# Analysis of endogenous peptides released from osteoarthritic articular cartilage unravels novel pathogenic markers

Patricia Fernández-Puente[a*], Lucía González-Rodríguez[a*], Valentina Calamia[a], Flor
5 Picchi[a], Lucía Lourido[a], María Camacho-Encina[a], Natividad Oreiro[a], Beatriz Rocha[a],
Rocío Paz[a], Anabel Marina[b], Carlos García[b], Francisco J Blanco[a,c#] and Cristina Ruiz-Romero[a,d#]

[a] Proteomics Group-PBR2-ProteoRed/ISCIII, Grupo de Investigación de Reumatología
10 (GIR). Instituto de Investigación Biomédica de A Coruña (INIBIC), Complexo Hospitalario Universitario de A Coruña (CHUAC). As Xubias, 84, 15006 A Coruña, Spain.

[b] Centro de Biología Molecular Severo Ochoa, CSIC. Nicolás Cabrera, 1, 28049 Madrid, Spain.

15 [c] RIER-RED de Inflamación y Enfermedades Reumáticas, INIBIC-CHUAC, As Xubias 84, 15006 A Coruña, Spain.

[d] CIBER-BBN Instituto de Salud Carlos III, INIBIC-CHUAC, As Xubias 84, 15006 A Coruña, Spain.

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**[*] These authors contributed equally to this work.**

**[#]Address correspondence and reprint requests to:**

Francisco J. Blanco and Cristina Ruiz-Romero

INIBIC-Complejo Hospitalario Universitario A Coruña

25 As Xubias, 84; 15006-A Coruña, SPAIN

Phone: 34-981-176399; Fax: 34-981-176398

E-mail: francisco.blanco.garcia@sergas.es; cristina.ruiz.romero@sergas.es

**Running title:** Neopeptidomic analysis of osteoarthritic cartilage

## Abstract

Osteoarthritis (OA) is characterized by the loss of articular cartilage. In this study, we performed a peptidomic strategy to identify endogenous peptides (neopeptides) that are released from human osteoarthritic tissue, which may serve as disease markers. With this aim, conditioned media of osteoarthritic and healthy articular cartilages obtained from knee and hip were analyzed by shotgun peptidomics. This discovery step led to the identification of 1175 different peptides, corresponding to 101 proteins, as products of the physiological or pathological turnover of cartilage extracellular matrix. Then, a targeted multiple reaction monitoring-mass spectrometry method was developed to quantify the panel of best marker candidates on a larger set of samples (n=62). Statistical analyses were performed to evaluate the significance of the observed differences and the ability of the neopeptides to classify the tissue. Eight of them were differentially abundant in the media from wounded zones of OA cartilage compared to the healthy tissue ($p<0.05$). Three neopeptides belonging to Clusterin and one from Cartilage Oligomeric Matrix Protein showed a disease-dependent decrease specifically in hip OA, whereas two from prolargin (PRELP) and one from Cartilage Intermediate Layer Protein 1 were significantly increased in knee OA. The release of one peptide from PRELP showed the best metrics for tissue classification (AUC=0.834). The present study reveals specific neopeptides that are differentially released from knee or hip OA cartilage compared to healthy tissue. This evidences the intervention of characteristic pathogenic pathways in OA and provides a novel panel of candidates for biomarker development.

The proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011800.



**Key words:** osteoarthritis, cartilage, secretome, peptidomics, neopeptides, biomarkers

## 1. Introduction

Osteoarthritis (OA) is the most common arthritic disease [1]. It is already one of the 10 most disabling pathologies in developed countries, becoming even more prevalent as the population ages and obesity rates rise. This disease is clinically silent in most patients in
60 their early stages; thus the deterioration of cartilage (one of the hallmarks of OA) is already extensive at the time of diagnosis. Therefore, the development of strategies for early diagnosis and accurate monitoring of disease progression is among the major research goals in OA.

OA is characterized by the loss of structural constituents from the extracellular matrix
65 (ECM) of articular cartilage [2]. The ECM maintains and supports chondrocytes within their natural physicochemical micro-environment [3], and the degradation and release of cartilage proteins can vary according to the stage of the disease process. Therefore, the presence of cartilage-characteristic proteins and their degradation products in both proximal or peripheral body fluids, such as synovial fluid, blood or urine has been
70 extensively evaluated to asses their biomarker usefulness. As examples confirming this hypothesis, the increase of the type II collagen fragment CTXII in urine has demonstrated a predictive value for disease progression [4, 5], and elevated levels of cartilage oligomeric matrix protein (COMP) in serum are correlated with the presence of OA and disease severity [6]. Altogether, the ability to detect biomarkers of cartilage degradation
75 and/or inflammation in biological samples, such as cartilage, serum, urine or synovial fluid, may be helpful to improve OA diagnosis, predict its progression and/or develop effective therapeutic strategies. In this area, proteomics has emerged as a powerful tool for biomarker discovery in OA research [7, 8]. The term "peptidomics" was introduced as a branch derived from proteomics to define the quantitative and qualitative analysis of
80 endogenous peptides (also named neopeptides) in biological samples, primarily by liquid

chromatography (LC) or biochip platforms coupled to various forms of mass spectrometry (MS) [9]. A specific neopeptide can be released from a protein due to the existence or progression of a specific disease. Therefore, peptidomics has been appealing for biomarker studies because the knowledge that is generated may present a dynamic view of health status: peptides are created by a complex and fluid interaction of proteases, activators, inhibitors and protein substrates [10]. Due to many difficulties, biomarker discovery of endogenous peptides in complex samples is challenging and require systematic peptide extraction to achieve successful analysis [11].

In this work, we aimed to characterize the profile of neopeptides present in conditioned media (secretomes) from human articular cartilage, and quantitatively compare these profiles between healthy and osteoarthritic tissues. This would allow not only to identify potential neopeptide biomarker candidates, but also to foster the understanding of specific protease pathways that may be relevant for cartilage ECM destruction, which is the hallmark pathogenic process in OA.

