Quantification of Peripheral Hematopoietic Progenitor Cell Circulation Following Repeated Acute, Vascular Restriction Resistance Exercise Using the Delfi Tourniquet System

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Table of Contents

Abbreviations and Definitions

AE Adverse Event

ANOVA Analysis of Variance

AREF Andrews Research and Education Foundation

BFR Blood Flow Restriction

CBC Complete Blood Count

Cc Cubic Centimeter

CD Cluster Differentiation

CFR Code of Federal Regulations

EDC Electronic Data Capture

G-CSF Granulocyte Colony Stimulating Factor

HIPAA Health Insurance Portability and Accountability Act

HIT High-Intensity Training

HPC Hematopoietic Progenitor Cells

HSD Tukey's Honestly Significant Difference

HSC Hematopoietic Stem Cells

ICF Informed Consent Form

IRB Institutional Review Board

mL Milliliters

MSC Mesenchymal Stem Cells

NIRS Near-infrared Spectroscopy

NSAIDS Non-steroidal anti-inflammatory drugs

PI Primary Investigator

SAE Serious Adverse Event

SAS Statistical Analysis System

UP Unanticipated Problem

1-RM One-repetition Maximum

1 Scientific Rationale / Background

Intense interest in the areas of orthopedic surgery and recovery science has been placed on progenitor cell research. The ability to transplant these cells in successful patient engraftments has been sought after due to properties of hematopoietic stem cells (HSCs) that allow for long-term self-renewal and multipotency (1). The obstacle of an immunocompetent immune system has been a source of difficulty for some time within the scientific community (2). Using stem cells from different regions of the same patient prevents the need for long-term immune system suppression (2, 3). Currently, HSCs are collected through two primary methods: bone marrow aspiration and peripheral harvest following pharmaceutically induced mobilization using granulocyte colony-stimulating factor (G-CSF). These methods suffice but less invasive methods are preferred. By quantifying peripheral hematopoietic progenitor cell presence within the blood following repeated blood flow restriction exercise, we hope to uncover a more convenient method for harvesting these hematopoietic progenitor cells. We aim to accomplish this with the aid of exercise-induced elevation of progenitor cell concentration in peripheral blood (4, 5). Prior studies with BFR and stem cell mobilization have shown increased cell concentration immediately post a single bout of exercise (6).

Also present within peripheral blood following exercise are adult mesenchymal stem cells (MSCs). MSCs can monitor their local and systemic environments for pertinent stimuli. These stimuli such as often determine the fate and commitment of MSC differentiation (7). MSCs utilize paracrine effects to conduct this interaction with their surrounding (8). MSCs can also respond to changes in environmental conditions and hypoxia, as seen in animal studies (9), as well as exercise, seen in human studies (4). MSCs have the same primary methods of collection as HSCs.

Blood Flow Restriction Therapy (BFR) has been successfully implemented during the post-operative rehabilitation period, for patients with osteoarthritis or muscle atrophy, and in instances of limb salvage following injury (10-13). This type of training has been shown to also be effective in producing hypertrophic skeletal muscle growth that can parallel growth seen in High-Intensity Training (HIT), but without the mechanical muscle damage that occurs with HIT (13-18). Blood flow restriction training is an alternative method to traditional exercise for the goal of retaining and increasing muscle mass without the musculoskeletal stress placed on the body by traditional training methodologies. The application of BFR uses low-intensity resistance training with high repetition volume to mimic the exertion required of the body by higher-intensity, lower-repetition training (19).

The mechanism by which BFR training acts is not fully understood. Some of the leading theories for the mechanism are the metabolite theory, which includes, increased cellular swelling through the accumulation of metabolites and blood within the muscle cells, increased protein synthesis and growth hormone production, elevated metabolic stress,

