**CLINICAL STUDY PROTOCOL**

**Safety and Efficacy of Allogeneic MSCs in Preventing Wound Complications in Amputation**

**Protocol #: 1505714405**

**Indiana University School of Medicine 1801 N. Senate Blvd.**

**MPC2, #3500**

**Indianapolis, IN 46202**

**17-FEB-2023**

**ETHICS AND REGULATORY COMPLIANCE STATEMENT**

The procedures set forth in this protocol are designed to ensure that the sponsor(s) and principal investigator(s) abide by the International Conference on Harmonization (ICH) current Good Clinical Practice (cGCP) guidelines, current Good Laboratory Practice (cGLP) guidelines, the Declaration of Helsinki, and applicable local regulatory requirements and laws in the conduct, evaluation, and documentation of this study.

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| **Protocol Name** | A Clinical and Histological Analysis of Mesenchymal Stem Cells in Amputation |
| **Investigational Product Name** | Allogeneic mesenchymal stem cells |
| **Author** | Michael P. Murphy, MD |

**PROTOCOL HISTORY**

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CONTACT INFORMATION

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| **Sponsor**  Indiana University School of Medicine | **Primary Study Contact, Principal Investigator**    Michael P. Murphy, MD |
| **Protocol Author:** Michael P. Murphy, MD | |
| **Coordinating Investigator and Principal Investigators**  An updated list of Principal Investigators (PI), investigation sites, and institutions will be maintained separately. The definitive list will be provided in the clinical study report. | |

**PROTOCOL SIGNATURE PAGE**

**A Clinical and Histological Analysis of Mesenchymal Stem Cells in Amputation**

As an Investigator for this Study, I have read the Clinical Trial Protocol. I agree to make available to the Sponsor, Indiana University School of Medicine (or its designee), original source documents and all regulatory documents pertaining to this Study. I agree to cooperate fully with the Sponsor with the conduct of study-related audits.

By my signature below, I agree to conduct this Study in accordance with the Clinical Trial Protocol, current Good Clinical Practice (cGCP) and Good Laboratory Practice (cGLP) guidelines, obligations as set forth in Title 21 CFR Parts 812, 54, 5,6 and 11 (as applicable), and any applicable regulatory laws. I will make no changes to protocol-defined procedures without written permission from the Sponsor.

I understand that Investigational Use Products may be used **only** for the purposes explicitly described in this protocol.

I further agree to treat the results of this Study as confidential information and will not submit the results of the Study for publication without prior written authorization from the Sponsor.

Michael P. Murphy



17 February 2023

PRINTED NAME SIGNATURE DATE

## SYNOPSIS

|  |  |
| --- | --- |
| **Title of Study** | A Clinical and Histological Analysis of Mesenchymal Stem Cells in Amputation |
| **Objectives** | Determine the safety and explore efficacy of allogeneic Mesenchymal Stem Cells (MSCs) in preventing wound complications in major lower extremity amputations.  Define the retention of allogeneic MSCs in human skeletal muscle and examine the role of MSCs in recruiting proangiogenic hematopoietic cells into sites of ischemia.  Determine the effects of chronic limb threatening ischemia and diabetes on nerve function and to assess the effects of mesenchymal stem cell administration on their function. |
| **Planned Number of Subjects and Duration of Involvement** | Up to 26 active subjects, plus 3 sham procedures, to equal up to 29 active subjects  Up to 46 observation group 1 subjects,  Up to 20 observation group 2 subjects,  Up to 20 observation group 3 subjects,  Up to 10 healthy control group 4 subjects,  = Up to 125 total subjects  Duration of up to 24 weeks for Active Group (including sham group) and Observation Group 1.  No follow-up period in Observation Groups 2-4. |
| **Patient Population** | Critical limb threatening ischemia (CLTI) patients |
| **Investigational Product Name** | Allogeneic mesenchymal stem cells |
| **Methodology Overview**  **Objectives Overview** | This will be Phase I single center open label trial study that will enroll up to twenty-six (26) **Active Group** patients requiring semi-elective major lower extremity amputation within a 30-day period for complications related to CLTI. After enrollment, eligible **Active Group** patients will be enrolled into one of four arms. **Active Group Arm 1** will include up to 3 HLA A2- subjects who will undergo below knee amputation (BKA) at Day 3 after MSC injections. **Active Group Arm 2** will include up to 3 subjects who will undergo a sham treatment procedure 7 days prior to above knee amputation (AKA). **Active Group Arm 3** will include up to 3 (HLA A2+/-) subjects who will undergo a BKA at Day 14 after MSC injections. **Active Group Arm 4** will include up to four (4) HLA A2+ subjects who will undergo BKA at Day 21 after MSC injections.  Additionally, we will consent up to forty-six (46) subjects who have screen failed or decline participation in the Active Group (MSC injection treatment group) and whom we will observe after amputation. These subjects will serve in **Observation Group 1** where tissue will be collected at amputation day and wound healing assessment data will be collected at Day 3 (+2 days) after amputation, and at Week 2 (+/- 2 days), Week 6 (+/- 4 days), Week 12 (+/-7 days), Week 18 (+/- 2 weeks), and Week 24 (+/- 2 weeks).  **Observation Group 2** will be comprised of up to twenty (20) subjects where tissue and data will be collected at the time of amputation with no follow-up period.  **Observation Group 3** will include up to twenty (20) subjects undergoing a lower extremity artery bypass procedure where muscle tissue will be collected at regions near the bypass graft. There is no follow-up period for Observation Group 3.  **Control Group 4** will include “healthy” control subjects where we will collect muscle tissue via core needle biopsy during their standard of care surgical procedure or clinic visit to serve as control in up to ten (10) subjects. Group 4 subject participation will end after a one-week post-biopsy telephone call.  **Determine the safety and explore efficacy of allogeneic MSCs in preventing wound complications in amputations**. Allogeneic MSCs will be injected in the gastrocnemius muscle of up to twenty-six patients undergoing below knee amputation. Through a review of treatment related adverse events over 6 months we will test the hypothesis that allogeneic MSCs do not result in significant cardiovascular, respiratory, or infectious treatment related adverse events. Through an exploratory investigation, we will assess the efficacy of MSCs in promoting freedom from gangrene, revision of major amputation, and death after major amputation of the lower extremity. |
|  | **Define the retention of allogeneic MSCs in human skeletal muscle and examine the role of MSCs in recruiting proangiogenic hematopoietic cells into sites of ischemia.** Concurrent with injection of allogeneic MSCs above the point of amputation, we will inject allogeneic MSCs into the anterior tibialis muscle (ATM). After MSC injection, patients will be enrolled to undergo below knee amputation at Day 3, Day 14, or Day 21 after MSC injections. A sham treatment procedure is also included in the Active Group where up to 3 subjects will undergo a sham treatment procedure, with no injection of investigational product treatment. Skeletal muscle tissue from MSC injection sites in the ATM will be collected at the time of amputation and analyzed with gene and protein arrays, immunohistochemical (IHC) staining, and multiparametric flow cytometry. The gene and protein expression profiles and histological findings will be used to test the hypotheses that (1) MSCs have limited survival post-injection and (2) act to recruit CD34+CD133+ proangiogenic hematopoietic cells.  **Determine the effects of chronic limb ischemia and diabetes on nerve function and to assess the effects of mesenchymal stem cell administration on their function.** In the Active Group, Observation Group 1 and Observation Group 2, peripheral nerve tissue (sciatic, femoral, tibial, sural and/or plantar nerve segments) will be collected at the time of amputation. Patients with critical limb threatening ischemia, particularly with diabetes, have polyneuropathies that alter motor unit engagement and peripheral sensation leading to ambulatory dysfunction and pressure ulceration of the foot. |

**Abbreviations**

ABI Ankle-Brachial Index

ABMNC Autologous Bone Marrow Mononuclear Cells

AGA1 Active Group Arm 1

AGA2 Active Group Arm 2

AGA3 Active Group Arm 3

AGA4 Active Group Arm 4

AKA Above Knee Amputation

ATM Anterior tibialis muscle

BKA Below Knee Amputation

CLTI Critical Limb Threatening Ischemia

DFU Diabetic Foot Ulcer

FISH Fluorescent in situ hybridization HIF-1α Hypoxia Inducible Factor-1α HLA Human Leukocyte Antigen

ICA Indocyanine angiography

IHC Immunohistochemical staining

IM Intramuscular

LC-MS Liquid Chromatography- Mass Spectrometry MHC Major Histocompatibility Complex

