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Interactions between extracellular matrix proteins and the complement system

In the perspective of cartilage degradation in inflammatory joint diseases

Camilla Melin Fürst

DOCTORAL DISSERTATION
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Abstract: The joint diseases osteoarthritis and rheumatoid arthritis are characterized by destructive inflammatory processes that result in pathological changes of the joint tissues, including proteolytic degradation of cartilage and release of extracellular matrix proteins or fragments to the synovial fluid. The complement system, which is a part of the innate immune system, plays a central role in promoting the joint inflammation in these diseases. Potential activators of complement are certain extracellular matrix proteins that become exposed during cartilage degradation. Previous studies have revealed that several proteins from cartilage can activate or inhibit the complement system in vitro.

In the present work, we describe the interactions between the complement system and additional extracellular matrix proteins, with the aim to better understand the role of endogenous ligands in the inflammatory process in joint diseases. We also describe the interactions between complement proteins and cartilage explants that have been subjected to inflammation-induced degradation.

In paper I, we found that the G3 domain of aggrecan activates the classical pathway of complement. However, the activation of the terminal pathway is limited due to the simultaneous binding of factor H. Whether it activates complement when maintained in cartilage or when released into the synovial fluid remains to be elucidated.

In paper II and III, we found that both proline/arginine-rich end leucine-rich repeat protein (PRELP) and the domain NC4 of collagen type IX, inhibit complement by preventing the assembly of the membrane attack complex. Further, PRELP also inhibits the assembly of the alternative pathway C3 convertase, while NC4 enhances the cofactor activities of C4b-binding protein and factor H, in the factor I-mediated cleavage of C4b and C3b. NC4 and fragments of PRELP can be detected in the synovial fluid of rheumatoid arthritis patients. Located in the synovial fluid or exposed on the cartilage surface, they might be important for limiting the complement activation, induced by other extracellular matrix proteins or other potential triggers.

In Paper IV, we found that both the classical and the alternative pathways are activated on the surface of degraded cartilage explants, while components released from cartilage might have a weak, somewhat delayed, opposing role by inhibiting complement. The main activation seems to occur after the major loss of aggrecan from cartilage.

In sum, several proteins of the extracellular matrix, as well as degraded cartilage have the potential to interact with the complement system, and may regulate the inflammatory processes in joint diseases.

Key words: Complement system, arthritis, extracellular matrix, cartilage degradation, mass spectrometry
Interactions between extracellular matrix proteins and the complement system

In the perspective of cartilage degradation in inflammatory joint diseases

Camilla Melin Fürst
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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ADAMTS</td>
<td>a disintegrin and metalloproteinase with a thrombospondin type 1 motif</td>
</tr>
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<td>CAIA</td>
<td>collagen-antibody induced arthritis</td>
</tr>
<tr>
<td>CIA</td>
<td>collagen induced arthritis</td>
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<tr>
<td>COMP</td>
<td>cartilage oligomeric matrix protein</td>
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<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>C4BP</td>
<td>C4b-binding protein</td>
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<tr>
<td>DAF</td>
<td>decay accelerating factor</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FH</td>
<td>factor H</td>
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<td>IL</td>
<td>interleukin</td>
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<td>MAC</td>
<td>membrane attack complex</td>
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<td>MASP</td>
<td>MBL-associated serine protease</td>
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<tr>
<td>MBL</td>
<td>mannose-binding lectin</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NC4</td>
<td>non-collagenous segment 4</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>PRELP</td>
<td>proline/arginine-rich end leucine-rich repeat protein</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>SLRP</td>
<td>small leucine-rich repeat protein</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor -α</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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The complement system

The complement system is a crucial part of innate immunity and consists of a large number of proteins that cooperate in order to identify and protect us from invading pathogens and damaged or altered self-structures. It detects targets with high specificity and optimally initiates fine-tuned responses that ultimately lead to the removal of the initial trigger with minimal damage to the host. The complement system includes soluble proteins that are organized into a proteolytic cascade, where inactive enzyme precursor are cleaved and activated by upstream enzymes, as well as complement inhibitors that tightly regulate the activation of the cascade, and complement receptors. Activation of the complement cascade leads to various effector functions that involve close communication with other players of the immune system, to regulate various inflammatory and cellular responses as well as adaptive and innate immune responses. The direct effects of complement activation are e.g. anaphylaxis, opsonisation to facilitate phagocytosis and clearance of pathogens, immune complexes, apoptotic cells and other endogenous ligands, and lysis of bacteria (1-3).

Since complement is involved in such a variety of functions, it is easy to see that if any part of the system is not working properly, it might lead to disease. Disturbances in this system that lead to excessive, improper activation that is not sufficiently controlled, might cause inflammatory conditions that are harmful for the host and its tissues (1). That is what occurs in rheumatoid arthritis (RA) and osteoarthritis (OA), where pathological inflammation in the joints contributes to tissue damage and progression of disease (4,5).

The complement proteins exist either as in soluble or membrane-bound forms. The primary site of synthesis for most of the soluble complement proteins is the liver. Notably, for factor D the primary site is adipose tissues (6). Further, all tissues in the body have the capacity to produce some complement proteins, usually in response to inflammatory stimuli (6). The complement cascade can be initiated via three pathways, namely the classical, lectin and alternative pathways. The alternative pathway also function as an amplification loop of the two other pathways. Activation of any of the pathways can result in the formation of the enzymatic complexes C3 and C5 convertases, and finally the pathways are joined into one terminal pathway (3).
The classical pathway

The classical pathway is activated upon binding of the pattern recognition molecule C1q to certain ligands, such as immunoglobulins (IgM and IgG)(3), C-reactive protein (7,8) and certain pathogens (9,10), apoptotic cells (11,12) or endogenous structures (13,14). C1q is composed of multiple polypeptide chains (encoded by three separate genes (C1qA, -B and -C)(15)) that are organized into a six-armed molecule with globular heads in the C-terminal ends. The arms combine in the N-terminal region into a common collagen-like stalk region (16). C1q associates with the Ca$^{2+}$-dependent pro-enzymes C1r and C1s (two of each) to form the dynamic C1-complex (16).

Binding of C1q to a target results in a conformational change in the C1q molecule, which leads to the activation of C1r and C1s. C1s cleaves C4, which results in the release of a small fragment, C4a, and the covalent attachment of C4b to a nucleophile in the close proximity (usually the activating target) via a newly exposed and highly reactive thioester group (3,17). Upon binding of C2 to C4b, C1s further cleaves C2 into C2a that associates with C4b, while the C2b fragment is released. This long sequence of enzymatic events, results in the formation of the classical pathway C3 convertase (C4b2a) that has C3 as substrate. The enzymatic complex cleaves C3 into C3a and C3b. Similar to C4, a reactive thioester group become exposed upon cleavage of C3 that mediates the covalent binding of C3b to the activating surface (3). The C3a fragment is released and function as an anaphylatoxin, which role will be discussed later.

