Safety and Efficacy of Concentrated Autologous Concentrated Bone Marrow Aspirate (BMAC) in Preventing Wound Complications in Amputations (The MarrowCHAMP Study)

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CLINICAL STUDY PROTOCOL

Safety and Efficacy of Concentrated Autologous Concentrated Bone Marrow Aspirate (BMAC) in Preventing Wound Complications in Amputations (The MarrowCHAMP Study)

Protocol IRB# 1511774456

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05 Nov 2021

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.

Protocol Name	Safety and efficacy of concentrated autologous concentrated bone marrow aspirate (BMAC) in preventing wound complications in amputations
Protocol Number	1511774456
Investigational Product Name	**BioCue™ Platelet Concentration System (autologous concentrated bone marrow aspirate)**
Author	Michael P. Murphy, MD

PROTOCOL HISTORY

Version Date (dd-mmm-yyyy)	**Description**
24 Sep 2015	Initial Release
12 Apr 2016	Initial IRB Approval
25 May 2016	Amendment 1
15 Jul 2016	Amendment 2
10 Jan 2017	Annual Renewal + Amendment 3
15 Mar 2017	Amendment 4
24 May 2017	Amendment 5
26 Jul 2017	Amendment 6
05 Dec 2018	Amendment 7 – add 6 subjects
05 Nov 2021	Amendment 8 – change to BioCue™ device

CONTACT INFORMATION

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

Protocol #: 151177456

Safety and efficacy of concentrated autologous concentrated bone marrow aspirate (BMAC) in preventing wound complications in amputations

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.

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, 56 and 11 (as applicable), and any applicable regulatory laws. I will make no changes to protocol-defined procedures without written permission from the Sponsor.

In compliance with Title 21 CFR Part 812, I will disclose any investigation or research in which I was involved that was terminated, with and explanation of the circumstances that led to termination. IND 11429 was terminated on June 30, 2011 due to noncompliance with regulatory reporting requirements [21 CFR 312.44(b)(viii)].

I understand that Investigational Use Products may be used **only** for the purposes explicitly described in this protocol. The BioCue™ Platelet Concentration Kit is investigational.

CAUTION – Investigational device. Limited by Federal (or United States) law to investigational use.

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 proper authorization.

Michael P. Murphy	Michael P. Murphy	11/5/2021
PRINTED NAME	SIGNATURE	DATE

SYNOPSIS

Title of Study	Safety and efficacy of concentrated autologous concentrated bone marrow aspirate (BMAC) in preventing wound complications in amputations
Objectives	• Determine the safety and explore efficacy of BMAC in preventing wound complications in amputations. • Characterize the host tissue response to, paracrine activity of, and fate of transplanted BMAC. • 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.
Planned Number of Subjects and Duration of Involvement	12 patients, 12 month follow up period
Patient Population	Critical limb ischemia (CLI) patients scheduled for amputation
Investigational Product Name	Concentrated autologous bone marrow aspirate (BMAC)
Methodology Overview	This is an open label pilot trial. Up to twelve (12) patients scheduled for amputation will receive bone marrow cells concentrated (BMAC) via the BioCue™ Platelet Concentration Kit device injected IM at 25 sites in the leg proximal to the amputation in the index limb to prevent ischemic wound complications after surgery. BMAC will be injected into the anterior tibialis muscle (ATM) below the point of amputation for analytical purposes. Patients will be scheduled for amputation at Day 7 post injection. Safety will be evaluated by review of treatment related adverse events (AE) during the 12-month follow-up period. In subjects undergoing BKA, we will compare rates of wound complications and conversion from BKA to AKA to historical controls at our institution to assess trends in therapeutic efficacy. Patients will undergo amputation and injection sites will be harvested from the ATM at that time. Immunohistochemical staining (IHC) will determine capillary density and local host immune responses. Angiogenic and inflammatory cytokines will be quantified using a multiplex array system and quantitative polymerase chain reaction (PCR).

Abbreviations

ABMNC	Autologous Bone Marrow Mononuclear Cells
ACS	Acute coronary syndrome
AE	Adverse event
AKA	Above Knee Amputation
ATM	Anterior tibialis muscle
BKA	Below Knee Amputation
BMAC	Concentrated bone marrow aspirate
cGCP	Good Clinical Practice
cGLP	Good Laboratory Practice
CHAMP	**C**linical and **H**istological **A**nalysis of concentrated autologous concentrated bone **M**arrow aspirate (BMAC) in am**P**utations (MarrowCHAMP)
CLI	Critical Limb Ischemia
CTSI	Clinical and Translational Sciences Institute
DMSB	Data Monitoring and Safety Board
HIF-1α	Hypoxia Inducible Factor-1α
ICA	Indocyanine angiography
ICF	Informed consent form
ICH	International Conference on Harmonization
IHC	Immunohistochemical staining
IM	Intramuscular
LEADs	Lower Extremity Arterial Dopplers
LC-MS	Liquid Chromatography- Mass Spectrometry
MHC	Major Histocompatibility Complex
MSC	Mesenchymal Stromal Cell
PAD	Peripheral Arterial Disease
PCR	polymerase chain reaction
PHC	Proangiogenic Hematopoietic Cell
PI	Principal Investigator
SAE	Serious adverse event
SDF-1	Stromal Cell Derived Factor-1
TcPO2	Transcutaneous oxygen pressure

