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Collagen X Longitudinal Fracture Biomarker Suggests Staged Fixation in Tibial Plateau Fractures Delays Rate of Endochondral Repair

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Abstract

Objectives: To use a novel, validated bioassay to monitor serum concentrations of a breakdown product of collagen X in a prospective longitudinal study of patients sustaining isolated tibial plateau fractures. Collagen X is the hallmark extracellular matrix protein present during conversion of soft, cartilaginous callus to bone during endochondral repair. Previous preclinical and clinical studies demonstrated a distinct peak in collagen X biomarker (CXM) bioassay levels after long bone fractures.

Setting: Level 1 academic trauma facility.

Patients/Participants: Thirty-six patients; isolated tibial plateau fractures.

Intervention: (3) Closed treatment, ex-fix (temporizing/definitive), and open reduction internal fixation.

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Level of Evidence: Level II, prospective clinical observational study.

Main Outcome Measurements: Collagen X serum biomarker levels (CXM bioassay).

Results: Twenty-two men and 14 women (average age: 46.3 y; 22.6–73.4, SD 13.3) enrolled (16 unicondylar and 20 bicondylar fractures). Twenty-five patients (72.2%) were treated operatively, including 12 (33.3%) provisionally or definitively treated by exfix. No difference was found in peak CXM values between sexes or age. Patients demonstrated peak expression near 1000 pg/mL (average: male—986.5 pg/mL, SD 369; female—953.2 pg/mL, SD 576). There was no difference in peak CXM by treatment protocol, external fixator use, or fracture severity (Schatzker). Patients treated with external fixation ($P = 0.05$) or staged open reduction internal fixation ($P = 0.046$) critically demonstrated delayed peaks.

Conclusions: Pilot analysis demonstrates a strong CXM peak after fractures commensurate with previous preclinical and clinical studies, which was delayed with staged fixation. This may represent the consequence of delayed construct loading. Further validation requires larger cohorts and long-term follow-up. Collagen X may provide an opportunity to support prospective interventional studies testing novel orthobiologics or fixation techniques.

Keywords

biomarkers; fracture healing; collagen X; tibial plateau

INTRODUCTION

All primary clinical methods currently used to monitor fracture healing are *qualitative* in nature. Fracture healing is typically observed through serial radiographs that, in the case of long bone fractures, can be scored using the modified Radiographic Union Score for Tibial Fractures (mRUST) method.[1–3] Radiographs are complimented with subjective physical examinations, clinical experience, and visual comparisons between serial radiographs to determine whether fracture healing matches clinical expectations. In tibial plateau fractures, radiographs can be a poor indicator of healing, and the mRUST scoring method is not validated for use in metaphyseal injuries.[4] A further limitation of the mRUST method is that the peak signal occurs late in the healing process, limiting its utility for early predictions. With progressively complex periarticular reconstructions, the entirety of the osteosynthesis construct frequently obscures the metaphysis and interval healing is not visualizable.[5] Given that there are no validated quantitative methods to monitor tibial plateau fracture healing, there is an unmet clinical need to find novel technologies that can complement standard radiographs.

The introduction of such a method to measure fracture healing activity at a biological level would be a substantial advancement and could allow clinicians to personalize postfracture care and identify delayed healing or nonunion at early time points in care. In addition, a quantitative method could give clarity to interventional questions, such as the appropriate time to weight-bearing or the consequence of medications such as non-steroidal antiinflammatories (NSAIDs). Biomarkers used to date in fracture healing have primarily measured bone turnover markers (BTM) and are therefore considered later indicators of healing consistent with radiographic evidence of bone mineralization.[6,7] Importantly,

no studies to date have investigated BTMs after tibial plateau fractures. The ability of established BTMs to monitor fracture healing in diaphyseal fractures remains uncertain.[8,9]

