PROTOCOL DOCUMENT

N-Acetyl-Cysteine for Healing of Amputation Stumps in the Setting of Diabetes

Short Title: NAC

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N-ACETYL-CYSTEINE FOR HEALING OF AMPUTATION STUMPS IN THE SETTING OF DIABETES

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Introduction

Study Abstract

Approximately 2.5 million Americans currently have advanced critical limb ischemia (CLI) [1]. Patients with diabetes are at increased risk of advanced CLI and resultant complications such as wounds, gangrene, and severe infection. This leads to more than 130,000 extremity amputations in the United States per year [2]. Poor major amputation site healing in patients with CLI and diabetes is associated with surgical site necrosis, dehiscence, and infection. In 40% of these patients, subsequent limb re-amputation or surgical site revision is required with added significant patient morbidity, extended disability, and increased healthcare costs [2, 3].

N-Acetyl-Cysteine (NAC) is a well-known thiol precursor of reduced glutathione [4, 5] and has been in clinical use for over 40 years. It is Food and Drug Administration (FDA) approved as a mucolytic agent. However its current primary clinical use is the treatment of acetaminophen poisoning – which is potentially life saving [6, 7]. Multiple studies and clinical trials have investigated the use of NAC as an antioxidant given its free thiol group and its ability to restore glutathione levels [8-10].

Recent studies have demonstrated that NAC can blunt tissue oxidative stress and decrease insulin resistance [11, 12]. Preclinical studies in our laboratory have also demonstrated that NAC can augment tissue perfusion and arterial collateral maturation (arteriogenesis) in a novel murine hind limb ischemia – amputation model, as well as alter the phospholipid profile in vascular endothelial cells. Recent clinical trials have repeatedly demonstrated the safe administration of NAC [8-10]. **We therefore hypothesize that perioperative administration of NAC in patients with CLI, with and without diabetes, who are undergoing major lower extremity amputation will improve tissue stump perfusion and alter tissue lipidomics.**

Primary Hypothesis

Our primary hypothesis is that perioperative administration of NAC in patients with CLI, plus or minus diabetes, who are undergoing major lower extremity amputation will improve tissue stump perfusion and alter tissue lipidomics, ultimately reducing amputation associated morbidity with respect to wound breakdown, wound infection, and amputation revision.

Purpose of the Study Protocol

The purpose of this study protocol is to evaluate the safety and potential efficacy of NAC in the setting of peripheral arterial disease as a tissue augmentation agent in patients with CLI undergoing lower limb amputation. This will be a Pilot Clinical trial designed to facilitate a future power calculation for a larger, likely multicenter trial that can evaluate the potential clinical benefit of using NAC to improve healing and perfusion of amputation stumps in diabetic patients.

This study is highly innovative in that it will: i) explore a compelling new modality of therapy in a highly vulnerable, but frequently under-represented clinical trial patient population, and ii) represent the potential use of a low-cost, generic, well-established, and generally very safe pharmacological agent for an important clinical problem. This would otherwise not be pursued by drug pharmaceutical manufacturers due to anticipated low-profit margins from a generic pharmacological agent.

Background

Prior Literature and Studies

Diabetes and CLI: Nearly 30 million Americans suffer from diabetes – a chronic metabolic disorder characterized by inappropriate hyperglycemia and increased risk of cardiovascular disease [13]. Diabetes is associated with altered serum lipidomics, endothelial cell dysfunction, and impaired arterial collateral formation [13]. These pathophysiologies make diabetic patients particularly prone to developing a distinct pattern of diffuse and recalcitrant peripheral arterial disease that leads to advanced CLI. Chronic inadequate supply of oxygen and nutrients to the extremity of these patients can lead to end-stage complications like non-healing wounds, infection, and gangrene [14]. Despite recent advances in the treatment and management of diabetic patients, CLI remains highly prevalent in this patient population, and results in high rates of major extremity amputations. Even when major extremity amputations are performed, healing at the surgical site in patients with diabetes is impaired due to poor blood flow at the arterial cul-de-sac in the newly created amputation stump [15]. At the present time, there are no widely accepted treatment strategies to augment tissue perfusion in ischemic peripheral tissue

NAC, Angiogenesis, and Tissue Perfusion: First FDA-approved as a pulmonary mucolytic agent in the 1970s, NAC was later found to be an effective treatment for acetaminophen poisoning by restoring hepatic glutathione levels [6]. Recent studies demonstrate that exogenous administration of NAC can increase the total antioxidant capacity of the intra- and extra-cellular milieu in both murine models as well as human subjects [16]. In streptozotocin (STZ)-treated rodents, NAC scavenger-of-free-radical properties are found to induce PI3K/AKT signaling in cardiomyocytes, reduce cardiac ischemia-reperfusion injury, and increases ischemic hind limb tissue angiogenesis and perfusion [17]. Consistent with these findings, we have also observed improved amputation stump perfusion and healing in a novel preclinical murine hind limb ischemia-amputation model. We also observed increased AKT phosphorylation in ECs following NAC treatment. In addition to the well-characterized antioxidant properties of NAC, another proposed mechanism leading to the therapeutic benefits of NAC is inhibition of sarcolemmal phospholipid breakdown and therefore maintenance of cell membrane integrity [18]. *Therefore, these studies support the role of NAC in augmenting compensatory tissue perfusion in the setting of ischemia, and raise the possibility that this beneficial effect of NAC may be related to altered tissue phospholipid content*

NAC in CLI and Diabetes: Recent studies exploring the therapeutic benefits of NAC in the setting of CLI and diabetes, demonstrated that it can preserve insulin content, induce insulin gene expression, improve insulin sensitivity, and restore beta islet cell morphology and function [11, 12, 18]. The molecular mechanisms by which these effects occur are not yet understood, but it is proposed that NAC therapeutic benefits in the setting of diabetes may be a result of altering cellular lipid metabolism. Accordingly, NAC treatment was recently found to decrease serum free fatty acids, increase serum HDL, and alter the expression profile of specific cellular phosphatidylinositols that are known to be essential for normal glucose metabolism [19, 20]. Consistent with this notion, we recently observed that NAC not only affects the levels of key phospholipid synthesis enzymes such as choline-ethanolamine phosphotransferase 1 (CEPT1; which regulates the synthesis of the majority of phosphatidylcholines and phosphatidylethanolamines), but can also alter the phospholipid ratios (PC:PE, a marker of ER stress) in endothelial cells. *This body of evidence supports the potential important functions that*

NAC plays in the setting of CLI as well as potentially diabetes as well, and points to previously unknown effects of NAC on key phospholipid synthesis pathways.

Rationale for this Study

Aside from smoking cessation and physical exercise, there are currently no known effective medical treatments for patients with advanced non-surgically correctable CLI [13, 21]. In addition, the prevalence of amputations resulting from CLI among diabetic patients is disturbingly high [2]. Based on our recent preclinical studies, we are proposing a pilot clinical trial to further explore the potential therapeutic benefits of NAC in healing extremity amputation stumps in the setting of CLI, plus or minus diabetes. These studies are highly innovative in that they: i) explore a compelling new modality of therapy in a highly vulnerable, but frequently under-represented clinical trial patient population, ii) explore a completely novel and not previously reported NAC mechanism of action on phospholipid synthesis and expression, and iii) represent the potential use of a low-cost, generic pharmacological agent, which would otherwise not be pursued by drug pharmaceutical manufacturers due to low-profit margins. In short, this proposal may establish a conceptual infrastructure for future larger clinical trials exploring the use of NAC in patients with CLI and with and without diabetes, and identify a previously unknown novel mechanism of action for NAC.

We believe that previous literature supporting the role of NAC in augmenting compensatory tissue perfusion raises the possibility that this may be an advantageous therapeutic agent to favorably alter the tissue lipidomic profile of diabetic patients receiving NAC. We believe that NAC offers a new treatment strategy for patients with CLI undergoing lower extremity amputation with the intent to improve tissue perfusion, healing, and reduce morbidity and mortality associated with lower limb amputation.

Study Objectives

Primary Aim 1

Determine whether NAC can affect major lower extremity amputation stump perfusion by POD (Post-operative Day) 5

Primary Aim 2

Determine whether NAC can affect major lower extremity stump healing for 30 days following amputation.

Secondary Aim

Determine the effect size in diabetic patients that is necessary to power a larger clinical trial to determine whether NAC treatment can improve tissue perfusion and healing at major lower extremity amputation stumps.

Rationale for the Selection of Outcome Measures

In this pilot clinical study we propose to conduct a prospective, randomized, double-blinded, placebo-controlled clinical trial for 30 days for patients with CLI who undergo a major (above-knee or below-knee) lower extremity amputation. By exploring the primary endpoints we aim to

determine whether NAC can affect amputation stump perfusion and healing. Based on our preclinical data, we hypothesize that NAC will augment both amputation stump perfusion as well as healing. We will utilize the data from this trial to determine the true effect size that is necessary for a larger clinical trial to determine the clinical efficacy of NAC is healing surgical sites such as major lower extremity amputation stumps.