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Critical limb ischemia (CLI) is the endstage of atherosclerotic peripheral arterial disease (PAD) and occurs when there is insufficient blood flow to meet the resting metabolic demands of the leg. The clinical features of CLI are rest pain and gangrene, both heralding signs of limb loss.[1] Despite advances in surgical and endovascular revascularization techniques, approximately 40% of patients with CLI will require amputation of the affected extremity within one year.[2,3] Most surgeons approach amputation in neurologically intact patients with the goal to save as much limb length as possible to promote independent ambulation with a limb prosthesis. Consequently, below knee amputation (BKA) is the most common amputation procedure performed in the U.S.[4,5] However, contemporary studies and review of our own series demonstrate conversion rates from a BKA to AKA as high as 19% with the primary cause being ischemic wound complications of the myocutaneous flaps below the knee.[6,7] The physiological consequences of loss of the knee joint with AKA, as compared to BKA, is a 40-60% increase in energy expenditure in ambulation with a prosthesis.[8] This excessive effort results in a decreased rate of independent ambulation after AKA as compared to BKA (< 33% vs. > 65%, respectively) and translates into decreased quality of life, increased institutionalization, and increased health care costs for AKA patients.[9,10] Thus, a procedure that may improve perfusion and promote wound healing of a lower extremity amputation stump may provide significant benefits to this patient population.

Bone marrow derived progenitor cells have been shown to 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.[11] For example, evidence supports the hypothesis that the predominant mechanisms driving tissue repair in a heart post-infarction is angiogenesis orchestrated via the release of stem-cell derived paracrine factors.[12,13] Cumulative data regarding the bioactivity of bone marrow cells has been derived entirely from animal models of disease despite the fact that more than 120 clinical trials listed in *clinicaltrials.gov* use bone marrow derived cells for therapeutic endpoints. Furthermore, the translation of these 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.[16] To date, no clinical trials have been designed with the ability to harvest human tissue post-cell implantation at time points relevant to their tissue reparative activity and survival. Thus the *in vivo* biological activity and survival of transplanted bone marrow derived cells in diseased human tissue remains undefined.[14,15]

The BioCue™ Blood and Bone Marrow Aspirate (BMAC) Concentration System is used to process blood and bone marrow aspirate to produce an autologous PRP output for use in autograft and/or allograft therapies. The BioCue™ device is the exact same device as the MarrowStim™ PAD Kit that was used to treat subjects in prior versions of this protocol. Treatment includes removal of approximately 300 mL of bone marrow from the patient's posterior or anterior iliac crest, which is then mixed with an anticoagulant solution and processed in the BioCue™ device to concentrate the autologous bone marrow cell suspension for delivery. The subsequent treatment utilizes this BMAC at the point of care by injection to multiple sites in the affected leg. The BioCue™ BMAC product is hypothesized to function via angiogenic mechanisms. To date there are no comprehensive descriptive analyses of the fate of BioCue™ BMAC cells after transplantation in human tissue. Thus we propose a pilot trial, **Safety and Efficacy of Concentrated Autologous Concentrated Bone Marrow Aspirate (BMAC) in Preventing Wound Complications in Amputations (The MarrowCHAMP Study),** that will help discern the mechanisms of therapeutic action in man. MarrowCHAMP will address two critical areas simultaneously:

assessing the efficacy of BMAC in preventing wound complications in lower extremity amputations and providing tissue at BioCue™ injections sites from a controlled time-point for first in man biochemical and histological analyses.

2 DESCRIPTION OF THE INVESTIGATIONAL PRODUCT

2.1 Overview

The BioCue™ Kit contains six (6) disposable BioCue™ Separator for the preparation of autologous concentrated bone marrow aspirate (BMAC) at the patient's point of care. The BioCue™ Kit can be used with any legally marketed bone marrow aspiration (BMA) device and associated delivery accessories, provided separately (Table 1). The BioCue™ Kit is used in conjunction with the Biomet Biologics Centrifuge, a table-top swinging bucket centrifuge.

