Evaluation of early degeneration of cartilage in patients with anterior cruciate ligament injuries: analysis using urine CTX-II biomarker

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Abstract

Background: Anterior cruciate ligament (ACL) rupture occurs predominantly in young patients. About 30% of individuals with this lesion will present with osteoarthritis within 20 years following the trauma, despite successful treatment. This can be explained by metabolic changes occurring concomitantly with the trauma that results in the injury to this ligament. Using analysis of biomarkers related to cartilage degeneration, we aimed to determine that soon after the trauma leading to ACL rupture there are intra-articular metabolic changes in the knee that could lead to cartilage degeneration. Methods: A cross-sectional study was carried out in two groups: patients with an ACL ruptures and a control group (each group with ten male subjects, age range 18 – 35 years, body mass index below 30 kg/m²). In both groups, urine concentrations of a biomarker related to degradation of type II collagen (CTX-II) was measured. For the group with ACL rupture, a temporal relationship between time post-injury and amount of the biomarker was also examined. Results: There were significant differences in the concentrations of urinary CTX-II between the ACL group and the control group (p = 0.009). No significant relationship was observed between time of injury and the quantity of the biomarker. Conclusions: Patients with ACL injury had higher concentrations of urinary CTX-II biomarker than those with no ACL injury (p = 0.009). However, there was no correlation between the concentration of this biomarker and the elapsed time post-injury (p > 0.05). Keywords: osteoarthritis; biomarkers, ACL injury, CTX-II

Background

Anterior cruciate ligament (ACL) injury causes knee instability. Other intra-articular lesions commonly accompany ACL tears, especially those of cartilage and menisci. Treatment of these lesions involves surgical reconstruction in order to reestablish the
anatomy and biomechanics of the native ligament, mitigating symptoms and allowing for return to activity.  

One of the main post-operative sequelae of ACL injury is not completely eliminated after ligament reconstruction: the onset of osteoarthritis (OA) of the knee. On average, signs and symptoms appear 10 to 15 years following ligament reconstruction, with incidence ranging from 0% to 86% of cases.  

The diagnosis of OA is clinical. Imaging tests have low sensitivity and specificity for detecting early changes and monitoring disease progression during short-term follow up. Visible radiographic changes occur on average two years after the onset of the disease. The lack of a universal measurement standard with adequate sensitivity and specificity makes it difficult to measure the early degenerative processes after injury or ACL reconstruction, making a more accurate, short-term screening modality desirable. The use of biomarkers allows for an early, non-invasive measurement of the degenerative cartilage processes. These biochemical markers of connective tissue are released into the systemic circulation and can be measured in blood, urine or synovial fluid. One of the main biomarkers for the diagnosis and prognosis of OA is C-telopeptide of type II collagen (CTX-II). This biomarker is released during the dynamic process of type II collagen degeneration, and consequently correlates with the destruction and formation of cartilage. 

Mourtizen et al. showed that CTX II has a specificity for knee OA. Rotational trauma and intra-articular bleeding associated with ACL rupture are thought to cause an acute metabolic alteration of cartilage and subchondral bone, resulting in the onset of the long-term degeneration of the articular cartilage. One of the biomarkers of this process is CTX-II. The aim of this study was to quantify the urinary concentration of CTX-II biomarker in
patients who suffered an isolated ACL injury and to compare concentrations found in this population with a control group of patients with no knee injury. Our hypothesis was that urine concentrations of CTX-II would be higher in patients with ACL rupture and therefore the biomarker would be useful as a prognostic indicator of OA development.

Methods

Study Design

This study was evaluated and approved by the Research Ethics Committee at the author’s institution. This was a cross-sectional, observational, single-center study comparing the presence of a type II collagen degradation urinary biomarker in patients with ACL injury of the knee and healthy patients without knee injury (control group).

Between June 2017 and February 2018, ten male subjects with a history of isolated ACL lesion (Group 1—treatment) and ten males with no history of knee ligament injury (Group 2—control) were evaluated and included in the study.

