Title: A Randomized, Single Blinded Study of the Augmentation of Anterior Cruciate Ligament Reconstruction using Stump-Derived Mesenchymal Stem Cells Versus Standard of Care Anterior Cruciate Ligament Reconstruction

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Abbreviations and Definitions

ACL Anterior Cruciate Ligament

AE Adverse Event

AREF Andrews Research and Education Foundation

BTB Bone-Patellar Tendon-Bone

FTA Functional Testing Algorithm

HIPAA Health Insurance Portability and Accountability Act

ICF Informed Consent Form

IRB Institutional Review Board

LDSC Ligament Derived Stem Cell

MRI Magnetic Resonance Imaging

MSC Mesenchymal Stem Cell

PCL Posterior Cruciate Ligament

PI Principal Investigator

PRP Platelet Rich Plasma

SAE Severe Adverse Event

SOC Standard of Care

TDSC Tendon Derived Stem Cell

Tg Transgenic

UP Unanticipated Problem

1 Background / Scientific Rationale

The anterior cruciate ligament (ACL)'s anatomical importance is to act as a stabilizer of the knee joint during physical activity. This ligament is vitally important during high intensity sports. It is estimated that 250,000 ACL injuries occur in the United States per year (1, 2). Leathers Et. AL. found that patients who elect to undergo ACL reconstruction are at a six times greater risk of tearing the reconstructed graft within the first two years of ACL reconstruction surgery or within their return to sports in comparison to uninjured counterparts (3). This translates to approximately 8.5% of females and 10.5% of males having a tear of the ipsilateral ACL graft within two years of repair or their return to sports. With such high recurrence rates, it is paramount to develop methods to improve outcomes in patients who undergo ACL reconstruction. Bioaugmentation of ACL reconstruction may provide a method to achieve improved outcomes in this patient population.

Orthopedic clinicians and researchers have taken interest in the underlying biology of healing, specifically regarding the application of orthobiologics to improve patient outcomes. Orthobiologics are a class of therapies that originate from biological products that can be applied at the point of care. Recent studies have focused on using a patient's own tissues to improve current clinical and surgical methods (4, 5). With an increased focus on cell therapy to adjunct current practices, the orthopedic community is determined to develop the least-invasive, convenient, and cost-effective methods to harvest and use stem cells.

Mesenchymal stem cells (MSCs) were first isolated from bone marrow, but they also can be isolated and are contained in synovial tissue, adipose tissue, injured ACL stump tissue, cartilage, and the ACL injury effusion (6-9). MSCs have shown exceptional promise regarding cellular proliferative potential, multipotentiality, and the ability to improve healing in animal models (10-17). In humans, harvesting stem cells from tissues inside the knee joint has recently become a method of interest. Utilizing already available tissues which contain stem cells during arthroscopic knee surgery is particularly beneficial compared to trying to use cells from bone marrow, as bone marrow aspiration for harvest would require patients to undergo a longer duration of anesthesia and positional readjustment over the course of an operation.

During surgery, injury effusion fluid, synovial tissue, and fat tissue found inside the knee are typically removed in order to visualize and repair structures. We recently evaluated this waste tissue in 30 individuals undergoing ACL reconstruction surgery. Culture analysis showed that the knee effusion contained 181 progenitor cells per ml, byproduct tissue contained a concentration of 429 progenitor cells per ml of tissue, and the byproduct fluid contained a

concentration of 14 cells per ml (14). Animal and human trials are emerging to suggest that autologous augmentation of ACL reconstruction deserves further development (21, 22). We recently completed a study describing the quantity of stem cell available from various waste tissues of ACL surgery: ACL stump, fat pad, bone debris, and cartilage debris. We determined that ACL stump tissue has the most potential to augment ACL reconstruction surgery.

A review of recent studies shows that there is growing evidence in support of bioaugmentation for ACL repairs. Berdis Et. Al. recently published their results for 109 knees in 101 adolescent patients who underwent hamstring ACL reconstruction with bioaugmentation with PRP contained in a porous bovine collagen matrix carrier (23). A total of 132 patients (92%) returned to their preinjury level of competition, while 7 patients sustained a reinjury requiring revision surgery (5%). They concluded that biologic augmentation with hamstring autograft in ACL reconstruction led to a decreased rate of subsequent ACL injury, specifically regarding ACL revision surgery. Patients in this study also showed the ability to return to preinjury level of competition at a faster rate when compared to data from other studies (23, 24).

