syndrome, intravenously administered unacylated ghrelin significantly improved endothelium-dependent brachial artery FMD within one hour after ghrelin infusion (8). Patients with PAD have significantly impaired brachial artery FMD compared to individuals without PAD (43). Thus, we expect that subcutaneously administered unacylated ghrelin will improve FMD in PAD patients.

Theoretical Framework.

Figure 1. Theoretical model for the GIFT Trial



F. The purpose of this pilot study is to preliminarily test whether subcutaneously administered unacylated ghrelin improves brachial artery FMD and reduces levels of inflammatory biomarkers in patients with PAD and to identify the most optimal dose of subcutaneously administered unacylated ghrelin in patients with PAD that may have therapeutic activity in people with PAD. We will also assess the safety of subcutaneously administered unacylated ghrelin in people age 55 and older with PAD.

Importance of the GIFT Study. Effective therapies are urgently needed to prevent mobility loss in older people with PAD. The GIFT Trial will provide evidence for the optimal dose of subcutaneous ghrelin, which can later be used to design a pilot trial of unacylated ghrelin in older people with PAD.

Our proposed work will also facilitate subsequent studies of unacylated ghrelin therapy administered in the outpatient setting to older people with chronic diseases.

3.0 Inclusion and Exclusion Criteria

3.1 Screening for eligibility.

Recruitment. We will identify six PAD participants age 55 and older meeting eligibility criteria for our proposed pilot study. For this pilot study, we will contact six PAD patients who participated in our previous research studies and agreed to be contacted for future research studies conducted by Dr. McDermott.

We may also use the Enterprise Data Warehouse to identify six consecutive patients with PAD to participate in this pilot study.

3.2 Criteria.

Inclusion Criteria. All participants will be age 55 and older. All will have an ABI $\leq$ 0.90 at baseline. ABI $\leq$ 0.90 is a well-accepted standard for defining PAD (44-47). In addition, people with a history of lower extremity revascularization for PAD will be eligible.

Exclusion Criteria.

1. Above or below-knee amputation, critical limb ischemia, and wheelchair confinement.

2. Cardiovascular event during the previous three months. [Note: Participants who have undergone coronary revascularization for a cardiac event during the previous three months may still qualify.]

3. Major medical illnesses including renal disease requiring dialysis, or cancer requiring treatment in the previous year.

4. Participation in another clinical trial or completion of a clinical trial in the previous month, unless they were in the control group of the previous trial.

5. Unwilling to attend nine study visits over approximately six months.

6. Surgery including lower extremity revascularization or orthopedic surgery in the previous month or anticipated surgery in the next three months.

7. Greater than 15 mmHg difference in blood pressure in both arm pressure measurements during the ABI, diagnosis of Raynaud's phenomenon, or unable to have the blood pressure checked in both arms.

8. Blood pressure < 90/50 at baseline.

9. Non-English speaking, a visual impairment that limits ability to read the consent, or a hearing impairment that interferes with study participation.

10. In addition to the above criteria, investigator discretion will be used to determine if the trial is unsafe or not a good fit for the potential participant.

3.3 Special Populations.

Vulnerable populations (fetuses, pregnant women, children, prisoners, and institutionalized persons) and adults unable to consent will not be included in this study.

4.0 Study-Wide Number of Subjects

NA

5.0 Study-Wide Recruitment Methods

NA

6.0 Multi-Site Research

NA

7.0 Study Timelines

Each participant's part in this study will last up to approximately six months.

Visit 1.

The initial visit will last approximately 2 hours. Informed consent will be provided and an ankle brachial index (ABI) measurement will be obtained to ascertain eligibility. Questionnaires will be administered to obtain information about their health and medical history. The six-minute walk test and the four-meter walking velocity at usual and fastest pace may be obtained. An electrocardiogram (ECG) will be performed and a blood sample will be obtained. Blood will be tested for liver and kidney function, electrolytes, and a complete blood count (CBC). A physical exam will be performed and medical history will be obtained. Height and weight will be measured. Vitals may be obtained. Vitals include blood pressure, heartrate, respiratory rate, temperature, and oxygen level.

Visits 2, 5, and 8.

Visits 2, 5, and 8 will last approximately 8-12 hours and will be separated by at least one week. We plan to collect data for each dose on all six participants before proceeding to the next dose. At visits #2, #5, and #8, respectively, participants will receive a subcutaneous injection of unacylated ghrelin of the following doses:

Visit 2: 10ug/kg
Visit 5: 20 ug/kg
Visit 8: 40 ug/kg

Prior to the injection. Prior to the injection, blood will be drawn and measured for levels of unacylated ghrelin and acylated ghrelin, inflammatory biomarkers, liver and kidney function, electrolytes, and CBC. Blood pressure and pulse will be obtained seated and in the standing position. Brachial artery flow-mediated dilation (FMD) will be measured.

30 minutes, 60 minutes, 90 minutes, and 3 hours and 6 hours post-injection. A blood sample will be obtained and measured for levels of unacylated ghrelin and acylated gherlin. Blood pressure and pulse will be obtained seated and in the standing position. A brachial artery FMD may be measured at 6 hours (e.g. if we do not see a change in brachial artery FMD 8-12 hours post-injection, we may want to move the collection time point to 6 hours).

