Changes in Circulating Biomarkers of Muscle Atrophy, Inflammation, and Cartilage Turnover in Patients Undergoing Anterior Cruciate Ligament Reconstruction and Rehabilitation

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Background: After anterior cruciate ligament (ACL) reconstruction, there is significant atrophy of the quadriceps muscles that can limit full recovery and place athletes at risk for recurrent injuries with return to play. The cause of this muscle atrophy is not fully understood.

Hypothesis: Circulating levels of proatrophy, proinflammatory, and cartilage turnover cytokines and biomarkers would increase after ACL reconstruction.

Study Design: Descriptive laboratory study.

Methods: Patients (N = 18; mean age, 28 ± 2.4 years) underwent surgical reconstruction of the ACL after a noncontact athletic injury. Circulating levels of biomarkers were measured along with Short Form–12, International Knee Documentation Committee, and objective knee strength measures preoperatively and at 6 postoperative visits. Differences were tested using repeated-measures 1-way analysis of variance.

Results: Myostatin, TGF-β, and C-reactive protein levels were significantly increased in the early postoperative period and returned to baseline. Cartilage oligomeric matrix protein levels decreased immediately after surgery and then returned to baseline. CCL2, CCL3, CCL4, CCL5, EGF, FGF-2, IGF-1, IL-10, IL-1α, IL-1β, IL-1ra, IL-6, myoglobin, and TNF-α were not different over the course of the study.

Conclusion: An increase in potent atrophy-inducing cytokines and corresponding changes in knee strength and functional scores were observed after ACL reconstruction.

Clinical Relevance: Although further studies are necessary, the therapeutic inhibition of myostatin may help prevent the muscle atrophy that occurs after ACL reconstruction and provide an accelerated return of patients to sport.

Keywords: myostatin; transforming growth factor-β; cartilage oligomeric matrix protein; C-reactive protein; muscle atrophy; ACL reconstruction

Anterior cruciate ligament (ACL) tears are among the most frequent knee injuries in physically active people, with tear rates in the United States up to 250,000 per year.¹⁹ Despite improvements in postoperative rehabilitation, many patients who suffer from ACL tears have persistent atrophy and weakness of their quadriceps muscles after ACL reconstruction (ACL-R). Several studies have reported persistent weakness exceeding 20% in quadriceps muscles after ACL-R.³² This weakness appears to occur for all types of ACL grafts, including hamstring and patellar tendon autografts as well as allografts.³² In addition to reducing physical performance and increasing the susceptibility to repeated injuries,²⁴ many studies have indicated that this loss of strength can alter knee kinematics in a way that promotes the development of early-onset osteoarthritis (OA) in younger patients.²⁸,³⁵,⁴² Surgical reconstruction of torn ACLs, while helpful in restoring some joint kinematics and proprioception, does not appear to modify the likelihood of the development of OA in ACL-reconstructed patients.³⁰ Developing new therapeutic interventions to prevent muscle atrophy and weakness...
after ACL-R is likely to reduce postinjury performance deficits, protect from reinjuries, and possibly reduce the likelihood of developing OA.

The cause of muscle atrophy and persistent dysfunction after ACL-R has not been fully characterized. Several studies have suggested a loss of proprioception and impaired neuromuscular control after ACL-R to be responsible for persistent muscle activation deficits and atrophy. Changes in neurological function, however, provide only a partial explanation for the observed strength deficits after ACL surgery. In many different types of neuromuscular diseases and injuries, there is atrophy and weakness of muscle fibers that reflect alterations in signaling pathways that regulate muscle protein synthesis and degradation. Identifying changes in atrophy- or hypertrophy-inducing signaling molecules that occur after ACL tears could provide new pharmacological options to prevent weakness and enhance the recovery and safe return to sport for patients who suffer ACL tears.

