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Monomeric CRP regulates inflammatory responses in human intervertebral disc cells

**C. Ruiz-Fernández,
D. Ait Eldjoudi,
M. González-Rodríguez,
A. Cordero Barreal,
Y. Farrag,
L. García-Caballero,
F. Lago,
A. Mobasheri,
D. Sakai,
J. Pino,
O. Gualillo**

From University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain

Aims

CRP is an acute-phase protein that is used as a biomarker to follow severity and progression in infectious and inflammatory diseases. Its pathophysiological mechanisms of action are still poorly defined. CRP in its pentameric form exhibits weak anti-inflammatory activity. The monomeric isoform (mCRP) exerts potent proinflammatory properties in chondrocytes, endothelial cells, and leucocytes. No data exist regarding mCRP effects in human intervertebral disc (IVD) cells. This work aimed to verify the pathophysiological relevance of mCRP in the aetiology and/or progression of IVD degeneration.

Methods

We investigated the effects of mCRP and the signalling pathways that are involved in cultured human primary annulus fibrosus (AF) cells and in the human nucleus pulposus (NP) immortalized cell line HNPSV-1. We determined messenger RNA (mRNA) and protein levels of relevant factors involved in inflammatory responses, by quantitative real-time polymerase chain reaction (RT-qPCR) and western blot. We also studied the presence of mCRP in human AF and NP tissues by immunohistochemistry.

Results

We demonstrated that mCRP increases nitric oxide synthase 2 (NOS2), cyclooxygenase 2 (COX2), matrix metalloproteinase 13 (MMP13), vascular cell adhesion molecule 1 (VCAM1), interleukin (IL)-6, IL-8, and Lipocalin 2 (LCN2) expression in human AF and NP cells. We also showed that nuclear factor- κ B (NF- κ B), extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphoinositide 3-kinase (PI3K) are at play in the intracellular signalling of mCRP. Finally, we demonstrated the presence of mCRP in human AF and NP tissues.

Conclusion

Our results indicate, for the first time, that mCRP can be localized in IVD tissues, where it triggers a proinflammatory and catabolic state in degenerative and healthy IVD cells, and that NF- κ B signalling may be implicated in the mediation of this mCRP-induced state.

Cite this article: *Bone Joint Res* 2023;12(3):189–198.

Keywords: Monomeric CRP, Inflammation, Intervertebral disc, Annulus fibrosus, Nucleus pulposus, Low back pain

Article focus

■ This study aimed to verify the pathophysiological relevance and mechanism of action of monomeric CRP (mCRP) in healthy and degenerative intervertebral disc (IVD) cells.

Key messages

■ mCRP can be present in IVD tissues and induces the expression of multiple

proinflammatory and catabolic factors in human annulus fibrosus (AF) and nucleus pulposus (NP) cells.

■ The effect of mCRP is persistent and sustained, regardless of the proinflammatory environment, as it was similar in healthy and degenerative human primary AF cells.

■ mCRP effects in healthy and degenerative AF cells are mediated by phosphoinositide

Correspondence should be sent to Oreste Gualillo; email: oreste.gualillo@sergas.es

doi: 10.1302/2046-3758.123.BJR-2022-0223.R1

Bone Joint Res 2023;12(3):189–198.

3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor- κ B (NF- κ B) p65 signalling pathways.

Strengths and limitations

- This is the first experimental article that provides evidence for the functional activity of mCRP in healthy and degenerative human AF and NP disc cells.
- This is the first article demonstrating the localization of mCRP in intravertebral disc cells of the AF and NP.
- Further studies are needed to confirm the role of mCRP in IVD degeneration. A larger number of subjects studied would give more strength to our results; the limited access to primary human disc cells is a limitation of this study.

Introduction

Low back pain (LBP) is the main cause of disability worldwide, affecting around 700 million people.¹ It has been estimated that 70% to 85% of the global population has LBP at some time in their lives, and it can limit the activity of people under 45 years of age, generating a huge socioeconomic impact.² The aetiology of LBP is complex, multifactorial, and still unclear. However, histological and MRI data associate chronic LBP with lumbar intervertebral disc (IVD) degeneration.³ IVDs are embedded between the vertebrae and provide flexibility to the spine. They consist of three anatomical parts: an inner cell-sparse gelatinous nucleus pulposus (NP), an outer fibrous region, the annulus fibrosus (AF), and, limiting above and below the AF, the hyaline cartilaginous end plates (CEPs).⁴ In a healthy adult IVD, homeostasis between extracellular matrix (ECM) synthesis and degradation is balanced by growth factors and cytokines. An imbalance towards catabolic processes contributes to structural degeneration of the IVD, likely contributing to the development of LBP.^{3,5}

IVD degeneration leads to multiple anatomical, mechanical, and biochemical disc changes. These alterations decrease mechanical stability and shock absorber functions, contributing to osteophyte formation, annular fissures, and decreased motion of spinal segments.³ Genetic risk has an important influence on IVD degeneration, however mechanical trauma, injuries, smoking, obesity, and ageing also play a role.⁶ In response to inflammation and degeneration, vascular and nerve ingrowth into the avascular IVD occur from the outer layers of the AF, extending into the NP.^{7,8}

IVD degeneration and the degeneration of joint cartilage as seen in osteoarthritis (OA) show marked similarities, although they are barely discussed simultaneously. Striking similarities are seen on plain radiographs: the loss of disc height or joint space, sclerosis of the subchondral bone, and the development of osteophytes. Furthermore, in both IVD and joint cartilage, the ECM is composed of similar constituents (although in another ratio), and similar ECM-degrading machinery is present in the process of degeneration. There are also differences

such as the type of forces applied to the ECM, and the absence of synovial fluid in IVDs.⁹⁻¹²

CRP is an acute-phase inflammatory protein whose plasma levels sharply increase in response to injury, infection, and inflammation. It is a member of the pentraxin family, and circulates prevalently in blood in its homopentameric form.¹³ CRP plasma levels increase from around 1 μ g/ml to over 500 μ g/ml within 24 to 72 hours upon severe tissue damage.¹⁴ Thus CRP levels are a well-established biomarker for monitoring the inflammatory status in infections and other multiple diseases, including IVD herniation¹⁵ and rheumatic diseases such as OA.¹⁶

