




# Evaluation of the CTX-II Biomarker in Patients with Anterior Cruciate Ligament Tear: Pilot Study\*

## *Avaliação do biomarcador CTX-II em pacientes com ruptura do ligamento cruzado anterior: Estudo piloto*

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### Abstract

**Objective** The aim of the present study was to quantify the urinary concentration of the C-terminal cross-linked telopeptide of type-II collagen (CTX-II) biomarker in patients who suffered an isolated ACL injury, and to compare the concentrations found in this population with a control group of patients with no metabolic changes in the knee that could lead to cartilage degeneration.

**Methods** A cross-sectional pilot study was performed in two groups: patients with ACL tears and a control group (each group with 10 male subjects, with an age range between 18 and 35 years, and body mass index below 30 kg/m<sup>2</sup>). In both groups, urine concentrations of a biomarker related to the degradation of type-II collagen (CTX-II) was measured. For the group with ACL tears, a temporal relationship between the time after the injury and the 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 the time after the 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 after the injury ( $p > 0.05$ ).

### Keywords

- ▶ osteoarthritis
- ▶ biomarkers
- ▶ anterior cruciate ligament injuries

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**Resumo**

**Objetivo** Quantificar a concentração urinária do biomarcador telopeptídeo C de colágeno de tipo II (*C-terminal cross-linked telopeptide of type-II collagen*, CTX-II) em casos de lesão isolada do ligamento cruzado anterior (LCA), e comparar as concentrações observadas nessa população com um grupo controle composto por pacientes sem alterações metabólicas no joelho que possam levar à degeneração da cartilagem.

**Métodos** Este é um estudo piloto transversal com dois grupos: pacientes com ruptura do LCA e grupo controle (cada grupo era composto por 10 indivíduos do sexo masculino, com 18 a 35 anos de idade, e índice de massa corporal inferior a 30 kg/m<sup>2</sup>). Nos dois grupos, as concentrações urinárias de um biomarcador relacionado à degradação do colágeno de tipo II (CTX-II) foram medidas. No grupo com ruptura do LCA, a relação entre o tempo pós-lesão e a quantidade do biomarcador também foi analisada.

**Resultados** Houve diferenças significativas nas concentrações urinárias de CTX-II entre o grupo LCA e o grupo controle ( $p = 0,009$ ). Não foi observada relação significativa entre o tempo de lesão e a quantidade do biomarcador.

**Conclusões** Pacientes com lesão do LCA apresentaram maiores concentrações urinárias do biomarcador CTX-II do que aqueles sem lesão do LCA ( $p = 0,009$ ). No entanto, não houve correlação entre a concentração desse biomarcador e o tempo decorrido após a lesão ( $p > 0,05$ ).

**Palavras-chave**

- ▶ osteoartrite
- ▶ biomarcadores
- ▶ lesões do ligamento cruzado anterior

**Introduction**

Anterior cruciate ligament (ACL) injury causes knee instability. Other intra-articular lesions commonly accompany ACL tears, especially those of the cartilage and menisci. The treatment of these lesions involves surgical reconstruction to reestablish the anatomy and biomechanics of the native ligament, mitigating symptoms and enabling the return to activities.<sup>1</sup>

One of the main postoperative sequelae of ACL injury is not completely eliminated after ligament reconstruction: the onset of osteoarthritis (OA) of the knee. On average, the signs and symptoms appear 10 to 15 years following ligament reconstruction, with an incidence ranging from 0% to 86% of cases.<sup>2-6</sup>

