DR DAVID WILKINSON (Orcid ID : 0000-0002-6262-3575)

DR KENNETH RANKIN (Orcid ID : 0000-0001-6302-0269)

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Matrix metalloproteinase-13 is fully activated by neutrophil elastase, and inactivates its serpin inhibitor, alpha-1 antitrypsin: Implications for osteoarthritis

David J. Wilkinson[ab#*], Adrian M.D. Falconer[b], Helen L. Wright[a], Hua Lin[b], Kazuhiro Yamamoto[a], Kathleen Cheung[b], Sarah H. Charlton[b], Maria del Carmen Arques[b], Sabina Janciauskiene[c], Ramsay Refaie[b], Kenneth S. Rankin[d], David A. Young[b], Andrew D. Rowan[b]

a - Institute of Life Course and Medical Sciences, William Henry Duncan Building, University of Liverpool, 6 West Derby St, Liverpool, L7 8TX

b - Skeletal Research Group, Institute of Genetic Medicine, Newcastle University, Central Parkway, Newcastle upon Tyne, NE1 3BZ, UK

c - Department of Respiratory Medicine, Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Deutsches Zentrum für Lungenforschung, Hannover Medical School, Hannover, Germany

d - Newcastle Centre for Cancer, Translational and Clinical Research Institute, Paul O'Gorman Building, Framlington Place, Newcastle upon Tyne, NE2 4AD

* - Correspondence should be addressed to Dr David Wilkinson: david.wilkinson@liverpool.ac.uk.

- Current address - Institute of Life Course and Medical Sciences, William Henry Duncan Building, University of Liverpool, 6 W Derby St, Liverpool, L7 8TX

Running title: Neutrophil elastase activates MMP-13

Abbreviations

MMP – Matrix Metalloproteinase; Serpin – serine proteinase inhibitor; AAT- alpha-1 antitrypsin; OA – osteoarthritis; APMA - 4-Aminophenylmercuric acetate; SDS- sodium dodecyl sulphate; Tris - tris(hydroxymethyl)aminomethane; BSA - Bovine serum albumin; HRP- horseradish peroxidase; TPM – transcripts per million; ANOVA – Analysis of variance; SD – Standard deviation; NOF– Neck of femur; OHP – hydroxyproline; GAG – glycosaminoglycan.

Keywords: neutrophil elastase, MMP, SERPINA1, alpha-1 antitrypsin, collagen, osteoarthritis

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Data accessibility statement

The data supporting the conclusions of this article are included within the article (and its additional files). The RNAseq dataset used has been published previously [39] and is publicly available (GSE111358). Data are available from the corresponding author upon reasonable request.

Abstract

Matrix metalloproteinase-13 is a uniquely important collagenase that promotes the irreversible destruction of cartilage collagen in osteoarthritis (OA). Collagenase activation is a key control point for cartilage breakdown to occur, yet our understanding of the proteinases involved in this process is limited. Neutrophil elastase is a well-described proteoglycan-degrading enzyme which is historically associated with inflammatory arthritis, but more recent evidence suggests a potential role in OA. In this study, we investigated the effect of neutrophil elastase on OA cartilage collagen destruction and collagenase activation. Neutrophil elastase induced significant collagen destruction from human OA cartilage *ex vivo*, in an MMP-dependent manner. *In vitro*, neutrophil elastase directly and robustly activated proMMP-13, and N-terminal sequencing identified cleavage close to the cysteine switch at ^{72}MKKPR, ultimately resulting in the fully active form with the neo-N terminus of ^{85}YNVFP. Mole-per-mole, activation was more potent than by MMP-3, a classical collagenase activator. Elastase was detectable in human OA synovial fluid, and OA synovia which displayed histologically graded evidence of synovitis. Bioinformatic analyses demonstrated that, compared with other tissues, control cartilage exhibited remarkably high transcript levels of the major elastase inhibitor, alpha-1 antitrypsin (AAT; gene name *SERPINA1*), but these were reduced in OA. AAT was located predominantly in superficial cartilage zones, and staining enhanced in regions of cartilage damage. Finally, active MMP-13 specifically inactivated AAT by removal of the serine proteinase cleavage/inhibition site. Taken together, this study identifies elastase as a novel activator of proMMP-13 that has relevance for cartilage collagen destruction in OA patients with synovitis.

Introduction

Osteoarthritis (OA) is a highly prevalent, degenerative joint disease which causes significant pain and disability. The progressive and irreversible destruction of the articular cartilage is the central characteristic of the disease. Cartilage is composed of two major components, the highly charged proteoglycan aggregate aggrecan and type II collagen, which is the major structural component of cartilage exhibiting a tightly interlocking triple helix, making it particularly resistant to proteolysis. Due to slow rates of synthesis, the loss of collagen is an essentially irreversible process [1], making it a critical therapeutic intervention point to prevent further cartilage breakdown. The crucial effector proteinases in this destruction are matrix metalloproteinases (MMPs) - specifically a sub-group known as the classical collagenases (MMP-1, -8 and -13). A wealth of evidence implicates MMP-13 (collagenase-3) with OA development and progression [2]. Indeed, this collagenase has the highest efficiency for cleavage of type II collagen [3], and MMP-13-deficient mice are protected from cartilage destruction in experimental OA [4]. However, MMP inhibition has failed clinically, with patients presenting with a range of off-target effects including arthralgia - referred to as 'musculoskeletal syndrome'[5]. This failure is attributed to a lack of inhibitor selectivity, which is a major hurdle, due to a common active-site architecture amongst different MMPs, many of which are considered 'anti-targets' – proteins to be avoided for therapeutic inhibition [6]. Although new generations of MMP-13 inhibitors exhibit markedly improved selectivity [7], none have been clinically successful thus far.

An essential control point in the degradation of cartilage collagen is the activation of proMMPs [8-11] and may represent a novel method to reduce or block cartilage breakdown. Although some MMPs, such as MMP-3 are capable of collagenase activation [3], serine proteinases have long been postulated as putative *in vivo* activators [12, 13] and are more amenable for drug development [14, 15]. These enzymes are often assumed to be cartilage-derived, but the synovium can also be a source of proteinases [16]. Despite historically being referred to as a 'non-inflammatory' arthritis, recent evidence suggests a significant inflammatory component to OA progression, at least within certain sub-groups of patients [17]. Several studies have recently reported that activated macrophages and neutrophils within the synovium contribute to the severity of knee OA, and that the serine proteinase neutrophil elastase may act as a functional biomarker for OA progression in this joint [18-22].

Neutrophil elastase is located primarily in azurophilic granules of polymorphonuclear leukocytes and released upon cell degranulation [23]. The proteinase cleaves preferentially after small hydrophobic residues, and has a range of substrates, and well-established roles such as pathogen digestion and cellular extravasation [24, 25]. The major inhibitor of elastase is alpha-1 antitrypsin (AAT), a member of the serine proteinase inhibitor (serpin) superfamily, encoded by the *SERPINA1* gene. This is perhaps most evident in patients with AAT deficiency, who often present with early-onset emphysema, thought to be driven by

uncontrolled neutrophil elastase activity in the lung [26]. This inflammatory proteinase has been proposed as a drug target in numerous disorders, and a commercially available inhibitor, Sivelestat has been approved for acute respiratory distress syndrome (ARDS), but only in specific jurisdictions [27, 28].

The potent, direct cleavage of cartilage proteoglycan by neutrophil elastase has been demonstrated previously, both *in vitro* and *in vivo* [29, 30]. Indeed, a role for neutrophil elastase in cartilage destruction in rheumatoid arthritis (RA) has long been postulated [31-33]. More recently, it has been demonstrated that neutrophil elastase inhibition reduces chronic pain in mice that have undergone monoiodoacetate injection to induce OA [34], and reduces structural changes in rats following induction of post-traumatic knee OA [35]. Considering these observations, we investigated the effect of neutrophil elastase in OA cartilage collagenolysis, and in particular explored the biochemical interplay with the major collagenase in OA, MMP-13.

Results

Neutrophil elastase induces collagen release from human OA cartilage in explant culture in an MMP-dependent manner.

To investigate the effect of neutrophil elastase on human OA knee cartilage, explant culture experiments were performed. Neutrophil elastase potently and reproducibly induced the destruction of cartilage proteoglycan and collagen. To investigate whether this destruction was direct or indirect, a broad-spectrum MMP inhibitor, GM6001 was utilised. Elastase induced collagen release was markedly reduced in the presence of this inhibitor (Fig.1A) which correlated with reduced MMP activity in the conditioned medium (Fig.1B), whereas proteoglycan release was unaffected (Fig.1C). GM6001 does not affect the activity of neutrophil elastase directly (Fig.1D) and its vehicle control (DMSO) had no effect on cartilage matrix destruction. When the serpin neutrophil elastase inhibitor AAT was also included ECM degradation was abrogated, demonstrating the requirement for elastolytic activity to induce cartilage degradation, both directly (proteoglycan release) and indirectly (collagen release).

