





RESEARCH ARTICLE

Clusterin secretion is attenuated by the proinflammatory cytokines interleukin-1 β and tumor necrosis factor- α in models of cartilage degradation

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Abstract

The protein clusterin has been implicated in the molecular alterations that occur in articular cartilage during osteoarthritis (OA). Clusterin exists in two isoforms with opposing functions, and their roles in cartilage have not been explored. The secreted form of clusterin (sCLU) is a cytoprotective extracellular chaperone that prevents protein aggregation, enhances cell proliferation and promotes viability, whereas nuclear clusterin acts as a pro-death signal. Therefore, these two clusterin isoforms may be putative molecular markers of repair and catabolic responses in cartilage and the ratio between them may be important. In this study, we focused on sCLU and used established, pathophysiologically relevant, in vitro models to understand its role in cytokine-stimulated cartilage degradation. The secretome of equine cartilage explants, osteochondral biopsies and isolated unpassaged chondrocytes was analyzed by western blotting for released sCLU, cartilage oligomeric protein (COMP) and matrix metalloproteinases (MMP) 3 and 13, following treatment with the proinflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α . Release of sulfated glycosaminoglycans (sGAG) was determined using the dimethylmethylene blue assay. Clusterin messenger RNA (mRNA) expression was quantified by quantitative real-time polymerase chain reaction. MMP-3, MMP-13, COMP, and sGAG release from explants and

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osteocondral biopsies was elevated with cytokine treatment, confirming cartilage degradation in these models. sCLU release was attenuated with cytokine treatment in all models, potentially limiting its cytoprotective function. Clusterin mRNA expression was down-regulated 7-days post cytokine stimulation. These observations implicate sCLU in catabolic responses of chondrocytes, but further studies are required to evaluate its role in OA and its potential as an investigative biomarker.

KEYWORDS

apolipoprotein J, articular cartilage, biomarker, chondrocyte, clusterin, osteoarthritis (OA), proteomics, secretome

1 | INTRODUCTION

Osteoarthritis (OA), the leading cause of physical disability in adults, is a degenerative disease of synovial joints characterized by low-grade inflammation, subchondral bone remodeling, and progressive degradation and loss of articular cartilage.¹ There is an acute need for robust biomarkers which can identify the onset of the disease during the earliest stages, enable monitoring of progression, and assess efficacy of emerging therapeutic interventions in clinical trials. Identification of molecular alterations that can serve as early markers of degenerative changes in OA is paramount. This is especially important since early degenerative changes in the joint can be asymptomatic for years, and patients often show clinical signs once joint damage is extensive and cartilage loss may be irreversible.² Newly synthesized molecules secreted by chondrocytes in early stages of the disease, along with breakdown products of cartilage extracellular matrix (ECM), are potential biochemical markers of early degenerative changes in OA. To date, ECM degradation products have not yet yielded clinically robust biomarkers of early OA.³ Neo-epitopes of type II collagen breakdown serve as excellent markers of cartilage degradation, but only in later stages of the disease. In our laboratory we have focused our efforts on identifying secreted molecules in the secretome of cartilage, which may be indicators of cellular and molecular alterations in the early stages of OA.

We have previously used proteomics to identify clusterin (CLU) in the secretome of an equine explant model of cartilage degradation.⁴ CLU, also known as apolipoprotein J, is a secreted glycoprotein constitutively expressed in a broad spectrum of tissues, and has been functionally implicated in numerous physiological processes and age-related diseases, including OA.⁵ CLU exists in two isoforms, a glycosylated secretory form (secreted form of clusterin [sCLU]) and a non-glycosylated cytoplasmic form, which can translocate to the nucleus under stress conditions and initiate apoptosis (nuclear clusterin [nCLU]),⁶⁻⁸ although some of the published studies did not confirm nuclear localization of clusterin isoforms.⁷ sCLU has cytoprotective functions and binds to numerous proteins preventing their aggregation, and initiating disposal by phagocytic cells.⁹⁻¹²

CLU was identified in the secretome of human healthy and OA cartilage explants as an endogenous protein released at significantly lower amounts from hip OA samples.¹³ Lower levels of CLU were

found to be associated with greater cartilage lesion size.¹⁴ We are particularly interested in the biology of this protein and clinical potential of sCLU as an exploratory biomarker in OA, due to its presence in serum and synovial fluid.¹⁵ Alterations in CLU expression have previously been described in OA cartilage and synovial fluid.^{4,15-23} However, these studies have reported contrasting results, with both increases and decreases in CLU expression. We believe this is due to the existence of the two CLU isoforms, with opposing biological functions, which have not previously been distinguished in OA.

In this study, we focused exclusively on the expression and secretion of sCLU in models of in vitro cartilage degradation through targeted biochemical analysis. We utilized cartilage explants, osteochondral biopsies and primary chondrocyte model systems to determine sCLU secretion with and without addition of the proinflammatory cytokines interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α). We also used IL-1 β and TNF- α in combination with dexamethasone, a steroidal anti-inflammatory drug commonly used to treat joint inflammation,^{24,25} to ascertain the potential of sCLU to monitor anti-inflammatory drug efficacy. Cartilage degradation in these models was determined by the quantification of known catabolic markers matrix metalloproteinases (MMP) 3 and 13, loss of cartilage oligomeric protein (COMP) and release of sulfated glycosaminoglycan (sGAG).⁴ Our aim was to validate sCLU, a protein previously implicated in OA pathophysiology, as a putative biomarker of early degenerative changes induced by proinflammatory cytokines implicated in low-grade inflammation in OA using three in vitro culture model systems.

2 | METHODS

2.1 | Chemicals and reagents

All reagents were supplied by Sigma-Aldrich UK unless otherwise stated.

2.1.1 | Cartilage and osteochondral explant culture

Macroscopically normal equine articular cartilage was obtained from the weight bearing region of the metacarpophalangeal joint of nine

horses. Animals were sourced from an abattoir and euthanized for purposes other than research. The study received the full approval of the local ethics committee and was exempt from review by animal welfare authorities as abattoir tissue was used.

Six-millimeter diameter explants of cartilage of equal thickness were harvested aseptically using a sterile biopsy punch (Steifel), and 10 mm osteochondral explants were isolated using an osteochondral autograft transfer system (Arthrex). Explants were placed into low glucose (1 g/L) Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 mM glutamine, 500 µg/mL penicillin, 500 IU/mL streptomycin, and 50 µg/mL gentamicin for 20 minutes before being washed twice in low glucose DMEM supplemented with 200 mM glutamine, 200 µg/mL penicillin, 200 IU/mL streptomycin, and 50 µg/mL gentamicin (control medium).

