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Synovial fluid peptidase activity as a biomarker for knee osteoarthritis clinical progression

A CROSS-SECTIONAL STUDY

J. Seco-Calvo,
S. Sánchez-Herráez,
L. Casis,
A. Valdivia,
I. Perez-Urzelai,
J. Gil,
E. Echevarría

From Institute of Biomedicine (IBIOMED), León, Spain

Aims

To analyze the potential role of synovial fluid peptidase activity as a measure of disease burden and predictive biomarker of progression in knee osteoarthritis (KOA).

Methods

A cross-sectional study of 39 patients (women 71.8%, men 28.2%; mean age of 72.03 years (SD 1.15) with advanced KOA (Ahlbäck grade ≥ 3 and clinical indications for arthrocentesis) recruited through the (Orthopaedic Department at the Complejo Asistencial Universitario de León, Spain (CAULE)), measuring synovial fluid levels of puromycin-sensitive aminopeptidase (PSA), neutral aminopeptidase (NAP), aminopeptidase B (APB), prolyl endopeptidase (PEP), aspartate aminopeptidase (ASP), glutamyl aminopeptidase (GLU) and pyroglutamyl aminopeptidase (PGAP).

Results

Synovial fluid peptidase activity varied significantly as a function of clinical signs, with differences in levels of PEP ($p = 0.020$), ASP ($p < 0.001$), and PGAP ($p = 0.003$) associated with knee locking, PEP ($p = 0.006$), ASP ($p = 0.001$), GLU ($p = 0.037$), and PGAP ($p = 0.000$) with knee failure, and PEP ($p = 0.006$), ASP ($p = 0.001$), GLU ($p = 0.037$), and PGAP ($p < 0.001$) with knee effusion. Further, patients with the greatest functional impairment had significantly higher levels of APB ($p = 0.005$), PEP ($p = 0.005$), ASP ($p = 0.006$), GLU ($p = 0.020$), and PGAP ($p < 0.001$) activity, though not of NAP or PSA, indicating local alterations in the renin-angiotensin system. A binary logistic regression model showed that PSA was protective ($p = 0.005$; Exp (B) 0.949), whereas PEP ($p = 0.005$) and GLU were risk factors ($p = 0.012$).

Conclusion

These results suggest synovial fluid peptidase activity could play a role as a measure of disease burden and predictive biomarker of progression in KOA.

Cite this article: *Bone Joint Res* 2020;9(11):789–797.

Keywords: Peptidase, Synovial fluid, Knee ostheoarthritis

Article focus

- We examined whether synovial fluid peptidase activity has a potential role as a measure of disease burden and predictive biomarker of progression in knee osteoarthritis (KOA).

Key messages

- Synovial fluid peptidase levels varied significantly between patients requiring total knee arthroplasty (TKA) and others with advanced KOA managed conservatively.

Strengths and limitations

- To our knowledge, these are the first data to suggest that synovial fluid peptidase activity could play a role as a measure of disease burden and predictive biomarker of progression in KOA.

Correspondence should be sent to Jesús Seco-Calvo; email: dr.seco.jesus@gmail.com

doi: 10.1302/2046-3758.911.BJR-2020-0022.R2

Bone Joint Res 2020;9(11):789–797.

- Limitations of the study include failure to consider potential differences in conservative treatment, and the sample size being relatively small, limiting the reliability and validity of the results.

Introduction

Osteoarthritis (OA) is a chronic inflammatory joint disease that frequently coexists with other comorbidities, reducing joint range of motion and generating pain, functional limitations, and a high economic burden.[1–3] Although there are some partially effective methods,[4] it is generally accepted that treatments do not completely prevent disease progression,[3] which is associated with cartilage erosion and joint inflammation, leading to disability.[1]

Several OA phenotypes have been described,[5] and RNA expression patterns have been found in knee osteoarthritis (KOA) cartilage,[6] indicating the importance of developing new biomarkers to improve the management of this condition. Regarding potential biomarkers, Bauer et al[7] proposed the Burden of Disease, Investigative, Prognostic, Efficacy of Intervention and Diagnostic (BIPED) classification and this has been used in subsequent research;[8] nonetheless, early biomarkers for clinical progression of OA have yet to be established.[9]

Several local renin-angiotensin systems have been described,[10] including one in the synovial fluid and synovium.[11] Animal models indicate its involvement in KOA,[12] and that it promotes periarticular osteopenia by increasing bone resorption and decreasing bone formation.[13] Moreover, research in humans suggests intra-articular renin and angiotensin-converting enzyme may contribute to progression in rheumatoid arthritis (RA).[11] Indeed, aliskiren, a renin inhibitor, has had positive effects in rat models of OA[14] and osteoporosis.[15] Angiotensin II is a known potent proinflammatory mediator,[16] and angiotensin receptor blockers show anti-inflammatory effects in animal models of arthritis.[17] Further, articular chondrocyte angiotensin II type 1 receptor was implicated in KOA progression in a mechanical stress mouse model[18] and activation of the renin-angiotensin system might be involved in the pathogenesis of this condition.[12] Nonetheless, the association between angiotensin-converting enzyme and KOA remains controversial.[19]

Peptidases can degrade bioactive peptides, modifying their physiological actions, and consequently may regulate cell growth and differentiation, and signal transduction.[20] Specifically, serum peptidases, such as angiotensin-converting enzyme (ACE), angiotensin II-converting enzyme (ACE2), neutral aminopeptidase (NAP), and aminopeptidase A (APA), are important elements of the renin-angiotensin system.[21] For example, ACE is essential for degradation of angiotensin I, to obtain angiotensin II, and regulate blood pressure.[22]

Abnormal peptidase levels have been observed in a range of conditions, from renal cancer (ACE and NAP)[23] to chronic tonsillitis (APA and dipeptidyl-peptidase IV).[24] In OA, proteomic studies have shown inflammatory cytokines and proteases in synovial fluid,[25,26] and evidence suggests the renin-angiotensin system may play a role in the pathophysiology of arthritis.[13,27] Specifically, dipeptidyl-peptidase IV could be involved in OA inflammation.[28] Other synovial peptidases implicated in inflammatory processes (e.g. aminopeptidase N, expressed by fibroblast-like synoviocytes) are present in synovial fluid and could play a role in OA.[29] As de Silveira et al[30] demonstrated, in an animal model of arthritis, joint inflammation was induced by activation of the renin-angiotensin system, but reduced by activation of angiotensin-converting enzyme-2/Ang-(1–7)/Mas receptor pathway.