The diagnosis of OA is clinical. The imaging tests have low sensitivity and specificity to detect early changes and monitor the progression of the disease during a short-term follow-up. Visible radiographic changes occur on average two years after the onset of the disease.<sup>1,6</sup> 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.<sup>4</sup> The use of biomarkers enables an early, non-invasive measurement of the processes of cartilage degeneration. These biochemical markers of connective tissue are released into the systemic circulation, and can be measured in the blood, the urine or the synovial fluid. One of the main biomarkers for the diagnosis and prognosis of OA is C-terminal cross-linked telopeptide of type-II collagen (CTX-II).<sup>7-11</sup> This biomarker is released during the dynamic process of type-II collagen degeneration, and, consequently, it correlates with the destruction and formation of cartilage.<sup>8,12-18</sup> Mouritzen et al<sup>19</sup> showed that CTX-II has a specificity for OA of the knee. Rotational trauma and intra-articular bleeding associated

with ACL tear are thought to cause an acute metabolic alteration of the 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.<sup>8</sup>

In the literature, no study has evaluated the correlation of the CTX-II biomarker in a homogeneous sample with isolated ACL injury, what makes the study unique and innovative. The aim of the present study was to quantify the urinary concentration of the CTX-II biomarker in patients who suffered an isolated ACL injury and to compare the concentrations found in this population with those of a control group of patients with no knee injury. Our hypothesis was that the 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 the development of OA.

**Methods****Study Design**

The present study was evaluated and approved by the Ethics in Research Committee of the author's institution. The present was a cross-sectional, observational, single-center, pilot 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, 10 male subjects with a history of isolated ACL lesion (group 1-treatment) and 10 males with no history of knee ligament injury (group 2-control) were evaluated and included in the study.

The inclusion criteria were: male gender; age between 18 and 35 years; body mass index (BMI) lower than 30 kg/m<sup>2</sup>; isolated ACL lesions or absence of knee injuries; and patients who did not practice sports, since some previous studies suggest

an increase in the levels of CTX-II after the practice of sports in certain conditions.<sup>20,21</sup> 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 of the associated cartilage (bone bruises on the magnetic resonance imaging [MRI] scans were not considered cartilage lesions, and were included in the study); any previous ACL surgical treatment; and use of nonsteroidal anti-inflammatory drugs for at least 1 month before the evaluation. 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.

The patients were selected from the outpatient care population with clinical and radiologic findings of the ACL tear. The anterior drawer, Lachman, and pivot-shift maneuvers were used during the physical examination, along with MRI diagnostic confirmation of ACL rupture for Group-1 subjects. These evaluations were performed just before the urine collection, excluding all pathologies that could interfere in the analysis (meniscal lesions, cartilage lesions, osteoarthritis). The maximum time postinjury was established as 2 years.

### 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 previous urination (exactly as described in the CTX-II kit instruction manual), during the middle urination stream (neglecting the 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 frozen at  $-20^{\circ}\text{C}$  for the period (1 week to 7 months) prior to the analysis. In the ACL group, the urine samples were obtained in the patients' first visit to the 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 CTX-II. An enzyme-linked immunosorbent assay (ELISA, Elabscience, Houston, TX, US) was performed on each sample. This ELISA kit used the ELISA-sandwich principle for sample analyses. Using this methodology, the supplied ELISA plate is precoated with an antibody specific for human CTX-II. The collected samples (urine) were poured into the wells of the ELISA plate and homogenized with the specific antibody, forming a conjugate (antigen-antibody complex).

Then, a biotinylated detection antibody specific for avidin-horseradish peroxidase (HRP) conjugate was added to the plate and incubated. The 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 a stop solution, and the color then became yellow. Immediately following this, the optical density (OD)

was measured spectrophotometrically at a wavelength of  $450 \text{ nm} \pm 2 \text{ nm}$  (EZ Read 400 Biochrom spectrometer, Cambridge, UK). The value of the OD is proportional to the concentration of human CTX-II present in the sample. The calculation of the concentration of CTX-II in the samples was then performed by comparing the values calculated based on a standard curve.