One osteochondral explant, or approximately 60 mg explanted cartilage was placed into one well of a 24-well plate, per treatment, and equilibrated overnight in 1 mL of control medium at 37°C and 5% CO₂. This was considered day 0. Cartilage explants were weighed in a sterile 1.5 mL tube with enough sterile filter paper at the bottom of the tube to absorb media. Then, the tubes were centrifuged at full speed in a benchtop centrifuge to remove excess media from the explants. Explants were then placed into 1 mL of control media in a 24-well plate whilst tube + media/filter paper (no explant) were weighed to determine explant weights. Explants were then placed into fresh media with volumes adjusted so that the ratio was exactly 20 µL per mg explant for each well. The same exact volumes were applied at subsequent media changes so that the ratio was maintained throughout the course of the experiment. Osteochondral explants were cultured in 1 mL medium for 14 days. Explanted cartilage was cultured in 20 µL media/mg explant for 7 days. Previous studies from our laboratory have demonstrated chondrocyte viability and metabolic activity in explants for culture periods of up to 28 days with medium changes.

2.2 | Chondrocyte isolation and culture

Cartilage explants were also used for the isolation of primary equine chondrocytes and collected as described above. Explants were diced and then digested by sequential incubation with pronase (70 U/mL for 1 hour at 37°C; Roche) and collagenase (0.2% wt/vol, overnight at 37°C; Gibco). Isolated passage 0 chondrocytes were seeded at high density (105 000 cells/cm²—which was still a monolayer) and incubated in high glucose (4.5 g/L) DMEM supplemented with 200 mM glutamine, 200 µg/mL penicillin, 200 IU/mL streptomycin, 50 µg/mL gentamicin, 1 mM sodium pyruvate, 10 µg/mL insulin, 5 µg/mL transferrin, and 6.7 ng/mL selenium (Gibco) (control medium), for 7 days.

2.2.1 | Experimental design

Cartilage explants and chondrocytes were subjected to the following treatments; corresponding control medium in the presence or

absence of 0.1, 1.0 or 10 ng/mL TNF-α (R&D Systems) and 0.1, 1.0, or 10 ng/mL IL-1β (R&D Systems). Cartilage explants were also treated with the anti-inflammatory dexamethasone (at 100 µM). Osteochondral explants were incubated in control medium in the presence or absence of 10 ng/mL TNF-α and 10 ng/mL IL-1β. Media was collected and replenished every 3.5 days from cartilage explants/osteochondral grafts, and then combined to reflect the average release over the culture period (7 or 14 days). Protease inhibitor cocktail (Halt, Thermo Fisher Scientific) was then added to collected media. Monolayer chondrocytes were lysed, and protein extracted, by 50 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate (wt/vol), 10% glycerol (v/v) and protease inhibitor cocktail (Halt, Thermo Fisher Scientific). Cell lysates were syringed with a 25 G needle to shear nuclear DNA and centrifuged at 12 000g for 5 min to pellet cell debris. Cell lysates, supernatants and spent media were stored at -80°C prior to analysis. The “n” numbers refer to separate experiments using explants/cells from individual animals (biological replicates) in all cases.

2.3 | Western blotting

Cell lysate total protein was determined by using the Pierce bicinchoninic acid assay (Thermo Fisher Scientific), following the instructions of the manufacturer. Media and cell lysate samples were subjected to western blotting under reducing conditions. Explant media loading volumes were normalized to explant wet weight. Cell media and cell lysate loading volumes were normalized to total cell protein. Generated by pooling samples, positive control standards (STD) were used to normalize between blots. Collagen type II standard (TII STD) was generated by extraction of cartilage explants in 4 M guanidine hydrochloride and the insoluble pellet reconstituted in 0.5 M acetic acid, and further diluted with 50 mM Tris-acetate (pH 7.4). Samples and standards were loaded onto 4% to 12% Tris-glycine or 10% Bis-Tris acrylamide gels (NuPAGE, Invitrogen), then transferred onto 0.45 µm nitrocellulose membrane. Membranes were blocked with 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline 0.1% Tween-20 (vol/vol) (TBS-T), or Odyssey blocking buffer (Licor), for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies diluted in 5% BSA/TBS-T or Odyssey blocking buffer with 0.2% Tween-20; anti-CLU (Aviva Systems Biology ARP61142_P050), anti-COMP (Abcam ab128893), anti-MMP-3 (Aviva Systems Biology ARP42042_P050), anti-MMP-13 (Aviva Systems Biology ARP56350_P050), anti-collagen type II (DSHB II-II6B3), rabbit normal immunoglobulin G (IgG) control (12-370; Sigma-Aldrich) or mouse normal IgG control (12-371; Sigma-Aldrich). Bound primary antibodies were detected with goat anti-rabbit or goat anti-mouse antibodies conjugated with alkaline phosphatase (Cambridge Bioscience), IRDye 680RD or IRDye 800CW (LI-COR Biosciences). Positive bands were imaged using the LI-COR Odyssey FC imaging system (LI-COR Biosciences) or by incubation in alkaline phosphatase NBT/BICP buffer (Promega), and then quantified by densitometry analysis (Image J, NIH). Blots were imaged using the LI-COR Odyssey FC imaging system and quantification of detected bands was performed

using the Image Studio software. Positive cell lysate bands were normalized to total protein post transfer, detected by REVERT Total Protein Stain (LI-COR Biosciences). For explants, equal volumes of media were loaded onto the gel since the experimental media volumes were already normalized to explant weight. Cell media volumes loaded onto the gel were normalized to total cell protein. REVERT was not used to correct for explant media protein or cell media protein since we expected media protein levels to change with cytokine treatment, due to increased release of matrix proteins; therefore, using REVERT to normalize densitometry values would have negated any cytokine effects.

2.4 | Dimethylmethylene blue assay

sGAG content was determined by using the dimethylmethylene blue (DMMB) colorimetric assay as previously described²⁶ and chondroitin sulfate C, from shark cartilage, was used as a standard. Absorbance readings were taken at a wavelength of 525 nm on a Tecan SPARK 10 M plate reader.

2.5 | Real-time quantitative polymerase chain reaction

Cartilage explants were homogenized in TRI reagent using a dismembrator (Braun Biotech). RNA was isolated from explant homogenate and chondrocyte lysate by using the Qiagen RNeasy RNA isolation kit and following the manufacturer's instructions. First strand cDNA was then synthesized using SuperScript III reverse transcriptase (Thermo Fisher Scientific), and real-time polymerase chain reaction (RT-PCR) conducted using the Techne Prime Pro thermal cycler with GoTaq qPCR Master Mix (Promega). Quantitative RT-PCR (RT-qPCR) reactions were carried out in 10 μ L volumes containing 3.5 mM MgCl₂, 200 μ M dNTPs, 0.3 μ M of sense and antisense primers, 0.025 U/ μ L GoTaq polymerase enzyme and 1:66,000 SYBR Green-1 (Thermo Fisher Scientific). CLU (NM_001081944) Forward: CTA-CTT-CTG-GAT-CAA-CGG-TGA-CC and Reverse: CGG-GTG-AAG-AAT-CTG-TCC-T primers produced a 144 base pair (bp) product. Clusterin gene expression was normalized to the housekeeping gene ribosomal protein S18 (RPS18, XM_001497064) using Forward: CAC-AGG-AGG-CCT-ACA-CGC-CG and Reverse: AGG-CTA-TCT-TCC-GCC-GCC-CA primers (119 bp product).

