State of the mineralized tissue comprising the femoral ACL enthesis in young women with an ACL failure

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Running Title: Femoral ACL enthesis tissue post-injury


Abstract

Despite poor graft integration among some patients that undergo an ACL reconstruction, there has been little consideration of the bone quality into which the ACL femoral tunnel is drilled and the graft is placed. Bone mineral density of the knee decreases following ACL injury. However, trabecular and cortical architecture differences between injured and non-injured femoral ACL entheses have not been reported. We hypothesize that injured femoral ACL entheses will show significantly less cortical and trabecular mass compared to non-injured controls. Femoral ACL enthesis explants from 54 female...
patients (13 – 25 years) were collected during ACL reconstructive surgery. Control explants (n = 12) were collected from 7 donors (18 - 36 years). Injured (I) femoral explants differed from those of non-injured (NI) controls with significantly less ($p \leq 0.001$) cortical volumetric bone mineral density (vBMD) (NI: 736.1 – 867.6 mg/cc; I: 451.2 – 891.9 mg/cc), relative bone volume (BV/TV) (NI: 0.674 – 0.867; I: 0.401 – 0.792) and porosity (Ct.Po) (NI: 0.133 – 0.326; I: 0.209 – 0.600). Injured explants showed significantly less trabecular vBMD ($p = 0.013$) but not trabecular BV/TV ($p = 0.314$), thickness ($p = 0.412$), or separation ($p = 0.828$). We found significantly less cortical bone within injured femoral entheses compared to non-injured controls. Lower cortical and trabecular bone mass within patient femoral ACL entheses may help explain poor ACL graft osseointegration outcomes in the young and may be a contributor to the osteolytic phenomenon that often occurs within the graft tunnel following ACL reconstruction.

**Introduction**

Between 2016 and 2017, ~3.4 million girls participated in organized high school athletics$^1$ and ~150,000 women participated in organized collegiate athletics. In a subset of this female demographic Beynnon et al.$^2$ reported a non-contact ACL injury incidence rate (IR) of 0.077 across all high school sports and an IR of 0.1425 across all collegiate sports per 1000-person days, which after adjusting for sex, level of play, and sport was more than double the injury risk than that of males. Approximately 70% of these injuries are surgically reconstructed using an auto- or allo-graft$^3$, and of these primary reconstructions as many as 5.8% of them will suffer a primary ACL graft failure within 5 years$^4$-$^6$. In those under the age of 20 years the probability of a primary ACL graft failure is estimated to be 6 times higher compared to individuals over 20 years of age$^5$. This steep increase in primary graft failure incidence rates, particularly in the young, is a major health concern. ACL revision surgery is inferior to the primary ACL reconstruction$^7$-$^8$ it often requires two surgeries to repair the bone and then place the
secondary graft, it drastically increases the likelihood of developing osteoarthritis\textsuperscript{9}, and it comes at a considerable economic cost\textsuperscript{10}.

Several prospective studies have determined that biologic factors resulting in an acute traumatic graft failure account for upwards of 43\% of all primary ACL graft failures\textsuperscript{12-16}. This type of failure is largely due to insufficient osseoligamentous integration of the graft\textsuperscript{17} resulting from tunnel osteolysis\textsuperscript{18} (refer to Fig. 1 for an example X-ray of a patient with tunnel osteolysis following ACL reconstructive surgery). Biologic failures are a more common occurrence with tendinous auto/allo-grafts with no associated bony component (e.g., hamstring tendon). In patients under 25 years of age, the failure rate for soft tissue grafts is at least 25\% higher compared to bone-patellar tendon-bone (BTB) grafts\textsuperscript{19}. However, integration failure is also a significant factor in BTB graft failures\textsuperscript{8}.

Despite the looming possibility of poor graft osseointegration among young patients that have received an ACL reconstruction, it is surprising that there has been little consideration of the condition of the mineralized tissue into which the ACL tunnel is drilled and the ACL graft is placed. It is well known that clinically measured bone mineral density (BMD) within the entirety of the distal femur and proximal tibia drastically decreases following an initial ACL injury\textsuperscript{20-22}, which may be directly related to altered loading and kinematics in the injured limb. However, dual-energy X-ray absorptiometry (DXA)-derived BMD is a two-dimensional surrogate for what is actually a three-dimensional volume that is manipulated algorithmically to approximate a true volumetric density measure (i.e., apparent BMD and volumetric BMD)\textsuperscript{23}. Additionally, DXA-based BMD does not have the resolution to assess bone morphology details to differentiate the contributions of cortical and trabecular tissues; which are important variables to consider in growing individuals to understand the structural and compositional state of the mineralized matrices. More importantly, DXA-based BMD measures are unable to adequately inform on the mineralized tissue architecture in a small region of interest (e.g., 50 mm\textsuperscript{3}) such as that adjacent to the femoral ACL enthesis,
where ~95% of non-contact ACL ruptures occur\textsuperscript{24}. These structural and compositional details are needed to understand the role mineralized tissue quantity and quality might play in the post-operative osseointegration of repair grafts, which may help inform decisions on the optimal timing for ACL reconstruction relative to the injury date.