Table 1. Aspiration Devices and Delivery Accessories Compatible with BioCue™ Kit

Description	FDA Clearance
H.S. Hospital Service Bone Marrow Biopsy Needle, or equivalent	K013070
Ranfac Bone Marrow Aspiration Needle, or equivalent	501(k) Exempt Product Code GAA
StemCor Systems MarrowMiner Biopsy Instrument, or equivalent	K071732
BD 60 ml Luer Lock Syringe, or equivalent	K980987
BD 30 ml Luer Lock Syringe, or equivalent	K980987
BD 10 ml Luer Lock Syringe, or equivalent	K980987
BD 22 G x 1 1/2 in. hypodermic needle, or equivalent	K021475
Terumo 23 G x 1 1/2 in. hypodermic needle, or equivalent	K771203
Merit Medical 20 ml Luer Lock Syringe, or equivalent	K981417

The BioCue™ Platelet Concentration Kit is indicated for use in the clinical laboratory or intraoperatively at the point of care for the safe and rapid preparation of platelet poor plasma and platelet concentrate (platelet rich plasma, or PRP) from a small sample of blood and bone marrow mixture. The design, materials, and processing methods are identical to the previously cleared GPS® II Platelet Concentration System in BK090008 and to the MarrowStim™ Concentration Kit and MarrowStim™ Mini Concentration Kit in K071934. The BioCue™ Separator is also identical in design to the MarrowStim™ Separators previously utilized in this investigation.

The BioCue™ Separator (**Figure 1**) has a 60 ml capacity and includes a density-tuned dual buoy separation system designed for the isolation and separation of nucleated cells.

Figure 1. BioCue™ Separator

The use of the BioCue™ device and Zimmer Biomet Biologics centrifuge to concentrate autologous bone marrow aspirate is illustrated in **Figure 2**. Anticoagulated bone marrow aspirate (60 ml) is loaded into the separator through the center port and covered with the white cap (left picture). The separator is placed into the centrifuge and spun at 3,200 RPM for 15 minutes (center picture). During centrifugation, the marrow's nucleated cells collect between the two buoys within a fixed volume of fluid (6 ml). The red blood cells pack below the bottom buoy and the remaining plasma collects above the top buoy. Following centrifugation, the concentrated bone marrow aspirate (BMAC - right picture) containing the nucleated cells is re-suspended within the fixed volume of fluid. The BMAC is then recovered from the separator by connecting a syringe to the red capped port and extracting the contents.

Figure 2. Separator (left); Centrifuge (center); BMAC (right)

The operating specifications for the Zimmer Biomet Biologics centrifuge are provided in **Table 2**.

Table 2. Operating Specifications for Zimmer Biomet Biologics Centrifuge

Principle of Operation	Separation based on density of liquids
Table Top	Yes
Refrigerated	No
Swinging Bucket	Yes
Automatic Decanting	No
Micro-Processor Controlled	Yes
User Programmable	Yes, preset program automatically displayed when switched on
Speed Control	Preset, adjustable
Acceleration and Braking	Current-controlled
Maximum RPM	3,500 RPM
Maximum RCF	2,200 g
Tube Capacity	Four 60ml disposable tubes
Imbalance Detector	Yes
Construction	Anti-torsion construction, metal housing and rotor

2.2 Proposed Intended Use Statement

Human bone marrow preparations induce angiogenesis, decrease muscle fiber apoptosis, and stimulate re-epithelialization of wounds in ischemic hindlimb models in mice. Prior to amputation, bone marrow aspirate will be isolated from patients and concentrated using a BioCue™ device (BMAC), thus presenting a more clinically feasible and potent cell population that may improve tissue perfusion and mitigate ischemic wound complications after amputation.

Concentrated, enriched, autologous, bone marrow aspirate is offered as an option to prevent wound complications after amputation. The BioCue™ device is intended for investigational use only to concentrate BMA by selected investigators familiar with their use and experienced in conducting clinical studies. Human BMAC may only be administered IM to human subjects participating in clinical studies sponsored/approved by Indiana University School of Medicine, and who meet all inclusion criteria and have provided formal written consent.

3 STUDY OBJECTIVES

The principal objectives of this study are as follows:

Determine the safety and explore efficacy of BMAC in preventing wound complications in amputations. In a Phase I trial, BMAC (up to 5 mL) will be injected into the ATM below the point of amputation in the index limb in an area of approximately 3 x 10 x 3 cm. At the same time, BMAC (1 mL) will be injected at approximately 25 sites in the proximal leg to promote tissue perfusion before

amputation (**Figure 4**). Patients will be scheduled for amputation at approximately one week post injection. Safety will be evaluated by review of treatment related Adverse Events during the 12-month follow-up period. Efficacy will be assessed by rates of wound complications and revision of amputation to historical controls at our institution to assess trends in therapeutic efficacy.