The pentameric protein is characterized by a discoid configuration of five identical non-covalently bound subunits of about 23 kDa each. It is synthesized primarily in liver hepatocytes but has also been reported to be produced in other cell types such as smooth muscle cells,¹⁷ macrophages,¹⁸ endothelial cells,¹⁹ lymphocytes, and adipocytes.²⁰ The monomeric CRP (mCRP) differs from the pentameric CRP (pCRP) in its different antigenic, biological, and electrophoretic mobility,²¹ and in its expression of different neoepitopes.²² These two distinct forms of CRP showed distinct biological functions in the inflammatory process. It has been proved that pCRP suppresses the adherence of platelets to neutrophils, whereas mCRP enhances these interactions.²² This difference in function can be explained by the two isoforms binding to differing types of Fc γ -receptors involved in the signalling process. The mCRP isoform uses the low-affinity immune complex binding immunoglobulin G (IgG) receptor called Fc γ RIIIb (CD16b) on neutrophils and Fc γ RIIa (CD16a) on monocytes, while pCRP binds to the low-affinity IgG receptor Fc γ RIIa (CD32).²³ Evidence shows that pCRP tends to exhibit weak anti-inflammatory activities. In contrast, mCRP has marked proinflammatory properties both *in vitro* and *in vivo* by promoting monocyte chemotaxis and the recruitment of circulating leucocytes to areas of inflammation via Fc γ -RI and Fc γ -RIIa signalling.^{24,25} However, the pathophysiological role of CRP as a regulator of inflammatory processes remains obscure.

Some of the published IVD disease literature assesses CRP as a reference biomarker of an ongoing inflammatory process.^{15,26,27} Ruiz-Fernández et al²⁴ recently investigated the inflammatory effect of mCRP in chondrocytes, proving that mCRP triggers a sustained proinflammatory and catabolic state in healthy and OA cartilage. However, the role of mCRP in disc pathophysiology has not yet been clarified. In this current study, we evaluated the functional role of mCRP as a modulator of the inflammatory response in NP and AF human cells, assessing some proinflammatory and catabolic factors and the pathways involved in the signal transduction upon mCRP stimulation.

Methods

Reagents. Fetal bovine serum (FBS), lipopolysaccharide (LPS), and human recombinant interleukin (IL)-1 β

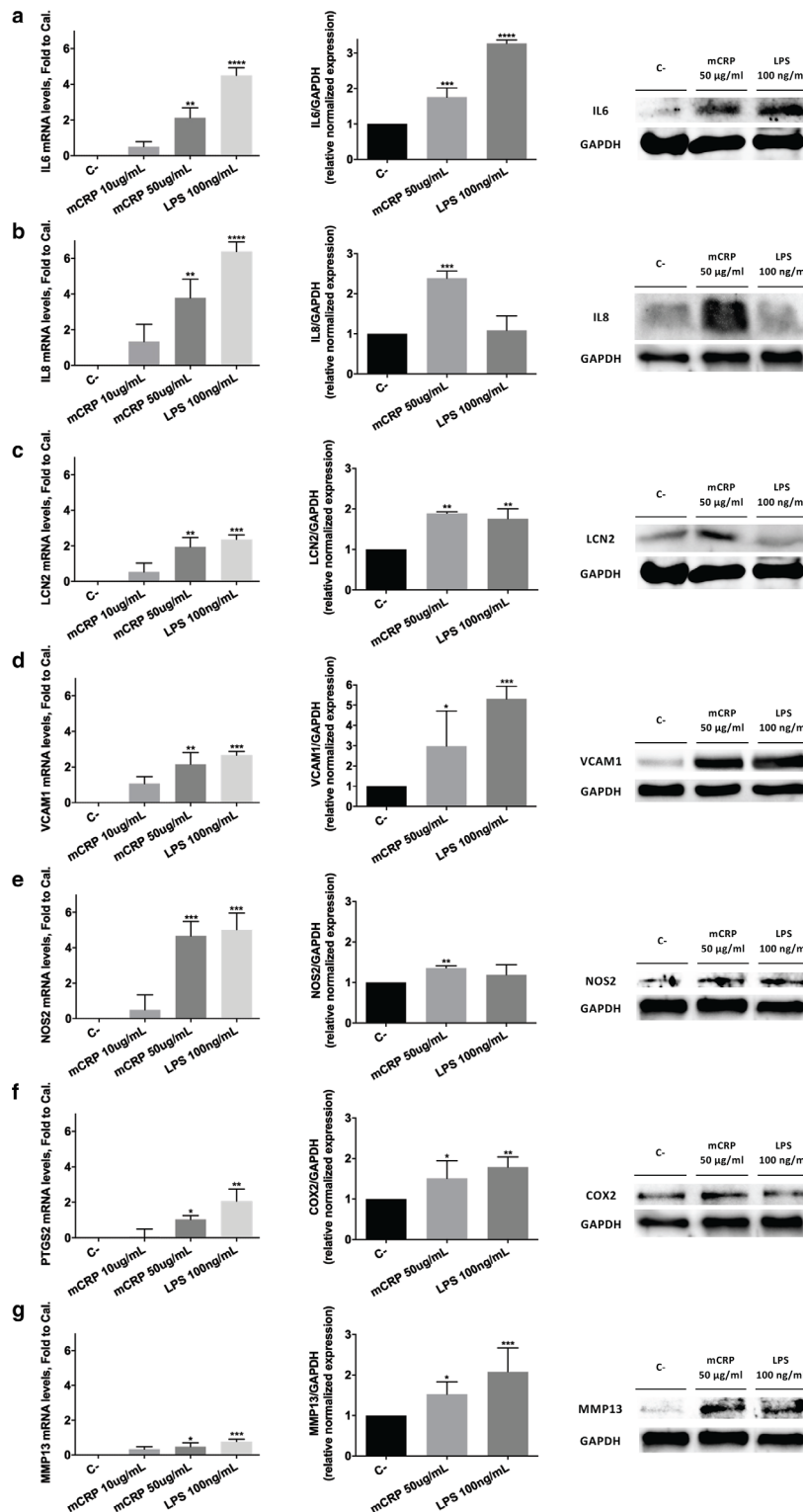


Fig. 1

Monomeric CRP (mCRP) proinflammatory effect on human primary degenerative intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells derived from degenerative IVD in culture were challenged with 10 or 50 $\mu\text{g/ml}$ mCRP, or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 24 hours, after four hours of serum starvation. a) to g) Left panels. Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). a) to g) Middle and right panels. Protein expression was measured by western blot. Data from densitometric analyses normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown as relative-to-control (C-) values. Data are expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Cal., calibrator (refers to control (C-) condition); COX2, cyclooxygenase 2; IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