## 2. Materials and methods

### 2.1 Cartilage samples

Articular cartilage for the proteomic analysis was obtained either from femoral heads or condyles of patients with OA undergoing hip or knee replacement, and donors with no history of joint disease (N). All tissue samples were provided by the Tissue Bank and the Autopsy Service at Hospital Universitario de A Coruña. The study was approved by the local Ethics Committee (Galicia, Spain). OA patients were diagnosed following the criteria determined by the American College of Rheumatology [12]. Cartilage samples from 4 patients were used for the shotgun analysis (2 OA and 2 N), 21 were employed for MRM development (13 OA and 8 N), and 40 in the validation studies (22 OA and 18 N). The demographic characteristics of the donors are detailed in Table 1.

**Table 1.** Characteristics of the articular cartilage explants employed in this work. Two different explants were obtained per OA tissue (one from the UZ and another from the WZ). Thus, the
110 number of samples analyzed is duplicated for OA cartilage.

| ***Screening*** | | | | | |
|---|---|---|---|---|---|
| | **Dx** | **n** | **% Female** | **Age (mean±SD)** | **Mankin (mean)** |
| | N | 2 | 33.3 | 77.33±4.16 | 1.5 |
| | OA | 2 | 0 | 66±11.31 | 2.5 (UZ) 7.6 (WZ) |
| *Total number of samples* | *6* | | | | |
| ***MRM Development*** | | | | | |
| | **Dx** | **n** | **% Female** | **Age (mean±SD)** | **Mankin (mean)** |
| **Hip** | N | 6 | 33.3 | 77.67±8.16 | 1.5 |
| | OA | 5 | 100 | 82.2±6.02 | 3.6 (UZ) 6.2 (WZ) |
| **Knee** | N | 2 | 0 | 56±2.83 | 1.5 |
| | OA | 8 | 62.5 | 82.5±9.26 | 3.2 (UZ) 9 (WZ) |
| *Total number of samples* | *34* | | | | |
| ***Validation*** | | | | | |
| | **Dx** | **n** | **% Female** | **Age (mean±SD)** | **Mankin (mean)** |
| **Hip** | N | 13 | 38.46 | 76.38±12.24 | 1.7 |
| | OA | 10 | 70 | 77.8±9.02 | 3.3 (UZ) 9.3 (WZ) |
| **Knee** | N | 5 | 40 | 70.6±13.6 | 2.6 |
| | OA | 12 | 41.67 | 73.93±6.97 | 5 (UZ) 9.8 (WZ) |
| *Total number of samples* | *62* | | | | |

UZ: Unwounded zone of OA cartilage; WZ: Wounded zone of OA cartilage.

## 2.2 Histological-histochemical grading of cartilage

A modified Mankin score [13] was employed for the histopathological classification of
115 the severity of lesions on all the cartilage samples employed in this work. Briefly, tissue sections (4 μm) were stained with hematoxylin and eosin to evaluate cellular architecture, and toluidine blue and safranin O/fast green to visualize the matrix proteoglycan content. Three different aspects of the score were determined and summed up: cartilage structure (0-7 points), cellular abnormalities (0-2 points) and matrix staining (0-4 points), leading
120 to a scale that ranges between 0 and 13. The Mankin score 0–2 represents normal

cartilage, 3–5 superficial fibrillation, 6–7 moderate cartilage destruction, 8–10 severe damage of cartilage, and over 10 complete loss of cartilage.

**2.3 Explants Culture**

Tissue explants were obtained from the dissection of N and OA hip and knee cartilages
125 as described previously [14]. Among the OA samples, we differentiated the wounded zones (WZ) from those corresponding to the area adjacent to the lesion, or unwounded zones (UZ). Three 6-mm explants were cut from each zone/condition using a sterile biopsy punch. After extensive washes with PBS, the discs were placed into 96-well plates (one disc/well), containing 200 μL of serum-free DMEM supplemented with 100
130 units/mL penicillin and 100 μg/mL streptomycin to avoid contamination. Plates were incubated overnight at 37 °C, 5% $CO_2$. The collection time line of conditioned media (secretomes) was optimized based on our previous experience [14] and after appraising representative peptidomic profiles along 7 days. Secretomes from day 1 were discarded and replaced with fresh medium. Then, they were collected at days 2 and 5 from each
135 explant culture. Protein concentrations were determined by the Bradford assay, and the samples were frozen at -80$^{O}$ C until processing.

**2.4 Secretome Processing**

Secretomes from the same donor and condition (WZ, UZ or N) collected at days 2 and 5 were mixed together in a total volume of 1200µL. The endogenous peptides were
140 concentrated by ultrafiltration using Amicon Ultra-4 devices (10 kDa MWCO, Merck Millipore, Bedford, MA). The resulting eluted volumes (fractions comprising peptides of < 10 kDa), were dried in a vacuum concentrator. The samples were cleaned twice prior to LC-MS/MS analysis, first by homemade Stage Tips containing six C18 Solid Phase Extraction Disks (Empore), and then using NuTip C18 (Glygen).

145 **2.5 Preparation of samples for MRM quantification**

Heavy stable synthetic isotope-labeled peptides (SIS peptides, crude purity) were purchased from Thermo Scientific, (USA). These peptides incorporated a fully atom labeled $^{13}C$ and $^{15}N$ isotopes at the different amino acids (labeled position; mass shift) as Alanine ($^{13}C_3$,$^{15}N$-Ala; +4 Da) (A), Proline ($^{13}C_5$,$^{15}N$-Pro; +6 Da) (P), Valine ($^{13}C_5$,$^{15}N$-Val; +6 Da) (V), Leucine ($^{13}C_6$,$^{15}N$-Leu; +7 Da) (L), Lysine ($^{13}C_6$,$^{15}N_2$-Lys; +8 Da) (K), or Arginine ($^{13}C_6$,$^{15}N_4$-Arg; +10 Da) (R). Individual stocks of each peptide ranging from 2.25-19.5 μg/μL were made. Then, equal volumes of each peptide were mixed to make the standard mixture solution. Finally, a dilution of 1/5000 of this mixture was made as the stock solution in a concentration range of 1.78-17.6 pmol/μL of each peptide. Aliquots were kept at -20□C. The processed cartilage secretome samples used to develop the targeted MRM method were reconstituted in 7 μL of buffer A (0.1% Formic acid in 5% acetonitrile), whereas the set of samples used for the validation was reconstituted in 7 μL of the peptide stock solution.