Characterize the host tissue response to, paracrine activity of, and fate of transplanted BMAC. Patients will undergo amputation and injection sites at the anterior tibial muscle (ATM) will be harvested at that time. Collection of up to 10 cm of the soleus muscle will serve as control. IHC staining will determine capillary density and local host immune responses. Angiogenic and inflammatory cytokines will be quantified using a multiplex array system and quantitative PCR.

The expected outcomes will provide the framework for the design of a Phase II randomized clinical trial and critical insights that may direct a future trial to test multi-dosing regimens. 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 survival. [16]

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. Peripheral nerve tissue (sciatic, femoral, tibial, sural and/or plantar nerve segments) will be collected at the time of amputation from the amputated portion of the lower limb. 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.

4 STUDY OVERVIEW

4.1 Study Approach

This protocol is designed to describe a Phase I single center open label trial study that will enroll up to 12 patients requiring semi-elective amputation within a 30-day period for complications related to CLI.

4.2 Study Duration

It is expected that the study will be completed within 5 years. Each patient will be followed for 52 weeks.

5 STUDY POPULATION

5.1 Sample Size and Target Study Population

5.1.1 Sample size

The total number of individual subjects is expected to reach approximately twelve (12).

5.1.2 Study population

The study population will be representative of adults at least 40 years of age who require major amputation, as determined by an independent vascular specialist. It is anticipated that Indiana School of Medicine will enroll up to 12 qualifying adults for this study.

5.1.3 Alignment with intended study population

The study population includes patients likely to benefit BMAC transplantation.

5.2 Recruitment Methods

5.2.1 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 not be offered, and no subject recruitment materials will be used. Each subject will be informed that no personally relevant clinical information will be derived from the collected data. Medications will be documented, and certain medications may be held. 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.

5.2.2 Duration of study activities

Enrollment is anticipated to continue for approximately 3 years. In the event that additional studies are going to be conducted, new protocols will be developed specifically for those studies.

5.3 Patient Selection

The eligibility criteria for prospective enrollment of subjects are shown in **Table 1**.

Table 3. Inclusion/Exclusion Criteria for Enrollment of Subjects

Inclusion criteria	1. Be ≥ 40 and ≤90 years of age. 2. Patients requiring lower extremity major 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 30 days after screening, as determined by an independent vascular or orthopedic surgeon. 5. 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 (ACS) 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. 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.

6 STUDY MATERIALS

6.1 Investigational Product

6.1.1 Identity of the investigational products

The BioCue™ Platelet Concentration Device

6.1.2 Safety issues

Safety of the BioCue™ device to concentrate BMA for the prevention of wound complications in amputation will be determined by review of treatment related adverse events (AE) and serious adverse events (SAE) during the study period of 12 months by the Data Safety Monitoring Board (DMSB) appointed by the Clinical and Translational Sciences Institute (CTSI) at the Indiana School of Medicine or Data Safety Committee (DMC) designated by the Principal Investigator.

The BMAC dose proposed in this trial was selected based on prior clinical trials.[17] We may not see any evidence of efficacy in our composite endpoint, which may reflect an inadequate dose or sample size. In this case we would propose a future study exploring escalating doses of BMAC. In the instance of evolving infection, we would re-assess our inclusion/exclusion criteria and evaluate the contribution of pre-amputation procedures on the lower leg. Patients may request earlier amputation due to worsening rest pain or anxiety. Consideration for amputation sooner than one week post injections will be determined by the PI based on the severity of the subject's condition, so as to offer this research as an option to all eligible subjects.

6.1.3 Handling, storage, accountability

Autologous heparin-anticoagulated bone marrow aspirate (360 ml) is loaded into 6 Biocue™ separators through their center ports. The separators are placed into the centrifuge and spun at 3,200 RPM for 15 minutes. Following centrifugation, the BMAC containing the nucleated cells is re-suspended within the fixed volume of fluid. The BMAC is then recovered from the separator by connecting a syringe to the red capped port and extracting the contents. All steps (marrow harvest, BioCue™ processing, BMAC administration) are performed at the point-of-care within a single procedure.

Output characterization will be performed on a sample from a single BioCue™ separator chosen randomly. A sample will be pulled from one tube, assuming it is representative of all tubes. The number of cells will be enumerated from one separator and multiplied by six (6). In the case that a positive culture should result from the BioCue™ product, the PI will notify the patient and schedule an examination within 48 hours. The patient will be assessed for evidence of infection including evidence of erythema at the site of the injection, fever (temperature > 100.9 F), and/or leukocytosis (white blood count >11K). In the event of these clinical and laboratory findings, the appropriate antibiotic or antifungal agent will be instituted based on the culture results and recommendations of an Infectious Disease specialist. Follow up examinations and laboratory work will be scheduled based on the severity of symptoms and laboratory findings at the discretion of the PI and per Indiana University School of Medicine procedures.