The lectin pathway

The lectin pathway resembles the classical pathway in many ways. A protein complex, consisting of a recognition molecule and associated proteases, forms the first step of the pathway (18). Upon binding of the complex to a target, the pathway is activated by the cleavage of C4 and C2 that form the same C3 convertase (C4b2a).

The recognition molecules are mannose-binding lectin (MBL) or ficolins (ficolin-1, -2 and -3) that associate with various mannose-binding lectin-associated serine proteases (MASPs) (For review see (18,19)). Like C1q, the recognition molecules are oligomers composed of several polypeptide chains, that are organized to form protruding arms in one end, and a common, assembled collagen-like stalk region (19). MBL and ficolins recognize a wide variety of pathogens via binding to highly conserved carbohydrate structures. MBL can bind for example lipopolysaccharide of Gram-negative bacteria and lipoteichoic acids of the cell
wall of Gram-positive bacteria (18). Ficolins were first identified to bind specifically to acetylated carbohydrates and proteins (20,21). However, ficolin-2 has later been shown to have broader substrate specificity, as it can bind for example DNA (22) and lipoteichoic acid (23). Further, MBL and ficolins are also involved in the phagocytosis of apoptotic and necrotic cells and mitochondria (24) (22,25,26). The lectin pathway can also be activated by interactions between MBL and IgA (27).

The alternative pathway

In plasma, a minimal amount of C3 is hydrolysed by a nucleophilic attack on the thioester by H₂O, which results in the formation of C3(H₂O) (for review see (28,29)). The spontaneous hydrolysis occurs at a constant, slow rate (30) that might be accelerated in vivo by the help of various biological surfaces, such as activated platelets, lipid surfaces and gas bubbles (29). The hydrolysis changes the conformation of C3 so that it resembles the conformation of C3b. That allows factor B to bind, and subsequently factor D cleaves factor B into Bb and Ba (31), creating the Mg²⁺-dependent C3 convertase C3(H₂O)Bb (28). The convertase can then cleave C3 molecules into C3b that covalently attaches to amine or hydroxyl groups of proteins and carbohydrates in nearby surfaces via its exposed, reactive thioester (28,32,33). Deposited C3b can now work as platforms to form new C3 convertases (C3bBb). The convertase has a short half-life, which is increased ten-fold by the binding of properdin (factor P) (34), which slows down the dissociation of Bb from C3b.

Moreover, properdin can also bind directly to certain structures (pathogen-associated molecular patterns and damage-associated molecular patterns) on pathogens (35) and apoptotic cells (36,37), and forms a platform to which fluid-phase C3b is recruited which initiates the formation of the C3 convertase (35). Further, C3 and C5 can be activated by specific proteases and not only by the convertases. Several enzymes involved in coagulation (e.g. kallikrein, thrombin, factor Xa), and certain intracellular enzymes (Granzyme B and certain cathepsins) can cleave either C3 or C5, or both, which could play an especially important role in trauma and sepsis (38).

Due to the spontaneous hydrolysis of C3, host cells as well as pathogens are targets for C3b deposition. However, host cells are protected by the presence of membrane-bound complement inhibitors, as well as soluble inhibitors (factor H, FH) recruited to cell surfaces, which rapidly inhibits further activation (2).
The terminal pathway

As a result of increasing concentrations of deposited C3b on surfaces, C3b associates with the C3 convertases to form C5 convertases (classical/lectin pathway C4b2a3b or alternative pathway C3bBb3b) (2,3). The C5 convertase obtains substrate specificity for C5, and by cleaving C5, the anaphylatoxin C5a is released, while C5b remains associated with the convertase (3). This enzymatic event is the first step of the terminal pathway (39). C5b recruits C6 and C7 from plasma and form the metastable complex C5b-7. The complex can be irreversibly inhibited by hydrolysis or by binding of C8 or complement inhibitors. A few C5b-7 molecules avoid attack and can bind tightly onto the membrane. Subsequently, C8 and several C9 molecules binds to form the membrane attack complex (MAC; C5b-9). The complex undergoes a conformational change that leads to the
formation of a lytic pore in the membrane (for review of MAC formation, see (39,40)).

Complement inhibition

Inhibitors tightly control the complement system, which is necessary to ensure a balanced level of activation, suitable for the specific situation. Otherwise, there would be excessive activation and inflammation that would be harmful for host tissues. The complement inhibitors can together control all stages of the complement cascade. Most of the complement inhibitors consist of structurally similar domains, called complement control protein domains.

The C1 inhibitor acts on the classical and lectin pathway initiation via interactions with proteases C1r, C1s and MASP2s (41). The next step where inhibitors could act is at the level of the C3 and C5 convertases. Formed C3b and C4b can be cleaved by factor I to iC3b and iC4b, with the support from cofactors, and are therefore prevented from forming the C3 convertases (2). Membrane-bound inhibitors, membrane cofactor protein (MCP; CD46) and complement receptor 1 (CR1), can act as cofactors, as well as soluble factor H (FH) and C4b-binding protein (C4BP) (42). The membrane-bound inhibitors serve as cofactors for cleavage of both C3b and C4b, while FH only acts on C3b and C4BP preferentially acts on C4b and to a lesser extent on C3b (43-45). C3b and C4b are cleaved sequentially by factor I and result in e.g. iC3b, C3c, C3dg, C3d and iC4b, C4c, C4d respectively (46,47). C3d and C4d are the final cleavage products that remain bound to the target, while C3c and C4c are released (46,47). CR1 is the only cofactor that can assist in the cleavage to obtain C3dg (48).

Further, complement inhibitors can also directly inhibit the formation of the C3 and C5 convertases, as well as accelerate their decay. The membrane-bound inhibitor decay accelerating factor (DAF; CD55) (2,49) as well as CR1 (50), FH (51) and C4BP (52,53) accelerate the convertase decay, by binding C3b or C4b that destabilize the convertases. FH and C4BP selectively accelerate the decay of either the alternative or the classical/lectin pathway convertases, while DAF and CR1 act on convertases derived from all pathways. DAF, FH and C4BP can also inhibit the actual formation of the convertases (2,51-53).