2.6 | Statistics

Statistical tests were performed using the R statistical package (CRAN). Data sets were checked for Gaussian distribution using Shapiro-Wilks tests and equal variance using Bartlett k^2 tests; log transformations were conducted to correct normality or unequal variance if required. Tests for statistical significance were performed using Student's paired *t* test and One-way analysis of variance with Tukey's HSD pair-wise comparisons. Effect size was calculated using

Cohen's *d* test (with effect sizes classified as small ($d \geq 0.2$), medium ($d \geq 0.5$), large ($d \geq 0.8$) and very large ($d \geq 1.3$).²⁷ Mean values are reported as \pm standard error of the mean.

3 | RESULTS

3.1 | sGAG release from cartilage explants is elevated by cytokine stimulation

To validate our in vitro model of cartilage degradation, we first looked at the sGAG content of the explant secretome using DMMB analysis. sGAG release was increased three-fold following stimulation with 10 ng/mL IL-1 β and TNF- α compared with the control with a very large effect size (fold change increase: FC = 2.98; $P = .0058$, $d = 2.3561$; Figure 1A). Levels of the cartilage degradation marker cartilage oligomeric matrix protein (COMP) in the explant secretome were increased two-fold with 10 ng/mL IL-1 β and TNF- α stimulation versus the control (FC = 1.94, $P = .0218$, $d = 1.7963$; Figure 1B), as revealed by western blot analysis, demonstrating the catabolic effect of cytokine treatment in this model. Uncropped blot images for COMP are shown in Figure S1.

3.2 | Clusterin release from cartilage explants is attenuated by cytokine stimulation

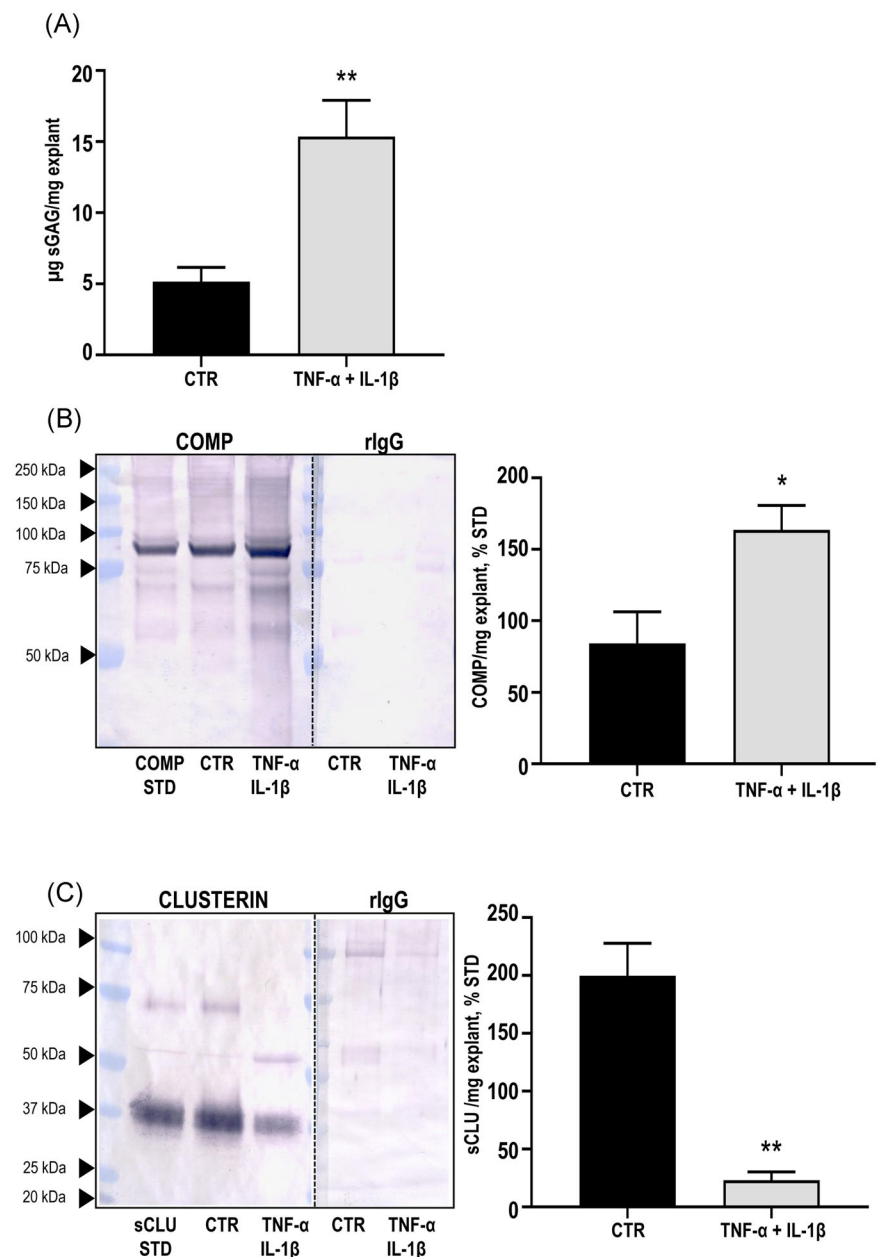
Mature sCLU is secreted as a heterodimeric protein with an apparent molecular weight (MW) of 75 to 80 kDa. Under reducing conditions, extracellular sCLU appears as α - and β -chains of 34 to 36 kDa and 37 to 9 kDa, respectively.⁷ The α - and β -bands usually appear as a smear, rather than sharp bands, due to varying degrees of glycosylation.⁸ Intracellular forms of clusterin include the 60 kDa precursor secretory clusterin (psCLU), the alternatively spliced 49 kDa precursor nuclear CLU (pnCLU), and its 55 kDa post-translationally modified nCLU.^{6,8}

Western blot analysis of the articular cartilage explant secretome in reducing conditions identified the glycosylated α - and β -chains of extracellular secreted sCLU between 34 and 39 kDa (Figure 1C). Cartilage explants stimulated for 7 days with 10 ng/mL IL-1 β and TNF- α showed a 9-fold reduction in sCLU release compared with control (FC = -8.69; $P = .00603$, $d = 1.2455$). Uncropped blot images for CLU are shown in Figure S1.

3.3 | Clusterin release from osteochondral explants is attenuated by cytokine stimulation

sGAG release from osteochondral explants was also increased three-fold following stimulation with 10 ng/mL IL-1 β and TNF- α compared with the control, with a very large effect size (FC = 3.01; $P = .002$; $d = 5.8973$; Figure 2A), indicating a similar response to cartilage explants. Western blot analysis showed that sCLU expression was downregulated 363.9-fold in the osteochondral biopsy secretome

FIGURE 1 Release of sulfated glycosaminoglycans (sGAG), cartilage oligomeric matrix protein (COMP), and secreted isoform of clusterin (sCLU) into the secretome of articular cartilage explants, following stimulation with 10 ng/mL tumor necrosis factor α (TNF- α) and 10 ng/mL interleukin-1 β (IL-1 β) for 7 days. A, sGAG quantified by dimethylmethylene blue (DMMB) analysis. B, COMP; C, sCLU, and rabbit immunoglobulin G (rlgG) controls, detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 5$. * and ** indicate significant differences ($P < .05$ and $P < .01$, respectively) as determined by Student's t test versus the control (CTR) [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/jor.24814)]



following 14 days of stimulation with 10 ng/mL IL-1 β and TNF- α compared with the control ($P = .0011$; $d = 3.3291$; Figure 2B). As observed in the cartilage explant secretome, the glycosylated α - and β -chains of secreted sCLU were identified in the control osteochondral secretome between 34 and 39 kDa. Uncropped blot images are shown in Figure S2.