MSC Mesenchymal Stromal Cell

OG1 Observation Group 1

OG2 Observation Group 2

OG3 Observation Group 3

OG4 Observation Group 4

PAD Peripheral Arterial Disease

PHC Proangiogenic Hematopoietic Cell POHS Protection of Human Subjects

RT-PCR Real-time reverse-transcription polymerase chain reaction SDF-1 Stromal Cell Derived factor-1

TcPO2 Transcutaneous oxygen pressure US Ultrasound

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2. INTRODUCTION

The incidence of critical limb ischemia (CLTI) in Western societies is approximately 220 new cases per million per year, and with a progressively aging population, persistent rates of tobacco abuse, and an increase in diabetes, a steady growth of the population at risk is expected.13 Although there have been significant improvements in restoring perfusion in the critically ischemic limb over the past two decades, below knee amputation (BKA) rates for complications related to CLTI have consistently remained between 9 and 12.5 per 100,000 population since 1990.7 A major complication of BKA is ischemic necrosis of the myocutaneous flap created to cover the terminal portion of the tibia. The need to perform a more proximal above knee amputation (AKA) due to gangrene occurs in up to 19% of patients within 4 months of BKA.8,9 The physiological consequences of loss of the knee joint with AKA is a 40-60% increase in energy expenditure in ambulation with a prosthesis.12 The social consequences of AKA are that patients have restricted mobility and are unable to complete their activities of daily living independently.8 Long term institutionalization rates for AKA patients are three times greater than BKA and are associated with a significantly increased incidence of sacral decubitus ulceration, urinary, and respiratory tract infections.10 This study will provide a novel therapeutic strategy that may prevent ischemic wound complications after BKA and subsequently prevent conversion to AKA. The result will have a dramatic impact on health care costs, morbidity, and quality of life for this unfortunate patient population.

The proposed research will assess the safety and explore efficacy of a more therapeutically potent and clinically feasible cell population for CLTI**.** The Therapeutic Angiogenesis using Cell Transplantation (TACT) Trial demonstrated that IM injection of autologous bone marrow mononuclear cells (ABMNCs) into critically ischemic legs increased perfusion with significant improvement in ankle-brachial indices, transcutaneous oxygen pressures , and rest pain.32 Subsequent to this vanguard study there have been multiple Phase I/II clinical reports on the efficacy of ABMNCs, including our own, in patients with CLTI.13,33-37 Although these small early phase studies report promising results, there are significant limitations to an autologous cell approach in the CLTI population. First, isolation of autologous bone marrow or adipose derived cells requires a harvesting procedure under anesthesia which places patients with CLTI, usually of advanced age and with multiple comorbidities, at risk. Second, autologous stem cells from patients with cardiovascular disease have demonstrated limited potency in models of neovascularization and experimental stroke.14-16 We will perform a comprehensive assessment of the safety of allogeneic MSCs, derived from healthy donors, that mitigates tissue collection from CLTI patients. We will assess the efficacy of allogeneic MSCs in preventing wound complications after lower extremity major amputation. This investigation will deliver a more potent and clinically feasible stem cell source, as well as provide preliminary evidence that MSCs may have further applications in diabetic and non-diabetic foot wounds, CLTI, and claudication.

Initial interest in the utility of MSCs for cardiovascular disease focused on the hypothesis that MSCs could engraft, differentiate, and replace damaged tissue. Subsequent research indicates that MSC engraftment actually proceeds at low levels with rates < 0.1% of injected cells.38,39 The current paradigm is that MSCs respond to crosstalk with injured cells to limit tissue destruction or enhance repair by a variety of mechanisms that include (a) secretion of bioactive proteins that act in a paracrine or autocrine fashion; (b) upregulation of genes that modulate excessive inflammatory and immune reactions; and (c) transfer of vesicular components that contain mitochondria and microRNAs.40 Accumulating evidence supports the hypothesis that the predominant mechanisms driving tissue repair in the heart post-infarction is angiogenesis orchestrated via the release of stem-cell derived paracrine factors.41,42 These cumulative data regarding the bioactivity of MSCs has been derived entirely from animal models of disease despite the fact that more than 120 clinical trials listed in *clinicaltrials.gov* use

MSCs for therapeutic endpoints. Furthermore, the translation of this data to human conditions is limited by the genomic discordance between mouse and human responses in pathological conditions. Seoka and colleagues found that among genes that changed significantly in humans in response to inflammatory stresses, the murine orthologs were close to random in matching their human counterparts.26 To date, no clinical trials have been designed with the ability to harvest human tissue post-MSC implantation at time points relevant to their tissue reparative activity and survival. Thus the *in vivo* biological activity and survival of MSCs in diseased human tissue remains undefined.26,43 Using multi-programmatic and high throughput analyses, we will define critical mechanisms through which MSCs promote tissue repair. These discoveries will have broad application in developing more effective strategies in regenerative approaches to cardiovascular disease.

1. DESCRIPTION OF THE INVESTIGATIONAL PRODUCT
   1. Overview

Human MSC induce angiogenesis, decrease muscle fiber apoptosis, and stimulate re-epithelialization of wounds in ischemic hindlimb models in mice. MSCs do not express MHC class II antigens and purportedly escape allogeneic rejection.20-22 MSCs can be isolated from healthy donors and thus present a more clinically feasible and potent cell population that may improve tissue perfusion and mitigate ischemic wound complications after major amputation.

* 1. Proposed Intended Use Statement

Allogeneic MSCs are a novel therapeutic option to prevent wound complications after major amputation. Human MSCs are intended for investigational use only by selected investigators familiar with their use and experienced in conducting clinical studies. Human MSCs may only be administered intramuscularly to human subjects participating in clinical studies sponsored/approved by Indiana University School of Medicine, and who have provided formal written consent.

1. STUDY OBJECTIVES

The principal objectives of this study are as follows:

**Determine the safety and explore efficacy of allogeneic MSCs in preventing wound complications in BKA**. In a Phase I trial, allogeneic MSCs will be injected in the proximal leg of up to twenty-six (26) patients undergoing major amputation. Through a review of treatment related adverse events over 6 months we will test the hypothesis that allogeneic MSCs do not result in significant cardiovascular, respiratory, or infectious treatment related adverse events. Through an exploratory investigation, we will assess the efficacy of MSCs in promoting freedom from gangrene, rates of revision of major amputation, and death after major amputation.

**Define the retention of allogeneic MSCs in human skeletal muscle and examine the role of MSCs in recruiting proangiogenic hematopoietic cells into sites of ischemia.**

In **Active Group Arm 1**, we will inject up to 110 million MSCs into the lower leg of up to 3 subjects with HLA A2- phenotype 3 days prior to below knee amputation to determine if MSCs are still present and to obtain an earlier analysis of the mononuclear cell infiltrates.

In **Active Group Arm 2**, we will perform a sham procedure in the lower leg of up to 3 subjects 7 days prior to above knee amputation to analyze muscle tissue shear stress due to the mechanics of needle injections.

In **Active Group Arm 3**, we will inject up to 110 million MSCs into the lower leg of up to 3 subjects with HLA A2+ or HLA A2- phenotype 14 days prior to below knee amputation to determine a later analysis of cell responses and skeletal muscle responses.

In **Active Group Arm 4**, we will inject up to 110 million MSCs into the lower leg of up to 4 subjects with HLA A2+ phenotype 21 days prior to below knee amputation to describe later phases of skeletal muscle responses.

Skeletal muscle from MSC injection sites in the ATM will be collected at the time of amputation and analyzed with gene and protein arrays, immunohistochemical (IHC) staining, and multiparametric flow cytometry. The gene and protein expression profiles and histological findings will be used to test the hypotheses that (1) MSCs have limited survival post-injection and (2) act to recruit CD34+CD133+ proangiogenic hematopoietic cells.

**Determine the effects of chronic limb ischemia and diabetes on nerve function and to assess the effects of mesenchymal stem cell administration on their function.** Patients with critical limb threatening ischemia, particularly with diabetes, have polyneuropathies that alter motor unit engagement and peripheral sensation leading to ambulatory dysfunction and pressure ulceration of the foot.

In the Active Group and Observation Group 1 and Observation Group 2, peripheral nerve tissue (sciatic, femoral, tibial, sural and/or plantar) will be collected from the amputated portion of the limb at the time of amputation.