Our overarching hypothesis is that tunnel osteolysis as a result of graft loosening within the tunnel is facilitated by the loss of structural integrity within the mineralized ACL entheseal structures (i.e., the calcified fibrocartilage, subchondral cortical bone, and adjacent trabeculae) following native ligament failure. To investigate this association, we first need to test whether localized mineralized tissue adjacent to the enthesis is lower in those that had an ACL injury compared to those that have not injured. Herein, we tested the hypothesis that injured femoral ACL entheses will show significantly less cortical and trabecular bone mass compared to non-injured controls. We characterized the mineralized tissue comprising the femoral ACL enthesis in young females that suffered an ACL injury and experienced a range of time intervals from the point of ACL failure until they were physically and physiologically ready for reconstructive surgery (on average 8 – 12 weeks post-injury). Our findings suggest there is substantially less mineralized tissue within the femoral ACL enthesis at the time of reconstructive surgery in patients that had an ACL failure. Whether this significantly lower mineralized tissue volume impacts osseointegration of the ACL graft following reconstruction will be the focus of future studies. Moreover, this low tissue volume may signify active bone resorptive activity, which may in part influence early tunnel osteolysis frequently observed with auto- and allo-grafts following primary and revision surgery.

**Methods**

**Sample population**

Femoral ACL enthesis explants were collected from the injured knee of 54 female patients during ACL reconstructive surgery. Subjects ranged in age from 13 to 25 years. The sex and ages were chosen since females are twice as likely to suffer an ACL injury.
compared to males, and because young individuals are 30% more likely to suffer a primary ACL graft failure within 5 years of surgery\textsuperscript{2-5}. De-identified data recorded for all patients included age, sex, activity at moment of injury, time from injury to surgery and location of injury (Fig. 2). Patient explants were collected by one surgeon (EMW) to minimize variation in arthroscopic techniques. For explant extraction, a 10 mm diameter trephine was employed using standard ‘outside-in’ surgical practices (Fig. 3), which removes ~50% of the entire native enthesis. Upon extraction, femoral explants were stored at 4$^\circ$ C in 1x phosphate-buffered solution (PBS) and imaged within 72 hours. In addition to the patient tissue, 12 control femoral explants were acquired from paired knees of five female cadaveric donors and two additional unpaired knees from two donors ranging in age from 18 – 36 years from the Gift of Life Michigan and the University of Michigan Medical School. These donor knees, which exhibited no signs of prior knee surgery or visible scars around the joint, were harvested within 48 hours following death, sealed in plastic bags and frozen at -20$^\circ$ C until ACL femoral explants could be extracted. Donor explants were extracted using equipment and techniques identical to that used in the clinical setting. All control tissue was stored at 4$^\circ$ C in 1x PBS and imaged three-dimensionally within 72 hours. Use of patient and cadaveric tissues was approved by the University of Michigan Institutional Review Board under an exempt status designation.

\textbf{Scanning preparation and acquisition}

High resolution (14 um voxel size) scans of femoral ACL explants were acquired using a nanotom-s computed tomography system (phoenix|x-ray, GE Measurement & Control; Wunstorf, Germany) and consistent acquisition parameters (80 kV, 300 $\mu$A, 68 minutes, 1000 ms exposure time, 1000 images, 0.012” aluminum filter). During the course of this study the nanotom-s was upgraded to a nanotom-m (phoenix|x-ray, GE Inspection Technologies; Skaneateles, NY, USA), resulting in 22 of the femoral ACL explants included in this study being scanned on the newer system (70 kV, 300 $\mu$A, 34
minutes, 5000 ms exposure time, 1000 images, 0.012” aluminum filter) at the same resolution. The image acquisition parameters of the new system were adjusted so the two systems generated similar grey values (< 2% difference). Femoral explants were imaged in a 5 mL polypropylene scintillation vial, surrounded by polyurethane foam to prevent movement and saturated in 1x PBS to maintain tissue hydration. A calibration phantom containing air, water and a hydroxyapatite mimicker (1.69 mg/cc; Gammex, Middleton, WI, USA) was included in each scan. Image volumes were reconstructed using datos|x reconstruction software (phoenix|x-ray, GE Sensing and Inspection Technologies, GmbH, Wunstorf, Germany).

