Matrix metalloproteinases in arthritis: towards precision medicine

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Abstract

Proteolysis of structural molecules of the extracellular matrix (ECM) is an irreversible post-translational modification (PTM) in all arthropathies. Common joint disorders, including osteoarthritis (OA) and rheumatoid arthritis (RA), have been associated with increased levels of matrix remodelling enzymes, including matrix metalloproteinases (MMPs). MMPs, in concert with other host proteinases and glycanases, destroy proteoglycans, collagens and other ECM molecules. MMPs may also control joint remodeling indirectly by signalling through cell-surface receptors or by proteolysis of cytokines and receptor molecules. After synthesis as pro-forms, MMPs can be activated by various types of PTMs, including proteolysis. Once activated, MMPs are controlled by general and specific tissue inhibitors of metalloproteinases (TIMPs). In RA, proteolysis of the ECM results in so-called remnant epitopes that enhance and perpetuate autoimmune processes in susceptible hosts. In OA, the considerable production of MMP-13 by chondrocytes, often concurrent with mechanical overload, is a key event. Hence, information about the regulation, timing, localization and activities of MMPs in specific disease phases and arthritic entities will help to develop better diagnostics. Insights into beneficial and detrimental effects of MMPs on joint tissue inflammation are also necessary to plan and execute (pre)clinical studies for better therapy and precision medicine with MMP inhibitors. With the advances in proteomics and single-cell transcriptomics, two critical points need attention: neglected neutrophil MMP biology and the analysis of net proteolytic activities as the result of balances between MMPs and their inhibitors.

[H1] Introduction

According to the registers of the Centers for Disease Control, ~23% of the US population will receive a diagnosis of arthritis at least once in their life[1], although this number might vary in other global regions. Host genetics, lifestyle, professional activity, age and sex are critical determinants of why and in which joints individual patients develop arthritis. Within specific patient cohorts, degenerative osteoarthritis (OA) and autoimmune rheumatoid arthritis (RA) are common disease entities, alongside forms arising due to trauma and other, less common causes, including septic arthritis and genetic defects. Correct differential diagnosis is important because preventive measures and dedicated therapies are possible for many arthritis entities.

Even with the introduction of new treatment options, some patients have an inadequate response to therapy and other patients might not benefit from novel therapies because of concurrent conditions, including infections. These findings stimulate the continuation of basic and clinical research efforts to find alternative and precise solutions. Although arthritis is a clinical diagnosis, it comes in many varieties, each one with different treatment and prognosis. Therefore, having additional biomarkers for discrimination of diagnostic entities could lead to adequate prevention of adverse effects and to the application of specific therapies, greatly improving the morbidity associated with arthritis.

Joint disorders including OA, RA and septic arthritis have been associated with increased concentrations of matrix remodelling enzymes, including matrix metalloproteinases (MMPs), in synovium and cartilage as well as in synovial fluid and serum. Proteolysis of structural molecules of the extracellular matrix (ECM) is an irreversible posttranslational modification (PTM) in all arthropathies. MMPs, in concert with other host proteinases and glycanases, destroy proteoglycans, collagens and other structural ECM molecules; MMPs can also control joint remodeling indirectly by acting as ligands for the activation of cell surface receptors or by proteolysis of cytokines and receptor molecules and thereby changing local processes. MMPs are produced as pro-enzymes, which then require activation via various types of PTMs to enable their proteolytic activity. Once activated, MMPs are controlled by general and specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The need for proteinase inhibition is illustrated in septic arthritis, in which, without adequate treatment, proteinases from joints and invading microorganisms cause serious damage. In RA and OA, proteinases produced by leukocytes and tissue-resident cells also need tight control in the synovium. In disease conditions with net proteolytic activities of MMPs and other proteases, their inhibition has been suggested as a therapeutic approach.

We here discuss six aspects of MMPs in relation to joint diseases to better understand opportunities for precision medicine and to improve early diagnosis, linked to more efficient therapies.

These aspects include: MMP genetics for primary arthritis and genetic susceptibilities for arthropathies; the location, timing and regulation of proteolysis; arthritogenic epitopes in RA; caveats of MMP analysis; the critical cells producing MMPs; and MMPs in relation to other potential target molecules in unbiased omics studies.

[H1] The emergence of MMPs in arthritis

MMPs were first discovered in the early 1960s by Lapière and Gross, who isolated a new enzyme that degrades collagen and is active at the moment of metamorphosis from tadpoles to frogs and, hence, named it collagenase[2]. Their discovery marked a time-point in biology and medicine research from which the pendulum has been swinging until today[2-7]. It initiated the purification of many MMPs, cloning of cDNAs and genomes, discoveries of activation cascades, endogenous and exogenous inhibitors and substrate repertoires and identification of posttranslational modifications (PTMs). A timeline of key discoveries in MMP biology in relation with other diagnostic and therapeutic breakthroughs in arthritis research is provided in **Figure 1**.

With nearly 60,000 entries in the PubMed database, MMPs are presently an established proteinase family and represent key players in the arthritis literature (4,350 entries), alongside other proteinase families, including serine proteinases (2,000 entries), cysteine proteinases (1,350 entries) and other metalloproteinase members (for example, a dysintegrin and metalloproteinase (ADAM) with 910 entries and ADAM with thrombospondin motifs[5] (ADAMTS) with 660 entries). MMPs are equipped with a characteristic metallic Zinc ion in their active site. This Zinc ion coordinates with a specific cysteine sulfhydryl group, essential for the latency of the MMP proforms by the so-called cysteine switch [GI] mechanism[6]. After removal of the propeptide cysteine, either proteolytically or chemically, and activation of MMPs, the Zinc ion also withholds the hydrolytic water molecule in the right position within the active site to enable the catalytic function of MMPs (ref.[7,8]). In the human species, approximately 20 MMPs exist with functional names and numbers: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), proteoglycanases, also named stromelysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26), metalloelastase (MMP-12), enamelysin (MMP-20), RASI (MMP-19) and six membrane-type MMPs (MMP-14 (MT1-MMP), MMP-15 (MT2-MMP), MMP-16 (MT3-MMP), MMP-17 (MT4-MMP), MMP-24 (MT4-MMP), MMP-25 (MT6-MMP)). Some peculiarities need to be mentioned about MMPs. Firstly, the MMP numbering scheme is based on the sequence of their discovery[3], with interstitial collagenase being named MMP-1 (**Figure 1**). By some unfortunate glitches in historical enzyme naming, MMP-4, MMP-5 and MMP-6 do not exist in the human species. Secondly, gelatinases (35,000 entries in the PubMed database) are relatively overrepresented in the literature, probably artificially owing to the frequently used supersensitive zymography technique[9]. A

third critical issue about the biological context of MMPs is their redundancy. No single mammalian MMP seems essential for life: general gene deficiencies in mice yield viable and (sub)fertile offspring. Genetic deletion phenotypes, including those with musculoskeletal effects, were previously reviewed for MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-11, MMP-12, MMP-13, MMP-14, MMP-19, MMP-20 and MMP-24 (ref.[7]). Here we complement this information and reinforce the aspect of redundancy in two ways. **Supplementary Table 1** contains follow-up information, from 2007 onwards, about general genetic deletions of *Mmp1*, *Mmp10*, *Mmp15*, *Mmp16*, *Mmp17* and *Mmp25* and the double deletion of *Mmp-9* and *Mmp14* in mice. All MMP genetic knockout experiments result in viable and reproducing mouse lines. However, spontaneous or induced joint-disease phenotypes are not yet well studied in these general gene knockout experiments. In addition, tissue-specific or cell-specific MMP gene knockout studies, having fewer problems with mouse viability and being more precise in dissecting cellular or molecular effects, are only now emerging (see later discussion). Musculoskeletal phenotypes[7] in mice were described for MMP-2, MMP-9, MMP-12, MMP-13 and MMP-14. Genetic deletion of mouse MMP-17 protects against inflammatory cartilage degradation[10] (**Supplementary Table 1**). In human diseases, primary arthropathies have occasionally been associated with MMP gene alterations (**Table 1**). Remarkably and so far, the most common MMP gene defect is found for MMP-2, a homeostatic and ubiquitously expressed MMP (see below) and several hitherto described MMP gene defects in medical syndromes are associated with some form of arthropathy (**Table** 1). Precision medical analysis with present-day exome sequencing techniques will increase the discoveries of more such genetic MMP defects in an unbiased way, even when these defects are ultrarare. This prediction was proven with the discovery of a new compound heterozygous defect in the *MMP9* gene [11]. In conclusion, the discovery of interstitial or fibroblast collagenase (MMP-1), about 60 years ago, marked a breakthrough in MMP and arthritis research that continues now with the tools of precision medicine.

[H1] Location and regulation of proteolysis

Proteolysis by microbial or host enzymes in the internal body milieu, including all joint tissues, is an intrinsically dangerous PTM. Indeed, proteinases are destructive for critical biomolecules and change the functions of structural and functional (synovial) proteins. Two important biological control mechanisms are essential to keep detrimental proteolysis in check: compartmentalization and inhibition.