The expected outcomes will provide the framework for the design of a Phase II randomized clinical trial and critical insights that may direct this future trial to test multi-dosing regimens, compare different cell types, or sources. This investigation will establish a unique clinical model that can expedite safety and mechanistic analyses of projects in PAD that include bioscaffolds to enhance cell survival28, combinations of cells (i.e., MSCs and endothelial progenitor cells)29,30, and induced pluripotent stem cells.31

1. STUDY OVERVIEW
   1. Study Approach

This protocol is designed to describe a Phase I single center open label trial study that will enroll up to 29 patients requiring semi-elective lower extremity major amputation for complications related to CLTI. Up to twenty-six (26) patients will be enrolled in the active group where MSC injections will occur at Day 3, Day 14, or Day 21 prior to below knee amputation. Additionally, up to three (3) patients will be enrolled to undergo a sham procedure comprising four (4) blank needle punctures into the ATM with no MSC product treatment. Subjects in the Sham Group will undergo their standard of care above knee amputation at Day 7 post-sham procedure.

Up to forty-six (46) patients undergoing BKA will consent to Observation Group 1 whom have declined participation or who are not eligible to enroll in any MSC injection treatment groups (Active Group). Follow up for this group will be up to 24 weeks, or 6 months, after BKA.

Up to twenty (20) patients undergoing BKA or AKA will consent to Observation Group 2 whom have declined participation or who are not eligible to enroll in the Active Group of this study. There will be no follow up for Observation Group 2 subjects after amputation.

Up to twenty (20) patients already scheduled for a standard of care lower extremity arterial bypass procedure will be enrolled in Observation Group 3. There will be no follow up for Observation Group 3.

Control Group 4 will comprise up to ten (10) “healthy” subjects already scheduled for a planned surgery monitored by anesthesia or who have a planned clinical appointment. Tissue will be collected at the time of surgery or clinic appointment and no follow-up will occur.

* 1. Study Duration

It is expected that the study will be completed within 5 years. Each subject in the Active Groups and Observation Group 1 will be followed for 24 weeks. Each subject in Observation Group 2, Observation Group 3, and Control Group 4 will have no follow up period after their procedure.

1. STUDY POPULATION
   1. Sample Size and Target Study Population
      1. Sample size

The total number of individual subjects in the active group is expected to reach approximately twenty-nine (29).

The total number of subjects in Observation Group 1 is expected to reach up to forty-six (46).

The total number of subjects in Observation Group 2 is expected to reach up to twenty (20).

The total number of subjects in Observation Group 3 is expected to reach up to twenty (20).

The total number of subjects in Control Group 4 is expected to reach up to ten (10).

For the entire study, the expected number of participants is expected to reach up to one-hundred twenty-five (125).

* + 1. Study population

The study population in the Active Group (including sham group subjects) will be representative of adults 40 – 90 years of age who require lower extremity major amputation, as determined by an independent vascular specialist. The study population for Observation Group 1, 2, and 3 will be representative of adults 40 - 90 years of age who require above knee amputation, below knee amputation, lower extremity bypass grafting, other surgical procedure or have a clinic appointment, as defined later in this protocol. The study population in Control Group 4 will be representative of adults 30-50 years of age who will undergo a planned surgical procedure monitored under anesthesia sedation or who have a clinic appointment in a hospital setting. It is anticipated that Indiana School of Medicine will enroll at minimum, approximately 1-3 qualifying adults per month for up to 5 years.

* + 1. Alignment with intended study population

The study population includes patients likely to benefit from allogeneic MSC transplantation. It also includes other patients who may provide controls.

* 1. Recruitment Methods
     1. Recruitment for Study

Eligible patients will be invited to participate in the study on a first-come basis, subject to Indiana School of Medicine weekly recruitment goals. They will be informed of the possible risks of the procedure and will be required to give informed consent before study-specific procedures can proceed.

A minimal financial inducement will be offered to subjects enrolled in the sham procedure (Active Group Arm 2) and in Group 4 – Healthy Subjects. Subjects consented and enrolled in the Sham Procedure Group will be offered a one-time stipend of $200.00 for their participation. Control Group 4 subjects will be offered a one-time stipend of $200 for their participation in the research study. Each subject will be informed that no personally relevant clinical information will be derived from the collected data, and that the only possible benefit to the subject is an improved chance to avoid a further revision of their amputation. Medications will be documented and certain medications may be held peri-operatively.

Each subject’s involvement in the study will be limited to the period between signing of the informed consent form (ICF) and the completion of study specific procedures.

* + 1. Duration of Study Activities

Enrollment is anticipated to continue for approximately 5 years. In the event that additional studies are going to be conducted, new protocols will be developed specifically for those studies.

* 1. Patient Selection

The eligibility criteria for prospective enrollment of subjects are shown in **Tables 1-4.**

**Inclusion/Exclusion Criteria for Enrollment of Subjects in the Active Groups**

**Table 1. Inclusion/Exclusion Criteria for Enrollment of Subjects in Active Group Arm 1:**

|  |  |
| --- | --- |
| **Inclusion criteria** | 1. Be ≥ 40 and ≤90 years of age. 2. Patients with **HLA A2-** phenotype. 3. Patients requiring **below** knee amputation, as determined by an independent vascular specialist. 4. If ulceration or gangrene is present, it is distal to malleoli (to allow adequate length of ATM area of approximately 3 cm x 10 cm x 3 cm). 5. Amputation can safely be performed at Day 3 post-MSC procedure. 6. Females of childbearing potential must be willing to use one form of birth control for the duration of the study. Female participants must undergo a blood or urine pregnancy test at screening. |
| **Exclusion criteria** | 1. Patients who are pregnant, planning to become pregnant in the next 12 months, or lactating. 2. CHF hospitalization within the last 1 month prior to enrollment.\* 3. Acute coronary syndrome in the last 1 month prior to enrollment.\* 4. HIV positive or active, untreated HCV as determined by review of medical records. 5. History of cancer within the last 5 years, except basal cell skin carcinoma. 6. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). 7. Concurrent enrollment in another clinical investigative trial that may affect the outcome of this study. 8. Any condition requiring immunosuppressant medications (e.g., for treatment of organ transplants, psoriasis, Crohn’s disease, alopecia areata). 9. Presence of any clinical condition that in the opinion of the PI or the sponsor makes the patient not suitable to participate in the trial.   \*As defined by the standard definitions of CHF and ACS by the American Heart Association. |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

**Table 1.1 Inclusion/Exclusion Criteria for Enrollment of Subjects in Active Group Arm 2 (Sham):**

|  |  |
| --- | --- |
| **Inclusion Criteria** | 1. Be ≥ 40 and ≤90 years of age. 2. Patients requiring **above** knee amputation, as determined by an independent vascular specialist. 3. If ulceration or gangrene is present, it is distal to malleoli (to allow adequate length of ATM area of approximately 3 cm x 10 cm x 3 cm) 4. Amputation can safely be performed up to 7 days post-Sham procedure. |
| **Exclusion Criteria** | 1. HIV positive or active, untreated HCV as determined by review of medical records. 2. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

**Table 1.2 Inclusion/Exclusion Criteria for Enrollment of Subjects in Active Group Arm 3:**

|  |  |
| --- | --- |
| **Inclusion Criteria** | 1. Be ≥ 40 and ≤90 years of age. 2. Patients with either **HLA A2+ or HLA A2-** phenotype. 3. Patients requiring **below** knee amputation, as determined by an independent vascular specialist. 4. If ulceration or gangrene is present, it is distal to malleoli (to allow adequate length of ATM area of approximately 3 cm x 10 cm x 3 cm). 5. Amputation can safely be performed up to 14 days post-MSC procedure. 6. Females of childbearing potential must be willing to use one form of birth control for the duration of the study. Female participants must undergo a blood or urine pregnancy test at screening. |
| **Exclusion Criteria** | 1. Patients who are pregnant, planning to become pregnant in the next 12 months, or lactating. 2. CHF hospitalization within the last 1 month prior to enrollment.\* 3. Acute coronary syndrome in the last 1 month prior to enrollment.\* 4. HIV positive or active, untreated HCV as determined by review of medical records. 5. History of cancer within the last 5 years, except basal cell skin carcinoma. 6. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). 7. Concurrent enrollment in another clinical investigative trial that may affect the outcome of this study. 8. Any condition requiring immunosuppressant medications (e.g., for treatment of organ transplants, psoriasis, Crohn’s disease, alopecia areata). 9. Presence of any clinical condition that in the opinion of the PI or the sponsor makes the patient not suitable to participate in the trial.   \*As defined by the standard definitions of CHF and ACS by the American Heart Association. |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