**2.5 Discovery phase analysis by shotgun LC/MS-MS**

Six secretome desalted samples (n=6, 2 N, 2 UZ, 2 WZ) were dried, resuspended in 10 μL of 0.1% formic acid (FA) and analyzed by LC-MS/MS in an Easy-nLC II system coupled to LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Scientific). The peptides were concentrated by reverse phase chromatography using a 0.1mm × 20 mm C18 RP precolumn (Proxeon), and then separated using a 0.075mm x 100 mm C18 RP column (Proxeon) operating at 0.3 μL/min. Peptides were eluted using a 90-min gradient from 5 to 40% solvent B (Solvent A: 0,1% FA in water, solvent B: 0,1% FA, 80% acetonitrile in water). ESI ionization was performed using a Nano-bore emitters Stainless Steel ID 30 μm (Proxeon) interface. The Orbitrap resolution was set at 30.000. Peptides were detected in survey scans from 400 to 1600 amu (1 μscan), followed by ten data dependent MS/MS scans (Top 10), using an isolation width of 2 m/z units (in mass-to-

charge ratio units), normalized collision energy of 35%, and dynamic exclusion applied during 30 seconds periods. The mass spectrometry proteomics data obtained from this analysis have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD011800.

### 2.7 Design and development of the Multiple Reaction Monitoring (MRM) method

The target peptides were chosen based on three criteria: 1) peptides with the highest Xscore (>3) using the Proteome Discoverer 1.3 software, 2) peptides present in at least 4 of the 6 secretomes analyzed in the discovery phase and 3) peptides belonging to cartilage ECM proteins. 54 peptide precursors and fragment ion masses were selected on this basis and assayed for MRM analysis. The five most intense transitions for each suitable precursor were selected based on data deposited in the MS/MS library using the Skyline software [15]. Endogenous and SIS peptides were analyzed by LC-MS/MS using a nanoLC system (TEMPO, Eksigent) coupled to a 5500-QTRAP instrument (Sciex). After desalting with a C18 precolumn (5µm, 300A, 100µm*2cm, Acclaim PepMap, Thermo Scientific, USA) and a flow of 3 µL/min during 10 minutes, peptides were separated on C18 nanocolumns (75 µm id, 15 cm, 3µm, Acclaim PepMap 100, Thermo Scientific, USA) at a flow rate of 300 nL/min. The total 70 min gradient for the MRM method starts with 5% buffer B (0.1% Formic acid in 95% acetonitrile) for 3 min, 35% B from 3 until 45 min, 95% B for 1 minute, hold for 10 minutes, and finally, equilibration of the column with 5% B during 15 min. The mass spectrometer was interfaced with nanospray sources equipped with uncoated fused silica emitter tips (20 µm inner diameter, 10 µm tip, NewObjective, Woburn, MA) and was operated in the positive ion mode. Skyline was used to predict and optimize collision energies (CE) and declustering potential (DP) for each peptide [15]. Q1 and Q3 were set to unit/unit resolution (0.7 Da) and the pause

195 between mass ranges was set to 3 ms. MRM analysis was conducted with up to 152 transitions per run (dwell time, 15 ms; cycle time 3s).

For the validation analyses, 23 peptides were selected and included in the final method based on the following criteria: good signal in the MRM method, co-elution of at least 3 transitions and detection using the MIDAS workflow. With this aim, the best MRM 200 transitions for these peptides were pooled in one scheduled-MRM method with a 45-min gradient, using retention times extracted during the assay refinement. Different detection windows were used and the signal was compared with the MRM-IDA acquisition methods. The detection window of 300 gave the best sensitivity with a time window of ±2.5 minutes due to the possible small differences in RT between different days. The 205 signal was defined as the detection of all the transitions from the endogenous peptide exactly co-eluting with all the transitions from the stable isotope–labeled peptide. Table 2 shows the final list of peptides quantified in this work, whereas Supplementary Table 1 enumerates all transitions and settings for their analysis. All data obtained in this targeted proteomics MRM-based analysis have been uploaded to PeptideAtlas and can be accessed 210 via http://www.peptideatlas.org/PASS/PASS01294

**Table 2.** Endogenous peptides quantified by LC-MRM in articular cartilage secretomes. Bold letters indicate the stable isotope-labeled amino acid in each peptide.

| Sequence | Protein Name | UNIPROT Acc No. |
|---|---|---|
| NANTFISPQQ**R**<br>NTFISPQQ**R** | Matrix Gla protein | sp\|P08493\|MGP |
| AEPGIQ**L**KAV<br>AVAEPGIQ**L**K<br>V**L**NQGREIVQT | Cartilage oligomeric matrix protein | sp\|P49747\|COMP |
| DEGDTFPL**R**<br>NLEPRTGF**L**SN<br>STATAAQTD**L**NFIN | Cartilage intermediate layer protein 1 | sp\|O75339\|CILP1 |
| DSNKIETI**P**N<br>SDGVFK**P**DT<br>SSD**L**ENVPH<br>DLENVPHL**R** | Prolargin | sp\|P51888\|PRELP |
| SSGSGPFTDVRA**A**<br>TSSGSGPFTDVRA**A** | Fibronectin | sp\|P02751\|FINC |
| DAVEDLESVG**K**<br>ENAGEDPGLA**R** | Dermcidin | sp\|P81605\|DCD |
| ASHTSDSDVPSGVTE**V**<br>ASHTSDSDVPSGVTEV**V**<br>GEDQYYLRVTT**V** | Clusterin | sp\|P10909\|CLUS |
| SEDGTKASAATTAI**L**<br>AVAQTDLKEP**L**KV | Glia-derived nexin | sp\|P07093\|GDN |
| AGPPGPVG**P**AGGP<br>AGPSGPRGPPG**P**VGP | Collagen alpha-1(II) chain | sp\|P02458\|CO2A1 |

## 2.8 Data analysis

Peptide identification from raw data from the LTQ-Orbitrap was carried out using the SEQUEST algorithm (Proteome Discoverer 1.3, Thermo Scientific). The following constraints were used for the searches: no enzyme and tolerances of 10 ppm for precursor ions and 0.8 Da for MS/MS fragment ions. Search against decoy database (integrated decoy approach) using false discovery rate (FDR) < 0.01. Data from the 5500 QTRAP were analyzed with ProteinPilot 4.0 (Sciex), using the Paragon algorithm as default search

program using no enzyme and modifications criteria. Raw files were imported to Skyline and integration was manually inspected to ensure correct peak detection and accurate integration. After the unambiguously detection of selected peptides in the secretome
225 samples, synthetic standard peptides were used for confirmatory analyses and quantitation. The Protease Specificity Prediction Server (PROSPER) tool [16] was employed to search enzymes putatively involved in the cleavage of the endogenous peptides that had been identified in this work.