The desired benefit of MSC therapy is to promote improved bone-to-tendon healing with better biomechanical properties and increased tissue maturity (25). Ligament derived stem cells (LDSC), such as those found in the ACL stump, and tendon derived stem cells (TDSC) have shown better tendon/ligament lineage-specific differentiation when compared to bone marrow counterparts (26). Studies on MSC-based therapies have provided growing evidence that damaged tissues express signaling molecules that act as chemoattractants to aid in the migration, adhesion, and infiltration of MSCs to sites of injury (27). Takeuchi Et. Al. studied the fate of endogenous cells in ACL graft infiltration in transgenic (Tg) pigs. They determined that eventually the endogenous cells proceeded to differentiate into spindle-shaped fibroblast-like cells with a uniform distribution, closely resembling the natural histology of the ACL (28). Nonetheless, future studies tracking MSC differentiation into fibroblasts and other cell types remain necessary.

A key to determining the efficacy of bioaugmentation methods for ACL reconstruction is possessing the ability to accurately evaluate the healing and rehabilitation processes of the patient. Functional testing algorithms (FTA) are used for clinical decision making based on quantitative and qualitative testing and assessment to make clinical judgements to safely return patients back to pre-injury activity and strength. Therapists have developed a systematic guideline and criteria to return to sport based on systematic literature reviews and clinical experience (29). Evaluation with FTA provides the quantitative and qualitative criteria needed to

make an informed decision on progress towards returning to sports activity. In addition to FTA, magnetic resonance imaging (MRI) is a key analytical method used to provide quantifiable data on the quality of the ACL graft following reconstruction. Evaluation with MRI enables the physician to assess the integrity of the graft, placement within the tunnel, tunnel healing, and healing of the donor site. This information coupled with the functional assessment enables the healthcare team to determine a patient's readiness to return to activity.

2 Objectives

2.1 Primary Objective

The primary objective of this study is to develop a cost-effective, autologous biologic augmentation technique for ACL reconstruction. The technique involves applying tissue harvested with the GraftNet from the patient's ACL stump to an ACL autograft. This study is key to determining a reproducible and effective autologous biologic augmentation technique that can be utilized at the point-of-care during ACL reconstruction surgery.

2.2 Primary Effectiveness Endpoint

FTA results as well as measurements from MRI evaluation will be recorded and utilized to quantify the healing and ACL graft maturation processes. MRI evaluation will be performed in accordance with accepted ACL imaging protocols. This data will then be compared to FTA results and MRI evaluation from patients who did not undergo the bioaugmentation technique for ACL reconstruction.

3 Hypotheses

3.1 Null Hypothesis

H_0 – Participants undergoing the proposed bioaugmentation technique for ACL reconstruction will not have improved healing times and graft maturation as evidenced by functional assessment scores and MRI evaluation when compared to matched participants who only underwent ACL reconstruction without bioaugmentation.

3.2 Alternative Hypothesis

H_1 – Participants undergoing the proposed bioaugmentation technique for ACL reconstruction will have improved healing times and graft maturation as evidenced by functional assessment scores and MRI evaluation when compared to matched participants who only underwent ACL reconstruction without bioaugmentation.

4 Study Design and Procedures

Study design will be a prospective, single-blinded, randomized, single-center trial. Patients at the Andrews Institute who meet the inclusion criteria will have the study explained in detail and informed consent will be obtained as outlined below. Fifty patients will be blinded, randomized, and undergo an ACL reconstruction surgery. Twenty-Five randomized patients will receive standard of care (SOC) ACL reconstruction surgery. Twenty-Five randomized patients will receive ACL reconstruction surgery augmented with the patient's ACL stump tissue harvested with the GraftNet device (Arthrex, Naples, Fl).

4.1 Intraoperative Tissue Harvest and Application

A tissue sample will be harvested from each participant with the GraftNet device (one per surgery) during ACL reconstruction from the ACL stump. During the intra-articular preparation phase of the reconstruction, the tissue sample collected from the ACL stump will be applied to the ACL graft on the back table during the graft preparation phase.

4.2 Post-operative Evaluation and Treatment Plan

Each of the participants will be qualified for the study due to falling within all inclusion criterion and no exclusion criterion. The screening of the inclusion criterion will occur at visit one of the study. Visit one will take place prior to or at the same time as surgery. At this time, the AREF Research Team will review and collect informed consent from each participant. Visit two will occur three (3) months after each participant's ACL reconstruction surgery. At this time, an evaluation by the treating physician will occur along with functional movement assessment and MRI. Visit three will occur six (6) months after each participant's ACL reconstruction surgery. At this time, an evaluation by the treating physician will occur along with functional movement assessment and MRI. Visit four will occur nine (9) months after each participant's ACL reconstruction surgery. At this time, an evaluation by the treating physician will occur along with functional movement assessment and MRI. Visit five will occur twelve (12) months after each participant's ACL reconstruction surgery. At this time, an evaluation by the treating physician will occur along with functional movement assessment and MRI. Visit six will occur eighteen (18) months after each participant's ACL reconstruction surgery. At this time, an evaluation by the treating physician will occur along with functional movement assessment and MRI. Visit seven will occur twenty-four (24) months after each participant's ACL reconstruction surgery. At this time, an evaluation by the treating physician will occur along with functional movement assessment and MRI. At each time point, the treating physician or the therapist performing the functional movement

assessment can choose to limit the functional movement assessment if there are concerns that the patient may not be ready for the expected load of one or more elements of the functional movement assessment.