**Table 1.3 Inclusion/Exclusion Criteria for Enrollment of Subjects in Active Group Arm 4:**

|  |  |
| --- | --- |
| **Inclusion Criteria** | 1. Be ≥ 40 and ≤90 years of age. 2. Patients with **HLA A2+** phenotype. 3. Patients requiring **below** knee amputation, as determined by an independent vascular specialist. 4. If ulceration or gangrene is present, it is distal to malleoli (to allow adequate length of ATM area of approximately 3 cm x 10 cm x 3 cm). 5. Amputation can safely be performed up to 21 days post-MSC procedure. 6. Females of childbearing potential must be willing to use one form of birth control for the duration of the study. Female participants must undergo a blood or urine pregnancy test at screening. |
| **Exclusion Criteria** | 1. Patients who are pregnant, planning to become pregnant in the next 12 months, or lactating. 2. CHF hospitalization within the last 1 month prior to enrollment.\* 3. Acute coronary syndrome in the last 1 month prior to enrollment.\* 4. HIV positive or active, untreated HCV as determined by review of medical records. 5. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

**Inclusion/Exclusion Criteria for Enrollment of Subjects in the Observation/Control Groups**

|  |  |
| --- | --- |
| **Inclusion criteria** | 1. Be ≥ 40 and ≤90 years of age. 2. Patients requiring below knee amputation or above knee amputation. 3. If ulceration or gangrene is present, it is distal to malleoli (to allow adequate length of ATM area of approximately 3 cm x 10 cm x 3 cm). |
| **Exclusion criteria** | 1. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). 2. Severe concomitant disease(s), which the investigator feels constitute(s) criteria for exclusion of a particular subject. |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

**Table 2.** **Inclusion/Exclusion Criteria for Enrollment of Subjects in Observation Groups 1 and 2.**

**Table 3.** **Inclusion/Exclusion Criteria for Enrollment of Subjects in Observation Group 3.**

|  |  |
| --- | --- |
| **Inclusion criteria** | 1. Be ≥ 40 and ≤90 years of age. 2. Patients requiring lower extremity bypass graft. |
| **Exclusion criteria** | 1. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). 2. Severe concomitant disease(s), which the investigator feels constitute(s) criteria for exclusion of a particular subject. |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

**Table 4.** **Inclusion/Exclusion Criteria for Enrollment of Subjects in Control Group 4.**

|  |  |
| --- | --- |
| **Inclusion criteria** | 1. Be ≥ 30 and ≤50 years of age. 2. Patients requiring a non-vascular surgical procedure monitored with anesthesia, or who have a clinic appointment. |
| **Exclusion criteria** | 1. Diagnosis of diabetes mellitus. 2. Diagnosis of peripheral artery disease. 3. Current smoker, or history of smoking within 10 years prior to enrollment. 4. Inability to provide written informed consent due to cognitive or language barriers (interpreter permitted). 5. Severe concomitant disease(s), which the investigator feels constitute(s) criteria for exclusion of a particular subject. |
| **Participant Withdrawal Criteria** | At the discretion of the investigator or at the request of the participant. |

STUDY MATERIALS

* 1. Investigational Product
     1. Identity of the Investigational Product

Clinical grade HLA-A2+ CD45- CD105+ MSCs required for this Phase I study will be provided by Case Western Reserve University located in Cleveland, Ohio. MSCs will be cultured from bone marrow mononuclear cells from healthy unrelated allogeneic HLA-A2+ female donors.

* + 1. Safety issues

The MSC dose proposed in this trial was selected from mouse models (based on body surface area) and prior clinical trials, notably the POSEIDON Trial which injected MSCs directly into the myocardium. We may not see any evidence of efficacy in our composite endpoint, which may reflect an inadequate dose. In this case, we would propose a future study exploring escalating doses of MSCs.

Subjects eligible for the Active Group will be scheduled at the screening visit, which may be on the same day as the MSC injection or sham procedure.

* + 1. Handling, storage, accountability

MSCs will be transported from Case Western Reserve University in Cleveland, Ohio to Indiana University in Indianapolis, Indiana using a liquid nitrogen dry shipper validated below -150 degrees Celsius for 4 days to maintain temperature for long-term storage. The temperature is continuously logged during shipment and the temperature trace becomes part of the official shipping documentation. The integrity and temperature of the cell containers are checked on arrival and results documented.

* + 1. Required training

There are no specific training requirements for investigators or laboratory personnel to receive or prepare MSCs for administration.

* + 1. External studies

In the event that plans are made to conduct additional studies, new protocols describing the relevant handling, storage, accountability, and training procedures will be prepared specifically for those studies.

* 1. Other Study Materials
     1. Materials to be provided by study site

Study notebooks to maintain study documents, including signed ICFs, all applicable information, and additional forms to be collected and retained by the study institution during the course of the study. Example spreadsheets for tracking patient information may be created.

* + 1. Materials to be provided by external testing facilities. There are no external testing facilities associated with this study.

1. STUDY PROCEDURES
   1. Workflow

**MSC typing and preparation.**

Female Donors: Typing for HLA-A2+ antigen expression and infectious disease testing will be performed by CLIA certified laboratories. Allogeneic donor infectious disease (ID) testing will include anti-HIV-1/2, anti-HTLV I/II, anti-HCV, HIV-1 (nucleic acid testing), HCV (nucleic acid testing), HBsAg, anti-HBc (IgG and IgM), anti-CMV, West Nile Virus (nucleic acid testing), T. cruzi (Chagas), RPR, EBV, CJD (screening only) (21CFR1271.90(a)). Testing will be repeated if it exceeds 30 days prior to bone marrow aspiration.

Potential donors whose testing is incomplete or not available within 30 days will not be eligible to donate bone marrow and testing will be repeated to confirm ID markers.

Potential donors testing positive for any of these infectious diseases will NOT be eligible. MSC products will be thawed and directly transferred to syringes ready for administration. Samples for gram stain, endotoxin, cell viability (i.e. Trypan Blue and/or 7AAD) and 14-day bacterial and fungal sterility testing will be obtained. Negative gram stain, endotoxin <5 EU/mL and cell viability >70% will need to be entered in the Certificate of Analysis before the product is released for administration. MSCs will be administered within 4 hours of thawing (i.e. stability studies indicate cell viability is >70% for up to 4 hours post-thaw).

Patients: Typing for HLA-A2 antigen will be performed as previously described.50 We expect that 65% of African-Americans, 50% of Caucasians, and 51% of Hispanics, the predominant racial/ethnic groups that constitute our patient population, will be HLA-A2-.50 Subjects enrolled to Active Group Arm 1 (up to 3 total) will be HLA A2-. Subjects in Active Group Arm 2 (up to 3 total) will undergo a sham procedure. Active Group Arm 3 subjects (up to 3 total) will either be HLA A2+ or HLA A2- , and Active Group Arm 4 subjects (up to 4 total) will be HLA A2+.

We will inject HLA-A2+ MSCs into HLA-A2- patients to permit identification of these cells with immunostaining techniques detailed in Aim 2, and previously described. Those patients who are HLA-A2+ will be treated with gender mismatched MSCs, to permit identification of these transplanted cells using fluorescent sex chromosome probes.

**FDA approval for the Use of Allogeneic Human Mesenchymal Stem Cells in this study.**

Dr. Murphy has full U.S. FDA approval (IND 16382). An authorization letter to cross reference FDA approved IND 16524 is on file.

**Scheduling Scheme and MSC Dosing**. After enrollment, 26 patients requiring below knee amputation for CLTI will be scheduled for amputation at either Day 3, Day 14, or Day 21 post-MSC injection.In addition,up to 3 subjects undergoing above knee amputation will be enrolled in the Sham Procedure Group (Active Group Arm 2). For subjects in Active Group Arm 1, Active Group Arm 3, and Active Group Arm 4, MSCs will be administered in one setting that will include the gastrocnemius muscle and the anterior tibialis muscle. See **Figure 1** for the scheduling scheme. Patients will receive IM injection of up to 10 x106 MSCs of gender mismatched or HLA-A2+ MSCs into the ATM below the point of amputation (see prelim data, and **Figure 2** below). MSCs of up to 100 x 106 will be injected IM at up to 10 sites in the gastrocnemius muscle.

The total dose of up to 110 million MSCs is based on the safety discussions with the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration during the approval process for the Investigational New Drug Application for this trial.