and heightened motor unit recruitment (20, 21). The influx of blood carrying metabolites during BFR to the area of injury facilitates elevation of growth hormone levels locally (21, 22). Local accumulation of metabolites has been indicated to influence the production of growth hormone more so than total metabolite production. (23, 24). These growth hormones can exhibit an anabolic effect on muscle. Increased metabolic stress results in the local accumulation of lactate, hydrogen ions among other metabolic byproducts (25, 26). Analysis of BFR from a physiological perspective have been consistent in determining that levels of lactate and growth hormone increase following BFR training, ultimately peaking in a time window immediately to 40 minutes after BFR (23, 27-29). Exercise using BFR has also been linked to extremely low oxygenation saturation (StO2) levels within the skeletal muscle (<10%) as measured via near-infrared spectroscopy (NIRS). This finding suggests that the working tissue experiences severe hypoxia during BFR exercise (30). There have been inconsistent findings on if exercise within this hypoxic state is better suited to stimulate greater gains in muscle strength and physiologic structure change (31, 32). Further investigation on muscular and physiologic response to hypoxic training conditions could provide insight into a new way of conditioning the body without needing excessive load, a more ideal approach for the therapeutic patient recovery process. The final theory behind the action mechanism of BFR is motor unit recruitment. Motor unit recruitment using Henneman's size principle theorizes that motor unit recruitment within a motor pool begins with the smallest motor unit(s) and progresses to larger and larger motor units depending on the targeted force. Recruiting progressively higher threshold motor units is influential in generating muscle hypertrophy as external load determines motor unit recruitment (21). This idea paired with accompanying adjustments in repetitions has been at the core of much of the traditional approaches to hypertrophy development within musculature. Thus, the aim of this study is to quantify the cellular content *(in vitro)* of mobilized hematopoietic stem cells extracted from peripheral blood before exercise and at 0-, and 20-minute intervals following exercise. This study will be useful in the continued advancement of localized, peripheral hematopoietic progenitor cell research and patient application, development of effective non-surgical or post-surgical therapeutic treatments, and incorporation of knowledge in the implementation of performance training.

2 Study Design

The proposed study is a prospective, quasi-experiment, dual-center (laboratory and exercise facility) study involving 10 healthy male and 10 healthy female volunteers. A potential subject must clear the screening, consent to the procedures of this study, and complete the medical interview before proceeding with the familiarization session. The familiarization process will include an introduction to the Delfi BFR Tourniquet System (Delfi Medical Innovations Inc., Vancouver, BC) and the exercises (seated leg extension,

semi-reclined leg press, prone hamstring curl) that the subject will complete. The weight each subject will use for each exercise will be determined during this time. All familiarization procedures must be completed prior to the scheduling and execution of the first of twelve experimental testing sessions.

The experimental testing sessions will occur twice a week for six weeks (12 sessions) and will involve the BFR training utilizing the Delfi BFR Tourniquet System, as further described in the Experimental Testing Session section of the treatment plan (6.2). At the beginning of the first, the sixth, and the twelfth testing sessions there will be a blood draw requiring 6 cc of blood to be taken prior to exercise. Post-exercise, at each testing session, additional 6cc blood draws will be conducted immediately post exercise (time point 0), and 20 minutes after the conclusion of the workout. Following every testing session workout, finger-prick blood samples for lactate testing will be taken. These blood samples are to be taken at Time point 0- (T 0), 10-, and 20-minutes following exercise. Once the twelfth experimental testing session has been completed, the subjects will have one final session that is to take place no more than five days following the twelfth testing session. This final session will include one final blood draw of 6 cc. Throughout the duration of the study, the blood drawn will be used for obtaining a complete blood count (CBC) and for cellular analysis to quantify peripheral hematopoietic progenitor cell concentration. The blood samples collected from subject's fingers throughout the study will be used for analysis using a portable lactate analyzer. Lactate analysis will allow researchers insight into each subject's exertion level reflected by the amount of lactate found within the samples collected.

3 Objectives

3.1 Primary Objective

The primary objective of this study is to quantify the cellular content *(in vitro)* of mobilized hematopoietic stem cells and CBC data extracted from peripheral blood before exercise and at 0-, and 20-minute intervals following exercise.

3.2 Secondary Objectives

Post-exercise lactate levels and demographic information will be recorded to provide insight into the fitness development and endurance shift of each subject. Each peripheral blood sample will also be analyzed for MSC concentration if enough sample is available after primary testing.

3.3 Primary Effectiveness Endpoint

The primary effectiveness endpoint will be the quantification of mobilized hematopoietic stem cells and CBC data extracted from peripheral blood before exercise and at 0- and 20-minute intervals following exercise.