In addition, we will obtain MR perfusion studies on 6 subjects (3 NAC and 3 placebo) conducted at approximately post-operative day (POD) 4. The study team will continue to remain blinded to their study drug randomization. The study pharmacist will notify the study team of which subjects will be eligible to have an MR perfusion study. For example, if the MR perfusion portion of the study has already recruited 3 patients from the NAC group, and the forth eligible patient also randomizes to NAC, the pharmacy will notify the study team that the patient is not eligible for the MR perfusion study.

MR perfusion studies will be performed by Dr. Jie Zheng and his research on a Siemens Prisma 3.0 T MR (Software version VE11c (latest software version), 40 mT/meter; 200 μsec rise time) in CCIR. The subject will lie down on a MRI table and The MRI scan will undergo only at rest. The MR study will be performed without using of any MR contrast media. The scan protocol will involve scout imaging to localize the calf and stump regions. This will be followed by perfusion, diffusion, and oxygenation imaging in these regions. The expected scan time will be less than 45 min.

MR perfusion studies will only be used for research purposes (i.e. will not direct medical/clinical therapy). MR perfusion will be used to assess skeletal muscle perfusion at the healing amputation stump. This data will be used in adjunct to assess overall stump perfusion along with the LAFA studies that are will already be performed on the patient.

Investigational Agent

Preclinical Data

Further studies exploring the therapeutic benefits of NAC in the setting of diabetes, demonstrated it can preserve insulin content, induce insulin gene expression, improve insulin sensitivity, and restore beta islet cell morphology and function [11, 12, 18]. The molecular mechanisms by which these effects occur are not yet understood, but it is proposed that NAC therapeutic benefits in the setting of diabetes may be a result of altering cellular lipid metabolism. Accordingly, NAC treatment was recently found to decrease serum free fatty acids, increase serum HDL, and alter the expression profile of specific cellular phosphatidylinositols that are known to be essential for normal glucose metabolism [19, 20]. Consistent with this notion, we recently observed that NAC not only affects the levels of key phospholipid synthesis enzymes such as choline-ethanolamine phosphotransferase 1 (CEPT1; which regulates the synthesis of the majority of phosphatidylcholines and phosphatidylethanolamines), but can also alter the phospholipid ratios (PC:PE, a marker of ER stress) in endothelial cells.

We recently performed a preclinical study to explore the effect of NAC in healing amputation stumps in the setting of diabetes. C57B6 adult mice with streptozotocin-induced diabetes underwent unilateral hindlimb femoral artery ligation, followed by a staged infrageniculate amputation of the malperfused limb one week after induction of ischemia. Compared to controls, mice treated with daily NAC demonstrated improved stump healing, increased hindlimb stump perfusion post-amputation, increased hindlimb adductor muscle neovascularization, and decreased adductor muscle fiber damage. NAC treatment stimulated EC migration and proliferation in a phospholipase C β (PLC β)-dependent fashion, and decreased Gαq palmitoylation. *In vivo*, NAC treatment also decreased Gαq palmitoylation in ischemic and non-ischemic hindlimbs, as well as in hindlimbs of acyl protein thioesterase-1 knockout mice with impaired palmitoylation dynamics. These findings are currently pending publication in a peer-reviewed scientific journal.

Clinical Data to Date

Currently, there is no clinical data examining the utility of NAC with regards to improving tissue stump vascular perfusion and altering lipidomic profiles in diabetic patients. Other clinical trials that previously explored the clinical efficacy of NAC, demonstrated that NAC was well tolerated by study patients.

Dose Rationale and Risk/Benefits

A variety of dosing regiments are well studied for the clinical use of NAC. The majority of these are using oral formulation. The most commonly studied oral dose is 600mg twice daily, though other studies evaluating 1200mg vs 600mg did show slightly better outcomes with the higher dose [22] for patients at risk of contrast nephropathy. Patient's requiring emergent coronary angiography or procedures, have received NAC safely.

For this study, we will administer NAC 1200mg twice-daily, intravenously.

Risks of NAC include rare side effects or adverse reactions. Severity of side effects are described as moderate, and include wheezing, tightness in the chest, or difficulty breathing, though the most commonly reported adverse effects for IV formulations include rash, urticaria, and pruritus. Other side effects of the oral formulation include nausea, vomiting, diarrhea, or constipation. Patients with asthma are at higher risk of developing bronchospasm with inhaled NAC. Patients with severe clinical asthma or end-stage liver disease will not be recruited to this study.

NAC has deemed to be LIKELY SAFE in women (Pregnancy Category B) who are pregnant and breast-feeding, though it does cross the placenta; however, there is currently no literature demonstrating teratogenicity to a fetus. Patients who are pregnant or are infant nursing will not be eligible to participate in the study.

Risks associated with MRI scan:

Likely: During the MR imaging study, the subject may experience a loud pounding noise coming from the scanner.

Less likely: The subject may experience some discomfort related to lying still in the scanner during the examination period (< 1 h). The subject may feel claustrophobic (anxious in small spaces) while lying in the MRI scanner. Typically, less than 5% of the subjects who have MRI exams feel claustrophobic. Should this occur, the subject will be allowed to get out of the scanner. Because of the powerful, high speed gradients in the MR system, some subjects (2-4%) experience slight tingling or tapping sensations in the arms and legs (mild peripheral neuromuscular stimulation). Tingling or tapping sensations stop when the machine is not scanning.

Study Design

Overview or Design Summary

In this pilot clinical study we propose to conduct a prospective, randomized, double-blinded, placebo-controlled clinical trial, in 50 human subjects with CLI who have undergone a major (above-knee or below-knee) lower extremity amputation. 25 patients will receive NAC 1200mg intravenously twice a day for 6 consecutive days following amputation. 25 patients will receive placebo saline intravenous infusion twice a day for 6 days following amputation. Post-amputation patients will be monitored for specific anthropometric parameters and stump perfusion assessments (using laser-assisted fluorescent angiography and transcutaneous oxygen pressure measurement). **The primary study endpoints are to determine if lower extremity stump healing and perfusion are affected by perioperative NAC administration. A secondary endpoint will be to determine the effect size that would be necessary to power a larger clinical trial to determine whether NAC treatment can affect tissue perfusion and healing at major lower extremity amputation stumps in patients with CLI.**

Subject Selection and Withdrawal

Inclusion Criteria

Inclusion criteria for recruited subjects will be as follows:

- Subject undergoing elective major (above-knee or below-knee) lower extremity amputation for CLI
- Both male and female patients
- All ethnic groups
- Between of the ages of 30-90 years old

1.a Exclusion Criteria

Exclusion criteria for patients will be as follows:

- Pregnant women, and women who are breastfeeding
- Known history of end-stage liver disease
- Severe asthma
- Heavy alcohol consumption (male > 2 drinks per day and women > 1 drink per day)
- Individuals actively receiving chemotherapy.

- Anticipated enrollment in another study that investigates another drug agent within 30 days from enrollment in this study.
- Patients receiving carbamazepine.
- Severe anemia (HCT < 22).
- Allergy to either NAC or ICG

All inclusion and exclusion criteria will be verified from patient chart in Epic and through self report and.or LAR report; in particular, alcohol consumption and participation in another drug research study to obtain the most current information. Email correspondence between RC, PI, and other study team members regarding patient enrollment may be included in the subjects chart to serve as a real time documentation of PI's approval to enroll participant, in addition to signature in CRFs.

Ethical Considerations

Ethical considerations for this particular clinical study include the issue of beneficence, non-maleficence, patient autonomy and the voluntary, competent decision making of the individual patients participating in this clinical trial [23]. For this reason, we believe that the following requirements will be satisfied for each patient enrolled in the clinical trial:

- Physical and psychological risks to subjects are minimized.
- Physical and psychological risks to subjects are reasonable in relation to anticipated benefits to those subjects and to the importance of the general knowledge that may reasonably be expected to result.
- Selection of subjects is equitable
- Informed consent will be obtained, including at least the following items being communicated to potential participants or their authorized surrogates:
    - Purposes of the research, its expected duration, and the nature of any interventions/experiments;
    - Anticipated risks and benefits of participation and the reasonable alternatives to participation in the research protocol;
    - Confidentiality provisions relating to the research records;
    - Any compensation and/or treatment available for research related injuries;
    - The right to not participate and to discontinue participation at any time without penalty.
    - Informed consent will be documented appropriately.

Subject Recruitment Plans and Consent Process

Subjects that meet study inclusion and exclusion criteria, who are undergoing an elective above-knee or below-knee amputation at the Washington University School of Medicine-affiliated Barnes-Jewish Hospital (BJH), will be approached for participation in this pilot clinical trial. Potential subjects will be identified by reviewing the weekly operative schedule and via referral from vascular surgery faculty study co-investigators.

Prior to the scheduled operation, the study investigator will screen eligible patients by reviewing the medical chart. Patients that meet study inclusion and exclusion criteria will be approached for informed consent. Informed consent will be obtained by a member of the vascular surgery research team in accordance with institutional regulations, prior to any further actions in the study. Per standard clinical procedures, all patients will also be evaluated by preoperative anesthesia services.