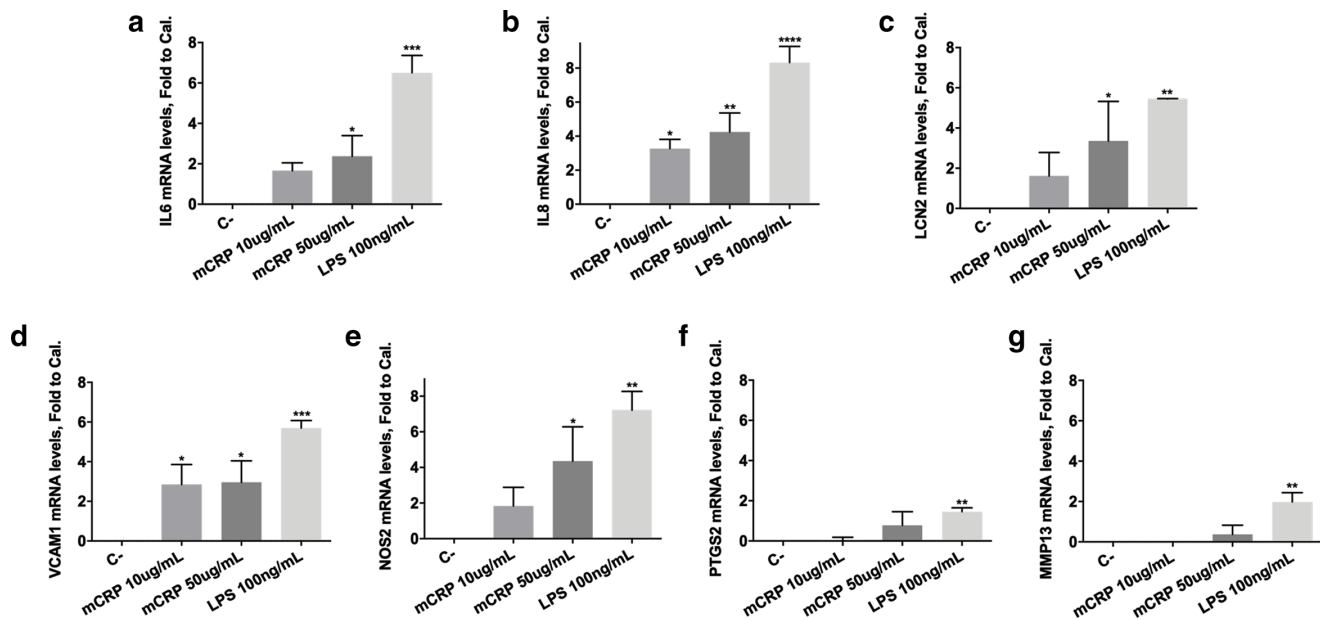


Fig. 2

Monomeric CRP (mCRP) proinflammatory effect on human primary healthy intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells from healthy IVD in culture were challenged with 10 or 50 $\mu\text{g}/\text{mL}$ mCRP, or with 100 ng/mL lipopolysaccharide (LPS) as positive control for 24 hours, after four hours of serum starvation. a) to g) Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). Data expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Cal., calibrator (refers to control (C-) condition); IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

were obtained from MilliporeSigma (USA). Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium, trypsin-ethylenediaminetetraacetic acid, L-glutamine, and penicillin-streptomycin mixture were obtained from Lonza Group (Switzerland). Pronase and collagenase P were obtained from Roche Diagnostics (Germany).

Recombinant mCRP (Roosevelt University, College of Pharmacy, USA) was produced in *Escherichia coli*, had both cysteines replaced with alanine residues and solubilized by an acylation technique (C-rmCRP) in 25 mM NaPBS (pH7.4), and was purified and tested in several bioassays to confirm its equivalence to biological mCRP from the pentameric form and free of contaminating endotoxin. The final endotoxin level of all protein solutions was below the detection limit 0.06 endotoxin units (EU)/ml of the assay.²⁸

Cell culture conditions and treatments. Human NP immortalized cell line HNPSV-1²⁹ (Tokai University School of Medicine; Japan) was cultured in DMEM/Ham's F12 supplemented with 10% FBS, 4 mM L-glutamine, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. In these experiments we used a cell passage range of 13 to 22.

Healthy IVD samples were obtained from deceased multi-organ donors. Degenerative IVD samples were obtained, under written patient consent, from patients undergoing discectomy. Inclusion criteria were: male or female patients undergoing discectomy diagnosed with spinal stenosis; adult degenerative scoliosis; disc herniation; and disc degeneration (Pfirrmann grade > 3

to 4). Exclusion criteria were: previous spinal malignancies or infections; previous spinal surgery; and patients diagnosed with metabolic diseases. All IVD samples were obtained under permission from the local ethics committee, according to the Declaration of Helsinki.³⁰ AF cells were isolated and cultured as previously described.³¹ Briefly, aseptically dissected AF tissue was diced and properly rinsed with phosphate-buffered saline (PBS). After removing PBS, tissue was incubated for 20 minutes with pronase at a final concentration of 1 mg/mL in DMEM/Ham's F12 at 37°C. Then, pronase was removed and the tissue was rinsed two to three times with PBS. After removing PBS, digestion continued with collagenase P at a final concentration of 1 mg/mL in DMEM/Ham's F12 with 10% FBS. Tissue was incubated for four to six hours in agitation at 37°C. The resulting cell suspension was filtered with a 40 μm nylon cell strainer (BD Biosciences Europe, Belgium) to remove debris. Cells were centrifuged, washed with PBS, resuspended in DMEM/Ham's F12 supplemented with 10% FBS, 4 mM L-glutamine, 50 units/ml penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin, counted, and plated in 12-well culture plates. Cells were maintained in culture for a maximum of ten days for expansion. All AF cells used in these experiments were at passage number 2.