In this study, we aimed to test the novel collagen X (ColX) biomarker (CXM) associated with the transient cartilaginous callus tissue present in fracture healing by endochondral ossification. The use of this biomarker is prompted by studies demonstrating that fractures heal through a mixture of endochondral (indirect) and intramembranous (direct) bone formation.[10–13] The relative contribution of these 2 repair processes is likely modulated by the strain experienced within the fracture field. We have previously validated the biomarker through preclinical work, demonstrating that the marker is measurable in serum during long bone healing.[14,15] During endochondral ossification, before conversion to bone tissue, chondrocytes within the soft callus undergo hypertrophic maturation and synthesize a provisional collagen X–rich extracellular matrix. This provisional matrix catalyzes bone formation by promoting matrix mineralization and angiogenesis such that the cartilage callus plays a critical role in vascularized bone regeneration.[16–20] The hypertrophic cartilaginous phase in fracture healing is transient, and the breakdown product of the homotrimeric ColX protein is detectable in serum using a sandwich ELISA-based assay (CXM) reactive to the intact trimeric noncollagenous 1 domain of ColX. The CXM assay was developed and validated as a measurement of skeletal growth velocities in children because growth plate activity requires expansion of the cartilaginous region.[15] This first publication included a 3-patient case series demonstrating that the CXM bioassay also displayed distinctive peaks during the endochondral phase of long bone fracture repair.[15] More recently, we have rigorously studied the CXM biomarker in a murine animal model of long bone fracture healing, validating that serum levels of the CXM biomarker followed expected patterns of *col10* gene and protein expression, consistently tracking with quantitative histomorphometric advancement of healing.[21]

Importantly, ColX is not normally expressed outside of the processes of limb growth and fracture healing. Baseline adult measurements are minimal.[15] Tibial plateau fractures are generally fixed with both absolute and relative stability concepts. Although the articular surface is treated with absolute stability goals and perhaps therefore intramembranous healing, we hypothesize that most plateau fractures heal in the metaphysis through large contributions from endochondral ossification. There are a wide range of treatments for plateau fractures, including nonoperative management, external fixation, primary open reduction internal fixation (ORIF), and staged fixation. Unicondylar and bicondylar fractures are addressed with different protocols resulting in differential durations of weight-bearing restrictions based on the injury pattern, surgeon preference, and patient adherence.[22] This produces an opportunity for detection and observation of differential CXM expression in a clinical cohort across varied strain microenvironments.

Therefore, the purpose of this study was to prospectively measure the CXM expression profile present in a cohort of patients sustaining tibial plateau fractures. We sought to understand the magnitude and timing of the CXM spike and to investigate fundamental differences in CXM expression as a function of age, sex, or basic fixation protocol during tibial plateau fracture healing. We hypothesized that age and sex would not demonstrate differences in expression within comparable treatment paradigms. The literature provides

clear evidence that strain modulation affects fracture healing and callus formation.[23,24] As such, we further hypothesized that the addition of external or internal fixation would change the strain environment and CXM response.

MATERIALS AND METHODS

Definitions

Collagen X: Spoken “Collagen type ten.” Refers to the endogenous matrix protein. Abbreviated “ColX.”

CXM: Spoken “C-X-M.” Refers to the results of the ELISA-based biomarker test, quantifying a breakdown product in serum of collagen X.

Patient Care and Exclusion

Institutional review board’s review and approval was obtained at all sites. All patients (2 Level 1 academic trauma centers, Level 3 trauma hospital, 2019–2021) sustaining isolated tibial plateau fractures presenting within 20 days of injury were offered inclusion. Standard of care was delivered by treatment teams independent of the research effort. Treatment strategies included (1) nonoperative management (“non-op”), (2) immediate open reduction internal fixation (“immediate ORIF”), and (3) staged open reduction internal fixation (“staged ORIF”). Patients were excluded for other acute concomitant fractures, prior fractures or major orthopaedic surgery within 12 months, pathologic fracture, genetic diseases of cartilage development/growth/metabolism, and known benign or malignant chondroid processes.