### 2.9 Statistical analysis

230 A $p < 0.05$ was considered statistically significant and all statistical tests were two-sided. GraphPad Prism 5.0 (Graphpad, San Diego, CA, USA) was used to compare medians among the three different conditions of patients and controls (WZ-UZ-Control), and a Kruskal–Wallis test's multiple comparison was used. Mann–Whitney U tests were performed to evaluate the significance of discrimination between the disease classes and
235 the control cohort. Receiver operator characteristic (ROC) analysis was performed to quantify the overall ability of a peptide to classify the tissue as OA or healthy. The ROC curves were smoothed, compared and threshold computed using the R package pROC 2018 [17].

240 ## 3. Results

### 3.1 Isolation and identification of endogenous peptides released from articular cartilage

The experimental workflow followed for the peptidomic profiling of articular cartilage degradation in OA is summarized in Figure 1. The studies were performed on conditioned
245 media from human articular cartilage explants, whose characteristics were assessed by Mankin scoring (Table 1). In the OA tissue, explants were obtained both from the macroscopically normal zone (unwounded zone, or UZ, with an average Mankin score of

3.52±0.92) and the lesion (wounded zone, or WZ, Mankin score of 8.38±1.47), to evaluate possible differences. Finally, the healthy cartilages analyzed in this work had a

250 Mankin score of 1.76±0.48.

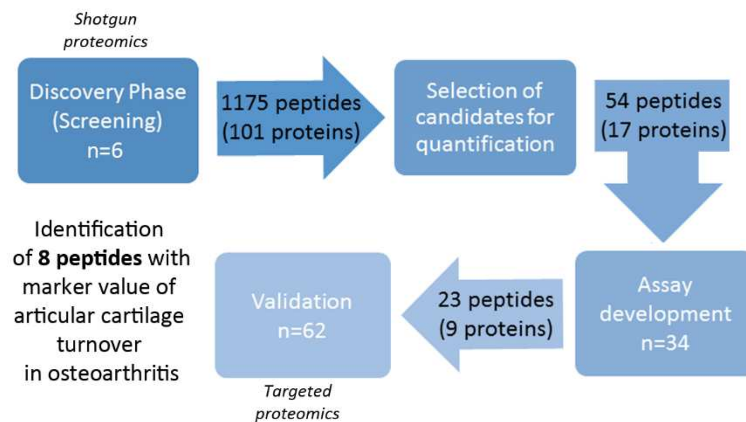


**Figure 1. Schematic workflow of the study.** *(1.5-column figure)*

To isolate the endogenous peptides present in the conditioned media, we explored

255 different combinations of ultrafiltration and solid phase extraction (SPE), which led to the final protocol described in the Methods section 2.2. Days 2 and 5 of culture were selected as the best points for the peptidomic analysis, showing the highest number of unique peptides and the lowest serum contamination in the conditioned media. The screening step led to the identification of 1175 different peptides corresponding to 101

260 unique proteins that were released from hip or knee articular cartilage to the conditioned media. The complete list of neopeptides that were identified, and their correspondent parent proteins, is shown in Supplementary Table 2. A higher number of peptides in OA compared to normal tissue was found, although the result was not statistically significant (p=0,17). The parent proteins identified with the highest score and highest number of

265 peptides were ECM structural constituents, such as COMP, PRELP or FINC. Several of

them were specifically characteristic of the articular cartilage ECM, such as COMP, CILP1 or PRG4.

### 3.2 Development of targeted methods for the quantitative analysis of endogenous peptides released from articular cartilage

270 The peptides that show the highest identification score (>3) in the screening phase, where identified in the majority of samples and belong to proteins expressed in articular cartilage were selected to develop a targeted analysis method based on MRM-mass spectrometry. The criteria for the selection of peptides in this phase is fully described in section 2.7. 54 endogenous peptides (belonging to 17 proteins) were explored for the development of the 275 method, which was carried out using secretome samples from eleven hip and 10 knee cartilages (Table 1). Then, the final MRM method was designed with the aid of SIS peptides for the detection and quantification of the 23 endogenous peptides showing the best performance (section 2.7), whose 9 parent proteins are expressed in human articular cartilage. These proteins are Matrix Gla Protein **(MGP),** Cartilage Oligomeric Matrix 280 Protein (**COMP),** Cartilage Intermediate Layer Protein 1 **(CILP1),** Prolargin **(PRELP**), Dermcidin (**DCD**), Fibronectin **(FINC),** clusterin **(CLUS),** Glia Derived Nexin **(GDN)** and Collagen Alpha-1 (II) Chain **(CO2A1).** The list of endogenous peptides included in this targeted analysis is detailed in Table 2.

The area under the curve for the endogenous peptides was plotted for each peptide in 285 samples from the UZ and WZ of OA and healthy donors. Certain peptides belonging to CILP1 (DEGDTFPLR) and PRELP (DSNKIETIPN, DLENVPHLR) were found to be mostly increased in the WZ of OA cartilages when compared to UZ and healthy donors. To confirm these results and normalise the data, we developed a scheduled MRM method and incorporated peptides labelled with heavy stable isotopes as internal standards for the 290 quantification.