At each follow up visit after ACL reconstruction, patient reported outcome measures (PROMs) will be collected by the research team in written or electronic format to assist in assessing the overall health and rehabilitation of each participant. A complete list of PROMs to be collected can be found in section 5.

4.3 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) will be used as a noninvasive assessment tool to obtain quantitative measures to assess progress in graft maturity and rehabilitation towards safe return to sport. As the injured ACL undergoes ligamentization, MRI will be used to monitor the progress of each participant through T2* analysis and volume at the following time intervals: 3months, 6months, 9months, 12months, 18months, and 24months. MRIs will review the integrity of each graft, placement within the tunnel, and tunnel healing, and healing of the donor site (30).

4.4 Functional Testing Algorithm

Using the Davies Functional Algorithm (29), the following tests will be performed: Basic Measurements, Sensorimotor System Testing: Balance/Proprioceptive Testing, Closed Kinetic Chain Testing, Open Kinetic Chain Testing, Functional Jump Tests, Functional Hop Tests, Lower Extremity Functional Tests, and Sport-Specific/Position-Specific Testing. Each test will be performed incrementally, and participants will not advance to the next series of tests until the prior assessment has been completed at a satisfactory level. The participants' results from the Functional Testing Algorithm (FTA) and the time required to complete the progression during the rehabilitation period will be quantified and analyzed according to demographic data and pre-injury activity level. The participants will be stratified according to activity level. Participant groups will include general orthopedic patients, recreational athletes, and competitive athletes and will be tested by the FTA accordingly.

5 Patient Reported Outcome Measures (PROMs)

Patient reported outcome measures will be used to quantify physical and psychological aspects of rehabilitation after ACL reconstruction monthly after informed consent has been obtained and surgery has occurred. The patient reported outcomes can be collected in paper format or through the REDCap system. The patient reported outcomes will follow the presented follow up visit schedule: 3months, 6months, 9months, 12months, 18months, and 24months.

5.1 Tampa Kinesiophobia Scale (TKS):

The fear of reinjury can be a barrier to the rehabilitation of competitive athletes after ACL reconstruction. To quantify the fear associated with reinjury, the Tampa Kinesiophobia (TSK) scale will be provided monthly to participants in the study (31). Kinesiophobia is defined as the fear of movement (31). This measure will be collected to evaluate progression in rehabilitation to take into account not only the physical health of the injured athlete but also the psychological effects and rehabilitation.

5.2 International Knee Documentation Committee Subjective Knee Evaluation Form (IKDC):

The International Knee Documentation Committee Subjective Knee Evaluation (IKDC) form will contribute to the comprehensive data set to evaluate each participant's confidence in performance on a monthly schedule. IKDC has 19 questions that will take approximately 3-5 minutes for each participant to complete monthly. IKDC data will be collected by the AREF research team and reviewed with the primary investigator.

5.3 Patient Reported Outcomes Measurements Information Systems (PROMIS):

The patient reported outcomes measurement information system (PROMIS) is a measurement system used monthly in this project to assess monthly the overall health functioning and rehabilitation levels of each participant (32). AREF will collect this data and alert the primary investigator if any adverse outcomes are found. The addition of the PROMIS data is required to ensure that a comprehensive assessment of overall functioning is reviewed in the determination of athlete readiness to return to sport.

5.4 Single Assessment Numeric Evaluation (SANE):

The SANE assessment is a single question patient reported outcome from a rating of 0-100 for each participant to score their current functioning in comparison to their pre-injury functioning level (33). This assessment will be collected monthly after ACL reconstruction by the AREF research team for each participant.

6 Participant Recruitment

A total of 50 participants will be recruited through the Andrews Institute physician practices. A recruitment flyer will be posted within the physicians' offices, on social media platforms and posted in community locations. Potential participants will be prescreened for inclusion and exclusion criteria through standard of care medical evaluations. Participants meeting the

inclusion criteria will have the study explained to them by one of the members of the research team, and they will be given an opportunity to participate if they are interested. Once a potential participant has agreed to be involved in the study, they will go through the described informed consent process.