Several cytokines and signaling molecules are known to control muscle fiber growth and strength, but little is known about the role that these molecules play in regulating the muscle atrophy that occurs after ACL tears. One of the most widely studied atrophy-inducing signaling molecules is myostatin (GDF-8). Myostatin is a member of the transforming growth factor-β (TGF-β) superfamily of cytokines and induces muscle atrophy by activating the ubiquitin-proteasome pathway. Closely related to myostatin is TGF-β, which also potently induces muscle atrophy and weakness via activation of the ubiquitin-proteasome pathway. Insulin-like growth factor 1 (IGF-1), which activates the Akt/mTOR protein synthesis pathway, is among the most well-studied hypertrophy-inducing factors. It is not known, however, if myostatin, TGF-β, and IGF-1 levels change in patients who undergo ACL-R.

We hypothesized that myostatin and TGF-β would increase immediately after ACL-R and remain elevated during the early postoperative period, and that IGF-1 levels would decrease after surgery and remain depressed in the early postoperative period. We measured circulating levels of myostatin, TGF-β, and IGF-1 in patients with ACL tears immediately after surgery and at 6 postoperative clinical follow-up visits until discharge to return to full activity. In addition to myostatin, TGF-β, and IGF-1, we also measured other circulating signaling molecules that are known regulators of muscle atrophy or hypertrophy that act either by directly signaling in muscle fibers or indirectly via the recruitment of macrophages or modulation of muscle stem cell activity. The atrophy-related factors measured include chemokine (C-C motif) ligands 2, 3, 4, and 5 (CCL2, CCL3, CCL4, and CCL5); interleukin 1 α (IL-1α); interleukin 1 β (IL-1β); interleukin 1 receptor antagonist (IL-1ra); interleukin 6 (IL-6); and tumor necrosis factor α (TNF-α). The hypertrophy-related factors evaluated were epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), and interleukin 10 (IL-10). Additional circulating proteins that were measured include myoglobin, which is a marker of muscle fiber damage, C-reactive protein (CRP), which is an acute-phase protein that increases because of inflammation, and cartilage oligomeric matrix protein (COMP), which is a marker of cartilage degradation. Short Form–12 (SF-12) and International Knee Documentation Committee (IKDC) surveys and knee strength measurements were performed to assess subjective and objective functions.

**MATERIALS AND METHODS**

**Patients**

This study was approved by the University of Michigan Medical School's institutional review board. All patients provided written informed consent to participate in this study. Patients who were 16 to 55 years of age (written parental consent was obtained for patients <18 years of age), who had unilateral complete ACL tears and who consented to surgical repair by an orthopaedic surgeon, were eligible to participate in the study. Patients who were undergoing revision ACL-R, had gone through menopause, had a previous injury to their involved knee, or had a history of myopathy or a rheumatological disorder were excluded from participation in the study.

**Study Design**

An overview of the study design is presented in Figure 1. Approximately 1 week before surgery, patients reported to our clinic for a preoperative history and physical screening, and initial study measures were performed at this point.
time. Patients were asked to complete the IKDC survey to evaluate knee function and the SF-12 survey to measure general health-related quality of life. After the surveys were completed, approximately 4 mL of venous blood was collected from an antecubital vein into K2EDTA tubes (BD, Franklin Lakes, New Jersey) and spun down at 100g for 10 minutes. Plasma was immediately collected from the tubes, placed in 150-µL aliquots, and stored at −80°C for further analysis. After the blood draw, knee strength measurements were performed.

Surgical Repair of ACL Tears

All patients sustained noncontact, traumatic ACL injuries during athletic activity and desired to return to their respective sport. All patients underwent preoperative rehabilitation to improve range of motion and control effusion before surgical reconstruction for a period lasting approximately 6 to 8 weeks. Gift choice for ACL-R was a bone-patellar tendon–bone (BTB) autograft in 15 patients, an Achilles tendon allograft in 2 patients, and a Tibialis anterior tendon allograft in 1 patient. Surgery was performed by a single, fellowship-trained sports medicine surgeon using a single-bundle, anatomic reconstruction to recapitulate native ligament footprints and ligament obliquity. An anteromedial portal reaming technique was used for preparation of the femoral socket. All grafts were tensioned and fixed in 10° of flexion. The BTB grafts were fixed with collinear interference screws to achieve aperture fixation. Allografts were fixed with suspensory fixation on the femoral side (EndoButton, Smith & Nephew, Andover, Massachusetts) and collinear interference fixation on the tibial side (IntraFix, DePuy Mitek, Raynham, Massachusetts).