The specifications of the ELISA test for the detection of the degradation of type-II collagen (CTX-II kit) according to the manufacturer were as follows: sensitivity: 0.10 ng/mL; detection range: 0.16 ng/mL to 10 ng/mL; reproducibility: coefficient of variation  $< 10\%$

All analyses were performed simultaneously in the same laboratory (at the Molecular Biology Division) 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 the characteristics of the patients and the biomarker concentrations within each group. The Mann-Whitney test was used to compare the groups in relation to the concentration of CTX-II. The Spearman correlation was used to evaluate the relationship between the time after the injury and the presence of urinary CTX-II. Values of  $p$  lower than 5% (0.05) were defined as statistically significant differences.

### Results

The patients in the ACL group had a mean age of 20.8 years and a mean BMI of  $25 \text{ kg/m}^2$ . The patients in control group had a mean age of 28.2 years and a mean BMI of  $24.5 \text{ kg/m}^2$ . The individuals in the ACL group were younger than those of the control group ( $p < 0.001$ ), and there was no significant difference between the groups in terms of BMI ( $p > 0.05$ ) (►Table 1).

The mean concentration of CTX-II in the ACL group was  $8.9 \pm 0.7 \text{ ng/mL}$  (range: 7.7 ng/mL to 9.8 ng/mL), which was higher than that of the control group:  $6.7 \pm 2.6 \text{ ng/mL}$  (range 0.7 ng/mL to 9.4 ng/mL;  $p = 0.009$ ) (►Table 2 and ►Fig. 1). The time postinjury varied between 2 and 18 months, and there was no difference between the time post-injury and the CTX-II levels for the ACL group ( $p = 0.521$ ;  $r = -0.231$ ) (►Fig. 2 and ►Table 1).

### Discussion

Our hypothesis was supported by the results of this study. Subjects with ACL rupture had significantly higher concentrations of urinary CTX-II than the subjects without injury ( $p = 0.009$ ), regardless of the time postinjury. 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, as seen in other previous studies.<sup>4,14,15,17,22-25</sup>

Interleukins (IL-6, IL-8) and matrix metalloproteases (MMP-3 and MMP-13) are some of the cytokines thought

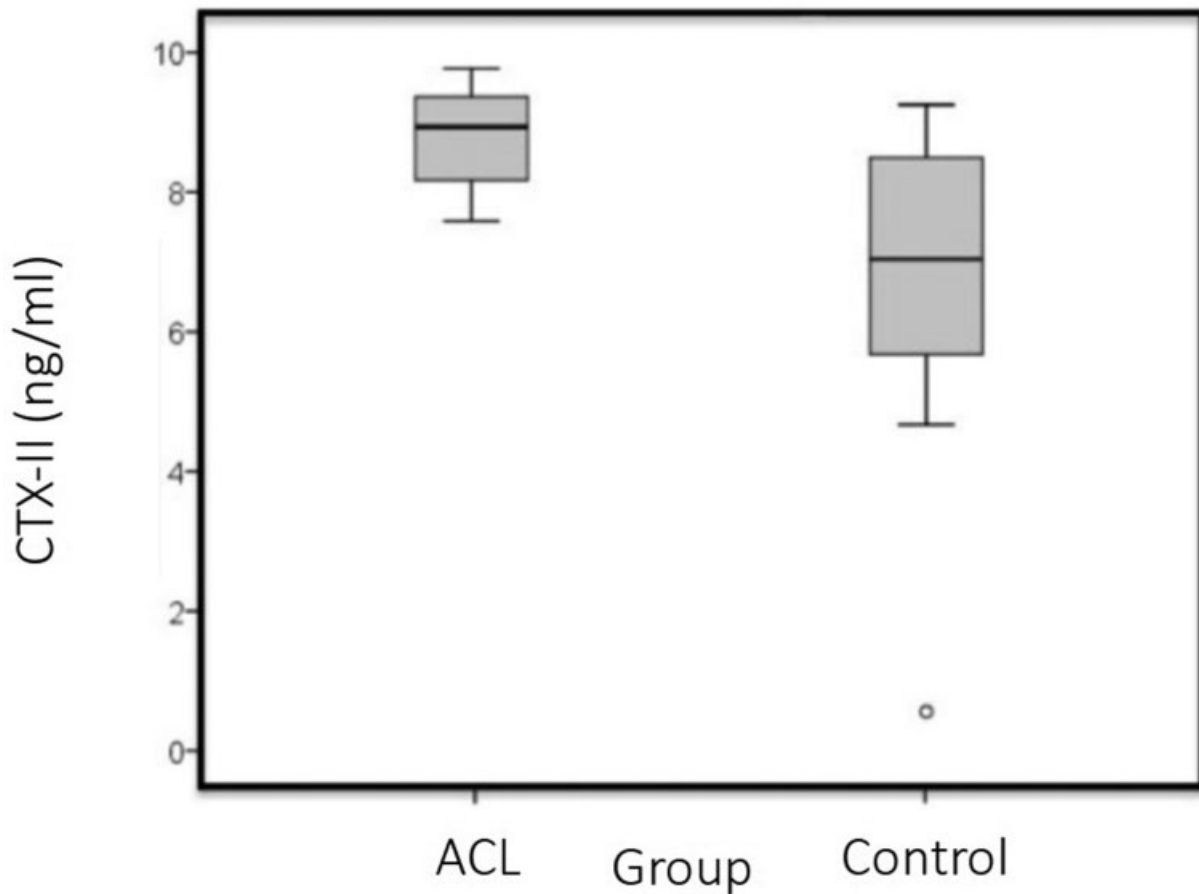
**Table 1** Descriptive characteristics evaluated according to each group and results of the comparative testing