AF cells and HNPSV-1 cells were seeded in six-well plates (2.5×10^5 cells/well) and treated with 10, 25, or 50 $\mu\text{g}/\text{mL}$ mCRP, 100 ng/mL LPS, or 0.1 ng/mL IL-1 β , after serum starvation for four hours. All the concentrations

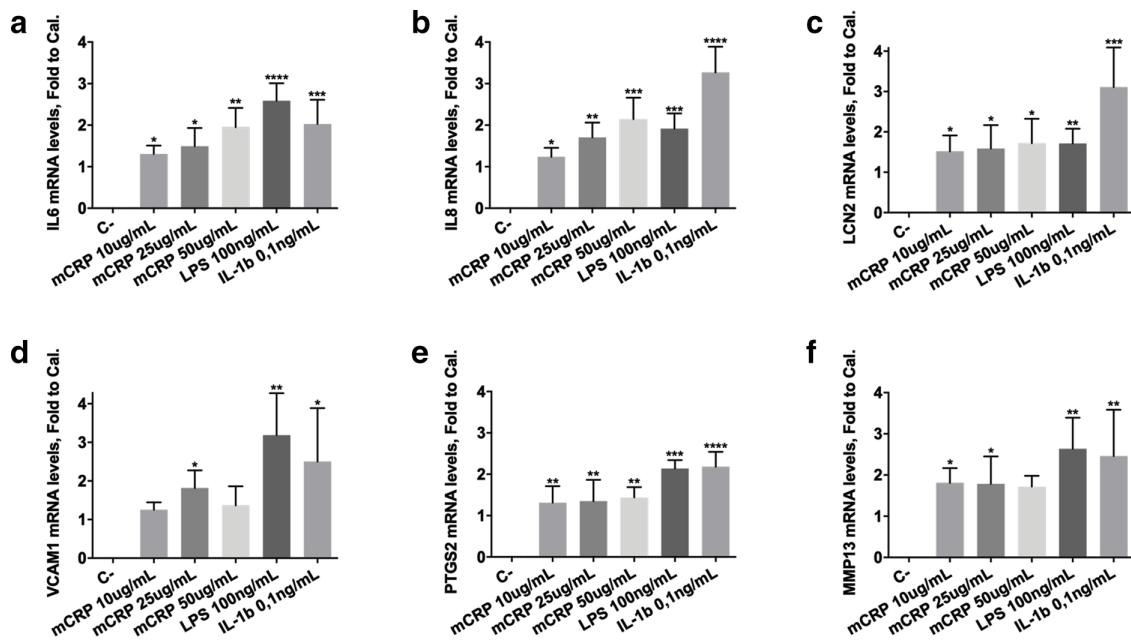


Fig. 3

Monomeric CRP (mCRP) proinflammatory effect on human intervertebral disc (IVD) nucleus pulposus (NP) cell line. HNPSV-1 human NP cells were challenged with 10, 25, or 50 $\mu\text{g}/\text{mL}$ mCRP, or with 100 ng/mL lipopolysaccharide (LPS) or 0.1 ng/mL IL-1b as positive controls for 24 hours, after four hours of serum starvation. a) to f) Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). Data expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Cal., calibrator (refers to control (C-) condition); IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

were selected based on a previous study²⁴ and in the absence of IVD cell toxicity. All treatments were done in serum-free conditions after four hours of serum starvation and lasted 24 hours for the proinflammatory response experiments (Figures 1 to 3), or 15 minutes for the intracellular signal transduction experiments (Figure 4).

Immunohistochemical assays. Sections 4 μm thick obtained from human AF or NP tissue were mounted on silanized coated slides (Dako-Agilent; USA). Epitope retrieval was performed in a PT-Link (Dako-Agilent) at high pH for 20 minutes. Then, the slides were automatically immunostained in an Autostainer-Link 48 (Dako-Agilent), employing the monoclonal anti-CRP antibody, clone CRP-8 (MilliporeSigma), diluted 1:100, and 30-minute incubation time. As a detection system, we used EnVision FLEX/HRP (Dako-Agilent), for 30 minutes, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen, for five minutes.

RNA isolation and real-time reverse transcription-polymerase chain reaction. Total RNA was isolated from cell culture with NZYol (NZYTech, Portugal) and E.Z.N.A. Total RNA Kit I (Omega Bio-tek, USA) according to the manufacturer's instructions, and reverse-transcribed using NZY First-Strand cDNA Synthesis Kit (NZYTech). Then, SYBR-green-based quantitative real-time polymerase chain reaction (RT-qPCR) was performed in Stratagene MX3005P thermal cycler as previously described,³² using a standard protocol with RT² SYBR Green qPCR Mastermix and specific PCR primers (Qiagen, Germany)

(human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 175 bp, PPH00150E, reference position 1287, Gen-Bank accession no. NM_002046.3; human nitric oxide synthase 2 (NOS2), 132 bp, PPH00173E, reference position 3962, Gen-Bank accession no. NM_000625.4; human matrix metalloproteinase 13 (MMP13), 61 bp, PPH00121B, reference position 1380, Gen-Bank accession no. NM_002427.3; human vascular cell adhesion molecule 1 (VCAM1), 141 bp, PPH00623E, reference position 2980, Gen-Bank accession no. NM_001078; human Lipocalin 2 (LCN2), 87 bp, PPH00446E, reference position 626 to 644, Gen-Bank accession no. NM_005564.3; human IL-6, 98 bp, PPH00560C, reference position 816, Gen-Bank accession no. NM_000600.3; human IL-8, 126 bp, PPH00568A, reference position 326, Gen-Bank accession no. NM_000584.3; human prostaglandin-endoperoxide synthase 2 (PTGS2), 63 bp, PPH01136F, reference position 1502, Gen-Bank accession no. NM_000963.2). No-template controls were included to eliminate any non-specific amplification, and melting curves were generated to ensure a single gene-specific peak. Gene expression changes were determined by the comparative $\Delta\Delta\text{Ct}$ method in MxPro qPCR Software version 4.10 (Stratagene, USA), and expressed as relative fold changes compared to control (C-) and normalized to GAPDH housekeeping gene.