Sterility testing of the BioCue™ output will be performed in accordance with 21 CFR 610.12. This information will be recorded on a source document and kept in the subject's research chart. Bone

marrow samples remaining after cell characterization may be subject to additional assessments by outside laboratories. Any samples sent outside the site will be identified by subject number only and will not contain any identifying information.

All sterility failures will be reported to the IRB and FDA as adverse events within 30 calendar days. The report will include results of the investigation into the cause of the event and any and all corrective actions taken to mitigate risks to future study subjects.

Following the BioCue™ procedure, laboratory analyses will be performed on unconcentrated and concentrated bone marrow aspirate samples. Total white blood cell (WBC) count, mononuclear cell (MNC) count, mononuclear cell recovery (concentrated MNC/unconcentrated MNC), granulocyte count, platelet count, cell viability, and cell purity (MNC/WBC) will be recorded. In addition, total aspiration volume, total BioCue™ input volume, total BioCue™ output volume, and total delivery volume will be recorded. Additional information, such as CD marker characterization, may also be performed.

6.1.4 Required training

Operating room personnel will be instructed on the use of the BioCue™ Kit and the collection and transfer of bone marrow preparations. The study coordinator will be trained in the operation and management of the centrifugation system.

6.1.5 External studies

No additional studies are planned at this time.

6.2 Other Study Materials

6.2.1 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.
- Hank's Balanced Salt Solution
- Sodium Heparin

6.2.2 Materials to be provided by device manufacturers

1) The BioCue™ Platelet Concentration Device

6.2.3 Materials to be provided by external testing facilities

There are no external testing facilities associated with this study. The cellular characterization work will be performed at the Indiana University School of Medicine core flow cytometry laboratory.

7 STUDY PROCEDURES

7.1 Workflow

The necessity for amputation will be determined by a vascular or orthopedic surgeon not participating in the study. After that decision has been rendered, the patient will be screened for enrollment. After enrollment, 12 patients requiring major amputation for CLI will be scheduled for amputation approximately one week post-BMAC injection in the ATM. Autologous BMA will be retrieved per standard institution procedures. The BioCue™ device will be used per manufacturer instructions. Patients will receive IM injections of BMAC into the ATM below the point of amputation and up to 25 mL

of BMAC in the proximal leg above the point of amputation. Delivery of up to 25 sites of up to 1 mL each of BMAC will be performed in 2 cm increments along the lateral and medial aspects of the upper leg and gastrocnemius to ensure an even distribution. Patients will then undergo amputation approximately one week after injections. Patients will be examined at Baseline, BMAC Injection Day, Day of Amputation, Day 3 post-op amputation, and Weeks 2, 6, 12, 18, and 24 after amputation. At any time during a subject's participation, photographs of current wounds (as applicable at baseline visit) and photographs of the subject's amputation site (at follow-up visits) may be taken to assess wound healing. There will be a week 52 phone call to assess the patient for primary endpoints. Incidence of wound infection, minor (debridement of skin), and major revision (debridement of muscle and bone) of the amputation stump, and revision of amputation will be recorded. Rates of revision of amputation at 12 months will be compared to historical controls. The initiation of treatment will be staggered by a minimum of one week for the first 3 subjects to ensure that potential study treatment-related risks to subjects are minimized.

Determine the safety and explore efficacy of BMAC in preventing wound complications in amputations. In a Phase I trial, BMAC (up to 3 mL) will be injected into the ATM below the point of amputation in the index limb. At the same time BMAC (1 mL) will be injected at up to 25 sites in the proximal leg to promote tissue perfusion before amputation (**Figure3, Figure 4**). Patients will be scheduled for amputation approximately one week post-injections. Safety will be evaluated by review of treatment related AEs during the 12-month follow-up period. Efficacy will be assessed by rates of wound complications and revision of amputation to historical controls at our institution to assess trends in therapeutic efficacy.