Acting on the last section of the complement pathway, membrane-bound CD59 and soluble clusterin, inhibit the MAC assembly and the formation of the pore (3). The released anaphylatoxins C3a and C5a are rapidly inhibited by carboxypeptidase N in plasma by cleavages at the C-terminal arginine that results in C3a-desArg and C5a-desArg. The biological activities of those new fragments are clearly reduced; C3a-desArg looses the ability to interact with C3aR, while C5a-desArg obtains a 90% reduction in its pro-inflammatory activity (42).
Effector functions

Anaphylaxis

Anaphylatoxins work as chemoattractants and exert various modulatory effects on cells of myeloid origin but also on other cell types, via signalling through receptors for C3a and C5a (C3aR and C5aR)(3). Activation of these cells by anaphylatoxins leads to different responses depending on the cell type (for review see (1)). Mast cells respond to anaphylatoxins by releasing histamine, cytokines, such as TNF-α, chemokines and proteases. Macrophages also increase the release of cytokines and chemokines, while neutrophils and monocytes increase the eicosanoid production (e.g. prostaglandins and leukotrienes). Several of these released inflammatory mediators cause vascular changes, such as vasodilation and increased vascular permeability. Further, the leukocyte extravasation (migration through the vascular wall) is increased as the anaphylatoxins cause an up-regulated expression of adhesion molecules in endothelial cells as well as in leukocytes. Importantly, the anaphylatoxins work as chemoattractants that attracts leukocytes towards the site of complement activation and anaphylatoxin release, by binding to their corresponding receptors (1). The anaphylatoxins also influence the capacity of dendritic cells to present antigens, as well as the T-cell response and differentiation (54).

Opsonisation

Complement activation results in opsonisation of the target that promotes recognition and engulfment by phagocytic cells. C3b and its cleavage fragments, C4b, C1q, MBL and ficolins all act as opsonins (2). The C3b cleavage fragments can mediate different effects, depending on what type of leukocyte and receptor they bind to. CR1 not only act as cofactor for the factor I-mediated cleavage of C3b and C4b described previously, but also act as a receptor recognising C3b and C4b, with lower affinity iC3b and C3dg, as well as MBL and C1q (55). CR1 expressed on erythrocytes binds to C3b-labeled targets and transport them to the liver and spleen where they are phagocytosed by tissue macrophages (48). This mechanism supports a safe removal of immune-complexes. CR1 is also expressed in other peripheral blood cells, such as neutrophils and monocytes, and can promote phagocytosis upon ligand binding (55), as well as promote secretion of pro-inflammatory molecules such as IL-1α and IL-1β (56). However, other complement receptors appear more important for phagocytosis than CR1 (48). CR1 as well as CR2 are expressed on follicular dendritic cells and support antigen presentation to B cells (3). CR2 is also expressed on B cells and recognizes C3d and C3dg fragments (48). When B-cells simultaneously encounter antigens and
complement opsonins, the threshold for activation is greatly lowered and therefore enhances the B-cell response (57). CR3 and CR4 are expressed on macrophages, neutrophils and natural killer cells, and enhance the phagocytosis upon binding of the cleavage fragment iC3b (48). CR1g is another receptor that is important for phagocytosis, and can be expressed by some variants of tissue-resident macrophages (58).

**Formation of MAC**

Gram-negative bacteria are susceptible for lysis by MAC, a mechanism that is especially important for the killing of Neisseria species. However, gram-positive bacteria are protected from MAC-mediated lysis due to its very thick outer wall, which prevents MAC from reaching the inner, sensitive plasma membrane (59).

Under normal conditions, host cells can sufficiently restrict the pore formation by the membrane-bound inhibitor CD59, but can be challenged if there is excessive MAC formation (60). If a pore is formed, it results in the leakage of water and ions into the cell. Nucleated cells may also counteract this by using ion-pumps and therefore have the ability to survive. This is however not the case for erythrocytes, which do not have ion pumps and will therefore lyse. Nucleated cells can also remove MAC-induced lesions by exocytosis or endocytosis (61). Even if the nucleated cells manage to protect themselves from lysis, MAC formation can initiate several cell-signalling events via influx of extracellular calcium ions, or releases of intracellular calcium or via interactions with cell-surface molecules. The sublytic levels of MAC can affect granule release, protein synthesis, and regulate apoptosis and proliferation in a positive or negative direction. MAC can induce release of pro-inflammatory cytokines from various cell types, such as neutrophils, macrophages and retinal epithelial cells (39,59). If there is excessive MAC formation and influx of calcium, the metabolism of mitochondria will be disturbed by the loss of the mitochondrial transmembrane potential (62).
Organisation of the extracellular matrix in the articular cartilage

The synovial joint consists of two bones that are joined together by a joint capsule. Smooth layers of hyaline cartilage cover the bone ends that minimize friction during motion and absorb shock. The space between the capsule and bone is filled with synovial fluid, which lubricates the joint surfaces and supplies cartilage with nutrition. The joint capsule consists of an outer fibrous membrane, and an inner, vascular synovial membrane. The inner layer of the synovial membrane (the intima) consists of a thin layer of macrophage-like and fibroblast-like synoviocytes. The fibroblasts produce hyaluronan and lubricin, which are the lubricating components of the synovial fluid. Many synovial joints also contain additional structures, such as articular discs that absorb compression forces and ligaments that stabilize the joints (63). See Figure 3 for a simplified picture of the joint.

The major components of cartilage are the proteoglycan aggrecan and collagen fibrils forming a complex network together with various smaller molecules, including collagen IX, proteins of the short leucine-rich repeat protein (SLRP)-family and matrilins (64). Aggrecan is heavily glycosylated by long chains of negatively charged glycosaminoglycans, which retain water in the tissue, and contributes to the ability of the tissue to resist compressive load (65). The collagen fibrils are formed from collagen type II and small amounts of collagen type XI. The collagen fibril network contributes to the mechanical tensile strength of cartilage (66). Selected extracellular matrix (ECM) proteins forming the complex structure of cartilage are shown in Figure 2. Chondrocytes are the cells of cartilage and are sparsely distributed. The presence and organisation of ECM proteins differs in different zones of cartilage. The zones can be defined based on the distance to the chondrocytes, with the pericellular matrix being closest to the cell, followed by the territorial and interterritorial matrices. The organisation and composition of the ECM in articular cartilage also varies with the distance to the subchondral bone (67).

Several of the proteins of the ECM of cartilage have the ability to interact with the complement system. Some of them are studied in the papers included in this thesis and are therefore given additional attention below.
Aggrecan

Aggrecan monomers assemble into very large aggregates, via binding of its N-terminal globular domain G1 to link protein and the glycosaminoglycan hyaluronan (68). Aggrecan contains three globular domains, with G2 close to G1, while G3 is positioned in the C-terminal region. The protein is heavily substituted with glycosaminoglycan chains. The region between G1 and G2 is rich in keratan sulphate chains, while the region between G2 and G3 contains mainly chondroitin sulphate chains. The negatively charged glycosaminoglycan chains try to repel
each other, as well as attract water into the tissue. The swelling forces created by the osmotic pressure are restrained by the collagen network, which keeps the aggrecan molecules slightly compressed (65). The G3 domain can bind to other ECM proteins like fibulin, tenascins and fibrillin, which can assemble into other networks (68-71). Aggrecan is very important during the skeletal development and some mutations could lead to an abnormal development, including dwarfism (72).