Collagen X Sampling, ELISA, and CXM

Patients gave blood samples at first presentation and at standard follow-ups (3, 6, and 12 weeks postoperatively and all further visits). Blood was captured using Whatman 903 Protein saver cards, creating dried blood spots (DBS) by lancet finger pricks. Protein cards were stored in a −20°C freezer until assay. Human DBS were sampled (3.1 mm punch in duplicate, 250 μL of sample diluent, and extracted overnight at 4°C), before assay per previously published procedure,[21] resulting in measurements in pg/mL. Biomarker performance metrics were measured including each patient’s peak CXM value, the time-to-peak CXM, and “ΔCXM,” which was defined as the peak CXM subtracted from their initial “injury” CXM value.

Patient and Injury Details

Demographic information and clinical details (mechanism, injury and surgery dates, operative notes, and radiographs) were collected. Injury mechanism was classified by previous methodology[25,26] into bins for suspected high-energy and low-energy groups.

Statistical Analysis

Summary statistics and frequencies were calculated (Stata) for demographics and each experimental outcome. All data were plotted in Prism GraphPad v8.2.1 with each individual data point representing an enrolled patient. Statistical calculations were performed for

subgroup analysis using paired and unpaired *t* tests with Welch correction and Pearson correlations ($P < 0.05$). One-way Brown–Forsythe analyses of variance with Welch corrections were performed for 3 or more group comparisons. Linear regressions or a cubic spline curve (5 knots, smoothing fit) were computed for all scatter plots.

RESULTS

Thirty-six patients with tibial plateau fractures and longitudinal collagen X biomarker measurements were enrolled (patient demographics: Table 1). Twenty-two patients (61.1%) were male and 14 (38.9%) were female, with an average age of 46.3 years (range 22.6–73.4; SD 13.3). Twenty-six patients (72.2%) were treated operatively, including 12 (33.3%) provisionally or definitely treated with external fixation. Fracture severity was grouped using the Schatzker classification (16 unicondylar and 20 bicondylar fractures). An XY scatter of all individual CXM values in time shows highest values tend to fall between 20 and 60 days (Fig. 1A), with median time-to-peak CXM at 30.5 days (Fig. 1B).

CXM values were segregated by sex. Both men and women demonstrated peak expression near 1000 pg/mL (average peak: male—986.5 pg/mL, SD 369; female—953.2 pg/mL, SD 576; Fig. 2A, $P = 0.849$). Neither time-to-peak CXM (Fig. 2E, $P = 0.726$) nor ΔCXM ($P = 0.867$) values were different by sex. Smoking and diabetes were tested using unpaired *t* tests with the Welch correction. There was no difference in peak CXM, regardless of smoking (Fig. 2B, $P = 0.886$) or diabetic status (Fig. 2C, $P = 0.655$). Time-to-peak CXM by smoking (Fig. 2F, $P = 0.056$) approached significance, whereas time-to-peak CXM by diabetes did not (Fig. 2G, $P = 0.376$). CXM values were tested against age. No correlation was demonstrated between age and CXM behavior, including peak CXM (Fig. 2D, $P = 0.652$), time-to-peak CXM (Fig. 2H, $P = 0.560$), or ΔCXM (not shown, $P = 0.564$).

There was no difference in peak CXM (Fig. 3A, $P = 0.271$) or time-to-peak CXM (Fig. 3B, $P = 0.404$) by fracture severity (unicondylar vs. bicondylar). Mechanism of injury (low vs. high) demonstrated no difference in peak CXM (Fig. 3C, $P = 0.217$) or time-to-peak CXM (Fig. 3D, $P = 0.527$).

The use of external fixation yielded no significant difference in peak CXM (Fig. 4A, $P = 0.843$), but time-to-peak CXM was significantly delayed with the use of external fixators (Fig. 4E; 37 days without and 90 days with, $P = 0.050$). No differences were found in peak CXM values and treatment protocol (Fig. 4B, $P = 0.844$). However, the time-to-peak CXM was significantly delayed in patients receiving staged ORIF (32 days with nonoperative care, 28 days with immediate ORIF, and 90 days with staged ORIF; Fig. 4F; $F_{2,19} = 3.66$, $P = 0.0458$). There was no correlation between the time to definitive surgery and either peak CXM (Fig. 4C, $P = 0.855$) or time-to-peak CXM (Fig. 4G, $P = 0.305$). Time to weight-bearing correlated with peak CXM for all patients although not significant (Fig. 4D, $P = 0.134$). Time to weight-bearing was strongly correlated with time-to-peak CXM (Fig. 4H, $P = 0.0089$), demonstrating later peaks with later clearance for weight-bearing. Zero patients experienced a loss of reduction or failure of hardware during the clinical follow-up.