Figure 3. Scheduling Scheme for Amputation Date and MSC Injections

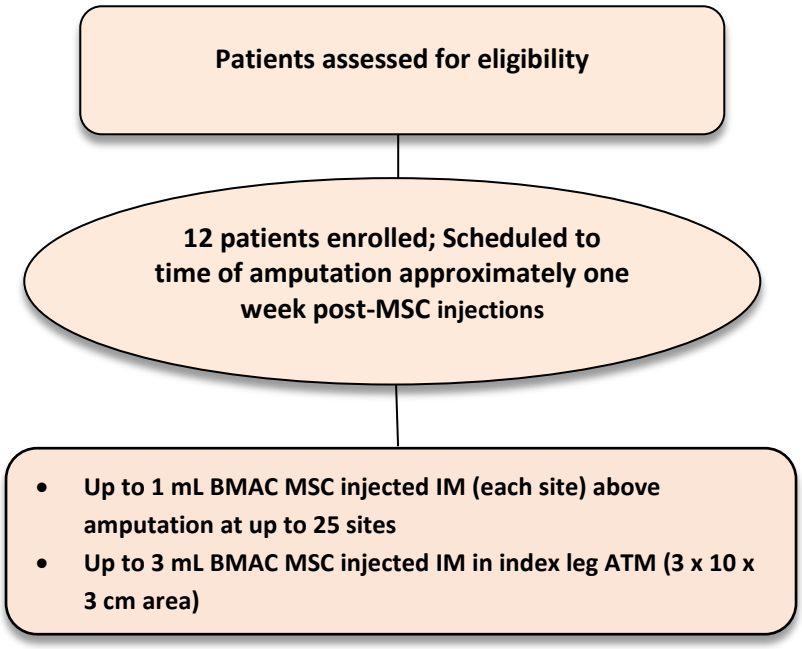

Figure 4. Diagram of Injection Sites of BMAC in Below Knee Amputations (BKA).

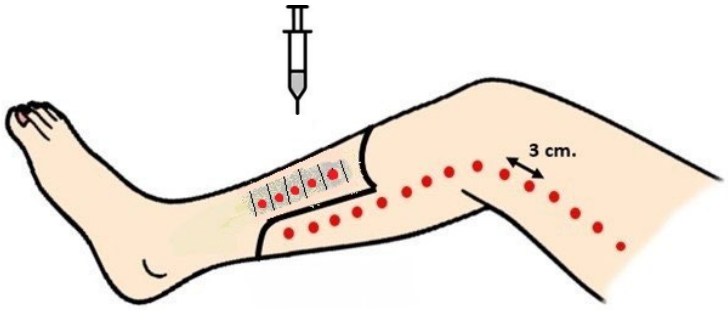

Figure 4.

Upper Leg and Gastrocnemius Muscle Injections: BMAC will be injected 2-3 cm. deep into the muscle at approximately 15 sites 3 cm apart medially and approximately 10 sites laterally, delivering 1 mL of BMAC at each site.

ATM Injections: BMAC (up to 3 mL) will be injected into the ATM below the anticipated point of amputation in up to 5 sites within the area of 3 x 10 x 3 cm.

BMAC Administration: Anterior Tibialis Muscle.
On the day of BMAC injection in the operating room of the Richard L. Roudebush VA Medical Center, the index limb will be marked for amputation. The injection site area is marked on the anterior compartment of the lower leg below the point of amputation. The BioCue™ product is injected along a 3 cm tract into the anterior tibialis muscle (ATM). The injection site(s) will be marked with permanent ink on the ATM. The BMAC suspension will be injected over 3 minutes with the needle retracted 5 mm simultaneously to permit infiltration into the muscle.

Ability to Recover BioCue™ Injection Sites in Anterior Tibialis Muscle.
Retrieval of the BioCue™ injection sites is accomplished at the time of amputation. The area demarcated by the surgical marker is excised for histological analysis.

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.

Baseline Screening and Follow-Up Schedule. The following evaluations will be carried out at baseline to determine if the patient is eligible for study: (1) Baseline blood tests (complete blood count, serum chemistries, eGFR, hsCRP); (2) medical history and physical exam; (3) concomitant medications; (4) pregnancy test for women of childbearing age; and (5) 12 lead ECG; and (6) LEADs and/or TcPO2 of index leg, as indicated. In the interest of time to major amputation, we may collect data retroactively to determine eligibility. If eligibility can be determined based on recent prior clinical notes and laboratory values, we will use those values for our study purposes and omit corresponding tests from the baseline

evaluation. It will not be considered a protocol deviation if labs are not finalized prior to cell injection and amputation; subjects will still be able to participate in the study.