Randomization Method and Blinding

Upon consent and enrollment in the study, relevant subject information will be relayed to the Barnes Jewish Hospital (BJH) Investigational Pharmacy. Prior to proceeding to the operating room for lower extremity amputation, the patients will be randomized 1:1 by the BJH Investigational Pharmacy to either placebo saline infusion (twice daily) or a standard adult intravenous dose of NAC (1200mg twice daily) for 6 days post-amputation during the usual post-operative inpatient extremity off-loading and stump monitoring period. The pharmacy will maintain blinding, with no member of the investigational team having access to the record until the conclusion of the trial.

Early Withdrawal of Subjects

Patients unable to tolerate the administration of the drug will be withdrawn from the study at any point during their hospitalization and will continue to be monitored until their condition stabilizes. Patients unable to participate in the Post-operative Day 0 Non-invasive Laser Assisted Fluorescence Angiography (LAFA) will be withdrawn from the study at that time point and will not receive the investigational product. Subjects for which continuous IV access is not medically indicated during the 6 day study drug administration period may be withdrawn from the study. Patients unable to participate in follow-up appointments will also be withdrawn from the study.

When and How to Withdraw Subjects

Patients unable to tolerate the administration of the drug will be withdrawn from the study trial at any point during their hospitalization. Patients unable to participate in follow-up appointments will also be withdrawn from the study. They will continue to be monitored for adverse events as inpatients for the duration of their hospitalization. Patients unable to participate in the Post-operative Day 0 Non-invasive Laser Assisted Fluorescence Angiography (LAFA) will be withdrawn from the study at that time point and will not receive the investigational product. No further study follow up will be required.

Data Collection and Follow-up for Withdrawn Subjects

Patient data will be collected up until time of withdrawal from the study and pooled into the collective analysis along with all other patients.

Study Drug

Description

N-acetyl cysteine (NAC) ((2R)-2-acetamido-3sulfanylpropanoic acid) is an amino acid derivative of L-cysteine, which is a precursor to glutathione and cysteine. Historically, NAC has been used to treat acetaminophen overdose [6], as well as mucous secretions in patients with cystic fibrosis or chronic obstructive pulmonary disease [24].

Pharmacokinetics including the following [24] :

Distribution: The steady-state volume of distribution (Vdss) and the protein binding for acetylcysteine were reported to be 0.47 liter/kg and 83%, respectively.

Metabolism: N-acetyl-cysteine may form cysteine, disulfides, and conjugates in vivo (N, N'-diacetylcysteine, N-acetyl cysteine-cysteine, N-acetyl cysteine-glutathione, N-acetyl cysteine-protein, etc). Based on published data, it was reported that after an oral dose of 35S-acetylcysteine, about 22% of total radioactivity was excreted in urine after 24 hours. No metabolites were identified.

Elimination: After a single intravenous dose of N-acetyl cysteine, the plasma concentration of total N-acetyl cysteine declined in a poly-exponential decay manner with a mean terminal half-life (T1/2) of 5.6 hours. The mean clearance (CL) for N-acetyl cysteine was reported to be 0.11 liter/hr/kg and renal CL constituted about 30% of total CL.

Treatment Regimen

Upon study enrollment, patients will be randomized 1:1 by BJH Investigational Pharmacy to either placebo ½ normal saline infusion (twice a day) or a standard adult intravenous dose of NAC (1200mg twice a day) for 6 days post-amputation. The BJH Investigational Pharmacy will perform this with knowledge of the patient's medical record number (MRN), and without knowledge of any pre-existing medical conditions, comorbidities, past medical history, prior interventions, etc. The first infusion will be administered within three hours from leaving the operating room. The second infusion will be a minimum of 6 hours after the first infusion, or at 21:00 in an attempt to be consistent with routine hospital BID dosing times. Patients will receive their scheduled infusions as long as they are hospitalized according to the chart below (Table 1). If medication is not administered within that window, dose will not be given to not interfere with the rest of the doses.

Table 1:

Post-operative Day (POD)	Infusion Schedule
POD 0	1. Within 3 hours from the conclusion of the operation 2. Approximately 6 hours after first infusion, or at 21:00 +/- 6 hours
POD 1	1. 9:00 +/- 6 hours 2. 21:00 +/- 6 hours
POD 2	1. 9:00 +/- 6 hours 2. 21:00 +/- 6 hours
POD 3	1. 9:00 +/- 6 hours 2. 21:00 +/- 6 hours
POD 4	1. 9:00 +/- 6 hours

	2. 21:00 +/- 6 hours
POD 5	1. 9:00 +/- 6 hours 2. Anticipated discharge, second infusion may be given if patient is still inpatient or missed any doses

Preparation and Administration of Study Drug

All investigational doses will be prepared by the research pharmacy and prepared in a manner that both active and placebo doses appear identical to protect the blind. Preparation of each dose will be performed using aseptic technique, and within a laminar flow hood. Each dose will be a total volume of 50 mL and will be administered over a period of 30 minutes through an IV. N-acetyl-cysteine will not be infused in the same intravenous line as iron-products, cefepime, or ceftazidime.

Subject Compliance Monitoring

The Barnes-Jewish Hospital inpatient electronic medication administration record (eMAR) will be used to assess compliance; significant patient noncompliance, defined as refusal or inability to adhere to the protocol requirements, will lead to subject withdrawal.

Prior and Concomitant Therapy

Use of any investigational agent within 30 days before the first dose of study drug and until after the last evaluation will not be allowed. Since concomitant use of N-acetyl-cysteine and carbamazepine may cause decrease carbamazepine plasma concentrations resulting in an increased risk of seizures, patients who are receiving carbamazepine will not be enrolled in the study.

Concurrent use of N-acetyl-cysteine and nitroglycerin may result in enhanced hypotension and nitroglycerin-induced headache. Therefore patients who receive both agents will be monitored by members of the study team for any potential anticipated complications.

All other concomitant medications that are necessary for the health and well-being of the patient that are not specifically prohibited by the protocol are permitted.

Packaging

Acetadote (acetyl-cysteine) Injection is available as a 20% solution (200mg/mL) in 30 mL single dose glass vials. Acetadote is sterile and can be used for I.V. administration.

Blinding of Study Drug

All intravenous doses will be prepared in identical evacuated IV containers with a total volume of 50mL. N-acetyl cysteine is a clear and colorless solution, and no additional blinding material will be required.

Receiving, Storage, Dispensing and Return

Medications will be prepared by the research pharmacy and delivered to the treating nurse; study drug will be blinded to the research team and the study participant. The research pharmacy will

maintain documentation of medication procurement and preparation, as well as randomization, dispensing, and accountability logs. All medications will be stored in a secure location within the research pharmacy and maintained under controlled environmental conditions. Unused doses will be returned to the research pharmacy, whereas used or unwanted partially infused doses will be destroyed on the nursing unit per policy.

Study Procedures

Screening for Eligibility

As discussed in 2.c, subjects that meet study inclusion and exclusion criteria, who are undergoing an elective above-knee or below-knee amputation at the Washington University School of Medicine-affiliated BJH, will be included in this pilot clinical trial.

Potential subjects will be screened by reviewing the weekly operative schedule, and via referral from vascular surgery faculty study co-investigators. Prior to the scheduled operation, eligible patients will be approached for informed consent. Informed consent will be obtained by a member of the vascular surgery research team in accordance with institutional regulations, prior to any further actions in the study. After informed consent is obtained, a baseline clinical evaluation will be performed including lower extremity anthropometric evaluation, medical history and vital signs documentation. Per standard clinical procedures, all patients will also be evaluated by preoperative anesthesia services. Subjects may participate in the study a second time on ipsilateral or contralateral site if all Inclusion /Exclusion is reviewed and verified prior to second surgery. Patient records from an outside hospital or facility may be obtained and used for screening and/or follow up in patient care.

Schedule of Measurements

A. Non-invasive Laser Assisted Fluorescence Angiography (LAFA)

Location of Procedure:

- Barnes-Jewish Hospital Operating Rooms
- 4400/8300/7400/7500 Inpatient Hospital Units
- Other Hospital Units also housing vascular surgery patients

Time of Data Collection:

- **Postoperative Day 0**: Obtained in the operating room after amputation, but before placement of dressings.
- **Postoperative Day3**: Obtained following dressing removal at bedside in the inpatient hospital unit.
- **Postoperative Day5**: Obtained at the bedside in the inpatient hospital unless the patient is discharged.

Contraindications to LAFA:

- Patient refusal/inability to precipitate in measurement before or during data collection.