8-12 hours post-injection. A blood sample will be obtained and measured for levels of unacylated ghrelin and acylated gherlin as well as inflammatory markers.

Blood pressure and pulse will be obtained seated and in the standing position. Brachial artery FMD may be measured.

Additional vitals will be obtained at the discretion of the CRU nurse throughout the visit. Vitals include blood pressure, heartrate, respiratory rate, temperature, and oxygen level. Participants will be administered a questionnaire to assess symptoms. Participants may be asked to take their medications in the evening rather than the morning on the day of injection visits.

Participants may also be asked to repeat the six-minute walk and/or four-meter walks performed at baseline.

Visits 4 and 7.

At visits 4 and 7, a blood sample will be obtained and tested for liver and kidney function, electrolytes, and CBC. The physical exam and medical history may be repeated. Weight may be re-measured.

Visits 3, 6, and 9.

Visits 3, 6, and 9 will occur approximately 24 hours after the subcutaneous injections given at visits 2, 5, and 8, respectively. A blood sample will be obtained and measured for levels of unacylated ghrelin and acylated gherlin as well as inflammatory markers and liver and kidney function, electrolytes, and CBC. Blood pressure and pulse will be obtained seated and in the standing position. Brachial artery FMD will be measured and an ECG will be performed. Vitals may be obtained at the discretion of the CRU nurse. Participants will be administered a questionnaire to assess symptoms.

Prior to FMD measures, the participant will be asked to consume a low calorie, low fat meal provided to the participant by CRU bionutrion services.

The table below lists timing of the outcome and safety measures.

Measures	Prior to Injection *	30 minutes	60 minutes	90 minutes	3 hours	6 hours	8-12 hours	24 hours
Safety Measures								
Electrocardiogram	X*							X
Comprehensive chemistry panel	X*							X
CBC	X*							X
Pulse and blood pressure	X	X	X	X	X	X	X	X
Orthostatic pulse and blood pressure	X	X	X	X	X	X	X	X

Efficacy outcome measures								
Unacylated and acylated ghrelin levels	X	X	X	X	X	X	X	X
Inflammatory biomarkers	X						X	X
Brachial artery FMD	X					X	X	X

*The ECG, comprehensive chemistry panel (liver and kidney function and electrolyes), and CBC will be obtained at visit #1 for the 10 ug/kg dose.
**The FMD will be performed at either 6 hours or 8-12 hours.

We will test doses of 10 ug/kg, 20 ug/kg, and 40 ug/kg of unacylated ghrelin on three separate days for each participant. These doses were selected based on published literature and the experience of our consultants Dr. Jenny Tong, who has an ongoing study of intravenously administered unacylated ghrelin in healthy people (R01-DK097550) and Dr. Anne Cappola, who has an ongoing study of subcutaneously administered acylated ghrelin in older frail people without PAD (R21-AG040488).

Justification for proposed doses of unacylated ghrelin. Unacylated ghrelin is a synthetic 28 amino-acid protein. The ½ life of intravenously administered unacylated ghrelin is 30-34 minutes, which is comparable to but slightly longer than the ½ life of insulin and glucagon (9). In comparison, the ½ life of intravenously administered acylated ghrelin is 9 to 11 minutes (9). A portion of intravenously administered acylated ghrelin is converted to unacylated ghrelin (9). In contrast, unacylated ghrelin is not converted to acylated ghrelin (9). In healthy people, circulating levels of unacylated ghrelin are higher than those of acylated ghrelin, in a ratio that is typically 4:1 but ranges from 2:1 to 9:1 (9,11,48). In a study of healthy people, a 4X higher dose of intravenously administered unacylated ghrelin achieved a CMax that was twelve times higher than that for intravenously administered acylated ghrelin (9). Because of the longer ½ life of unacylated ghrelin and the substantially greater CMax achieved with unacylated ghrelin as compared to acylated ghrelin, we will begin with a subcutaneous unacylated ghrelin (10 ug/kg) dose that is comparable to that selected by Dr. Cappola for her study of subcutaneously administered acylated ghrelin. Next, we will test a subcutaneous dose of unacylated ghrelin that is 2X the starting dose (20 ug/kg). Finally, we will test a subcutaneous dose of unacylated ghrelin that is 4X times the starting dose (40 ug/kg). Four times the starting dose was selected as the maximal dose to test because circulating levels of unacylated ghrelin are approximately four times higher than circulating levels of acylated ghrelin and because of the higher CMax of unacylated as compared to acylated ghrelin (9,11,48). Although the GIFT Trial will administer unacylated ghrelin subcutaneously, we anticipate that, similar to intravenous administration, the maximum concentration of unacylated ghrelin will be greater than that for acylated ghrelin.

We anticipate that we will enroll all study subjects within one year of obtaining IRB approval. We anticipate that we will complete data collection within thirteen months of obtaining IRB approval.