3.4 | Clusterin release from cartilage explants is reduced by physiologically relevant levels of cytokines

Levels of IL-1 β and TNF- α found in the synovial fluid of patients with OA are in the region of 0.1 ng/mL.^{28,29} We therefore aimed

to determine whether a lower concentration of IL-1 β and TNF- α had any effect on clusterin release in this model. sGAG release into the explant secretome was gradually increased by 0.1, 1.0, and 10 ng/mL IL-1 β and TNF- α treatment compared with the control (fold change increases: FC = 2.28, FC = 3.55, FC = 3.67; $P = .0283$, $P = .0067$, $P = .0044$; and $d = 1.4534$, $d = 1.746$, $d = 1.8914$, respectively; Figure 3A). In contrast, clusterin release was significantly reduced following 0.1, 1.0, and 10 ng/mL IL-1 β and TNF- α treatment versus the control explants (FC decreases: FC = -1.32, FC = -2.62, FC = -6.3; $P = .0027$, $P = .0158$, $P < .0001$; and $d = 1.1179$, $d = 2.0148$, $d = 5.9987$, respectively). Uncropped blot images are shown in Figure S3.

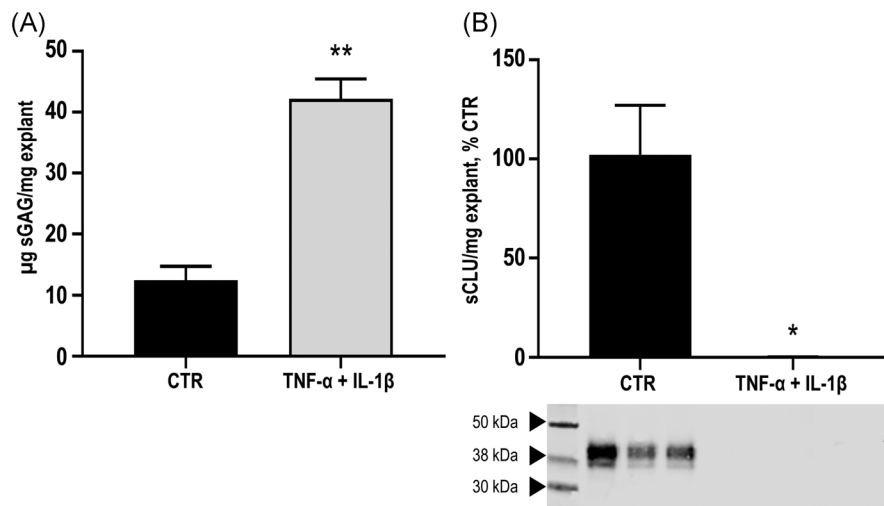


FIGURE 2 Release of sGAG and sCLU into the secretome of osteochondral explants, following stimulation with 10 ng/mL TNF- α and 10 ng/mL IL-1 β for 14 days. A, sGAG quantified by DMMB analysis. B, sCLU, detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 5$. * and ** indicate significant differences ($P < .05$ and $P < .01$, respectively) as determined by Student's t test versus the CTR. CTR, control; DMMB, dimethylmethylene blue; IL-1 β , interleukin-1 β ; sCLU, secreted isoform of clusterin; sGAG, sulfated glycosaminoglycans; TNF- α , tumor necrosis factor α

3.5 | Clusterin release and gene expression in cartilage explants is unaffected by MMP inhibition

To determine whether the loss of clusterin release upon proinflammatory cytokine stimulation was due to increased proteolytic activity in the cartilage explant, and/or degradation of secreted clusterin by matrix proteases, explants were treated with 10 ng/mL TNF- α , IL-1 β , and 100 μ M dexamethasone in combination. Dexamethasone is an anti-inflammatory steroidal drug known to reduce MMP activity in cartilage.²⁵ Release of clusterin from cartilage explants, and cartilage explant clusterin gene expression as detected by RT-qPCR, were both significantly attenuated by treatment with IL-1 β and TNF- α with very large effect sizes (FC reduction: FC = -109.61, $P = .0009$, $d = 4.8357$; and FC = -596.57, $P = .0002$, $d = 3.0197$ vs the control, respectively; Figure 4A,B), and the same effect was observed when IL-1 β and TNF- α were applied in combination with dexamethasone (FC reduction: FC = -79.44, $P = .0009$, $d = 4.4183$ and FC = -204.65, $P = .0005$, $d = 3.01$ vs the control, respectively). In contrast, whilst IL-1 β and TNF- α significantly increased MMP13 and MMP3 release into the explant secretome (FC = 10.73 increase, $P = .0004$, $d = 6.9966$ and FC = 4.24 increase, $P = .002$, $d = 4.8831$ vs the control, respectively; Figure 4C,D), co-application of dexamethasone with the cytokines abolished the increased levels of both metalloproteinases in this explant model (FC = -9.61 reduction, $P = .021$, $d = 5.59$ and FC = -1.95 reduction, $P = .041$, $d = 3.37$ vs proinflammatory cytokine treatment, respectively; Figure 4C,D). These data indicate that loss of secreted clusterin is unlikely due to enhanced matrix protease activity since inhibition of MMP-3 and MMP-13 by dexamethasone does not rescue cytokine-induced reduction of either clusterin release or clusterin gene expression. Uncropped blot images are shown in Figure S4.

3.6 | Clusterin secretion from chondrocytes is reduced by cytokine stimulation

Next, we looked at clusterin release in primary (unpassaged equine) articular chondrocytes. Cells were cultured for 7 days in serum-free medium at high density. Secretion of sGAG and collagen type II was maintained in the control cells for the duration of the experiment, confirming the chondrocyte phenotype, as revealed by DMMB assay and western blot analysis of the chondrocyte secretome, respectively (Figure 5A,B). The α - and β -chains of sCLU were present in the chondrocyte secretome, and sCLU secretion was down-regulated by almost four-fold with 10 ng/mL IL-1 β and TNF- α stimulation, compared with the control (FC = -3.79 reduction, $P = .0138$, $d = 1.62$), as revealed by western blot analyses (Figure 5C). Treatment with different concentrations (0.1, 1, and 10 mg/mL) of the cytokines resulted in a dose-dependent trend of reduced sGAG secretion (Figure 5D). A gradually attenuated release of sCLU (Figure 5E) was detected on western blots performed on the secretome following stimulation with different concentrations of the proinflammatory cytokines, similar to what has been observed with cartilage explants. Uncropped blot images are shown in Figure S5.