By keeping microbial and digestive proteinases outside the internal milieu and compartmentalized within, for example, the luminal compartment of the gastrointestinal tract, the human body can convert proteins into single amino acids for safe resorption as building blocks for all

protein synthesis. The importance of the location of proteinase activity is best illustrated in pancreatic surgery and acute pancreatitis[12]: after surgical cutting of pancreas tissue and in for example viral pancreatitis, pancreatic proteinases seep into wrong locations, including the blood circulation, which can lead to life-threatening inflammation with extensive involvement of neutrophil MMPs (ref.[13]). Owing to the specialized epithelial structures of the gastrointestinal tract and various types of barriers, including a thick mucus layer and tight junctions between cells, a unique luminal compartment is created for full-blown proteolysis. In this compartment, microbial proteinases are also key players.

The internal environment of the synovium is sterile and proteolysis in this interior milieu is, because of its dangerous nature, heavily controlled by various types of inhibition: latency of pro-proteins and endogenous inhibition. Latency is the proteinase-intrinsic inhibition by the pro-form state of proteinases, which protects primarily the proteinase-producing cells against auto-proteolysis and secondarily other cells and tissues, until the pro-form is activated [6,8]. Inhibition of proteinases is also executed by endogenous inhibitors, which come in two types: general (or 'emergency') inhibitors and specific or regulatory tissue inhibitors. For the 20 or so human MMPs, four specific inhibitors exist; these are named tissue inhibitors of metalloproteases (TIMPs) because they originate mainly from local tissue cells, including synovial membrane fibroblasts and endothelial cells, chondrocytes and eventually also chemoattracted mononuclear leukocytes. In humans and mice, the gene encoding TIMP-1 is X-linked and TIMP-2, TIMP-3 and TIMP-4 genes reside on different other chromosomes. So far, no arthropathy has been associated with any TIMP gene and the quadruple gene knockout is viable in mice[14]. One emergency-type inhibitor, which migrates among the serum α-globulins upon electrophoresis in agarose gels, is α-2-macroglobulin (α_2M). This positive acute-phase molecule is made primarily by hepatocytes and is present in blood at a considerable concentration of ~275 mg/dl (ref.[15]). α_2M is a tetrameric molecule with an exposed 'bait region' that acts as a trap for most types of proteinases. When any proteinase snips this bait region, the tetramer immediately folds into a cage surrounding the proteinase, thereby preventing cleavage of macromolecular substrates unable to enter the α_2M cage. In agreement with this model mechanism, a remarkable observation has been made with MMP-9 within the past decade. MMP-9 is produced by most cells, including neutrophils and mononuclear leukocytes, mostly as monomeric proteoforms **[G]** and ~30% as trimers, which possess a higher affinity for TIMP-1 and less angiogenic activity than the monomers[16]. In contrast to MMP-9 monomers (92 kDa), the covalently linked trimers (>250 kDa) are too large to be accommodated within the α_2M cage. Therefore, the proteolytic activity of MMP-9 monomers is completely neutralized by α_2M, whereas the active trimers in complex with α_2M retain net proteolytic activity. This is an important observation for all types of inflammation because it implies that, when serum proteins including α_2M enter tissues as inflammatory exudates, MMP-9 monomers are

inactivated by α_2M whereas active trimers are not[17]. Understandably, caging of proteinases by α_2M is a very efficient way to block proteolysis within the circulatory system, thus avoiding for example intravascular coagulation[18]. No direct association between any of the named MMP inhibitor genes (encoding TIMPs and α_2M) and arthritis has been described to date.

Considering the existence of MMP latency (that is, the necessity to activate pro-MMPs produced by all cells[8]), the omnipresence of α_2M in exudates[18] and the tissular production of TIMPs together with MMPs by all mononuclear cells, including leukocytes, fibroblasts, synoviocytes and even chondrocytes during inflammation, it is hard to envisage how net proteolytic activity could be generated in joint tissues, except when polymorphonuclear leukocytes or neutrophils enter the synovium or are abundantly present in synovial fluid[19]. We contemplate that at the time when neutrophils are chemoattracted into tissues, including in the joints, net ECM proteolysis occurs because of the presence of these cells. First, neutrophils produce pro-MMPs, in particular pro-MMP-8 and pro-MMP-9, in considerable amounts and in the absence of the endogenous inhibitor TIMP-1 (ref.[19,20]). Second, neutrophils degranulate quickly after activation and release reactive oxygen species (ROS) for chemical activation of pro-MMPs (ref.[21,22]). It is understood that in all inflammation, and thus also in arthritis, this neutrophil activation can be additionally executed by various types of other host mediators, including chemokines, leukotrienes and complement C3a and C5a. Furthermore, in septic arthritis all bacteria provide formyl peptides for neutrophil activation, whereas specific bacteria, viruses and other types of microorganisms might also act directly through various Toll-like receptors to indirectly induce cytokines and chemokines, which will activate additional neutrophil receptors[19]. Third, proteolytic activation of neutrophil MMPs in the microenvironment, if not yet executed by ubiquitous plasmin in exudates[23,24], can also be locally achieved by neutrophil serine and other proteinases[8]. Fourth, even when α_2M diffuses into tissues during an inflammatory reaction of any kind, it will not be capable of terminating the activity of MMP-9 trimers (as discussed above). Full inhibition of all MMP-9 activity will only happen at a subsequent stage when mononuclear leukocytes increase in numbers and sufficient TIMP-1 is brought into the local microenvironment[19]. Therefore, chemoattracted and thereby activated neutrophils are the only cell type that — with certainty — usher net MMP activity and the presence of neutrophils in synovial fluid thereby constitutes an important alarm signal in all severe forms of arthritis (see later discussion). These insights into septic arthritis have critical consequences for precision medicine in clinical rheumatology. Today, septic arthritis is commonly diagnosed by bacterial culture of synovial fluid. However, in the absence of a positive microbial culture and in the presence of synovial fluid neutrophilia, many other microorganisms, including viruses, could be involved. With existing technologies it is straightforward to develop gene arrays for all common viruses and to apply these to testing of synovial fluid samples. With such an

approach it is possible that more patients with viral forms of septic arthritis will be discovered and these patients could benefit from dedicated antiviral therapies. In summary, the balances between leukocyte types and between active proteinases and inhibitors are critical to understanding whether net proteolysis exists in tissues.

Another aspect of balance is knowledge of which MMP function is beneficial, detrimental or without effect on joint tissues. Here we discuss some aspects of these balances as they concern proteolytic activities; other functions, including receptor-mediated signalling and bioavailability by internalization are covered in other sections. For details about the biology of individual proteinases, including MMPs and their inhibitors, we refer the reader to the Handbook of Proteolytic Enzymes[25], to specific reviews[7,14,18] and to **Supplementary Table 1**. Three aspects are important to reiterate from these overviews: homeostasis versus induction, phase-specificity and interactive networks.

The preeminent homeostatic MMP in physiology is MMP-2. It is constitutively present in all (normal) body fluids, whereas the inducible companion gelatinase MMP-9 is temporarily expressed only under pathological conditions, e.g. of arthritis [7,26]. Because genetic knockout of MMP-2 exacerbates experimental arthritis in mice whereas deletion of the MMP-9 gene alleviates it[27] (as discussed in more detail below), a possible interpretation is that ubiquitous and constitutive MMP-2 helps to prevent adhesions and tissue fibrosis, including in musculoskeletal diseases. Development of and recovery from arthritis is intrinsically a four-phase-specific process: initiation; progression, concurrently with induction of specific MMPs; re-balancing, involving MMP inhibitors; and recovery to steady-state homeostasis. Specific MMPs, such as MMP-2, MMP-8 and MMP-9, can convey detrimental effects in the initiation phase (see below), whereas they can be beneficial in the resolution phase (for example, for the avoidance of fibrosis). Finally, MMPs and TIMPs interact in networks, thereby acting together with other proteinases and their inhibitors. This interaction network is further elaborated in **Box 1**.

In summary, MMP proteolytic activities are continuously subject to regulatory balances, the biological functions of MMPs vary according to the phase of arthritis and MMPs interact with many other proteinases, thereby complicating the use of MMP inhibitors for the pharmacological treatment of arthritis.

[H1] Proteinases and remnant epitopes in rheumatoid arthritis

Autoimmune diseases have many similarities with cancer at the molecular and cellular levels. Both types of disease are based on the genetic susceptibilities of the host and depend on environmental

influences, including host microbiomes. RA has previously been compared with cancer because in both diseases molecular tissue microenvironments with hypoxia and metabolic changes lead to similar cellular effects, such as neovascularization, synovial proliferation and leukocyte chemotaxis. By these processes, normally acellular synovial tissue transforms into an invasive tumour-like 'pannus'[28]. Cancer and autoimmune RA both represent chronic diseases, influenced by cell growth factors and inhibitors and by proteinases and proteinase inhibitors. However, the genetic instability of cancer cells is a prominent difference between cancer and autoimmune diseases. MMP inhibitor research has not provided solutions in oncology, as the use of MMP inhibitors resulted in severe adverse effects affecting joint tissues. The intrinsically complex proteinase networks, which can be influenced by the genetic instability of cancer cells and in function of location and time, have been invoked to explain the failure of MMP inhibitors for the treatment of invasive cancers (**Box 1).** The stable genetic constitution of host cells, however, diminishes uncertainties about therapeutic outcomes of MMP inhibition in autoimmune diseases, including RA.