Collagen type IX

Collagen type IX is covalently bound to the surface of collagen fibrils by crosslinking to collagen II (73). Collagen type IX consists of three polypeptide chains (α-1, α -2, α -3) that fold into a triple helix. The helix forms four non-collagenous segments (NC1 to 4) with three intervening collagenous domains (73). NC4 consists of the α-1 chain only. The collagenous domain 3 and NC4 project out from the fibril mediating contact with other molecules, such as fibromodulin (74) and COMP (75).

Proteins of the SLRP-family

Proteins of the SLRP-family are small, biologically active proteoglycans, which can be found in the extracellular matrix of various tissues, such as cartilage, skin and tendons (64). Several of them are considered to be important for the regulation of the organisation of ECM structure and collagen fibril formation (64,76,77). Some SLRPs can also bind to various cytokines and modulate their biological functions, e.g. transforming growth factor-beta, tumour necrosis factor-alpha (TNF-α) and insulin-like growth factor-I (64). When SLRPs are not bound to the ECM, they might also work as signalling molecules binding to various cell receptors, including integrin α2β1 and toll-like receptors (TLRs), influencing e.g. cellular proliferation and differentiation, and immunity (78,79).

The SLRPs all have a characteristic region of 10-12 leucine-rich repeats and cystein loops at both sides of this region and usually at least one glycosaminoglycan side chain (80). Examples of SLRPs that can be found in cartilage are fibromodulin (81), chondroadherin (82), decorin (83,84), biglycan (83) and proline/arginine-rich end leucine-rich repeat protein (PRELP) (81). Osteoadherin is normally expressed in bone (85), but can be found expressed by OA-derived chondrocytes (86). All of those mentioned SLRPs are known to interact with the complement system in different ways, and some of them will be discussed later.
Fibromodulin can via its N-terminal part - rich in sulphated tyrosines - interact with the before-mentioned NC4 domain of collagen type IX, and also PRELP and chondroadherin (74). The N-terminal part of PRELP, with its high content of arginine and proline amino acids, gives the protein basic properties, which clearly distinguishes it from the other members of the SLRP-family (87). PRELP is suggested to anchor the basement membrane with underlying connective tissue by binding basement membrane perlecan and collagens (88). PRELP can via its N-terminal part also interact with heparin and heparan sulphate present on cells, which suggests that PRELP also mediates the binding between the ECM and cells (89). Further, peptides originating from the N-terminal part have shown several other interesting properties, such as the ability to inhibit osteoclastogenesis and bone loss in mice (90,91).
Joint diseases

Osteoarthritis

Osteoarthritis (OA) is a disease of synovial joints, involving all tissues of the joint. One or more joints can be affected in a patient. There is a progressive damage or pathological changes of the joint structures, clearly seen by degradation of articular cartilage, thickening of subchondral bone, osteophyte formation and inflammation in the synovial membrane (synovitis) (92,93). Normal repair mechanisms in response to joint damage are out of balance, with catabolic activities overcoming anabolic factors. The exact trigger and mechanisms of the disease are not yet clear, but mechanical and inflammatory stimuli are considered important (93,94). The pathological changes in joint structures can cause joint pain, stiffness and loss of joint function, especially at later stages of the disease (95,96). The available treatments of today reduce pain but do not halt the joint damage (93).

Several mechanical problems in joints seem to play a role in the initiation of OA. For example, an injury to the meniscus (97) or anterior cruciate ligament (98) is a risk factor for the development of disease, which could be due to either the trauma itself or the resulting instability of the joint (67). Further, hip dysplasia induced cartilage degradation in an animal model (99), indicating the effect of abnormal loading for the initiation of disease. Further, mutations in structurally important molecules of the articular cartilage extracellular matrix, such as collagen type II and type IX, COMP and matrilins (67) increase the incidence of OA. This emphasizes the importance of a properly organized and functional extracellular matrix for the maintenance of a healthy cartilage.

Other risk factors for OA are obesity and age. Adipokines, which are substances secreted from white adipose tissue, promote a low-grade inflammation in obese people, which might contribute to the damage of cartilage (94). With age there is for example a decreased synthesis of cartilage matrix proteins and accumulation of advanced glycation end products that can alter the tensile strength of cartilage making it more brittle (100).

Chondrocytes, and also synovial cells in the osteoarthritic joint can sense and respond to signals of stress or inflammation. Abnormal (e.g. mechanical or
inflammatory) stimuli could evoke an exaggerated response, including elevated expression of genes associated with catabolism and inflammation, and apoptosis.

Figure 3. Schematic presentations of a healthy joint and joints affected by RA or OA.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterized by systemic and synovial inflammation, and damage of the articular cartilage and underlying bone. It affects up to 1% of adults in industrialised countries, most commonly women and elderly people (101). RA patients are a heterogeneous group that can be classified into different subsets (102). The goal of RA treatments is to keep patients in remission (non-active disease) to minimize joint damage, disability as well as comorbidities such as cardiovascular disease (101).

Genetic risk factors contribute strongly to the development of disease (101). Genetic variations in certain genes increase the susceptibility to disease, such as genes that affect T-cell reactivity or citrullination of proteins (103-105). Interestingly, several polymorphisms in the TRAF1/C5 locus, encoding TNF receptor-associated factor 1 and complement protein C5, are associated with an increased risk of developing RA (106,107). Further, there are several suggested environmental risk factors, smoking being the strongest contributor (101,108). The importance of e.g. air pollutions (109) and vitamin D status (110) is under discussion.

In RA, there is a clear inflammation of the synovial membrane, which is infiltrated by macrophages, granulocytes and T- and B-cells. The lymphoid cell profile in RA differs from that found in OA, where a milder synovitis can be found (111).
In the synovial membrane produce pro-inflammatory cytokines and the fibroblast-like synoviocytes show an abnormal behaviour as they invade cartilage and contribute to the cartilage degradation. Moreover, activated osteoclasts in bone lead to bone erosion (101).

In a preclinical phase, before detectable joint inflammation and damage, autoantibodies, such as rheumatoid factor and anti-citrullinated protein antibodies can be detected in blood. Also increased levels of cytokines and chemokines precede disease onset, indicating the presence of systemic inflammation. It is not fully understood how various factors, such as autoantibodies, immune cells, innate immunity as well as genetic and environmental factors interplay and contribute to disease progression (112).

Molecular alterations in cartilage extracellular matrix in OA and RA

Methods for studying molecular alterations in cartilage

Molecular changes in arthritic cartilage can be studied by using cartilage obtained from OA and RA patients, undergoing joint replacement. Usually the cartilage represents late stage disease and pronounced molecular changes have already occurred. To be able to study early changes in cartilage, cartilage explants of human or mammalian origin can be cultured in vitro together with pro-inflammatory cytokines to study inflammation-induced degradation caused by matrix-degrading proteases (113,114). In vitro cultures can give valuable information about changes in cartilage structure, which cytokines and ECM molecules are released, and which proteases are involved in the cleavages of ECM molecules (114,115). The culturing and stimulation of cartilage with cytokines probably reflects the situation in OA better than in RA, due to the absence of synoviocytes, which contribute largely to RA (see later section in thesis). Anyhow, many of the enzymes responsible for matrix degradation are considered to be involved in both OA and RA, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS)-4 and -5 (116).