**Volumetric analysis**

Grey values from each reconstructed image were converted to Hounsfield units using the calibration phantom as described previously. Each explant was reoriented along the anteroposterior and mediolateral anatomical axes using MicroView 2.0 software (Parallax Innovations, Inc., Ilderton, ON, Canada), and the calcified fibrocartilage and cortical (true mineralized enthesis) were manually segmented into one volume of interest (VOI) while the adjacent trabeculae wherein mechanical stress dissipates was included as a second VOI. (Fig. 4). Each 10 mm diameter and ~6 mm long explant contained a 2.5 mm diameter guide-pin hole directly through the center of the explant due to the tissue extraction technique. Thus, the total measured volume for each specimen was ~440 mm$^3$ after digitally subtracting the ~30 mm$^3$ volume where the guide-pin passed through the center for VOI analyses. True volumetric bone mineral density (vBMD), relative bone volume (BV/TV) and porosity (Ct.Po) were quantified for the cortical VOI. For the trabecular VOIs, analyses were standardized to 3.5 mm of trabecular bone relative to the most inferior aspect of the cortical matrix. Trabecular variables quantified included vBMD, BV/TV, trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp).
**Histologic analysis**

Following three-dimensional imaging of the femoral ACL explants, all explants from patients that underwent reconstructive surgery between 4 to 15 weeks post-injury were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for ten days, dehydrated through graded alcohols, and embedded in paraffin. For each tissue block, sagittal 5-µm serial sections were taken across the entire ACL enthesis. Paraffin sections were then stained with 1% toluidine blue (Sigma-Aldrich, St. Louis, MO, USA), a monochromatic stain that highlights the 4 matrix zones of the ACL enthesis (i.e., ligament proper, uncalcified fibrocartilage (UF), calcified fibrocartilage (CF), and bone) and is sensitive to mineralization (i.e., proteoglycan) differences between tissue structures. Stained histologic sections were brightfield imaged at 10x magnification using a Nikon Eclipse (Ni; Nikon, Melville, NY, USA) microscope affixed with a digital color camera (DS-Ri2; Nikon, Melville, NY, USA). For each tissue section, the presence/amount of disordered cartilaginous matrix, matured calcified fibrocartilage, cortical bone, and mineral resorption was ranked for each patient as follows: disordered cartilaginous matrix (1 = >50%, 2 = 25-50%, 3 = <25%), mature calcified fibrocartilage (1 = >50%, 2 = 25-50%, 3 = <25%); cortical bone (1 = thick, 2 = thin); mineral resorption (1 = present, 2 = absent). Histomorphometry was qualitatively analyzed since the precise location each explant was removed from the patient cohort was specific to their anatomy and surgical needs, and thus the sampled region could not be standardized.

**Statistical analysis**

All data were analyzed using RStudio Team (2015) (RStudio, Inc., Boston, MA). A nonparametric Wilcoxon t-test was conducted to determine if there were significant asymmetric differences between femoral explants harvested from the left and right knees of the control cadaveric donors (n=5). Results from control donors with explants from paired knees were averaged since there was no significant difference in cortical or trabecular results (see Results). Individual data points acquired from each patient variable

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and each cadaveric control variable were combined to create injured (n=54) and non-injured (n=7, unpaired explants from 2 cadavers and 5 cadavers with bilateral explants) groupings. This was necessary to account for the inherent biological variation between individuals since the distribution of demographic data was not always well powered (see Fig. 2). Once the individual data points were combined, an unpaired t-test with Welch’s correction was performed to test whether the cortical and trabecular parameters differed significantly between patient and control explants. Linear regression analysis as well as unpaired t-test with Welch’s correction in groups of different time frames (1 - 7, 8 - 11, 12 - 16, and 17 + weeks) from injury to operation was used to test how time from injury to operation affected the architectural parameters derived from the cortical and trabecular VOIs. Since the majority of ACL reconstructions performed on our patient cohort were done around 4, 8, and 12 weeks post-injury, these time point ranges were chosen to encompass the first 3 months following an injury, which correspond with the inflammatory, regenerative, proliferations, and remodeling phases within the ligament following an injury. In order to assess the effect of age on bone microstructure, a multivariate regression including age, time from injury to operation, and the interaction between the two was performed to identify significant independent predictors of bone microstructure. An alpha of 0.05 was used for all statistical analyses to identify significant differences between groups.