DISCUSSION

This study describes the prospective clinical observation of a circulating collagen X biomarker, analyzed longitudinally in dried blood spots and quantified using a validated CXM protein ELISA,[15] in a cohort of patients sustaining tibial plateau fractures. CXM differs from other BTMs used in fracture healing because CXM measures the serum concentration of a collagen X breakdown product created during the intermediate cartilaginous phase in fracture healing. To date, clinical validation of the CXM biomarker has been performed primarily in children, proving equivalence to height growth velocity based on collagen X breakdown activity in growth plates. In a preclinical murine tibia fracture model, we have characterized the temporal expression pattern of collagen X and the CXM biomarker during normal fracture repair. We found that *col10a* gene expression spikes early postfracture and is followed by a commensurate rise in collagen X protein presence in the matrix of the fracture callus. A rise in CXM serum concentration follows, which peaks at 14 days before the process of bone remodeling finishes.[21] Similar peaks in CXM have been shown in a pilot human cohort.[15,27] However, the heterogenous and nuanced nature of fracture care will likely require investigation at each anatomical fracture site to make comparisons between CXM assays of relevant patients.

The most critical findings from this cohort of tibial plateau patients are that there were no differences in CXM expression based on patient demographics (sex and age). These are fundamental findings which imply, but do not confirm at this early stage, that there may be no quantifiable difference in tibial plateau fracture healing between men and women. Similarly, we observed no correlation in the peak CXM value between young and old across a well-distributed age span from 22 to 73 years. These findings need to be validated in a larger cohort of metaphyseal tibial plateau fractures but suggest the potential power of this biomarker to answer basic questions on the influence of demographics in rate of fracture repair that remain unclear.

The literature is unclear regarding whether sex-based differences in fracture healing exist. Fundamentally different hormonal environments and body sizes could give rise to differential phenotypic healing rates.[28–30] Preclinical studies to date have not provided definitive clarity with conflicting results. There are studies demonstrating more rapid bone generation in male rats and mice,[31,32] but other studies refute this.[33] Deng et al[32] found that male mice formed larger bone calluses than female mice during tibial fracture healing because of increased IGF-1 expression, stronger activation of Wnt/β-catenin signaling pathway, and more osteoblasts during callus formation. More recently, Haffner-Luntzer et al[34] showed that male mice demonstrated differences in fracture healing, starting with a more prominent cartilaginous callus and ending with higher tissue mineral density and bending stiffness at day 21, attributed mechanistically to greater activation of Wnt/β-catenin signaling. The authors hypothesized that the differences found in fracture healing associated with sex could be attributable to differences in mouse size alone. In our preclinical validation of the CXM biomarker, we did not find significant differences in temporal biomarker expression according to sex but did not quantify functional bone repair using CT or biomechanics.[21] The lack of difference by sex of CXM levels in this study would support the idea that differences in endochondral ossification as a function of sex may be

small or insignificant. An important difference between clinical and preclinical studies to consider is that it is standard in human treatment to have a period of 6–8 weeks without weight-bearing, whereas small animal preclinical studies allow immediate ambulation of the animals and thus may amplify differences in loading. Sex differences in humans may not be relevant in fractures initially treated with protected modes of weight-bearing. Finally, the preclinical studies investigating sex differences were all based on long bone fractures, which may not match metaphyseal behavior.