Further, preoperatory predictive markers of knee joint infection have been proposed,[31–33] but it remains unclear whether the activity of any specific enzymes can be considered a reliable measure of disease burden or predictor of progression in KOA. In this context, we considered that analyzing potential biomarkers could contribute to this line of research. In particular, we explored the potential role of synovial fluid peptidase activity as a measure of disease burden and predictive biomarker of progression in KOA, as this would be useful in clinical practice.

Methods

This cross-sectional study was approved by the Ethics Committee of the University of León, Spain (Agency code: AVPD; ref.: 280310015, 7 October 2011) and conducted in accordance with the Declaration of Helsinki (2013, revised May 5, 2015), ethical regulations and Spanish Laws for Data Protection (15/1999), and Biomedical Research in Human Participants (14/2007). All participants gave written informed consent to arthrocentesis and inclusion in this study.

The study is reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology statement.[34] Patients with advanced KOA were recruited by non-probability sampling through the Orthopaedic Department at CAULE, Spain, between 2011 and 2019.

Inclusion criteria were a diagnosis of KOA (Ahlbäck grade $\geq$ 3)[35] and clinical indications for arthrocentesis and related treatment, i.e. the extraction of synovial fluid and intra-articular injections for the local treatment of peripheral joint disease. We excluded patients with contraindications to arthrocentesis, biochemical markers of inflammatory activity, or inflammatory comorbidities. A sample size calculation indicated we needed to recruit at least 38 patients. Overall, we included 39 patients (women 71.8%, men 28.2%) with advanced KOA and a mean age of 72.03 years (SD 1.154; 69.69 to 74.36) (see Supplementary table i).

We assessed the activity of the following peptidases: puromycin-sensitive aminopeptidase (PSA) (EC 3.4.11.14, cytosolic form), NAP (EC 3.4.24.11), aminopeptidase B (APB) (EC 3.4.11.6), PEP (EC 3.4.21.26), aspartate aminopeptidase (ASP) (EC 3.4.11.21), glutamyl aminopeptidase

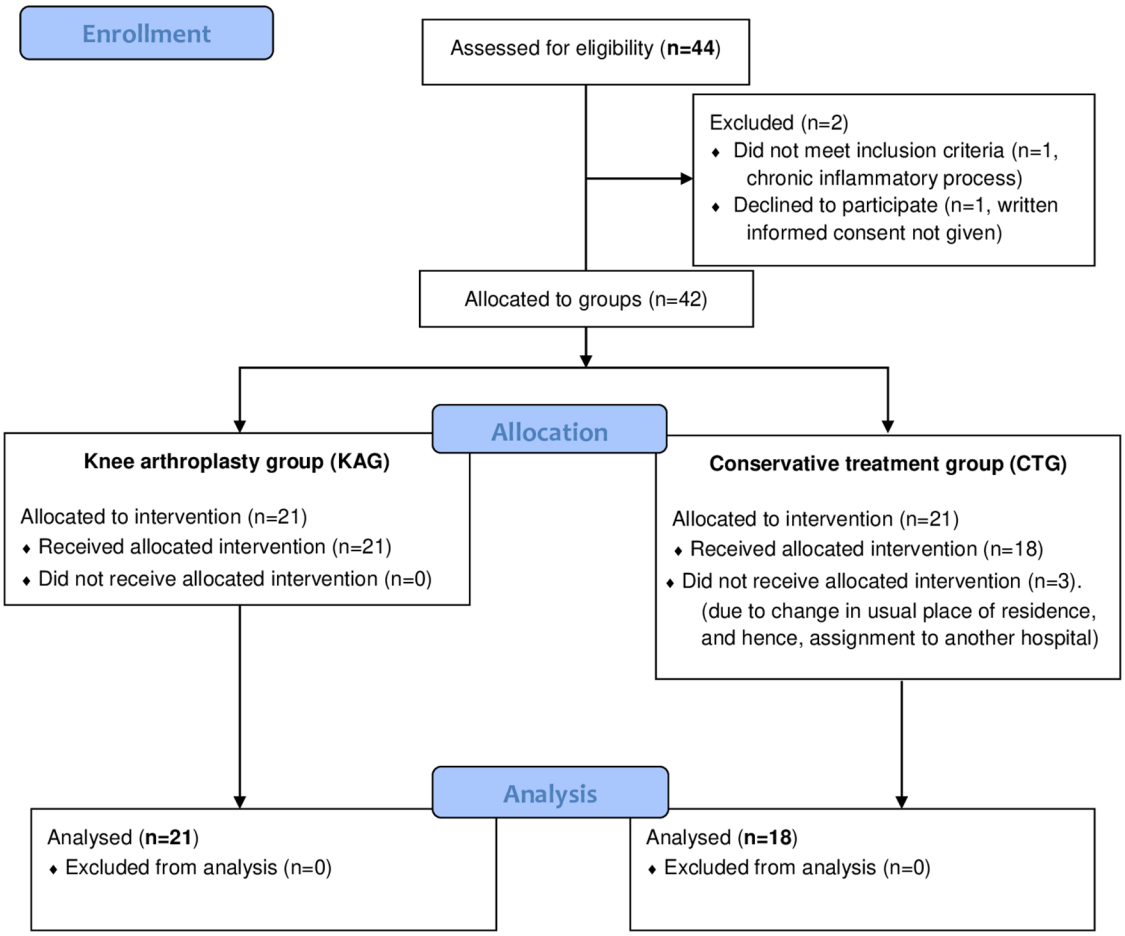

Fig. 1

Flow of patients through the study (Consolidated standards of reporting Trials (CONSORT) flow diagram).

(GLU) (EC 3.4.11.7), and pyroglutamyl aminopeptidase (PGAP) (EC 3.4.19.3). At enrolment, synovial fluid samples (blood-free and ≥ 10 ml) were collected from all patients by standard arthrocentesis, centrifuged, and frozen until blind analysis (see Supplementary Material 2). In all cases, samples were collected ≥ six months after the most recent intra-articular injections.

Further, we retrieved data on MRI and laboratory test results (e.g. knee cartilage thickness and blood counts) from health records and collected data on sociodemographic and clinical characteristics through clinical examinations and interviews. Lastly, patient-reported outcome measures ((PROMs) quality of life, physical status, and functional status) were assessed at the most recent visit (see Supplementary Material).