Proteomic analysis of cartilage and synovial fluids from patient with joint disease, as well as of cultured cytokine-stimulated cartilage explants, contribute to the research trying to understand the mechanisms behind cartilage degradation, by describing changes in the ECM composition or identifying new factors involved in disease (115,117-119). A description of mass spectrometry in proteomic research will follow in a later chapter.
Further, mouse or rat models of OA and RA can also be used to study molecular mechanisms behind disease. Models of OA include rupture of the anterior cruciate ligament (120) or removal of meniscus (121) for the induction of OA. Commonly used models resembling RA are collagen-induced arthritis (CIA), where collagen type II is injected in susceptible mice (122), and anti-collagen antibody-induced arthritis (CAIA), where anti-collagen-II antibodies are used to initiate disease (123). Additionally, knockout mice models of e.g. selected ECM components (124) or matrix-degrading proteases (125) can also give valuable information about their individual importance for ECM structure and stability and disease progression.

The progressive degradation of cartilage

Early events
The cartilage degradation has mainly been studied in the context of OA, but it is probable that similar pattern of degradation is seen in RA. An early event in the disease progression is the appearance of fibrillation at the cartilage surface (126) and swelling of cartilage. The swelling of cartilage correlates with collagen degradation (seen by denaturation of surface collagen) and a decreased tensile strength and stiffness due to a weakened collagen network (66). At the same time, when there is only little fibrillation present, aggrecan fragments are released from cartilage, which is compensated by new synthesis (at least in knee-joint), maintaining a quite constant total amount in the OA cartilage (99,127). In the early phase with only little cartilage damage, fragments of the glycosaminoglycan-rich region of the core aggrecan are found in synovial fluid (of RA patients), while in patients with severe cartilage destruction, fragments containing the hyaluronan-binding region are mostly detected. This indicates that the part of the aggrecan molecule bound to hyaluronan is initially retained in the tissue (128). In the early OA cartilage with surface fibrillation, there is also an altered turnover (degradation and synthesis) of other proteins, such as COMP, fibronectin and cartilage intermediate layer protein-1 (CILP-1) (127).

Breakdown of the collagen network
The collagen fibers form a network together with various small proteins, which mediate interactions between fibers and participating in the fibril formation and organisation. Many of these proteins can be targets for proteolytic cleavage, such as collagen type IX (114,129), fibromodulin (113,130), COMP (131,132) and decorin (133). Upon cleavage, the stability of the collagen network could be affected (67), and moreover, the collagen fibers may be left accessible for enzymatic cleavage (130).
Interestingly, the cleavages of collagen type IX and fibromodulin are both caused by MMP-13, upon stimulation of cartilage explants with IL-1, and precede the loss of collagen type II (113,114,129). Almost the complete NC4 domain of collagen type IX is released (114), which usually interacts with several other ECM molecules, e.g. COMP (75) and fibromodulin (74). In the case of fibromodulin, it is the N-terminal part rich in sulphated tyrosines that is released, which normally interacts with NC4, PRELP and chondroadherin (74) in the cartilage matrix. The remaining fragment of fibromodulin is retained in cartilage (130). Furthermore, the released N-terminal fragment of fibromodulin and the NC4-domain can be detected in synovial fluids of OA and RA patients (67).

The major loss of collagen fibers, and a compensatory collagen synthesis, might appear at a late stage in the disease progression (118,127), after the major loss of aggrecan and proteolytic modifications of fibromodulin (118,130).

**Proteases involved in cartilage degradation**

MMP-1 and -13 are identified as the collagenases being responsible for the cleavage of collagen type II. They co-localize with collagen type II cleavage epitopes in OA cartilage (134) and can cleave collagen type II in vitro, however MMP-13 does it with a much higher rate (135). MMP-13 can also cleave aggrecan (136) and COMP (137), and the previously mentioned collagen IX and fibromodulin, which renders it an important protease in arthritis. Interestingly, MMP-13-deficient mice were protected from cartilage erosion in a model of surgically induced OA (138).

ADAMTS- 4 and -5 are considered to be the enzymes responsible for the pathological cleavage of aggrecan in the interglobular domain, located near the N-terminus between globular domains G1 and G2, at the bond Glu373-Ala374 (139). The cleavage releases a large part of the glycosaminoglycan-containing region, probably having drastic effects on cartilage ability to manage compressive load. Cleavage at a nearby site (Asn341-Phe342) can be mediated by MMPs, which is believed to occur later in the degradation process (140). The importance of especially ADAMTS-5 has been shown using ADAMTS-5-deficient mice. Deficient mice developed less cartilage damage, in a surgical OA model (125) and an antigen-induced arthritis model (141).

Other MMPs, ADAMS and some serine and cysteine proteinases (e.g. cathepsins) are possible mediators of cartilage degradation (139), but will not be discussed in detail here. However, it is worth mentioning that C1s of complement have some properties unrelated to the function in the complement system. C1s can cleave collagen type I and II (142) and activate MMP-9 in vitro (143). C1s activity in the joint indirectly lead to a decrease of insulin-like growth factor-1 concentration by cleaving insulin-like growth factor binding protein-5, which leads to increased cartilage damage in an OA model (144).
Inflammatory processes behind cartilage degradation in OA and RA

Inflammatory processes lies behind the cartilage degradation seen in OA and RA. Chondrocytes, and also synovial cells and cells in other tissues in the arthritic joint can sense and respond to signals of inflammation. The inflammatory signals are cytokines, chemokines and various ligands for TLRs. The response includes increased production of cytokines, matrix-degrading enzymes, prostaglandins and nitric oxide (NO) that are all contributing to the catabolic processes (94). Complement activation is also involved in the inflammatory processes and will be discussed in a later section.

In both RA and OA, the increased expression and activity of proteolytic enzymes is responsible for the degradation of articular cartilage. However, the origin of proteinases differs; the chondrocytes or synovium constitute the main source in OA and RA, respectively (145). The proteases involved are mainly overlapping in the two diseases, including MMPs, ADAMTS-4 and -5, and cathepsins (146). In OA, MMP-13 and ADAMTS-5 are considered most important (139,146). In RA, the membrane-bound MTI-MMP (MMP-14) has a crucial role in the cartilage destruction mediated by the fibroblast-like synoviocytes, promoting their invasiveness (146,147).