- Allergic reaction previously documented to Indocyanine green (ICG) fluorescent dye (*a contraindication to LAFA does not exclude the patient from other measurements within the study protocol*).
- Patient unable to lie supine and immobile for at least 10 minutes during the imaging procedure.
- End-stage liver disease
- Severe clinical asthma

LAFA Procedural Steps:

1. Each patient is entered into the Novadaq SPY *Elite*® computer system, with pre-set optimized settings for appropriate visualization for LAFA.
2. Utilizing a standard protocol per the manufacturer (Novadaq SPY *Elite*® system), a 7.5mg (3mL) solution of ICG (reconstituted with sterile saline) is injected intravenously into each patient.
3. The patient is asked to lie supine, immobile for a period of approximately 10 minutes
4. The device is placed over the limb/amputation stump being studied and centered to allow full visualization of the entire stump
    a. Prop up and immobilize stump
    b. Obtain a still color image of the stump
    c. Start an image buffering time of between 10-30 seconds
    d. ICG is intravenously injected by the patient's nurse or a member of the study team.
    e. ICG injection is followed by a 10mL of sterile saline flush in the same intravenous line.
    f. A member of the study team will obtain LAFA imaging of the stump for a total of 150-180 seconds.
    g. All study images will be stored on the Novadaq SPY *Elite*® system hard drive. Study images, including only the study ID and date of exam, will be loaded onto a jump drive and then immediately downloaded onto the WUSTL Department of Surgery/Vascular network drive. The images will then be deleted from the jump drive..
5. Each study patient will be carefully monitored by a physician, nurse practitioner, physician assistant or nurse for signs and symptoms of allergic reaction or intolerance to this non-invasive procedure until discharge.

B. Measurement of Transcutaneous Oxygen Pressure ($TcPO_2$)

Location of Procedure:

- Barnes-Jewish Hospital Operating Rooms
- 4400/8300/7400/7500 Inpatient Hospital Units
- Other Hospital Units also housing vascular surgery patients

Time of Data Collection:

- **Day 0 (Day of Operation)**: If possible and/or equipment available, obtained prior to the operation at the anticipated level of the amputation.
- **Postoperative Day3**: If possible and/or equipment available, obtained following dressing removal at bedside in the inpatient hospital unit.
- **Postoperative Day5**: If possible and/or equipment available, also obtained at bedside in the inpatient hospital unit if the patient is still in-house.

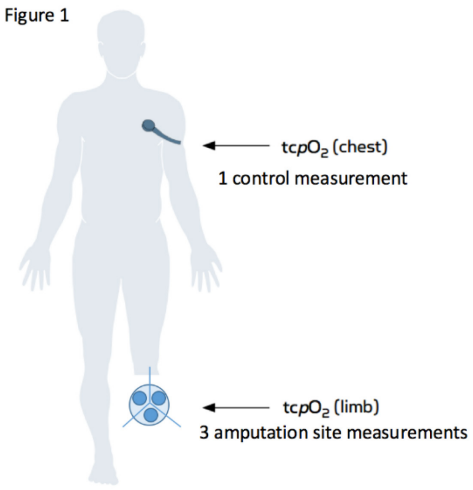
Contraindications to Measurement:

- Patient refusal/inability to participate in measurement before or during data collection
- Patient SpO_2 < 92%, Patient is febrile >38˚C, unstable vitals
- Patient unable to lie supine for at least 20 minutes during measurement collection
- Patient unable to breathe normobaric oxygen
- *A contraindication to $TcPO_2$ measurements does not exclude the patient from other measurements within the study protocol

Protocol Steps:

1. Ensure patient meets the eligibility criteria listed above.
2. Keep patient covered, and expose the anticipated amputation site or at the amputation stump. Do not elevate the site throughout the measurement collection.
3. Select 4 collection sites as follows:
    1. 3 sites on the anticipated amputation site or at the stump site, one per section. (Figure 1)
    2. 1 control site on the left anterior chest in area indicated on figure. (Figure 1)
4. Electrodes will be placed as per TCM Manual instructions, replicated below with the following specifications:
    1. Temp. set to 45˚C.
    2. Do not place probes on scar tissue or dermatologically compromised sites.
    3. Do not place probe over bone.
5. After 15-20 minutes, record the $TcPO_2$ numbers in all 4 sites. Mark the location of the sites for future measurements to ensure a consistent location for measurement and control (days 2 and 4).
6. Remove probes from patients.

Figure 1



Data Analysis:

After $TcPO_2$ measurements have been collected, the 3 amputation site measurements will be averaged to calculate a mean $TcPO_2$. The control site measurement on the operated leg will be used to calculate a Regional perfusion index (Mean $TcPO_2$ / Chest/Control $TcPO_2$). The non-operated leg measurement site (compared against the operated site) will be used to determine if lower $TcPO_2$ values in the amputation site are due to regional or systemic oxygenation.

How to place an electrode

Step	Action
1.	Calibrate the tcpO_2 electrode.
2.	Clean the selected measuring site with alcohol or other skin-preparation solution.
3.	Dry the site well with a gauze pad.
4.	Take a standard fixation ring.

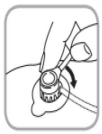
5. Remove the fixation ring from the protective film.

6. Apply the fixation ring to the measuring site as follows:
   - Press the center of the fixation ring onto the measuring site with a finger.
   - Run a finger around the rim circumference.

Note: Press firmly to prevent leaks.

7. Fill the hole in the fixation ring with 3-5 drops of the contact liquid.

8. Affix the electrode into the fixation ring as follows:
   - Align the arrow on the electrode with one of the marks on the fixation ring.
   - Turn the electrode 90° clockwise to fasten it in the fixation ring.



9. Repeat steps 1 to 8 if more electrodes are to be applied.

Wait for a stable reading after the electrode has been affixed to the patient.

Note: The physiological stabilization time of a patient is 15-20 minutes for the tcpO_2 reading. During this time the electrode will slowly heat the skin, making the arteries dilate. Longer time may indicate an incorrect attachment of the electrode or a poorly selected measuring site.

C. Anthropometric Measurements

<u>Location of Procedure</u>:

- Barnes-Jewish Hospital Operating Rooms
- Inpatient Hospital Units
- Other Hospital Units also housing vascular surgery patients

<u>Measurements</u>:

Measurements will be collected as long as the patient is hospitalized, including:

1. Temperature (daily maximum and minimum)
2. Blood Pressure Range

3. Heart Rate Range
4. SpO_2 Range
5. Respiratory Rate Range
6. Amputation wound assessment on POD 3 (if stump dressings are off and still hospitalized) and POD 5 (if stump dressings are off and still hospitalized).

In addition, a blood sample (~50mL; 3-4 table spoons) may be obtained for:

1. Hospital laboratory evaluations that may be obtained per standard of care, at standard of care time points during the hospital stay
    1. CBC
    2. BMP
    3. HbA1C Maybe obtained at some point during the course of the 6 day study if the patient did not have a value obtained within 3 month prior to the amputation)
    4. Pre-albumin (May be obtained at some point during the course of the 6 day study if the patient did not have a value obtained within 1 week prior to the amputation)

2. Research laboratory evaluation
    1. One sample within 12 hours from the time of the amputation operation, if possible
    2. One sample within 12 hours prior to discharge from the hospital or by POD5, if possible
    3. Samples will be taken to the research laboratory to evaluate:
        1. Serum Insulin level
        2. Serum Thiol Level
        3. Serum L-Cysteine level
        4. Serum lipidomics
        5. Serum nicotine and cotinine levels
        6. Other essential plasma/serum proteins or biomarkers

Contraindications to Measurement:

- Patient refusal/inability to participate in measurement before or at time of data collection (*a contraindication to anthropometric measurements does not exclude the patient from other measurements within the study protocol*).
- Severe anemia (Hematocrit < 22), that develops following enrollment in the study.

Data Analysis:

Anthropometric data will be analyzed to determine its effects on patient amputation stump healing score, qualitative peak perfusion, and/or quantitative peak perfusion.

D. MRI Measurements

Location of Procedure:

- Center for Clinical Imaging Research

Time of Data Collection:

- Postoperative Day 4, If possible and/or equipment available and if the patient is still in-house.

Contraindications to Measurement:

- Patient refusal/inability to participate in measurement before or during data collection
- Patient unable to lie supine for at least 30 minutes during measurement collection
- Contraindication to MR scanning such as intracranial vascular clips, pacemaker or intraocular metal.
- Patient is hemodynamically unstable, pregnant, are claustrophobic.

MRI Procedural Steps:

1. The patient is asked to lie supine on a narrow bed in the MRI scanner. An MRI scanner is a large donut-shaped machine that is used by thousands of hospitals to make pictures of various parts of the body. The patient is given ear plugs or earphones to dampen the loud noise the scanner makes when taking pictures. The patient is also given a squeeze ball which can activate an alarm at the scanner operator's console, so the patient can let the operator know to have a question or need assistance.
2. The patient is scanned under an existing non-contrast MRI protocol to obtain blood flow and tissue oxygenation pictures at rest.
3. Each study patient is carefully monitored by a physician, nurse practitioner, physician assistant or nurse for signs and symptoms of allergic reaction or intolerance to this non-invasive procedure until discharge.