Neutrophil elastase activates proMMP-13.

As collagen release was MMP-dependent we hypothesised that neutrophil elastase could activate proMMPs which promote collagenolysis. To determine whether elastase can directly activate procollagenases, *in vitro* incubation experiments were performed using recombinant enzymes followed by an MMP-specific fluorogenic peptide substrate. In this system, neutrophil elastase induced very modest activation of proMMP-1 (Fig.2A) and no activation of proMMP-8 (Fig.2B). However when incubated with proMMP-13 it induced robust activation of the collagenase, to a similar level as the chemical activator APMA (Fig.2C). Remarkably, neutrophil elastase induced approximately 3 fold higher MMP-13 activity than an equimolar

amount of active MMP-3, while matriptase and hepsin failed to significantly activate proMMP-13 (Fig.2D), in line with previous observations [36, 37].

Neutrophil elastase fully activates MMP-13, generating the ^{85}YNVFP neo-N-terminus.

To demonstrate that proMMP-13 was processed into its active form, an activation time-course was performed. Neutrophil elastase rapidly converted proMMP-13 into a 45 kDa species which corresponds to the fully active form as demonstrated by treatment with APMA (Fig.3A+B). APMA also generated an additional band of a lower molecular weight, likely due to continued autoproteolysis of MMP-13. A dose-response experiment was conducted to assess the kinetics of processing in more detail. We observed optimum activation at a 1:5 enzyme to substrate ratio, but processing and increased MMP activity were observed at as low as 1:100. A 1:1 ratio resulted in slightly lower MMP activity, consistent with a reduced level of active MMP-13, suggesting a degree of non-specific proteolysis at this higher concentration (Fig.3C+D). The inclusion of the metalloproteinase inhibitor GM6001, led to the formation of an intermediate species (Fig.3E). N-terminal sequencing was performed to identify cleavage sites. Neutrophil elastase alone generated the ^{85}YNVFP neo-N-terminus, whilst the predominant form in the presence of GM6001 was ^{72}MKKPR (Fig.3F). To demonstrate the activation of proMMP-13 following neutrophil degranulation, blood-purified neutrophils were primed with GM-CSF then subsequently stimulated with a degranulation cocktail of cytochalasin B and the bacterial peptide stimulant fMLP. Neutrophil elastase (Fig.4A) and proteolytic activity against an elastase-specific quenched fluorescent peptide MeOSuc-AAPV-AMC (Fig.4B) were detectable in supernatants from degranulated neutrophils. When degranulation supernatants were incubated with proMMP-13 they induced rapid activation, as determined by an MMP-specific fluorogenic assay and a shift to the active 45 kDa form, an effect that was abrogated by the inclusion of AAT (Fig. 4C +D).

Neutrophil elastase is detectable in inflamed OA synovium and OA synovial fluid.

Neutrophil elastase is poorly expressed in cartilage [38, 39], so to determine whether neutrophil elastase could be detected in the OA joint, an immunohistochemical examination of human OA synovial tissues was performed. In total, 17 human OA synovia were scored histologically using a previously described synovitis scoring system [37, 40]. Neutrophil elastase was virtually undetectable in tissues with no synovitis but was detectable in invading leukocytes and in blood vessels of samples categorised as having ‘slight’ or ‘moderate’ synovitis. MMP-13 however was detectable at all levels of synovitis, although staining was observed predominantly in fibroblasts in no/low synovitis samples and often in invading leukocytes in higher grades (Fig.5A). To determine the presence of neutrophil elastase and MMP-13 in OA synovial fluid, ELISAs were performed on fluid which was taken at the time of joint arthroplasty. Neutrophil elastase was detectable in all OA synovial fluids tested although this varied significantly

between patients (mean (±SD): 6374 (± 5745) pg/ml, n=8; Fig.5B). MMP-13 was detectable in all except 3 OA synovial fluid samples (mean (±SD): 584 (± 760) pg/ml, n=8; Fig.5C). In this small sample size, neutrophil elastase did not correlate with MMP-13 (Fig.5D) and neither proteinases significantly correlated with erthyrocyte sedimentation rate (ESR), a marker of systemic inflammation (Fig.5E+F).

The neutrophil elastase inhibitor, AAT is abundant in cartilage and is observed predominantly in the superficial layer.

Serine proteinase activity is regulated by endogenous inhibitors, the largest family of which are the serpins. To determine relative serpin expression in human cartilage we interrogated our previously published RNAseq data (GSE111358; [39]). In non-OA control human hip cartilage, several serpins were highly abundant, with 10 serpins having transcripts per million (TPM) counts of 10 or more. Of these, the *SERPINA1* gene, encoding AAT, the major neutrophil elastase inhibitor, was particularly abundant with a TPM count exceeding 7000. Indeed, *SERPINA1* was the 9th most abundant transcript within this dataset (Fig.6A). Using the human protein atlas, cross-tissue expression levels were determined. Interestingly, *SERPINA1* expression was markedly enriched in cartilage, second only to expression within the liver (Fig.6B). When comparing macroscopically intact hip OA cartilage to control non-OA (NOF fracture) hip cartilage, levels of *SERPINA1* transcript were markedly downregulated (p_{adj}=0.004; Fig.6C). To determine specific localisation of the AAT protein within human cartilage, immunohistochemistry revealed that the staining was particularly strong in the upper regions of the cartilage matrix, consistent with expression predominantly in the superficial cartilage layer. In undamaged cartilage the staining was principally localised to the chondrocyte, but interestingly, staining intensity was markedly increased in areas of damaged ECM, with intense AAT staining in the most damaged areas of OA cartilage (Fig.6D).

MMP-13 inactivates AAT at two sites, removing the reactive-centre loop

MMPs have previously been demonstrated to inactivate AAT by removing the reactive-centre loop required for irreversible inhibition of target proteinases [41]. Here, we demonstrate that *in vitro* incubation of AAT with active MMP-13 results in the generation of a single protein band - even after a 16-hour incubation - with a lower molecular size than that of native AAT (Fig.7A), and AAT is no longer able to inhibit neutrophil elastase (Fig.7B). A time-course experiment was performed, and products separated using tris-glycine (protein) or tris-tricine (peptide) SDS-PAGE (Fig.7C). N-terminal sequencing revealed that MMP-13 cleaved AAT at two sites, ^{377}LEAIP and ^{382}MSIPP (Fig.7D), upstream of the canonical neutrophil elastase cleavage site. Thus, active MMP-13 induces a specific inactivation of AAT, removing the inhibitory bait region of the serpin.

Discussion

In this study we demonstrate that neutrophil elastase is a potent activator of the major OA collagenase, MMP-13. Historically described as non-inflammatory arthritis, it is becoming clear that OA is a disease of the whole joint, and that inflammation contributes to the disease processes, at least in a significant subgroup of patients [16]. The importance of the synovium in OA is becoming increasingly apparent, making factors often thought of having a role only in inflammatory arthritis progression - including proteinases - of renewed interest for study in OA.

Serine proteinases have been shown to be important in the proteolytic cascades leading to cartilage destruction previously [8, 9, 12, 36, 37, 42], although identifying the specific proteinases involved is challenging. Indeed, the conversion of proMMP-13 into its active form is an essential, yet often overlooked, control point for the breakdown of cartilage collagen in OA. To our knowledge, the only described direct serine proteinase activators of MMP-13, are trypsin [3] and plasmin [43], with the former lacking physiological relevance to the joint, while the latter has a complex and debated role in arthritis [44]. This is the first study to describe the direct and full activation of proMMP-13 by neutrophil elastase. This serine proteinase has often been linked to the pathophysiology of RA and has been previously shown to be present in patient synovial fluid [45-47]. Indeed, systemic administration of human plasma AAT, reduced arthritis scores and joint destruction in murine collagen-induced arthritis [48], and mice deficient for neutrophil elastase and the related proteinase cathepsin-G, are also protected in this model [32]. As a relatively broad-spectrum serine proteinase the role of neutrophil elastase has been considered one of matrix destruction, and it has been shown to directly degrade proteoglycans [30, 49]. The data in our study are consistent with this, as neutrophil elastase-induced proteoglycan release in human OA cartilage was insensitive to metalloproteinase inhibition, and therefore direct. Elastase has previously been shown to directly cleave type III collagen *in vitro*, but was unable to cleave type I collagen [50]. The hydroxyproline assay is a sensitive and specific way to measure collagen breakdown from cartilage in explant culture [37, 42, 51]. Cartilage is composed overwhelmingly of type II collagen (>90%; [52]), but does include other minor collagens such as type IX and type XI, which elastase has been shown to degrade directly, at least *in vitro* [53]. In our study we observed up to 15% collagen release from human OA cartilage, which was mediated by metalloproteinase activity, not the direct activity of elastase. The unique structure of triple-helical collagen makes it susceptible to significant proteolysis from only a very restricted number of collagenases, likely due to important interactions of exosites required for unfolding of the triple-helix [54, 55]. Although proteoglycan breakdown by neutrophil elastase is well established, the finding that this proteinase can induce MMP-dependent collagen degradation is important, as this is an irreversible step in the process of joint destruction [1]. Indeed, explant data in this study indicate that a pool of latent collagenases exist within diseased OA cartilage, something which has been observed in other studies through the addition of MMP activators to the diseased tissue [37, 56]. Correlation between elastase