### 3.3 Quantification of endogenous peptides in cartilage secretomes

The validation study was carried out using the scheduled MRM method and stable isotope labelled peptide standards on 62 secretome samples obtained from hip (n=33) and knee (n=29) cartilage. All the quantification data (expressed as peak area ratios of light/heavy peptides) from the different peptides in the secretome of different zones of OA cartilage (UZ and WZ) and healthy donors in the different joints are showed in **Supplementary Table 3**. After statistical analysis of the results, four endogenous peptides were found to be differentially released from OA cartilage compared to healthy tissue with a significant p-value. Among these, two peptides from PRELP (DSNKIETIPN and DLENVPHLR) and one from MGP (NTFISPQQR) were differentially released independently of the OA cartilage zones (Figure 2A). Furthermore, the same tendency was found in the OA WZ compared to control donors for these peptides and the peptide DEGDTFPLR from CILP1. All of them were found increased in the OA WZ vs healthy cartilage (Figure 2B). Finally, the peptide DSNKIETIPN (PRELP) was differentially released in the UZ compared to normal cartilage, and also between the two OA cartilage zones.

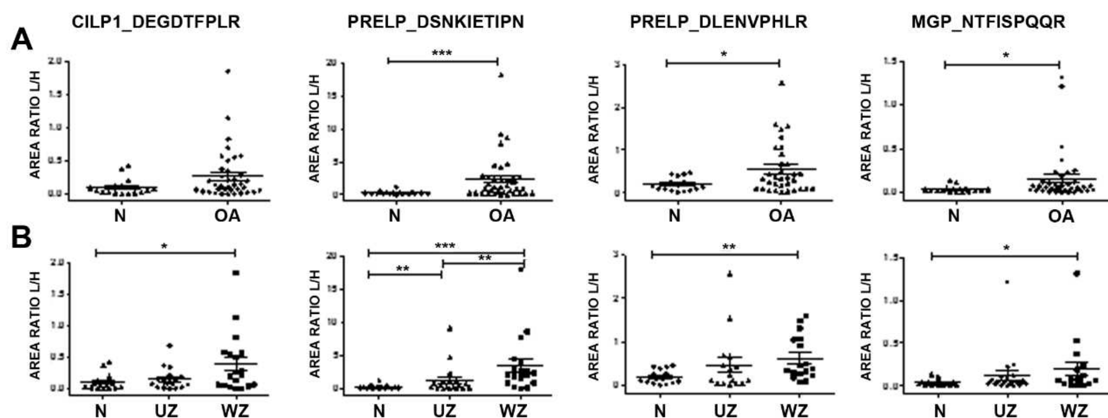


**Figure 2. Differential endogenous peptides released from osteoarthritic articular cartilage.** Scattering plots representing the different abundance of each peptide in the cartilage secretomes. ***A)*** Comparison between OA (n=44) and normal tissue (n=18). ***B)*** OA samples were classified into those from the unwounded zone of the tissue (UZ, n=22) and from the wounded (WZ, n=22). The results are expressed as area ratios (light/heavy,

L/H). Data were analyzed using Mann-Whitney test and plotted as means ± SEM for each condition. *p*<0.05, p**<0.005 p***<0.0005. (Two-column figure).*

315 **3.4 Differential release of endogenous peptides from knee and hip articular cartilages**

The targeted peptide quantification evidenced a differential release of certain neopeptides depending on the joint that was studied ($p<0.05$), which are shown in the Supplementary Table 4 and Supplementary Figure 2. In all cases, the release was higher from the knee

320 tissue. Comparison of the conditioned media of all knee (n=29) and hip (n=33) cartilage samples demonstrated the increased release from knee of endogenous peptides corresponding to the MGP (NANTFISPQQR and NTFISPQQR), COMP (AEPGIQLKAV) and PRELP (DSNKIETIPN), with fold changes ranging from 2.29 to 5.11 (Supplementary Figure 2A). In OA cartilage, the peptide AEPGIQLKAV (COMP)

325 has a remarkable 8-fold change ratio higher in knee vs hip, while DSNKIETIPN from PRELP and GEDQYYLRVTTV and ASHTSDSDVPSGVTEV from CLUS also showed significant differences (Supplementary Figure 2B). Considering only the healthy tissues (knee n=5 and hip n=13), one peptide was increased in the knee samples (NTFISPQQR, from MGP) with a fold ratio of 3.54 (Supplementary Figure 2C).

330 Given these joint-characteristic profiles, the differences in the release of peptides were examined independently in hip and knee samples. In hip, two peptides from CLUS were increased in the conditioned media of healthy cartilage compared to OA tissue: ASHTSDSDVPSGVTEVV and GEDQYYLRVTTV (Figure 3A). When the different zones in the diseased cartilage were taken together (Figure 3B), these two peptides

335 showed a significant lower release from the wounded zone of the tissue (WZ). The same happens with another peptide from CLUS, ASHTSDSDVPSGVTEV, and the peptide AEPGIQLKAV from COMP.

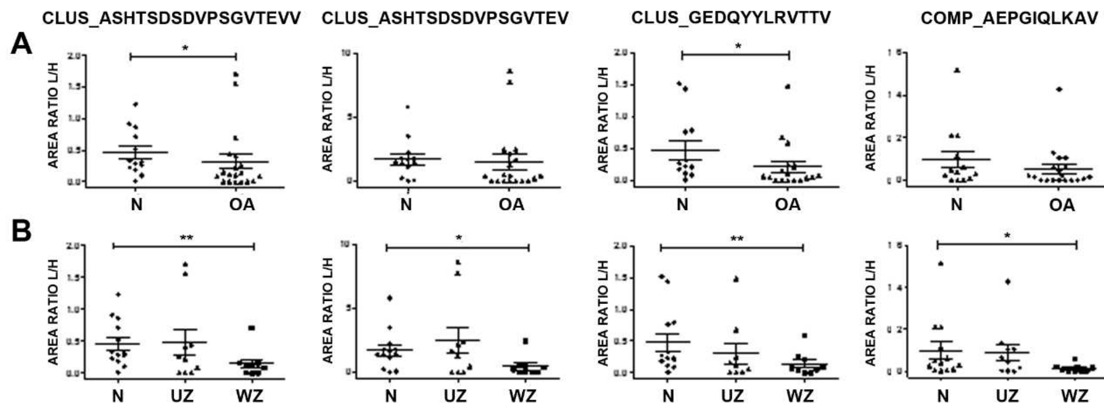


**Figure 3. Differential endogenous peptides released from hip articular cartilage.** Scattering plots showing the abundance of each peptide in hip cartilage secretomes. ***A)*** Comparison between OA (n=20) and normal tissue (n=13). ***B)*** OA samples were classified into those from unwounded zones (UZ, n=10) or wounded zones (WZ, n=10). The results are expressed as area ratios (light/heavy, L/H). Data were analyzed using Mann-Whitney test and plotted as means ± SEM for each condition. *P*<0.05* and *p**<0.005. (two-column figure).*

In knee samples, two endogenous peptides from PRELP were significantly increased in the conditioned media of OA tissue: DSNKIETIPN and DLENVPHLR (Figure 4A). Considering the two zones of OA tissue separately, these two peptides showed an enhanced release specifically from the WZ (Figure 4B). Interestingly, the peptide DSNKIETIPN exhibited the most significant differences, which were also detectable in samples from the UZ of OA tissue. The peptide DEGDTFPLR from CILP1 displayed a similar tendency.