3.4 Secondary Effectiveness Endpoints

Secondary effectiveness endpoints include post-exercise lactate levels, participant demographics, and MSC marker concentrations.

4 Hypotheses

4.1 Null Hypothesis:

H_0 - Subjects will have equal levels of hematopoietic progenitor cells (HPCs) before and after undergoing twelve experimental testing sessions with the Delfi BFR tourniquet system.

4.2 Alternative Hypothesis:

H_1- Subjects will not have equal levels of hematopoietic progenitor cells (HPCs) before and after undergoing twelve experimental testing sessions with the Delfi BFR tourniquet system.

5 Participant Recruitment and Screening

The research study team intends to enroll a total of 25 volunteers (with 5 expected screen fails or withdraws)

Subject recruitment will occur through the Andrews Institute physician practices and physical therapy departments via word of mouth. Recruitment of subjects will also utilize a flyer displaying the opportunity for subjects to enroll in this study. Flyers will be placed in easily viewed spaces around the Andrews Institute campus in Gulf Breeze, FL. Potential study subjects will undergo a prescreening process covering inclusion and exclusion criteria through standard of care medical evaluations. Potential subjects meeting all inclusion criteria will have the study described to them by a member of the investigating team. This description will include potential risks, discomforts, and benefits. Subjects will then be provided an opportunity to read and sign an approved informed consent form. The subject will be provided sufficient time to consent and sign the informed consent form (ICF). The principal investigator (PI) will be available to answer any questions or provide clarifications during the informed consent process. This process will be completed before conducting any study related activities.

5.1 Screening Process

Once consented, each participant can be scheduled for the initial screening visit. During the initial visit, a screening form will be completed and reviewed. If an individual answers "yes" to any of the initial screening exclusion questions, they will be informed that they do not qualify, and they will be informed that they can keep their screening form. If all answers are "no" then the form will be placed in the study documents. A

medical interview will also be conducted. This medical interview will include a complete physical examination and subjects will also be asked to fill out the Tegner Activity Level Scale to obtain a measure of their physical activity. The physical examination will include head/neck, cardiovascular, lung, and abdominal examinations. Standard vitals will also be taken.

5.2 Participant Eligibility

5.2.1 Inclusion Criteria

1. Volunteer population of healthy males and females aged 18-45.

2. Subjects consent to attending a total of 14 visits over a time span of seven to nine weeks. The breakdown of the 14 visits is as follows: 1 familiarization session, 12 experimental testing sessions, 1 final session.

5.2.2 Exclusion Criteria

Volunteers who have medical history involving one or more of the following medical conditions:

1. Uncontrolled diabetes (accepted methods of control include medication, diet, exercise, or another form of intervention following guidelines from the American Diabetes Association)(33).

2. Uncontrolled hypertension (accepted methods following guidelines from the International Society of Hypertension)(34).

3. Autoimmune disorders

4. Blood disorders

5. Ongoing infectious disease

6. Cancer

7. Any disorder requiring immunosuppression treatment

8. Steroid usage

9. Significant cardiovascular, pulmonary, hepatic, or renal disease.

10. Volunteers where 20 minutes of intense exercise is contra-indicated will also be excluded from the study.

11. Positive pregnancy test

5.3 Informed Consent 21 CFR 50

In adherence to the 21 CFR 50, Protection of Human Subjects Guidelines, the informed consent process will be performed by one of the study investigators or research team member, in the physician clinic or research office that has received training on the informed consent process. No aspects of the study will be conducted prior to obtaining informed consent from each subject. The purpose and methods of the study along with the expected effects will be reviewed with each potential subject. Each subject will be provided a copy of the consent and sufficient time will be given for the opportunity to read and ask any questions about the study. After signing of the informed consent document, subjects will be given a copy for their records.

The designee will review with each subject that they are free to refuse to the study or to withdraw from it at any time.

5.4 Consent Withdrawal:

During the informed consent procedure, subjects will be informed that if at any point during the study, consent may be withdrawn. To withdraw consent, subjects can request via phone, email, or in writing to withdraw HIPAA authorization and the research site will not use or provide any health information to researchers. At this time, the link between the subject's health information will be severed with the research team. This process for consent withdrawal will be reviewed with each subject and identified barriers will be addressed at the time of informed consent. Any samples obtained as part of the study will be destroyed per the site's standard operating procedures, unless broad consent was obtained.

