Bone & Joint Research

Supplementary Material

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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.

References

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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.