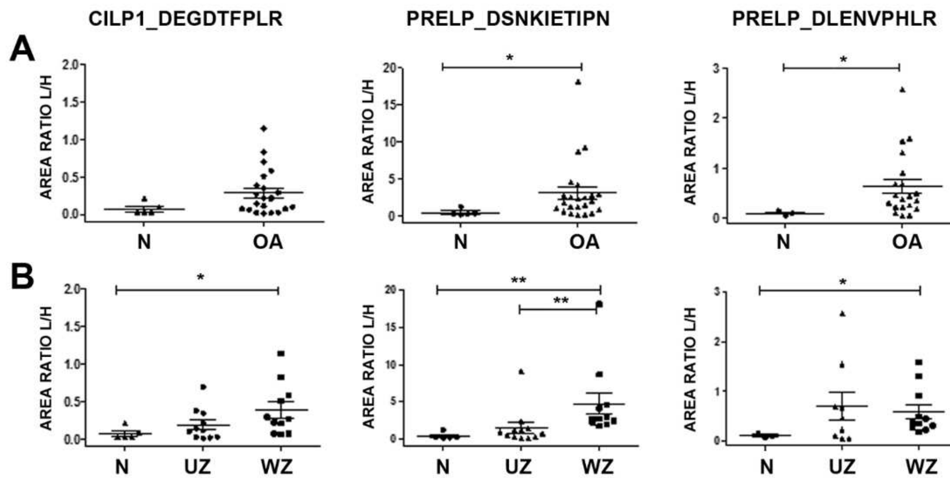


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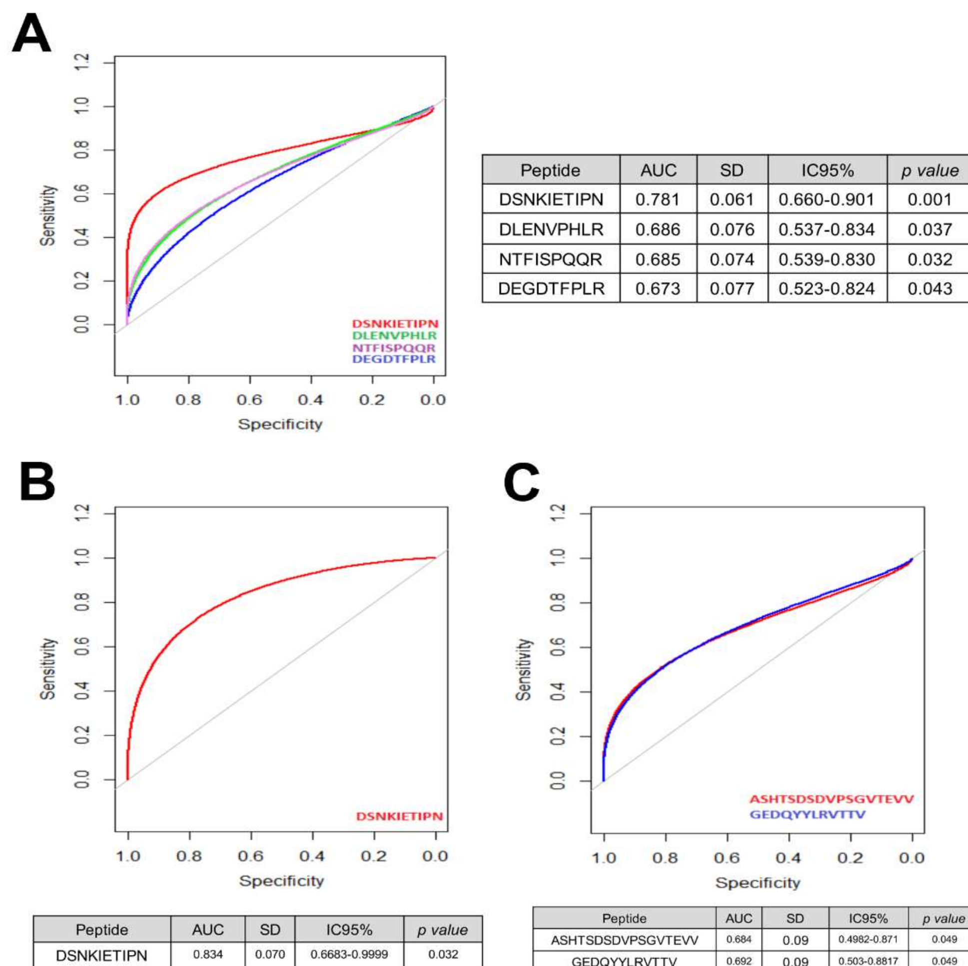
**Figure 4. Differential endogenous peptides released from knee articular cartilage.** Scattering plots showing the abundance of each peptide in knee cartilage secretomes. ***A)*** Comparison between OA (n=24) and normal tissue (n=5). ***B)*** OA samples were classified into those from unwounded zones (UZ, n=12) or wounded zones (WZ, n=12). The results

360 are expressed as area ratios (light/heavy, L/H). Data were analyzed using Mann-Whitney test and plotted as means ± SEM for each condition. *P*<0.05* and *p**<0.005. (Two-column figure).*

### 3.5 Value of the identified peptides as biomarkers of articular cartilage degradation

365 To evaluate the putative biomarker value of the endogenous peptides that have been identified, an analysis by receiver operator characteristic (ROC) curves was performed. As illustrated in Figure 5A, the peptide DSNKIETIPN showed an area under the curve (AUC) of 0.781 [IC 95%: (0.660-0.901), *p=0.001*], being the best candidate to discriminate healthy vs OA tissue independently of the target joint. Considering only the