Results

Sample Population

Basketball (31%) and soccer (24%) were the most prevalent activities resulting in ACL injury in our cohort. The location of these injuries was most prevalent adjacent to the femoral enthesis and the range of time from injury to operation varied from 4 to 78 weeks. The majority of primary reconstructive surgeries occurred within the first 16 weeks (85%). The median time in which ACL reconstructive surgery was completed post-injury was 10 weeks.
Cortical Results

The paired left and right femoral explants of the five control non-injured (NI) cadaveric donors showed no significant difference in the cortical measures (vBMD, $p = 0.313$; BV/TV, $p = 0.313$; Ct.Po $p = 0.438$). The injured (I) explants showed significantly lower vBMD (NI: 736.1 - 867.6 mg/cc; I: 451.2 - 891.9 mg/cc; $p < 0.001$), BV/TV (NI: 0.674 - 0.867; I: 0.401 - 0.792; $p = 0.001$), and Ct.Po (NI: 0.133 - 0.326; I: 0.209 - 0.600; $p = 0.001$) compared to the non-injured femoral explants (Fig. 5). F-tests comparing variances between non-injured and injured femoral explants was not significant for any cortical parameters (vBMD $p = 0.112$, BV/TV, $p = 0.490$; Ct.Po, $p = 0.489$). Linear regressions for patient explants showed no significant positive association between any parameters and time between surgery and injury (vBMD, $p = 0.284$; BV/TV, $p = 0.175$; Ct.Po, $p = 0.177$). Explants were grouped by time from injury to operation (Control, 1-7, 8 – 11, 12 – 16, and 17 + weeks from injury to operation). The injured (I) explants in all groups showed significantly lower vBMD (1 - 7 weeks, $p<0.001$; 8 - 11 weeks, $p = 0.007$; 12 - 16 weeks, $p = 0.002$; 17 + weeks, $p = 0.005$), BV/TV (1 - 7 weeks, $p<0.001$; 8 - 11 weeks, $p = 0.006$; 12 - 16 weeks, $p = 0.002$; 17 + weeks, $p = 0.008$), and Ct.Po (1 - 7 weeks, $p<0.001$; 8 - 11 weeks, $p = 0.007$; 12 - 16 weeks, $p = 0.002$; 17 + weeks, $p = 0.008$) compared to the non-injured femoral explants (Fig. 6). However, despite a qualitatively lower cortical vBMD and BV/TV and greater Ct.Po in relation to time from injury, no significant quantitative difference was found between patient groups.

Trabecular Results

There were no significant differences in the trabecular measures between the paired left and right femoral explants removed from the five control non-injured (NI) cadaveric donors (vBMD, $p = 0.125$, BV/TV, $p = 0.313$; Tb.Th, $p = 0.313$; Tb.Sp, $p = 1.000$). The injured (I) vBMD was significantly lower (NI: 364.5 - 424.3 mg/cc; I: 246.7 - 529.6 mg/cc; $p = 0.013$) (Fig. 7) compared to the non-injured femoral explants. Non-significant differences in BV/TV ($p = 0.314$), Tb.Th ($p = 0.412$), and Tb.Sp ($p = 0.828$) between
non-injured and injured femoral explants were observed, suggesting that trabecular bone quality may not be significantly affected by ACL injury in our patient cohort. F-tests comparing variances between non-injured and injured femoral explants were significant for vBMD ($p = 0.016$) but for no other trabecular variable (BV/TV, $p = 0.301$; Tb.Th, $p = 0.841$; Tb.Sp, $p = 0.543$). In addition, linear regressions showed no association between vBMD, BV/TV, Tb.Th, and Tb.Sp at the time that had elapsed post-injury and prior to reconstructive surgery (vBMD, $p = 0.391$; BV/TV, $p = 0.284$; Tb.Th, $p = 0.157$; Tb.Sp, $p = 0.569$). Explants were grouped by time from injury to operation (Control, 1 - 7, 8 - 11, 12 - 16, and 17 + weeks from injury to operation) for vBMD. The injured (I) explants only showed significantly lower vBMD at 17 weeks or greater from injury to operation ($p = 0.018$) but for no other time frame (1 - 7 weeks, $p = 0.153$; 8 - 11 weeks, $p = 0.132$; 12 - 16 weeks, $p = 0.145$), compared to the non-injured femoral explants. No significant difference was found in vBMD between patients grouped by time from injury to operation.