Visit 1

Following patient discharge, amputation stumps will be evaluated within 20-50 days during a routine post-operative visit using the same anthropometric parameters (LAFA and TcPO2 will not be performed at this time point).

Safety and Adverse Events

Safety and Compliance Monitoring

All study patients are inpatients at BJH in St. Louis Missouri and are cared for by appropriately trained nurses on the inpatient wards. Vascular surgery Attending Physicians, Vascular Surgery Fellows, Surgical Residents, and a team of Nurse Practitioners oversee their overall care. Inpatient ward nurses routinely assess patients at least every 4 hours with vital signs, intake and output measurements, pain assessment, nutrition assessments, medication review and administration, etc.

Medical Monitoring

Medical monitoring of the study will be performed by the Principal Investigator. The goal of monitoring this study is to enhance human subject protection and to monitor the quality of the clinical trial data by focusing investigator oversight on the most important aspects of study conduct and reporting. The PI will provide regular oversight to ensure adequate protection of the rights, welfare, and safety of the human subjects and the quality of the clinical trial data.

Definitions of Adverse Events

An adverse event (AE) is defined as any untoward medical occurrence in a subject administered a pharmaceutical product or who has undergone study procedures and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of the investigational product, whether or not related to the medicinal (investigational) product.

All AEs will be recorded in the CRF from the time of consent through the 30 days post-operative visit. AEs will be followed to resolution or stabilization.
An abnormality identified during a medical test (e.g., laboratory parameter, vital sign, physical exam) should be defined as an AE only if the abnormality meets one of the following criteria:
● Induces clinical signs or symptoms
● Requires active intervention
● Requires interruption or discontinuation of study medication
● The abnormality or investigational value is clinically significant in the opinion of the investigator.

An AE is considered “serious” if, in the view of the Investigator, it results in any of the following outcomes:

- Results in death
- Is life threatening (an AE is considered “life threatening” if, in the view of the investigator, its occurrence places the subject at immediate risk of death. It does not include an AE that, had it occurred in a more severe form, might have caused death)
- Results in persistent or significant disability/incapacity or substantial disruption of the ability to conduct normal life functions
- Results in congenital anomaly, or birth defect
- Requires inpatient hospitalization or leads to prolongation of hospitalization (hospitalization for treatment/observation/examination caused by AE is to be considered as serious)
- Other medically important events

Medical and scientific judgment should be exercised in deciding whether expedited reporting is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the other outcomes listed in the definition above. These events, including those that may result in disability/incapacity, should also usually be considered serious. Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm or blood dyscrasias or convulsions that do not result in hospitalization.

Events related to the medicinal products administered to the subject as part of the study (e.g., study drug, comparator, background therapy) that may require expedited reporting, but are not limited to:

- Overdose of the medicinal product(s)
- Inadvertent or accidental exposure to the medicinal product(s)
- Medication error involving the medicinal product(s)

All AEs and events related to the medicinal product administered to the subject as part of the study will be recorded in the CRF from the time of consent through the 30 days post-operative visit. AEs should be followed to resolution or stabilization.

Classification of Events

AEs are classified as *serious or non-serious; expected or unexpected; and study-related, possibly study-related, or not study-related.*

Relationship

Defining Relationship

Adverse events that fall under either "Possible" or "Probable" should be defined as "AEs whose relationship to the study drugs could not be ruled out." Relationship to study drug will be assessed using the following criteria:

Not Related	A clinical event, including laboratory test abnormality, with a temporal relationship to drug administration, which makes a causal relationship improbable, and/or in which other drugs, chemicals or underlying disease provide plausible explanations.
Possible	A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, but which could also be explained by concurrent disease or other drugs or chemicals.
Probable	A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, unlikely to be attributed to concurrent disease or other drugs or chemicals, and which follows a clinically reasonable response on re- administration (re-challenge) or withdrawal (de-challenge).

Severity

Defining Severity

Grade 1 - Mild AE: Asymptomatic or mild symptoms, clinical or diagnostic observations noted; intervention not indicated.

Grade 2 - Moderate AE: Local or noninvasive intervention indicated.

Grade 3 - Severe AE: Medically significant but not immediately life threatening hospitalization or prolonged hospitalization

Grade 4 - Life-threatening: Life threatening consequences, urgent intervention indicated or disabling AE

Grade 5 – Death: Death related to AE

Expectedness

All AEs that are previously unobserved or undocumented are referred to as “unexpected,” in that their nature and severity are not consistent with information provided in the relevant product information (e.g., approved professional package insert or product label). Determination of expectedness is made by the Investigator on a case-by-case basis.

Data Collection Procedures for Adverse Events

All adverse events must be documented in the subject’s medical record and/or Case Report Form (CRF). In order to prevent bias collection of AEs, subjects should not be questioned regarding specific events that might be anticipated while on the study. AEs should be spontaneously reported or elicited from a subject using open-ended questioning during examination or evaluation.

The AE CRF will include the start date (and time if applicable; e.g. It may be important to time the AE, especially with infusion reactions), a detailed description of the event, any treatment for the AE (e.g., no treatment needed, further testing to diagnosis event, hospitalization, dose reduction, holding of study intervention), Investigators assessment of the AE (serious or not serious, expected or unexpected, related or unrelated) and the date the AE resolved. If an ongoing AE worsens or improves in its severity or its relationship to the study drug changes, documentation should be collected as above.

Reporting Procedures

Unanticipated problems and unexpected adverse drug events are defined below and will be reported to the local IRB according to local policy.

An Unanticipated problem involving risks to participants or others:

1. Are unexpected (in terms of nature, severity, or frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied.
2. Are related or possibly related to participation in the research.
3. Suggest that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

An unexpected adverse drug event:

1. Any adverse drug experience (associated with the use of the drug), the frequency, specificity, or severity of which is not consistent with the current investigator brochure or package insert; or
2. If an investigator brochure or package insert is not required or available, the specificity or severity of which is not consistent with the risk information provided to the participants and the IRB

Adverse Event Reporting Period

Per local IRB policy an individual adverse event that meets the definition of an anticipated problem involving risk to participants or others or an unexpected adverse drug event will be reported to the IRB within 10 working days. If an unexpected adverse drug event results in a death the event

will be reported to the IRB within 1 working day. Individual adverse events that do not meet the definition of an unanticipated problem involving risks to participants will be reported to the IRB at the time of continuing review (annual renewal).

Post-study Adverse Event

All AEs will be recorded in the CRF from the time of consent through the 30 days post-operative visit however all AEs will be followed to resolution or stabilization.

Study Outcome Measurements and Ascertainment

As described previously, study subjects will undergo evaluations using LAFA, TcP02, and anthropometric measurements.

Statistical Plan

Sample Size Determination and Power

The purpose of this study is to obtain sufficient preliminary data to determine the sample size necessary for a future large definitive clinical trial. For this pilot study, we plan to enroll 25 patients in each group, allowing for a dropout rate of 10% per group.

We emphasize that effect size (the expected difference in primary outcome between the treatment and control groups) should not be determined by the pilot study. Rather it is determined by the minimum clinically important difference. However, the effect size from the pilot give us some ideas about possible achievable difference.

Interim Monitoring and Early Stopping

An interim analysis will be conducted once >30 patients are enrolled in the study. Analysis will focus on determining differences between NAC and control group. Interim analysis will help determine early differences between the study groups and safety profile of study intervention. The investigators may end the study early depending on study findings, safety profile of the data, and/or if data is adequate for a peer-reviewed publication.

Analysis Plan

Data analysis include (1) the partition of variation of outcome measurements into within and between patient components to obtain standard deviation of measurements, intra-class correlation of the measurements, (2) use of appropriate regression model (GEE or mixed effect regression) to compare the outcome measurements between two groups, while accounting for the intra-class correlation. Interpretation of analysis should be made within the context of a pilot study where we have a pre-specified hypothesis but do not have power analysis for testing the hypothesis.

Statistical Methods

One of the main study outcomes (and in the future large study) is the stump perfusion assessment, which is a continuous variable measured three times (D0, D3, and D5) after

treatment. Based on this longitudinal repeated measurement study design, the preliminary data needed for designing future study include: (1) variance of single measurement, (2) covariance (correlation) among the repeated measurements over time within an individual, (3) level of outcome measurements in patients with and without the treatment.

Missing Outcome Data

Not applicable

Unblinding Procedures

The BJH Investigational Pharmacy will maintain a record of each patient's medical record number and to which arm of the study they were assigned. No member of the vascular surgery investigational team will have access to this information. This will serve to maintain blinding and reduce any unwanted during the trial and subsequent analysis of collected data. Un-blinding of patient data will occur during the interim analysis, and at the end of the study. For un-blinding the pharmacy will then un-blind the study to the investigators such that appropriate conclusions can be made regarding the efficacy of the investigational agent.

Data Handling and Record Keeping

Confidentiality and Security

All patient data and demographic information will be analyzed on computers on the Washington University in St. Louis (WUSTL) network. This network is only accessible through appropriately credentialed employees, physicians, and Washington University of St. Louis medical students. Additionally, data is stored on the WUSTL network drive, with separate encryption levels, only accessible by users granted permissions by the appropriate administrator. This prevents unauthorized users, i.e. those not immediately involved in specific projects, from gaining access to patient sensitive information.