For secondary analysis, the sample was divided into a conservative treatment group (CTG) (n = 18; women 66.7%; mean age 71.33 years (SD 1.926; 67.31 to 75.35)) and knee arthroplasty group (KAG) (n = 21; women 77.8%, mean age 72.83 years (SD 1.135; 70.44 to 75.23)). The CTG contained patients who responded satisfactorily to conservative management, and the KAG those selected for knee arthroplasty following usual criteria: namely, knee locking, failure and/or effusion, pain and joint cartilage damage regardless of the presence of deformity, and others who did not respond satisfactorily to conservative management (see Supplementary Material). Figure 1 shows the flow of patients through the study.

Statistical analysis. All statistical analyses were performed using SPSS Statistics for Windows v25 (IBM, Armonk, New York, USA). The Shapiro-Wilk test was used to check whether quantitative data were normally distributed. Sociodemographic and clinical characteristics of all patients were summarized using descriptive statistics (medians, means, and SDs) and comparisons made using independent-samples *t*-test or Mann-Whitney U tests for quantitative variables, and chi-squared tests for qualitative variables.

Binary logistic regression models were built to explore the effects of the peptidase activities studied on the dependent binary variable of interest (namely, knee locking, failure, or effusion) with all the samples, and in secondary analysis, the need for arthroplasty (inclusion in the KAG). All enzymes were initially considered candidates and the best model was identified with a backward stepwise procedure, using the Wald statistic. Further, B

Table I. Comparison between peptidase activity and patient-reported outcome measures (PROMs): state of the articular cartilage (as assessed by MRI) and pain on movement and range of motion (as assessed with the modified Knee Society Score) (n = 39).[36]

Peptidase activity (U/mg prot)*	Median (IQR)	p-value†	p-value‡	p-value§
NAP	87.8259 (23.42 to 338.79)	0.565	0.051	0.051
PSA	173.0000 (40.56 to 367.44)	0.502	0.154	0.154
ABP	46.8974 (9.00 to 276.54)	0.776	0.103	0.103
PEP	15.3273 (3.00 to 64.15)	0.266	0.103	0.103
ASP	13.7366 (4.93 to 32.99)	0.734	0.205	0.205
GLU	14.0000 (5.00 to 46.88)	0.738	0.564	0.564
PGAP	7.0000 (3.00 to 17.24)	0.426	0.256	0.256

*Peptidase activity reported as units of enzyme per milligram of protein; Mann-Whitney U test ($p < 0.05$).
†Comparison between peptidase activity and state of the articular cartilage (assessed by MRI). Mann-Whitney U test.
‡Comparison between peptidase activity and pain on movement (as assessed with the modified Knee Society Score).
§Comparison between peptidase activity and range of motion (as assessed with the modified Knee Society Score).
APB, aminopeptidase B; ASP, aspartate aminopeptidase; GLU, glutamyl aminopeptidase; IQR, interquartile range; NAP, neutral aminopeptidase; PEP, prolyl endopeptidase; PGAP, pyroglutamyl aminopeptidase; PSA, puromycin-sensitive aminopeptidase.

Table II. Comparison between peptidase activity (reported as units of enzyme per milligram of protein) and clinical signs: knee locking, knee failure, and knee effusion (n = 39).

Peptidase activity (U/mg prot)*	Median (IQR)	p-value†	p-value‡	p-value§
NAP	87.8259 (23.42 to 338.79)	0.251	0.489	0.489
PSA	173.0000 (40.56 to 367.44)	0.392	0.735	0.735
ABP	46.8974 (9.00 to 276.54)	0.041	0.076	0.076
PEP	15.3273 (3.00 to 64.15)	0.020	0.006	0.006
ASP	13.7366 (4.93 to 32.99)	< 0.001	0.001	0.001
GLU	14.0000 (5.00 to 46.88)	0.071	0.037	0.037
PGAP	7.0000 (3.00 to 17.24)	0.003	< 0.001	< 0.001

*U/mg prot.: Peptidase activity reported as units of enzyme per milligram of protein; Mann-Whitney U test ($p < 0.05$).
†Comparison between peptidase activity and knee locking.
‡Comparison between peptidase activity and knee failure.
§Comparison between peptidase activity and knee effusion.
APB, aminopeptidase B; ASP, aspartate aminopeptidase; GLU, glutamyl aminopeptidase; IQR, interquartile range; NAP, neutral aminopeptidase; PEP, prolyl endopeptidase; PGAP, pyroglutamyl aminopeptidase; PSA, puromycin-sensitive aminopeptidase.

coefficients were calculated for the predictive equation, as well as Exp(B) values and corresponding 95% confidence intervals for the peptidase activities included. Additionally, having performed Levene's test to confirm homoscedasticity, independent-samples *t*-tests were used for between-group comparisons, and standardized means were calculated to assess effect sizes (Cohen's d).

Results

The synovial fluid peptidase levels studied did not vary significantly with knee cartilage thickness as measured by MRI. Nonetheless, we observed clinically significant differences in NAP as a function of pain and range of motion (Table I). Further, peptidase levels did vary significantly as a function of clinical signs, with differences in levels of PEP, ASP, and PGAP associated with knee locking, PEP, ASP, GLU, and PGAP with knee failure, and PEP, ASP, GLU, and PGAP with knee effusion (Table II). Moreover, the knee locking model showed an influence of PSA, ASP, and GLU, while NAP and PGAP were significant in knee failure and effusion models (Table III). In addition, knee locking, failure, and effusion were all significantly associated with the need for knee arthroplasty (Table IV).

Regarding the secondary analysis by need for arthroplasty (CTG vs KAG), the groups were similar, with no significant differences in sociodemographic or the majority of clinical variables (Tables V and VI and Supplementary table iii). Nonetheless, as expected, there were significant differences in variables commonly used as criteria for indicating knee arthroplasty (Table VII). Specifically, KAG patients had not substantially benefited from previous treatment, such as hyaluronic acid injections, and had highly inflamed knee joints, with recurrent severe distention of the joint capsule, intense pain, and joint effusion, locking, and failure in all cases.

All peptidases studied were detected in all patients, and several between-group differences were found. Specifically, KAG patients showed significantly higher activity of APB, PEP, ASP, GLU, and PGLU, but not PNA or PSA (Table VIII).