5.5 Benefits

There are no direct benefits for subjects who participate in the study. Indirect benefits include additional medical care and physical therapy care not associated with any medical procedures or injury. Results from the study may help clinicians to develop a better understanding of BFR and its effects on blood metabolites and the mobilization of peripheral hematopoietic progenitor cells (aspirational benefit).

5.6 Compensation

Participants will be provided a stipend for their time spent participating in this study. A $20.00 gift card will be provided at each of the 14 sessions described below (Introductory, 12 Experimental, Final).

6 Treatment Plan

Each subject will attend a total of 14 sessions.

Subjects will attend a familiarization session three to fourteen days prior to the beginning of the experimental sessions during which testing will be conducted.

Following the familiarization session, subjects will attend two experimental testing sessions per week for six weeks.

During the seventh week following the first experimental testing session, subjects will attend the final session for a blood draw.

Sessions will not occur within 24 hours of another session. Blood samples will be collected from the same visit within the week if possible (either the first in the week or second in the week depending on scheduling).

6.1 Familiarization Session:

- Subjects will undergo a standard physical examination that includes answering questions pertaining to their medical history to rule out any exclusion criterion.

- Subjects assigned female at birth with no history of surgical sterilization, including but not limited to hysterectomy, tubal ligation, or oophorectomy, must have a negative pregnancy test at screening prior to enrollment.

- Subjects will also be asked to fill out the Tegner Activity Level Scale to obtain a measure of their physical activity.

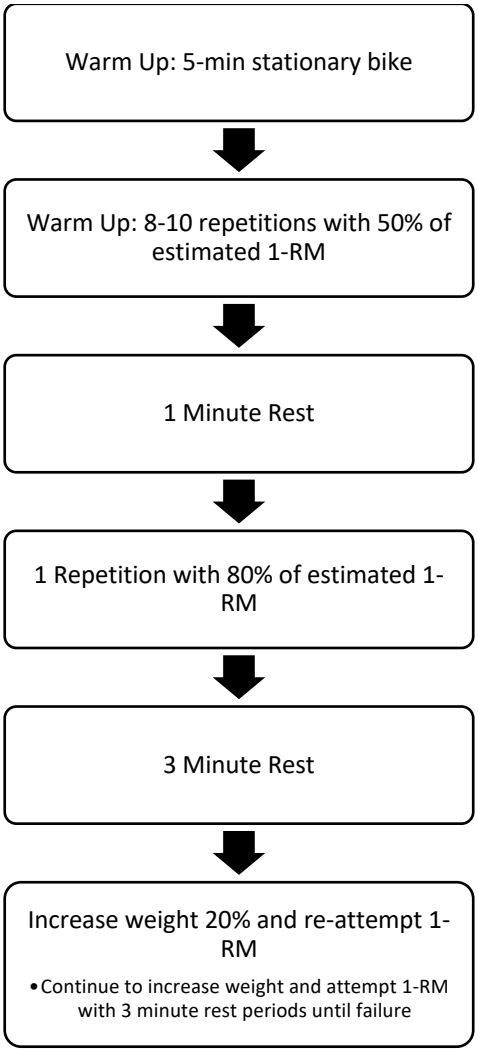
- Subjects will be introduced to each of the exercise machines and will be taught how to properly use the equipment by a study team member. Subjects will be instructed on a seated leg extension machine, a semi-reclined leg press machine, and a prone hamstring curl machine.

- The Delfi BFR tourniquet system will be introduced to the subject. Subjects will become familiarized with the pressure that the tourniquet system will produce on their legs during the experimental testing session.

- The study team will find the one repetition maximum (1-RM) for each exercise (Figure 1). This process will be performed by the physical therapist trained in the use of the exercise equipment. The resistance of each exercise will be modified as needed until the physical therapist finds the suitable amount of weight required for the subject to be able to perform one and only one repetition. The progression for obtaining a 1-RM weight will be repeated for each exercise (seated leg extension, semi-reclined leg press, prone hamstring curl.)

- The study team will answer any questions the subject may have about any of the exercises or the Delfi tourniquet system.

Figure 1. Protocol for determining 1-RM.