Determination of survival of BioCue™ cells and Quantify angiogenic activity of transplanted BMAC cells. Harvested ATM from each time point fixed in 4% paraformaldehyde will be embedded in paraffin and sectioned (5μm thickness). Using IHC, sections of ATM will be stained with fluorescent monoclonal antibodies against CD34 and CD133 to quantify Proangiogenic Hematopoeitic Cells (PHC) at each injection site and each time point. We will further enumerate PHCs in ATM using multiparametric flow cytometry (MFC) for $CD133^{+}CD34^{+}CD45^{+}CD16^{-}CD14^{-}CD235^{-}$ cells, as previously described.[16,17] Hypoxia-inducible factor-1α (HIF-1α), will be quantified and localized in ATM sections with immunostaining, as previously performed in ischemic human muscle.[16] mRNA for HIF-1α, VEGFF, PDGF, and FGF will be quantified using qPCR.[16] Chemokine protein expression from muscle homogenates and plasma from blood samples will be further quantified with multiplex chemokine analyte panels and liquid chromatography-mass spectrometry (LC-MS), focusing on Angiogenin, Angiopoietin-1, Angiopoietin-2, Endostatin, FGF (acidic and basic), PDGF-AA, PDGF-BB, PIGF, Thrombospondin-2, VEGF, VEGF-D, Activin, Follistatin, GCSF, TGF-B, SDF-1, and HGF, in addition to other proteins associated with the BioCue secretome, as previously reported.[15]

Capillary density will be quantified using IHC for CD31. Angiogenic and inflammatory cytokines will be quantified using multiplex protein arrays from muscle homogenates and peripheral blood samples (collected at baseline through week 24 follow up visit). Comparison between BioCue™ injection sites and controls will be made. Levels of circulating cytokines from peripheral blood will be compared to baseline. Changes in skeletal muscle morphology will be described as compared to healthy controls. It will not be considered a protocol deviation if peripheral blood specimens are missed or outside the window ranges of study visits.

Table 4. Schedule of Events

Procedures	Baseline Visit 1	BMAC Injection Visit 2	Amp Day Visit 3	Day 3 F/U + 5 days Visit 4	Week 2 F/U +/- 2 days Visit 5	Week 6 F/U +/- 4 days Visit 6	Week 12 F/U +/- 7 days Visit 7	Week 18 F/U +/- 2 weeks Visit 8	Week 24 F/U +/-2 weeks Visit 9	Week 52 Phone Call
Informed Consent	X									
Medical History	X									
Physical Exam	X	X	X	X	X	X	X	X	X	
Vital Signs	X	X	X	X	X	X	X	X	X	
Con. Medications	X	X	X	X	X	X	X	X	X	
AE/SAE Evaluations		X	X	X	X	X	X	X	X	
Laboratory evaluations	X[1]				X[2]	X[2]	X[2]	X[2]	X[2]	
12 Lead ECG	X				X		X		X	
LEADs / TcPO2	X[3]				X[4]	X[4]	X[4]	X[4]	X[4]	
Photograph of index leg / amputation site	X[5]				X[6]	X[6]	X[6]	X[6]	X[6]	
Schedule Amputation	X									
Research Blood Specimen[7]		X	X		X	X	X	X	X	
Evaluate for infection, gangrene				X	X	X	X	X	X	X
Evaluate necessity for amputation revision				X	X	X	X	X	X	X

Screening Laboratory Tests[1]: CBC w/diff/plt; CMP (Sodium, Potassium, Chloride, CO2, Glucose, Calcium, BUN, Creatinine); eGFR, hsCRP

Baseline only: Serum pregnancy testing (childbearing females)

Follow-up labs[2]: hsCRP

TcPO2: Transcutaneous Oxygen Pressure; LEADs or Foot TcPO2[3] will be collected as a baseline measurement, TcPO2 of medial and lateral stump site[4] will be collected at follow up visits

Photographs[5,6]: Index leg at baseline[5], BKA stump site at follow up visits[6]. It is not considered a protocol deviation if photographs were not performed.

Research blood specimens[7]: Collected prior to induction of surgery (BMAC Injection Day, and Amputation Day) from peripheral vein of up to 30 mL, and at follow-up visits at weeks 2 through week 24. It is not considered a protocol deviation if research blood collection is not performed.

7.2 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, subjects must be at least 40 years of age.

7.2.1 Collection of data

At the discretion of the Investigator and subject to Indiana University School of Medicine requirements, data will be collected from each eligible subject providing signed informed consent.

7.3 Procedures for Study Closure

7.3.1 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.).

7.3.2 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 and research related event based on the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0. Enrollment will be temporarily suspended for any related or possibly related post-BMAC injection and post-amputation infection, bleeding or tissue reaction at the index site that are ≥ Grade 3. 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 with an explanation of the circumstances that led to termination.

8 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.

9 STATISTICAL METHODS

9.1 Determination of Sample Size

The study is not powered for efficacy in preventing wound complications of revision of amputation. The maximum number of tissue specimens we hope to collect will be twelve (12). Quantification of BioCue™, capillary density and cytokines will be compared between injection sites (between escalating doses and control) using a paired Student's t-test. Serum cytokine levels post BioCue™ injection will be compared to baseline levels using a paired Student's t-test.

9.2 Bias Minimization

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

9.3 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.