**Age and Time from Injury to Operation Effects on Bone Microstructure**

For cortical variables, time from injury to operation (vBMD, $\beta = 6.7$, $p = 0.283$; BV/TV, $\beta =-4.8e-4$, $p = 0.932$; porosity, $\beta = 6.7$, $p = 0.283$), age (vBMD, $\beta = 12.1$, $p = 0.113$; BV/TV, $\beta = 3.8e-4$, $p = 0.956$; porosity, $\beta = 12.1$, $p = 0.113$), and the time from injury to operation-age interaction (vBMD, $\beta = -0.4$, $p = 0.212$; BV/TV, $\beta = -2.5e-5$, $p = 0.932$; porosity, $\beta = -0.4$, $p = 0.211$) were non-significant predictors of cortical bone microstructure (vBMD, $R^2[\text{adj.}] = 0.02$, $p = 0.295$; BV/TV, $R^2[\text{adj.}] = -0.02$, $p = 0.612$; porosity, $R^2[\text{adj.}] = 0.02$, $p = 0.295$). For trabecular variables, time from injury to operation (vBMD, $\beta = 0.64$, $p = 0.869$; BV/TV, $\beta = 3.2e-3$, $p = 0.301$; Tb.Th, $\beta = -1.3e-3$, $p = 0.509$; Tb.Sp, $\beta = 5.4e-3$, $p = 0.251$), age (vBMD, $\beta = 1.1$, $p = 0.818$; BV/TV, $\beta = 5.2e-3$, $p = 0.179$; Tb.Th, $\beta = -2.2e-3$, $p = 0.352$; Tb.Sp, $\beta = 1.8e-3$, $p = 0.756$), and the time from injury to operation-age interaction (vBMD, $\beta = 9.4e-3$, $p = 0.963$; BV/TV, $\beta = 1.5e-4$, $p = 0.349$; Tb.Th, $\beta = 5.3e-5$, $p = 0.609$; Tb.Sp, $\beta = -2.7e-4$, $p = 0.286$) were non-
significant predictors of cortical bone microstructure (vBMD, R²[adj.] = -0.04, p = 0.832; BV/TV, R²[adj.] < 0.01, p = 0.397; Tb.Th, R²[adj.] < 0.01, p = 0.407; Tb.Sp R²[adj.] = -0.02, p = 0.585).

**Enthesial Histomorphometry and Time from Injury to Operation**

Qualitative histomorphometric data of the femoral ACL enthesis for all included patients are provided in Figure 8. Only 25% of patients between that injured between 1 - 7 weeks post-injury demonstrated a high degree (> 50%) of disordered cartilaginous matrix in their enthesis, with those further out from injury demonstrating a higher prevalence (40% of patients between 8 - 11 weeks and 38% of patients between 12 - 16 weeks post-injury). More than 50% of mature calcified fibrocartilage was observed within the enthesis of 67% of patients between 1 - 7 weeks post-injury, 40% of patients between 8 - 11 weeks post-injury, and 50% of patients between 12 - 16 weeks post-injury. A thick cortical shell was only observed within the enthesis of 33% of patients between 1 - 7 weeks post-injury, 40% of patients between 8 - 11 weeks post-injury, and 38% of patients between 12 - 16 weeks post-injury. Patients within the shortest elapsed time from injury to operation demonstrated the greatest evidence of osteoclastic activity with 67% of those between 1 - 7 weeks post-injury showing recent mineral resorption. Patients further out from injury demonstrated lower resorptive activity with 40% of patients between 8 - 11 weeks post-injury and 25% of patients between 12 - 16 weeks post-injury showing recent mineral resorption.

**Discussion**

The data supported the hypothesis that there would be substantial bone loss within the femoral ACL enthesis. The femoral explants removed at the time of surgery revealed extensive and significant differences in cortical vBMD, BV/TV, and Ct.Po between patient and control explants. Patient explants also showed a significantly lower trabecular vBMD compared to controls. However, this difference in vBMD could not be explained based on details of the trabecular architecture, but instead may be due to changes in the...
organic matrix of the bone at the site of an ACL injury. On average, complete cortical and trabecular bone turnover takes 120 and 200 days, respectively\textsuperscript{27}. Therefore, much of the lower bone volume observed in our patient population may be reflective of active bone resorption during the early phase of the bone remodeling cycle. This timing corresponds with the histological presence of osteoclastic activity along the calcified fibrocartilage, cortical and trabecular surfaces, and is in agreement with the rapid bone resorption occurring early on following mechanical unloading\textsuperscript{28} and the inflammatory phase of the ruptured ligament characterized by an increase in neutrophilic, lymphocytic, and macrophagic cellular activity\textsuperscript{26}.