After ACL-R, patients were seen in our clinic for postoperative follow-up appointments 3 days after surgery (first postoperative visit), 2 weeks after surgery (second postoperative visit), 5 weeks after surgery (third postoperative visit), 12 weeks after surgery (fourth postoperative visit), 18 weeks after surgery (fifth postoperative visit), and 26 weeks after surgery (sixth postoperative visit). Measurement of Knee Strength

Bilateral strength measurements were performed with the patient seated on a dynamometer (BioDex System 3, Shirley, New York). Maximum isometric knee flexion and extension force measurements were performed at 45° and 90° of knee flexion, and isokinetic knee flexion and extension force measurements were performed over a range of 0° to 90° of knee flexion for each knee at each time point. Because of patient safety and comfort, isometric measurements at 45° of knee flexion were deferred at the first postoperative visit, and isometric measurements at 90° of knee flexion and isokinetic measurements were deferred at the first and second postoperative visits. For each measurement, the highest force from a series of 5 repetitions was used. All force data of the involved side were normalized to the uninvolved side at each visit.

Measurement of Biomarkers

The concentration of circulating biomarkers from plasma samples was analyzed in duplicate for each time point, and manufacturer instructions were followed for each assay. The coefficient of determination for all standards was greater than 0.99. Any replicate samples that had greater than 3% variation between values were analyzed again. Plasma was selected over serum, as platelets contain high levels of many of the proteins evaluated in this study. A Luminex-based Milliplex multiplex assay (Millipore, Billerica, Massachusetts) was used to measure circulating levels of CCL2, CCL3, CCL4, CCL5, EGF, FGF-2, IL-1α, IL-1β, IL-1ra, IL-6, IL-10, and TNF-α in a MagPIX system (Luminex, Austin, Texas). Enzyme-linked immunosorbent assays (ELISAs) were performed to measure circulating levels of COMP (R&D Systems, Minneapolis, Minnesota), high-sensitivity CRP (Calbiotech, Spring Valley, California), IGF-1 (R&D Systems), myoglobin (Calbiotech), myostatin (Alpco, Salem, New Hampshire), and TGF-β (R&D Systems). The ELISA plates were read in a SpectraMax microplate reader (Molecular Devices, Sunnyvale, California).

Statistical Analyses

Results are presented as mean ± standard error. Prism 6.0 software (GraphPad Software, La Jolla, California) was used to conduct statistical analyses. Differences between time points were tested using a repeated-measures 1-way analysis of variance (ANOVA) (α = .05), followed by Dunnett post hoc sorting to identify differences from the preoperative visit.

RESULTS

There were 18 patients (12 male, 6 female) who completed the study. The mean age of the patients was 28 ± 2.4 years (range, 16-50 years), and the mean body mass index was 26 ± 0.7 kg/m² (range, 20-32 kg/m²). All patients in the study tolerated the surgical reconstruction well and progressed along the accelerated rehabilitation plan without complications. For the SF-12 physical component summary (PCS), scores decreased immediately after surgery, returned to preoperative values by the third postoperative visit, and had values significantly higher than the preoperative scores by the fifth postoperative visit (Figure 2A). No
differences in SF-12 mental component summary (MCS) scores were detected throughout the study (Figure 2B). A nearly identical relationship to that of SF-12 PCS scores was observed for IKDC scores, except values became significantly higher than the preoperative score by the fourth postoperative visit (Figure 2C).