Historically, antibodies and adaptive immune mechanisms by T cells were discovered by experimental studies of infections and these preceded the discovery of autoimmune diseases. The major proteinases in the studies of how adaptive immune reactions are established were all intracellular proteinases for protein antigen processing and presentation to MHC class I and II. For the presentation of intracellular antigens, the proteasome **[G]** acts as a threonine proteinase and creates, in partnership with other proteinases, intracellularly processed antigens that are presented by MHC class I molecules for the activation of cytotoxic $CD8^+$ T cells[29]. Extracellular antigens are endocytosed and intracellularly processed by lysosomal proteinases of various catalytic categories and loaded into the grooves of MHC class II molecules for $CD4^+$ helper T cell activation and the formation of neutralizing antibodies [30]. In contrast to intracellular proteolytic antigen processing, it took considerable time and effort to better understand the contributions and roles of extracellular proteinases in (auto)antigen generation and presentation and how infections might relate to autoimmune diseases.

The role of cytokine-regulated extracellular proteolysis of host molecules in autoimmune diseases, the links with infection and inflammation and the term remnant epitopes **[G]** for autoantigens were described a few decades ago[31] and this concept has since then been further developed[32]. To date, it remains underappreciated that extracellular endoproteinases and exoproteinases in the internal milieu are a critical link in associations with all autoimmune disorders and in understanding quantitative aspects in autoimmune RA. Molecular mimicry was defined as cross-reactivity of T or B cell receptors between microbial and host peptide antigens and this mechanism provides insight into how infections could underlie autoimmune reactions[33], but it does not reveal how the host peptide antigens are generated. For the latter to happen, extracellular proteolysis and intracellular processing

of host molecules into remnant epitopes and MHC loading provide a solution. In fact, molecular mimicry is not essential for a susceptible host to develop an autoimmune disease. It is sufficient that MHC molecules, capable of presenting a remnant epitope, are laden with such autoantigen and that endogenous or therapeutic immunosuppression mechanisms are insufficient to block specific lymphocyte activation. Any low-grade infection or inflammation will induce cytokine-regulated proteolysis of host molecules that can (re)activate autoreactive lymphocytes and lead to autoimmune disease[32].

MMPs are established enzymes for ECM protein degradation[34]. As an example, early events in the initiation phase of RA are the influx and stimulation of neutrophils to release MMP-8 and MMP-9 as inducible pro-inflammatory proteinases. By the combined action of MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B) on triple-helical type II collagen in the joint, more than 100 remnant peptides are generated. Specifically, collagenases (MMP-1, MMP-8 and MMP-13) cleave triple-helical collagen substrates into a large (three-quarter) fragment and a small (one-quarter) fragment[35,36]. These two fragments, which are still triple-helical, consequently relax and partially unwind into denatured collagen or gelatin. In a next step, inducible MMP-9 cleaves each of these relaxed triple-helical fragments into multiple peptides (~40 cleavages times three fragments). In addition, some of these peptides are protected against proteolysis by attached O-linked oligosaccharides and can thereby persist for longer time intervals to be presented in MHC class II and cross-presented in MHC class I as autoantigenic remnant epitopes[35,36]. Type II collagen can also be cleaved by MMP-2 (ref.[37]) and MMP-14 (ref.[38,39]), the latter of which is a true collagenase with subtle differences with MMP-1 (ref.[40]). It is not yet known whether and which possible antigenic remnant epitopes for T cell stimulation are generated by MMP-2 or MMP-14 in humans or other species. Furthermore, whether active MMP-2 or MMP-14 possess enzymatic activity in vivo or in vitro to generate remnant epitopes in the presence of α_2M are additional interesting questions to solve. As stated above, the trimer of MMP-9 escapes the inhibition by α_2M (ref.[17]).

Aside from RA as an autoimmune disease with type II collagen peptides as remnant epitopes[35,36], other autoimmune diseases exist with symptoms of arthritis. Systemic lupus erythematosus (SLE) and psoriasis are notorious examples of the latter. In these conditions, too, extracellular proteolysis of host glycoproteins into remnant epitopes (including collagens) takes place and leads to altered autoantigenic repertoires, causing disease in susceptible hosts. Of note, whereas MMP-9 can be detrimental in RA by generating remnant epitopes, it may be beneficial in SLE by inducing clearance of intracellular substrates that enter the internal milieu by cytolysis[41]. Consequently, both quantitative changes (relative abundancies of autoantigens after proteolysis) and qualitative changes (PTMs, such

as proteolysis, citrullination and glycosylation) in autoantigen repertoires could break tolerance mechanisms, as has been reviewed elsewhere[32].

The relevance of these findings about MMP-mediated proteolysis for autoimmune arthritis is reinforced by evidence regarding MMP inhibition as the mechanism of action of the historical RA drugs D-penicillamine and minocycline. Both these drugs inhibit MMPs, and for minocycline this action is independent of its antibiotic activity[7,42,43]. Minocycline was used in a small number of RA studies, but because of the fear of the development of antibiotic resistance its use was soon surpassed by that of methotrexate, which was first used prudently in low doses and later at high doses. Meanwhile, the use of minocycline as a potential MMP inhibitor was also broadened beyond RA with success in clinical trials of multiple sclerosis[44]. Concurrently and in a preclinical setting, a selective inhibitor of MMP-14 was effective against experimental arthritis and acted in synergy with blockade of TNF (ref.[45]). In conclusion, remnant epitopes of autoantigenic proteins are formed in RA by MMPs at disease onset[32] and MMP inhibitors are available to be used in a disease-phase-specific way, maybe not only for RA, but also for other common forms of arthritis.

[H1] Converging evidence for MMPs in arthritis

Divergent causes can lead to joint inflammation and these include physical, infectious, chemical, immunological and neoplastic processes (**Box 2**).

Since MMP-1, MMP-2 and MMP-3 were suggested to be functional enzymes in cartilage destruction and arthritis in the 1980s [ref. [46]], the popularity of this topic for drug and diagnostics development has varied. This was mirrored — with the advent of gene-deficient mouse models — by the discoveries of beneficial and detrimental functions of specific MMPs. Notoriously, the two gelatinases MMP-2 and MMP-9 have divergent functions in an animal model of anti-collagen antibody-induced autoimmune arthritis. The homeostatic MMP-2 protects mice against arthritis whereas inflammation-associated and inducible MMP-9 leads to severe arthritis and both enzymes seem to compensate each other functionally in this animal model[27]. In a preclinical animal model of autoimmune RA, induced by injection of collagen antigens for T helper cell activation, inhibition of MMP-14 reduced cartilage degradation and improved preclinical disease parameters in synergy with TNF blockade[45]. In patients with various forms of arthritis, MMP concentrations, in particular those of MMP-3, are increased in plasma or sera and in synovial fluids and can be used for diagnostic purposes of systemic and local disease, respectively[47]. Although it is known that all forms of arthritis lead to both local and systemic immunological reactions, the discrepancy between local versus systemic forms of arthritis are foremost clinically relevant and greatly determine patient comfort in the short-term and

over long time periods. Having good biomarkers for disease diagnosis, prognosis and the effects of and resistance to specific forms of therapies will be helpful for patients and their physicians. Therefore, before specifying MMPs as disease markers, it is imperative to have an idea of their normal and pathological levels in accessible body fluids, such as serum, plasma and synovial fluid. **Table 2** outlines these data.

If MMPs are to be used in the future as biomarkers for specific arthritis entities, it is also relevant to address some information about currently used markers and to evaluate how MMPs might be compared with these markers and whether the combination of markers enhances sensitivity or selectivity. For RA diagnosis, the presence of antibodies against cyclic citrullinated peptide (CCP) is an established marker[48]. In 2003, the first studies appeared in which both MMPs and citrullination were named together[49]. Around that period, the discoveries of RA being associated with immunoglobulins without galactose[50] or with anti-CCP antibodies [51] led to their clinical use as experimental biomarkers for RA. Since 2003 and as analyzed for all MMPs, the association with any form of arthritis is, by far, best documented for MMP-3. Indeed, MMP-3 has been described in ~2,000 PubMed-registered studies on arthritis. Two collagenases, MMP-1 and MMP-13, and a gelatinase, MMP-9, follow in this ranking, each described in about 1,000 manuscripts related to arthritis. For MMP-2 (gelatinase A) (<600 entries) and any other member of the MMPs (each with <100 entries), including the membrane-type MMPs, the literature associated with various forms of arthritis is more limited. The records of all PubMed manuscripts with the entries “citrullination and MMP” indicated that, out of a total of 117 items, 93 are about arthritic diseases, whereas the remaining manuscripts mention other inflammatory diseases (such as pancreatitis or meningitis) or neoplastic diseases. The detection of antibodies against citrullinated peptides and against CCP was observed in 15 and 62 studies, respectively. Laboratory medicine studies reveal that MMP-3 was found to be associated with arthritis in 81 out of the traced 117 studies (**Supplementary Table 2**). In the complete literature on PubMed until the end of 2020, not a single mention exists about citrullination of any MMP in arthritis. After the discovery of MMP citrullination[52], a small cohort study confirmed that this PTM is also observed in synovial fluid samples from patients with RA[53]. Thus, citrullinated MMP-9 occurs as a proteoform associated with RA.