It seems that the disrupted cartilage homeostasis have different origins in OA and RA. The initial degradation of cartilage seems to start in different regions in the joint. In OA, the initial cleavage and denaturation of collagen type II appears at the surface of cartilage, and in the pericellular region in deeper zones (134). In RA, the proteolytic activity is seen in the cartilage-pannus region and in deeper zones of cartilage (145).

In OA, the chondrocytes undergo a phenotypic shift towards hypertrophy, where the chondrocytes metabolism and catabolic responses are changed, which results in cartilage destruction and bone remodelling (146). On the other hand, in RA, chronically activated fibroblast-like synoviocytes seem to mediate the destruction of articular cartilage (146).

Chondrocytes in OA

Several cytokines, such as IL-1 beta and TNF-a are present in tissues and fluids in OA (148,149), and the level of cytokines in serum correlates with OA cartilage damage (146). Chondrocytes respond to these cytokines by up-regulating the protein expression or regulating the activity of the matrix degrading enzymes, which are various MMPs and ADAMTS-1, -4 and -5 (145,150).
In response to stimulation of chondrocytes by cytokines IL-1 beta and TNF-α, chondrocytes produce more of those cytokines, as well as several other cytokines, including IL-6, IL-17, IL-18 and chemokines such as IL-8 (94). Several of these cytokines synergize to strengthen the inflammatory signalling in chondrocytes, as shown in culture models (151,152). Upon stimulation of chondrocytes with pro-inflammatory cytokines, the cells release other inflammatory mediators as well. These are prostaglandin E2, which modulates inflammatory responses (94,153) and NO, which creates an oxidative environment and could mediate chondrocyte apoptosis (154). Further, IL-1β and TNF-α also suppress the expression of aggregan and type II collagen in chondrocytes (155).

Chondrocytes can sense on-going degradation and damage of cartilage by the binding of certain ECM molecules via receptors, which can lead to the production of proteinases, cytokines and chemokines (94). These receptors include integrins, which bind to fragments of fibronectin and collagen type II, and discoidin domain receptor 2 that senses only fibrillar collagen type II and induces specifically MMP-13 production. The fibrillar collagen can only reach the cell after previous degradation of components in the pericellular matrix. Discoidin domain receptor 2 are found up-regulated in OA cartilage, constituting an important contributor in the catabolic processes (94).

Syndecan-4 is a transmembrane heparin sulphate proteoglycan, which is increasingly expressed in chondrocytes during OA. Up-regulation of syndecan-4 seems to increase the sensitivity of chondrocytes to IL-1 stimulation, and is involved in the positive regulation of ADAMTS-5 by controlling the synthesis of MMP-3 (156,157).

**Biomechanical stress affecting chondrocytes**

Chondrocytes are responsive to not only inflammatory stimuli, but also biomechanical stimuli. A non-injurious cyclic compression would increase the chondrocytes synthesis of matrix components and even inhibit any IL-1 induced matrix degradation. On the other hand, when the cartilage is exposed to a traumatic injury, or an extreme static compression, chondrocytes will respond by increased expression of inflammatory mediators (TNF-α) and matrix-degrading proteases (MMP-13, ADAMTS-5) (158), and release of reactive oxygen species (159). This shows that biomechanical stress is connected to an inflammatory response, which could contribute to an altered cartilage homeostasis or the development of OA.
**Fibroblast-like synoviocytes in RA**

During the disease process of RA, cells of the inflamed synovium, especially the fibroblast-like synoviocytes, attack and destroy the cartilage (160,161). The cells produce high levels of MT1-MMP in RA (162), which participates in the invasion into cartilage and the degradation of cartilage (147,163). The close contact that is created between synovial membrane and cartilage seems to be important for the invasiveness of cells and ECM destruction in RA; the enhanced adhesion of the fibroblast-like synoviocytes to cartilage in RA is mediated via integrins and syndecans, which are increasingly expressed in RA. The cell attachment seems crucial for the catabolic events, since the loss of individual adhesion molecules in the synoviocytes lead to reduced cartilage degradation (164,165).

Similar to chondrocytes, the fibroblast-like synoviocytes are sensitive to stimulation by cytokines, such as TNF, IL-1 and IL-6, which induce the production of cytokines and release of MMPs (161) and contribute to cartilage degradation in the cartilage-synoviocyte junction. Further, matrix-degrading enzymes are present in the synovial fluid in sufficiently high amount to induce cartilage damage, and could participate in the degradation (166).

Interestingly, proteoglycans are lost from cartilage before the attachment of inflamed synovial tissue to cartilage (164). In SCID (severe combined immunodeficiency) mice, arthritis was spread to unaffected joints via synovial fibroblasts; however not until cartilage damage was initiated by injection of collagenase. This indicates important properties of damaged cartilage that promote fibroblast attachment and activation (167). However, the mechanisms behind this are not known. It is suggested that degraded components of ECM, such as fibronectin, or factors bound to cartilage, such as CXCR2-specific chemokines and fibroblast growth factor 2, could stimulate the fibroblast-like synoviocytes when released (146).

**Complement in RA**

*Evidence of complement activation in human RA*

There is plenty of evidence that complement is activated in RA. Elevated levels of complement cleavage products can be detected in RA synovial fluid, such as C3a, C3c, C3d, C5a, sC5b-9, Bb and C1-C1 inhibitor (168-172). The level of C3d is higher in the synovial fluid from “active”, inflamed RA joints compared to “inactive” RA joints (169). Also the presence and amount of C1q-C4 complexes in RA plasma, a marker of classical pathway activation, can be correlated to RA disease activity (173). Further, deposition of complement proteins (C3c, C9, C5b-9) can be detected in RA synovial membranes, indicating complement activation...
in those tissues. (174,175). Additionally, CD59 expression is lower in synovial-lining cells compared to in endothelial cells from synovial tissues of RA patients (174). Inversely, pronounced C5b-9 deposition can be detected on synovial membranes, but less on vessel endothelial cells (175), indicating that the synovial lining-cells are extra sensitive to MAC formation.

Complement proteins present in the joint may come from infiltrating plasma due to increased permeability caused by inflammation, as well as from local production by cells of the synovial tissues, chondrocytes and infiltrated immune cells (115,176-182). The cells can together produce most of the proteins required for complement activation.

Possible inducers of complement activation

There are several possible ways that complement could be activated in the RA joints. Autoantibodies targeting several types of antigens can be present in RA patients, but some autoantibodies can also be detected in healthy individuals or in individuals of other inflammatory diseases (for review see (183)). Moreover, RA patients have increased levels of immune complexes in plasma as well as in synovial fluid (184). These immune complexes, present and potentially formed in the joint space (i.e. on cartilage or synovial membrane), could locally activate the classical pathway of complement. Autoantibodies against collagen type II, present in 30% of RA patients (183), could potentially target damaged cartilage. The antibody has a proven pathogenicity at least in mice, where it can induce arthritis (123). Rheumatoid factors are autoantibodies reactive to the Fc part of IgGs and are present in a majority of RA patients (183). Immune complexes containing rheumatoid factor can be detected in RA plasma, and these could potentially activate the classical pathway (185). Moreover, antibodies reactive to citrullinated peptides/proteins have the potential to activate both the classical and alternative pathways in vitro (186), and the presence of these antibodies are highly predictive for the development of RA (183).