Strength values also followed an expected pattern of change (Figure 3). Isometric knee flexion and extension values at 45° of knee flexion decreased immediately after surgery and were significantly greater than preoperative values by the time of discharge (Figure 3, A and B). For isometric values at 90°, knee flexion strength returned to preoperative levels by the fourth postoperative visit, while knee extension strength returned at the sixth postoperative visit (Figure 3, C and D). Isokinetic knee flexion force values were significantly higher than preoperative values by the fifth postoperative visit, and the strength of the involved knee nearly returned to the strength of the uninvolved knee (Figure 3E). For isokinetic knee extension, strength values returned to preoperative levels by the fifth postoperative visit, but patients were discharged with a strength deficit of 29% relative to the uninvolved limb (Figure 3F).

The panel of biomarkers evaluated in this study is presented in Figure 4. The acute-phase proinflammatory protein CRP was highly elevated immediately after surgery but then returned to preoperative levels by the second postoperative visit (Figure 4F). For atrophy-related biomarkers, myostatin levels were elevated at the first postoperative visit, and both myostatin and TGF-β were elevated at the second postoperative visit, but both cytokines returned to preoperative levels by the third postoperative visit (Figure 4, P and Q). Other atrophy-related biomarkers, CCL2, CCL3, CCL4, CCL5, IL-1α, IL-1β, IL-1ra, IL-6, and TNF-α, did not change throughout the study (Figure 4, A-D, J-M, and R). No differences in the hypertrophy-related biomarkers IGF-1, EGF, FGF-2, and IL-10 were observed (Figure 4, G-I and N). The cartilage turnover biomarker COMP decreased immediately after surgery but then returned to preoperative levels (Figure 4E). The intramyocellular protein myoglobin, used as a marker of physical muscle damage, did not change over the course of the study (Figure 4O).

**DISCUSSION**

The purpose of this study was to measure circulating levels of biomarkers involved in muscle atrophy and hypertrophy, inflammation, and cartilage turnover during the postoperative recovery after ACL-R. In the current study, myostatin and TGF-β levels significantly increased in the early postoperative period after ACL-R. Patients perceived a premature return of knee function as their IKDC and SF-12 PCS scores returned to their preoperative baseline level by 5 weeks postoperatively. Despite the improvement in subjective function, however, patients continued to have a substantial deficit in force production compared with their uninvolved limb. The results of this study suggest that the cause of muscle atrophy after ACL surgery may be multifactorial and that elevated levels of myostatin and TGF-β postoperatively may significantly contribute to the delayed return of strength and dynamic joint stability. Furthermore, the study supports the notion that objective improvements in strength and muscle function lag behind patient-perceived functional recovery.

The mechanisms that induce muscle atrophy after ACL-R have not been fully characterized. Many studies have evaluated knee proprioception and neuromuscular control in patients with ACL injuries, but little is known about the role of cytokines or hormones that may directly or indirectly regulate muscle mass change after ACL-R. Myostatin is a cytokine that potently induces muscle atrophy. Mice, dogs, cattle, pigs, sheep, trout, and humans with mutations in myostatin have marked increases in muscle mass, and systemic administration of myostatin in mice leads to profound muscle atrophy and weakness. Intramuscular levels of myostatin are elevated after hindlimb unloading, and in a rat model of ACL injury, increases in intramuscular myostatin levels correlated with tear-induced quadriceps atrophy. Myostatin and its primary receptor, ActRIIB, are expressed in ACLs, and recombinant myostatin can increase cultured ACL fibroblast proliferation and type I collagen expression, but the role of myostatin in the ACL graft integration and ligamentization process is unknown. TGF-β is
a cytokine closely related to myostatin and also directly induces muscle atrophy and dramatically reduces muscle force production.\textsuperscript{20} Consistent with the elevation of myostatin and TGF-\(\beta\) in the immediate postoperative period, there was a reduction in knee strength, SF-12 PCS scores, and IKDC scores. While increases in myostatin and TGF-\(\beta\) were anticipated and confirmed after ACL-R, of interest are several factors that did not demonstrate significantly different plasma levels after ACL-R. IL-1\(\alpha\), IL-1\(\beta\), IL-6, and TNF-\(\alpha\) can directly cause muscle atrophy by activating proteolytic systems in muscle.\textsuperscript{20,26,27} However, no differences in serum levels of IL-1\(\alpha\), IL-1\(\beta\), IL-6, and IL-1 regulatory protein (IL-1ra) were detected at any time point in the current study. IL-1ra were detected at any time point in the current study.