370 knee, the AUC of this peptide increased up to 0.834 (Figure 5B). On the other hand, two peptides from CLUS (ASHTSDSDVPSGVTEVV and GEDQYYLRVTTV) displayed significant AUCs when analyzing the hip tissue exclusively (Figure 5C).




| Peptide | AUC | SD | IC95% | *p value* |
|---|---|---|---|---|
| DSNKIETIPN | 0.781 | 0.061 | 0.660-0.901 | 0.001 |
| DLENVPHLR | 0.686 | 0.076 | 0.537-0.834 | 0.037 |
| NTFISPQQR | 0.685 | 0.074 | 0.539-0.830 | 0.032 |
| DEGDTFPLR | 0.673 | 0.077 | 0.523-0.824 | 0.043 |

| Peptide | AUC | SD | IC95% | *p value* |
|---|---|---|---|---|
| DSNKIETIPN | 0.834 | 0.070 | 0.6683-0.9999 | 0.032 |

| Peptide | AUC | SD | IC95% | *p value* |
|---|---|---|---|---|
| ASHTSDSDVPSGVTEVV | 0.684 | 0.09 | 0.4982-0.871 | 0.049 |
| GEDQYYLRVTTV | 0.692 | 0.09 | 0.503-0.8817 | 0.049 |

**Figure 5. Receiver operator characteristic (ROC) curves of the biomarker peptides**
375 **identified in this work.** ***A)*** The release of four peptides discriminates OA vs healthy articular cartilage with significant p value (*p<0.05*), ***B)*** The peptide DSNKIETIPN from prolargin differentiates knee OA from healthy tissue, and ***C)*** Two peptides from clusterin discriminate hip OA from healthy tissue. *(1.5 or two-column figure)*

380 Finally, we also performed this analysis by splitting the OA tissue in zones (Supplementary Figure 3). In this case, again the best results were obtained for the peptide DSNKIETIPN in knee, showing a good biomarker value (AUC=0.783) in OA but macroscopically normal cartilage. Comparing healthy knee tissue with the damaged zones of knee OA, this AUC increased up to 0.891. In hip, the performance of
385 GEDQYYLRVTTV was worse, but still significant (AUC normal vs WZOA=0.761).

## 4. Discussion

Peptides are constantly generated *in vivo* either by active synthesis and proteolytic processing of larger precursor proteins, often yielding protein fragments that mediate a

390 variety of physiological or pathological functions. Given that abnormal proteolysis is a hallmark of various diseases, many studies have now turned to the focus on the peptidome [18] as a source of biomarkers. The investigation of peptides in a system-wide manner could facilitate the identification of potential biomarkers, the identification of protease-substrate relationships and the profiling of pathological degradation processes.

395 Considering that the process of articular cartilage ECM degradation is a hallmark for OA, we aimed to perform the first neo-peptidomic profiling of this pathological situation without the use of any *in vitro* stimulus. Previous studies on endogenous peptides in OA have all employed models using either well known OA-related proteinases [19] or inducers of cartilage degradation such as mechanical damage or proinflammatory

400 cytokines [20, 21]. Our two-step peptidomic analysis started with a first discovery phase on conditioned media from cartilage explants, identifying 1175 different peptides corresponding to 101 unique proteins. This is, to our knowledge, the deepest characterization of cartilage neopeptides. Interestingly, in general we detected more peptides and with higher signals in secretomes from knee samples than from hip

405 (Supplementary Figures 1 and 2), which depicts the differences between these two joints and also indicates a higher turnover in the knee that could not been revealed in previous proteomic analyses performed directly on the tissue [3, 22]. Data mining showed that most of the identified proteins were cartilage ECM proteins or proteins with well-established matrix functions, such as collagens and proteoglycans. Although some of the

410 parental proteins of many of these neopeptides have been reported for the first time in cartilage-derived samples (such as salivary acidic proline-rich phosphoprotein 1/2) most

of them had been previously associated with OA: type II collagen, proteoglycan 4, fibronectin or cartilage oligomeric matrix protein. Notably, our list of neopeptides includes the detection of previously known OA biomarkers, such as CTXII (peptides
415 GPDPLQYMRA, DPLQYMRA and SAFAGLGPRE, from the C-telopeptide fragment of type II collagen). Altogether, this further evidences the usefulness of secretome analysis as a source of cartilage-characteristic biomarkers [14, 21, 23].

Next, in a second validation step, we selected a panel of these endogenous peptides and developed a targeted method for their quantification in secretomes. Then, this method
420 was applied for an exhaustive analysis on 62 secretomes from articular cartilage, which allowed to obtain statistically significant results of the differences. Eight endogenous peptides were found to be differentially released from OA compared to healthy tissue. The metrics obtained in this study are summarized in Table 3.

**Table 3.** Endogenous peptides identified as putative OA biomarkers in human articular
425 cartilage. Numbers show the p-value calculated in each case.

| ***Peptide markers of OA*** | | | | | |
|---|---|---|---|---|---|
| **Peptide** | **Protein** | **N vs OA** | **N vs UZ** | **N vs WZ** | **UZ vs WZ** |
| DEGDTFPLR | CILP1 | | | 0.0233 | |
| DSNKIETIPN | PRELP | 0.0008 | 0.049 | 0.0001 | 0.0094 |
| DLENVPHLR | PRELP | 0.0376 | | 0.0047 | |
| NTFISPQQR | MGP | 0.0327 | | 0.0202 | |
| ***Peptide markers of Knee OA*** | | | | | |
| **Peptide** | **Protein** | **N vs OA** | **N vs UZ** | **N vs WZ** | **UZ vs WZ** |
| DEGDTFPLR | CILP1 | | | 0.0235 | |
| DSNKIETIPN | PRELP | 0.0226 | | 0.0022 | 0.0012 |
| DLENVPHLR | PRELP | 0.04 | | 0.0127 | |
| ***Peptide markers of Hip OA*** | | | | | |
| **Peptide** | **Protein** | **N vs OA** | **N vs UZ** | **N vs WZ** | **UZ vs WZ** |
| ASHTSDSDVPSGVTEVV | CLUS | 0.0383 | | 0.0076 | |
| ASHTSDSDVPSGVTEV | CLUS | | | 0.0162 | |

| | | | | | |
|---|---|---|---|---|---|
| GEDQYYLRVTTV | CLUS | 0.0237 | | 0.0277 | |
| AEPGIQLKAV | COMP | | | 0.0194 | |

N: healthy tissue; UZ: unwounded zone of OA cartilage; WZ: wounded zone of OA cartilage.