Apoptotic and necrotic cells, and microparticles originating from them are also potential activators of the classical and lectin pathways. Apoptotic cells can bind C1q and MBL (12,24,187,188), which might promote their phagocytosis (11,187). Complement activation products are present on apoptotic cells (189) and microparticles from RA synovial fluid carry high levels of C1q, C4 and C3 indicating classical pathway activation (190). However, the activation on dying cells is limited by the simultaneous binding of C4BP and FH (191,192). Under normal conditions, binding of C1q and MBL likely promotes a beneficial phagocytosis of cells. In RA, there might be excessive apoptosis, insufficient clearance of apoptotic material and excess complement activation.
Upon cartilage degradation, certain proteins might become exposed and be possible targets for complement activation, such as fibromodulin. *In vitro*, fibromodulin can activate the classical pathway by a direct binding to C1q (193). The ability of proteins of cartilage to interact with complement proteins will be discussed more thoroughly in the section “The present investigation”.

There are also possible targets for alternative pathway activation in the joint. Collagen II purified from human cartilage might activate the alternative pathway directly *in vitro*, as seen by C3 consumption (194). However, complement activation by collagen II could not be confirmed by (5), where recombinant collagen II was incubated with serum followed my measurements of soluble C5b-9. In juvenile RA, the plasma levels of Bb fragments correlates with the level of immune complexes (195) as well as soluble C5b-9 (196) suggesting an important role of the alternative pathway in this form of arthritis. Further, immune complexes containing IgA RF can be detected in juvenile RA (197), and IgA antibodies can activate the alternative pathway (*in vitro*) (27). Taken together, these complexes might contribute to the alternative pathway activation (4).

Whether the lectin pathway contributes to RA has not been given much attention. However, IgGs, bearing an altered glycosylation pattern in the Fc region are increasingly detected in RA patients, which show an increased ability to bind MBL and activate the lectin pathway (198). This interaction might also contribute to the local complement activation in the joint. It is not agreed on which complement pathway is the most important one for arthritis. However, the alternative pathway acts as an amplification loop for the other two initiating pathways and can always contribute to the activation, no matter what pathway is initiated first.

*Effects of complement activation*

When complement is activated in the joint, C3a and C5a are released. Those anaphylatoxins can likely recruit and activate macrophages and granulocytes (e.g. neutrophils) into synovial tissues and fluids that are increasingly detected in the RA joint (199). The anaphylatoxins can also contribute to vasodilation and increase the vascular permeability (171,200). Released anaphylatoxins could probably also affect synoviocytes as well as chondrocytes in some way, since they can express C3aR and C5aR (177,201-203).

Moreover, C3b and iC3b can interact with neutrophils present in the synovial fluid via their CR1 and CR3 receptors (204), which would promote further processing and phagocytosis. MAC formation can result in lysis of cells, but also at sublytic concentrations it can affect cells. MAC formation on synovial fibroblast was shown to induce collagenase production (205), while MAC formation on chondrocytes could induce the expression of proteinases and chemokines (5).
Evidence from animal models

The importance of complement for disease progression has been shown in various ways, using CIA and CAIA mouse models of RA. Mice with C3 and FB deficiency clearly developed less severe or no arthritis in these models (206-208). In the CIA model, C5 deficiency (209) as well as treatment with monoclonal anti-C5 antibodies protected against disease onset (210). The antibody could also ameliorate already established disease, by inhibiting the cleavage of C5 to C5a, which indicates the importance of C5a release for disease progression (210). Further, endogenous FH plays an important role in the regulation of the alternative pathway in a CAIA model (211). Administration of an inhibitor that prevented binding of FH to tissues increased the complement deposition seen on cartilage extracts and synoviocytes.

Complement in OA

The involvement of complement in OA disease progression has not been studied as much as in RA. Signs of complement activation are generally less pronounced in OA than in RA patients, such as the level of the complement activation product C3d in the synovial fluid (169). The level of complement activation may reflect different episodes of disease, since higher levels of C3a can be detected in the synovial fluid of patients with early OA than in healthy or end-stage OA individuals (5). On the other hand, sC5b-9 levels in synovial fluid are higher in both OA states compared to in healthy individuals (5). Moreover, C5b-9 can also be detected deposited in OA cartilage around the chondrocytes (5). In other studies, the level of complement activation reflected the disease activity of the joint, with more deposited C3c, C9 or C5b-9 in inflamed than in non-inflamed synovial tissues (174,212).

Further, the importance of complement for the development of OA has been shown in mouse models of OA, where joint instability was induced by removal of the medial meniscus. Mice deficient in C5 or C6 were partially protected against the development of OA (5). Also administration of a monoclonal anti-C5 antibody in wild type mice showed the same protective effect. CD59 deficient mice, on the other hand, obtained a more severe OA, indicating the importance of the formation of the membrane attack complex (5).

The same authors also showed that there was a clear difference in gene expression of complement proteins in the synovial membrane comparing tissues from osteoarthritic and healthy individuals. Expression of C4, C5, C7, C9 and FB was higher in the OA membrane, while the expression of complement inhibitors such as FH and C4BP was lower, indicating the involvement of the synovial membrane in the inflammatory process (5).
TLRs in OA and RA

TLRs are continuously expressed on e.g. macrophages (213), but the expression can also be induced in other cell types, including chondrocytes (214). The receptors can sense tissue damage and stress by binding endogenous molecules with damage-associated molecular patterns (DAMPs). In response to this, inflammatory mediators (e.g. IL-1beta and chemokines) are produced and released, via activation of intracellular pathways (215). Therefore, TLR activation might contribute to joint inflammation and damage in OA and RA. TLRs can be detected in the synovial membranes from RA and OA patients (much less in the latter) (216), as well as on chondrocytes in articular cartilage lesions (214). There are several possible TLR ligands found in the joint upon inflammation and tissue damage, which could serve to activate the TLRs. To name a few, there are extracellular matrix components like tenascin C (217), fibronectin fragments (218) and biglycan (219), as well as certain plasma proteins (148). Activation of TLR in OA and RA could via release of cytokines and chemokines, lead to an increased infiltration and activation of macrophages, granulocytes and lymphocytes (220), contributing to synovitis as well as cartilage damage. An activated complement system can further enhance the TLR activation, via binding of the complement anaphylatoxins to C3aR and C5aR (221), which could possibly occur in the synovial tissues.
Mass spectrometry in proteomics

High-resolution mass spectrometry (MS) in proteomic research is a useful method for identification and quantification of proteins in biological samples, in order to answer research questions about protein modifications and localization, change in expression pattern and identifying interaction partners. Shotgun tandem MS is used to identify any possible proteins, while targeted MS is used to quantify only predefined proteins, which are selected based on previous knowledge or interest. The protein identifications are made from analyses of the protein’s peptides after proteolytic processing.