8.0 Study Endpoints

8.1 Primary and secondary study endpoints.

We selected brachial artery FMD as an endpoint because unacylated ghrelin increases brachial artery FMD within hours of intravenously administered unacylated ghrelin (8). This immediate effect on brachial artery FMD provides a short-term therapeutic endpoint for identifying an optimal unacylated ghrelin dose. The brachial artery FMD will be measured at baseline and at six or 8-12 hours after each dose of unacylated ghrelin is administered.

Additional endpoints for this pilot study include a) circulating levels of unacylated ghrelin; b) levels of acylated ghrelin; c) biomarkers of inflammation.

8.2 Primary or secondary safety endpoints.

Unacylated ghrelin has been administered intravenously to more than 70 humans without any serious adverse effects (4,8-14). Two participants in one study experienced mild facial swelling after receiving unacylated ghrelin intravenously (14). Symptoms resolved within hours (14). Dizziness was reported in one study in which intravenous acylated and unacylated ghrelin were administered simultaneously over the short term (12). However, this adverse effect was not observed when unacylated ghrelin was administered alone (12). We will monitor participants for symptoms of facial swelling and dizziness. We will monitor participants for blood pressure and signs of facial swelling. An electrocardiogram will be obtained at visit 1 and before the 20 ug/kg and 40 ug/kg injections and 24 hours after each injection. Renal function, liver function, electrolytes, and CBC will be obtained at baseline, before the 20 ug/kg and 40 ug/kg injections, and approximately 24 hours after each injection.

9.0 Procedures Involved

9.1 Study design.

STUDY OVERVIEW. We will identify the lowest subcutaneous dose of unacylated ghrelin that maximizes brachial artery flow mediated dilation (FMD). These PAD participants will receive three different subcutaneous doses of unacylated ghrelin on three different days and will undergo measurement of unacylated ghrelin levels prior to the injection and at specified time intervals. Each dose will be separated by a wash-out period of $\geq$ one week. Data will be obtained for all six participants at each dose before moving to the next dose. We will test doses of 10 ug/kg, 20 ug/kg, and 40 ug/kg of unacylated ghrelin on three separate days for each participant. These doses were selected based on published literature and the experience of our consultants Dr. Jenny Tong, who has an ongoing study of intravenously administered unacylated ghrelin in healthy

people (R01-DK097550) and Dr. Anne Cappola, who has an ongoing study of subcutaneously administered acylated ghrelin in older frail people without PAD (R21-AG040488).

(See Section 7.0 above). Justification for proposed doses of unacylated ghrelin. Unacylated ghrelin is a synthetic 28 amino-acid protein. The ½ life of intravenously administered unacylated ghrelin is 30-34 minutes, which is comparable to but slightly longer than the ½ life of insulin and glucagon (9). In comparison, the ½ life of intravenously administered acylated ghrelin is 9 to 11 minutes (9). A portion of intravenously administered acylated ghrelin is converted to unacylated ghrelin (9). In contrast, unacylated ghrelin is not converted to acylated ghrelin (9). In healthy people, circulating levels of unacylated ghrelin are higher than those of acylated ghrelin, in a ratio that is typically 4:1 but ranges from 2:1 to 9:1 (9,11,48). In a study of healthy people, a 4X higher dose of intravenously administered unacylated ghrelin achieved a CMax that was twelve times higher than that for intravenously administered acylated ghrelin (9). Because of the longer ½ life of unacylated ghrelin and the substantially greater CMax achieved with unacylated ghrelin as compared to acylated ghrelin, we will begin with a subcutaneous unacylated ghrelin (10 ug/kg) dose that is comparable to that selected by Dr. Cappola for her study of subcutaneously administered acylated ghrelin. Next, we will test a subcutaneous dose of unacylated ghrelin that is 2X the starting dose (20 ug/kg). Finally, we will test a subcutaneous dose of unacylated ghrelin that is 4X times the starting dose (40 ug/kg). Four times the starting dose was selected as the maximal dose to test because circulating levels of unacylated ghrelin are approximately four times higher than circulating levels of acylated ghrelin and because of the higher CMax of unacylated as compared to acylated ghrelin (9,11,48). Although the GIFT Trial will administer unacylated ghrelin subcutaneously, we anticipate that, similar to intravenous administration, the maximum concentration of unacylated ghrelin will be greater than that for acylated ghrelin.

Preparation of subcutaneous unacylated ghrelin. Synthetic unacylated ghrelin powder will be supplied by the CS Bio Company and prepared by Northwestern Hospital's pharmacy for subcutaneous injection, using methods successfully implemented by Dr. Cappola for subcutaneously administered acylated ghrelin.

Selecting the dose of unacylated ghrelin for future study. We will select the lowest subcutaneous dose of unacylated ghrelin that maximizes brachial artery FMD and minimizes circulating levels of oxidative stress. We will also use unacylated ghrelin levels to select the unacylated ghrelin dose. In selecting the dose, greater increases in brachial artery FMD and lower levels of oxidative stress will be prioritized over the level of unacylated ghrelin. For example, if the dose of 40 ug/kg results in substantially higher levels of unacylated ghrelin compared to the dose of 20 ug/kg, but improvement in brachial artery FMD is similar between the 20 ug/kg and the 40 ug/kg doses, then we will select a dose of 20 ug/kg for use in a possible future trial of unacylated ghrelin in PAD patients.