We also evaluated changes in circulating factors that induce muscle hypertrophy. Studies in cultured muscle cells and animal models have identified IGF-1 as a major regulator of muscle growth. IGF-1 activates protein synthesis within muscle fibers and also promotes the proliferation of muscle stem cells, which are critical to enhance the growth of atrophied muscle.\textsuperscript{22,56} EGF and FGF-2 also stimulate muscle growth through similar mechanisms.\textsuperscript{12,22,38,45} IL-10 is an anti-inflammatory cytokine that deactivates proinflammatory, atrophy-inducing macrophages in skeletal muscle.\textsuperscript{41} We anticipated that hypertrophy-associated factors would be reduced in the immediate postoperative period when muscle atrophy is greatest and slowly increase as patients progress through rehabilitation and regain strength. Surprisingly, we found no change in circulating levels of IGF-1, EGF, FGF-2, or IL-10 throughout the course of the study. It is possible that these factors do not play a role in determining changes in muscle size after ACL-R or that local changes of these factors within the muscle are not reflected in the circulation.

In addition to atrophy- and hypertrophy-associated factors, we evaluated circulating levels of myoglobin, CRP, and COMP. Myoglobin is a protein found within the cytosol of muscle fibers, and elevations in circulating levels of myoglobin occur as a result of physical injury to muscle fibers and disruption of the sarcolemma.\textsuperscript{40} Myoglobin levels did not change over the course of the study, suggesting that minimal direct injury to muscle tissue occurred during the ACL-R procedure or secondary to the use of a lower extremity tourniquet. Also, CRP is an acute-phase protein that plays a central role in initiating the systemic response to inflammation.\textsuperscript{7} and acute increases in CRP have been reported after hip and knee arthroplasty.\textsuperscript{3,37} Normative values have yet to be established for most of the biomarkers evaluated in this study, but CRP values between 1 and 3 \(\mu\)g/mL are considered average by American Heart Association and Centers for Disease Control and Prevention guidelines.\textsuperscript{31} In the current study, CRP levels went up over 10-fold immediately after surgery and then returned to baseline over the course of the study. It is possible that these factors do not play a role in determining changes in muscle size after ACL-R or that local changes of these factors within the muscle are not reflected in the circulation.