3.7 | The sCLU isoform, but not the nCLU isoform, could be detected intracellularly

Western blotting performed on total lysates of primary unpassaged chondrocytes cultured for 7 days in serum-free medium at high density revealed the presence of intracellular clusterin (Figure 6A). Uncropped blot images are shown in Figure S6. The MW of the band corresponding to clusterin was confirmed at 41.1 kDa (± 0.2),

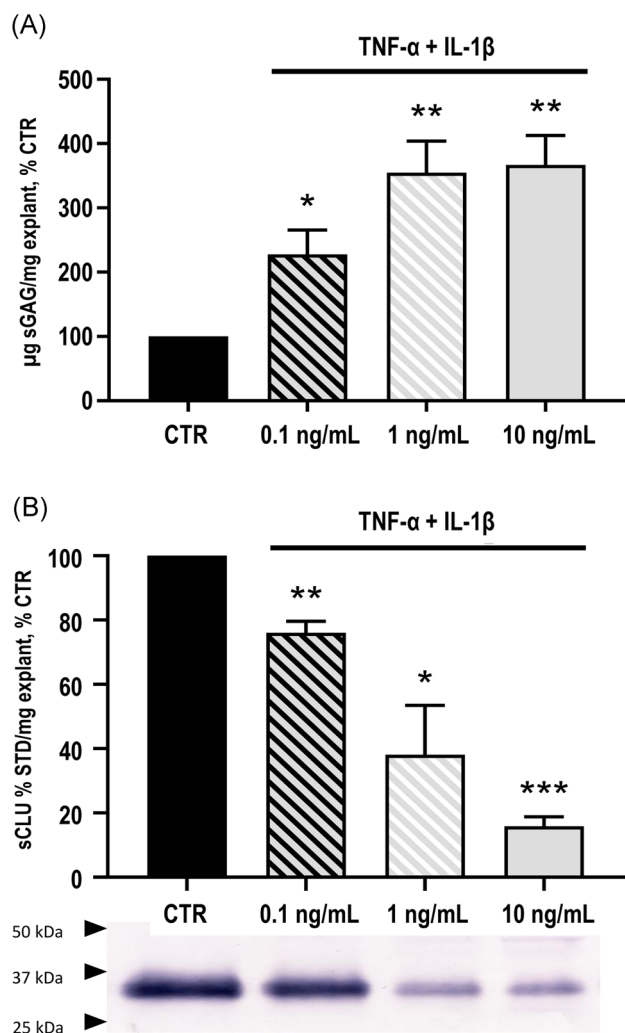


FIGURE 3 Release of sGAG and sCLU into the secretome of cartilage explants, following stimulation for 7 days with 0.1, 1, or 10 ng/mL TNF- α and 0.1, 1, or 10 ng/mL IL-1 β . A, sGAG quantified by DMMB analysis. B, sCLU, detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 3$. *, **, and *** indicate significant difference ($P < .05$, $P < .01$, and $P < .001$, respectively) as determined by hypothetical mean t test versus the CTR. CTR, control; DMMB, dimethylmethylene blue; IL-1 β , interleukin-1 β ; sCLU, secreted isoform of clusterin; sGAG, sulfated glycosaminoglycans; TNF- α , tumor necrosis factor α [Color figure can be viewed at wileyonlinelibrary.com]

indicating the presence of the intracellular CLU isoform, whilst no positive bands were detected at 49 kDa or 55 kDa (see Figure S7), which correspond to the predicted MWs of nCLU. It should be noted that the anti-clusterin primary antibody employed in this study recognizes an epitope in a sequence which spans both exons 6 and 7 (NM_001831.4). sCLU and nCLU both share exons 6 and 7 and differ only in the presence or absence of exons 1 and 2.⁷ Treatment with the proinflammatory cytokines IL-1 β and TNF- α at 10 ng/mL significantly reduced the levels of the intracellular CLU isoform (FC = -1.45 reduction, $P = .0173$, $d = 1.2402$), compared with the control (Figure 6B).

3.8 | Clusterin gene expression in chondrocyte cultures is decreased by cytokine stimulation

Clusterin gene expression was investigated using RT-qPCR analysis of primary equine articular chondrocytes cultured in a monolayer. Clusterin gene expression in chondrocyte cultures was unaffected following 3 days of stimulation with 10 ng/mL IL-1 β and TNF- α ; however, a 1.85-fold decrease was evident 7 days post stimulation compared with the control ($P = .005$, $d = 8.6747$), as shown in Figure 6C.

4 | DISCUSSION

The aim of this study was to validate sCLU, a protein previously implicated in OA pathophysiology, as a candidate biomarker of the early degenerative changes induced by proinflammatory cytokines using three in vitro culture model systems. The ultimate aim of this study was to determine if sCLU may be proposed as a potential investigative biomarker of early OA. Clusterin was identified as a putative biomarker candidate by our previous proteomic study, using an in vitro model of cartilage degradation,^{4,16} and proteomic and transcriptome studies carried out by other groups, which analyzed serum, plasma, synovial fluid and articular cartilage.^{13-15,17,18,20-23} Increases and decreases in clusterin levels have been reported with disease, and these contradictory findings are perhaps due to the existence of two isoforms of clusterin, and varying disease stage of donor tissue. The difference is critical due to the opposing roles of the two isoforms, and the relevance of isoform function in OA pathology. By considering only sCLU, and by using a targeted approach in a controlled in vitro model, we validated this candidate marker and further elucidated its role in three in vitro culture model systems frequently used to study early degenerative changes in articular cartilage stimulated in response to pathophysiologically relevant concentrations of proinflammatory cytokines implicated in low-grade inflammation in OA.

We used an equine articular cartilage explant model to monitor sCLU release in response to the proinflammatory cytokines IL-1 β and TNF- α . In order to maintain consistency with previous work from our laboratory,^{4,16} the proinflammatory cytokines were applied at the pathophysiologically relevant concentration of 10 ng/mL to evaluate early responses to cytokine-stimulation in articular cartilage. However, given that the levels of IL-1 β and TNF- α found in the synovial fluid of patients with OA are in the realm of 0.1 ng/mL,^{28,29} we also aimed at determining whether lower concentrations of IL-1 β and TNF- α had any effect on sCLU release in this model of cartilage degradation. Incorporation of the osteochondral model in our workflow meant that the influence of crosstalk, at the bone-cartilage interface, on sCLU release could be ascertained.³⁰ Analysis of the secretome of primary chondrocytes removed the complication of pre-existing sCLU present in the cartilage matrix, enabling quantification of only newly secreted sCLU.