Variable	Groups	Total	p-value	
<b>Age (years)</b>	Control (n = 10)	Anterior cruciate ligament (n = 10)	(n = 20)	< 0.001
Mean ± standard deviation	28.2 ± 3.8	20.8 ± 2.6	24.5 ± 5	
Median (minimum; maximum)	28.5 (22; 35)	20 (18; 25)	24 (18; 35)	
<b>Body mass index (Kg/m<sup>2</sup>)</b>				0.853
Mean ± standard deviation	24.5 ± 3.4	25 ± 2.3	24.8 ± 2.8	
Median (minimum; maximum)	24.5 (18; 29)	24 (22; 29)	24 (18; 29)	
<b>Time postinjury (months)</b>				
Mean ± standard deviation		6.8 ± 4.7	6.8 ± 4.7	
Median (minimum; maximum)		5.5 (2; 18)	5.5 (2; 18)	

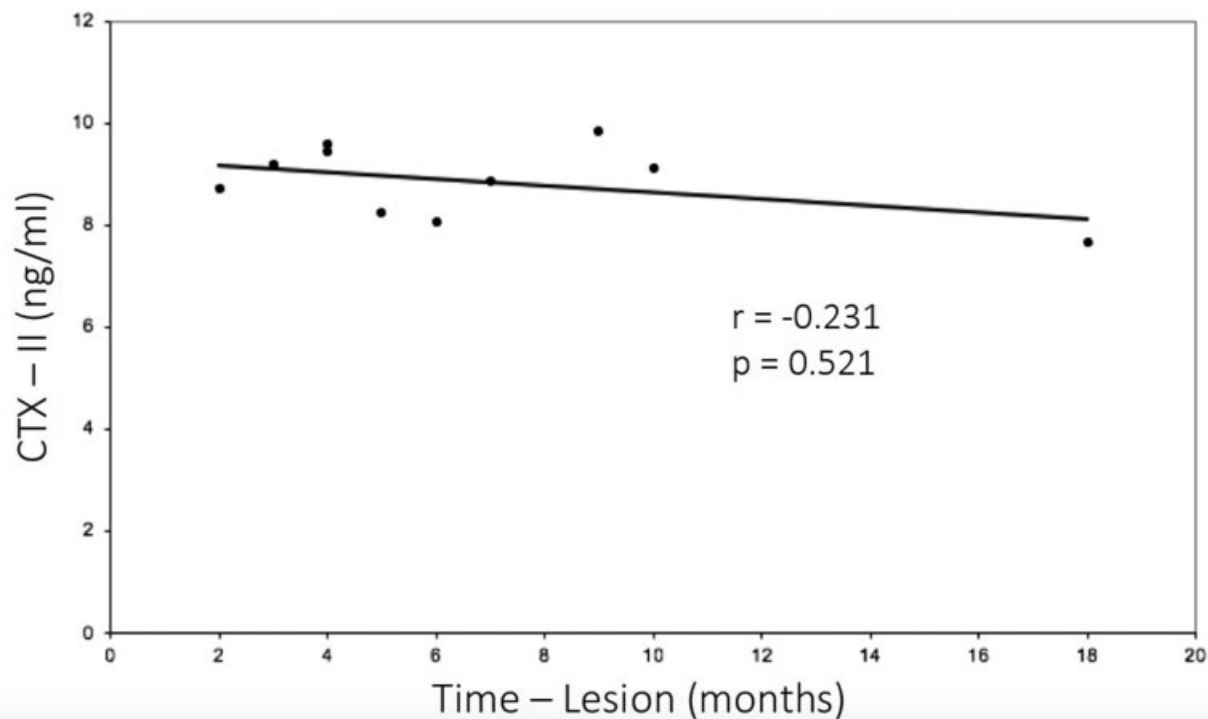
**Table 2** Quantitative analysis of the CTX-II urine biomarker according to each group and results of the comparative testing

Groups	CTX-II Urine Concentration (ng/mL)						p-value
	n	Mean	SD	Median	Minimum	Maximum	
ACL (1)	10	8.9	0.7	9	7.7	9.8	
Control (2)	10	6.7	2.6	7.2	0.7	9.4	p = 0.009
Total	20	7.8	2.1	8.4	0.7	9.8	

Abbreviations: ACL, anterior cruciate ligament; CTX-II, C-terminal cross-linked telopeptide of type-II collagen; SD, standard deviation.



**Fig. 1** Mean value of the G-terminal cross-linked telopeptide of type-II collagen (CTX-II): anterior cruciate ligament (ACL) group versus control group. The mean value of the CTX-II in the ACL group was of 8.9 ± 0.7 ng/mL (range: 7.7 ng/mL to 9.8 ng/mL), which was higher than that of the control group: 6.7 ± 2.6 ng/mL (range 0.7 ng/mL to 9.4 ng/mL) (p = 0.009).



**Fig. 2** Relationship between time postinjury and CTX-II concentration. There was no difference between the time postinjury and the CTX-II concentration in the ACL group ( $p = 0.521$ ;  $r = -0.231$ ).

to be intimately related to the degradation of type-II collagen.<sup>1,5</sup> On average, OA becomes symptomatic and hinders the patients from performing activities 10 to 15 years after the initial traumatic event.<sup>7,9,12</sup> However, this degenerative process does not affect all patients with a history of ACL tear, as evidenced by the wide range of incidence in this population (0% to 86%).<sup>2-6</sup>