9.2 Describe all research procedures.

Please see sections 7.0, 8.1, 8.2, and 9.1 above.

Ankle Brachial Index (ABI). After the participant rests supine for five minutes, the right brachial, dorsalis pedis (DP), posterior tibial (PT) and left DP, PT, and brachial artery pressures are measured using a hand-held Doppler probe. Pressures are measured twice. The ABI is calculated for each leg by dividing the average of the DP and PT pressures by the average brachial pressure.

Questionnaire Administration. Participants will be administered study questionnaires by a trained and certified health interviewer.

Brachial Artery Flow-Mediated Dilation (FMD). Brachial artery imaging will be performed by a Registered Diagnostic Cardiac Sonographer according to established protocol (44,45). Changes in FMD will be read by Dr. James Stein's University of Wisconsin Atherosclerosis Imaging Research Program Core Laboratory by a single reader blinded to participant characteristics. We have more than a decade of experience working with Dr. Stein's reading center (44,47,48). Measurement reproducibility in Dr. Stein's laboratory has a median FMD difference of 0.02% (inter-quartile range: -0.03 to 0.04).

Six-minute walk. Participants will be asked to walk back and forth along a 100-foot hallway for six minutes. They will be instructed that the purpose of the six-minute walk test is to measure how long a distance they can walk in six-minutes. A script will be read to describe the study procedure. Participants will be asked whether they feel the test is safe to try and whether they have any questions. The six-minute walk is a well validated measure of walking endurance that predicts mobility loss and mortality in PAD populations (19,22,49,50) and improves in response to therapeutic interventions in older people with PAD (49-53). The intra-class correlation coefficient for the test-retest reliability of the six-minute walk test among 156 PAD participants in our SILC exercise trial was 0.90 ($p<0.001$) when two six-minute walks were completed 1-2 weeks apart (51,54). Participants may be asked to perform the six-minute walk at baseline and may be asked to repeat it approximately four-eight hours after each subcutaneous ghrelin injection.

Four-Meter Walk. Participants are timed walking a four-meter distance in a corridor at their usual and fastest pace. Because of a learning effect, the four-meter walk is performed twice, and the fastest walk in each set is used in analyses. Participants are read a script by the study coordinator, describing the test. The test is demonstrated for the participant by the study coordinator- both at usual and at fastest pace. Participants may be asked to perform the four-meter walk at baseline and may be asked to repeat it approximately four-eight hours after each subcutaneous ghrelin injection.

Blood pressure and pulse measurement. We will monitor blood pressure and pulse changes after the unacylated ghrelin injection. We will measure both sitting and standing blood pressure and pulse prior to the injections and at the intervals described above.

Electrocardiogram. An electrocardiogram will be obtained using standard procedures at baseline, prior to the 20 ug/kg and 40 ug/kg doses, and approximately 24 hours after each injection.

Blood collection. Participants will have approximately 300 mls of blood drawn over the course of the study to test levels of unacylated ghrelin and acylated ghrelin, inflammatory biomarkers, CBC, liver and kidney function, and electrolytes. Some blood will be stored for possible later analyses. At visits 2, 4, and 6 a peripheral intravenous line may be inserted for the purpose of obtaining blood samples more easily without multiple venipunctures.

9.3 **Adequacy of protection against risks and methods to minimize potential risks.** *Overview of protection against risks.* Prior to beginning data collection and study interventions, all study coordinators undergo training and are certified by Dr. McDermott using a detailed checklist for each data collection element. Research coordinators are certified in each element of the study visit including obtaining informed consent, administering questionnaires, protecting confidentiality of collected data, performing the six-minute walk, and measuring the ABI. Dr. McDermott re-certifies coordinators every six months to ensure continued adherence to protocol. Those not adhering to all aspects of the protocol undergo additional training followed by re-certification.

All research staff members have completed human subjects training required by Northwestern's institutional review board (IRB). This training includes education about the importance of maintaining confidentiality of personal health information. Dr. McDermott or a co-investigator is available to answer questions that arise during the informed consent process as needed.
Participants are asked to sign a study consent form prior to data collection. The research coordinator reviews study procedures, including risks and benefits associated with study participation. The research coordinator answers participants' questions. Dr. McDermott and other study investigators are available to answer participants' questions. Both the participant and the individual administering the consent form will sign the consent form. Dr. McDermott's pager, direct telephone line, and home telephone number are provided to participants.

Minimizing risks related to unacylated ghrelin. The six participants who receive the three injections of increasing doses of unacylated ghrelin will be observed at Northwestern Medical Center for approximately 8-12 hours after each injection. Participants will be observed for side effects. Participants will also return at 24-hour follow-up for assessment. The three injections will be separated by intervals

of at least one week. Safety will also be monitored by our data safety monitoring board (DSMB) (see below).

Minimizing risk related to baseline and follow-up testing. All study coordinators undergo baseline training and are certified by Dr. McDermott before beginning data collection. Training and certification involves ensuring that coordinators are trained in methods to help minimize falls. Dr. McDermott re-certifies coordinators every six months to ensure continued adherence to study protocol. Those who are not adhering to protocol undergo additional training followed by re-certification.