While differences in trabecular vBMD were observed, bone architectural differences between injured and controls were most evident in the cortical tissue comprising the femoral ACL enthesis. Bone microstructure of the patient and control explants varied considerably. However, when cortical vBMD, BV/TV, and Ct.Po data were sub-grouped by time from injury to operation, there was no significant difference between any of the sub-groups compared to controls. If the various findings are occurring post-injury, it would suggest there is a rapid significant decrease in cortical bone volume following ACL injury combined with an increase in bone remodeling activity (i.e., Ct.Po) that when compared with the controls appears to not be recovered prior to ACL reconstructive surgery. Interestingly, finding trabecular differences in vBMD but in no other architectural measure (BV/TV, thickness, or separation) in our patient group compared to controls suggests the organic matrix (inorganic, organic, or water component) may be changing in the bone at the site of ACL injury\textsuperscript{29}. When trabecular vBMD was sub-grouped, there was only a significant difference in the 17 week and greater post-injury on time frame compared to controls. If this lower vBMD is due to injury, it may suggest that the underlying mechanism of trabecular vBMD bone loss differs from cortical bone and may occur at a slower, steadier rate and increases with time following injury.
Our patient population also demonstrated that there is considerable inter-individual variation, despite many falling well below (Tb.vBMD, Ct.vBMD, and Ct.BV/TV) or above (Ct.Po) the non-injured cohort. Time from injury to operation alone could not explain the difference observed in this study. However, considering that the majority of the explants analyzed were from patients that were 8 to 12 weeks from injury, our results suggest that many patients have mineralized matrices that still may not be optimal for adequate osseointegration of an ACL auto-/allo-graft following reconstruction. Our tissue harvesting protocol attempted to remove the central portion of the femoral ACL enthesis by placing a guide pin under arthroscopic visualization. Considering the fact that we only harvested ~50% of the entheseal surface area (i.e., 10 mm), we are reasonably certain that the harvested tissue is a good representation of the surrounding, remaining enthesis. The remaining entheseal tissue (i.e., calcified fibrocartilage, cortical bone, trabecular bone) is the optimal location for graft healing and the eventual restoration of mechanical function (see Fu et al. for review). If osseointegration does not occur at the orifice of the femoral tunnel located in the enthesis, osteolysis via the ‘windshield wiper effect’, is more likely to occur resulting in a change in the position of the ACL attachment to bone. This alteration could easily result in abnormal graft function. Future studies need to characterize the viability of osteogenic cells in this region in conjunction with the inflammatory activity occurring in the adjacent synovium. Further, future research could characterize this site in animal models to better understand the early biological response to ACL tears in a setting where variables are better controlled (e.g., mechanism of tears, activity post injury, sex, age, etc.). Nonetheless, clinically these findings may allude to an important biological parameter that may affect ACL reconstruction success.

Bone loss following an ACL injury, as measured using clinical dual-energy X-ray absorptiometry (DXA), has been observed both pre- and post-surgery around the knee joint. However, most of these studies focused on regional changes after extensive time had passed (~1 to 12 years post-surgery) and were largely focused on the cancellous
bone within the distal femoral and proximal tibial metaphyses, avoiding the more variable subchondral bone that may be sclerotic and less homogenous after injury.22

Altered weight bearing and/or limb disuse could explain the outcomes of these DXA-based studies since they were largely focused on cancellous bone, which is the most affected tissue following prolonged immobilization and weightlessness (see LeBlanc et al.33 for review). However, this explanation may not be the only factor contributing to the differences among bone measures observed in our patient population since we observed significant structural and compositional differences present in the subchondral bone early after injury and only vBMD differences in the adjacent trabecular regions more than 3 months after injury, compared to controls. Moreover, Rittweger et al.34 observed significant reductions in volumetric bone mineral content in patients that had an ACL reconstruction using a BTB graft more than five years earlier but had a full recovery of knee extensor strength and patellar tendon stiffness. This would also suggest that a lack of weight-bearing activity and kinematic changes may not be the only mechanism driving mineralized tissue changes following ACL injury. All patients included in this study underwent pre-surgical rehabilitation to improve range of motion, and stave of thigh, knee, and hip muscle wasting. These prescribed loading bouts are enough to largely maintain bone volume, since only a few mechanical strain cycles are needed to generate an osteogenic stimulus.35 However, based on the location of the femoral ACL enthesis this region would not be directly subjected to these weight-bearing loads, suggesting that the localized mineralized tissue differences observed between patients and controls is attributable more to ACL unloading and the localized inflammatory response within the synovium rather than gross load-bearing differences post-injury.

Neither age nor the interaction between age and time from injury to operation significantly predicted differences in cortical or trabecular bone microarchitecture in our patient cohort. Our patient cohort ranged in age from 13 to 25 years of age (i.e., before and after skeletal maturity is reached) due to fact that females are twice as likely to suffer

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an ACL injury compared to males, and because females in the age-range are at particularly high risk of ACL graft failures following surgery\textsuperscript{2,5}. While the authors recognize that age is an important factor to consider in understanding bone microarchitecture, our findings suggest that age-related differences cannot explain the variability in bone architecture across the patient groups or between these patient and control groups.

To better understand the mineralized tissue variation in our patient cohort, we qualitatively inspected the surrounding fibrocartilaginous enthesis of a subset of the patient and control explants using histological slides with toluidine blue staining. A healthy fibrocartilaginous enthesis is characterized by a zone of uncalcified fibrocartilage, calcified cartilage, and bone\textsuperscript{36}. A “tidemark” delineates the uncalcified and calcified fibrocartilaginous zones (Fig. 8A). This feature was disrupted in a large number of patients due to mineral resorption activity as defined by osteoclastic scalloping between tissue zones. Moreover, our patient cohort demonstrates variable volumes of calcified fibrocartilage anchoring the ACL to the femur (Fig. 8C), which is much lower than that observed among controls across all ages. Further, many patients showed signs of new cartilaginous matrix formation between the ACL and femur (Fig. 8B) that is similar to reparative matrices formed following tibial tubercle avulsion in Osgood-Schlatter patients\textsuperscript{37}. In conjunction with new matrix, many of these same patients demonstrated osteophyte formation at the ACL-cortical bone junction suggesting that an inflammatory process probably plays an important role, in addition to disuse, in the underlying mechanism of bone loss following injury. Future work should focus on characterizing the entire enthesis complex post-injury under controlled settings in animal models.