activity and collagen neo-epitopes generated by a collagenase has been observed in the synovial fluids of patients with non-infectious knee joint synovitis previously [57]. Although neutrophil elastase has been shown to be an activator of other MMPs [13, 58], here we demonstrate that it is a direct activator of the collagenase most commonly associated with OA, MMP-13. [12]. Under the described experimental conditions, neutrophil elastase was unable to significantly activate proMMP-8 while providing only modest activation of proMMP-1, further supporting a role for MMP-13 in neutrophil elastase-induced collagen destruction. Degranulation experiments further demonstrated a rapid activation of proMMP-13, which could be blocked in the presence of AAT. Neutrophil degranulation products have previously been shown to induce cartilage breakdown in porcine cartilage explants, with neutrophil elastase implicated in the process [59]. Neutrophil elastase was detectable in all synovial fluid samples tested. While these levels are likely to be significantly lower than concentrations observed in RA [45], it is important to recognise that OA is disease of slow progression. Furthermore, the timing of sample collection will likely be of critical importance. For example, levels of neutrophil elastase have been shown to be high immediately after sports injury, but then gradually reduce over a 90 day period [60]. Periodic, localised inflammation could contribute to temporary release of neutrophil elastase into the OA joint, which could contribute to collagen degradation through MMP-13 activation.

The serpins are a superfamily of structurally similar proteins, the majority of which inhibit serine proteinases, and we have recently described their emerging role in cartilage [61]. In this study, we have identified remarkably high transcript expression levels of *SERPINA1* gene in human cartilage. Indeed, others have demonstrated high levels of the AAT protein within synovial fluid previously [62, 63]. This serpin is a serum protein with levels in the blood of approximately 0.9-2 mg/ml [64], the vast majority of which is produced in the liver [65]. Here, we show that levels of *SERPINA1* transcript in human cartilage are second only to those in the liver. An interesting study which used microdissection and iTRAQ proteomics to investigate the protein composition of human cartilage layers found AAT to be mainly located in the upper levels of cartilage [66]. Indeed, this is something we also observed in our current study. It is important to recognise that AAT has multiple proteinase targets, although neutrophil elastase is by far the most kinetically favourable (K_a = 6.5 x10^7 $M^{-1}s^{-1}$; [24, 67]). It is plausible that AAT may act as a 'first line of defence' against damaging immune cell-derived serine proteinases following degranulation. We also observed that AAT localises to damaged cartilage regions which requires further investigation. Possible reasons could include a localised damage response or that greater levels of AAT from the synovial fluid are able to penetrate the cartilage following significant degradation of the proteoglycan matrix (as evidenced by loss of safranin-O staining in these areas).

High *SERPINA1* expression in cartilage raises important questions about the physiological relevance of neutrophil elastase deposition from an inflamed synovium. Indeed, using the total protein ELISAs undertaken in this study, it is not possible to determine the level of active neutrophil elastase or the level of functional AAT protein. However, it has been previously demonstrated that cartilage-bound neutrophil elastase is protected from the activity of proteinase inhibitors [68]. Moreover, serpins can be inactivated by other proteinases, as well as by oxidation, both of which have been demonstrated in the RA joint previously [69-71]. These mechanisms suggest total serpin levels may not be representative of the effective inhibitor concentrations within the joint. In this study, we describe the inactivation of AAT by MMP-13. Whilst this finding is novel, it is likely that *in vivo,* several MMPs may contribute to the inactivation of this inhibitor. Indeed, this serpin has been shown to be inactivated by MMP-1, MMP-2 and MMP-3 previously and the inactivated form has been observed in the synovial fluid of arthritis patients [70, 71]. Here we have identified cleavage sites generated by MMP-13 which correspond to regions N-terminal of the canonical cleavage site of neutrophil elastase (M^{382} is the neo-N-terminus generated following this cleavage/inhibition). Both sites have been identified as cleavage sites for other MMPs [71]. Future studies could involve the generation of neo-epitope antibodies which specifically recognise MMP-cleaved AAT. Such antibodies could be used to determine the level of AAT inactivation within the synovial joint of OA patients and the likely proteolytic source. Alternatively, the relative levels of different AAT (cleaved/uncleaved or complexed) could be determined by mass spectrometry approaches. The disarming of cognate inhibitor(s) will make joints more susceptible to the damaging effects of serine proteinases which present during periods of localised inflammation. A working hypothesis regarding the interplay between MMP-mediated AAT inactivation and proMMP-13 activation by neutrophil elastase is outlined in Fig.7E. A corollary of this hypothesis is the crucial role of functional AAT in mediating joint protection. To our knowledge, studies investigating the susceptibility of cartilage for degradation in OA (or indeed other arthritic diseases), in patients with alpha-1 antitrypsin deficiency has not been thoroughly investigated previously. This too should be the focus of future endeavours. The high level of *SERPINA1* expression within cartilage suggests mutations within the *SERPINA1* gene (rendering a weaker/non-functional AAT) may have significant consequences for the integrity of the tissue during periods of inflammation.

There are limitations to this study. Although our observations regarding cleavages (and cleavage sites) are novel and important, we must be candid about extrapolating biochemical observations to the OA joint. To determine pathophysiological importance of neutrophil elastase on MMP-13 activation in OA, future studies would need to: 1) determine of the level of *active* neutrophil elastase in the OA joint; 2) assess how this compares to the level of proMMP-13 in the cartilage; 3) establish the degree of AAT inactivation and therefore the viable inhibitor pool in the OA joint; 4) How these change with stage and severity of OA (and the presence or absence of synovitis). Larger cohorts of human clinical samples and the use of different

preclinical models of OA will prove important in this regard. Recently, Kaneva and colleagues demonstrated that AAT protected cartilage in two animal models of inflammatory arthritis, including one induced by neutrophil elastase [72]. Our study both supports and compliments these observations and provides novel insights into the proteolytic interplay between AAT, neutrophil elastase and MMP-13, with potential consequences for the destruction of OA cartilage. AAT can have a role beyond proteinase inhibition [61], however, and the authors conclude that AAT was not only chondroprotective but also anti-inflammatory and has a significant effect on pain [72]. This study and our own highlight the need to also investigate the effects of AAT administration using *in vivo* models of OA, with direct quantification of cartilage degradation, pain and inflammation.

The importance of synovial inflammation in OA progression is contested, and we agree with the central role of dysregulated chondrocyte biology in OA cartilage destruction. However, we and others have demonstrated a variable degree of synovitis in OA patients (reviewed in [73]), and in these instances, it is credible that neutrophil elastase - given its activation potency and ability to generate a fully active MMP-13 - will contribute to OA cartilage collagen destruction through direct activation of latent MMP-13. The search for *in vivo* activators of MMP-13 is important, particularly as this represents a key control point for cartilage destruction and may represent a novel approach for therapy. The rapid, full activation of MMP-13 by neutrophil elastase may have significance in a subset of OA patients where an inflamed synovium contributes to the progression and exacerbation of the disease process.