Remarkably, we found decreased amounts of three neopeptides from CLUS and one from COMP in hip OA samples (Figure 3). This is in accordance with the disease-related
430 significant decrease of these two proteins in articular cartilage that has been described recently [3]. CLUS, also known as Apolipoprotein J, is a secreted protein that regulates apoptosis and inflammation. A few studies have observed elevated CLUS in cartilage and synovial fluid in early OA [24, 25]. Furthermore, increased CLUS levels in SF and serum showed statistically significant associations with joint space narrowing after adjustment
435 for age and sex [26]. However, IL-1α-stimulated cartilage explants have shown to produce decreased levels of CLU compared to untreated cartilage [3, 27]. An analogous discrepancy happens with COMP: although this protein is decreased in knee and hip OA articular cartilage (p=0.007) [3], it is well known that its elevated levels in serum are associated with OA severity [6, 28]. An explanation for this might be that these higher
440 levels of CLUS and COMP in OA SF and plasma could represent the activation of a compensatory, but ultimately ineffective, protective pathway.

In knee, we observed the disease-related increase of one neopeptide from CILP1 and two from PRELP. This increase was significant from the WZ zones of the tissue in all cases, but in the case of the peptide DSNKIETIPN from PRELP it was also detectable in the
445 macroscopically normal zone. Furthermore, the ROC analysis showed the best results for this peptide (Figure 5), with and AUC of 0.834 for the classification of the tissue as OA or healthy, with a high specificity (0.821) for OA. Interestingly, DSNKIETIPN was identified in a previous study as the relatively most abundant peptide from an *in vitro* digestion with ADAMTS4 [19]. The contribution of the aggrecanases ADAMTS4 and
450 ADAMTS5 to cartilage destruction in OA has been widely established [29, 30], although

it has not been resolved completely. PRELP is a small leucine-rich proteoglycan highly abundant in cartilage [31, 32] that binds the basement membrane heparan sulfate proteoglycan perlecan through its N-terminal region, and collagens (type I and II) through its 12 leucine-rich repeat (LRR) domains. An increase in DSNKIETIPN, localized in the
455 7$^{th}$ LRR domain of the protein, denotes PRELP breakage with a loss of half its LRR domains for collagen binding. Thus, the statistically significant increase of this neopeptide in OA cartilage that we demonstrate in the present work depicts the role of PRELP as mediator of ADAMTS4 catabolic effects in articular cartilage.

## 5. Conclusions

460 We have performed a peptidomic analysis for the discovery and validation of novel neopeptides associated with the degradation of articular cartilage ECM in osteoarthritis. This work has enabled not only to obtain an exhaustive neopeptidome profile of healthy and diseased tissues, but also the identification and validation of a panel of eight differential endogenous peptides that are released in the pathogenic process. The peptide
465 DSNKIETIPN, from Prolargin, showed the best metrics as a biomarker of OA cartilage, proving to be the most promising candidate for the development of assays aimed at its detection and quantification in biological fluids.

## Author contributions

470 Conception and design: CRR, FJB, PFP

Acqusition, analysis and interpretation of data: PFP, LGR, VC, FP, LL, MCE, NO, BR, RP, AM, CG

Drafting the article: CRR, PFP, LGR, VC

Final approval of the article: All authors

475 **Competing interests statement**

Authors declare no competing interests.

**Role of the funding source**

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

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**Supplementary Data**

**Supplementary Figure 1. Multiple Reaction Monitoring (MRM) - mass spectromety quantification of endogenous peptides. A)** Representative chromatograms of the endogenous peptide PRELP_DSNKIETIPN in a pool of secretome samples (n=3) from hip (upper row) and knee (lower row). The endogenous peptides (light) are represented in red, whereas the heavy peptide standards (SIS) are displayed in blue. The amount of SIS spiked into each sample was kept constant. B) Chart plot representing the peak area ratio normalized to the heavy peptide standard for each type of sample. WZH, wounded zone from hip OA; UZH, unwounded zone from hip OA; NH, healthy hip; WZK, wounded zone from knee OA; UZK, unwounded zone from knee OA; NK, healthy knee.

**Supplementary Figure 2. Differential release of endogenous peptides from hip and knee articular cartilages.** Scattering plots showing the distribution of the Area light/heavy (L/H) ratios of representative endogenous peptides. The data were analyzed using Mann-Whitney test and plotted as means ± SEM for each condition. A) Knee (n=29) vs hip (n=33), B) OA knee (n=23, 12 WZ and 13 UZ) vs OA hip (n=20, 10 WZ and 10 UZ), and C) Healthy knee (n=5) vs healthy hip (n=13). $p^* < 0.05$, $p^{**}<0.005$ $p^{***}<0.0005$.

**Supplementary Figure 3. Receiver operator characteristic (ROC) curves of the best biomarker peptides differentiating disease and zone in knee (A) or hip (B) articular**

510 **cartilage.** The inset tables show the metrics obtained for each peptide in normal (healthy), unwounded (UW) or wounded (W) zones of OA tissue from each joint.

**Supplementary Table 1. Targeted proteomics design.** MRM mass spectrometry transitions analyzed in this work, and settings for their analysis.

**Supplementary Table 2. Full results from the discovery phase. A)** Endogenous
515 peptides identified in the secretomes of human articular cartilage. **B)** Unique proteins corresponding to the endogenous peptides identified in this work.

**Supplementary Table 3. Quantification data obtained for the panel of peptides analyzed by MRM mass spectrometry.** Results are expressed in peak area ratios of abundance (light/heavy peptides), with a confidence level of p<0.05*.

520 **Supplementary Table 4. Fold changes of endogenous peptides differentially released from knee and hip articular cartilage with a significant p-value (<0.05).** Data obtained using the MS stats tool from Skyline software.

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