MMP-3 is detectable in blood (plasma and/or serum) and synovial fluid (**Table 2**). Functionally, stromelysins have a broad repertoire of ECM substrates: proteoglycans, collagens type IV and IX, fibronectin, laminin and gelatin[54]. Stromelysins convey positive feedback loops by activating other MMPs in an interconnected proteinase network and by modulating the biological activities of cytokines and chemokines in positive (activation or potentiation) or negative (that is, by proteolytic degradation) ways. In preclinical rabbit models, injection of pro-inflammatory IL-1 in one joint induced MMP-3

mRNA and protein expression in synovium and cartilage; this effect was not observed following injection of PBS in the contralateral joint[55]. In patients with RA, both MMP-1 and MMP-3 are produced by the same cell types in joint tissues; however, in serum only the MMP-3 titre is significantly increased in RA (**Table 2**). From comparisons of serum and synovial fluid, it is reasonable to accept that the blood concentrations of MMP-3 (and MMP-1) in RA, which are 10-fold to 100-fold higher in synovial fluid than in serum, probably represent leakage from inflamed joints into serum, also because no additional endothelial barrier exists in synovial tissue and inflamed tissues have leaky basement membranes that enable molecular diffusion out of and into the vascular lumen. The original findings about synovial and vascular MMP concentrations have been confirmed in other studies, mainly with the use of ELISA measurements[56-61], which also have serious limitations. Indeed, with the present details about the existence of specific MMP-9 proteoforms (see below), reflection about the techniques used remains mandatory. With the development of alternative methods, such as fast and quantitative zymography[9] and mass spectrometry technology[62,63], better insights into biological processes are imminent.

In order to degrade collagen and generate remnant epitopes by proteinases such as MMP-9 to occur in the initiation or perpetuation of the autoimmune process in RA, cartilage type II collagen must first be cleaved by one of the three collagenases (MMP-1, MMP-8 and MMP-13). In RA, this process is most probably executed by MMP-8, which is abundant and activated in inflamed joints[64,65]. MMP-8 is also produced in limited amounts by chondrocytes in the context of inflammation[66], possibly in OA. Collagen is also cleaved by MMP-1, which is produced by a wide range of cells after stimulation by, for example, IL-1 or TNF (ref.[67,68,69]). MMP-13 also contributes to collagenolysis, because the constitutive levels of this enzyme are increased by IL-1 and TNF in OA cartilage and synovial tissues[70-72]. It has been well established that MMP-13 is the major type II collagen-degrading MMP in mouse models[73] and in human OA (ref.[74]). Particularly when pro-inflammatory cytokines seem not to be prominent, MMP-13 is induced by non-inflammatory mechanisms. These elements form a solid basis to develop selective MMP-13 inhibitors for OA treatment[75]. Finally, and as written above, MMP-14 is also a true collagenase[39] and is capable, together with membrane-bound ADAM-17, to cleave and shed the LDL receptor-related protein 1 (LRP-1) into a fragment that can sequester the aggrecanase ADAMTS-5 and MMP-13, thereby preventing these OA-associated metalloproteinases from elimination by endocytosis[76].

Collectively, these findings imply that various collagenases and stromelysins, both of which are abundantly present in RA as well as OA (**Table 2**), execute collagen and proteoglycan degradation in joints. Not only in RA, but also in septic and gout arthritis, neutrophils that are chemoattracted by IL-8 (CXCL8) (ref.[77]) contribute to the degradation of collagen by MMP-8 and the formation of gelatin. With the help of the cytokine-regulated MMP-9, gelatin is completely destroyed or broken down into

immunogenic remnant epitopes. PTMs other than proteolysis and including citrullination and alterations of glycosylation might further influence these processes in RA, dependent on the contexts of host genetics and of specific disease phases[32]. OA can be viewed as a disturbance of predominantly chondrocytes, which produce relatively small amounts of MMP-9 (ref.[78,79]), but instead rely on considerable proteolysis by ADAMTS-5 and MMP-13. Biomechanical joint damage causes OA *in vivo* and leads to MMP-13 production in an experimental mouse model[80]. The role of mechanical overload in the development of slowly degenerative human OA is in line with the experimental findings[81].

[H1] Synovial MMP-producing cells in arthritis

In terms of cellular composition, a diarthrodial joint is composed of a bony structure covered with cartilage, which consists mainly of proteoglycans and type II collagen. Within cartilage, hydrated proteoglycans dampen physical shocks by their water content. A normal synovial space is limited and filled with only a few millilitres of synovial fluid, which acts as lubricant against friction in all joints and particularly in heavily loaded joints, such as the hips and knees (**Figure 2**). In contrast with body fluids in other virtual internal compartments, such as the pleural and peritoneal space, synovial fluid is not just a serum ultrafiltrate; instead, it is rich in mucopolysaccharides from synovial membrane cells and it is poor in cells. A major component of synovial fluid is lubricin (proteoglycan 4), which is mainly locally produced by chondrocytes. In analogy with the production of mucins by goblet cells in the gastrointestinal tract, considerable amounts of lubricin are deposited by chondrocytes on cartilage as superficial zone protein as a protective barrier. Other synovial cell types also produce lubricin as a protectant[82]. Lubricin is a highly O-glycosylated protein, and in fact a mucin-type protein. By sialylation of its repetitive glycans it prevents self-association of its bottle-brush-like structure and it adsorbs a vast number of water molecules, thereby functioning as a biochemical shock absorber. Synovial fluid of a healthy individual contains an extremely low number of cells; only ~100 cells per ml, mainly mononuclear cells such as macrophages and lymphocytes and <10 neutrophils per ml. In sharp contrast, human blood in adults contains 10^7 leukocytes per ml with about 40–70% being neutrophils[83]. In all forms of exudative arthritis and in experimental animal models, neutrophils are the first and major leukocyte cell type to enter the synovial space[19,64,65]. Neutrophils bring along serine proteinases such as neutrophil elastase and proteinase-3 and also MMPs, mainly MMP-8 and MMP-9, and thereby control proteinase balances[84]. Neutrophils are also exceptional cells because they do not produce the homeostatic MMP-2, nor TIMP-1 (ref.[19,20]). All other cell types in the articular lining, including the macrophage-derived type A synoviocytes and the mesenchymal fibroblast-derived type B synoviocytes, produce MMP-2 and co-produce TIMP-1 and MMP-9 when stimulated by infectious

agents or pro-inflammatory cytokines[85]. Primary pro-inflammatory cytokines (IL-1 and TNF) stimulate the local production of MMPs and of chemokines[31]. In humans, the major neutrophil chemokine acting in the synovial fluid is IL-8 (CXCL8) (ref.[77]); other chemokine ligands participate in leukocyte recruitment and their concentrations have been compared in various forms of arthritis[86]. Activated neutrophils release proteinases, thereby generating autoantigenic peptides in RA. Citrullination and other PTMs of these remnant epitopes lead to further neo-epitope formation and T and B cell activation[32]. In animal models of OA, neutrophils can also enter into the synovial fluid and counts of these cells in synovial fluid correlate with disease parameters[87]. Although the relative amounts of MMPs produced by neutrophils overshadow those of mononuclear leukocytes[19], implying that a small number of neutrophils can cause more proteolytic damage than monocytes, in OA research the study of chemotaxis and chemokines is still skewed towards mononuclear cells[88]. Unbiased and detailed studies of all synovial fluid leukocyte types and their relative contributions in MMP production could yield new insights.

Unbiased information about MMPs from cells in synovial fluids by protein marker analysis or RNA sequencing is limited to studies from the past 4 years about OA and RA in patients and animal models of arthritis. In OA, specific cells, mainly fibroblast-like synoviocytes and recruited macrophages expressing CD14 and CD163, are needed for MMP expression, as reviewed elsewhere[89]. In the inflammatory process of OA, these cells produce MMP-3 to degrade proteoglycans and to activate the procollagenases pro-MMP-1 and pro-MMP-13 and pro-MMP-9. Fibroblast-like synoviocytes and chemoattracted macrophages also produce aggrecanases[89]. Studies using cytometry and RNA sequencing techniques have established the existence of molecular crosstalk of local chondrocytes and synoviocytes with recruited leukocytes, providing insights for future means of intervention at cellular and molecular levels[90]. Comparisons of normal cells and molecules with those of OA and RA are also relevant for precision medicine (**Figure 2**). In a 2019 publication [91] leukocyte-poor and leukocyte-rich subtypes of RA were discriminated on the basis of mass cytometry and marker analysis and compared with OA. By use of single-cell RNA sequencing (scRNA-seq) and massive data analysis, fine details of cytokines, in particular of interferons, and chemokine expression became evident, whereas information about metalloproteinases was limited to MMP-1 and TIMP-2 (ref.[91]). However, autoimmune B cell subsets were discovered in patients with RA, but not in those with OA, corroborating some findings in animal model studies. One caveat of the studies to date is that information about neutrophils is limited, even though these cells are the main producers of MMPs, particularly in RA. In comparison with neutrophils, B and T cells, even after appropriate stimulation, produce orders of magnitude less MMP-9 and co-produce TIMP-1 (ref.[19]). In selective case studies, germinal centres with lymphocytes have been detected in RA pannus tissue, but their importance in

MMP biology is not clear[92], whereas their role in the autoimmune process of RA is evident[32]. Indeed, a 2022 study profiled subpopulations of B cells from within tertiary lymphoid tissue in RA synovial tissue and provided molecular evidence of *in situ* differentiation[93]. Serendipitously, we had previously described the expression of MMP-9 in ectopic lymphoid tissue in chronic synovitis and illustrated that the MMP-9 protein is produced mainly by morphologically characterized dendritic cells, rather than lymphocytes[94]. In relation to OA studies, synovial tissues of posttraumatic joints contain B and T lymphocytes in association with specific chemokine production and progression to OA (ref.[88]).