The effect of age on fracture healing has a strongly supported mechanistic foundation at the preclinical level, with multiple studies demonstrating delayed healing with increased age associated with increased systemic inflammation and reduced vascularization.[35] Lu et al[36] demonstrated differences in healing between juvenile (4 weeks old), middle-aged (6 months old), and elderly (18 months old) mice. They concluded that there was a significant change in fracture healing between juvenile and middle-aged animals, with a much smaller decrease in healing from the middle-aged to the elderly mice. This seemed to be due to slower chondrocyte maturation and decreased vascular invasion leading to delayed endochondral repair in older mice. Later, the team investigated vascularization, demonstrating a higher density of blood vessels and greater expression of proangiogenesic factors (hypoxia-inducible factor-1 alpha protein and transcripts of vascular endothelial growth factor) in juvenile mice compared with older-aged animals.[37] More recent murine studies build on these initial mechanistic data to show senescent periosteal progenitors and decreased proliferation within fracture callus, and chronic inflammation contribute to delayed fracture healing in aged animals.[38–43] Taken together, these data suggest that the elderly may exhibit a baseline level of inflammation (inflamm-aging[44]) that interferes with the catabolic stages of fracture healing. One of the major challenges, however, of interpreting preclinical research on aging is mapping the mouse to human life span. Our data suggest that older patients trend toward a longer time to peak and difference in CXM (DCXM), but a larger patient cohort will be needed to clarify the impact of age on tibial plateau healing.

Important secondary findings of our research include the delay to peak CXM in patients who received staged ORIF of their plateau fractures relative to nonoperative or immediate ORIF and the delay to peak CXM associated with delayed time to weight-bearing. We hypothesize that the delayed definitive surgery and a prolonged period of non–weight-bearing (up to 8 weeks after definitive fixation) associated with this protocol may limit early strain at the fracture and delay endochondral ossification. This has interesting implications regarding the differential rate of healing between operative and nonoperative fractures, as well as the potential pros and cons of the protocols such as early total care[45,46] versus staged periarticular fixation.[47,48] Finding appropriately matched control patients with similar fracture patterns may prove difficult, but these data justify additional work to increase the power of this observation in a larger cohort.

Research quantifying fracture healing in human patients is limited fundamentally; no methods exist for direct quantification of biological healing. The excellent multicenter work that yielded first the RUST[3] followed by the mRUST scoring system[1,2,49,50] is the current standard for fracture healing and is a qualitative scoring system. The mRUST has been

adapted into multiple other systems for other anatomic locations (hip[51] and humerus[52,53] fractures), but this system has not been used successfully or validated for any metaphyseal anatomic location to date. Wojahn et al[50] performed an elegant study that characterized the spread of tibial healing performance across the mRUST spectrum, demonstrating remarkably large ranges for time(s) to score(s). A simple interpretation of this study is that tibias demonstrate a wide range of phenotypic healing. A fair criticism of all literature on fracture healing scoring is that none of the analyses have been segmented to consider the effect of age or sex. In many ways, this is remarkable because application to large-scale grant funding is not possible without consideration of these factors, yet the orthopaedic literature remains exclusively focused on parameters such as fracture geometry and size and construct factors. These are all important but are perhaps drowned out in effect size by central and nonmodifiable patient factors. Our current study found no differences in CXM levels as a function of age or sex in patients with tibial plateau fractures.

The strength of our study is the exploration of a unique diagnostic opportunity through quantifying a novel serum biomarker in patients with isolated tibial plateau fractures. No previous investigation exists linking biomarkers to endochondral ossification in a clinical cohort of patients with bone fractures. Weaknesses of the research presented here center around the exploratory and novel nature of the collagen X bioassay. In addition, there is no validated scoring system for metaphyseal healing, and we have no qualitative scores to compare CXM values with tibial plateau fractures. Finally, this work faces a common limitation of biomarker studies in that the resolution of sampling can greatly affect the conclusions. Collagen X may prove to be a superior biomarker based on ease of capture alone; DBS sampling is superior in this regard to serum collection in that it only requires a finger prick rather than a phlebotomist for blood acquisition and has potential applicability for austere environments.