Materials and Methods

Reagents

Unless stated otherwise all reagents were of the highest purity and purchased from Sigma Aldrich (Gillingham, UK). Human neutrophil elastase (EC 3.4.21.37) purified from human sputum was purchased from Elastin Products (SE663; MO, USA). AAT protein was purified as described previously [74]. Human recombinant proMMP-1 (E.C. 3.4.24.7) and proMMP-13 (E.C. 3.4.24.B4) were expressed using an insect cell expression system (and purified) as previously described [11, 37]. Recombinant human proMMP-8 (E.C. 3.4.24.34) was purchased from R&D Systems (908-MP-010; Abingdon, UK). Recombinant human active MMP-3 EC (3.4.24.17) was expressed and purified as previously described [75]. Recombinant human matriptase (E.C. 3.4.21.109) and hepsin (E.C. 3.4.21.106) were expressed and purified as previously described [37]. Recombinant human full-length tissue-inhibitor of metalloproteinase (TIMP)-1 was a kind gift from Celltech Pharmaceuticals, Slough, UK. Elastase fluorogenic substrate (CAS 72252-90-5), MMP fluorogenic substrate (FS-6; SCP0193) and GM6001 were purchased from Merck Millipore (Watford, UK). Neutrophil elastase (ab68672) and MMP-13 (Ab75606) antibodies were from Abcam (Cambridge, UK). MMP-13 and neutrophil elastase DUOset ELISA kits were from R&D systems. Ficoll-Paque was from GE

healthcare (GE Healthcare, Chalfont St Giles, UK). Cytochalasin B and f-Met-Leu-Phe (fMLP), were from Sigma Aldrich. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was from Roche Applied Biosciences (Burgess Hill, UK).

Human tissue and cartilage explant culture

All tissue was taken with informed consent from patients undergoing total joint replacement at the Freeman Hospital in Newcastle upon Tyne. All OA tissues (cartilage, synovium and synovial fluid) were handled according to Newcastle University guidelines with ethical approval from NHS Health Authority NRES Committee North East – Newcastle and North Tyneside (REC14/NE/1212). Neutrophils were isolated from consenting healthy donors at the University of Liverpool, approved by the University of Liverpool Research Ethics Subcommittee for physical interventions (Ref: 1672). For cartilage explant cultures, macroscopically intact human OA cartilage was cut from the tibial plateau (approximately 2 mm^3 pieces) and cultured overnight in 24 well plates (3 chips per well) in serum-free Dulbecco's Modified Eagle's Medium (DMEM) (supplemented with glutamine (2 mM), penicillin/streptomycin (200IU/ml and 200 µg/ml, respectively) and nystatin (40 IU/ml)). The following day, explants were stimulated with neutrophil elastase for the time period and concentrations indicated. At the end of the experiment, medium was harvested and remaining cartilage digested with papain as previously described [51]. For both medium and digested cartilage, hydroxyproline (OHP) assays were performed to assess percentage collagen destruction, whilst proteoglycan destruction was assessed by measuring glycosaminoglycan (GAG) levels using dimethyl methylene blue (see [51] and references therein).

In vitro incubations, SDS-PAGE and N-terminal sequencing

For *in vitro* incubation experiments, neutrophil elastase (20 nM) was incubated with proMMP-13 (100 nM) in 'reaction buffer' (100 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.01% (w/v) Brij35) for the indicated time points at 37°C, in a final volume of 20 µl. 4-Aminophenylmercuric acetate (APMA) was used a positive control (0.67 mM). For inactivation experiments, AAT (50 nM) was incubated with MMP-13 (10 nM), APMA, or both for 16 hours at 37°C. The incubation products (10 µl) were subsequently incubated with neutrophil elastase to determine inhibitory capacity (see below for assay details). For protein gels, digestion products (10 µl for AAT inactivation and 20 µl for proMMP-13 activation experiments) were mixed with 5x sample buffer (625 mM Tris HCl pH 6.8, 10% (w/v) SDS, 40% (v/v) glycerol, 0.5% (w/v) bromophenol blue, 3% (v/v) ß-mercaptoethanol) and separated by 10% SDS-PAGE as described previously [37]. Gels were subsequently stained using a PlusONE silver staining kit according to the manufacturer's instructions (GE healthcare). For low molecular mass products of AAT digests, peptides were detected by separating reaction products by Tris-Tricine SDS-PAGE as described previously [76]. For N-terminal sequencing (both activation and inactivation experiments), gels were blotted onto PVDF and

stained with Coomassie brilliant blue for 2 minutes. Bands were excised with a clean scalpel blade and sent for N-terminal sequencing (Alphalyse, Denmark).

Enzyme activity assays

To test MMP activation, proMMP-1, -8 and -13 were incubated with elastase as described above, for 4 hours. As a negative control, an excess of AAT (1.14 mg/ml) was included, which demonstrated the requirement for elastase activity. Incubation products (5 µl) were added to white-walled plates and diluted to 80 µl in MMP assay buffer (reaction buffer with 0.1% (v/v) polyethylene glycol 6000). MMP fluorogenic substrate (FS-6) was added to start the reaction at a final concentration of 10 µM, to a total well volume of 100 µl. Reaction velocity was calculated by linear regression of initial rates of progress curves. For mole-per-mole comparison experiments: neutrophil elastase was titrated with freshly resuspended AAT protein (Merck Millipore; 178251); MMP-3 was titrated with full-length TIMP-1; while matriptase and hepsin were titrated with 4-methylumbelliferyl 4-guanidinobenzoate (MUGB). ProMMP-13 (100 nM) was incubated with 20 nM of each enzyme for 4 hours in the reaction buffer described above prior to conducting the MMP activity assay. To measure MMP activity in human OA cartilage conditioned media, 50 µl of medium was used with FS-6 substrate as described above. To test AAT inhibitory capacity following MMP-13 incubation, elastase activity assays were performed in a similar manner using elastase activity buffer (100 mM Tris pH 7.6, 150 mM NaCl, 0.01% (w/v) Brij 35) and an elastase fluorogenic substrate (MeOSuc-AAPV-AMC) at a final concentration of 20 µM. Neutrophil elastase was used at a final concentration of 5 nM.

Neutrophil isolation, degranulation and MMP-13 activation

Neutrophils (purity typically >97%, assessed by cytospin) were isolated from heparinised whole blood using HetaSep and Ficoll-Paque as previously described [77] and resuspended at $5x10^6$ cells/ml in 1 ml of RPMI 1640 media supplemented with HEPES (25 mM) and L-glutamine (2 mM). Neutrophils were incubated with GM-CSF (5 ng/ml) for 30 min followed by cytochalasin B (6 µg/ml) and f-Met-Leu-Phe (fMLP, 1 µM) for a further 30 min. Following incubation, cells were pelleted at 1000 x g for 3 min and supernatant decanted into clean tubes. Degranulation products (supernatant; 5 µl) from either treatment were incubated with proMMP-13 (100 nM) for 2 hours at 37°C. Controls of the degranulation stimulants (Cytochalasin/GM-CSF/fMLP mix in the absence of neutrophils) were also incubated with proMMP-13. AAT (50 nM) was included as a negative control. After the incubation, 5 µl was added to a white-walled 96-well plate and an MMP-activity assay was undertaken as described above. Data are presented as mean ± standard deviation using 3 independent donors of neutrophils. For SDS-PAGE, 10 µl of this incubation was

mixed with 5x sample buffer (as above), boiled, and kept at -20°C until the products were separated by SDS-PAGE and western blotting performed (see below).

Western Blotting

For western blotting, samples were separated by SDS-PAGE using 10% (w/v) precast acrylamide gels and transferred onto Polyvinylidene fluoride (PVDF) using the Transblot Turbo apparatus (BioRad, Watford, UK). Blots were blocked in 5% (w/v) non-fat dry milk for 1 hour, washed in tris-buffered saline-Tween20 (TBS-T), prior to overnight incubation at 4°C with an MMP-13 antibody (sc-30073, Santa Cruz, USA), diluted 1:2000 in 5% (w/v) bovine serum album (BSA). Blots were then further washed in TBS-T prior to incubation with an anti-rabbit secondary (GE healthcare, Bucks, UK) at 1:2000 dilution in milk for 1 hour. Blots were washed again in TBS-T and detected using enhanced chemiluminescent substrate (ECL; ThermoFisher, Loughborough, UK).

Enzyme-Linked Immunosorbent assay (ELISA)

Levels of MMP-13 and neutrophil elastase in human OA knee synovial fluids (8 samples; 6 females/2 males; age 69.5±10.4) were determined by ELISA according to the manufacturer's instructions (R&D Systems). Briefly, Maxisorp plates (ThermoFisher) were incubated with coating antibody overnight at 4ºC. Plates were washed in phosphate buffered saline (PBS) with Tween (0.1% v/v) and blocked in 300 µl of 1% (w/v) BSA in PBS for 1 hour. Plates were washed and standards and samples (100 µl; neat or diluted in PBS) were loaded onto the plates and incubated overnight. Plates were washed and detection antibody diluted in PBS with 0.1% (w/v) BSA added to the wells. Plates were washed and streptavidin-horseradish peroxidase (HRP) diluted in PBS with 0.1% (w/v) BSA. Colour development was initiated by the addition of o-phenylenediamine dihydrochloride substrate solution, the reaction stopped with 3M HCl and the absorbance read at 490 nm.