6.2 Experimental Testing Sessions (12 Total):

- Subjects will be asked to refrain from consumption of alcohol and caffeine for 12 hours before testing and strenuous exercise 24 hours prior to testing.

- Subjects will be advised to avoid the consumption of non-steroidal anti-inflammatory drugs (NSAIDS) in response to muscle soreness. NSAIDS include but are not limited to ibuprofen, high dose aspirin, naproxen, and diclofenac.

- Data will be collected upon presentation for testing and the final experimental testing session. Data collected will include height, weight, and seated blood pressure.

- At the first, sixth, and twelfth experimental testing sessions, subjects will then have 6 cc of blood collected via forearm/arm vein access once 15 minutes of rest in the sitting position have elapsed. The first sample will be used to establish a pre-BFR complete blood count (CBC) with differential and flow cytometry to analyze the concentration of HPCs and MSCs (if available) in the blood.

- During all experimental testing visits, subjects will, under the supervision of an individual trained in BFR, undergo a BFR session. The Delfi Personalized Tourniquet System (PTS) (Delfi Medical Innovations Inc., Vancouver, BC) is the BFR system that will be utilized. Bilateral proximal thigh bands will be applied and inflated to a pressure of 80% of occlusive pressure as determined by the automated tourniquet. The three exercises (seated leg extension, semi-reclined leg press, prone hamstring curl) will be formatted with 4 sets of 30-15-15-15 repetitions per exercise. The resistance will be set as 30% of one repetition maximum (1-RM).

- At the first, sixth, and twelfth experimental testing sessions, after the BFR session concludes, additional 6 cc blood draws will be obtained immediately post-exercise and after 20-minutes have elapsed. Subjects will be offered the insertion of a peripheral intravenous blood draw or multiple percutaneous blood draws. The blood collected post-BFR will also be analyzed using a CBC with differential and flow cytometry.

- Following all experimental testing sessions, blood samples will be taken for lactate testing. These samples will be collected prior to the exercise session, immediately after exercise, 10 and 20-minute time points post-exercise and analyzed via a portable lactate analyzer. To acquire the blood samples, a finger will be cleaned with an alcohol swab and a single-use lancet will be used to puncture the finger for blood testing. Both sides of the puncture site will be gently pressed to develop a drop of

blood. Care will be taken to avoid a vigorous squeeze. The first drop of blood will be wiped off using a sterile cotton swab as it may contain interstitial fluid. When the second drop of blood develops, the test strip with the lactate meter will be touched to the blood drop until the lactate meter beeps. Different testing sites (fingers) will be used for each lactate test.

All samples will be handled under Universal Precautions. Samples will be stored and disposed of according to site standard operating procedures.

6.3 Final Session:

- The final session will take place no sooner than six weeks after the first experimental session and within five days of the twelfth and final experimental session.

- Within the seventh week following completion of the familiarization session, subjects will be brought in for a blood draw post-BFR. Subjects will be asked to refrain from strenuous exercise 24 hours leading up to the final session. Subjects will also be asked to refrain from the consumption of alcohol and caffeine for 12 hours before the blood draw.

- Subjects will be advised to avoid the consumption of non-steroidal anti-inflammatory drugs (NSAIDS) in response to muscle soreness. NSAIDS include but are not limited to ibuprofen, high dose aspirin, naproxen, and diclofenac.

- Data will be collected upon presentation for the blood draw. Data collected will include height, weight, and seated blood pressure.

- Subjects will then have 6 cc of blood collected via forearm/arm vein access once 15 minutes of rest in the sitting position have elapsed. This sample will be utilized to obtain a post-BFR CBC with differential and flow cytometry to evaluate the concentration of HPCs and MSCs (if available) within the blood.

All samples will be handled under Universal Precautions. Samples will be stored and disposed of according to site standard operating procedures.

7 Review of Safety

7.1 Adverse Event (AE)

An adverse event is any untoward or unfavorable medical occurrence in the human subject, including any abnormal sign, symptom, or disease, temporally associated with

the subject's participation in the research, whether considered related to the subject's participation in the research.

Subjects must agree to notify the investigator or study team member of potential or confirmed pregnancy, either of which may result in being withdrawn from the study or having limited participation in the study. Subjects who become pregnant while in the study will be followed for AEs/SAEs that may occur for up to 30 days following the birth of the child.