This study represents a first clinical cohort of fractures observed for behavior of CXM levels after an isolated fracture and demonstrates that in vivo CXM levels spike initially and subsequently resolve, which is related to resorption of the cartilage callus and consolidation of the bone matrix. The CXM spike magnitude was not affected by patient age or sex or fracture type. Differences were found within the treatment protocols because staged treatment and delayed weight-bearing resulted in a delayed peak CXM. In the future, we hope to expand on these findings by investigating larger cohorts of other fracture types to uncover granular associations between patient and injury factors and CXM expression.

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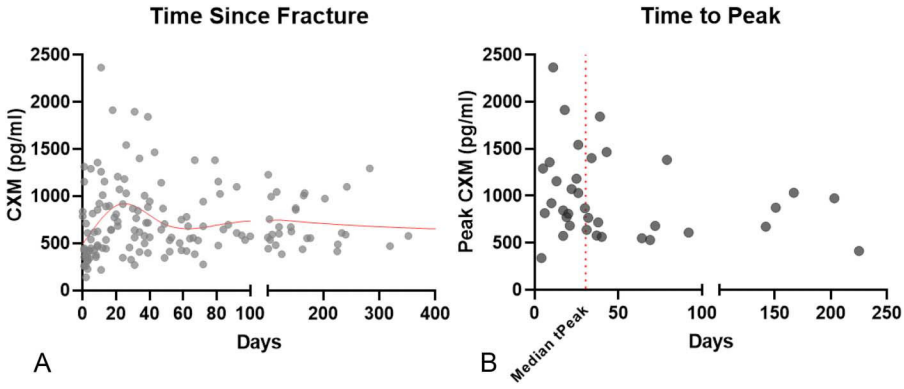

FIGURE 1.

Overall CXM values suggest the median time to peak at 31-day postinjury. A, Individual CXM levels (pg/mL) with a cubic spline curve fit (red line) over time suggest a peak followed by a resolution back toward the baseline. Each dot represents an individual patient value. B, Time-to-peak CXM value for each patient. Median time to peak (t_{peak}) = 30.5 days (red line).