An outstanding study about systemic juvenile idiopathic arthritis reinforces the power of the present analytical platforms and at the same time addresses a pivotal role of neutrophils and osteoclasts[95]. Knowledge of the critical role of neutrophils could lead to novel types of intervention, including upstream blocking of the action of granulocyte colony-stimulating factor or downstream inhibition of pivotal proteinases: neutrophil elastase, MMP-8 and MMP-9 (ref.[95]). Osteoclasts are also well-known producers of MMPs (ref.[96]), by which they help in bone resorption. With the use of precision basic research, Zhu and colleagues searched for critical osteoclast MMP substrates with the expectation of finding type I collagen as a major ECM component and MMP substrate of bone. Serendipitously, they discovered that osteoclast MMP-14 and MMP-9 cooperate in cleaving galectin-3, a ligand of LDL-related protein-1, and thereby disables a signalling pathway in mouse osteoclasts. By selective knockout of *Mmp-9* and *Mmp-14* in osteoclasts and by MMP protein inhibition, these researchers provide evidence for transcriptional changes in skeletal tissues by the actions of MMPs and thereby reinforce the concept that specific MMPs display not only tissue effects on structural ECM proteins, but also catalyze important regulatory signalling events [97]. When (re)purposing MMP inhibitors for clinical investigations in arthritis, it is important to take these insights into consideration.

Inhibition of MMPs in rheumatic diseases will directly interfere with the irreversible process of proteolysis. Indeed, once collagen is clipped by neutrophil MMP-8 or osteoclast MMP-14, it is destroyed, denatured and prepared for further clipping into remnant epitopes. As stated above, this process will increase the risk of developing autoimmune diseases in susceptible hosts. However, neutrophils might not only digest collagen; they might also carry gelatin fragments through the circulation to distant sites to modulate wound fibrosis or regeneration, including in joint tissues. This was proven in a 2022 report by use of scRNA-seq and chemical and genetic screening approaches[98].

In conclusion, unbiased single synovial cell counting and phenotyping (with inclusion of neutrophils and T and B cells) are providing novel and precise information that will benefit individual patients with arthritis, including those whose disease is resistant to present treatments.

[H1] Limitations of present omics-profiling in arthritis

Based on the above considerations about MMP studies of arthritis, unbiased transcriptomics technologies are providing interesting new insights. However, these investigations need to be complemented with other 'omics', namely analyses of proteins, oligosaccharides and lipids. Indeed, transcriptomics studies, even at the single-cell level, provide only critical information about mRNA steady-states from cells in body fluids and tissue extracts. Although it is useful to discover the master switches of biological control, bioinformatic analysis of transcriptomes is insufficient to grasp the biology of arthritis, in which proteoforms, as the multiple presentations of single gene products (see earlier discussion), and proteolysis as well as other PTMs have essential roles (**Figure 3**). Another essential addition is 'data crunching' for each arthritis entity and comparison with large data sets with the use of high-end computing, from deep learning to artificial intelligence applications. We here exemplify these issues with the use of recent research, from simple and complex applications.

In diagnostics, it has become an adage that the combination of biomarkers could lead to better selectivity and higher sensitivity. This view can be applied in rheumatology concerning the use of MMP analysis. For instance, the combination of increased concentrations of MMP-9 in arthritis, which has long been established[99], and citrullination, a known PTM in RA (ref.[48,51]), might lead to simple tests of citrullinated MMPs, such as MMP-1 and MMP-9, in RA (ref.[53]). Indeed, MMP-9 and MMP-1 are readily citrullinated *in vitro*, whereas MMP-3 and MMP-13 are less so and MMP-2 and MMP-8 are barely altered by this PTM. Furthermore, hypercitrullinated MMP-9 has a higher affinity for gelatin, is more rapidly activated by MMP-3 and is less efficiently inhibited by a therapeutic MMP inhibitor than unmodified MMP-9 (ref.[52]). In overviews about proteomic profiling of synovial fluids in arthritic diseases, the analysis of various MMPs was placed in such context. MMP-8 and MMP-9 seem to have relatively high clinical specificity for RA (in comparison with OA). As reported, however, the selectivity still seems low in proteomic analysis[61,63]. To enhance specificity and owing to the possibilities of multiplexing and unbiased proteomic testing, the use of biomarker combinations is gradually gaining importance. However, such types of analysis remain expensive and labour-intensive. For this reason, we presently advocate alternative approaches, such as the combined analysis of covalently linked molecules or the combination of a specific analyte in association with a disease-specific PTM (ref.[53]).

Time-dependent variation of parameters in individual patients remains an issue when using artificial intelligence systems to compare large data sets. These individual parameters will also vary according to diurnal changes in metabolic states and, conversely, arthritis could influence metabolic

changes[100]. Integration of metabolomic data in arthritis diagnosis and treatment thus becomes a challenge for precision medicine. Two studies with applications of precision medicine by molecular profiling, mentioning MMPs as possible biomarkers, are provided here[101, 102]. In a descriptive study, selected cytokine, chemokine and MMP proteins were compared in the synovial fluid and serum of patients with RA and OA with the aim of improving diagnosis [101]. To enhance the success of expensive treatment with biologic agents, biopsy-obtained synovial tissue samples were profiled to identify humoral immune response gene signatures associated with response to rituximab and tocilizumab, and a stromal cell/fibroblast signature in patients with disease refractory to all medications. This study supports the notion that disease phenotypes are driven by diverse molecular pathology pathways in the diseased tissue and illustrates that it is important to integrate molecular signatures into clinical algorithms to improve the use of existing medications and to stimulate research to help those patients in whom present drugs do not work[102].

For specific arthritis entities, in particular those based on irreversible events, the timing of treatment initiation is known to be essential. This is known as 'the window of opportunity'[103,104]. Ideally, the best arthritis treatment is prevention. This approach is possible for traumata and stress overload in OA-susceptible individuals. Smoking, which is preventable, possibly contributes to loss of cartilage and OA development. For RA too avoidance of smoking alongside other healthy lifestyle changes are critical preventive measures[105]. Susceptibility to RA is presently determined by analysis of genetic markers and anti-CCP antibodies and this is possible long before disease onset and the development of clinical symptoms. In an ideal situation, immediate and adequate treatment at the time of the first symptoms could prevent the development or deterioration of the autoimmune process[31,32].

For known and often unknown reasons, inflammatory reactions in the joint do not lead to a successful healing, but instead result in an ongoing process with tissue damage and partial repair (**Box 3**). Effective biologic therapies, but unfortunately no cure, already exist for RA (ref.[106]), whereas the lack of therapies for OA could be attributable to its heterogenous nature[107]. If MMPs are to be targeted for arthritis therapy, clear views are needed about which MMPs are causes and which are consequences of RA and OA. One of the reasons for the failure in clinical practice of inhibitors that target the active site of MMPs is that we lack insight into the network of interactions between MMPs and inhibitors in biology (**Box 1**). One of the assumptions made about MMP inhibitors was the need for selectivity towards one key MMP. However, whereas for OA such selectivity towards MMP-13 might be useful, selectivity for a single MMP might be superfluous in RA. Indeed, dual-specificity

inhibitors against MMP-8 and MMP-9 could become useful for neutrophil-driven forms of arthritis, including crystal arthritis, septic arthritis and RA (ref.[108]).

[H1] Conclusions

Arthritis remains a diagnostic and therapeutic challenge. For diagnosis, rheumatologists need to determine which of the more than 100 arthritis entities best fits with each patient, with OA and RA constituting considerable cohorts. In the near future, the clinical diagnosis of arthritis will be reinforced by patient-specific accessible molecular and cellular biomarkers of disease entities. The analysis of MMP proteoforms in blood (plasma and/or serum) and synovial fluid could help with diagnosis, alongside genetic information and cell (immune)phenotyping. Gradually, with the development of validated analytes in individual patient samples, including transcriptome and proteome data about PTMs and with global integration of large data sets, precision diagnosis and treatment will become a reality for treatment-naïve patients. Proteolysis by MMPs in arthritis is one such irreversible PTM and is accessible for analysis. Any medical diagnosis becomes stronger with more biomarkers; hence, combinations of validated biomarkers are helpful for the time being. This approach started from arthritis research[53] and could be broadened to other spectra of inflammatory and autoimmune diseases. The insight that some biomarkers can be altered before clinical signs of autoimmune arthritis become evident creates a window of opportunity for preventive measures. Novel preclinical and clinical studies of MMP inhibition need to complement present insights and it is possible that inhibition of relevant pathogenic MMPs, which generate remnant epitopes in RA or degrade cartilage constituents in OA, could be used in the future. In such ways, precision medicine will fulfill its role for prevention, new therapies and cures of arthritic diseases.