**Sham Procedure.** Three subjects meeting Active Group Arm 2 eligibility will be enrolled in the Sham Procedure. Subjects requiring above knee amputation will undergo four (4) blank needle punctures in the index leg ATM using a 22-gauge needle, duplicating the actual cell injection scheme, however, no investigational product will be injected into the subject’s leg.

Needle biopsies may be performed below the knee at the time of cell injections or above the knee at the time of amputation to obtain skeletal muscle samples for histological and biochemical analysis. There will be no additional MCS injections for this purpose.

**Figure 1. Scheduling Scheme for Enrollment and Procedures**

TISSUE COLLECTION PROCEDURE ENROLLMENT

**Patients assessed for eligibility in Active Groups**

**MSC Injection Procedure (n=26)**

* 100 x 106 MSCs injected IM in the gastrocnemius muscle at up to 10 sites
* 10 x 106 MSCs injected IM below amputation site in ATM at up to 2 sites

**Sham Procedure (n=3)**

* Four (4) blank needle punctures into the ATM
* No MSC product delivered

**Above knee amputation** and collection of ATM and soleus, healthy muscle, nerve, wound, skin, and bone marrow specimens from the amputated limb at **14 days** **post-Sham procedure**

**Below knee amputation** and collection of ATM and soleus, healthy muscle, nerve, wound, skin and bone marrow specimens from amputated limb at **3, 7, or 21 days** **post-MSC injections**

**MSC Administration: Injection into the anterior tibialis muscle.**

On the day of MSC injection, the index limb will be marked for amputation. Up to two injection sites will be marked with permanent ink at the ATM, then we will inject up to 10 million MSCs, 2-3 cm. deep at this location (**Figure 2**).

**MSC Administration: Injection into the gastrocnemius muscle.**

For subjects undergoing below knee amputation, MSCs (up to 100 million divided in 1 mL aliquots) will be injected in the gastrocnemius muscle 2-3 cm. deep, approximately 3 cm apart as depicted in **Figure 2**.

At the Investigator’s discretion and as MSC volume permits, MSCs may be injected in the proximity of present wounds in the index extremity. There will be no increase in the amount of MSCs used for this purpose.

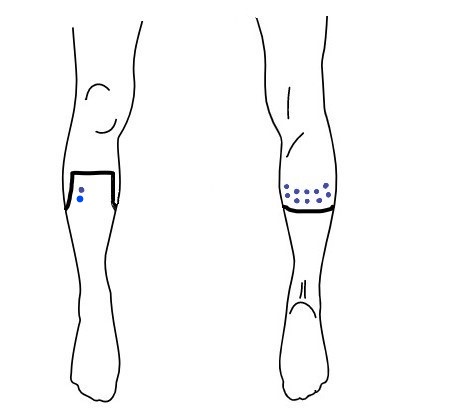
**Sham Procedure: Blank Needle Punctures into the anterior tibialis muscle.**

On the day of the sham treatment procedure, the index limb will be marked for amputation. The sham procedure will be performed in a similar manner to the MSC injection treatment into the ATM; however, no cell product will be delivered intramuscularly. The sham procedure will be sterilely performed by making up to four (4) percutaneous skin punctures into the anterior tibialis muscle with the aid of a 22-gauge needle. There will be no blank needle punctures performed above the knee.

**Sham Procedure Follow-Up.** Subjects in the sham treatment group will be followed for 24 weeks after above knee amputation (**Table 1.1**), the same follow-up plan for the Active treated subject groups and Observation Group 1.

**Figure 2. Below Knee Amputation Injection Scheme (with amputation marked)**

1. (Anterior view) Up to 1 mL total of MSCs will be injected into the ATM, delivered at up to 2 sites.
2. (Posterior view) MSCs will be injected 2-3 cm deep into the muscle at approximately 10 sites, 3 cm apart, delivering 1 mL of MSCs (approximately 10 million MSCs per site) at each site in the gastrocnemius muscle.



Posterior Leg View

Anterior Leg View

**Amputation Day Tissue Collection.** As indicated throughout this document, tissue collection will occur during the time of amputation. All tissue collected will be derived from the amputated portion of the limb and analyzed at Indiana University and/or VA Medical Center. Portions of the index leg anterior tibialis muscle and soleus muscle, wound, normal skin, and bone marrow tissue may be collected.

Segments from sciatic, femoral, sural, and/or plantar nerves may be collected. The purpose of collecting these nerve bundles is to determine the effects of chronic limb ischemia and diabetes on nerve function and assess the effects of mesenchymal stem cell administration on their function. Patients with critical limb threatening ischemia, particularly with diabetes, have polyneuropathies that alter motor unit engagement and peripheral sensation leading to ambulatory dysfunction and pressure ulceration of the foot.

Approximately 15 grams of healthy skeletal muscle will be collected for secondary research not associated with this study. De-identified healthy skeletal muscle samples from the amputated portion of the limb (up to 15 grams) will be collected and sent to IU School of Medicine collaborator, Harikrishna Nakshatri, PhD, for research analysis under their specific IBC protocol.

**Baseline Screening and Follow-Up Schedules.** The following evaluations will be reviewed at baseline to determine if, and to which group(s) the subject is eligible: (1) Blood tests (complete blood count, serum chemistries, eGFR, hsCRP, HLA); (2) presence of infectious disease (active, untreated Hepatitis C, positive HIV) as determined by review of medical records; (3) medical history and physical exam; (4) concomitant medications; (5) pregnancy test for women of childbearing age; (6) 12 lead ECG; and (7) TcPO2 of the index leg. In the interest of imminent amputation, we may collect retroactive data prior to consent date to determine eligibility.

Baseline screening/physical exam for all groups enrolled may occur on the procedure day, prior to start of the procedure. In this instance, the time of consent will be documented on the informed consent form.

In the event of a positive sterility test result after product administration, the physician and subject will be informed, and the results reported to the IRB and FDA, either as a promptly reportable event, if applicable, or at the next annual renewal date, as determined by the Investigator.

Should cultures for detection of bacteria and fungi become positive, the Principal Investigator (PI) of the study will be notified. The PI will then notify the subject and the appropriate antibiotics will be started based on sensitivities of the identified organism.

A physical examination of the subject will be conducted within 24 -48 business hours of discovery of positive culture, at which time a complete blood count will be obtained. Should there be an elevated white blood cell count (> 11.5K), the patient will be admitted to the hospital for intravenous antibiotics or antifungal medications. Should there be no clinical evidence of infection the patient will be treated for 7 days with oral antibiotics or anti-fungal medication.

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| **Table 5. Follow up Data Collection for Active Group (including Sham Group)** | | | | | | | | | |
| **Procedures** | **Baseline** | **MSC Injection Day** | **Amputation Day** | **Day 3** + 2 days | **Week 2** +/- 2 days | **Week 6** +/- 4 days | **Week 12** +/-7 days | **Week 18** +/-2 weeks | **Week 24** +/-2 weeks |
| Informed Consent | X |  |  |  |  |  |  |  |  |
| Medical History | X |  |  |  |  |  |  |  |  |
| Physical Exam1 | X | X | X | X | X | X | X | X | X |
| Vital Signs | X | X | X | X | X | X | X | X | X |
| Concomitant  Medications | X | X | X | X | X | X | X | X | X |
| Serum Pregnancy Test (if indicated) | X |  |  |  |  |  |  |  |  |
| AE/SAE Evaluations |  | X | X | X | X | X | X | X | X |
| Laboratory Tests | X2 |  |  |  | X3 | X3 | X3 | X3 | X3 |
| HLA-A2 typing3 | X4 |  |  |  |  |  |  |  |  |
| 12 Lead ECG7 | X | X8 |  |  | X |  | X |  | X |
| TcPO25 | X |  |  |  | X | X | X | X | X |
| Blood Sample for Aim 26 |  | X | X |  | X | X | X | X | X |
| 1. Physical exams will be standard of care; baseline exam may be on the morning of amputation.  2. Screening Laboratory Tests\*: CBC w/diff/plt; CMP (Sodium, Potassium, Chloride, CO2, Glucose, Calcium, BUN, Creatinine); eGFR, hsCRP, serum pregnancy testing (childbearing females), if indicated.  3. Follow-up laboratory tests: hsCRP  4. Typing for HLA-A2\*: (presence or absence) will occur prior to subject’s scheduling of treatment to determine into which group they will enroll.  5. TcP02: Transcutaneous oxygen pressure will be measured at the foot at baseline. Following the amputation, TcPO2 will be measured at the medial and lateral amputation stump site.  6. Blood Sample for Aim 2: Collected on the day of surgical procedures for MSC Injection Day and Amputation Day. Blood sample collection will be up to 30 mL at each time point. It will not be considered a protocol deviation if research blood draws are not collected.  7. For safety purposes, 12 Lead ECG will be performed on subjects receiving MSC treatment only. The Sham Group (AGA2) will not have 12 Lead ECG performed.  8. 12 lead ECG on MSC Injection Day will be assessed after treatment has been completed.  \*If any of these measurements have been performed within one month prior to enrollment, we will use these measurements to determine eligibility. | | | | | | | | | |

**Observation Group 1 - Study Procedures**

Observation Group 1 will include up to forty-six (46) subjects undergoing BKA who decline participation in the Active Group(s) or who are deemed ineligible for the study product treatment. Tissue and blood collection in Group 1 will serve as control. The following evaluations will be reviewed at baseline to determine if the subject is eligible for enrollment in the study: (1) Baseline blood tests (WBC and HbA1C, if indicated); (3) medical history and physical exam; and (4) concomitant medications. Subjects in Group 1 will be followed for 24 weeks after amputation.