Histology and Immunohistochemistry

Human synovial tissues (17 patients, 14 Female/3 male, mean age = 65 years ± 9.2) were fixed in 10% formalin, embedded in paraffin and sections were taken at 4 µm intervals. Haematoxylin and eosin (H&E) staining was performed as previously described [11] and synovitis scored according to a previously described system [37, 40]. Immunohistochemistry was performed essentially as previously described [37], except antigen retrieval was performed using a mini autoclave (Aptum 2100 retriever; Aptum, Southampton, UK) for 30 minutes. Primary antibodies for neutrophil elastase and MMP-13 were used at 1:100 dilution (both 10 µg/ml) for 1 hour, and staining conducted using Vectastain Elite ABC HRP Kit according to the manufacturer's instructions (Vector Laboratories; CA, USA). To visualise AAT human

OA cartilage samples from patients undergoing total hip replacement (mean age 61.3 ± 7.5, 3 x Female) or from hip replacement following fracture (NOF; mean age 74 ± 8.4, 1 x male, 1 x female) were fixed, embedded and sectioned as described above. Serial sections were stained with safranin-O or underwent IHC staining using an antibody against AAT protein (16382-1-AP; Proteintech, Manchester, UK) at 1:150 dilution (3.2 µg/ml) for 2 hours, followed by signal detection using Vectastain Elite ABC HRP Kit. In all cases, sections treated in the same manner in the absence of a primary antibody, or with an isotype control antibody (R&D systems; AB-105-C) at the same concentration as the corresponding primary antibody, served as negative controls. Images were taken using a Leica Biosystems DM4000B light microscope.

Bioinformatic analyses and protein visualisation

Transcripts per million (TPM) values in control cartilage (NOF) samples were generated using Salmon as described [39] whilst Human Protein Atlas TPMs were retrieved from the Human Protein Atlas ([78]; www.proteinatlas.org). R software was used to generate figures from RNA sequencing data in Figures 6A and 6B. For structural images, AAT was visualised using PyMol software (version PyMOL v1.7.6.6 Enhanced for Mac OS X) using the structure of the intact protein (Protein Data Bank (PDB) ID 1QLP; [79]).

Statistics

Statistical analyses where multiple comparisons are required were performed using a one-way ANOVA with a Tukey's post-hoc test. In all cases, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and data are presented as mean ± standard deviation (SD). RNAseq statistical analysis was performed using DESeq2 where p-values were adjusted using the Benjamini-Hochberg method which controls for the false discovery rate (<0.05).

Declarations

Ethics approval and consent to participate

All tissue was taken with informed consent from patients undergoing total joint replacement at the Freeman Hospital in Newcastle upon Tyne. All OA tissue (cartilage, synovium and synovial fluid) was handled according to Newcastle University guidelines with ethical approval from NHS Health Authority NRES Committee North East – Newcastle and North Tyneside (REC14/NE/1212). Neutrophils were isolated from healthy donors at the University of Liverpool. This study was approved by the University of Liverpool Research Ethics Subcommittee for physical interventions (reference 1672). All participants gave written, informed consent in accordance with the declaration of Helsinki.

Author contributions

Conceptualisation; DJW, ADR; investigation: DJW, AMDF, HLW, HL, KY, KC, SLC, MdCA, , SJ, RR, KSR, DAY, ADR; formal analysis: DJW, AMDF, HL, KC, ADR; writing-original draft: DJW, AMDF, ADR; writing-review and editing: DJW, AMDF, HLW, HL, KY, SLC, MdCA, SJ, RR, KSR, DAY, ADR; Supervision: DJW, KSR, DAY, ADR; Funding acquisition: DJW, DAY, ADR

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Figure Legends

Figure 1. Neutrophil elastase-induced collagen release is MMP-dependent

Neutrophil elastase (NE) was added to human OA cartilage for a period of 7 days in the presence or absence of the MMP inhibitor GM6001 (10 μM), DMSO vehicle control (0.02% v/v) or AAT protein (100 μg/ml), after which the medium was removed and remaining cartilage digested with papain as described previously [51]. A) Collagen breakdown was determined by hydroxyproline assay. B) MMP activity was determined in conditioned medium using an MMP-specific quenched fluorescent peptide. C) Proteoglycan release was determined by measurement of sulphated glycosaminoglycan (GAG) using a DMMB assay. D) GM6001 or its vehicle DMSO had no effect on neutrophil elastase activity, as determined in vitro using the elastase specific quenched-fluorescent peptide, MeOSuc-AAPV-AMC. Matrix breakdown is expressed as a percentage of the total, which is determined by level of GAG or hydroxyproline in the conditioned medium and the remaining papain-digested cartilage. Cartilage matrix degradation by elastase is representative of at

least three independent experiments, while inclusion of the metalloproteinase inhibitor, GM6001 was conducted twice. Data are presented as mean ± SD (n=4) with statistical analyses performed using a One-way ANOVA with Tukey's post-hoc test where *= $p<0.05$, **=$p<0.01$ and ***= $p<0.001$; ns = not significant.

Figure 2. Neutrophil elastase potently activates MMP-13 *in vitro*

Neutrophil elastase (NE) was incubated with A) proMMP-1, B) proMMP-8 and C) proMMP-13 in a 1:5 enzyme to substrate ratio at 37°C for 4 hours. Activation with the chemical activator APMA (0.67 mM) was used as a positive control, whilst neutrophil elastase in combination with an excess of AAT (1.14 mg/ml) served as a negative control, demonstrating the requirement of elastolytic activity. After 4 hours, MMP activity was determined using MMP fluorogenic substrate (final MMP concentration was 5 nM). D) Mole-per-mole comparisons of neutrophil elastase with MMP-3, matriptase and hepsin for their MMP-13 activation capability. Reaction velocities were calculated by linear regression of progress curves. Data are presented as mean ± SD of 3 independent experiments with statistical analyses performed using a One-way ANOVA with Tukey's post-hoc test where *** = $p<0.001$ compared to proMMP alone, ### = $p<0.001$ compared to proMMP-13+NE, ns = not significant compared to proMMP alone (n=3).

Figure 3. Neutrophil elastase cleavage results in an N-terminus indicative of a fully active MMP-13.

A) For time course experiments, neutrophil elastase (NE) was incubated with proMMP-13 in a 1:5 enzyme substrate ratio in a final volume of 20 µl for the time points indicated. Reactions were stopped by the addition of 5x sample buffer and the products separated by SDS-PAGE and stained with silver. B) Densitometry of the activation timecourse calculated as percentage of active MMP-13 compared to proMMP-13 at each timepoint. Data presented as mean ± SD from three independent experiments. C) Dose response experiments were conducted over 4 hours with 1:1000, 1:100, 1:50 ,1:20, 1:10, 1:5 and 1:1 neutrophil elatase:MMP-13 ratios, followed by silver stain (C) and MMP activity assay (D). E) The inclusion of the broad-spectrum metalloproteinase inhibitor GM6001 was used to determine direct and indirect cleavage. F) To identify cleavage sites, similar experiments were performed, with products separated by SDS-PAGE and blotted onto PVDF. Blots were briefly stained with Coomassie and both the full cleavage product (*full*) and the intermediate species (*int*) generated in the presence of GM6001 were subject to analysis by N-terminal sequencing. Sequencing gave 5 unambiguous amino acid reads at each site which correspond to the cleavage sites as depicted. Solid arrow corresponds to the final N-terminus in the absence of metalloproteinase inhibition, whereas the dashed arrow corresponds to cleavage by neutrophil elastase alone in the absence of metalloproteinase activity (i.e. in the presence of metalloproteinase inhibitor, GM6001).

Figure 4. MMP-13 is activated by neutrophil degranulation products

Purified neutrophils were primed with GM-CSF for 30 minutes, stimulated with cytochalasin B + fMLP for a further 30 minutes, and supernatants harvested. A) Neutrophil elastase was detected in supernatants from degranulated neutrophils and elatase activity could be detected in these samples using an elastase-specific quenched fluorescent peptide, MeOSuc-AAPV-AMC (B; timepoints shown as mean ± SD). Degranulation products were incubated with proMMP-13 for 2 hours for donors 1 and 3 (blue and red, respectively) and 30 minutes for donor 3 (green), due to high proteolytic activity in degranulated neutrophils this donor. MMP activity was measured with an MMP-specific quenched fluorescent substrate (FS-6; C, upper panel) and neutrophil elastase activity was also determined using an MeOSuc-AAPV-AMC (C, lower panel). MMP-13 activation was further demonstrated using western blotting (D, upper panel), and a neutrophil elastase western blot was performed to act as a loading control (D, lower panel). Treatment with stimulation cocktail alone did not cause MMP-13 to be activated, and inclusion of AAT blocked processing of proMMP-13. Activity shown from 3 independent donors (bars represent mean ± SD) while the western blots are representative of all 3 donors. Statistical analyses of MMP activity were performed using a One-way ANOVA with Tukey's post-hoc test where ***= $p<0.001$ compared to proMMP-13 alone, ###=$p<0.001$ compared to proMMP-13 with degranulation products (n=3).