Protein extraction and western blot analysis. After treatment, cells were rapidly washed with ice-cold PBS and scraped in lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM

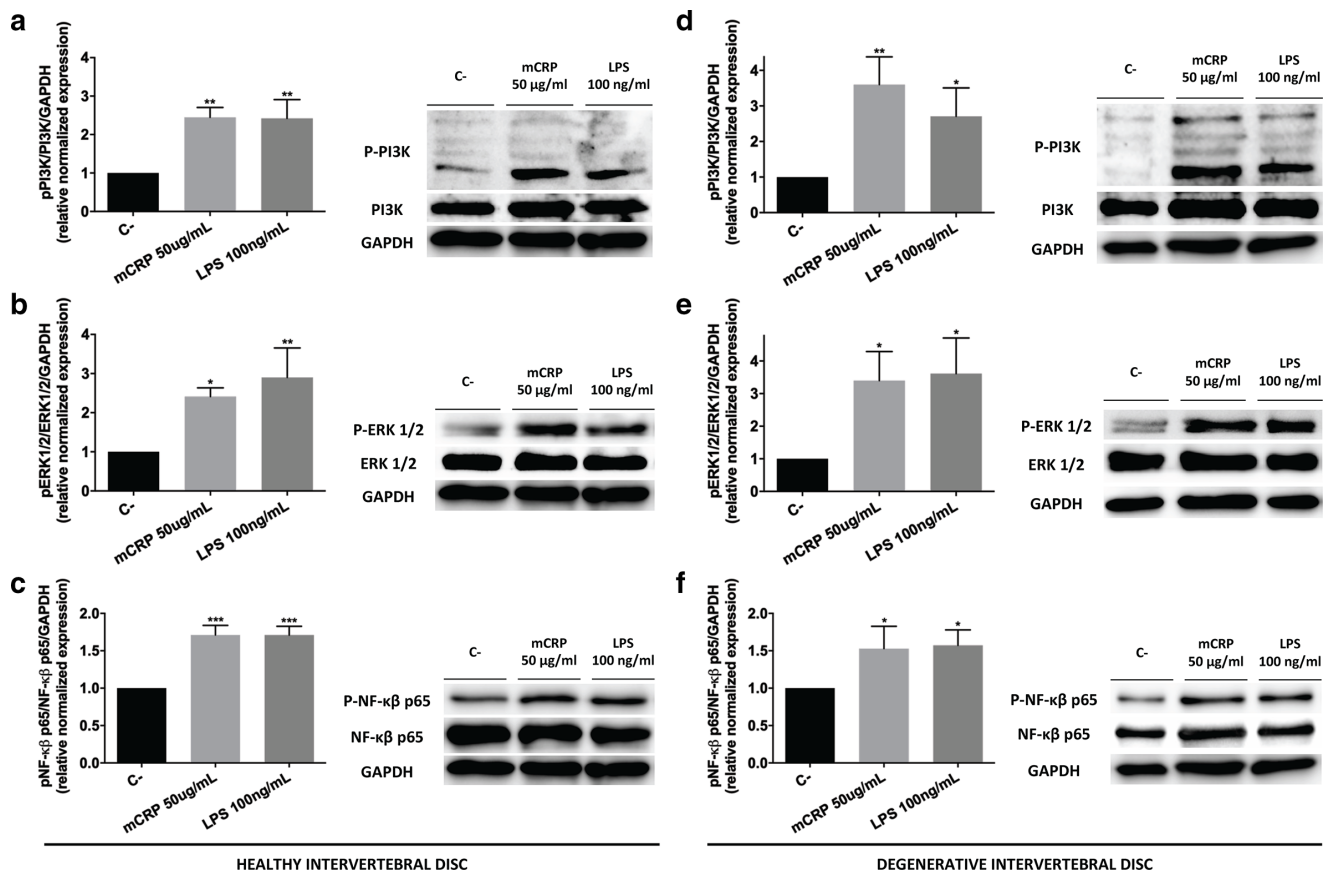
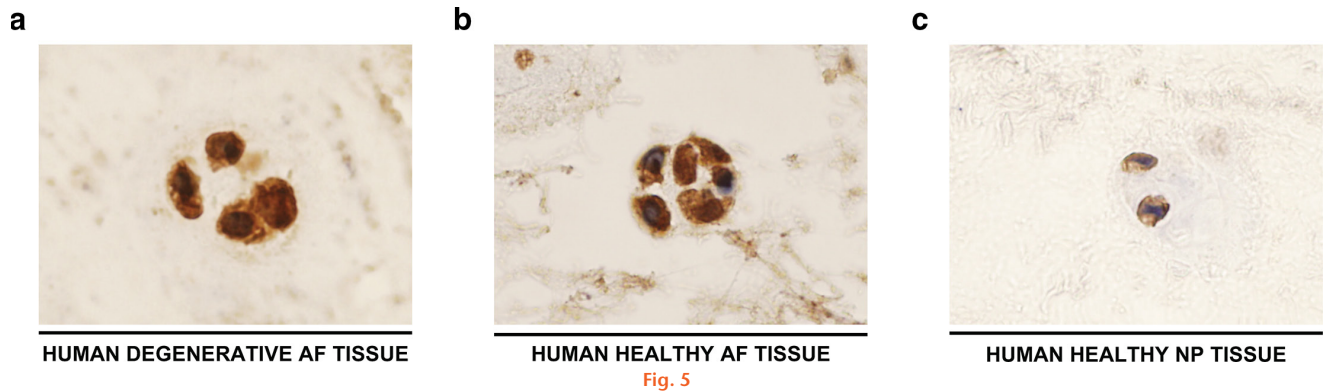