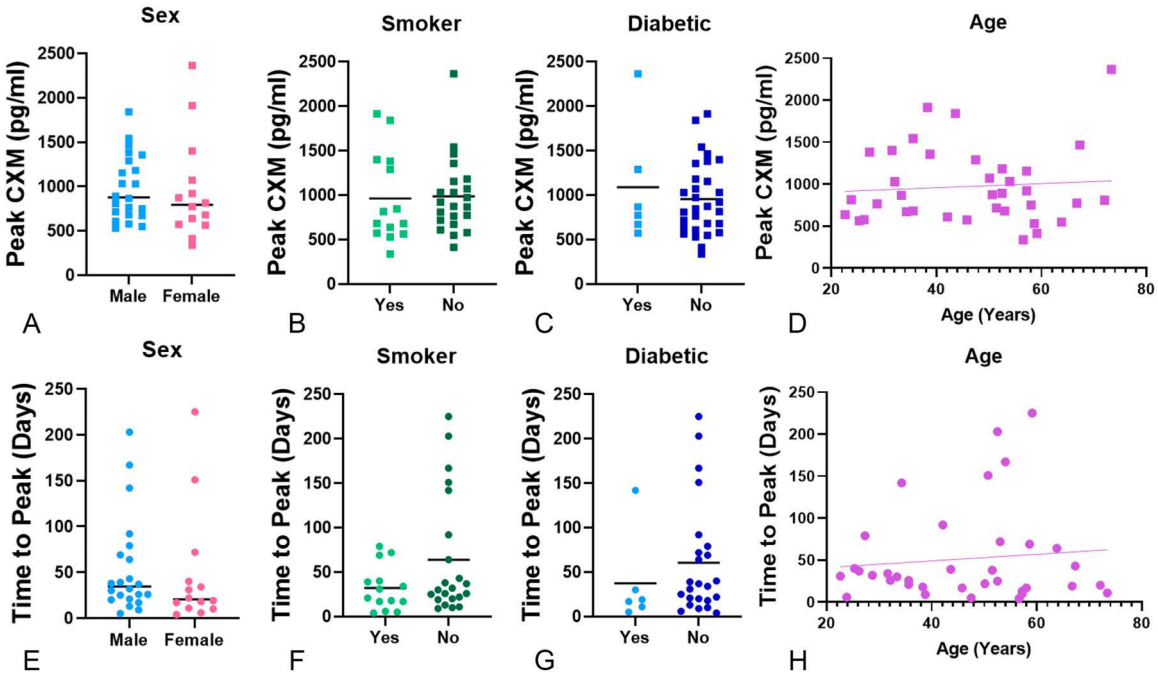

FIGURE 2.

CXM values do not segregate by patient demographics. There was no difference based on sex and (A) peak CXM ($P = 0.849$) or (E) time-to-peak CXM ($P = 0.726$). Graphs represent individual CXM values for men (N = 22) versus women (N = 14). There was no difference between smoking status and (B) peak CXM ($P = 0.886$); however, (F) time-to-peak CXM ($P = 0.056$) neared significance. Diabetic status (C), peak CXM ($P = 0.655$), and (G) time-to-peak CXM ($P = 0.376$) showed no significance. Similarly, there was no correlation between age and (D) peak CXM value ($P = 0.652$) or (H) time-to-peak CXM ($P = 0.560$).

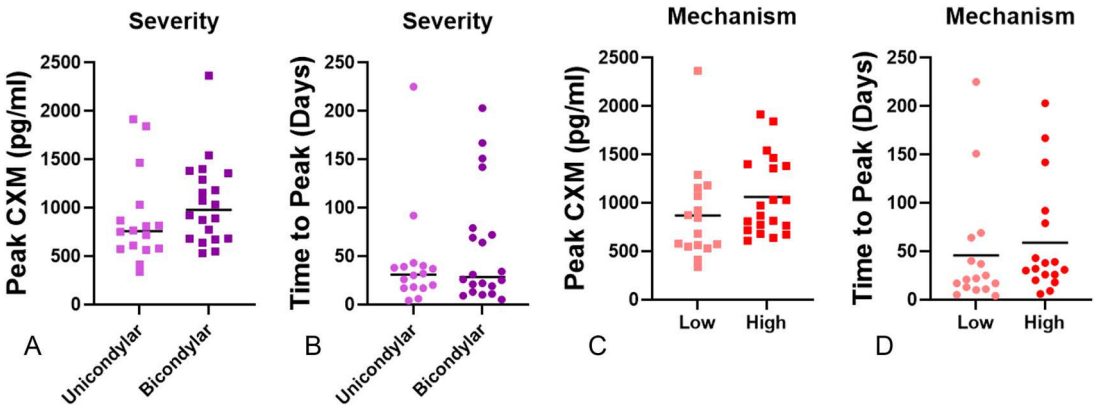

FIGURE 3.

Injury severity and mechanism have no effect on CXM peak or time-to-peak CXM. Unicondylar and bicondylar injuries demonstrated no difference in (A) peak CXM (P = 0.271) or (B) time-to-peak CXM (P = 0.404). Mechanism of the injury demonstrated no significant difference for (C) peak CXM (P = 0.217) or (D) time-to-peak CXM (P = 0.527).