Inclusion criteria were: male gender; 18 to 35 years of age; with a body mass index of less than 30 kg/m²; and isolated ACL lesions or absence of knee injuries. The exclusion criteria were: female gender; presence of degenerative knee or other joint disease; systemic, autoimmune or infectious diseases; other knee ligament injuries; history of knee surgery; lesions of the meniscus or associated cartilage. With these selection criteria we tried to make our sample as homogeneous as possible and to obtain the lowest risk to present idiopathic OA or other pathologies such as osteoporosis.

Patients were selected from the outpatient care population, with clinical and radiologic findings of the ACL rupture. The anterior drawer, Lachman, and pivot-shift maneuvers were used during physical examination, along with magnetic resonance imaging diagnostic confirmation of ACL rupture for Group 1 subjects.
Urine Sample Collection

A single, clean-catch urine collection of all participants was performed, following the same aseptic protocol: after the genital region was topically sterilized, collection of either the first urination of the day or urination two hours after the prior urination, during the middle urination stream (neglecting initial and final phases), using a sterile flask was completed. These urine samples were kept in a refrigerated environment for a maximum duration of 12 hours before centrifugation and freezing at -20 ºC for a period (one week to seven months) before analysis. In ACL group the urine sample was obtained in their first visit to office before any treatment.

Urinalysis and presence of CTX-II

The urine samples were thawed simultaneously at room temperature for thirty minutes prior to the quantitative measurement of the collagen degradation biomarker type II (CTX-II - Cross Linked C-telopeptide of Type II Collagen). An enzyme-linked immunosorbent assay (ELISA, Elabscience, Houston, TX, United States - catalog number E-EL-H0837) was performed on each sample. This ELISA kit used the ELISA-sandwich principle for sample analysis. Using this methodology, the supplied ELISA plate is precoated with an antibody specific for human CTX-II. The collected samples (urine) were added to the wells of the ELISA plate and homogenized with the specific antibody, forming a conjugate (antigen-antibody complex).

Thereafter, a biotinylated detection antibody specific for Avidin-Horseradish Peroxidase (HRP) conjugate was added to the plate and incubated. Free components were removed during a wash. The substrate solution was added to each well and only the wells containing human CTX-II / conjugate would appear blue. The enzyme-substrate reaction was terminated by the addition of stop solution and the color then became yellow.
Immediately following this, the optical density (OD) was measured spectrophotometrically at a wave length of 450 nm ± 2 nm (EZ Read 400 Biochrom spectrometer). The OD value is proportional to the concentration of human CTX-II present in the sample. The calculation of CTX-II concentration in the samples was then performed comparing the values calculated by a standard curve.

The specifications of the ELISA test for detection of degradation of type II collagen (CTX-II kit) according to the manufacturer were as follows: Sensitivity: 0.10 ng/mL; Detection Range: 0.16–10 ng/ml; Reproducibility: coefficient of variation <10%

All analyses were performed simultaneously in the same laboratory (Molecular Biology Department) on the same equipment. The results were evaluated and compared between groups.

Statistical analysis

Summary statistics (mean, standard deviation, median, minimum, and maximum) were used to describe patient characteristics and biomarker concentrations within each group. The Mann-Whitney test was used to compare the groups in relation to the concentration of CTX-II. Spearman correlation was used to evaluate the relationship between injury time and the presence of urinary CTX-II. A p-value of less than 5% (0.05) defined a significant difference.

Results

The patients in the ACL group had a mean age of 20.8 years and a mean BMI of 25 kg/m².