Figure 5. Neutrophil elastase is detectable in OA synovial tissue and synovial fluid

A) Human synovial tissue from OA patients (N=17, 14 Female/3 male, mean age = 65 years ± 9.2) was embedded and sectioned. Sections of different depths were stained with haematoxylin and eosin and the level of synovitis scored according to a previously described scoring system [40] where samples were categorised as having no synovitis (0-1), slight synovitis (2-3), moderate synovitis (4-6) or strong synovitis (7-9). Immunohistochemistry was performed using antibodies to neutrophil elastase and MMP-13. Sections where no primary antibody was included, as well as an isotype control antibody at the same concentration as primary antibody, served as negative controls. Neutrophil elastase was absent from samples categorised as having 'no synovitis' (synovitis score <1; 0/3), but present in the majority of samples with a synovitis score >1 (11/14). MMP-13 was detectable in all synovial samples regardless of synovitis grade. Synovial fibroblasts are indicated with red arrows, invading leukocytes with green arrows and blood vessels with orange arrows. Neutrophil elastase mainly stained positive in leukocytes and blood vessels. MMP-13 was detectable in fibroblasts and invading leukocytes. Images taken at 40x magnification and scale bars represent 50 μm. In synovial fluid samples from different patents, levels of neutrophil elastase (B) and MMP-13 (C) were determined by sandwich ELISA. Data points are from 8 individual donors (N=8, 6 females/2 males; age 69.5±10.4) with bars representing mean ± SD. D) MMP-13 levels did not correlate with neutrophil elastase levels, and neither proteinase significantly correlated with erthyrocyte sedimentation rate (ESR; E and F).

Figure 6. *SERPINA1* gene expression is abundant in human cartilage and the alpha-1 antitrypsin (AAT) protein localised predominantly in the superficial layer.

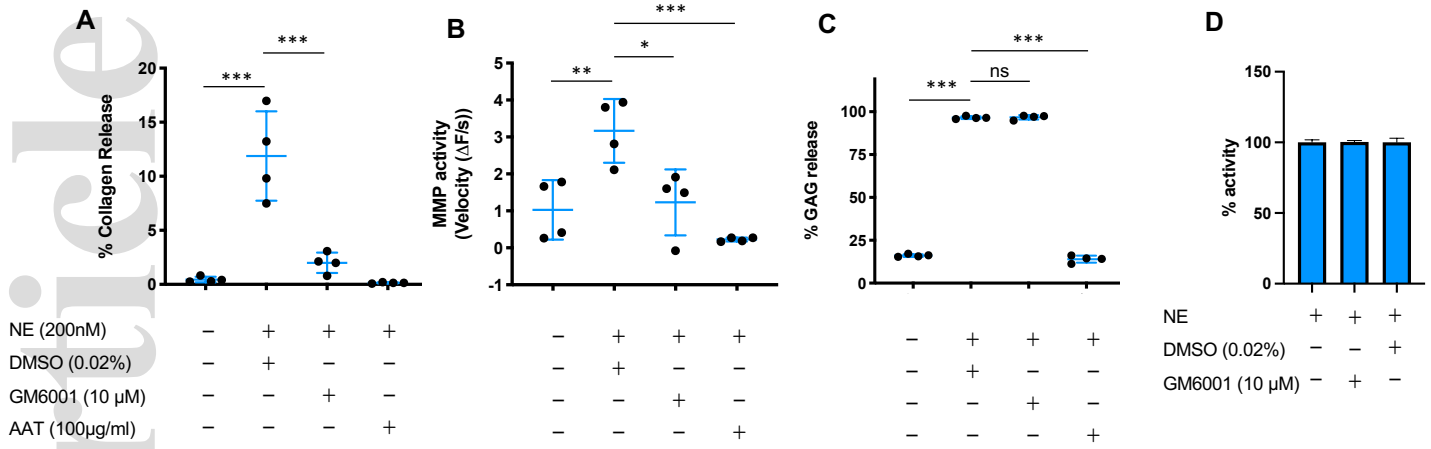
A) The top 50 most abundant genes within a control non-OA cartilage dataset (GSE111358). When ordered by transcript per million (TPM), *SERPINA1* is the 9th most abundant transcript in hip cartilage. B) Comparison of *SERPINA1* expression in human tissues using TPM values in control non-OA (neck of femur fracture; NOF) hip cartilage samples were generated using Salmon as described [39] and Human Protein Atlas TPMs were retrieved from the HPA website [78]. C) *SERPINA1* transcript expression is significantly reduced in macroscopically intact OA cartilage compared to intact control NOF fracture cartilage. Data shown as individual TPM from each donor with bars representing mean ± SD. Adjusted p-value shown is calculated by DESeq2 analysis using a Benjamini post-hoc test. D) Immunohistochemistry of damaged regions from cartilage samples (both OA and NOF controls) shows that AAT staining localises to areas of damage and staining intensity increases with level of cartilage destruction. Sections where no primary antibody was included, as well as an isotype control antibody at the same concentration as primary antibody, served as negative controls. Cartilage proteoglycan loss was determined by safranin-O (Saf-O) staining. AAT is localised predominantly to chondrocytes in the superficial and upper middle zone of the tissue. Images taken at 20x magnification and scale bars represent 100 μm.

Figure 7. MMP-13 inactivates alpha-1 antitrypsin (AAT)

A) Human purified AAT was incubated with proMMP-13 in a 1:5 ratio for 16 hours at 37ºC in a final volume of 20 μl. ProMMP-13 was activated by APMA (0.67 mM). Products were separated by SDS-PAGE (10 μl) and stained with silver. B) In similar experiments, 5 μl of incubation products was added to activity assays, in which neutrophil elastase (NE; 5 nM) was incubated with an elastase specific fluorogenic substrate (20 μM). Reaction velocities were calculated from linear progress curves of 3 independent experiments, and data plotted as mean ±SD. Statistical analysis calculated by One-Way ANOVA with a Tukey's post-hoc test where *** = $p<0.001$ (n=3). C) A time course experiment was performed and incubation products were separated by both (i) 10% tris-glycine (protein) and (ii) 20% tris-tricine (peptide) SDS-PAGE. All gels and activity assays are representative of three independent experiments. D) Cleavage sites were identified by N-terminal sequencing of the peptide products. MMP-13 cleaves upstream of the neutrophil elastase (NE) cleavage site in two positions, disarming the inhibitor, as depicted in both protein sequence (upper panel) and structural view (lower panel). SERPINA1 was visualised using PyMol software (PDB ID: 1QLP). The flexible reactive centre loop (bait region) is shown in green, and P1 residues at cleavage sites are shown for both MMP-13 (blue - Phe^{376} and Pro^{381}) and neutrophil elastase (NE; red - Met^{382}). Images taken using Ray Trace mode. E) Schematic showing working hypothesis for the interplay

between neutrophil elastase, MMP-13 and AAT in cartilage. Neutrophil elastase fully activates MMP-13, which can result in collagen breakdown. Rapid and specific inactivation of AAT by MMP-13 (along with other MMPs) may add to the proteolytic burden, and further enhance activation of other latent MMP-13 within the cartilage.

Figure 1.