A binary logistic regression model showed effects of PSA, PEP, and GLU on the need for arthroplasty. PSA was a protective factor, whereas PEP and GLU were risk factors (Table III) (Nagelkerke R^2 p = 0.812; Hosmer-Lemeshow p = 0.222; Omnibus $p < 0.001$).

There was a significant difference between groups (Levene's p = 0.138; independent-samples *t*-test p = 0.011); specifically, the difference between means was –8.22763 (95% confidence interval (CI) –1.98798 to –14.46727), with higher levels in the KAG than the CTG. The effect sizes obtained were 0.36, 0.79, and 0.64 for PSA, PEP, and GLU, respectively.

Discussion

To our knowledge, this study provides the first evidence for the role of synovial fluid peptidase activity as a measure of disease burden and predictive biomarker of progression in KOA. Specifically, we found significant differences in biomarker levels as a function of pain and range of motion, with somewhat different patterns in each case.

To date, few studies have investigated synovial fluid aminopeptidase activity in intra-articular inflammatory processes in humans. Some years ago, human articular chondrocytes obtained from patients with OA undergoing arthroplasty were found to express angiotensin II receptors.[39] In line with this, losartan showed anti-inflammatory action in human arthritis.[40] Further, Cobankara et al[11] have suggested that the local articular

Table III. Binary logistic regression models.

Category	B	SD	Wald	Sig.	Exp(B)	95% CI for Exp(B)
Peptidase activity and knee locking*						
PSA	–0.016	0.008	4.268	0.039	0.984	0.970 to 0.999
ASP	0.246	0.091	7.268	0.007	1.279	1.069 to 1.529
GLU	0.155	0.086	3.203	0.073	1.167	0.985 to 1.383
Constant	–2.127	1.328	2.565	0.109	0.119	N/A
Peptidase activity and knee failure†						
NAP	–0.020	0.010	3.888	0.058	0.980	0.961 to 1.001
PGAP	0.930	0.337	7.643	0.006	2.536	1.311 to 4.904
Constant	–3.273	1.529	4.581	0.032	0.038	N/A
Peptidase activity and knee effusion‡						
NAP	–0.020	0.010	3.888	0.058	0.980	0.961 to 1.001
PGAP	0.930	0.337	7.643	0.006	2.536	1.311 to 4.904
Constant	–3.273	1.529	4.581	0.032	0.038	N/A
Peptidase activity and inclusion in the KAG§						
PSA	–0.052	0.019	8.012	0.005	0.949	0.915 to 0.984
PEP	0.414	0.149	7.738	0.005	1.513	1.130 to 2.026
GLU	0.423	0.169	6.250	0.012	1.527	1.096 to 2.128
Constant	–3.625	2.077	3.046	0.081	0.027	N/A

*Omnibus p < 0.005; Nagelkerke R^2 = 0.577; Hosmer-Lemeshow p = 0.808.
†Omnibus p < 0.005; Nagelkerke R^2 = 0.520; Hosmer-Lemeshow p = 0.893.
‡Omnibus p < 0.005; Nagelkerke R^2 = 0.520; Hosmer-Lemeshow p = 0.893.
§Omnibus p = 0.000; Nagelkerke R^2 = 0.812; Hosmer-Lemeshow p = 0.222. This shows the influence of PSA, PEP, and GLU, as independent variables, on a dependent binary variable indicating inclusion in the Knee Arthroplasty Group. PSA was a protective factor, whereas PEP and GLU were risk factors.
ASP, aspartate aminopeptidase; CI, confidence interval; GLU, glutamyl aminopeptidase; KAG, Knee Arthroplasty Group; N/A, not applicable; NAP, neutral aminopeptidase; PEP, prolyl endopeptidase; PGAP, pyroglutamyl aminopeptidase; PSA, puromycin-sensitive aminopeptidase.

Table IV. Between-group comparison of qualitative clinical variables commonly used in routine clinical practice as criteria for indicating knee arthroplasty (n = 39). All p-values were < 0.05 (chi-squared test).

Qualitative clinical variable	Conservative treatment group (n = 18)	Knee arthroplasty group (n = 21)
Knee locking, n (%)	4 (16)	21 (84)
Knee failure, n (%)	5 (19.2)	21 (80.8)
Knee effusion, n (%)	5 (19.2)	21 (80.8)

renin-angiotensin system is involved in joint destruction in RA, and recent studies support the view that this system plays a role in the pathophysiology of arthritis.[13,27]

We have found significant differences in synovial fluid levels of some biomarkers as a function of clinical signs: knee locking, failure, and effusion, although not as a function of PROMs (visual analogue scale, modified Knee Society Score, or EQ-5D score). Moreover, in patients with advanced KOA, synovial fluid peptidase analysis revealed significant differences between patients requiring TKA and those managed conservatively, the former (those with the greatest functional impairment) having significantly higher peptidase activities, indicating alterations in the local renin-angiotensin system in the cases of APB, PEP, ASP, GLU, and PGAP, but not NAP or PSA. This is consistent with the higher APB activity in synovial fluid from swollen knees in a rat model of RA[41] and synovial fluid NAP activity inducing T-cell chemotaxis in a similar animal model.[42] Further, NAP seems to be involved in the pathogenesis of RA[43] and possibly also OA.[29]

The predictive model for knee locking showed the influence of PSA, ASP, and GLU, while APN and PGLU were significant in knee failure and effusion models (Table III). Further, the model for the need for arthroplasty (Table III) showed the influence of PSA, PEP, and GLU. Specifically, this model indicated that PSA activity was a protective factor, whereas PEP and GLU activities were risk factors. We could have studied other inflammatory cytokines and matrix metalloproteinases, but opted to focus on synovial soluble peptidases involved in the articular renin-angiotensin system, which has been associated with clinical progression in KOA.

Although between-group differences were not significant for NAP or PSA, both were considered candidates for inclusion in this model, given their clinical relevance, and the results indicated a role as a predictive factor for PSA, but not for NAP. The potential importance of NAP is related to its expression by fibroblast-like synoviocytes in inflamed synovial tissue in humans, suggesting a role in acute inflammatory arthritis.[29] Regarding PSA, while there is a paucity of data, spinorphin, an endogenous enzyme inhibitor, has been reported to break down enkephalin and play a role in pain and inflammation.[44] The possible role of PSA in pain strengthens its clinical relevance, in that, as underlined by scientific societies, pain is an important clinical feature in deciding whether to indicate

Table V. Between-group comparison of quantitative sociodemographic and clinical variables.