Minimizing risk related to loss of confidentiality. The following methods will be employed to maintain confidentiality of participants. First, study recruitment letters will be mailed, using IRB-approved methods, only after receiving written permission from the participant's physician. The personal physician of each study participant will have the option of not allowing investigators to contact the potential participant. Lists of potentially eligible participants will be obtained by individuals who normally have access to these lists as part of their daily work requirements. Recruitment letters for potential participants identified from hospital and outpatient lists are prepared by research staff members whose job is to assist study investigators with recruitment. These research staff members have completed training in the ethical conduct of human subject research, including maintaining participant confidentiality. Recruitment letters to potential participants identified from medical center lists are mailed in sealed envelopes and addressed to the potential participant. All potential participants who receive mailed information about the study after the approval from their physician will have the opportunity to call a voice-mail system to ask not to be further contacted about this study. Secondly, only study investigators and key research staff will have access to the study database. Third, participants will be assigned a unique study identifier. Individual names will ultimately be removed from the study database and only the unique study identifier will be used to distinguish participants in the database. Fourth, collected data will be maintained in locked computer files and file cabinets to which only study investigators have access. Collected data will be used only for research purposes. Any published data will not contain any individual identifiers.

Data and Safety Monitoring Board (DSMB). We have identified three individuals: Dr. William Hiatt, Professor of Medicine at University of Colorado, Dr. Lawrence Frohman, Professor of Endocrinology at the University of Illinois at Chicago, and Dr. Sally Freels, Professor of Statistics, University of Illinois at Chicago who have agreed to serve on the DSMB for the GIFT Trial. Dr. Hiatt is an internationally recognized expert in vascular medicine and is a board-certified cardiologist. Dr. Frohman is an internationally recognized expert in endocrinology and is board certified in endocrinology. Dr. Freels is an internationally recognized statistician with expertise and experience with clinical trials and also missing data. All have provided letters of support, agreeing to

participate in the GIFT Trial. The DSMB held their first meeting by telephone conference on Monday June 27, 2016. The DSMB will meet at least every six months during the study or more frequently as requested by the DSMB. The DSMB will convene by telephone conference call to review and approve the protocol prior to beginning data collection. The biostatisticians and data manager will work closely with the DSMB to perform interim analyses. Adverse events will be monitored continuously throughout the study and will be reported to the DSMB and IRB in a timely manner according to pre-specified requirements. Adverse event rates and interim study results will be reviewed and discussed by the DSMB at their regularly scheduled meetings. At least four categories of adverse events will be defined: a) Death; b) cardiovascular events (myocardial infarction, stroke, and coronary arrhythmias) c) lightheadedness/dizziness; d) swelling. We will report all hospitalizations to the DSMB in a timely fashion. We will use a designated data collection form to record these events and they will be reported to the Institutional Review Board according to local IRB requirements. They will be reported to the DSMB as required by the DSMB charter. As noted above, however, to date unacylated ghrelin has been found to have little to no toxicity in humans.

The purpose of this pilot study is to determine the potential therapeutic potential of subcutaneously administered unacylated ghrelin. The purpose is also to identify the most optimal dose of subcutaneously administered unacylated ghrelin for improving brachial artery flow-mediated dilation and reducing levels of inflammation in patients with PAD. An Investigational New Drug application to the FDA has been approved (IND# 130513).

We will use standardized questionnaires to collect data about each study participant. Please see attached data collection forms. We may use medical records to determine whether the patient was diagnosed with PAD prior to study enrollment.

10.0 Data and Specimen Banking

10.1 Storage of specimens.

Blood specimens for long-term storage will be stored in a freezer belonging to Dr. McDermott's research program at Northwestern University, in the freezer farm in the basement of Olson Pavilion.

Specimens will be stored for up to 70 years, after which they will be destroyed.

10.2 Data to be stored or associated with each specimen.

Specimens will be coded; meaning that a key will exist that can link the codes back to the direct subject identifiers. Each participant will be assigned a unique study ID number that can be traced back to the study participant. The blood

samples that are maintained in long-term storage will be labeled with this unique identifier and the date and time of the blood collection.

10.3 Procedures to release data or specimens.

Only Dr. McDermott has control over release of study data or specimens. Any investigators seeking to analyze blood specimens must contact Dr. McDermott for permission. Each request, if it occurs, will be considered on a case-by-case basis. Dr. McDermott will obtain IRB approval prior to releasing any blood specimens for analysis, other than those tests specifically named in this application.

11.0 Data and Specimen Management

11.1 Data management.

Data is recorded using preprogrammed instruments and an electronic case report form using secure, HIPAA-compliant REDCap database software on servers maintained by Northwestern's Clinical and Translational Sciences Institute. We have substantial experience with REDCap.

Data Safety Monitoring Board (DSMB). We have identified three nationally recognized experts to serve on the DSMB.