Fig. 4

Mediators involved in monomeric CRP (mCRP) intracellular signal transduction in human primary healthy and degenerative intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells from healthy or degenerative IVD in culture were challenged with 50 µg/ml mCRP or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 15 minutes, after four hours of serum starvation. a) to f) Protein expression was measured by western blot. Determination of the phosphorylated protein normalized to total protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data from densitometric analyses are shown as relative-to-control (C-) values. Data expressed as mean (standard error of the mean) of at least two or three independent experiments. Comparisons referred to control (C-): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NF- $\kappa\beta$ p65, nuclear factor- $\kappa\beta$ p65; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PI3K, phosphoinositide 3-kinase; pPI3K, phosphorylated PI3-kinase; pNF- $\kappa\beta$ p65, phosphorylated nuclear factor- $\kappa\beta$ p65.

ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF)), freshly supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, USA), then the total cell lysates were centrifuged at 14,000 g for 20 minutes. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting procedures were performed as previously described.³³ Immunoblots were incubated with the specific antibody against NOS2, cyclooxygenase 2 (COX2), phospho-phosphoinositide 3-kinase (PI3K) p85/p55, phospho-p44/p42 MAPK (extracellular signal-regulated kinase 1/2 (ERK1/2)), phospho-nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$) p65, or NF- $\kappa\beta$ p65 diluted 1:1000 (Cell Signaling Technology, USA), PI3K p85 or MAPK 1/2 (ERK1/2) diluted 1:1000 (MilliporeSigma), VCAM1 diluted 1:500 (EnoGene Biotech, USA), IL-6 or IL-8 diluted 1:100 (Santa Cruz Biotechnology, USA), MMP13 diluted 1:500 (Abcam, UK), and human LCN2 diluted 1:1,000 (R&D Systems, USA).

The immune complexes were detected using anti-rabbit, anti-mouse (both from GE Healthcare, USA), or anti-goat (Santa Cruz Biotechnology) horseradish-peroxidase-labelled secondary antibodies diluted 1:5,000 and visualized with Immobilon Western Detection kit (Millipore, USA). To verify equivalent protein loading, the membranes were stripped 15 minutes in stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7), blocked and incubated with anti-GAPDH antibody diluted 1:2,000 (MilliporeSigma). In all figures showing images of gels, the bands for each picture were obtained from the same gel, although they may have been spliced for clarity. The images were captured with ChemiDoc MP Imaging System and analyzed with Image Lab 6.0.1 Software, both from Bio-Rad Laboratories (USA) **Statistical analysis.** Data are reported as mean (standard error of the mean (SEM)) of at least three independent experiments. Statistical analyses were performed with GraphPad Prism 9.3.1 software (GraphPad Software, USA). Assuming a normal distribution, one-way analysis



Monomeric CRP (mCRP) localization in human degenerative and healthy intervertebral disc tissues. Immunohistochemical images showing mCRP protein presence (brown staining) in: a) human degenerative annulus fibrosus (AF) tissue; b) human healthy AF tissue; and c) human healthy nucleus pulposus (NP) tissue. Images taken at 60 \times magnification.

of variance (ANOVA) followed by Fisher's least significant difference (LSD) test were employed. As we performed few planned comparisons, *p*-values have not been corrected for multiple comparisons; they apply individually to each value reported and not to the entire family of comparisons. A *p*-value less than 0.05 was considered to be statistically significant.

Results

As shown in Figure 1, mCRP increases IL-6, IL-8, LCN2, VCAM1, NOS2, COX2, and MMP13 mRNA expression and protein levels in cultured human primary AF cells from patients with IVD degeneration, as confirmed by RT-qPCR and western blot analysis. The induction of proinflammatory mediators occurred in a dose-dependent manner after 24 hours.

The upregulation of these inflammatory and catabolic factors by mCRP was also determined in human primary AF cells from healthy patients, suggesting that mCRP can modify the healthy cell status by promoting the development of a proinflammatory environment in the IVD (Figures 2a to 2g). In all the experiments with human cells, LPS inflammatory stimulus was used as control of inflammatory induction.

To broaden the scope of our results, we also tested mCRP activity in HNPSV-1, a cell line derived from human NP cells that maintains the original architecture of the cells and their gene expression profile.²⁹ Figure 3 shows that mCRP at 10 μ g/ml, 25 μ g/ml, and 50 μ g/ml significantly increases IL-6, IL-8, LCN2, VCAM1, PTGS2, and MMP13 mRNA expression levels, as previously observed in human primary AF cells. LPS and IL-1 β , as well-known inflammatory stimuli, were used as controls of inflammatory induction.

For completeness, to elucidate which mediators might be implicated in mCRP intracellular signal transduction in both healthy and degenerative human disc cells, we analyzed NF- κ B signalling pathway upon mCRP challenge. Healthy or degenerative human primary AF cells were treated with 50 μ g/ml mCRP or 100 ng/ml LPS as a positive control, for 15 minutes. The results obtained

showed that mCRP induces PI3K phosphorylation, ERK1/2 phosphorylation, and NF- κ B p65 phosphorylation in healthy AF cells (Figures 4a to 4c) and degenerative AF cells (Figures 4d to 4f).

Finally, we performed immunohistochemical assays to investigate whether mCRP is located in IVD tissues. We assessed mCRP presence in human degenerative and healthy AF and healthy NP tissues, confirming its presence in physiological state in all of them. As we can observe in Figures 5a to 5c, mCRP location is predominantly cytoplasmic and perinuclear, being the more intense stain in the AF, especially in the degenerative tissue, than in the NP tissue.