Samples and/or data from this research may be shared with other researchers for future projects. All requests, inclusive of proof of review and approval by the Institutional Review Board as applicable, from potential research collaborators will be reviewed on a case by case basis, in writing, by the Principal Investigator(s) prior to the release of samples/data. The data and/or samples approved for release will then be provided to other investigators and will be de-identified (coded). Only the primary NAC study team members have access to the password-protected link to protect patient confidentiality.

Training

All individuals responsible for handling of patient information and/or the consenting process for this clinical trial have been approved and received appropriate training in line with the standards set for by the Washington University in St. Louis Office of the Vice Chancellor for Research. These individuals may include clinical trial nurse coordinators, clinical nurses, clinical fellows, research fellows, and vascular surgery attendings.

Case Report Forms and Source Documents

N/A

Records Retention

Records will be maintained for at least 7 years following the close of this study.

Study Monitoring

Study Monitoring Plan

Monitoring will be used as a quality control tool to be sure that the study is being conducted as planned and as approved by the institutional IRB. Monitoring activities will include regular communication between the PI and study staff; review of the study processes, procedures, and records; assurance of protocol compliance and verification of the accuracy of data collected by the study team. All data collected for the study will have a reliable source document to verify the source of the data. Review of the data for each enrolled subject will be performed on an ongoing basis by the study team (including participant recruitment, accrual and retention, review of documentation of proper informed consent, source document verification, adverse events identification and reporting activities). Conclusions of the ongoing monitoring activities will be reported to the PI. Ongoing monitoring findings will be evaluated to determine whether additional actions (e.g., training of clinical investigator and site staff, clarification of protocol requirements) are necessary to ensure human subject protection and data quality.

Any incidence of protocol noncompliance and all unanticipated problems will be reported to the IRB in accordance with local policy. The study Principal Investigator will perform continuous monitoring of participant safety based on regular review of adverse events captured in the clinical trial.

The PI will ensure monitoring of the conduct and progress of the study that ensures:
1) important information that may affect the safety or welfare of participants comes to light and is acted upon as quickly as possible, and 2) the validity and integrity of the data. The PI will determine based on ongoing safety review whether the study should continue as planned or should be modified/amended or stopped for safety concerns.

Study Administration

Organization and Participating Centers

The study will be performed at the Washington University School of Medicine-affiliated Barnes Jewish Hospital.

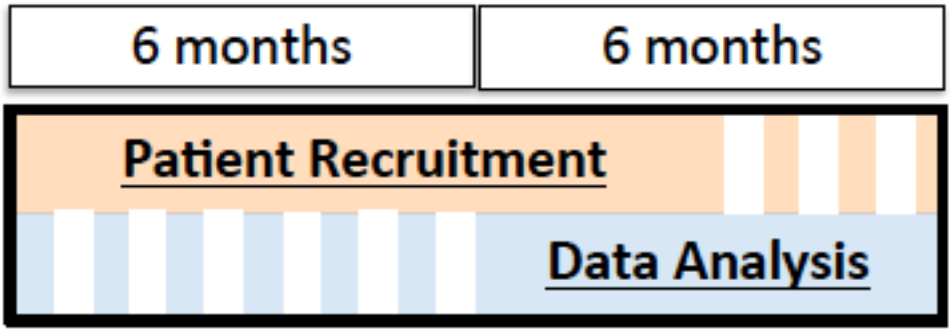
Funding Source and Conflicts of Interest

The study is funded by a Pilot and Feasibility Grant from the Diabetes Research Center. This is an NIH/NIDDK sponsored grant (P30 DK020579)

Committees
N/A

Subject Stipends or Payments
No subject stipends or payments will be offered or available for this clinical study

Study Timetable



Publication Plan

We plan to make clinical trial findings public and aim to publish the study findings in a peer-reviewed scientific journal.

Attachments

Tables
N/A

Informed consent documents
See attachment

Patient education brochures
N/A

Special procedures protocols
N/A

Questionnaires or surveys
N/A

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STATISTICAL ANALYSIS PLAN

Protocol Title: N-Acetyl-Cysteine for Healing of Amputation Stumps in the Setting of Diabetes

NCT03253328

Protocol Version 11.0
Date: March 26, 2020

Statistical Plan

Sample Size Determination and Power

The purpose of this study is to obtain sufficient preliminary data to determine the sample size necessary for a future large definitive clinical trial. For this pilot study, we plan to enroll 25 patients in each group, allowing for a dropout rate of 10% per group.

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Data analysis include (1) the partition of variation of outcome measurements into within and between patient components to obtain standard deviation of measurements, intra-class correlation of the measurements, (2) use of appropriate regression model (GEE or mixed effect regression) to compare the outcome measurements between two groups, while accounting for the intra-class correlation. Interpretation of analysis should be made within the context of a pilot study where we have a pre-specified hypothesis but do not have power analysis for testing the hypothesis.

Statistical Methods

One of the main study outcomes (and in the future large study) is the stump perfusion assessment, which is a continuous variable measured three times (D0, D3, and D5) after treatment. Based on this longitudinal repeated measurement study design, the preliminary data needed for designing future study include: (1) variance of single measurement, (2) covariance (correlation) among the repeated measurements over time within an individual, (3) level of outcome measurements in patients with and without the treatment.

Missing Outcome Data

Not applicable

Unblinding Procedures

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CONSENT DOCUMENT

Protocol Title: N-Acetyl-Cysteine for Healing of Amputation Stumps in the Setting of Diabetes

NCT03253328

Protocol Version 11.0
Date: March 26, 2020

Washington University in St. Louis

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INFORMED CONSENT DOCUMENT

Project Title: N-Acetyl-Cysteine for Healing of Amputation Stumps in the Setting of Diabetes

Principal Investigator: Mohamed Zayed, M.D. Ph.D.

Research Team Contact: Ashley Burgess, RN, BSN (314) 362-6257
Julie Wilson, RN (314) 747-7828
Vanetta Worthy, BS (314) 273-0872

If you are the legally authorized representative of a person who is being invited to participate in this study, the word "you" in this document refers to the person you represent. As the legally authorized representative, you will be asked to read and sign this document to give permission for the person you represent to participate in this research study.

This consent form describes the research study and helps you decide if you want to participate. It provides important information about what you will be asked to do during the study, about the risks and benefits of the study, and about your rights and responsibilities as a research participant. By signing this form you are agreeing to participate in this study.

- You should read and understand the information in this document including the procedures, risks and potential benefits.
- If you have questions about anything in this form, you should ask the research team for more information before you agree to participate.
- You may also wish to talk to your family or friends about your participation in this study.
- Do not agree to participate in this study unless the research team has answered your questions and you decide that you want to be part of this study.

WHAT IS THE PURPOSE OF THIS STUDY?

This is a research study. We invite you to participate in this research study because you are scheduled to undergo elective above-knee or below-knee amputation due to critical limb ischemia (CLI), with and without diabetes mellitus.

Diabetes is a significant factor for peripheral arterial disease (PAD) and critical limb ischemia (CLI). PAD is caused by atherosclerosis, the hardening and narrowing of the arteries, over time due to the buildup of fatty deposits called plaque. Critical limb ischemia (CLI) is a severe form of PAD, and is a chronic condition that results in severe pain in the feet or toes, even while resting. Complications of poor circulation can include sores and wounds that won't heal in the legs and feet.

The purpose of this research study is to evaluate the effectiveness and safety of using a drug called, N-

Acetyl-Cysteine (NAC) in the setting of PAD as a tissue augmenting agent in patients undergoing lower limb amputation with and without diabetes mellitus. NAC is approved by the U.S. Food and Drug Administration for acetaminophen (Tylenol) overdose, carbon monoxide poisoning, and multiple other uses including as a medication to thin mucous in respiratory conditions such as cystic fibrosis or bronchitis. NAC is considered investigational for its use in this study which means that it has not been approved by the U.S. Food and Drug Administration. This is a Pilot Clinical Trial study designed to establish if there is any benefit in using NAC for patients undergoing lower limb amputation with respect to improving stump circulation and perfusion (increase blood flow to the extremity), improving wound healing and serum lipidomics. Lipidomics is the study of the variety of fatty molecules in the body, their cellular and extracellular functions and interactions, and the diseases to which they contribute. We are evaluating NAC because prior research studies have shown it to be effective in improving wound healing.

This study will only be conducted at Washington University/Barnes-Jewish Hospital.

WHAT WILL HAPPEN DURING THIS STUDY?

In this study, you will have your above-knee or below-knee amputation surgical procedure according to standard care. This study will not change your surgery or how it is performed. Research procedures will occur during your recovery period after surgery.