Power Estimates. The primary aim of the GIFT Trial is to obtain preliminary data that will allow us to determine whether there is sufficient 'signal' from unacylated ghrelin to warrant additional study of unacylated ghrelin in older patients with PAD. In addition, the GIFT Trial will allow us to obtain an estimate of effect size and standard deviation of change in our study outcomes when unacylated ghrelin is administered to older PAD participants. Finally, the GIFT Trial will allow us to determine the feasibility of administering unacylated ghrelin to older patients with PAD. Because this is a preliminary study, no power calculations were performed.

Statistical Analyses. Simple statistics will be performed to assess changes in brachial artery FMD over time in response to each unacylated ghrelin injection. Simple statistics will also be performed to analyze changes in levels of unacylated ghrelin and acylated ghrelin and circulating levels of oxidative stress after each subcutaneous injection of unacylated ghrelin.

11.2 Power analysis.

A power analysis is not applicable, given the exploratory nature of this research.

11.3 Steps to secure data to maintain confidentiality during storage, use, and transmission.

First, all research assistants must complete training in protection of subject privacy and prevention of disclosure of identifying information.

Second, all data collection forms are maintained in a secure office space.

Third, our study databases are maintained in locked computer files or on secure hard-drives that are password protected; to which only authorized staff have access. Dr. McDermott or a study manager must provide permission for programmers and research assistants to access study databases.

Fourth, a study identification number will be assigned to each participant. This identification number will be used to label blood specimens, for example. In addition, most pages of our data collection forms will have only the study identification number listed (and not the participant's name, for example).

11.4 Quality control

As in our prior studies, health interviewers will be trained by a senior coordinator and certified by Dr. McDermott in each component of data collection, using a detailed checklist developed for this trial. Health interviewers are rigorously evaluated for adherence to protocol, prior to beginning data collection and every six months after initial certification.

12.0 Provisions to Monitor the Data to Ensure the Safety of Subjects

Serious adverse events will be reported to the DSMB within seven days of each serious adverse event. Adverse event data will be reported to the DSMB every six months during the study, and/or as requested by the DSMB. The DSMB will have the ability to stop the study at any time if there are concerns about safety.

13.0 Withdrawal of Subjects*

Subjects may withdraw from the research at any time. If they decide to leave the research, they should contact the investigator, Dr. Mary McDermott.

If they stop being in the research, already collected data may not be removed from the study database. They will be asked whether the investigator can collect data from their routine medical care. If the subject agrees, this data will be handled the same as research data.

Subjects who withdraw from the study will be replaced by another PAD participant(s) who meets eligibility criteria. The newly enrolled PAD participants will begin the study at the dose at which the withdrawn participant would have received next, if they had not withdrawn.

14.0 Risks to Subjects*

Overview of the GIFT Trial and anticipated characteristics of study participants. Six participants with PAD age 55 and older will participate in a

dose finding study, in which participants will receive three different doses of unacylated ghrelin (10 ug/kg, 20 ug/kg, and 40 ug/kg) on three different days. Each dose will be separated by at least one week. Based on our previous work involving older participants with PAD, we anticipate that the average age will be approximately 76 years, that at least 33% of participants will be minorities, and that approximately 50% will be women. For example, in our recently completed SILC randomized controlled clinical trial of exercise in patients with PAD participants included 52% women and 45% African-Americans (39). In general, older patients with PAD have a high prevalence of comorbid diseases, particularly coronary artery disease, cerebrovascular disease, diabetes mellitus, and pulmonary disease. Thus the patient population is likely to be of generally poorer health than that of older men and women without PAD in the general population. Inclusion and exclusion criteria are provided in section 3.2.

Sources of material. We will collect data on levels of circulating unacylated ghrelin at multiple defined time points within a 24-hour period after three increasing doses of subcutaneously injected unacylated ghrelin. We will also measure brachial artery flow-mediated dilation (FMD) at baseline and at six or 8-12 hours after each of the three doses of subcutaneously injected unacylated ghrelin. Information obtained from the levels of circulating unacylated ghrelin and the brachial artery FMD will be used to select the dose of subcutaneous unacylated ghrelin to use in future trials.

Potential Risks.

Risks associated with unacylated ghrelin. As described in section 8.2 above, unacylated ghrelin has been administered intravenously to more than 70 humans without any serious adverse effects (4,8-14). Two participants in one study experienced mild facial swelling after receiving unacylated ghrelin intravenously (14). Symptoms resolved within hours (14). Dizziness was reported in one study in which intravenous acylated and unacylated ghrelin were administered simultaneously over the short term (12). However, this adverse effect was not observed when unacylated ghrelin was administered alone (12). A participant who received 10 ug/kg in the pilot study presented 24 hours later in atrial flutter. However, this participant had a prior history of atrial fibrillation.

We will minimize risk by observing study participants for approximately 8-12 hours after each of the three doses of unacylated ghrelin. Participants will be observed at Northwestern Memorial Hospital and they will be monitored for adverse events. Dr. McDermott will monitor safety and all adverse events will be reported to the DSMB and IRB as described below. The study is being conducted under an Investigational New Drug application with the FDA and is linked to existing INDs held by Dr. Jenny Tong for intravenously delivered unacylated ghrelin and by Dr. Anne Cappola for subcutaneously administered acylated ghrelin.