Until now, there has not been a reliable, early prognostic marker for this process. As an established marker of type-II cartilage breakdown, CTX-II can be measured in the blood, the synovial fluid, and the urine because the molecule is not altered after renal filtration. The advantage of the urinary measurement is the ease of collection. Moreover, because it is considered a burden-of-disease type biomarker according to the burden of disease, investigative, prognostic, efficacy of intervention and diagnostic (BIPED) classification,<sup>10</sup> the correlation between the severity of the degenerative changes of the OA and the CTX-II biomarker concentrations support its use as a diagnostic and prognostic tool.<sup>10,25-27</sup>

Because of the intrinsic characteristics of each method of biomarker quantification (manufacturer, as well as gender, age, BMI and articular joint of the studied patients) there are no uniform or reference values described in the literature, and even the units of measurement differ according to the kit used.<sup>19</sup> Several biomarkers of inflammation quantification such as the cartilage oligomeric matrix protein (COMP), aggrecan degradation products (ARGS) and even inflammatory cytokines have been associated with degenerative processes in the knee cartilage.<sup>5</sup> Despite this variability in the analysis of biomarkers, previous studies in the literature concluded that there is a quantitative increase in biomarkers after an ACL injury.<sup>3,24</sup> However, these studies did not control

for isolated ACL injury, the stage of the disease, or use of the same biomarker.