7.2 Serious Adverse Event (SAE)

Serious adverse events are any events that:

- Result in death
- Is life threatening, or places the subject at immediate risk of death from the event as it occurred
- Requires or prolongs hospitalization
- Causes persistent or significant disability or incapacity
- Results in congenital anomalies or birth defects
- Is another condition which investigators just to represent significant hazards

7.3 Unanticipated Problem (UP):

Defined by DHHS 45 CFR part 46 as any incident, experience, or outcome that meets the following criteria.

- unexpected, in terms of nature, severity, or frequency, given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the study population.
- related or possibly related to participation in the research (in this guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research).
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

7.4 AE & SAE Collection and Reporting

Throughout the study the research team will monitor the occurrence of AE and SAE. Data will be collected if an instance occurs, and the PI will be notified. All AE data, such

as onset date, resolution date, outcome and treatments given will be documented in the source documents and will be recorded in the Electronic Data Capture (EDC) and analyzed for severity to follow reporting protocol if severity level.

Follow-up will occur using the provided safety monitoring form if AE occurs. The follow up will end either when the symptoms resolve or up to 30 days past the end of the study participation.

7.5 Risks and Discomforts

This study includes the risks of blood draws, mild exercise, and vascular occlusion. The risks associated with the taking of blood include pain, bruising at the point where the blood is taken, redness and swelling of the vein and infection, and a risk of fainting although rare. These risks will be mitigated via utilization of standard universal precautions. During the exercise, the following may occur abnormal blood pressure, fainting, dizziness, disorders of heart rhythm, and in very rare cases heart attack, stroke, or even death. Bodily injury is also a possible risk including, but not limited to, injuries to the muscles, ligaments, tendons, and joints of the body. Exercise could also cause fatigue and soreness of the muscles. The risk of vascular occlusion is discomfort. The aforementioned risks are minimized by conducting the proper assessments before exercise and having properly trained staff. With the implementation of the Delfi BFR tourniquet, nerve conduction deficits or pinching of the superficial skin may occur. However, these are transient effects and can be mitigated with the usage of a proper fitting cuff placed in the correct location and an adequate amount of pressure.

8 Data Management Procedures

All personal information is strictly confidential, and no names will be disclosed except as required by law. All information and data collected during this research will be recorded in spreadsheeting source and within the EDC. Records related to this study will be securely retained in a secure location for a period of 3 years after the completion of the study or longer as required by law. At that time, all records will be properly destroyed.

Data will be collected using the EDC system. Reports of data will be used by internal site monitors to ensure accuracy of data elements.

9 Data Analysis

All participant data will be entered into the Electronic Data Capture (EDC) system. The study team will meet at appropriate intervals to evaluate and analyze the data. All compiled data will be de-identified. A combination of Excel and Statistical Analysis System (SAS) Studio (Version 3.8 on SAS 9.4, SAS Institute Inc., Cary, NC) will be

used. Outcome measures will be those from pre and post exercise blood samples collected according to the study procedures (Section 5).

Outcome measures will be as follows:

Complete Blood Counts with differential: Red Blood Cell Count, White Blood Cell Count with differential, Total Nucleated Cell Count (number of nucleated cells per milliliter (mL)), Mononuclear Cell Count (number of mononuclear cells per mL), and Platelet Count (number of platelets per mL).

Flow Cytometry: Counts including cluster differentiation (CD) markers for HPCs and MSCs (if available).

Lactate Pro 2 Portable Lactate Analyzer (COSMED USA Inc., Concord, CA) Blood lactate level.

10 Statistical Considerations

Descriptive statistics will be compiled for all numeral measures. Analysis will be performed with a repeated-measures ANOVA and post-hoc Tukey's Honestly Significant Difference (HSD) for each numerical outcome variable. Significance will be set a priori at $p < 0.05$. With an alpha level of 5%, a beta of 10%, and an effect size of 200%, we calculated that a sample size of 10 is sufficient. Sufficient power has been confirmed on previous mobilization studies. The sample size of this study has been increased to 25 to account for potential subject withdrawal.

11 Quality Control and Assurance

All protocols will be monitored and analyzed data will be checked for accuracy by the principal investigator and/or a designated AREF study team member. All medical data will be kept in compliance with HIPAA guidelines.

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