We will recruit at least 50 participants: 25 patients will receive the study drug, NAC, at a standard, commonly used dose of 1200mg intravenously (IV) twice a day for 6 consecutive days after surgery (treatment group). The other 25 participants will receive placebo saline intravenous (IV) infusion twice a day for 6 consecutive days after surgery (control group). You will be randomly chosen, like the flip of a coin, to one of these two groups. Neither you nor the study doctor will know to which group you will be assigned.

After informed consent is obtained, a baseline clinical evaluation will be performed including lower extremity anthropometric evaluation, medical history and vital signs documentation. Anthropometric evaluation is the measurement of the size and proportion of your amputated stump.

As part of your standard care you will also have blood draws to assess laboratory values for a complete blood count (CBC) and a basic metabolic panel. As part of standard of care, you will have an HbA1C drawn if it has not been checked within 3 months and a pre-albumin level if it has not been checked within 1 week prior to amputation. These tests are done as standard care and will be done whether you participate in this study or not.

In addition, for research purposes, a sample of blood, approximately 3-4 teaspoons, will be taken within 12 hours of the amputation operation, if possible and another sample will be taken within 12 hours prior to discharge from the hospital, or by Day 5, if possible. A portion of your blood sample will be used to obtain laboratory evaluations of your serum insulin level, serum thiol level, serum L-cysteine levels,

serum lipidomics, serum nicotine and cotinine levels, and other essential plasma serum proteins or biomarkers.

Non-invasive Laser Assisted Fluorescence Angiography (LAFA)

We will assess blood circulation and wound healing in your stump by using a special angiogram. A dye, ICG, will be injected through your IV that will allow us to see blood flow and allow us to take images. You will be asked to lie still for about 10 minutes.

This imaging will occur at three (3) time points: in the operating room after your amputation, at your bedside on Day 3, and on Day 5 at your bedside if you are still in the hospital. If we are unable to obtain the image in the operating room, your study participation will end however you will continue to receive your standard medical care.

Measurement of Transcutaneous Oxygen Pressure (TcPO2)

This procedure assesses the amount of oxygen that reaches your skin through blood circulation. We will place three (3) adhesive sensors on your stump and one (1) adhesive sensor on your chest. Each sensor contains an electrode that can sense oxygen flow. This procedure will take about 15 minutes.

This procedure will be performed at three (3) time points: obtained prior to the operation, if possible, at your bedside on Day 3 if possible, and on Day 5 if possible, at your bedside if you are still in the hospital.

Throughout your hospitalization, we will collect information about your medical condition such as vital signs, changes in health (adverse events), medication use and amputated stump measurement information.

Following discharge, your amputated stump will be evaluated within 20-30 days during a routine postoperative visit. At this time we will collect information including records describing stump healing or care, any changes in your health, your temperature, blood pressure, heart rate, breathing rate and blood oxygen level as part of the research study.

Will you save my samples or research data to use in future research studies?

As part of this study, we are obtaining blood sample(s) and/or data from you. We would like to use the blood sample(s) and/or data for studies going on right now as well as studies that are conducted in the future. These studies may provide additional information that will be helpful in understanding peripheral arterial disease (PAD) and critical limb ischemia (CLI), or other diseases or conditions, including research to develop investigational tests, treatments, drugs or devices that are not yet approved

by the U.S. Food and Drug Administration. It is unlikely that what we learn from these studies will have a direct benefit to you. There are no plans to provide financial compensation to you should this occur. By allowing us to use your blood sample(s) and/or data you give up any property rights you may have in the blood sample(s) and/or data.

We will share your blood sample(s) and/or data with other researchers. They may be doing research in areas similar to this research or in other unrelated areas. These researchers may be at Washington University, at other research centers and institutions, or industry sponsors of research. We may also share your research data with large data repositories (a repository is a database of information) for broad sharing with the research community. If your individual research data is placed in one of these repositories only qualified researchers, who have received prior approval from individuals that monitor the use of the data, will be able to look at your information.

If you change your mind and do not want us to store and use your blood sample(s) and/or data for future research you should contact the research team member identified at the top of this document. The blood sample(s) and/or data will no longer be used for research purposes. However, if some research with your blood sample(s) and/or data has already been completed, the information from that research may still be used. Also, if the blood sample(s) and/or data has been shared with other researchers it might not be possible to withdraw the blood sample(s) and/or data to the extent it has been shared.

Please place your initials in the blank next to Yes or No for each of the questions below:

My blood sample(s) and/or data may be stored and used for future research as described above.

_____ Yes **_____ No**
Initials **Initials**

My blood sample(s) and/or data may be shared with other researchers and used by these researchers for the future research as described above.

_____ Yes **_____ No**
Initials **Initials**

In addition, you may be randomly chosen, like the flip of a coin, to have a magnetic resonance image performed on your stump to check blood flow and oxygen (for perfusion assessments). Neither the PI nor the study staff will know if you are from the group who will receive NAC or placebo. This MR imaging will take approximately 45 – 60 minutes and you will be asked to lie down on a narrow bed in the MRI scanner. An MRI scanner is a large donut-shaped machine that is used by thousands of hospitals to make pictures of various parts of the body. The MRI scanner does not expose you to ionizing radiation (x-rays). You will be given ear plugs to dampen the loud noise the scanner makes when taking pictures. You will be given a squeeze ball which can activate an alarm at the scanner operator's console so you can let the operator know you have a question or need assistance.

HOW MANY PEOPLE WILL PARTICIPATE?

At least 50 people will take part in this study conducted by investigators at Washington University.

HOW LONG WILL I BE IN THIS STUDY?

If you agree to take part in this study, your participation will last for approximately 30 days following your surgery.

WHAT ARE THE RISKS OF THIS STUDY?

You may experience one or more of the risks indicated below from being in this study. In addition to these, there may be other unknown risks, or risks that we did not anticipate, associated with being in this study.

Some risks described in this consent document, if severe, may cause death.

Risks of N-Acetyl-Cysteine (NAC)

Rare

Life Threatening

- Anaphylaxis (severe allergic) reaction to NAC

Serious

- Wheezing
- Tightness in the chest
- Difficulty breathing

Mild

- Rash
- Urticaria (hives)
- Pruritus (itching) reaction
- Infection at the site of the injection
- Venous thrombosis (clot)
- Phlebitis (inflammation of a vein) at the site of the IV
- Chance leakage of saline fluid at the site of the injection

Concurrent use of NAC and nitroglycerin may result in enhanced hypotension (low blood pressure) and nitroglycerin-induced headache. If you are on nitroglycerin, notify your doctor if you feel light headed or weak as this may be a sign of low blood pressure. Nitroglycerin is known to cause headaches but if your headache becomes worse notify your doctor. Adjustments to your medications may need to be made.

Risk of Saline Infusion

Rare

Mild

- Infection at the site of the injection
- Venous thrombosis (clot)
- Phlebitis (inflammation of a vein) at the site of the IV
- Chance leakage of saline fluid at the site of the injection

Risk of Indocyanine Green (ICG) for Laser Assisted Fluorescence Angiography (LAFA)

Rare

Life Threatening

- Anaphylaxis (severe allergic) reaction to ICG

Serious

- Difficulty breathing

Mild

- Urticarial (hives) reaction to ICG
- Flushing
- Itching

Risks associated with the blood draw may include:

Likely:

Mild

- Pain at the needle stick site

Less Likely / Less Common

Mild

- The blood draw may cause bleeding or bruising.

Rare

Serious

- Some people become dizzy or feel faint.
- There is also a rare risk of infection

Risks associated with MRI scan:

MRI

You may be uncomfortable inside the MRI scanner if you do not like ot be in closed spaces ("claustrophobia"). During the procedure, you will be able to talk with the MRI staff through a speaker system. You can tell them to stop the scan at any time.

The MRI scanner produces a loud hammering noise, which has caused hearing loss in a very small number of patients. You will be given earplugs to reduce this risk.

There is a risk of burns that could be serious.

If you have a device such as a pacemaker, bone hardware, or device placed in your uterus there may be additional risks. We will review what device you have and inform you of these risks. In general, these risks could be:

- Heating or movement of the device
- Device malfunction
- Damage to the tissue that surrounds the device

If you have a skin tattoo, including cosmetic tattoos (eye-liner, lip-liner) you could experience the following:

- Irritation, swelling or heating in the area of the tattoo(s)
- In rare instances a primary or secondary burn

If you have a tattoo we will offer you a cold wet washcloth to put over the tattoo to reduce this risk.

Breach of Confidentiality
One risk of participating in this study is that confidential information about you may be accidentally disclosed. We will use our best efforts to keep the information about you secure. Please see the section in this consent form titled *"How will you keep my information confidential?"* for more information.

WHAT ARE THE BENEFITS OF THIS STUDY?

You may or may not benefit from being in this study.

However, we hope that, in the future, other people might benefit from this study because information collected from this clinical trial may benefit a larger number of patients with critical limb ischemia (CLI) with and without diabetes who are at risk of poor amputation stump healing.

WHAT OTHER TREATMENT OPTIONS ARE THERE?

Before you decide whether or not to be in this study, your doctor will discuss the other options that are available to you. You do not have to participate in this study. If you choose not to participate, you will not receive NAC. Your decision will not impact your standard care.