Variable	Conservative treatment group (n = 18)			Knee arthroplasty group (n = 21)				
	Average (median or mean (SD))	IQR or 95% CI	p-value*	Average (median or mean (SD))	IQR or 95% CI	p-value*	p-value†	p-value‡
Age, yrs	72.83 (4.817)	95% CI 70.44 to 75.23	0.518	71.33 (8.828)	95% CI 67.31 to 75.35	0.317	0. 507	0.922
Duration of pain, yrs	9.50	2 to 30	0.007	9.00	6 to 22	0.005	0.563	0.282
Pain VAS (1 to 10)	8.00	4 to 10	0.003	8.00	6 to 10	0.029	0.205	0.174
mKSS ROM (0 to 100)	26.67 (13.284)	95% CI 20.06 to 33.27	0.096	28.33 (15.838)	95% CI 21.12 to 35.54	0.077	0.726	0.707
mKSS pain on movement (0 to 100)	22.17 (16.525)	95% CI 13.95 to 30.38)	0.091	13.00	0 to 69	0.018	0.919	0.967

*Shapiro-Wilk test.
†Independent-samples *t*-test.
‡Mann-Whitney U test.
CI, confidence interval; IQR, interquartile range; mKSS, Insall's modified Knee Society Score; ROM, range of motion; VAS, visual analogue scale.

Table VI. Between-group comparison of qualitative sociodemographic and clinical variables.

Variable	Conservative treatment group (n = 18)	Knee arthroplasty group (n = 21)	p-value*
Sex, n (%)			0.442
Female	14 (77.8)	14 (66.7)	
Male	4 (22.4)	7 (33.3)	
Surgical risk based on physical status (ASA class), n (%)			
No systemic conditions	6 (33.3)	5 (23.8)	0.700
Mild systemic disease	10 (55.6)	12 (57.1)	
Severe systemic disease	2 (11.1)	4 (19.0)	
Laterality, n (%)			0.882
Right	9 (50)	10 (47.6)	
Left	9 (50)	11 (52.4)	
Contralateral pain, n (%)			0.159
No	6 (33.3)	3 (14.3)	
Yes	12 (66.6)	18 (85.7)	
mKSS pain on movement (Insall's modified Knee Society Score), n (%)			
Acceptable (60 to 69)	0 (0)	1 (4.8)	0.348
Poor (< 60)	18 (100)	20 (95.2)	
mKSS ROM, n (%)			
Acceptable (60 to 69)	0 (0)	1 (4.8)	0.348
Poor (< 60)	18 (100)	20 (95.2)	
State of the articular cartilage (assessed by MRI), n (%)			
Ulceration (depth < 50%)	2 (11.1)	0 (0)	0.267
Ulceration (depth ≥ 50%)	8 (44.4)	12 (57.1)	
Exposure of subchondral bone	8 (44.4)	9 (42.9)	

*Chi-squared test.
ASA, American Society of Anesthesiologists; mKSS, Insall's modified Knee Society Score;ROM, range of motion.

arthroplasty, and this is what motivated us to include it in our study. Notably, patients with chronic pain associated with fibromyalgia have enzyme activity characterized by abnormally low serum enkephalin-degrading enzyme activity,[45] suggesting that it may influence musculoskeletal pain and that PSA might be involved in pain neuromodulatory mechanisms in OA. More studies are needed to clarify these issues.

Given the high specificity in the clinical characterization of these patients, and in line with the BIPED classification,[7,8] our results suggest a role for synovial fluid peptidase activity as a measure of disease burden and predictive biomarker of progression in KOA, which would be useful in clinical practice. Generally, patients with advanced KOA are seen by trauma and orthopaedic specialists every six months at outpatient appointments, in which arthrocentesis may be performed to drain synovial fluid, and intra-articular injections given. During such appointments, a small quantity of synovial fluid could be collected easily, inexpensively, and without causing bleeding, and sent for peptidase analysis, which is straightforward and quick. The use of PSA, PEP, and GLU

Table VII. Between-group comparison of qualitative clinical variables.

Clinical variable	Conservative treatment group (n = 18)	Knee arthroplasty group (n = 21)	p-value*
Degree of OA (Ahlbäck grade), n (%)†			
3	3 (16.7)	4 (19.0)	0.006
4	8 (44.4)	17 (81.0)	
5	7	0	
Change in quality of life over 12 months (EuroQol EQ-5D)†	18 (100)	21 (100)	< 0.001
BMI, kg/m²‡			
18.5 to 24.9 (normal weight)	4 (22.2)	5 (23.8)	0.981
25.0 to 29.9 (grade I and II overweight)	10 (55.6)	11 (52.4)	
30.0 to 39.9 (grade I and II obesity)	4 (22.2)	5 (23.8)	
History of surgery for OA in contralateral knee, n (%)			0.052
No	15 (83.3)	21 (100)	
Yes	3 (16.7)	0 (0)	
History of surgical interventions in the knee, n (%)			0.002
No	13 (72.2)	5 (23.8)	
Yes	5 (27.8)	16 (76.2)	
Knee locking, n (%)†			< 0.001
No	14 (77.8)	0 (0)	
Yes	4 (22.2)	21 (100)	
Knee failure, n (%)†			< 0.001
No	13 (72.2)	0 (0)	
Yes	5 (27.8)	21 (100)	
Knee effusion, n (%)†			< 0.001
No	13 (72.2)	0 (0)	
Yes	5 (27.8)	21 (100)	
History of treatment			
Only oral analgesia	11 (61.1)	9 (42.9)	< 0.001
Oral analgesia + physical therapy + intra-articular injections	7 (38.9)	12 (57.1)	

*Chi-squared test.
†Commonly used in routine clinical practice as criteria for indicating knee arthroplasty.
‡Classification as recommended by Spanish Society for the Study of Obesity[37] and Federation of European Nutrition Societies (FENS),[38] 2020.
BMI, body mass index; OA, osteoarthritis.

Table VIII. Between-group comparison of levels of activity of synovial fluid peptidases. Each column of p-values signify comparisons between groups.