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| **Table 6. Follow up Data Collection for Observation Group 1** | | | | | | | | |
| **Procedures** | **Baseline** | **Amputation Day** | **Day 3**  **+ 2 days** | **Week 2 +/- 2 days** | **Week 6 +/- 4 days** | **Week 12 +/-7 days** | **Week 18 +/-2 weeks** | **Week 24 +/-2 weeks** |
| Informed Consent | X |  |  |  |  |  |  |  |
| Medical History | X |  |  |  |  |  |  |  |
| Physical Exam1 | X | X | X | X | X | X | X | X |
| Concomitant  Medications | X |  |  |  |  |  |  |  |
| Laboratory Tests2 | X |  |  |  |  |  |  |  |
| ABI, TBI, TcPO23 | X |  |  | X | X | X | X | X |
| Blood Sample for Aim 24 |  | X |  | X | X | X | X | X |
| 1. Physical exams will be standard of care; baseline exam may be on the morning of amputation. 2. Laboratory Tests: WBC only (prior to BKA), Hgb A1c (if indicated) 3. ABI, TBI, and TcPO2: Transcutaneous oxygen pressure will be measured at the foot at baseline. Following the amputation, TcPO2 will be measured at the medial and lateral amputation stump site. It is not considered a protocol deviation if TcPO2 are not collected. If ABI and TBI have been performed within 30 days of enrollment, we will collect this data as baseline assessment data and will not repeat testing. 4. Blood Sample for Aim 2: Blood sample collection will be up to 30 mL at each time point. It will not be considered a protocol deviation if research blood draws are not collected. | | | | | | | | |

**Observation Group 2 - Study Procedures**

Observation Group 2 will include up to twenty (20) subjects undergoing BKA or AKA who decline participation in the Active Group(s) or who are deemed ineligible for the study product treatment. Tissue and blood collection in Group 2 will serve as control. The following evaluations will be reviewed at baseline to determine if the subject is eligible for enrollment in the study: (1) Baseline blood tests (WBC and HbA1C, if indicated); (2) medical history and physical exam; and (3) concomitant medications. There is no follow-up period for Group 2; study participation will end upon completion of Amputation Day activities.

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| **Table 7. Data Collection for Observation Group 2** | | |
| **Procedures** | **Baseline** | **Amputation Day** |
| Informed Consent1 | X | |
| Medical History | X |  |
| Physical Exam2 | X | |
| Concomitant  Medications | X |  |
| Laboratory Tests(WBC and Hgb A1c, if indicated) | X |  |
| ABI, TBI, TcPO2 \* | X |  |
| Blood sample for Aim 2 \* | X | |
| 1 Consent signing may occur on the morning of procedure, the time of consent will be documented in this case.  2 Physical exams will be standard of care; baseline exam may be on the morning of amputation.  \*It will not be considered a protocol deviation if these measurements and blood draw were not collected. | | |

**Observation Group 3 - Study Procedures**

Observation Group 3 will include up to twenty (20) subjects undergoing an elective lower extremity arterial bypass procedure. Tissue and blood collection in Group 3 will serve as control. The following evaluations will be reviewed at baseline to determine if the subject is eligible for enrollment in the study: (1) Baseline blood tests (WBC and HbA1C, if indicated); (2) medical history and physical exam; and (3) concomitant medications. Most recent measurements of perfusion including ABI, TBI, and TcPO2 will be collected from subjects’ medical charts to assess adequacy of healing. Collection of muscle tissue from the anterior tibialis muscle (ATM) of the index leg will occur during subjects’ standard of care lower extremity arterial bypass procedure. Tissue samples will include up to 2 cm x 0.5 cm from the ATM and 2 cm x 0.5 cm from the sartorius muscle. Blood collection of up to 26 mL will be collected on the day of the procedure to serve as control. There is no follow-up period for Group 3; study participation will end upon completion of the lower extremity bypass procedure.

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| **Table 8. Data Collection for Observation Group 3** | | |
| **Procedures** | **Baseline** | **Bypass Procedure Day** |
| Informed Consent1 | X | |
| Medical History | X |  |
| Physical Exam2 | X | |
| Concomitant  Medications | X |  |
| Laboratory Tests(WBC and Hgb A1c, if indicated) | X |  |
| ABI, TBI, TcPO2 \* | X |  |
| Blood sample for Aim 2 \* | X | |
| 1 Consent signing may occur on the morning of procedure, the time of consent will be documented in this case.  2 Physical exams will be standard of care; baseline exam may be on the morning of bypass procedure.  \*It will not be considered a protocol deviation if these measurements and blood draw were not collected. | | |
|  | | |

**Control Group 4 - Study Procedures**

Control Group 4 will include “healthy” control subjects who do not have diagnoses of diabetes mellitus, peripheral artery disease, obesity with BMI >30, nor who have smoked within the last ten years. Tissue and blood collection in Group 4 will serve as control in up to ten (10) subjects. The following evaluations will be reviewed at baseline to determine if the subject is eligible for enrollment in the study: (1) Baseline blood tests (WBC and HbA1C, if indicated); (3) medical history and physical exam; and (4) concomitant medications.

Collection of muscle tissue from the anterior tibialis muscle (ATM) of one lower leg via core needle biopsy will occur during subjects’ standard of care surgical procedure or clinic visit. Biopsies will include up to three locations in the ATM, with each biopsy measuring about 1/16 inch in diameter and 1/2 inch long. Blood collection of up to 30 mL will be collected on the day of biopsies to serve as control. Group 4 subject participation will end after a one-week post-biopsy telephone call to assess for safety and complications. The subject will be instructed to call the study team if there are concerns of infection or increased pain prior to the one-week phone call. If necessary, based on the PI’s recommendation, a follow-up visit with the PI will be scheduled, to address any adverse events.

If subjects are undergoing a standard of care surgical procedure under monitored anesthesia, up to three core needle biopsies will be collected from the ATM in the operating room during their routine procedure. The blood collection of up to 30 mL may be drawn through an IV access. Subjects will be monitored after their surgical procedure as standard of care.

If subjects do not have a planned surgical procedure, core needle biopsies will be performed in a clinic setting under sterile conditions. For pain control, prior to biopsies, subjects may have up to 1 mL of lidocaine injected near the biopsy site(s). Alternatively, topical anesthetic (EMLA cream) may be applied to this area for subjects who have a known allergy to lidocaine. The procedure is expected to last up to 15 minutes and subjects will be monitored for an additional 15 minutes for adverse effects after biopsy completion, prior to leaving the clinic.

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| --- | --- | --- | --- |
| **Table 9. Data Collection for Control Group 4** | | | |
| **Procedures** | **Baseline** | **Procedure Day (Group 4)** | **Week 1 Phone Call** |
| Informed Consent1 | X | |  |
| Medical History | X |  |  |
| Physical Exam2 | X | |  |
| Concomitant  Medications | X |  |  |
| Laboratory Tests(WBC and Hgb A1c, if indicated) | X |  |  |
| Blood sample for Aim 2 \* | X | |  |
| Safety Assessment Post-biopsy |  | | X |
| 1Consent signing may occur on the morning of procedure. The time of consent will be documented in this case.  2Physical exams will be standard of care; Baseline exam may be on the morning of the core needle biopsy research procedure.  \*It will not be considered a protocol deviation if the research blood draw was not collected. | | | |

* 1. Study Data

Eligible subjects will be invited to participate in the study on a first-come basis. Potential donors must provide informed consent to participate. To be eligible for participation in the Active treatment group and Observation Groups 1-3, subjects must be between 40 and 90 years of age. In Control Group 4, eligible “healthy” participants will be between 30 and 50 years of age.