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Key points

- After activation of pro-matrix metalloproteinases (pro-MMPs) via various post-translational modifications, MMPs irreversibly digest structural proteins in joint tissues.
- In rheumatoid arthritis (RA), MMPs from neutrophils and pannus tissue cleave cartilage glycoproteins into remnant epitopes that enhance autoimmune processes and thereby contribute to RA pathogenesis in susceptible hosts.
- MMPs from chondrocytes in concerted action with other metalloproteinases contribute to degenerative OA.
- Detection of proteoforms of MMPs from various cellular sources in blood and synovial fluids can inform the diagnosis of rheumatic diseases.
- Clinical use of MMP inhibitors first requires firm proof of the causal effects of MMPs and knowledge about the cells and molecules involved in specific arthritis entities.
- Development of precision diagnostics and therapeutics will be aided by the use of large data sets of analytes and patient cohorts and the integration of multi-omics data

Table 1. Primary arthritis in MMP gene variants

MMP	Affected (*n*)[a]	Variants (*n*)[b]	Alternative name	Chromosome	~~Ref.~~
MMP-2 (gelatinase A)	46	24	Multicentric osteolysis, and arthropathy	16q12	109, 110
MMP-9 (gelatinase B)	6[c]	4	Recessive metaphyseal anadysplasia	20q13.12	111-113
	1[d]	2			114
MMP-13 (collagenase-3)	10	3	Dominant metaphyseal anadysplasia	11q22.2	111
MMP-14 (MT1-MMP)	4	2	Severe osteolysis, Winchester syndrome	14q11.2	115
MMP-20 (enamelysin)	>19	19	Amelogenesis imperfecta	11q22.2	116-118

MMP, matrix metalloproteinase.

Footnotes: [a]Numbers of affected individuals for the indicated MMP gene. [b]Numbers of different gene variants, including missense and frame-shift mutations and nucleotide substitutions. [c]Homozygous children. [d]Compound heterozygous fetus.

Table 2. Reference concentrations of MMPs in health, RA and OA

Enzyme	Location	Condition			Ref(s)
		Normal	RA	OA	
Collagenase (MMP-1 (collagenase)	Serum (ng/ml)	6.6	8.2	n/a	[119] Manicourt et al., 1995
	Synovial fluid (ng/ml)	n/a	1,400	350	[67] Wolfe et al., 1993
	Cartilage (ng/mg-ww)	n/a	0.168	0.059	[68] Martel-Pelletier et al., 1994
MMP-3 (stromelysin-1)	Serum (ng/ml)	33	209	n/a	[119] Manicourt et al., 1995
		134	944	n/a	[56] Ishiguro et al., 1996
		n/a	348	68.8	[47] Sasaki et al., 1994
	Plasma (ng/ml)	50	187	115	[120] Zucker et al., 1994
	Synovial fluid (ng/ml)	n/a	13,200	1400	[67] Wolfe et al., 1993
		n/a	180,900	29000	[47] Sasaki et al., 1994
		n/a	140,000	n/a	[56] Ishiguro et al., 1996
	Cartilage (ng/mg-ww)	11.1	99.1	16.8	[68] Martel-Pelletier et al., 1994
MMP-2 (gelatinase A)	Serum (ng/ml)	800	n/a	n/a	[121] Garbisa et al., 1992
	Synovial fluid (nmol/l)	n/a	29	22	[122] Yoshihara et al., 2000
MMP-9 (gelatinase B)	Serum (ng/ml)	43	987	n/a	[123] Gruber et al., 1996
	Synovial fluid (ng/ml)	3	1,620	n/a	[123] Gruber et al., 1996
	Synovial fluid (nmol/l)	n/a	11	n/a	[122] Yoshihara et al., 2000
MMP-8 (neutrophil collagenase)	Serum (ng/ml)	n/a	n/a	>1	[101] Meehan et al., 2021
	Synovial fluid (ng/ml)	1	Active RA: 141 Controlled RA: 1.6	1	[101] Meehan et al., 2021

mg-ww, mg wet weight; MMP, matrix metalloproteinase; OA, osteoarthritis; RA: rheumatoid arthritis.

Figure 1: Timeline of MMP discoveries related to arthritis research.

During 6 decades, matrix metalloproteinase (MMP) research included the discovery of MMPs, cDNA cloning, identification of proteoforms by amino acid sequencing, and activation of MMPs by proteolysis and by reactive oxygen species (ROS) (yellow bullets). At the biochemical level, knowledge of MMP involvement in joint destruction emerged in the decade when aglycosyl IgG was discovered as a biomarker of rheumatoid arthritis (RA), whereas citrullinated peptides were later added for diagnosis (purple bullets)[48,50]. Purification and regulation of neutrophil MMP-9 and the discovery of the cysteine switch mechanism happened at the time when anti-TNF was introduced for RA treatment (blue bullets)[6,124]. The role of proteolysis by MMPs as a post-translational modification (PTM) in the origin of remnant epitopes as initiators and/or perpetuators of autoimmune diseases and of RA in particular[31] was later complemented by peptide citrullination as a key PTM for RA (ref.[51]) (orange bullets). The specific roles of neutrophils and their peculiar MMP biology (with no MMP-2 or TIMP-1 production[19,20]) and of MMP-9 trimers and MMP citrullination were refined relatively recently (dark blue bullets). Neutrophils, alongside osteoclasts, remain still insufficiently recognized as key players in arthritis in the present age of single-cell RNA sequencing, cell immunophenotyping and detection of citrullinated MMPs in patients with arthritis, and deserve further studies because their abundantly produced MMP-9 trimers are so far the only MMP proteoforms that escape inhibition by α-2-macroglobulin[17] (grey background).

Figure 2: Comparison of major producer cell types, MMPs and TIMPs in normal, RA and OA synovium.

In a normal joint (centre), the synovial space is almost virtual, with a few mononuclear cells (not shown) in a few milliliters of synovial fluid. Chondrocytes within the intact cartilage produce and embed collagens and proteoglycans within their extracellular matrix and secrete lubricin. In osteoarthritis (OA) (left), a degenerative process leads to slow cartilage thinning, mainly by chondrocytic MMP-13 and MMP-2 from within the cartilage, but also by matrix metalloproteinase (MMPs) from other mononuclear cells, primarily monocytes and macrophages, which are chemoattracted by CC chemokine ligands (CCLs), and local synoviocytes. The synovial fluid fills the enlarged synovial space, where MMPs from chondrocytes, mononuclear leukocytes, synovial fibroblasts and endothelial cells within the thickened synovial membranes are kept in check by co-expressed tissue inhibitors of metalloproteinases (TIMPs) and α-2-macroglobulin (α_2M) in any acute OA phase. In rheumatoid

arthritis (RA) (right), the amount of synovial fluid is considerable, with mainly neutrophils bringing along MMP-8 and MMP-9 without any MMP-2 or TIMP-1 (ref.[19,20]) and activating these MMPs via reactive oxygen species (ROS) (ref.[21,22]) . Mononuclear leukocytes entering the synovial space bring along TIMPs in order to restore MMP–TIMP balances (ref.[125]). Furthermore, MMP-9 trimers escape inhibition by α_2M [17], yielding net proteolytic activity within the synovial fluid, thereby attacking the cartilage surfaces and generating remnant epitopes from type II collagen (ref.[35,36]). In addition, MMP-9 potentiates the activity of the major neutrophil chemokine IL-8 (CXCL-8) 10-fold, further enhancing neutrophil influx[126]. Because the abundant mature neutrophils are short-lived cells with rather unstable mRNAs, in single-cell RNA sequencing profiles the presence of these cells seems rather underestimated in comparison with that of other leukocytes, in both OA and RA. Finally, in RA, erosion of bone and cartilage also takes place by the invasive pannus tissue, leading to painful and mutilating effects in severe RA. Resident cells, including osteoclasts and synoviocytes, as well as chemoattracted leukocytes in the pannus tissue produce MMP-14 (MT1-MMP) as a major collagenase[38,39,97] and constitutive MMP-2 and induced MMP-9 also here further degrade cartilage type II collagen and bone type I collagen into (auto)immunodominant epitopes. From these general views, the inhibition of MMP-13 in OA and dual-specific inhibition of MMP-8 and MMP-9 as well as inhibition of MMP-14 in RA warrant preclinical and clinical evaluation.

Figure 3: Post-translational modifications as control of MMP activities in arthritis

Cellular production of matrix metalloproteinases (MMPs) is under the form of catalytically inactive pro-MMPs (first level). The life cycles of inactive pro-MMPs are influenced by many post-translational modifications (PTMs). Pro-MMPs are endogenously produced as mixtures with variations in N- or O-linked glycosylation (PTM1), named glycoforms **[G]**. In general, glycosylation (indicated by large yellow branched structures) results in rather large PTMs in comparison with citrullination and phosphorylation and it fine-tunes the interactions of proteins with other molecules. At the second level, specific forms of pro-MMP activations are illustrated. Chemical oxidation of the cysteine sulfhydryl group (-SH) in the pro-peptide (light green) cysteine residue by neutrophil reactive oxygen species (ROS) (PTM2) (ref.[21,22]) or removal of this cysteine residue by proteolysis (PTM3) (ref.[8]) are established mechanisms of activation, yielding active MMPs with a hydrolytic water molecule (H_2O) interacting with the catalytic Zinc ion (Zn^{2+}) (red). Whereas the conversion of arginine residues to citrulline (indicated in dark green ball) is a known PTM in RA and also takes place on specific MMPs (PTM4) (ref.[52]), it is not yet established whether MMP nitrosylation or phosphorylation (not shown) play any role in arthritis. All MMPs are finally degraded by proteolysis (PTM5), for example by complement proteases, by MMPs and by other inflammatory proteases, resulting in degradation products (third level). Altogether, these PTMs are essential in MMP biology, with each individual

presentation form of an MMP, termed a proteoform, having specific activities. Hence, MMPs exist in arthritis always as protein mixtures. With the exception of nitrosylation, all of the PTMs illustrated here are established in arthritis, yielding various MMP proteoforms. The latter variations are not captured by transcriptomics or single-cell RNA-sequencing methods. In addition, most commonly used immunological MMP detection systems (ELISAs) do not distinguish proteoforms with different PTMs. With the development of dedicated proteomic platforms and tests for net MMP activities, some of these complications might be resolved and enable precision medicine in arthritic diseases.