Peptidase activity (U/mg prot)*	Conservative treatment group (n = 18)			Knee arthroplasty group (n = 21)			p-value‡
	Average (median or mean (SD))	IQR or 95% CI	p-value†	Average (median or mean (SD))	IQR or 95% CI	p-value†	
NAP	106.500	41.00 to 266.00	0.041	85.5089	23.42 to 338.79	0.001	0.223
PSA	202.2193 (87.06746)	95% CI 158.9217 to 245.516	0.222	152.8595	40.56 to 367.44	0.013	0.183
ABP	17.500	9.00 to 242.00	0.000	55.5021	28.11 to 276.54	0.000	0.005
PEP	12.8358 (5.55793)	95% CI 10.0719 to 15.5997	0.859	16.9785	9.06 to 64.15	0.000	0.006
ASP	8.500	5.00 to 22.40	0.023	19.0425	4.93 to 32.99	0.045	< 0.001
GLU	11.000	5.00 to 30.88	0.037	16.3267	8.79 to 46.88	0.003	0.020
PGAP	6.000	3.00 to 13.00	0.012	9.4819 (3.21187)	95% CI 8.0199 to 10.9439	0.080	< 0.001

*Peptidase activity is reported as units of enzyme per milligram of protein (U/mg prot).
†Shapiro-Wilk test.
‡Mann-Whitney U test.
APB, aminopeptidase B; ASP, aspartate aminopeptidase; CI, confidence interval; GLU, glutamyl aminopeptidase; IQR, interquartile range; NAP, neutral aminopeptidase; PEP, prolyl endopeptidase; PGAP, pyroglutamyl aminopeptidase; PSA, puromycin-sensitive aminopeptidase.

activities as indicators of disease burden and predictors of progression could help clinicians decide whether to indicate knee arthroplasty. This might not only reduce delays, avoiding patients waiting a further year and potentially experiencing functional deterioration, but also the risk of complications (such as infection) should surgery become necessary,[31–33] and the need for prosthesis arthroplasty, reducing surgical reintervention and failure rates.

This study has limitations, including failure to consider differences in conservative treatment, or other proteins and enzymes likely involved in KOA. Other limitations are the small sample size, and that we only analyzed samples from patients undergoing arthrocentesis. All the participants had a marked inflammatory component, and our results may not apply to less inflamed joints. Future studies are needed to explore whether enzyme activity is influenced by treatment type (anti-inflammatory corticosteroids, chondroprotective agents, or platelet-rich plasma), and confirm whether peptidase levels are indeed reliable bioindicators of KOA progression.

Supplementary material

In the supplementary material, we provide more information about inclusion and exclusion criteria, and other key parts of the Methods section such as sample size calculation and assay conditions for the enzymes studied. Sociodemographic and clinical variables in the entire sample, and between-group comparison of qualitative sociodemographic and clinical variables, are also provided in the tables.

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Author information:
- J. Seco-Calvo, MD, PhD, Visiting Professor and Researcher, Institute of Biomedicine (IBIOMED), University of León, University of the Basque Country, León, Spain.
- S. Sánchez-Herráez, MD, PhD, Orthopaedic Surgeon, Servicio de Cirugía y Traumatología Ortopédica, Complejo Asistencial Universitario de León (CAULE), León, Spain.
- L. Casis, MD, PhD, Head Professor
- A. Valdivia, PhD, Professor
- I. Perez-Urzelai, PhD, Professor

  Department of Physiology, Faculty of Medicine, University of the Basque Country, Leioa, Spain.
- J. Gil, PhD, Head Professor
- E. Echevarría, MD, PhD, Head Professor

  Department of Physiology, Faculty of Medicine, University of the Basque Country, Leioa, Spain; Biomedical Research Networking Center for Mental Health Network (CIBERSAM), Carlos III Health Institute, Madrid, Spain.

Author contributions:
- J. Seco-Calvo: Drafted the first version of the study protocol, Contributed to the data gathering, Drafted and revised the manuscript.
- S. Sánchez-Herráez: Contributed to the data gathering, Revised the manuscript.
- L. Casis: Analyzed and interpreted the data, Revised the manuscript.
- A. Valdivia: Analyzed and interpreted the data, Revised the manuscript.
- I. Perez-Urzelai: Analyzed and interpreted the data, Revised the manuscript.
- J. Gil: Analyzed and interpreted the data, Revised the manuscript.
- E. Echevarría: Analyzed and interpreted the data, Revised the manuscript.

Funding statement:
- No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

ICMJE COI statement
- None declared.

Acknowledgements
- We are grateful for help from Dr. J. A. Iglesias with synovial fluid sample processing, Dr. R. Ramos-Pascua with obtaining samples, and Arantza Perez Dobaran with enzyme substrate preparation.

Bone & Joint Research

Supplementary Material

<10.1302/2046-3758.911.BJR-2020-0022.R2>

Methods

A sample size calculation was performed a priori to estimate the number of patients required for testing differences between independent groups using G*Power 3.1.9.2 (Dusseldorf University, Germany) (as recommended in Faul et al).[1] For this calculation, we considered the difference in values of prolyl endopeptidase (PEP) found in a pilot study (n = 10) with two groups of ten patients conducted by Calvo-Lobo et al,[2] and the following parameters: mean: 114.0877 units of enzyme per milligram of protein (U/mg prot) (SEM: 52.87091) for the controls versus 102.6057 U/mg prot (SEM: 70.6356) in the experimental group; an α-error of 0.05, and a β error of 0.20. This calculation indicated that we needed at least 17 patients in each group (34 patients) and considering a possible dropout rate of 10%, the minimum sample size was set at 38.

Inclusion criteria were a diagnosis of KOA (Ahlbäck grade $\geq$ 3)[3] and clinical indications for arthrocentesis and related treatment, that is, the extraction of synovial fluid and also intraarticular injections of corticosteroids, anaesthetics, hyaluronic acid, interleukin-1 receptor antagonists, anti-TNF (infliximab) and/or platelet-enriched plasma, for the local treatment of peripheral joint disease. Patients were excluded if they had contraindications to arthrocentesis, including local infection at the injection site or blood clotting disorders (e.g. haemophilia), joint infection or bacteraemia, a history of adverse reactions to medications used in previous injections, or polyarthritis with active involvement of several joints; and additionally, if they had any biochemical markers of inflammatory activity (a high total white blood cell count or high levels of neutrophils, eosinophils or lymphocytes), or had inflammatory comorbidities (e.g. rheumatoid arthritis or sarcoidosis).