6.1 Screening Process

Once interested patients are identified and prescreened, a screening visit (visit 1) will be scheduled. During the screening visit, a screening form will be completed which includes the inclusion and exclusion criterion below. If an individual answers "yes" to any of the initial screening exclusion questions, they will be informed that they do not qualify for the study, and they will be informed that they can keep their screening form and their medical care will not be adversely affected by not enrolling in the study. If all answers are "no" then the form will be placed in the study documents and the enrollment process will continue.

Once the screening requirements are met, the informed consent form will be provided to the participant or electronically via REDCap for review ensure the volunteer understands the details of the study including the benefits and risk factors. The patient will be provided sufficient time to consent and sign the informed consent form (ICF). The principal investigator (PI) and study/research team will be available to answer any questions or provide clarifications during the informed consent process.

6.2 Participant Eligibility

6.2.1 Inclusion Criteria:

Patients between the ages of 14 and 50 who are scheduled to have ACL reconstruction by one of the investigating physicians will be pre-screened for participation in this study.

6.2.2 Exclusion criteria:

Exclusion criteria will include patients requiring ACL and posterior cruciate ligament (PCL) combined surgery, patients with a history of an autoimmune disease, diabetes, a blood/clotting disorder, or history of previous surgery on the injured knee. Patients will undergo informed consent and an unidentifiable patient study number will be created.

6.3 Benefits

Direct benefit to the intervention group participant may include improved outcome following the ACL reconstruction and earlier return to baseline functioning level. Indirect benefits to the

participant include increased surgical follow-up, and additional post-surgery monitoring. This study may also help direct clinical practice in developing preferred techniques to augment orthopedic surgical procedures (aspirational benefit).

6.4 Compensation

Compensation will not be provided to participants in this study.

7 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 staff, in the research office on paper or electronically via REDCap. REDCap is a secure web application for building and managing online surveys and databases. While REDCap can be used to collect virtually any type of data in any environment (including compliance with 21 CFR Part 11, FISMA, HIPAA, and GDPR), it is specifically geared to support online and offline data capture for research studies and operations. The REDCap Consortium, a vast support network of collaborators, is composed of thousands of active institutional partners in over one hundred countries who utilize and support their own individual REDCap systems. All participants will have the study described to them and will give as much time as they require to read an approved, stamped version of the informed consent document. After physical or electronic signing of the informed consent document, participants will be given a copy for their records. This process will take place only after the patient has consented to proceed with the study.

7.1 Consent Withdrawal:

During the informed consent procedure, participants will be informed that if at any point during the study, consent may be withdrawn. To withdraw consent, participants can request 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 participant's health information will be severed with the research team. This process for consent withdrawal will be reviewed with each participant and identified barriers will be addressed at the time of informed consent.

8 Review of Safety

8.1 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.

8.2 Serious Adverse Event (SAE)

Serious adverse events are any events that:

- Result in death
- Is life threatening, or places the participant 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

8.3 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 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 participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

8.4 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 system (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.

8.5 Expected Risks and Discomforts

Risks and Discomforts: As with any research involving human subjects there is the inherent risk of a breach in patient confidentiality. This will be minimized using participant code numbers and adhering to all HIPAA guidelines. Standard sterile precautions for surgical procedures will be utilized for all ACL surgeries. With any surgery there remains the risk of infection, bleeding, and swelling. The collection of soft tissue samples with the GraftNet device does not increase the risk associated with the surgical procedure. MSC transplantation has been deemed safe by the Food and Drug Administration (FDA) (34). However, as with all stem cell therapy, there is the inherent risk of tumorigenicity (35) (36). Long-term follow up is needed to further elucidate these risks. Use of the bovine collagen matrix presents minimal risk to the participant. Other studies utilizing this implant did not report any discomforts or adverse events attributed to the collagen matrix implant (23).

9 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 including patient reported outcomes measures during this research will be compiled into an Excel spreadsheet. This spreadsheet will not contain protected health information. The spreadsheet will be stored in a secure password protected folder on a laptop that only the study investigators will have access to and will be permanently deleted following publication of all manuscripts, if any, written as a result of this research. 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.

10 Data Analysis

Data will be compiled in Microsoft Excel and REDCap. All compiled data will be de-identified.

11 Statistical Considerations

Statistical analysis will be performed in excel and SAS Studio (Version 3.8 on SAS 9.4, SAS Institute Inc., Cary, NC). Descriptive statistics will be compiled for all numeral measures (outcomes).

12 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 research team member. All medical data will be kept in compliance with HIPAA guidelines.

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