Discussion

CRP in its pentameric form is an acute-phase serum protein and a well-established biomarker in a great number of infectious and inflammatory diseases, including IVD herniation.³⁴ It has also been evidenced as a mediator of inflammatory and immune responses in the context of local tissue injury.^{35,36} Serial CRP measures are useful not only for the follow-up of postoperative infection, but also for monitoring treatment efficacy and for the detection of relapses.^{27,37}

CRP monomeric isoform, although much less studied, has been found to have different biological functions in the inflammatory process. While clarifying its pathophysiological function needs further investigation, new insights could be extremely helpful in better understanding the inflammatory mechanism and how it works in the pathogenesis and disease progression.

There is evidence that the inflammatory process plays an important role in the development of disc degeneration, and there is a marked correlation between the levels of inflammatory markers and the degree of degeneration.³⁸ An association has been previously reported between high-sensitivity CRP (hs-CRP) levels and severe pain (VAS of > 40).³⁹ Sugimori et al⁴⁰ found markedly higher mean hs-CRP levels among patients with lumbar disc herniation (0.056 mg/dl (standard deviation (SD) 0.076)) than in the control group (0.017 mg/dl (SD

0.021)); no significant correlation between hs-CRP levels and herniation level was reported, but those patients with a higher concentration of hs-CRP before operation showed a poorer recovery. Of interest, some papers only quantified CRP levels in the high sensitivity range (i.e. hs-CRP), describing values below 10 µg/ml. According to several studies^{41–47} and to the guidance of the USA Food and Drug Administration (FDA),⁴⁸ the clinical significance of such values is questionable.

In cervical disc herniation, pain occurs as a result of the release of local inflammatory cytokines. The elevated plasma concentration of IL-6 produced by macrophages around the IVD tissue increments CRP levels.^{49,50} It has been shown that CRP levels were considerably higher in patients with cervical disc hernia and neck pain compared with those patients with neck pain but a normal cervical magnetic resonance.³⁴ A recent study found that plasma CRP levels in peripheral blood did not differ between healthy controls and non-herniated IVD degeneration patients.⁵¹

Recently, it has been demonstrated that only mCRP originated from local pCRP in the joint compartment may bind to receptor activator of nuclear factor-κB ligand (RANKL), neutralizing its activity and downregulating osteoclast differentiation.⁵² Ruiz-Fernández et al²⁴ recently reported the inflammatory effect of mCRP in chondrocytes, demonstrating that mCRP triggers a sustained proinflammatory and catabolic state in OA and healthy cartilage, and that the NF-κβ pathway is involved in its signal transduction. To demonstrate that this activity can also be exerted in IVD cells, we tested the effect of mCRP in primary culture of human AF cells isolated from patients with IVD degeneration or from healthy subjects. In order to show that these effects are not exclusive to AF cells, the human NP immortalized cell line HNPSV-1 was used as a NP cells model for comparison in order to study mCRP effects in the whole disc, since NP cells commonly exhibit chondrocyte-like characteristics.⁵³

As far as we know, this is the first experimental article that provides evidence for the functional activity of mCRP in AF and NP disc cells. To gain further insights into the action of mCRP and to verify whether this stimulation can result in sustained proinflammatory effects, we evaluated the dose-dependent activity of mCRP in master inflammatory and catabolic players in IVD degeneration. IL-8 and IL-6 are two of the proinflammatory cytokines strongly expressed in IVD degeneration.⁵⁴ In this work, we explored the ability of mCRP to modulate some of the most important factors involved in the disc degeneration inflammatory and catabolic processes. We also assessed LCN2, an adipokine implicated in the catabolic mechanism of IVD degeneration, MMP13, a catabolic enzyme, and the inflammatory mediators NOS2, cyclooxygenase 2 (COX2), and VCAM1. Nitric oxide synthase type II (iNOS) and COX2 are classically involved in the amplification of inflammatory responses. NO has cytotoxic effects, damages the IVD, and is involved in the upregulation of MMPs, among which MMP13 is one of

the major mediators of ECM degradation. COX2 induces prostaglandins that in turn increase the synthesis of other inflammatory mediators, including cytokines, to perpetuate tissue destruction. All these mediators are produced by IVD cells and are elevated in IVD degeneration.^{55–59}

Our present findings bring out important evidence regarding the modulation of IVD metabolism and functions exerted by mCRP, as well as the localization of mCRP in human IVD tissues. First, exposure of AF and NP cells to mCRP induced multiple proinflammatory genes, suggesting that its effects are not limited to one specific molecular target, rather, mCRP has a broader spectrum of action. The second important point is that the effect of mCRP is persistent and sustained for at least 24 hours, regardless of the proinflammatory environment. In fact, mCRP exerted its action on human primary AF cells coming not only from patients with IVD degeneration, but also from healthy subjects. In accordance with this, we found that mCRP can be localized physiologically both in healthy NP and AF tissues and, in addition, mCRP presence seems to increase in the degenerative AF tissue compared to the healthy one. Our finding suggests that elevated locally produced levels of mCRP may also trigger sustained multigenic inflammatory responses in normal tissues not previously exposed to a proinflammatory micro-environment, as in the case of AF cells from degenerated IVD. Another relevant aspect is that the effects of mCRP were observed both in AF and NP cells, showing that mCRP has multiple cell targets. The last important insight of this work is that mCRP effects in AF cells are mediated by the NF-κβ signalling pathway. We proved that upon mCRP challenge, PI3K, ERK1/2, and NF-κβ p65 were phosphorylated. These mediators were activated in a similar manner both in healthy and degenerative AF disc cells, confirming that mCRP action does not depend on a previous inflammatory state.

Our results are in agreement with those obtained by other groups working with other cell types. For instance, it has been observed in osteoclasts that mCRP acts through the PI3K signalling pathway.⁶⁰ Wang et al⁶¹ demonstrated that CRP increases IL-8 gene expression in human umbilical vein endothelial cells (HUVECs). Previous published results in cartilage showed the proinflammatory effect of mCRP in chondrocytes, clearly demonstrating that mCRP triggers a sustained proinflammatory and catabolic state in OA and healthy cartilage. These results also proved that NF-κβ has a role in mCRP signalling in cartilage.²⁴ This experimental design and the results observed were similar to those obtained in IVD cells.