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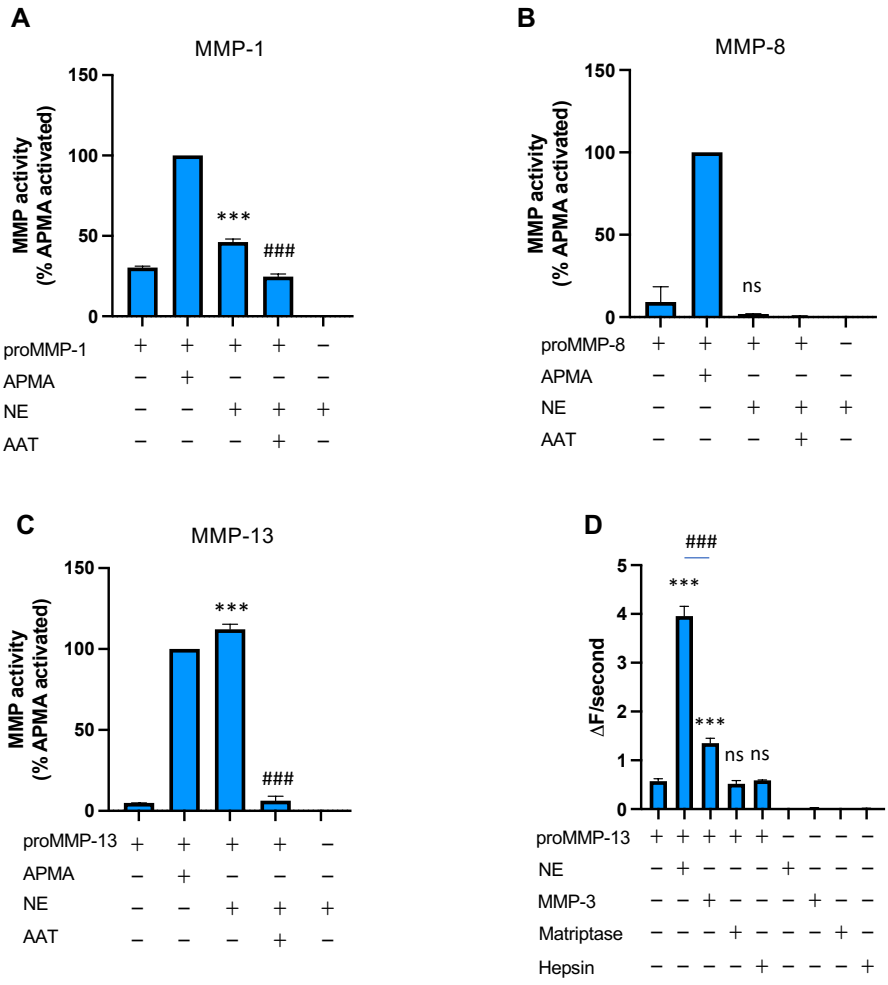

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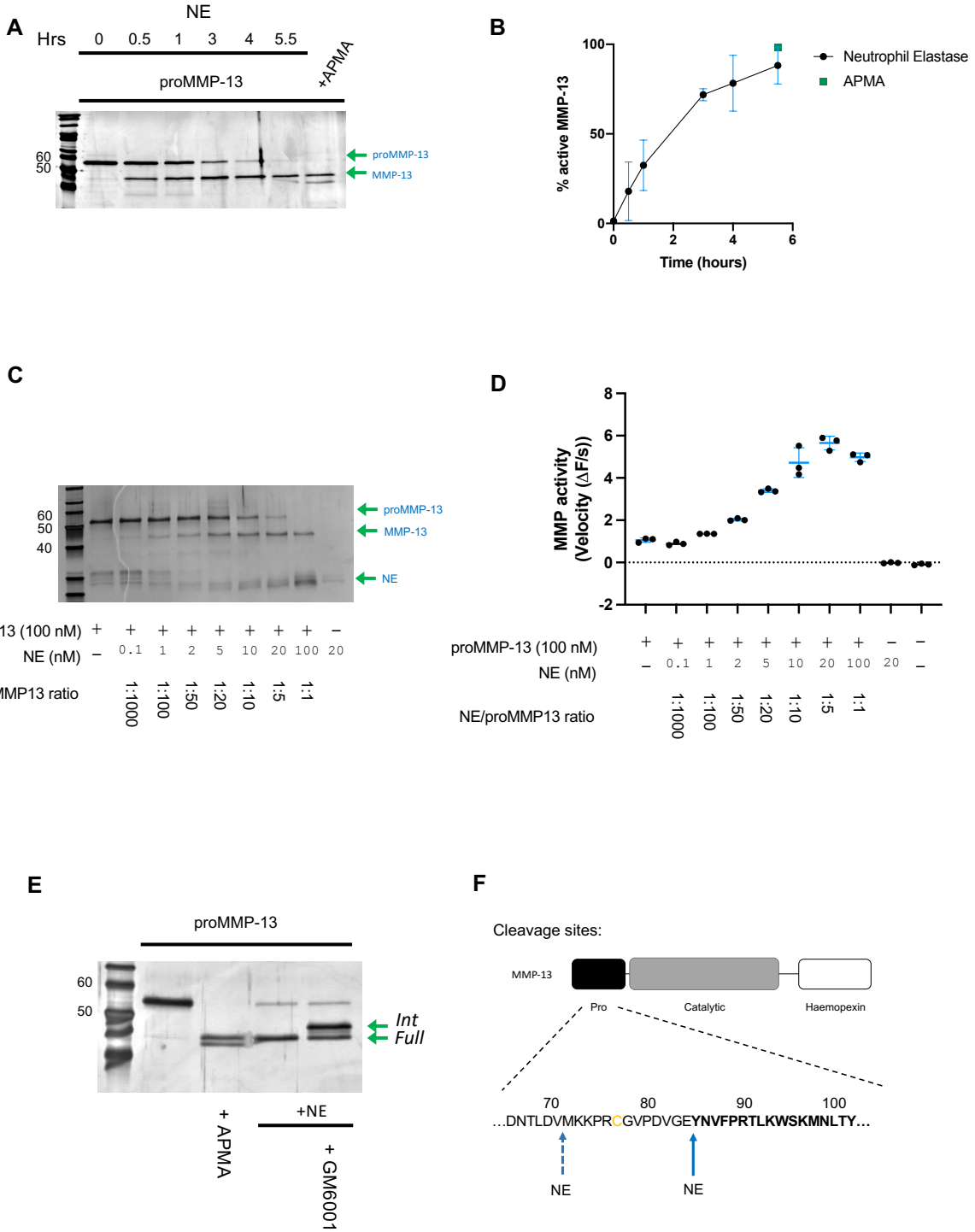

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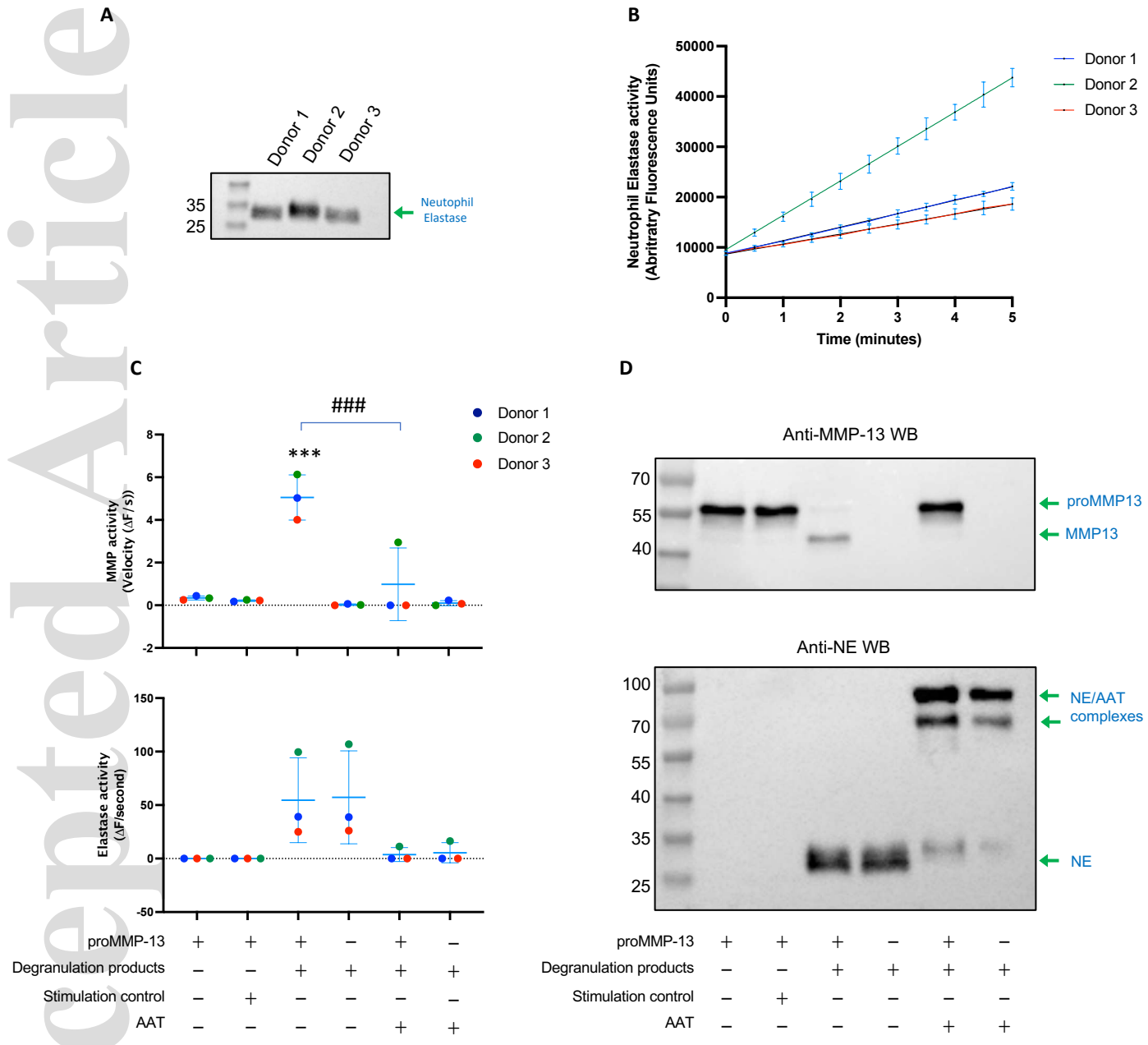