* + 1. Collection of data

At the discretion of the Investigator and subject to Indiana University School of Medicine requirements, data (**Tables 5-9**) will be collected from each eligible subject providing signed informed consent.

* 1. Procedures for Study Closure
     1. Routine study close-out

The study will end when Indiana University School of Medicine has obtained all data necessary to complete its studies of the test product. Study close-out will follow Indiana University School of Medicine standard procedures and may include, but is not limited to, review of regulatory documents, collection of completed case report forms, reconciliation of study records, removal or destruction of ancillary study supplies, and informing the Investigator of remaining obligations (e.g. record retention, final report submission to the IRB, financial disclosure updates, etc.).

* + 1. Suspension or premature termination of the study

This study may prematurely terminate at any time because of a regulatory authority decision, a change in opinion of the IRB, or at the discretion of the Investigator or Sponsor. The study will be terminated in the event of a Grade 4-5 unexpected, investigational product related event based on the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0. If this trial is temporarily suspended or prematurely discontinued, Indiana University School of Medicine will promptly notify the Investigator(s) and provide instructions. If the study is temporarily suspended, Indiana University School of Medicine will provide guidance on timing and procedures for resuming the study. If the study is prematurely discontinued, all study materials must be collected and all study forms completed to the extent possible. All such materials must be returned to Indiana University School of Medicine upon request.

1. DATA QUALITY ASSURANCE

The study site will be responsible for the accuracy of data. Indiana University School of Medicine or its agent may periodically conduct monitoring visits to ensure the quality of data collection.

1. STATISTICAL METHODS
   1. Determination of Sample Size

**SubAim 1A – Safety Assessment.** Primary attention in Aim 1 will focus on the safety of the administration of MSCs as measured by treatment-related adverse events. Treatment-related adverse events will be categorized overlapping systems and severities. Three categories of systems are cardiovascular, respiratory, or infectious. Two categories of severity will be serious adverse (SAE) and major adverse cardiac events (MACE). For completeness, instances of adverse events may appear in more than one category. Within each of these categories adverse events will be listed in descending order of frequency for the treatment-group. In addition, for each category, the sum and difference between the two routes of delivery of the proportions will be reported as percent incidence. Binomial confidence Intervals at the 95% confidence level and *p*-values for these four groups will be calculated. Since four previous trials have not reported adverse events with MSC treatment, confidence intervals will be generated by the method of the Wilson Score Interval because they are robust, with good coverage probability even for small number of trials and are not degenerate near zero. These intervals will be used to summarize the data rather than as any formal inferential statement and to assist the Data Monitoring Board in their deliberations. No adjustment for multiplicity will be made.

**SubAim 1B – Exploratory Efficacy**. Continuous confidence intervals at the 95% level will be constructed to explore the effect of administration of MSC on the composite endpoint at 6-months of death, AKA conversion and gangrene, and will be compared to historical cohorts at our institution. The null hypothesis of no change will be rejected if the pairwise confidence intervals does not include zero. The critical levels for the multiplicity adjustment will be determined by simple Monte Carlo simulation.

**Aim 2-** Quantities over time of MSC will be fit to an exponential decay curve using a residual pseudo- likelihood procedure and cell half-life (λ) will be estimated. Binomial confidence Intervals at the 95% confidence level and *p*-values for these four groups after MSC transplantation will be calculated for the presence or absence of MHC expression and SDF-1activation. Continuous confidence intervals at the 95% level will be constructed to explore the differences among the time-tiered administration of MSC for (1) the CD34+CD133+ pro-angiogenic hematopoietic cells recruitment of HIF-1α/SDF-1/CXCR4 to ischemic muscle, (2) the quantify of capillary density in muscle fibers using hematoxylin phloxin saffron and CD31 counts, (3) VEGF-A,C,D, hepatocyte growth factor, angiopoietin-1 to characterize angiogenic cytokine expression, (4) percent coverage, fiber diameter and cross-sectional area to examine changes in morphology. The correlation between capillary density (CD31 counts) with tissue perfusion (ICA) for each time point will be estimated by Spearman’s rank coefficient.

* 1. Bias Minimization

All subjects meeting the specified eligibility criteria will be enrolled on a first-come basis.

* 1. Planned Analyses

The clinical study report will contain only summary data analyses reflecting safety profiles, and summary data analyses of efficacy. All patient data will remain coded during analyses to maintain patient confidentiality.

Quantities over time of MSC will be fit to an exponential decay curve using a residual pseudo-likelihood procedure and cell half-life (λ) will be estimated.59 Binomial confidence Intervals at the 95% confidence level and *p*-values for these groups after MSC transplantation will be calculated for the presence or absence of MHC expression and SDF-1activation. Continuous confidence intervals at the 95% level will be constructed to explore the differences among the time-tiered administration of MSC for (1) the CD34+CD133+ pro-angiogenic hematopoietic cells recruitment of HIF-1α/SDF-1/CXCR4 to ischemic muscle, (2) the quantify of capillary density in muscle fibers using hematoxylin phloxin saffron and CD31 counts, (3) VEGF-A,C,D, hepatocyte growth factor, angiopoietin-1 to characterize angiogenic cytokine expression, (4) percent coverage, fiber diameter and cross-sectional area to examine changes in morphology. The correlation between capillary density (CD31 counts) with tissue perfusion (ICA) for each time point will be estimated by Spearman’s rank coefficient.60

1. ADVERSE EVENT REPORTING
   1. Adverse Events

Adverse events occurring during the enrollment period should be documented by the Investigator in progress notes but will not be collected or analyzed by Indiana University School of Medicine unless considered serious and research related by the Investigator. The MedDRA scale will be used to describe adverse events.

Only events that occur after the start of study treatment will be classified as adverse events. An *adverse event* (AE) is any untoward medical event in a subject that has been given any dose of a biologic product and does not necessarily have a causal relationship with the use of the product or product output.

Any untoward medical event that occurs outside the period of follow-up defined in the protocol is not considered a protocol adverse event.

Symptoms of a preexisting disease, such as hypertension or diabetes, should not be considered adverse events but must be documented in the medical history if clinically significant. New symptoms, however, as well as worsening of existing ones, are considered adverse events.

* 1. Serious Adverse Events

A serious adverse event (SAE) is any untoward medical occurrence that:

* results in death
* is life-threatening, i.e., an event that, in the view of the investigator, places the subject at immediate risk of death from the event as it occurred (it does not include an event that could have caused death if it had been more severe) requires inpatient hospitalization or prolongs the existing hospitalization
* results in persistent or significant disability or incapacity, where disability is defined as a substantial disruption of a person’s ability to conduct normal life functions, either reported or defined as per clinical judgment
* is a congenital anomaly or birth defect (if exposure to product just before conception or during pregnancy resulted in an adverse outcome in the child)
* is a medically important adverse event that can be regarded as serious even if it does not meet any of the above criteria. Such an important medical event may not be immediately life-threatening, or result in death or hospitalization, but based upon appropriate medical judgment, it may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed in the points above. These events should also be considered serious. Examples of such events include allergic bronchospasm requiring intensive treatment in

an emergency room or at home, and blood dyscrasias or convulsions that do not result in subject hospitalization.

**9.2.1** Reporting SAEs

Serious adverse events (SAEs) that are unanticipated and related to the research treatment that are encountered during study enrollment will be documented by the Investigator and reported to Indiana University School of Medicine within 48 hours of discovery.

**9.2.2** Potential SAEs

The following events are considered potential SAEs, given the nature of the investigational treatment and the health of potential subjects:

* anaphylactic reaction
* pulmonary embolus
* myocardial infarction
* cerebrovascular accident
* death

In the case of completion or termination of the study or an Investigator's role in the study, or at Indiana University School of Medicine request, all study materials must be returned to Indiana University School of Medicine.

Anticipated Adverse Events

An *anticipated adverse event* is an AE for which the nature and severity are consistent with the applicable product information (e.g. Investigator’s Brochure for an unapproved investigational product or package insert/summary of product characteristics for an approved product). This term relates only to the research treatment, not the subject’s underlying condition.