Comparing with previous studies involving CTX-II and OA, Garnerio et al<sup>28</sup> compared 67 patients with knee OA and 67 healthy subjects, and found a significant difference between both groups concerning the concentration of urinary CTX-II (mean value: 431 ng/mmol Creatinine and 345 ng/mmol Cr respectively). In another study comparing 17 healthy individuals and 329 patients with any kind of knee injury (ACL/ACL combined with another ligament or meniscus tear, or an isolated meniscus tear), Lohmander et al<sup>18</sup> found a significant difference in the serum CTX-II measurement (mean: 1.4 ng/mL and 9.5 ng/mL respectively). Saberi Hosniji et al,<sup>29</sup> in a large cohort study, evaluated 1,335 participants with and without OA and the relationship with several biomarkers, including CTX-II, and showed as result values of 2.4 ng/mmol Cr for the CTX-II in the group that developed OA, and of 2.27 ng/mmol Cr in the group without OA (a small but significant difference).

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<sup>26</sup> found no difference in CTX-II biomarker concentrations in the serum, urine, and synovial fluid when comparing the values before and after surgery. Chmielewski et al<sup>8</sup> 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 measured



accurately and reproducibly, with coefficients of variation lower than 10%. Because patient characteristics such as gender, age and BMI vary, it is necessary to minimize the variability between the compared groups, or to stratify the study according to the analyzed variable. The present study is unique in its methodology, because care was taken to evaluate only patients with isolated ACL injuries, establishing the specificity of this biomarker for post-ACL injury cartilage degeneration in a homogeneous group. According to Deshpande et al,<sup>30</sup> the risk of OA in male, non-obese patients, under 35 years of age is lower than 1%.

The temporal trend of the concentration of the 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 at a maximum of 2 years prior to enrollment, and the postinjury time ranged from 2 to 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 after the trauma sooner than our analysis. These levels could remain chronically elevated, unless a new event occurs, including surgical treatment or some other non-surgical treatment.<sup>26</sup>

Despite the fact that our inclusion criteria were designed to identify demographically similar patients, there was a significant difference between the mean ages of the groups. The control group had a higher average age ( $28.2 \pm 3.8$  years) than the ACL group ( $20.8 \pm 2.6$  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 the 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.<sup>8,26</sup> Prospective studies tend to provide better information regarding the temporal trends of biomarker concentrations and their related pathological severity. Another limitation of the present study is the 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 study with a larger sample will ultimately provide more conclusive data and may better elucidate the changes in 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. This analysis will be necessary in future studies with larger samples of patients of both genders.

Further studies may better elucidate how to inhibit the process of cartilage degeneration after an ACL injury or other joint conditions. Biomarker measurement may play an important role in achieving this goal, because they help in 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 OA.<sup>2-4,17,24</sup> Another way to show an alteration in cartilage metabolism after knee trauma is using biomarkers, such as COMP and CTX-II.<sup>5,15</sup> The importance of the present study is to show the increase in CTX-II concentrations after an ACL tear, and other studies are being conducted after these results. This also could be useful for other pathologies, such as meniscal or cartilaginous injuries and other ligament injuries. It may also be useful to test or compare surgical techniques, medications or interventions to prevent OA. C-terminal cross-linked C-telopeptide of type-II collagen, among other biomarkers, has the advantage of being measured in the 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 postinjury ( $p > 0.05$ ).

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### Conflict of Interests

The authors have no conflict interests to declare.

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