Regarding the division of patients into two groups, the definition of a satisfactory response to conservative treatment and indication for arthroplasty were based on recommendations of the Spanish Society of Orthopedic Surgery and Traumatology, European Board of Orthopaedics and Traumatology and European Union of Medical Specialists (Orthopaedics and Traumatology Section). Specifically, following recommendations of these groups, patients were considered to not respond satisfactorily to conservative management if they had persistent pain (non-steroidal anti-inflammatory drugs for $\geq$ six months) and limited functional capacity (use of sticks or other walking aids) and had received all other usual treatment options, namely, injections of corticosteroids, hyaluronic acid, and platelet-rich plasma, as well as physiotherapy. A poor response implies poor clinical progression of osteoarthritis and such patients tend to need arthroplasty. In contrast, patients considered to have "responded satisfactorily to conservative management" did not have such symptoms, and consequently, arthroplasty was not offered.

We retrieved data on magnetic resonance findings[4] and laboratory test results from patient health records and gathered data on sociodemographic and clinical variables, including comorbidities, body mass index, and pain on a visual analogue scale, through clinical examinations and interviews. Further, at the most recent visit, the EuroQol EQ-5D[5], American Society of Anesthesiologists (ASA) Classification[6], and a modified version of Insall's Knee Society Score were used to assess patient's quality of life, physical status, and functional status respectively.[7]

Synovial fluid samples (10 ml) were taken from patients in both groups at enrolment by standard arthrocentesis. They were collected into heparinized tubes and any contaminated with blood were discarded. Samples of at least 10 ml in volume and free of blood contamination were successfully obtained from all patients. The sample collection was not blinded, but samples were analyzed blindly. All samples were collected at least six months after any intra-articular injections, as such injections are routinely given at an interval of at least six months.

Following centrifugation (5,000 rpm, for three minutes), synovial fluid samples were separated and stored frozen at -80ºC until analysis. Peptidase activities were quantified by fluorescence spectroscopy, in discontinuous enzymatic assays, following the method described by Larrinaga et al,[8] modified from Mantle et al.[9]

In brief, aliquots of 10-30 UL (depending on the enzyme studied) of sample were incubated for 30 minutes at 37ºC in 1 ml of a saturating substrate solution for each enzyme activity determination assay, and each assay was performed in triplicate. Substrates were aminoacyl-β-naphthylamide derivatives, whose specific cleavage by each enzyme releases β-naphthylamine, a fluorescent compound, as a product. The substrate-to-product ratio was 1:1 in

all enzyme assays. We detected the fluorescence produced in each reaction assay using a Shimadzu RF-540 (Shimadzu Corporation, Kyoto, Japan) spectrofluorophotometer (excitation wavelength of 345 nm, emission wavelength of 412 nm). For neutral endopeptidase activity, we used a specific N-dansyl fluorogenic derivative (excitation wavelength of 342 nm, emission wavelength of 562 nm), dansyl-d-Ala-Gly-p-nitro-Phe-Gly. All the substrates were purchased from Sigma-Aldrich (St Louis, Missouri, USA), now Merck-Millipore, or Bachem Chemical (Bachem AG, Bubendorf, Switzerland). To determine enzyme activities, fluorescence results were compared to a β-napthylamide concentration versus fluorescence standard curve. To convert activity values into specific activity levels, total protein content in each sample was determined using the Bradford colorimetric method (1976).[10] Activity levels are presented as mean (SD) or medians, in units of enzyme activity per milligram of protein (U/mg prot).

In accordance with Standards for Reporting Enzymology Data guidelines (https://www.beilstein-institut.de/en/projects/strenda/guidelines), more details of these activity assays are provided in the tables below.

Regarding the binary logistic regression model, the omnibus p-value, Hosmer-Lemeshow statistic and Nagelkerke's R^2 were calculated.

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Table i. Sociodemographic and clinical variables in the entire sample. Peptidase activity is reported as units of enzyme per milligram of protein (U/mg prot).

Variable	**Total sample (n = 39)**		
	Average (median or mean (SD))	**IQR or 95% CI**	**p-value***
Age, yrs	72.03 (7.209)	95% CI 69.69 to 74.36	0.349
Duration of pain, yrs	9.00	2 to 30	0.005
Pain VAS (1 to 10)	8.00	4 to 10	0.004
mKSS ROM (0 to 100)	25.00	65 to 65	0.031
mKSS pain on movement (0 to 100)	17.00	69 to 69	0.003
NAP	87.8259	338.79 to 315.37	0.000
PSA	184.4932 (90.81701)	95% CI 155.0537 to 213.9326	0.061
ABP	46.8974	9.00 to 276.54	0.000
PEP	15.3273	3.00 to 64.15	0.000
ASP	14.7012 (6.75951)	95% CI 12.5101 to 16.8924	0.065
GLU	14.0000	5.00 to 46.88	< 0.001
PGAP	7.0000	3.00 to 17.24	0.034

*Shapiro-Wilk test.

APB, aminopeptidase B; ASP, aspartate aminopeptidase; CI, confidence interval; GLU, glutamyl aminopeptidase; IQR, interquartile range; mKSS, Insall's modified Knee Society Score; NAP, neutral aminopeptidase; PEP, prolyl

endopeptidase; PGAP, pyroglutamyl aminopeptidase; PSA, puromycin-sensitive aminopeptidase; ROM, range of motion; VAS, visual analogue scale.

Table ii. Assay conditions for the enzymes studied.

Enzyme			**Metallo enzyme (Y/N)**	**Assay conditions**				
Abbreviation	**EC number**	**International Union of Biochemistry and Molecular Biology name**		**Substrate name**	**pH**	**Buffer**	**Salts**	**Others**
NAP	3.4.24.11	Neprilysin (neutral endopeptidase)	Y	N-Dansyl-dala-Gly-p-nitro-phe-gly	7.4	Na-Phosphate 50 mM	N/A	BSA; Puromycin (PSA inhibitor, 40 µM) and captopril (ACE inhibitor)
PGAP	3.4.19.3	Pyroglutamyl peptidase-I	N	Pyroglutamylnaphthylamide	7.4	Na-Phosphate 50 mM	DTT 2 mM	BSA 0.15 mg/ml
ASP	3.4.11.21	Aspartyl aminopeptidase	N	Aspartyl-□-naphtylamide	7.4	Tris-HCl 50 mM	N/A	BSA 0.15 mg/ml