Six-minute walk and four-meter walk test. The walking tests may be associated with the risk of falling or coronary ischemia or dyspnea due to heart failure or lung disease. Rarely, falling during the walking tests may result in a fracture. However, the research assistant who will collect these data has been trained to prevent falling. The risk of a fracture secondary to a fall during the walking tests is less than 1 in 7,000.

Risks associated with ankle brachial index (ABI) measurement. The ABI measurement consists of measuring systolic blood pressure in each extremity using a hand-held Doppler. The ABI is non-invasive, safe and does not have any known lasting side effects. During the ABI test, participants may experience discomfort from the inflated blood pressure cuff. However, this discomfort resolves immediately when the cuff is released.

Risks associated with blood draws. The potential risks of drawing blood include a bruise at the site of vein puncture, inflammation of the vein, and infection. Participants may experience lightheadedness or dizziness or fainting after injections. Care will be taken to avoid these complications.

Risks associated with holding medications for study testing. Participants may be asked to take their medications in the evening rather than in the morning on the day of study drug injections. This change in medication dosing may result in higher levels of blood pressure during the day with potentially adverse consequences, including increased risk of stroke or need for evaluation in the Emergency Department. However, participants will be carefully monitored for their blood pressure level throughout the day.

Risks associated with questionnaire administration. Participation includes a risk of loss of confidentiality regarding personal health information. However, all research staff has undergone formal human subjects training. They are trained to protect the privacy of research subject participants.

In addition, administration of unacylated ghrelin may have risks to the subjects that are currently unforeseeable.

15.0 Potential Benefits to Subjects

15.1 The potential benefits that individual subjects may experience from taking part in the research.

Potential benefits of the proposed research.
Participants will receive no direct benefits from study participation. Information from this study may be used to develop a large randomized clinical trial using subcutaneously administered unacylated ghrelin to improve walking performance in patients with PAD.

Importance of knowledge to be gained. PAD is common in older men and women (9,10). The number of older people with PAD is expected to increase as the U.S. population lives to older ages and survives longer with chronic disease. Our prior work and that of others shows that patients with PAD have greater functional impairment, increased rates of functional decline, and increased mobility loss compared to persons without PAD (15,18-24). Recent evidence shows that chronic disability, such as that associated with PAD, accounts for more than half of United States health burden (28). Furthermore, therapeutic advances have not kept pace with the growing burden of disability from chronic disease (28). New therapies are urgently needed for patients with PAD. If we are able to determine the optimal dose of unacylated ghrelin, results will be used to design a randomized controlled trial of subcutaneously delivered unacylated ghrelin to improve lower extremity functioning and prevent mobility loss in the large and growing number of older people who are disabled by PAD.

15.2 *Indicate if there is no direct benefit. Do not include benefits to society or others.*

There are no direct benefits.

16.0 Vulnerable Populations

NA

17.0 Community-Based Participatory Research

NA

18.0 Sharing of Results with Subjects

Participants will receive results of their ankle brachial index (ABI) test results immediately after this testing is completed. They will be provided with a "result letter" at the end of their baseline visit. They will not be provided with other study results, because these results are not clinically relevant at this time (i.e. levels of unacylated ghrelin are not clinically relevant. Short term changes in brachial artery FMD are not clinically relevant, and the clinical significance of short term changes in measures of oxidative stress are unclear).

19.0 Setting

The research will be conducted at Northwestern Memorial Hospital.

20.0 Resources Available

Collaborating sites.
Dr. James Stein (University of Wisconsin) is internationally recognized for his expertise measuring vascular function (i.e. flow-mediated dilation). Dr. Stein directs the University of Wisconsin Atherosclerosis Imaging Research Program (UW AIRP), and has served as the reading center for multiple NIH-funded studies, including MESA (51,55,56). Dr. Stein's AIRP will read the brachial artery flow-mediated dilation results.

Drs. Michael H. Criqui (University of California at San Diego), Jack M. Guralnik (University of Maryland), and Luigi Ferrucci (National Institute on Aging) have worked with Dr. McDermott on PAD studies of functional impairment for over eleven years and bring expertise in functional assessment, PAD, and clinical trials to the study team. Dr. Anne Cappola (study consultant) is Associate Professor at University of Pennsylvania and a board-certified endocrinologist. She is currently leading a pilot randomized trial of subcutaneously administered acylated ghrelin in older, frail men and women (R21-AG040488). Her experience administering subcutaneous acylated ghrelin to older, frail individuals will be extremely helpful for the successful execution of the GIFT Trial. Dr. Jenny Tong (study consultant) is Associate Professor at the University of Cincinnati. Dr. Tong has extensive experience studying the pharmacodynamics of intravenously administered acylated ghrelin and unacylated ghrelin in humans. Dr. Tong's experience administering intravenous acylated and unacylated ghrelin in humans will also be particularly valuable to GIFT Trial investigators.

21.0 Prior Approvals

NA

22.0 Recruitment Methods

22.1 When, where, and how potential subjects will be recruited.

PAD participants will be identified from among individuals with PAD who have participated previously in research conducted by Dr. McDermott and/or who have expressed an interest in participating in future studies conducted by Dr. McDermott.