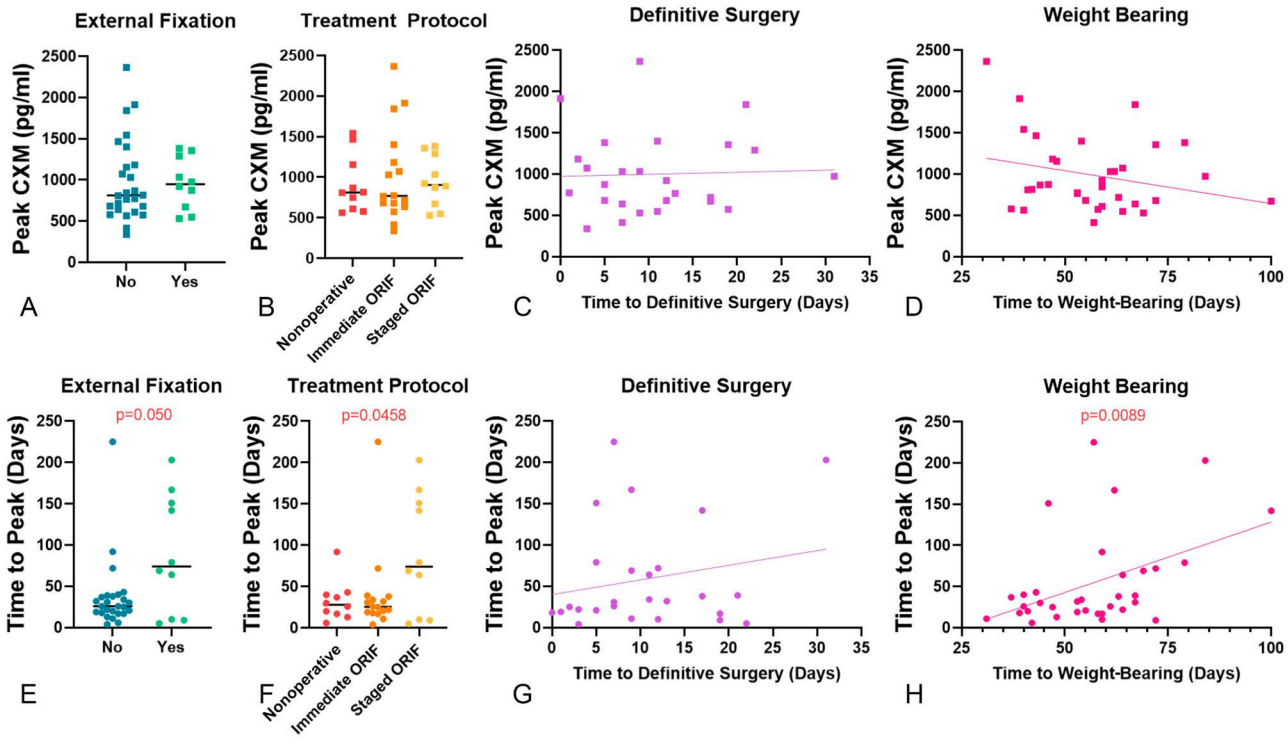

FIGURE 4.

Staged ORIF delays time to CXM peak. The use of external fixators did not affect (A) the peak CXM value ($P = 0.843$) but did delay (E) time to peak ($P = 0.050$). Surgical treatment (nonoperational, immediate ORIF, and staged ORIF) had no difference between groups for (B) peak CXM ($P = 0.844$), although it did show significance between groups for (F) time-to-peak CXM ($P = 0.0458$). Of those who received surgical intervention, time to definitive surgery showed no correlation between (C) peak CXM ($P = 0.855$) and (G) time-to-peak CXM ($P = 0.305$). Similarly, there was no correlation in time to weight-bearing (D) peak CXM ($P = 0.134$), but there was a significant correlation between time to weight-bearing and (H) time to peak ($P = 0.0089$).

TABLE 1.

Patient Demographics and Injury Patterns

	Unicondylar (n = 16)	Bicondylar (n = 20)	Total (n = 36)
	N (%)	N (%)	N (%)
Male	10 (62.5)	12 (60.0)	22 (61.1)
Female	6 (37.5)	8 (40.0)	14 (38.9)
Average age (range)	46.1 (23.8–72.1)	48.2 (22.6–73.4)	46.3 (22.6–73.4)
BMI (range)	28.8 (21.3–40.4)	30.9 (16.1–48.3)	29.9 (16.1–48.3)
Schatzker classification			
I			4
II			6
III			1
IV			6
VI			19
Management			
Operative	8 (50)	18 (84.2)	26 (72.2)
Nonoperative	8 (50)	2 (15.8)	10 (27.8)