PEP	3.4.21.26	Prolyl oligopeptidase (prolyl endopeptidase)	N	Z-Gly-Pro-β-naphthylamide	7.4	Na-Phosphate 50 mM	DTT 2 mM	BSA 0.15 mg/ml
APB	3.4.11.6	Aminopeptidase B (arginyl aminopeptidase)	Y (Zn)	Arginyl-□-naphthylamide	6.5	Na-Phosphate 50 mM	N/A	BSA 0.15 mg/ml; HCl; Puromycin (40 µM)
GLU	3.4.11.7	Glutamyl aminopeptidase	Y (Zn)	Glutamyl-□-naphthylamide	7.4	Tris-HCl 50 mM	N/A	BSA 0.15 mg/ml
PSA	3.4.11.14	Cytosol alanyl aminopeptidase (Puromycin-sensitive aminopeptidase)	Y (Zn)	Alanyl-□-naphthylamide	7.4	Na-Phosphate 50 mM	DTT 2 mM	BSA 0.15 mg/ml

ACE, angiotensin-converting enzyme; APB, aminopeptidase B; ASP, aspartate aminopeptidase; BSA, bovine serum albumin; DTT, dithiothreitol; GLU, glutamyl aminopeptidase; N/A, not available; NAP, neutral aminopeptidase; PGAP, pyroglutamyl aminopeptidase; PEP, prolyl endopeptidase; PSA, puromycin-sensitive aminopeptidase; Zn, Zinc.

Table iii. Data on the method used.

Enzyme	Localization	Description	Storage conditions	Assay temperature and pressure	Stopping procedure
NAP	Soluble/ membrane	Metalloendopeptidase	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2
PGAP	Soluble	Cysteine peptidase	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2
ASP	Soluble	Aminopeptidase	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2
PEP	Soluble	Serin protease	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2
APB	Soluble/membrane	Zn metallopeptidase	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2
GLU	Soluble	Zn metallopeptidase	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2
PSA	Soluble	Zn metallopeptidase (m1)	-80°C	37°C /atmospheric pressure	pH shock/ Na Acetate pH 4.2

APB: aminopeptidase B; ASP: aspartate aminopeptidase; GLU: glutamyl aminopeptidase; NAP: neutral aminopeptidase; PEP: prolyl endopeptidase; PGAP: pyroglutamyl aminopeptidase; PSA: puromycin-sensitive aminopeptidase; Zn: Zinc.

Table iv. Between-group comparison of qualitative sociodemographic and clinical variables.

Variable	**Conservative treatment group (n = 18)**	**Knee arthroplasty group (n = 21)**	**p-value***
Widowhood, n (%)			0.847
No	15 (83.3)	17 (81.0)	
Yes	3 (16.7)	4 (19.0)	
Diabetes, n (%)			0.493
No	16 (88.9)	17 (81.0)	
Yes	2 (11.1)	4 (19.0)	
Hypertension, n (%)			0.002
No	11 (61.1)	3 (14.3)	
Yes	7 (38.9)	18 (85.7)	
Heart disease, n (%)			0.233
No	16 (88.9)	17 (81.0)	
Yes	1 (5.6)	4 (19.0)	
Dyslipidemia, n (%)			0.907
No	14 (77.8)	16 (76.2)	
Yes	4 (22.2)	5 (23.8)	

Hyperuricemia, n (%)			0.348
No	18 (100)	20 (95.2)	
Yes	0 (0)	1 (4.8)	
Laterality, n (%)			0.882
Right	9 (50)	10 (47.6)	
Left	9 (50)	11 (52.4)	
Contralateral pain, n (%)			0.159
No	6 (33.3)	3 (14.3)	
Yes	12 (66.6)	18 (85.7)	
White blood cell count, n (%)			0.348
Normal	18 (100)	20 (95.2)	
Low	0 (0)	1 (4.8)	
Neutrophil count, n (%)			0.566
Normal	17 (94.4)	N/A	
Low	1 (5.6)	N/A	
Lymphocyte count, n (%)			0.549
Normal	14 (77.8)	18 (85.7)	
High	2 (11.1)	1 (4.8)	
Low	2 (11.1)	2 (9.5)	
Red blood cell count, n (%)			0.642
Normal	17 (94.4)	19 (90.5)	
High	0 (0)	1 (4.8)	
Low	1 (5.6)	1 (4.8)	
Haemoglobin level, n (%)			0.642
Normal	17 (94.4)	19 (90.5)	
High	0 (0)	1 (4.8)	

Low	1 (5.6)	1 (4.8)	
Haematocrit, n (%)			0.642
Normal	17 (94.4)	19 (90.5)	
High	0 (0)	1 (4.8)	
Low	1 (5.6)	1 (4.8)	
Platelet count, n (%)			0.505
Normal	16 (88.9)	19 (90.5)	
High	1 (5.6)	0 (0)	
Low	1 (5.6)	2 (9.5)	
Glucose level (normal)	18 (100)	21 (100)	< 0.001
Creatinine level, n (%)			0.274
Normal	17 (94.4)	21 (100)	
Low	1 (5.6)	0 (0)	
Estimated glomerular filtration rate (normal)	18 (100)	21 (100)	< 0.001
Aspartate aminotransferase activity (normal)	18 (100)	21 (100)	< 0.001
Gamma-glutamyl transferase activity (normal)	18 (100)	21 (100)	< 0.001
Total protein level, n (%)			0.274
Normal	17 (94.4)	21 (100)	
Low	1 (5.6)	0 (0)	
Chloride, n (%)			0.274
Normal	17 (94.4)	21 (100)	
High	1 (5.6)	0 (0)	
Sodium level, n (%)			0.274

Normal	17 (94.4)	21 (100)	
High	1 (5.6)	0 (0)	
Potassium level, n (%)			0.274
Normal	17 (94.4)	21 (100)	
Low	1 (5.6)	0 (0)	
Derived fibrinogen, n (%)			0.008
Normal	17 (94.4)	12 (57.1)	
High	1 (5.6)	9 (42.9)	
Urea, n (%)			0.001
Normal	10 (55.6)	21 (100)	
High	8 (44.4)	0 (0)	
Alanine aminotransferase, n (%)			0.052
Normal	15 (83.3)	21 (100)	
High	3 (16.7)	0 (0)	
Cholesterol, n (%)			< 0.001
Normal	9 (50)	21 (100)	
High	9 (50)	0 (0)	

*Chi-squared test.