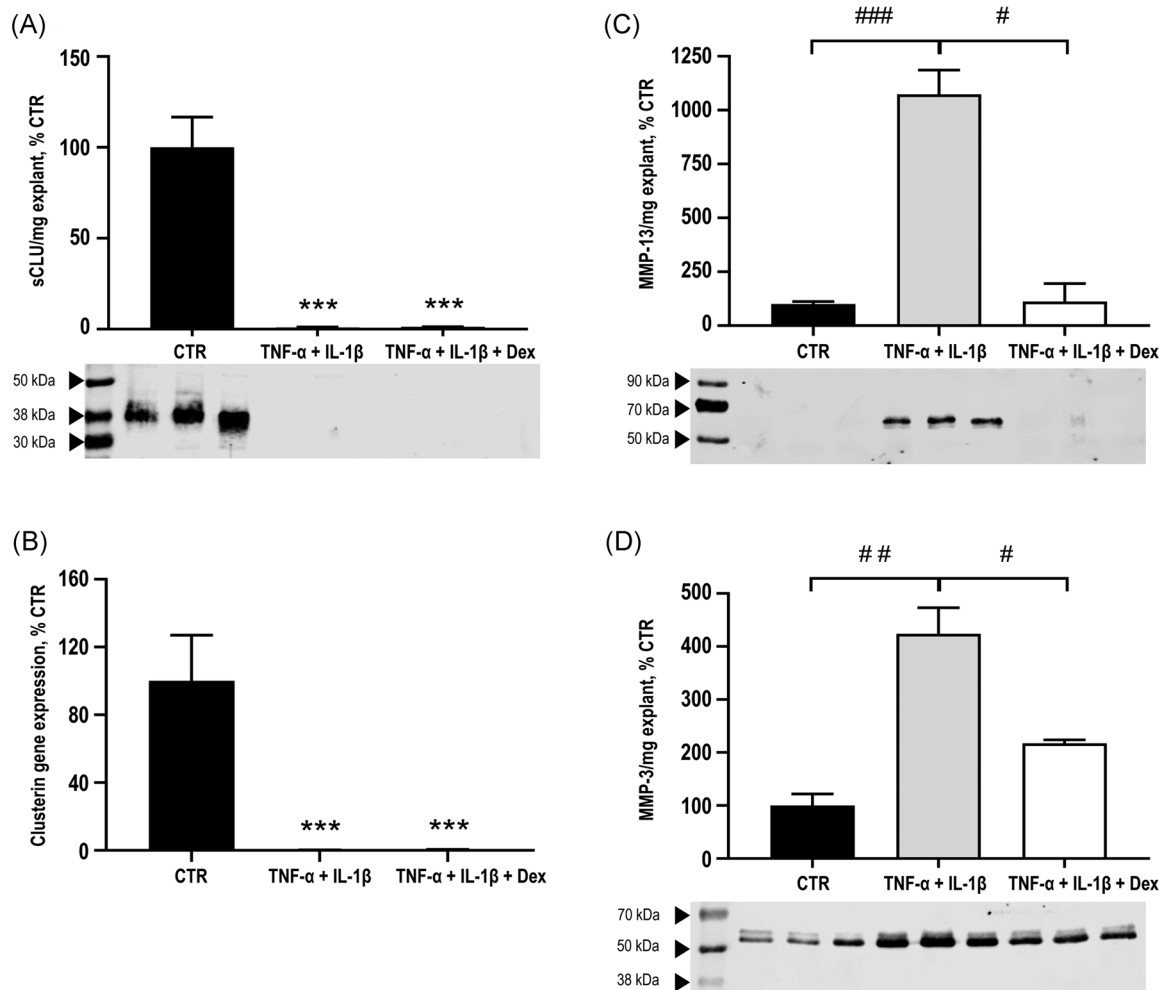


FIGURE 4 Clusterin (CLU) expression in cartilage explants, and release of sCLU and matrix metalloproteinase (MMP) into the secretome of cartilage explants, following stimulation for 7 days with 10 ng/mL TNF- α and 10 ng/mL IL-1 β \pm 10 μ M dexamethasone (DEX). A, sCLU detected by western blotting and quantified by densitometry. B, CLU gene expression quantified by real-time quantitative real-time-polymerase chain reaction (RT-qPCR) analysis and normalized to ribosomal protein S18 (RPS18). C, MMP-13, and (D) MMP-3 detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 3$. *** indicates significant difference ($P < .001$) as determined by one-way analysis of variance (ANOVA) versus the CTR. #, ##, and ### indicate significant difference ($P < .05$, $P < .01$, and $P < .001$, respectively) as determined by one-way ANOVA versus TNF- α + IL-1 β . CTR, control; IL-1 β , interleukin-1 β ; sCLU, secreted isoform of clusterin; TNF- α , tumor necrosis factor α

IL-1 β and TNF- α produced by the synovial membrane are key contributors to the catabolic signaling and low-grade inflammation implicated in the development of early OA.³¹ These proinflammatory cytokines suppress anabolic pathways whilst stimulating catabolic enzymes, namely MMPs and aggrecanases.^{32,33} Increased levels of MMPs results in intensified proteolysis, and the progressive loss of the ECM.³⁴ Proinflammatory cytokine induction of aggrecanases leads to the loss of the large aggregating proteoglycan aggrecan, and associated release of sGAG attachments.^{35,36} COMP mediates chondrocyte attachment to the ECM,³⁷ organizes ECM assembly, and is an established marker of OA due to its correlation with disease severity and presence in synovial fluid, serum and urine.³⁸⁻⁴⁰

To demonstrate the validity and biological relevance of the culture models of OA used in this study, we quantified MMP-3, MMP-13, COMP, and sGAG released into the secretome of the cartilage explant and osteochondral biopsy systems. Levels of

MMP-3 and MMP-13 were elevated in the cartilage explant model following treatment with IL-1 β and TNF- α . COMP levels also increased in the cartilage explant secretome following proinflammatory cytokine treatment. The sGAG content of cartilage explants was reduced with IL-1 β and TNF- α stimulation confirming early-stage cartilage degradation in this model. sGAG release into the secretome was elevated with proinflammatory cytokine stimulation, in both cartilage explant and osteochondral biopsy models to a similar extent, suggesting that aggrecanase-driven cartilage degradation was similarly induced in both models, highlighting the relevance of both models for identifying molecular markers that reflect early catabolic changes. The addition of the anti-inflammatory compound dexamethasone reduced proinflammatory cytokine-induced levels of MMP-3 and 13 in the cartilage explant model. These observed changes are in line with our previous work.^{4,16}