In addition, some PAD participants may be identified from among consecutive patients diagnosed with PAD in the non-invasive vascular laboratory at Northwestern Medical Group (NMG). Dr. Mark Eskandari is medical director of the non-invasive vascular laboratory at NMG and will assist with identifying potential participants from the non-invasive vascular laboratory. As director of the vascular laboratory at NMG, Dr. Eskandari formally reads many of the non-invasive vascular laboratory tests. He maintains all non-invasive vascular test results in his vascular laboratory. As director of the vascular laboratory, Dr. Eskandari could conceivably contact the patients whose test results are maintained in his laboratory. However, Dr. Eskandari prefers that the contact of potential participants in studies come from the physicians referring him for testing. Lists of patients who have undergone lower extremity arterial testing in the non-invasive vascular laboratory are generated monthly and e-mailed from NMG to Dr. McDermott using an encrypted file. A research assistant, working on behalf of Dr. Eskandari, will contact referring physicians of potential participants identified from the vascular laboratory via fax, phone, page, or electronic message (EPIC or e-mail), to ask for permission to contact their patient about the study. If a reply is not received within three weeks, up to five letters are mailed from Dr. McDermott

about the research study. We have substantial experience with our recruitment methods for our previous or ongoing studies.

We also propose to obtain lists of consecutive patients with a diagnosis of lower extremity peripheral arterial disease and individuals at high risk for peripheral artery disease from Northwestern's Enterprise Data Warehouse (EDW). EDW lists will be obtained by an individual who is employed by the Division of General Internal Medicine who has received training and permission to obtain the lists from the EDW.

Similar methods will be used as those described above, in which the patient's physician will be contacted via fax, telephone, page, or electronic message (EPIC or email) to ask for permission to contact their patient about the study. If a reply is not received within three weeks, up to five letters are mailed from Dr. McDermott about the research study.

In the recruitment letters, recipients are asked to call us if they are interested in participation or if they do not want to be contacted further. Potential participants who do not call us within three weeks of the first mailed recruitment letter may be telephoned by study staff and invited to participate.

22.2 Source of subjects.

Please see details regarding "source of subjects" in section 22.0 and 22.1.

22.3 Methods that will be used to identify potential subjects.

Please see details regarding methods used to identify potential subjects in sections 22.0 and 22.1.

22.4 Amount, timing, and method of any payments to subjects.

Participants will receive up to $150 for taking part in this research study. Participants will be paid in cash after completing visits 3, 6, and 9.

Participants will be given assistance and/or reimbursement for expenses related to travel such as parking, bus/train fare, taxi fare, and mileage, if requested. A receipt will be required for taxi fare reimbursement. Participants will be provided up to $50 per visit for travel reimbursement. If they require the use of our taxi service, we will estimate the fare on www.taxifarefinder.com. A one-way fare estimate must be less than or equal to $25.00 (i.e. round trip of $50) in order for the study to provide taxi service.

23.0 Local Number of Subjects

We will identify six PAD participants age 55 and older. We anticipate that we will have up to 20 screen failures.

24.0 Confidentiality

NA

25.0 Provisions to Protect the Privacy Interests of Subjects

Questionnaires will be administered in an enclosed space by a trained and certified research assistant. Dr. McDermott personally certifies study participants in data collection to help ensure that participants are treated with the highest level of professionalism. The study drug injection and phlebotomy will also take place in an examination room with the door closed to ensure optimal privacy.

All research staff undergo training (human subjects training) in the protection of participant confidentiality and privacy. Research staff have access to medical records only for the purpose of conducting research that is approved by the IRB.

26.0 Compensation for Research-Related Injury

If the subject needs medical care because of taking part in this research study, they should contact the investigator and medical care will be made available. Generally, this care will be billed to the subject, their insurance, or other third party. Northwestern University has no program to pay for medical care for research-related injury.

27.0 Economic Burden to Subjects

NA

28.0 Consent Process

The "SOP: Informed Consent Process for Research (HRP-090)" will be followed. Participants will be consented by a research assistant who has been trained and certified by Dr. McDermott in obtaining informed consent. Prior to attending their first study visit, a research assistant will explain the study to potential participants by telephone. When a potential participant arrives to the medical center for study participation, the research assistant will explain the full details of the research study, including risks and benefits. The informed consent process will take place first at initial study visit at Northwestern.

Potential participants will be provided plenty of time to read the consent form. The research assistant will answer study questions. However, if the participant would like more time to discuss the research study with their physician or family member, they will be allowed to do so. In this case, the study visit will not proceed. Dr. McDermott or another study investigator at Northwestern is also available to answer any questions that participants may have about the research.

Non-English speaking subjects, subjects who are not yet adults, cognitively impaired adults, and adults unable to consent will not be eligible for participation.

29.0 Process to Document Consent in Writing

The "SOP: Written Documentation of Consent (HRP-091): will be followed.

30.0 Drugs or Devices

Northwestern Hospital's pharmacy will prepare syringes of varying doses of unacylated ghrelin.

An Investigational New Drug application has been approved by the FDA (IND# 130513). Dr. McDermott is the holder of the IND.

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