Box 1: The search for synthetic MMP inhibitors

Many concepts of extracellular matrix (ECM)-degrading proteinases in rheumatology were built on knowledge about serine proteinases and date from 1975 onwards. The plasminogen–plasminogen activator system gained much attention because ECM proteolysis by plasmin is essential for tumour cells to invade surrounding tissues and eventually to enhance metastasis to distant organs[127]. With the discoveries of plasminogen activators and many new MMPs (**Figure 1**), these proteinases became interesting new targets for the development of metastasis inhibitors in cancer research[128]. Although many clinical studies with MMP inhibitors in oncology failed, investigations into why this happened[129] and excellent preclinical studies that gradually deciphered proteinase and proteinase inhibitor networks followed[130], providing information that is helpful for all studies of MMP inhibitors in specific phases of any disease. For example, the development of painful joints of patients treated with small-molecule MMP inhibitors for metastatic breast cancer could be explained by the inhibition of homeostatic MMP-2. Beneficial effects of MMP-2 according to disease phase are suggested by the reduced recovery from spinal cord injury and increased immune complex arthritis observed in *Mmp2*$^{-/-}$ mice[27].

Investments in MMP inhibitor research thus resulted in double positive effects. First, great expectations for broad applications to reduce metastasis stimulated many pharmaceutical companies to develop potent inhibitors of the plasminogen activation system and for almost every human MMP. These MMP inhibitors remain insufficiently explored for inflammatory joint diseases. Second, MMP inhibitors produced at industrial scale were already clinically tested in oncology, implying that detailed pharmacological and pharmacokinetics data already exist. Excellent anti-inflammatory drugs are available for rheumatic diseases[124,131] but therapy-resistant arthritis cohorts remain. With the development of new omics technologies, a renaissance of pharmacological MMP inhibition studies in arthritis research is imminent.

Box 2: Joint inflammation and MMPs

Physical stress of joints occurs in acute trauma and with chronic overload (as observed in tennis elbow or frozen shoulder), in burns and after accidental or therapeutic irradiation. Septic arthritis is commonly established via a haematogenic route or by non-sterile puncture of the synovial space. Chemical and/or biochemical irritation can occur in metabolic diseases, such as gout, and is also observed with the use of matrix metalloproteinase (MMP) inhibitors[132]. Rheumatoid arthritis (RA) is a prototypic autoimmune disease, in which autoantibodies or antigen-specific T cells against collagen antigens are involved[32, 133]. Even local neoplastic processes, such as observed in pathological fractures in Kahler's disease or osteosarcoma, or systemic malignancies, mainly leukaemias, can lead to joint inflammation. Almost all of the more than 100 rheumatic diseases are characterized by inflammation, and reduction of the degree of inflammation remains a pillar of treatment strategies. Although the clinical signs of inflammation can differ considerably in localization (from one joint to general arthritis in systemic lupus erythematosus) and in time (hyperacute septic arthritis *versus* years of fibrosis development in systemic sclerosis), the molecular processes always converge to chemical and biochemical reactions [7,17,18,21-24]. Important chemical principles in arthritis are pH modification and free radical formation, whereas the biochemical mechanisms include enzymatic hydrolysis of proteins, lipids and glycans, alongside the formation of cellular membrane pores by the complement and perforin systems. Intrinsic connections and fine-tuning processes exist between chemical and biochemical reactions. For instance, under purulent conditions with insufficient buffering, acidic conditions can develop and provide optimal catalytic conditions for aspartate proteinases such as cathepsin D. Pro-MMPs can be chemically activated *in vivo* by hypochlorous acid and other reactive oxygen species (ROS) from neutrophils[21,22]. Extracellular matrix-degrading proteinases belong to several catalytic classes and their homeostatic functions happen usually at neutral pH. MMPs do not act alone in this setting, but always in concert with other proteinases, for example those of the plasminogen activation system[23,24,134-136]. Under conditions of inflammation, the influx of plasma exudate can restore the pH from acidic conditions to neutral levels. Under such conditions, MMPs act optimally and activate each other in a proteinase cascade or proteinase network, until the actions of α-2-macroglobulin and locally induced tissue inhibitors of metalloproteinases stop the catalytic processes.

Box 3: MMPs in rheumatoid arthritis versus osteoarthritis.

Major progress has been made in the study of inflammation in rheumatoid arthritis (RA). Neutrophils are responsible for the release of matrix metalloproteinase (MMP)-8 (neutrophil collagenase) and MMP-9 (gelatinase B). Both MMPs are detected in considerable amounts in synovial fluids, collectively

induce cartilage collagen breakdown and generate type II collagen fragments, known as autoantigenic remnant epitopes. MMP-1 (collagenase) and MMP-3 (stromelysin-1) are also detected in synovial fluid and in cartilage tissue[54] and might be involved in local inflammation of cartilage, bone, synovium and tendons[137]**.** Unfortunately, neutrophils are often overlooked in single-cell transcriptomic analysis of synovial fluid or tissues [138-139].

In osteoarthritis (OA), by contrast, neutrophils and mononuclear leukocytes are not the core of the problem. Here, the first changes occur in cartilage and subchondral bone. Cartilage tissue consists mainly of chondrocytes, type II collagen and proteoglycans. During the normal ageing process, the number of chondrocytes decreases in cartilage, and the glycosaminoglycans in the proteoglycans become shorter and less abundant[140,141]. In OA cartilage, changes occur in the number and in the function of chondrocytes, resulting in decreases of collagen and aggrecan synthesis and increased secretion of proteinases, particularly MMP-3 (ref.[142]), MMP-13 (collagenase-3)(ref.[143,144]), ADAMTS-4 and ADAMTS-5 (ref.[145,146]). As most aggrecan fragments in OA synovial fluid are cleaved at a specific Glu373–Ala374 bond[147], ADAMTS-4 and ADAMTS-5, rather than MMP-3, are responsible for aggrecan degradation. Although both MMP-1 and MMP-13 are detected in OA synovial fluid, MMP-13 is the primary collagenase in OA (ref.[148]). OA cartilage breakdown is accompanied by sclerosis of the subchondral bone and by a mild synovitis with infiltration of lymphocytes and histiocytes and the appearance of MMP-1 (ref.[54]), MMP-3 (ref.[54,55]) and MMP-9 (ref.[59,60]), although in titres far below those detected in RA.

Although MMPs are involved in both of these common forms of arthritis, potential inhibitors need to be targeted to clearly different MMPs.

Glossary terms

Cysteine switch: Mechanism explaining the latency of matrix metalloproteinases by way of a cysteine residue in the propeptide; removal of the cysteine sulfhydryl from the zinc ion in the active site switches the proteinase on.

Glycoform: Presentation of a protein with specific oligosaccharides attached, implying that any glycoprotein exists as a mixture of one protein backbone with different attached sugars.

Proteasome: Macromolecular complex with threonine proteinase activity, which cleaves misfolded intracellular proteins into short peptides that can be presented by MHC class I.

Proteoform: Individual presentations of any protein, implying that all proteins exist as collections of variants with different posttranslational modifications, such as phosphorylation, citrullination and truncations.

Remnant epitope: Host-derived peptide after proteolytic processing by extracellular proteinases from the host or from invading microorganisms; these peptides can be altered by posttranslational modifications and presented as autoantigens, mainly in MHC class II molecules.

Editor's Summary

Matrix metalloproteinases (MMPs) contribute to irreversible joint remodelling in the pathogenesis of joint diseases including rheumatoid arthritis and osteoarthritis. This article reviews several aspects of MMP biology related to arthritis and discusses how they relate to opportunities for precision medicine and diagnosis.

