**Anteromedial and Posterolateral Bundles of the Anterior Cruciate Ligament Exhibit Different Microstructural and Material Properties**

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Introduction: Over the last two decades, the double-bundle anatomic model has become a widely used description of anterior cruciate ligament (ACL) anatomy.1 Surprisingly, however, the material properties of the ACL have not been characterized in great detail. Several studies have analyzed the macroscopic biomechanics of the whole ACL, but tissue level analyses have been limited by preparation techniques and imaging technology.2 No study has analyzed the microscopic forces and collagen alignment of the human ACL to determine if the two bundles have different properties on a tissue level. The goal of this project was to quantify the tensile mechanical properties and fiber alignment of the anteromedial (AM) and posterolateral (PL) bundles in the human ACL in real-time under dynamic loading using a novel polarization imaging technique.3 We hypothesized that there would be no difference in properties between the AM and PL bundles. Deeper understanding of bundle-specific properties will help optimize surgical ACL reconstruction techniques, inform graft choice, and guide the development of novel grafts and scaffolds.

Methods: Sixteen donor knees were acquired (11 male, 5 female; avg. age = 41; avg. BMI = 27) with no history of knee trauma, surgery, or instability. Sample preparation. All soft tissues were removed, leaving only the ACL and PCL intact. An oscillating saw was used to separate the femoral condyles starting in the trochlear groove. The lateral intercondylar ridge of the femur and associated lateral bifurcate ridge between the two bundles were identified. Each bundle was gently separated using forceps and sectioned into three pieces by making two longitudinal incisions with a scalpel, yielding a total of six samples per ligament. Free from the bony attachments, samples were sectioned to ~600 μm using a freezing-stage sliding microtome. Cross-sectional area (CSA) was measured with a 3D laser scanning system. Testing. After gluing four 0.5-mm diameter brass beads to the sample for strain analysis, samples were clamped to a tensile test machine integrated with a custom-built quantitative polarized light imaging system.3 Using a high-resolution division-of-focal-plane polarization camera, the average direction of collagen orientation (AoP = Angle of Polarization) and the degree to which the fibers are aligned in that direction (DoLP = Degree of Linear Polarization) were quantified for each sample throughout the testing protocol. Our analysis focused on average (AVG) DoLP and standard deviation (STD) of the AoP within the central portion of each tested specimen. Ligament samples were preloaded to 0.1N, then subjected to 10 cycles preconditioning at 3% strain, a ramp-and-hold stress-relaxation test (5% strain step and dwell time of 300 seconds), and a quasi-static ramp to failure at a strain rate of 0.1%/s. Images were acquired at 17 frames per second throughout testing for optical strain and fiber alignment analysis. Data Analysis. Surface beads were tracked to calculate 2D Lagrangian strain. Stress was calculated as force divided by initial CSA. A bilinear curvefit was used to quantify the
modulus in the toe- and linear-regions and failure stress was computed. For each image captured during the ramp-to-failure portion of the mechanical tests, AVG DoLP and STD AoP values were computed (Fig.1); these values were then interpolated to calculate alignment parameters corresponding to specific points on the stress-strain curve (zero, transition, linear, and failure strain). Significant differences between bundles were detected using unpaired t-tests. Significance was defined as p<0.05, and trends towards significance are reported for 0.05<p<0.1.

Results: Contrary to our hypothesis, AM and PL bundles demonstrated significantly different mechanical and microstructural properties. AM samples exhibited significantly larger toe- and linear-region moduli than PL samples (Fig.2A). For both bundles, linear-region moduli were ~4-5X larger than toe-region moduli, demonstrating significant mechanical non-linearity. Failure stress was significantly larger \( p=0.021 \) for the AM bundle (Fig.2B). In terms of collagen fiber alignment, AVG DoLP values (Fig.2C) were similar at low strain, but were larger for the AM bundle at the transition \( p=0.084 \), linear \( p=0.052 \) and failure strain \( p=0.039 \). STD AoP values (Fig.2D) were not different at zero strain, but smaller for the AM bundle than the PL bundle at transition \( p=0.058 \), linear \( p=0.018 \) and failure strain \( p=0.018 \). Both bundles showed similar changes in alignment as strain increased (i.e., increasing AVG DoLP and decreasing STD AoP).

Discussion: Several studies have described ACL anatomy but lack quantitative data to define anatomic bundles. Currently, the double-bundle anatomic model is the most widely accepted description of ACL anatomy. Some orthopaedists advocate for double-bundle reconstruction to more closely recreate the natural ACL anatomy, however advocates of the single-bundle reconstruction believe a properly placed single-graft functions similarly to the double-bundle reconstruction. The purpose of this study was to determine whether the AM and PL bundles of the ACL exhibit differences on the tissue level. The material properties of the AM and PL bundles were found to be significantly different, specifically in terms of mechanics and microstructural organization. The AM bundle exhibited larger moduli and failure stress compared to the PL bundle. Collagen fiber organization was inhomogeneous, with most realignment occurring in the toe-region of both bundles. At moderate to high levels of strain, the AM bundle demonstrated more highly aligned collagen fibers. As the stronger, stiffer bundle with more uniform fiber alignment, the AM bundle appears to be the “dominant” bundle. The implications for ACL reconstruction surgery are not immediately clear. For example, there could be an advantage to using a graft with material properties similar to those of the native ligament. If so, it is not clear if two bundles are necessary, or if a single bundle with varying properties would suffice. In addition, the material properties of certain allografts such as Achilles or tibialis tendons may not be ideal for ACL reconstruction. Besides informing reconstruction approaches, these results could be used to guide development of materials for ligament replacement.

Significance: Using a novel polarization imaging technique to quantify collagen fiber alignment during mechanical tensile testing, this study identified significantly different material and microstructural properties in the AM and PL bundles of the ACL. These insights into native ligament mechanics and organization can be used to assess graft options for ACL reconstruction, optimize surgical techniques, and assist in the design of novel grafts and bioscaffolds for ACL replacement.
Fig. 1: Example fiber alignment of an anteromedial (AM) sample: grayscale, DoLP and AoP images at zero and linear strain (DoLP = Degree of Linear Polarization; AoP = Angle of Polarization, where the horizontal axis corresponds to 90°).

A

B

C

D

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