![Figure 3](image-url) Changes in isometric and isokinetic strength over the course of the study. (A) Maximum isometric knee flexion force at 45° of knee flexion. (B) Maximum isometric knee extension force at 45° of knee flexion. (C) Maximum isometric knee flexion force at 90° of knee flexion. (D) Maximum isometric knee extension force at 90° of knee flexion. (E) Maximum isokinetic knee flexion force. (F) Maximum isokinetic knee extension force. All force values of the involved limb were normalized to the uninvolved limb at each time point. Vertical dashed line indicates the time of surgery. Values are expressed as mean ± standard error. N = 18 patients at each time point. *Significantly different from the preoperative time point (\(P < .05\)).
Figure 4. Changes in plasma levels of biomarkers over the course of the study. (A) CCL2, (B) CCL3, (C) CCL4, (D) CCL5, (E) cartilage oligomeric matrix protein, (F) C-reactive protein, (G) EGF, (H) FGF-2, (I) IGF-1, (J) IL-1α, (K) IL-1β, (L) IL-1ra, (M) IL-6, (N) IL-10, (O) myoglobin, (P) myostatin, (Q) TGF-β, and (R) TNF-α. Vertical dashed line indicates the time of surgery. Values are expressed as mean ± standard error. N = 18 patients at each time point. *Significantly different from the preoperative time point (P < .05).
to normal levels by the third postoperative visit. Further, COMP is a glycoprotein found in articular cartilage that helps to stabilize and align type II collagen molecules. When articular cartilage is broken down, COMP is released into the circulation, which makes it a useful marker of cartilage degeneration. Moreover, COMP is commonly used as a biomarker to track the progression of OA, and elevations in COMP are associated with the formation of knee osteophytes and joint space narrowing. As COMP is a sensitive biomarker for OA progression, and ACL tears are associated with an elevated risk of developing early-onset OA, we anticipated a slow and steady increase in COMP levels over time. However, we observed a significant decrease in COMP immediately after surgery, but otherwise, COMP levels were not different from the preoperative time point. The reason for this acute decrease in COMP levels is not known but may be caused by irritation of the joint and dilution during arthroscopic ACL-R. Given the association between OA and ACL tears, it is also not clear why COMP levels were not significantly elevated, but it is possible that the baseline preoperative levels are already high secondary to the traumatic joint injury from the index event.

There are several limitations to this study. While substantial muscle atrophy is known to occur after ACL-R, we did not directly measure muscle volume in our patients. We were not able to enroll patients in the study immediately after they suffered an ACL tear but instead enrolled patients at their preoperative time point after they had successfully completed a standard preoperative rehabilitation regimen. Patients were not followed past their 6-month time point, and it is likely that if we evaluated patients past this point, increases in COMP levels might be observed. While most patients are able to return to sport after ACL-R, the level at which patients can compete varies greatly, and it would be interesting to evaluate whether biomarkers of muscle atrophy and inflammation could identify which patients have greater deficits in athletic performance after return to play. Although it is likely that the muscles in the involved limb were the source of the increased myostatin and TGF-β observed in the circulation, we did not directly measure biomarker levels within the muscle secondary to potential morbidity associated with direct and serial muscle biopsy in athletes. Additional insight would be gained in future studies by looking at the changes in biomarkers immediately after suffering an ACL tear; examining changes across a wide variety of grafts, age ranges, and sexes; evaluating changes in biomarkers in the context of electromyography and muscle activation patterns; following patients for at least 2 years of follow-up; and exploring local changes in biomarkers via muscle biopsy.

CONCLUSION

Despite the limitations of our study, our results demonstrated significant increases in myostatin in the immediate postoperative period, and these changes corresponded to marked decreases in muscle strength and SF-12 PCS and IKDC scores. While it would be necessary to determine if intramuscular changes in myostatin levels occur in a similar fashion to what was observed in plasma, targeted inhibition of myostatin may have the potential to prevent muscle atrophy in the acute postoperative period after ACL repair. In a recent phase I clinical trial, the soluble decoy myostatin receptor ACE-031 was generally well tolerated, and administration of a single dose resulted in a small increase in lean mass 1 to 2 months after injection. However, there are some concerns within the sports medicine community as to the potential use of myostatin inhibitors as doping agents, although studies from mice suggest that myostatin inhibitors are not likely to be effective doping agents. The muscle fibers from otherwise healthy mice with a targeted inhibition of myostatin were bigger but not stronger than those from wild-type mice. As myostatin induces the expression of the main E3-ubiquitin ligases that serve as rate-limiting steps in protein degradation in muscle, the reason for an increase in fiber size with no change in force production was postulated to be an accumulation of nonfunctional proteins that would otherwise be targeted for degradation by the ubiquitin proteasome. For pathological conditions of muscle atrophy in which myostatin is elevated, the targeted inhibition of myostatin remains a potentially attractive target. If intramuscular levels of myostatin also increase after ACL-R, the pharmacological inhibition of myostatin may offer a way to safely restore patients to their preinjury physical abilities and accelerate the overall rehabilitation process.

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REFERENCES