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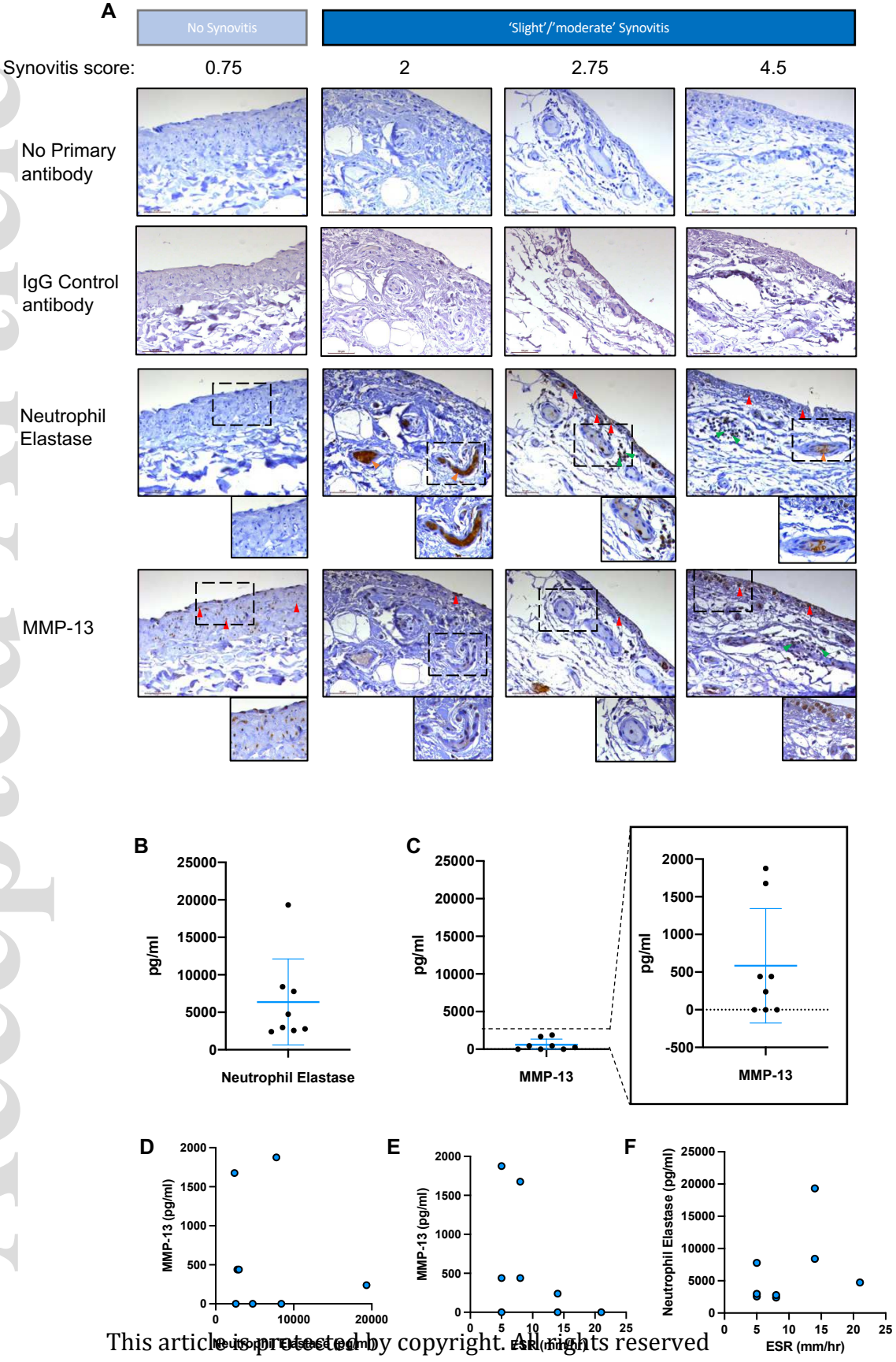

Figure 6.

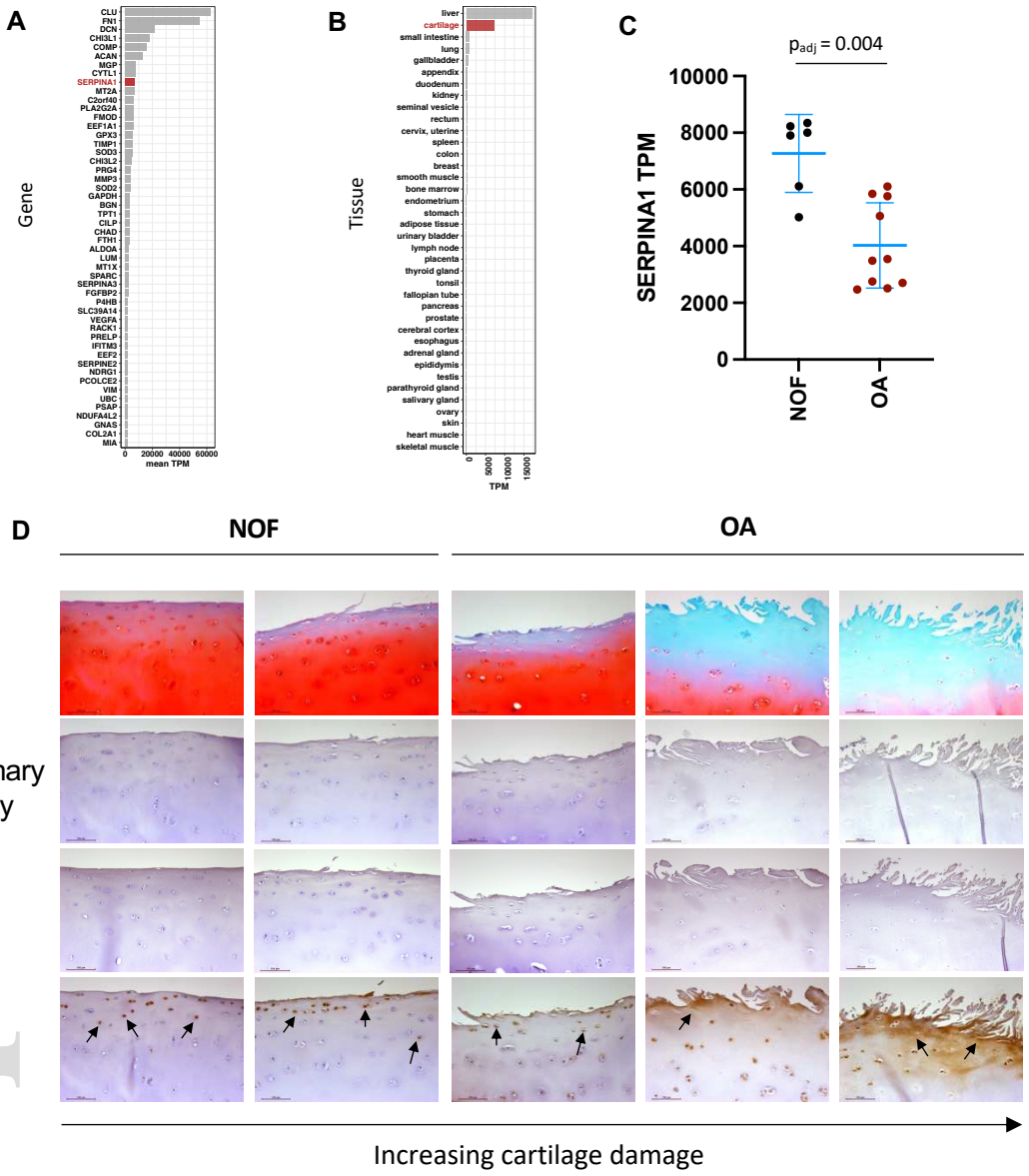

Figure 7.