Our findings are novel in that these are the first assessments of early differences between patients and controls in cortical and trabecular architecture at the exact location in which ACL graft fixation occurs at reconstruction. Further, we argue that our findings
are directly related to ACL injury and cannot be simply related to cohort (e.g., same age, sex, and procurement) effects because we predominately observed architectural differences in cortical but not trabecular tissue. Less is known about how the mineralized matrices in a small focal area such as at the osseoligamentous interface of the ACL enthesis structurally and functionally responds to the application, or lack thereof, of mechanical forces. Despite the complex physiological kinematics of the knee, where the distal femur experiences both joint contact (reaction) forces and tibiofemoral (bone-on-bone contact) forces, loads directly at the ACL origin are largely tension and shear forces from the ACL itself \(^3\). Thus, based on our findings, in the context of what has been previously reported by others, we hypothesize that the mineralized tissue differences observed in our patient femoral ACL explants represent highly localized early disuse osteoporosis resulting from the loss of ACL tension and shear loads \(^3\), in conjunction with the phagocytic and macrophagic activity in the synovium known to occur within the first few weeks following injury \(^2\). However, we cannot rule out that some of the mineralized tissue differences observed herein could also be the result of a microscopic subchondral avulsion of the calcified fibrocartilage in conjunction with the ACL rupture. Regardless of the mechanism responsible for these differences, our results indicate that the remodeling of mineralized tissue in the ACL enthesis may be significant and, based on our post-injury time points, occurs early following injury. This finding could be of great importance considering that within the first 3 months following injury the bone remodeling response appears to be one sided, with rapid osteoclastic- and/or inflammation-driven bone resorption, but not simultaneously coupled osteoblastic-driven bone formation response. Therefore, the physiologic imbalance between bone resorptive and formative activities may play a contributory role in tunnel widening and graft loosening due to an osteolytic tissue response. Thus, the timing of the re-establishment of tensile forces and weight-bearing activities on the ACL femoral enthesis following surgery could be an important factor to consider in evaluating ACL reconstruction.

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protocols. Supporting this conclusion, Tomita et al.\textsuperscript{40} demonstrated that the timing of graft integration and the restoration of near-native mechanical properties differ between soft tissue and BTB grafts in dogs. Osseointegration may be jeopardized if the mineralized regions through which the ACL graft passed has degenerated, particularly if a soft tissue graft or allograft is used. Secondly, BTB grafts appear to fail less often than soft tissue grafts that lack a bony interface\textsuperscript{41}. Additional research is needed to determine if the supply of viable bone cells (i.e., osteoblasts, osteocytes, chondrofibroblasts) in the BTB constructs placed into the post-injury native bone of the femoral tunnel explain this divergent outcome between graft types.

There is additional concern for bone integrity loss after ACL injury when considering the phenomenon of tunnel expansion or osteolysis seen frequently with various ACL reconstruction techniques, both in primary and revision surgeries\textsuperscript{42-44}. This gradual tunnel expansion that accompanies graft motion within the tunnel (i.e. ‘windshield wiper effect’) and in both auto- and allo-grafts, may be facilitated by the localized loss of bony structural integrity in the enthesis and adjacent matrices when the ACL fails. ACL remnant preservation techniques have been utilized in an attempt to prevent tunnel osteolysis by preventing synovial fluid leaks from the knee joint\textsuperscript{45}. However, the root cause for that bone degeneration may not only be leaking synovial fluid but also primary bone degeneration from the prolonged loss of tensile forces on the ACL enthesis.

Other regions of the ACL-complex have demonstrated cell loss following an ACL injury that is time dependent\textsuperscript{46}. Cell apoptosis following injury has been documented in the ruptured ligament itself\textsuperscript{47; 48}, but the cellular integrity of the mineralized matrices comprising the entheses remains an enigma. The reality may be that the same process and timetable is occurring in bone. For those prescribing ACL stump preservation during reconstructive surgery\textsuperscript{45}, the timing of ACL surgery has become more important to minimize ligament cellular apoptosis. The same scenario may be in play within the mineralized entheseal zones of the ACL.