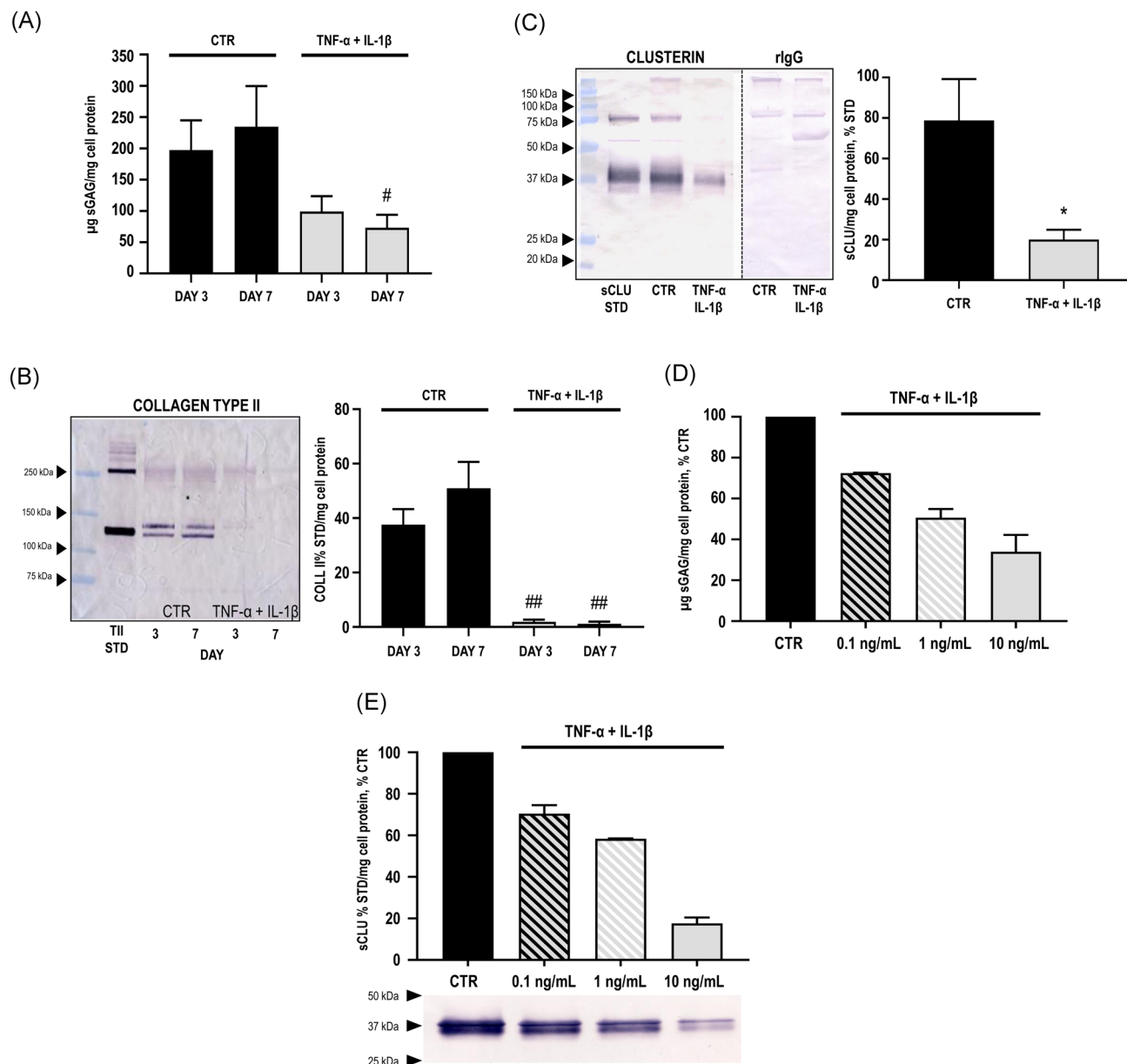


FIGURE 5 Release of sGAG, collagen type II, and CLU into the secretome of un-passaged primary articular chondrocytes, following stimulation for 3 or 7 days with TNF- α and IL-1 β . A, Release of sGAG following stimulation for 3 or 7 days with 10 ng/mL TNF- α and 10 ng/mL IL-1 β , quantified by DMMB analysis. B, Release of collagen type II (TII) following stimulation for 3 or 7 days with 10 ng/mL TNF- α and 10 ng/mL IL-1 β , detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 6$. # and ## indicate significant difference ($P < .05$ and $P < .01$, respectively), as determined by one-way ANOVA versus the CTR on day 3 or 7, respectively). C, Release of the secreted sCLU into the secretome, following stimulation for 7 days with 10 ng/mL TNF- α and 10 ng/mL IL-1 β , detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 6$. * indicates significant difference ($P < .05$) as determined by Student's t test versus the CTR. D, E, Release of sGAG and CLU into the secretome of monolayer chondrocytes, following stimulation for 7 days with 0.1, 1, or 10 ng/mL TNF- α and 0.1, 1, or 10 ng/mL IL-1 β . sGAG release was quantified by DMMB analysis; sCLU release was detected by western blotting and quantified by densitometry. Data shown are mean \pm SEM, $n = 2$. ANOVA, analysis of variance; CLU, clusterin; CTR, control; DMMB, dimethylmethylene blue; IL-1 β , interleukin-1 β ; sCLU, secreted isoform of clusterin; sGAG, sulfated glycosaminoglycans; TNF- α , tumor necrosis factor α [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/terms-and-conditions)]

To quantify the newly secreted sCLU without measuring pre-existing sCLU present in the cartilage matrix, we employed monolayer cultures of freshly isolated articular chondrocytes. sGAG release into the spent culture medium was higher in the control group, demonstrating the relevant biological activity associated with the chondrocyte phenotype in vitro. Stimulation with the

proinflammatory cytokines IL-1 β and TNF- α caused a decrease in sGAG content of the secretome, consistent with previous studies which also showed suppression of proteoglycan synthesis by cytokines.⁴¹ Levels of both MMP-3 and MMP-13 were elevated with proinflammatory cytokine treatment in the secretome of the chondrocyte monolayer model. This observation confirms the concordant