The data management center for this trial will be at the CTSI, or a Data Monitoring Committee (DMC) will be appointed by the Principal Investigator. We will use a web-based HIPAA compliant online system for data entry and data management, as we are currently doing in our current clinical trials. Data will be collected from each enrolled patient using a comprehensive case report form and uploaded into a specifically designed database in the OnCore Enterprise Clinical Trial Management System and VA REDCap system. All the data will be analyzed and generated using SAS.

Safety Assessment. Primary attention will focus on the safety of the administration of BMAC as measured by treatment-related AEs. Treatment-related AEs 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 AEs may appear in more than one category. Within each of these categories AEs 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 3 groups will be calculated. Since previous trials have not reported AEs with BMAC 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.

Exploratory Efficacy. Continuous confidence intervals at the 95% level will be constructed to explore the effect of administration of BMAC on the composite endpoint at 6-months of death, revision of amputation 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.

Quantities over time of BMAC will be fit to an exponential decay curve using a residual pseudo-likelihood procedure and cell half-life (λ) will be estimated.[18] Binomial confidence Intervals at the 95% confidence level and *p*-values for these four groups after BMAC transplantation will be calculated for the presence or absence of MHC expression and SDF-1 activation. Continuous confidence intervals at the 95% level will be constructed to explore the differences among the time-tiered administration of BMAC 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.[19]

10 ADVERSE EVENT REPORTING

10.1 Adverse Events

Reporting of adverse events will comply with federal regulations for IDE safety reporting. AEs 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.

Serious adverse events (SAEs) 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 72 hours upon discovery. SAEs are defined under current cGCP guidelines as events that result in one or more of the following:

- life-threatening illness or injury;
- permanent impairment of a body structure or a body function;
- medically necessary in-patient hospitalization;
- medical or surgical intervention necessary to prevent permanent impairment to body structure or function; or
- fetal distress, fetal death, or congenital abnormality.

Serious events that affect the rights, safety, or welfare of subjects must be documented on a form and be reported within 72 hours to Indiana University School of Medicine and to the Investigator's IRB according to that IRB's policies.

Any potential deficiencies with the BioCue™ devices (including, but not limited to deficiencies in packaging, labelling, and/or device processing) will be reported to Zimmer Biomet for evaluation.

10.2 Sponsor Contact for SAE Reporting

Michael P. Murphy, MD
1801 N. Senate Blvd.
MPC2, #3500
Indianapolis, IN 46202

11 RISK ANALYSIS

11.1 Potential Risks of the Investigational Product and Clinical Investigation

The types of risk associated with IM injection are notably rash, mild fever, and myalgia. These risks are mild and rare. These risks are all stated in the consent form.

Further risks include pain, bleeding, and infection following the surgical procedure. Additionally, as with any other surgical procedure, the investigational device may be dropped in the surgical theater. Flash sterilization will not be allowed, and additional sterile devices will be available to prevent procedure termination with minimal delay.

11.2 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.

11.3 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.

An additional risk is the event that a syringe containing the bone marrow product or the centrifugation tube is dropped. In this case the syringe or tube would be recovered, swabbed externally with alcohol, the needle/cap removed and replaced with a sterile needle, and then the sterile contents containing the bone marrow product will be emitted into a sterile container on the sterile surgical field and reloaded into a new sterile 10 cc syringe and needle. The dropped syringe will be discarded and not contact the sterile field(s). In the rare event that the investigator feels that the bone marrow product comes into contact with the non-sterile environment then the bone marrow product will be discarded, which is a total volume of 6 mL of the planned 30 mL to be injected. Additional bone marrow will not be aspirated and the patient will receive 25 mL rather than 30 mL of bone marrow product. It must be emphasized that the sterile field encompasses the area of the patient's limb continuous by sterile drapes to the operating room table where the bone marrow product is loaded into syringes, thus the likelihood of the syringe contacting a non-sterile surface is minimal. Likewise, the centrifugation tube is a closed sterile system with fixed caps and should it fall to the floor the contents would remain in the closed sterile system and recovered as described.

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.

12 INVESTIGATOR RESPONSIBILITIES

12.1 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 themselves 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;
- 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.
- Investigators, primary care physicians, and all enrolled diabetic subjects follow the American Diabetes Association guidelines for diabetic care throughout the trial.

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.

12.2 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.

12.3 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 cGLPs, 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.

12.4 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.

12.5 Deviations from the Study Protocol

An Investigator may not 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. If research blood draws (Research Blood Specimen†) are not obtained at time points specified in the schedule of events, they will not be considered protocol deviations.

12.6 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.

12.7 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.

13 ETHICS AND COMPLIANCE

13.1 Informed Consent and De-Identification

13.1.1 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 authorized study personnel 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.

13.2 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 GCP 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) (if applicable) 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.

13.3 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.

13.3.1 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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