The limited access to primary degenerative and healthy human disc cells is a limitation of this study. Further studies involving a larger number of subjects would be needed to fully understand the role of mCRP in IVD degeneration pathophysiology.

In conclusion, our present findings provide, for the first time, evidence of the mCRP localization in healthy and degenerative IVD tissues, and a novel molecular basis for the sustained action of mCRP in human AF and NP cells.

Taken together, these results suggest that mCRP exerts a lasting catabolic effect by increasing the expression of inflammatory mediators and proteolytic enzymes, being able to promote IVD cell breakdown and trigger inflammatory responses in healthy and degenerative AF cells and in the immortalized NP cell line. Altogether, these molecules can cooperate, resulting in the enhancement and perpetuation of the ECM-degrading processes at IVD level. In addition, mCRP by itself can trigger a severe increase in the expression of inflammatory mediators in healthy disc cells, suggesting that mCRP has a clear pathogenic role in disc disease. Our results indicate that this effect occurs both in AF and NP cells, and so affects the two main disc cellular compartments. Besides these findings, further studies on the pathophysiology of mCRP are needed to completely determine the role of mCRP in the pathogenesis and progression of IVD degeneration.

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Author information:

- C. Ruiz-Fernández, BS, MS, Predoctoral fellow, PhD candidate, SERGAS (Galician Healthcare Service) and NEIRID Lab (Neuroendocrine Interactions in Rheumatology and Inflammatory Diseases), Research Laboratory 9, IDIS (Health Research Institute of Santiago de Compostela), University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain; Doctoral Programme in Medicine Clinical Research,

International PhD School of the University of Santiago de Compostela (EDIUS), Santiago de Compostela, Spain.

- D. Ait Eldjoudi, BS, MS, PhD, Postdoctoral fellow
- M. González-Rodríguez, BS, Predoctoral fellow
- A. Cordero Barreal, BS, MS, Predoctoral fellow
- Y. Farrag, PhD, Postdoctoral fellow
- O. Gualillo, PharmD, PhD, Senior scientist group leader SERGAS (Galician Healthcare Service) and NEIRID Lab (Neuroendocrine Interactions in Rheumatology and Inflammatory Diseases), Research Laboratory 9, IDIS (Health Research Institute of Santiago de Compostela), University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain.
- L. García-Caballero, MD, PhD, Assistant professor, Department of Morphological Sciences, University of Santiago de Compostela, Santiago de Compostela, Spain.
- F. Lago, PhD, Senior scientist group leader, Molecular and Cellular Cardiology Group, SERGAS (Galician Healthcare Service) and IDIS (Health Research Institute of Santiago de Compostela), Research Laboratory 7, University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain.
- A. Mobasher, DPhil (Oxon), Full professor of musculoskeletal biology, Research Unit of Medical Imaging, Physics, and Technology, Faculty of Medicine, University of Oulu, Oulu, Finland; Department of Regenerative Medicine, State Research Institute Centre for Innovative Medicine, Vilnius, Lithuania; Departments of Orthopedic, Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands; World Health Organization Collaborating Center for Public Health Aspects of Musculoskeletal Health and Aging, University of Liège, Liège, Belgium.
- D. Sakai, MD, PhD, Orthopaedic surgeon, Department of Orthopedic Surgery, Surgical Science, School of Medicine, Tokai University, Isehara, Japan.
- J. Pino, MD, PhD, Orthopaedic surgeon, SERGAS (Galician Healthcare Service) and NEIRID Lab (Neuroendocrine Interactions in Rheumatology and Inflammatory Diseases), Research Laboratory 9, IDIS (Health Research Institute of Santiago de Compostela), University Clinical Hospital of Santiago de Compostela, Santiago de Compostela, Spain; Traumatology and Orthopedics Area, Department of Surgery and Medical-Surgical Specialties, University of Santiago de Compostela, Santiago de Compostela, Spain.

Author contributions:

- C. Ruiz-Fernández: Investigation, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing.
- D. Ait Eldjoudi: Investigation, Data curation.
- M. González-Rodríguez: Investigation, Data curation.
- A. Cordero Barreal: Investigation, Data curation, Writing – review & editing.
- Y. Farrag: Investigation, Formal analysis, Writing – review & editing.
- L. García-Caballero: Investigation, Resources, Writing – review & editing.
- F. Lago: Formal analysis, Data curation, Writing – review & editing.
- A. Mobasher: Data analysis, Writing – review & editing.
- D. Sakai: Data analysis, Writing – review & editing.
- J. Pino: Conceptualization, Supervision, Data curation, Writing – original draft, Writing – review & editing.
- O. Gualillo: Conceptualization, Supervision, Data curation, Writing – original draft, Writing – review & editing.
- J. Pino and O. Gualillo contributed equally to this work.
- J. Pino and O. Gualillo are joint senior authors.

Funding statement:

- The authors disclose receipt of the following financial or material support for the research, authorship, and/or publication of this article: funding from the ISCIII (Instituto de Salud Carlos III) and The European Regional Development Fund (ERDF- FEDER) (grant PI20/00902).

ICMJE COI statement:

- The authors declare no competing interests.

Acknowledgements:

- The authors kindly acknowledge Professor Lawrence Potempa and Dr Ibraheem Rajab (Roosevelt University, College of Pharmacy, USA) for providing them with the recombinant monomeric CRP.

Ethical review statement:

- The current study was approved by the Galician Ethics Committee (Comité Autonómico de Ética da Investigación de Galicia, Xunta de Galicia, Consellería de Sanidade. Código de Registro: 2017/279), and was conducted in accordance with the Declaration of Helsinki. Signed written informed consents were obtained from each donor.

Open access funding

- The authors report that they received open access funding for their manuscript by grant PI20/00902 funded by ISCIII (Instituto de Salud Carlos III) and The European Regional Development Fund (ERDF- FEDER).

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