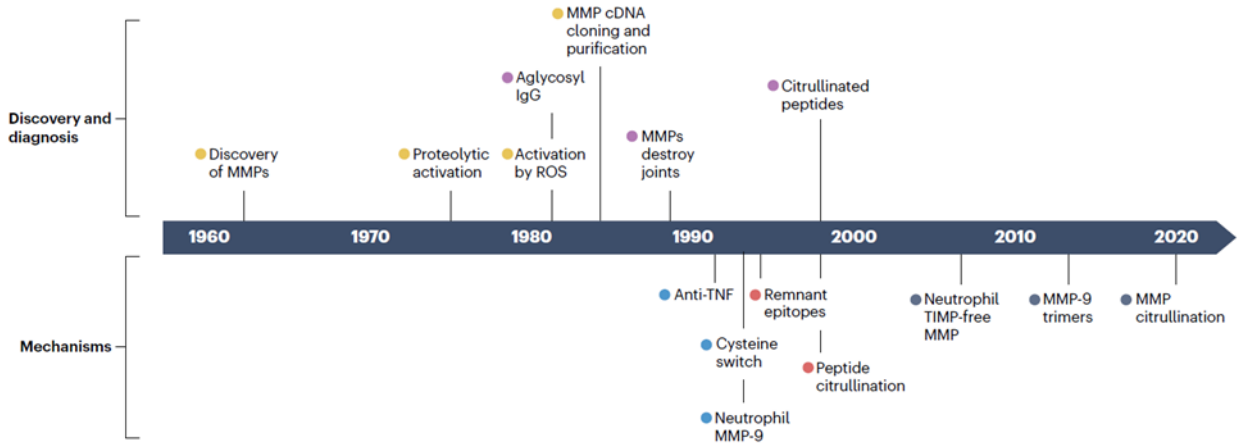

Figure 1

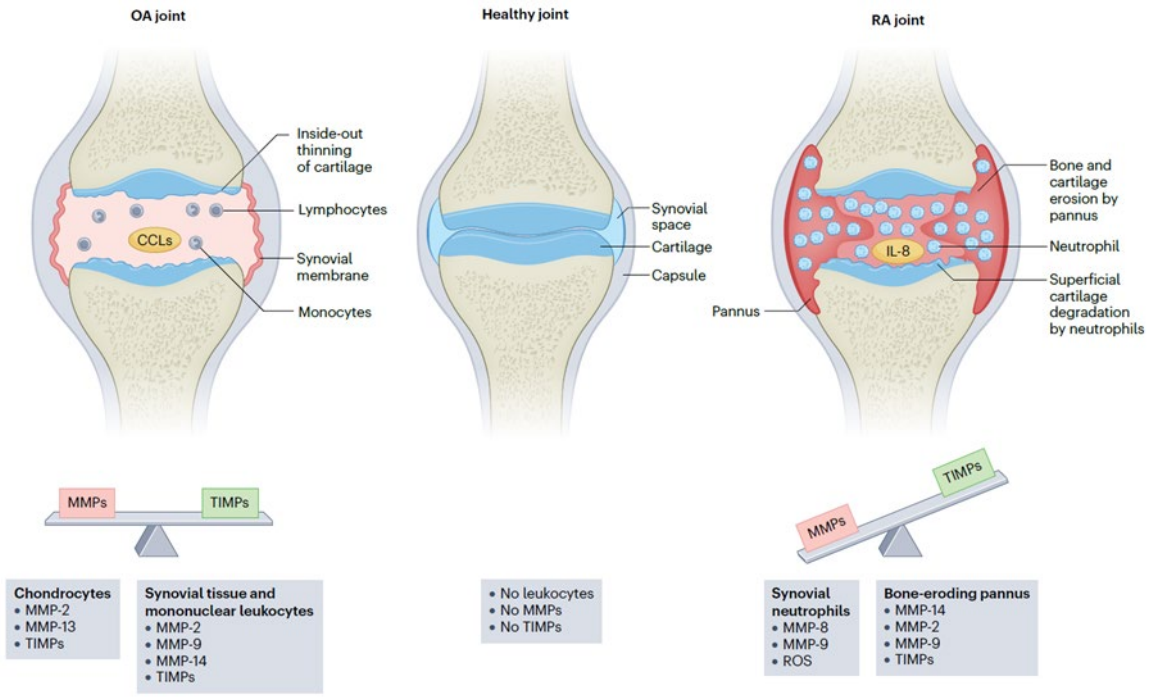

Figure 2

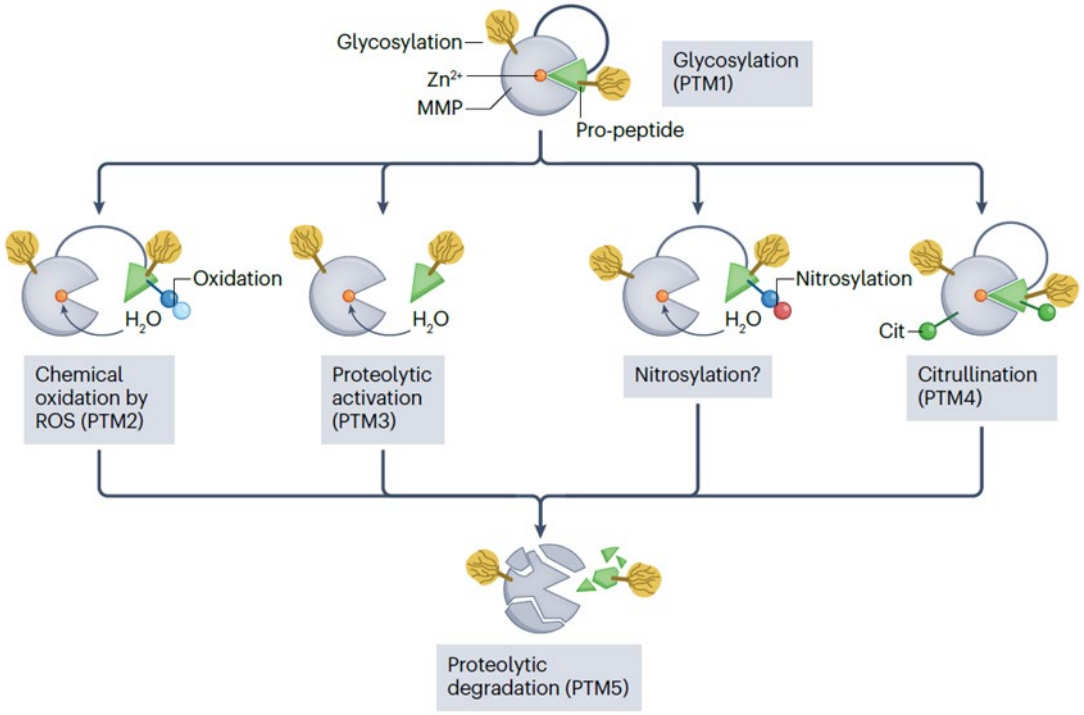

Figure 3

Supplementary Table 1. Experimental models of MMP gene deletions and phenotypes in mice, reported since 2007.

Mmp deficiency (alternative MMP name)	Phenotypic characteristics	Genetic background	Ref.
Mmp1a$^{-/-}$ (collagenase)	Viable, fertile, normal life-span; decreased susceptibility for chemical carcinogenesis; decreased angiogenesis in lung tumours	C57BL/6J	1,2
Mmp10$^{-/-}$ (stromelysin 2)	Viable, fertile, normal gross appearance; enhanced pulmonary inflammation after infection; delayed myotube regeneration after ischemia by vascular ligation	C57BL/6J	3,4
Mmp15$^{-/-}$ (MT2-MMP)	Normal viability through adulthood; increased beige/brown fat production in mammary gland tissue	C57BL/6J	5
Mmp16$^{-/-}$ (MT3-MMP)	Viable and fertile	C57BL/6	6
Mmp17$^{-/-}$ (MT4-MMP)	Viable and normal fertility and life-span; protection against cartilage aggrecan degradation by inflammatory (but not by mechanical) stress; predisposition for thoracic aortic aneurysms	C57BL/6	7,8,9
Mmp25$^{-/-}$ (MT6-MMP)	Viable, fertile and no spontaneous phenotype; defective innate immune response upon challenge	C57BL/6	10
Mmp9$^{-/-}$ and Mmp14$^{-/-}$ (MT1-MMP)	Double gene knockout in osteoclasts yields viable mice with altered osteoclast transcriptome mediated by galectin-3 cleavage	C57BL/6	11

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Supplementary Table 2. Literature information about MMPs and citrullination

Numbers of PubMed entries on "MMP and citrullination", according to publication year and in association with arthritis and corresponding details about antibodies against citrullinated peptides or presence of indicated MMPs.

Year	Citrullination	Arthritis (j)	ACP (k)	ACCP (k)	MMP (l)
2003	2	2	1	1	MMP-3
2004	2	2	2	0	MMP-3
2005	3	3	0	2	MMP-3
2006	2 (a)	3	0	2	MMP-3
2007	4	4	2	2	MMP-3
2008	3	3	0	3	MMP-1,-3,-9
2009	5 (b)	4	1	3	MMP-3
2010	6 (c)	5	1	5	MMP-3
2011	3	3	0	3	MMP-3
2012	5 (d)	5	2	3	MMP-3
2013	8 (e)	7	1	5	MMP-1,-3
2014	7 (f)	5	1	3	MMP-3
2015	6 (g)	5	0	4	MMP-3
2016	10 (h)	8	1	6	MMP-1,-3
2017	12 (i)	9	0	5	MMP-1,-3
2018	9	6	0	3	MMP-3,-9
2019	16	10	1	5	MMP-3,-9
2020	14	9	2	7	MMP-1,-3
2021	8	2	0	1	Many MMPs
TOTAL	125	95	15	63	

Footnotes:

(a) One study related to multiple sclerosis
(b) One *in vitro* study is with dental pulp cells
(c) One study is about meningitis
(d) One study is about liver fibrosis
(e) One study is about systemic sclerosis
(f) Two studies relate to cancer research
(g) One study is about inflammatory bowel disease
(h) Two studies are about cancer

(i) One study is about pancreatitis and two are about cancer research
(j) Arthritis refers to all forms of proven joint inflammation, including rheumatoid arthritis, osteoarthritis, traumatic and infectious arthritis and unclassified microgeodic syndrome of the hand articulations
(k) In specific studies, the antigens were defined as vimentin, fibronectin, enolase, filaggrin and in one study the conversion of arginine to citrulline was by NO synthetase; ACP presence of antibodies against citrullinated peptides; ACCP presence of antibodies against cyclic citrullinated peptides.
(l) Notice that the presence/identification of MMP-3 is present in all studies.