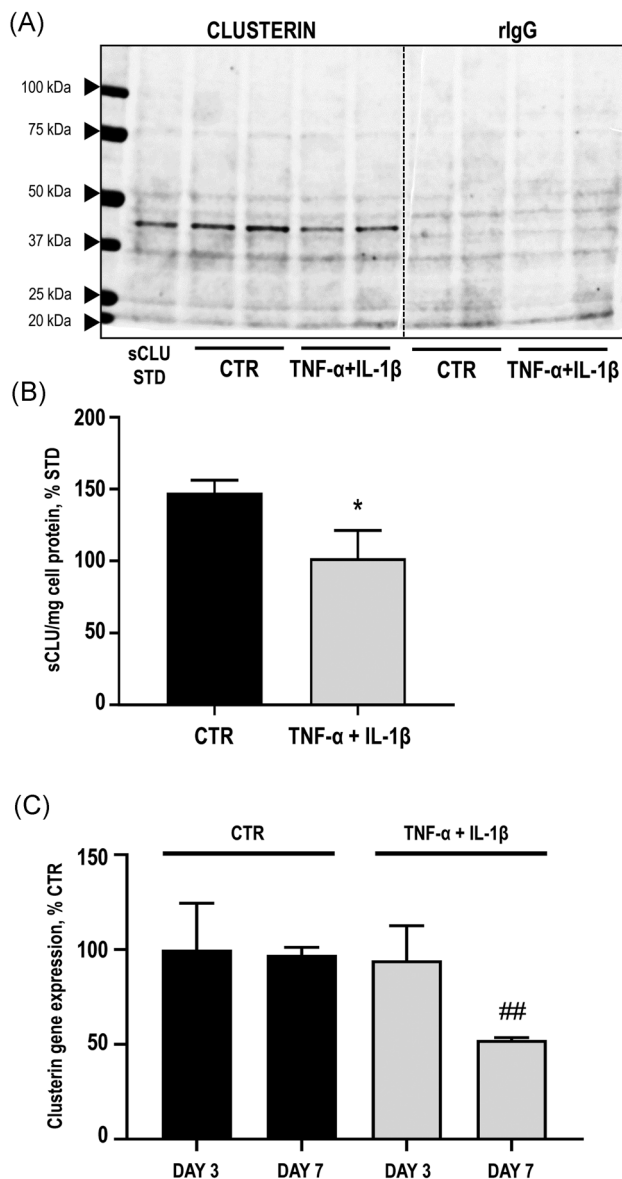


FIGURE 6 Clusterin protein and messenger RNA (mRNA) expression in un-passaged primary articular chondrocytes, following stimulation for 7 days with 10 ng/mL TNF- α and 10 ng/mL IL-1 β . A, B, Secreted sCLU, and rabbit IgG (rIgG) control, detected by western blotting and quantified by densitometry; normalized to total protein loaded. Data shown are mean \pm SEM, $n = 6$. * indicates significant difference ($P < .05$) as determined by Student's t test versus the CTR. C, CLU mRNA quantified by real-time RT-qPCR analysis, after being normalized to ribosomal protein S18 (RPS18). Data shown are mean \pm SEM, $n = 3$. ## indicates significant difference ($P < 0.01$) as determined by one-way ANOVA versus the control (CTR) on day 7. CLU, clusterin; CTR, control; IL-1 β , interleukin-1 β ; rIgG, rabbit immunoglobulin G; RT-qPCR, quantitative real-time transcription-polymerase chain reaction; sCLU, secreted isoform of clusterin; sGAG, sulfated glycosaminoglycans; TNF- α , tumor necrosis factor α

responses of all three culture models to proinflammatory cytokines. Furthermore, all three culture models exhibited a marked reduction in sCLU release with IL-1 β and TNF- α stimulation. We also showed that in monolayer chondrocytes and cartilage explants, clusterin gene

expression was downregulated following 7-days of stimulation with IL-1 β and TNF- α , which is in line with our previous data showing a reduction in CLU precursor release.¹⁶ The addition of dexamethasone did not affect sCLU release from cartilage explants, which suggests that the loss of clusterin is MMP-3 and 13 independent. In addition, sCLU secretion was just as marked in the osteochondral model despite reduced MMP release, suggesting bone-cartilage cross-talk, and that MMP levels have little influence on the fate of sCLU. Taken together, it is likely that the loss in sCLU secretion from cartilage is due to an interruption at transcriptional level, rather than degradation of extracellular sCLU. It has been reported that transforming growth factor beta (TGF- β) regulates the expression of CLU through the transcription factor c-Fos.⁴² c-Fos represses CLU expression in the absence of TGF- β , and conversely TGF- β inhibits c-Fos, resulting in increased CLU expression.⁴² IL-1 β and TNF- α have both been shown to exert a suppressive effect on TGF- β signaling⁴³ possibly accounting for the loss of sCLU observed in our study.

Clusterin is a protein that is constitutively produced and secreted by mammalian cells. It can function as a cytoprotective chaperone, aiding protein refolding, preventing aggregation under stress conditions, enhancing cell proliferation and cell-viability.⁷ In this study, we show that the proinflammatory cytokines IL-1 β and TNF- α interrupt the expression and secretion of sCLU, and may therefore interfere with the protection it offers to cells. The balance between IL-1 β , TNF- α and TGF- β signaling pathways is crucial for maintenance of articular cartilage homeostasis and its disruption likely plays a key role in the pathogenesis of OA. We propose that this may be, in part, due to the loss of a variety of cytoprotective factors, such as the secreted isoform of clusterin.

Both increases and decreases in clusterin levels have been reported in cartilage explants, synovium, synovial fluid and plasma of patients with OA, depending on the experimental design, and varying disease stage of donor tissue.^{13-15,17,19,20,23} Clusterin is increased at the transcriptional level in early OA cartilage, and decreased in advanced OA.¹⁷ Although detectable, clusterin is significantly reduced in OA samples compared with healthy.^{18,21} Clusterin is increased in plasma, and synovial fluid, from patients with OA, and peptide levels of clusterin have been suggested to be as predictive of OA progression as age²³; and may represent activation of a compensatory, but ultimately ineffective, protective pathway.¹⁵

The in vitro explant model stimulated with proinflammatory cytokines mimics the early events that occur during the onset of disease, the mechanisms of which may precede events observed in early clinical OA samples derived from bio-banks. Our group has previously reported decreased release of clusterin from cartilage explants stimulated with IL-1 β , using high-throughput mass spectrometry, which not only supports the findings in our current study, but also allowed us to develop a new hypothesis implicating sCLU in early catabolic changes.^{4,16} Another published study also showed decreased clusterin release with IL-1 β treatment in mouse femoral head cartilage.⁴⁴ Conversely, increased clusterin release from bovine articular cartilage has been observed following IL-1 β and TNF- α stimulation.⁴⁵

It is important to emphasize the novelty of the observations described in this paper. Only in our proteomic study has the issue of clusterin isoforms been identified.¹⁶ Previous studies have not explored or discussed this issue and therefore there is a need for further molecular studies that focus on clusterin isoforms. It is possible that the differences between the biological actions of sCLU and nCLU explain the current contradictory picture, and increases in nCLU with simultaneous decreases in sCLU may account for the variability in clusterin levels reported in the literature. The nuclear isoform has an antithetical function to sCLU, and acts as a pro-death signal, inhibiting cell growth and survival.^{8,46-48} Cytoplasmic pnCLU is posttranslationally modified in response to cell damage and cytotoxic events, and nCLU translocates to the nucleus where it induces apoptosis.^{6,8} Chondrocyte apoptosis is a key process in OA and is associated with disease progression, with late-stage exhibiting higher levels of cell death.^{49,50} Elevated levels of clusterin in late stage disease^{15,17,19,20,23} could be due to the release of intracellular nCLU following programmed cell death, whereas loss of the protective sCLU could reflect disease onset. It is essential that further future studies are carefully designed and carried out to distinguish between the actions of these two CLU isoforms.

5 | CONCLUSION

This study has, for the first time, identified specifically the secreted isoform of clusterin, sCLU, in three in vitro model systems that are used to model the low-grade inflammatory micro-environment in early OA. We concede that our in vitro models do not incorporate the mechanical signaling that is thought to be involved in early degenerative changes in OA but all three models consistently showed a loss of sCLU secretion with proinflammatory cytokine stimulation. Loss of cytoprotective sCLU in early OA could lead to increased cellular stress, which may promote further catabolic activity and contribute to OA progression. We propose that early OA biomarkers will be extremely challenging to identify if we resort only to prospective clinical studies with bio-banked samples. Ex vivo model systems have a valuable place in biological discovery in the context of OA biomarkers and three-dimensional biomimetic culture models can be used to identify candidate biomarkers for further consideration as investigative biomarkers. Further studies are needed to determine if the sCLU is a potential biomarker for the onset of OA.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Original observations were made by AW during his PhD training supervised by AM and DA. Funding for the original studies was acquired by AM and DA. Funding for the follow-up studies were secured by AM and CM. Acquisition, analysis and interpretation of data was performed by CM, CRF, HQ, AW, and BJ. The first draft of the manuscript was written by CRF, HQ, DA, CM, and AM. All authors made intellectual contributions to the development of the concept, edited the draft and revised the previous versions of the manuscript critically. All authors read and approved the final submitted version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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