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The primary limitation of this study was the lack of longitudinal data to be able to track physiological changes occurring within the tissue post-injury. Due to this limitation all we characterize is the state of the tissue at the time of surgery. Nonetheless, this snapshot taken at the time of surgery is important not only because the injured ACL tissue in humans is rarely analyzed, but also because this is the exact time and location wherein a primary graft is placed. Another limitation was in restricting analyses to young females that were injured. Young females were selected due to their high clinical risk of ACL injury and primary revision rates. Currently we are working to expand the sample population to include males and more post-injury time points to better account for biological and injury mechanism variation. A further limitation of this study was the de-identified nature of specimens that we collected under the ‘exempt’ category of Institutional Review Board approval. This precluded considering other important factors that could have contributed to the significantly lower bone volume among these patients compared to controls. Thus, although the volumes of interest we analyzed were localized to a small portion of the femoral ACL enthesis and adjacent trabeculae, we are unable to speak to the prevalence of knee effusions, synovitis, bone contusions, or inflammatory factors, and how these conditions may relate to the patient tissue we investigated. Another limitation is that the 10 mm diameter trephine only provides a core sample of a portion of the ACL enthesis (~50%) and the exact enthesal location of the sampling is patient specific and may vary (e.g., proximal vs. distal and medial vs. lateral). Lastly, due to the clinical needs of our patient cohort we have no data concerning the early post-injury that occur within the first month. We hypothesize that the dramatic bone loss we report most likely happened early post-injury. Future work will be required to test this. Despite these limitations precluding a more robust investigation into the remodeling state of the mineralized structures, the findings of this study suggest further investigations into post-ACL injury changes in vivo are needed to better account for changes occurring at the
time of injury, as well as during the subsequent inflammation and tissue remodeling responses within the ACL enthesis.

In conclusion, our results support the hypothesis that the condition of the mineralized tissue, particularly that of the cortical matrix, into which a femoral tunnel is drilled and the ACL auto- or allo-graft is placed, may not be in a homeostatic remodeling state at the time most surgical interventions take place (≈ 2 - 3 months following injury). Cortical bone volume and the density of the matrix were dramatically different 4 weeks out from injury and was increasingly observed in those patients undergoing reconstructive surgery at a later time point compared to non-injured individuals. Moreover, the early post-injury appearance of bone remodeling that occurred within this matrix may not provide a suitable population of bone-forming precursor cells (i.e., pre-osteoblasts) within the first 12 weeks following injury, potentially jeopardizing the success of graft osseointegration, and thereby leading to graft failure and/or osteolysis within the tunnel.

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Figures

Figure 1. Radiograph of the knee of a patient (24-year-old male) with an unstable bone-patellar tendon-bone autograft. Note the significant expansion of the original graft tunnel on the femoral side (arrows) due to osteolytic activity.
Figure 2: Histogram of patient (A) age, (B) time from injury to operation, and (C) activity at time of injury.

Figure 3. View of the 10 mm trephine (at left) and guide pin (at right) used in the extraction procedure in both patients and cadavers. A sample femoral ACL explant is also shown. Reproduced with permission from49.
Figure 4. Representative explant. (a) reorientation of the explant in the y – z plane; (b) segmentation of the cortical VOI; and (c) segmentation of trabecular VOI.

Figure 5: Unpaired t-test between injured and non-injured explants for cortical (A) vBMD, (B) BV/TV, and (C) Ct.Po. In this and the following figures, the box encompasses the first and third quartiles (the 25th and 75th percentiles, respectively) and is bisected by the median value. With the exception of potential outliers, the minimum and maximum values are visually presented as the line extending below and above the box (ns: p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001, ****: p <= 0.0001).
Figure 6: Unpaired t-tests between injured and non-injured explants grouped by time from injury to operation for (A) vBMD, (B) BV/TV, and (C) Ct.Po
Figure 7: Unpaired t-tests between injured and non-injured explants for trabecular (A) vBMD, (B), BV/TV, (C) thickness, and (D) separation.

Figure 8: Representative histology sections and data histomorphometric feature ranking. A) Uninjured ACL femoral enthesis demonstrating the fibrocartilaginous zones of the enthesis with uncalcified fibrocartilage (UF) delineated from the calcified fibrocartilage (CF) by a basophilic tidemark (TM). Note how the CF integrates with the mineralized bone (B). B) 6-weeks post-injury demonstrating an ACL femoral enthesis with active formation of new cartilaginous matrix (asterisks) as part of the reparative process. C) 12-weeks post-injury demonstrating an ACL femoral enthesis with extensive osteoclastic activity (asterisks) that effectively resorbed the CF matrix anchoring the ACL to the femur. Note the disordered collagen of the ACL at the ligament-bone interface. Patients in both B and C panels showed no sign of ACL avulsion from the femur at the time of surgery. Specimens stained with toluidine blue and imaged at 10x magnification.
C. References


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