Patients in control group had a mean age of 28.2 years and a mean BMI of 24.5 kg/m². The individuals in ACL group were younger than the patients in control group 2 (p < 0.001) and there was no significant difference between groups in terms of BMI (p > 0.05). (Table 1)
The mean value of the presence of CTX-II in the ACL group was 8.9 ± 0.7 ng/ml (range 7.7–9.8) higher than that of the control group 6.7 ± 2.6 mg/ml (range 0.7–9.4) (p = 0.009). (Table 2 and Figure 1). There was no difference between the time post-injury and the CTX-II level for ACL group patients. (p = 0.521; r = -0.231). (Figure 2) (Table 1)

Discussion

Our hypothesis was supported by the results of this study. Subjects with ACL rupture had significantly higher concentration of urinary CTX-II than did patients without injury (p = 0.009), regardless of the time post-injury. The difference suggests a predisposition to the development of OA in this patient population. This finding supports the notion that metabolic changes in the articular cartilage occurring soon after the initial ACL rupture appear to predispose patients to degenerative knee pathology.

Interleukins (IL–6, IL–8) and metalloproteases (MMP–3 and MMP–13) are some of the cytokines thought to be intimately related to degradation of type II collagen. On average, OA becomes symptomatic and activity-prohibiting in patients 10 to 15 years after the initial traumatic event. However, this degenerative process does not affect all patients with a history of ACL ruptures, as evidenced by the wide range of incidence in this population (0%-86%)..

Until now, there has not been a reliable, early prognostic marker for this process. CTX-II, an established marker of type II cartilage breakdown, can be measured in blood, synovial fluid, and urine because the molecule is not altered after renal filtration. The advantage of urinary measurement is the ease of collection. Moreover, because it is considered a burden-of-disease type biomarker according to the classification BIPEDs, the correlation between the severity of OA degenerative changes and the CTX-II biomarker concentrations support its use as a diagnostic and prognostic tool.
Because of the intrinsic characteristics of each biomarker quantification method (manufacturing company, gender, age, and BMI, and articular joint studied) there are no uniform or reference values described in the literature. Several biomarkers of inflammation quantification such as COMP, Aggrecan degradation products (ARGS) or even inflammatory cytokines have been associated with degenerative processes in the knee cartilage\textsuperscript{6,16}. Despite this variability of biomarker analysis, previous studies in the literature concluded that there is a quantitative increase in biomarkers after ACL injury\textsuperscript{3,8,32}. However, these studies did not control for isolated ACL injury, the stage of disease, or use of the same biomarker.

The present study measured only the preoperative, urine CTX-II concentrations; therefore, our study is unique in its aim to identify an early prognostic OA biomarker. Two other studies quantified CTX-II following surgical ligament reconstruction. Larsson et al. found no difference in CTX-II biomarker concentrations in serum, urine, and synovial fluid when comparing the values before and after surgery.\textsuperscript{37} Chmielewski et al.\textsuperscript{13} performed serial urine CTX-II measurements after surgery and found that the concentration decreased over time.

Nevertheless, to demonstrate the specificity of a biomarker and its prognostic power, the homogeneity of the sample is crucial. Furthermore, the markers must be accurate and reproducibly measured, with coefficients of variation of less than 10%. Because patient characteristics such as gender, age and body mass index (BMI) vary, it is necessary to minimize variability between compared groups, or to stratify the study according to the analyzed variable. The present study is unique in its methodology in that care was taken to evaluate only patients with isolated ACL injury, establishing the specificity of this biomarker for post-ACL injury cartilage degeneration in an homogeneous group. According
to Deshpande et al.\textsuperscript{40} the risk of OA in male, non-obese, under 35 years old is less than 1%.

The temporal trend of the concentration of CTX-II biomarker was also evaluated. However, no statistically significant trend was found (\(p > 0.05\)). One of the inclusion criteria for the study was an injury sustained a maximum of 2 years prior to enrollment, and post-injury time ranged between 2 and 18 months. The absence of a temporal trend can be explained in two ways: the small sample population, and the possibility of type II collagen degradation occurring sooner after the trauma than our study analyzed. These levels could remain chronically elevated, unless a new event occurs, including surgical treatment or some other non-surgical treatment\textsuperscript{37}.

Despite the fact that our inclusion criteria were designed to identify demographically similar patients, a significant difference existed between the mean ages of the groups. The control group had a higher average age (28.2 ± 3.8 years) than the ACL group (20.8 ± 2.6 years). If the result were the opposite, we might consider this an important bias, once CTX-II values are increased in older patients.