The following events are considered anticipated AEs, given the nature of the investigational treatment and the health of potential subjects:

* + - hemorrhage, seroma formation, infection, and/or persistent pain at the product injection site
    - hematoma, rash, or erythema at delivery sites
    - cellulitis of skin surrounding delivery sites
    - deep venous thrombosis in treated leg
    - lower extremity swelling and/or discomfort in treated leg
    - fever, flu-like syndrome, fatigue, diarrhea, vomiting
    - anemia

**9.3** Adverse Event Follow-up

All adverse events occurring within the follow-up period (whether serious, non-serious, unanticipated, or anticipated) will be monitored and documented according to the follow-up schedule outlined in Table 5 of Section 7 (Study Procedures).

To be compliant with ethical and regulatory principles, treatment-related SAEs that occur after follow-up is completed (outside the protocol) and are assessed by the investigator as “unanticipated” must be updated to the FDA via the MedWatch reporting system in accordance with FDA regulations.

Sponsor Contact for Serious Adverse Event Reporting

Michael P. Murphy, MD 1801 N. Senate Blvd.

MPC2, #3500

Indianapolis, IN 46202

1. RISK ANALYSIS
   1. Potential Risks of the Investigational Product and Clinical Investigation

Study sites will not be provided with an investigational device.

The types of risk associated with IM injection of allogeneic MSCs align with those associated with IM injection of autologous bone marrow mononuclear cells (ABMNCs), most notably rash, mild fever, and myalgia. These risks are mild and rare. These risks are all stated in the consent form**.**

Risks associated with PED-10 dressing include the rare possibility of thermal skin injury. Safety measures have been implemented with use of a power on/off switch and a ballast resistor, which should eliminate these risks.

* 1. Potential Benefits of the Investigational Product and Clinical Investigation

Subjects are not expected to benefit in any way from their participation in the study.

The studies made possible by the data collected in this study are expected to lead to the prevention of wound complications after major amputation.

* 1. Minimization of Risks

Although the risk to subjects participating in the study is anticipated to be minimal, the clinician, at his/her discretion, will not collect data from those individuals for whom collection is judged to pose an unusually high risk of physical or mental harm or discomfort.

Participation in this study poses no risk to study personnel other than that normally encountered during standard practice. These risks will be minimized by adherence to the following guidelines:

* Personnel should wear appropriate personal protective equipment to avoid contact of the eyes or skin with hazardous materials or products derived from biological sources.

1. INVESTIGATOR RESPONSIBILITIES
   1. Site Qualification and Study Oversight

The PI is responsible for general administration of the study. Before the study, the PI must:

* Obtain approval to conduct the study from the study site’s IRB;
* Sign the Protocol Signature Page him/herself and have all sub-investigators sign the Protocol Signature Page and return it to Indiana University School of Medicine;
* Provide financial disclosures to Indiana University School of Medicine for themselves and all sub- investigators participating in study conduct, per Title 21CFR 54 (see **Section 12.4** below).

During the study, the PI must ensure that:

* The study is conducted ethically; and
* Case report forms (CRFs), including Subject ICFs, are provided with each transfer of data requiring informed consent; and
* All other study forms are completed as instructed by Indiana University School of Medicine.
  1. Case Report Forms/Electronic Data Records

As used in this protocol, the term CRF should be understood to refer to either a paper form or an electronic data record or both, depending on the data collection method(s) used.

Original CRFs are the sole property of Indiana University School of Medicine and should not be made available in any form to third parties, except for authorized representatives of Indiana University School of Medicine or appropriate regulatory authorities, without written permission from Indiana University School of Medicine.

It is the PI's responsibility to ensure completion, review, and approval of all CRFs. CRFs must be signed by the PI or by an authorized staff member. These signatures serve to attest that the information contained on the CRFs is true. At all times, the PI has final personal responsibility for the accuracy and authenticity of all clinical and laboratory data entered on the CRFs.

* 1. Access to Source Documents

Indiana University School of Medicine or its agents and appropriate regulatory authorities shall be granted direct access to all study-related documents to perform verification that the protocol and all applicable current Good Laboratory Practices (cGLPs), Good Clinical Practices (GCPs), and regulations are being followed and to confirm that study documents are complete and accurate. It is important that Investigator(s) and their relevant personnel be made available during monitoring visits and any audits or inspections, and that sufficient time is allotted for the process.

* 1. Financial Disclosure

Investigators must provide Indiana University School of Medicine with sufficient, accurate financial information in accordance with local regulations to allow Indiana University School of Medicine to submit complete and accurate financial certification or disclosure statements to the appropriate health authorities. Investigators are responsible for providing information to Indiana University School of Medicine concerning their relevant financial interests during the course of the study and for 1 year after completion of the study. Conflicts of interest should be disclosed as required by law.

Financial support for this project is provided by Indiana Clinical and Translational Sciences Institute (CTSI) and the National Institutes of Health (NIH).

* 1. Deviations from the Study Protocol

An Investigator may not intentionally deviate from the study protocol without prior approval by Indiana University School of Medicine unless the deviations are necessary under emergency circumstances to protect the rights, safety, or well-being of human subjects or the scientific integrity of the clinical investigation.

These deviations must be documented and promptly reported to Indiana University School of Medicine and, if applicable, to the IRB providing oversight of the study. Protocol deviations may result in corrective and preventive actions and/or disqualification of the Investigator. Unintentional protocol deviations will be recorded in the regulatory files and submitted to the IRB and FDA on a regular basis, but no less than annually. If research blood draws “Blood sample for Aim 2” are not obtained at time points specified in the schedule of events, they will not be considered protocol deviations.

* 1. Record Retention

To enable evaluations and/or audits from regulatory authorities or Indiana University School of Medicine, the PI and all sub-investigators agrees to retain all study records, including copies of all CRFs, UADE forms, and source documents, for 3 years following completion of the project dependent upon the study data. The Investigator must obtain the Indiana University School of Medicine written permission before disposing of any records, even if retention requirements have been met.

If an Investigator relocates, retires, or for any other reason withdraws from the trial, Indiana University School of Medicine must be notified in advance, and study records must be transferred to a designee acceptable to Indiana University School of Medicine. This designee might be another Investigator, another institution, or Indiana University School of Medicine itself.

* 1. Publication Policy

The results of this study will be submitted for publication to a medical journal. The PI agrees that any publication of data from this study will comply with Indiana University School of Medicine publication policy, the instructions to authors outlined by the editor of the journal or conference proceedings where the data is to be published, and the spirit of recommendations made in the good publication practice guidelines (GPP2) of the International Society of Medical Publication Professionals. Indiana University School of Medicine has the right to review any manuscripts, presentations, or abstracts that originate from this study or that utilize these data before they are submitted for publication or other means of communication.

1. ETHICS AND COMPLIANCE
   1. Informed Consent and De-Identification
      1. Prospectively collected data

All subjects will be given a copy of the IRB-approved ICF to review before their study participation begins. The Investigator, or their designee, will explain all aspects of the study in lay language and answer all of the potential participant’s questions regarding the study. If the participant decides to participate in the study, they will be asked to sign and date the ICF. Subjects who refuse to participate or who withdraw from the study will be treated without prejudice.

* 1. IRB Review

The PI is required to obtain IRB oversight of the research study. The IRB must be provided with the Indiana University School of Medicine-approved study protocol. Performance of the study may not begin until written evidence of IRB approval has been provided to Indiana University School of Medicine.

The conduct and performance of this study will be in accordance with applicable Sponsor and Investigator responsibilities as described in Title 21 CFR 812 and other Good Clinical Practice guidance.

IRB/Ethics Committee oversight will be required as human subjects or data from humans are being used. This protocol and the associated informed consent document(s) must be submitted to the IRB for review and approval. Performance of the study at a given site may not begin until written evidence of IRB oversight has been provided to an Indiana University School of Medicine study manager. IRB Review and approval must comply with Title 21 CFR 812 Subpart D.

* 1. Confidentiality of Data and Patient Records

The study institution shall keep all records associated with this study for at least 3 years, as specified in **Section 12.6**. Investigators will keep all records associated with this study for at least 3 years.

**13.4** Provisions to Protect the Privacy Interests of Participants

The PI and/or study institution shall provide sufficient information to allow the IRB to evaluate the researcher’s provisions to maintain the confidentiality of data.

Privacy data will be maintained in accordance with HIPAA and other applicable policies and local law.

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