WILL IT COST ME ANYTHING TO BE IN THIS STUDY?

As part of this study you will receive tests and procedures that are similar to what you would receive during routine clinical care of your condition. Your health plan/insurance company will be billed for some or all of these costs, and you will be responsible for any co-pays and deductibles that are normally required by your health plan/insurance. Not all insurance plans cover the costs associated with being in a study. Even if they do, you may be responsible for more out-of-pocket expenses, such as co-pays and

deductibles, when there are more tests and procedures or more expensive tests and procedures involved in the study than if you were to receive routine clinical care outside the study.

If you wish to know whether there are more tests and procedures or more expensive tests and procedures in the study, you should ask your study doctor.

If you wish to know whether your insurance will pay, you should contact them directly, or speak with the study team about obtaining a financial pre-certification prior to enrolling in the study.

You will not have any costs associated with the MR imaging.

WILL I BE PAID FOR PARTICIPATING?

You will not be paid for being in this research study.

WHO IS FUNDING THIS STUDY?

The University and the research team are not receiving payment from other agencies, organizations, or companies to conduct this research study.

WHAT IF I AM INJURED AS A RESULT OF THIS STUDY?

Washington University investigators and staff will try to reduce, control, and treat any complications from this research. If you feel you are injured because of the study, please contact the investigator at (314) 362-5707 and/or the Human Research Protection Office at 1-(800)-438-0445.

Decisions about whether payment for medical treatment for injuries relating to your participation in research will be made by Washington University. If you need to seek medical care for a research-related injury, please notify the investigator as soon as possible.

HOW WILL YOU KEEP MY INFORMATION CONFIDENTIAL?

We will keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people such as those indicated below may become aware of your participation in this study and may inspect and copy records pertaining to this research. Some of these records could contain information that personally identifies you.

- Government representatives, (including the Office for Human Research Protections) to complete federal or state responsibilities
- The U.S. Food and Drug Administration

- Your primary care physician if a medical condition that needs urgent attention is discovered
- Hospital or University representatives, to complete Hospital or University responsibilities
- The last four digits of your social security number may be used in hospital or University systems to track billing information for research procedures
- Information about your participation in this study may be documented in your health care records and be available to your health care providers who are not part of the research team.
- Washington University's Institutional Review Board (a committee that oversees the conduct of research involving human participants) and the Human Research Protection Office. The Institutional Review Board has reviewed and approved this study.

To help protect your confidentiality, we will only collect data as it pertains to this study. You will be assigned a study code and your data will be collected using the study code. All of your study records will be maintained by the study staff, kept in a locked file within a locked office, and access will be limited to only those on the study team. We will maintain an electronic enrollment log that will contain identifiable information along with your specific study code. This electronic enrollment log will be stored behind a firewall on a Washington University server. All computers are password protected and only those on the research team will have access to this electronic data. Blood samples collected as part of the research will be labeled with your study code and will be maintained in a locked -80 degree celsius freezer contained in the study investigator's laboratory. If we write a report or article about this study or share the study data set with others, we will do so in such a way that you cannot be directly identified.

This consent form or similar documentation that you are participating in a research study will be included in your health care record. Anyone with access to your health care record, including your health insurance company will be able to see that you are participating in a research study.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

Are there additional protections for my health information?

Protected Health Information (PHI) is health information that identifies you. PHI is protected by federal law under HIPAA (the Health Insurance Portability and Accountability Act). To take part in this research, you must give the research team permission to use and disclose (share) your PHI for the study as explained in this consent form. The research team will follow state and federal laws and may share your health information with the agencies and people listed under the previous section titled, "How will you keep my information confidential?"

Once your health information is shared with someone outside of the research team, it may no longer be protected by HIPAA.

The research team will only use and share your information as talked about in this form or as permitted

or required by law. When possible, the research team will make sure information cannot be linked to you (de-identified). Once information is de-identified, it may be used and shared for other purposes not discussed in this consent form. If you have questions or concerns about your privacy and the use of your PHI, please contact the University's Privacy Officer at 866-747-4975.

Although you will not be allowed to see the study information, you may be given access to your health care records by contacting your health care provider.

If you decide not to sign this form, it will not affect

- your treatment or the care given by your health provider.
- your insurance payment or enrollment in any health plans.
- any benefits to which you are entitled.

However, it will not be possible for you to take part in the study.

If you sign this form:

- You authorize the use of your PHI for this research
- This authorization does not expire.
- You may later change your mind and not let the research team use or share your information (you may revoke your authorization).
- To revoke your authorization, complete the withdrawal letter, found in the Participant section of the Human Research Protection Office website at https://hrpo.wustl.edu/participants/withdrawing-from-a-study/ or you may request that the investigator send you a copy of the letter.
    - **If you revoke your authorization:**
        - The research team may only use and share information already collected for the study.
        - Your information may still be used and shared as necessary to maintain the integrity of the research, for example, to account for a participant's withdrawal from the research study or for safety reasons.
        - You will not be allowed to continue to participate in the study.

IS BEING IN THIS STUDY VOLUNTARY?

Taking part in this research study is completely voluntary. You may choose not to take part at all. If you decide to be in this study, you may stop participating at any time. Any data that was collected as part of your participation in the study will remain as part of the study records and cannot be removed.

If you decide not to be in this study, or if you stop participating at any time, you won't be penalized or lose any benefits for which you otherwise qualify.

What if I decide to withdraw from the study?

You may withdraw by telling the study team you are no longer interested in participating in the study or

you may send in a withdrawal letter. A sample withdrawal letter can be found at https://hrpo.wustl.edu/participants/withdrawing-from-a-study/under Withdrawing from a Research Study.

Will I receive new information about the study while participating?

If we obtain any new information during this study that might affect your willingness to continue participating in the study, we'll promptly provide you with that information.

Can someone else end my participation in this study?

Under certain circumstances, the investigator might decide to end your participation in this research study earlier than planned. This might happen for no reason, or if you are found in the judgement of the investigation team to be developing a side effect or adverse reaction to the medication. The study may also be terminated if funding for the research study has ended.

WHAT IF I HAVE QUESTIONS?

We encourage you to ask questions. If you have any questions about the research study itself, please contact: **Dr. Zayed at (314) 362-5648 or a study team member at (314) 362-5707**. If you experience a research-related injury, please contact: **Dr. Zayed at (314) 362-5648 or a study team member at (314) 362-5707.**

If you have questions, concerns, or complaints about your rights as a research participant, please contact the Human Research Protection Office at 660 South Euclid Avenue, Campus Box 8089, St. Louis, MO 63110, 1-(800)-438-0445, or email hrpo@wustl.edu. General information about being a research participant can be found on the Human Research Protection Office web site, hrpo.wustl.edu. To offer input about your experiences as a research participant or to speak to someone other than the research staff, call the Human Research Protection Office at the number above.

This consent form is not a contract. It is a written explanation of what will happen during the study if you decide to participate. You are not waiving any legal rights by agreeing to participate in this study. As a participant you have rights and responsibilities as described in this document and including:

- To be given enough time before signing below to weigh the risks and potential benefits and decide if you want to participate without any pressure from the research team or others.
- To understand all of the information included in the document, have your questions answered, and receive an explanation of anything you do not understand.
- To follow the procedures described in this document and the instructions of the research team to the best of your ability unless you choose to stop your participation in the research study.
- To give the research team accurate and complete information.
- To tell the research team promptly about any problems you have related to your participation, or

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if you are unable to continue and wish to stop participating in the research study.
Your signature indicates that this research study has been explained to you, that your questions have been answered, and that you agree to take part in this study. You will receive a signed and dated copy of this form.

Do not sign this form if today's date is after $STAMP_EXP_DT.

_____ _____

(Signature of Participant) (Date)

_____

(Participant's name – printed)

Legally Authorized Representative's Name and Relationship to Participant:

Do not sign this form if today's date is after $STAMP_EXP_DT.

_____

(Participant's name – printed)

_____ _____

(Signature of Legally Authorized Representative) (Date)

_____ _____

(Name of Legally Authorized Representative - printed) (Relationship to Participant – printed)

Who should sign as the Legally Authorized Representative (LAR)?
If the participant has a legal guardian or attorney-in-fact this individual must sign as the LAR.

If there is no legal guardian or attorney-in-fact the individuals listed below may sign in order of priority.

(1) Spouse unless the participant has no spouse, or is separated, or the spouse is physically or mentally incapable of giving consent, or the spouse's whereabouts is unknown or the spouse is overseas;
(2) Adult child;
(3) Parent;
(4) Brother or sister;

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(5) Relative by blood or marriage.

Statement of Person Who Obtained Consent

The information in this document has been discussed with the participant or, where appropriate, with the participant's legally authorized representative. The participant has indicated that they understand the risks, benefits, and procedures involved with participation in this research study.

_____ _____

(Signature of Person who Obtained Consent) (Date)

_____

(Name of Person who Obtained Consent - printed)

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