We acknowledge limitations of the present study. This cross-sectional pilot study provided a statistical evaluation of biomarker concentration in both injured and control populations. Biomarker concentrations are known to change with time and stage of cartilage degeneration. In addition, biomarker concentrations are known to vary with interventions, both surgical and/or clinical\textsuperscript{13,37}. Prospective studies tend to provide better information regarding the temporal trends of biomarker concentrations and their related pathological severity. Another limitation of this study is small sample size (\(n = 20\)). While this limitation may reduce the robustness of the conclusions, the sample size provided ample power to provide statistically significant results. A larger sample study analyzing more
individuals will ultimately provide more conclusive data and may better elucidate the changes of CTX-II concentrations over time. The final limitation is the exclusion of female patients, because the main objective of the selection criteria was to homogenize the sample. For future studies with larger samples both gender, analysis will be necessary. Further studies may better elucidate how to inhibit the degenerative cartilage process after ACL injury or other joint conditions. Biomarker measurement may play an important role in achieving this goal, because they help aid the early diagnosis of metabolic alterations both qualitatively and quantitatively, in terms of disease severity. Finally, they may be used in therapeutic studies to evaluate treatment efficacy.

Perspective

Previous studies have shown that inflammatory cytokine levels increase after ACL rupture and that this phenomenon may be associated with osteoarthritis. Another way to show an alteration of cartilage metabolism after knee trauma is using biomarkers, such as COMP and CTX-II. The importance of this study is to show CTX-II increasing after ACL rupture. This could be useful for other pathologies such as meniscal or cartilage injuries and other ligament injuries. It may also be useful to test or compare surgical techniques, medications or interventions to prevent OA. CTX-II, among other biomarkers, has the advantage of being measured in urine; therefore, it is an easy and non-invasive way to show and follow cartilage formation and degradation.

Conclusion

Patients with ACL injury had higher concentrations of urinary CTX-II than those with no ACL injury ($p = 0.009$). Nevertheless, there was no correlation between the concentration of this biomarker and the elapsed time post-injury ($p > 0.05$). The CTX-II may be a useful prognostic indicator for the development of OA in patients with a history of ACL injury.
Declarations

- Ethics approval and consent to participate

Sao Paulo Federal University (UNIFESP) Ethics Committee approval—number 2.422.141

All participants signed consent forms for participation

- Consent for publication

Not applicable

- Availability of data and material

All data generated or analysed during this study are included in this published article

- Competing interests

The authors declare that they have no competing interests

- Funding

No financial support. Funding for design of the study and collection, analysis, interpretation of data and writing were done exclusively by authors.

- Authors’ contributions

APN was responsible for conception and design, acquisition of the study patients, analysis and interpretation of the data, drafting of the article, and final approval of the submitted version.

NSBM was responsible for conception and design, logistical support, drafting of the article, and final approval of the submitted version.

JLD was responsible for conception and design, drafting of the article, statistical expertise, technical support and final approval of the submitted version.

JJK was responsible for conception and design, drafting of the article, critical revision of the article, and final approval of the submitted version.

BE was responsible for conception and design, drafting of the article, and final approval of the submitted version
MC was responsible for conception and design, drafting of the article, and final approval of the submitted version.

DCA was responsible for conception and design, analysis and interpretation of the data, draft the article, critical revision, acquisition of the study patients, drafting of the article, and final approval of the submitted version.

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section

Figures
Figure 1

CTX-II mean value: ACL group vs control group The mean value CTX-II in the ACL group was $8.9 \pm 0.7$ ng/ml (range 7.7-9.8) higher than that of the control group $6.7 \pm 2.6$ mg/ml (range 0.7 – 9.4) ($p = 0.009$)
There was a difference between the time post-injury and the CTX-II level for the ACL group patients. ($p = 0.521; r = -0.231$)

**Supplementary Files**

This is a list of supplementary files associated with the primary manuscript. Click to download.

Table 1.docx
Table 2.docx