Samples can be very complex, consisting of thousands of proteins that are present in a wide range of concentrations, which is an analytical challenge. On-going advances in all steps of the proteomic workflow, from sample preparation to instrumentation and data processing tools, allow increasing numbers of proteins to be identified with increasing confidence. Identification of thousands of proteins is now possible in one shotgun tandem MS experiment.

Common steps in MS-based proteomics

Sample preparation

Generally, proteins are extracted from a biological sample and denatured by using chaotropic salts, such as guanidium hydrochloride or urea. This is followed by cleavage of the disulphide bonds using reducing agents, and irreversible alkylation of the free cysteines in order to prevent them from forming new disulphide bonds (222). This is performed to enhance the protein sequence coverage (223). The proteins are digested into peptides using proteases, in most cases those that cleave specifically next to certain amino acids (222). With trypsin, one obtains peptides with charged amino acids (the basic residues lysine or arginine) in the new C-terminal and with a suitable mass range, beneficial for the mass spectrometric analysis (224). Finally, the peptide mixture is desalted and concentrated (222).
Peptide separation and MS analysis

The prepared peptides are separated according to hydrophobicity, using an analytical column carrying an hydrophobic stationary phase, together with a gradient of increasing proportion of organic solvent, in a (ultra)high-pressure liquid chromatography (UHPLC or HPLC) system. The column is coupled online with the mass spectrometer so that peptides leaving the column are immediately ionized by electrospray ionization (ESI) and transferred into gas phase before entering the mass spectrometer. Depending on the MS technique, the components of the mass spectrometer are different, as well as the procedure to obtain information about the proteins in the sample. However some principles are the same. The mass spectrometer measures and use the information of mass over charge (m/z) values of peptides (the precursor ions), performs selection and fragmentation of some precursor ions based on certain criteria, such as intensity, and measures the intensity of all or some of the resulting product or fragment ions. After the data collection, proteins are identified and quantified with suitable processing tools (222,225).

Figure 4. Overview of the MS workflow.

Shotgun tandem MS

For shotgun proteomics, various mass spectrometers are available with different constructions, e.g. in what types of mass analysers are used. Generally, the mass spectrometer first records the mass and intensity of the entering precursor ions, which results in a MS or survey spectrum that shows the intensity and m/z on the y- and x-axes. Then the mass spectrometer selects and isolates individual precursor ions sequentially, and transfers them to a collision cell, where they are fragmented into smaller peptide fragments (product ions) by colliding with a gas. The intensity and m/z of the product ions are recorded during a second scan, and
results in a MS/MS or tandem MS spectrum. Since the cycle time of a survey scan and a tandem MS scan is fast compared to the chromatographic elution time of a particular peptide, several MS/MS spectra can be collected after one survey scan. The cycles of MS and MS/MS spectra generation are repeated over and over again throughout the chromatographic elution (222,225).

Figure 5. Principles of shotgun tandem MS.

The precursor ion selection can be based on the signal intensity of the ions, where the most abundant ones are selected for further fragmentation and MS/MS analysis. It is set in advance how many different precursor ions will be selected from each survey scan. After data collection, the intensity of a precursor ion can be used for quantification of the peptide, while the MS/MS spectrum together with the precursor m/z value is used to identify the amino acid sequence of the original peptide (222).

For the shotgun MS experiments in paper IV, the mass spectrometer Q Exactive (Thermo Scientific) (226) was used. In this instrument, the selection of precursor ions is done with a quadrupole. The quadrupole consists of four parallel metal rods. By varying the voltage applied over them, one can control which peptides pass through and which is stopped as they collide with the rods. In the MS/MS mode, the quadrupole only lets ions of selected mass (m/z) to pass through to the so-called higher-energy collision dissociation cell, where they are fragmented (226). The fragment ions are transferred to an Orbitrap analyser that determines their masses (m/z), from their frequencies of oscillation as the move around a central electrode (227). In paper IV, the ten most abundant precursor ions per
survey scan were selected for further fragmentation and generation of MS/MS spectra.

In shotgun proteomics, the sampling of peptides is incomplete and not completely reproducible, since the instrument do not have the capacity to select and identify all precursor ions detected in a survey scan. This is especially true in very complex samples containing several thousands of proteins present in a wide range of concentrations. One may miss less abundant peptides, especially if they are detected in the same survey scan as other more abundant peptides. The absence of a protein in an MS experiment does not mean that it is not present; it may simply just not be detected or chosen for sequencing (222,225).

**Analysis of shotgun tandem MS data**

A MS/MS spectrum can be used to perform *de-novo* sequencing, which means that the amino acid sequence is determined directly from the spectrum. Since that is very computationally demanding, one usually compares the spectrum with theoretical spectra of peptides from a protein sequence database, in order to find a matching peptide (222). Many peptides can have nearly the same mass, however their fragmentation pattern differs due to their different amino acid sequences. Therefore, the fragmentation pattern of an unidentified peptide is compared to the theoretical fragmentation patterns of those peptides that have nearly the same mass (228). From all the identified peptides the original proteins are inferred (229).

There is always some uncertainty that an identified peptide is really a correct identification, since the MS/MS spectrum can be of insufficient quality or the observed peptide is actually not present in the database and is therefore assigned another, wrong identity. To get an estimation of the theoretical error of the spectral-peptide matches, one can use a so-called target-decoy database, which contains the target protein sequences (for example the human proteome) and all the reversed sequences of those proteins (called decoys). When searching a dataset against the database, some MS/MS spectra will be identified as decoy-peptides, i.e. false positives, which give an estimation of the number of peptides assigned to a false identity in the whole dataset (230).

In paper IV, the software tools included in the Trans-Proteomic Pipeline workflow are used in the analysis of the shotgun MS data, in which the tools PeptideProphet and ProteinProphet are important parts (231). The PeptideProphet statistically validates the peptide identifications made by MS/MS and the subsequent search in a protein sequence database, and computes the probability of each peptide assignment of being correct (229).
ProteinProphet then uses the list of identified peptides and their calculated probabilities, to estimate the probability that a protein is present in the sample. It deals with the problem that peptides can be associated to more than one protein entry in the database. When the protein probabilities are calculated, adjustments are done that consider the tendency that proteins only identified by one peptide is less likely to be correct than proteins identified by several peptides (232). Also, peptides that are shared across multiple proteins contribute less (are given less weight) to the proteins final probability score (233). The ProteinProphet results contain predicted sensitivity and false positive identification error rates (232) of proteins.

However, the estimates obtained from ProteinProphet may not be completely accurate, when performing large experiments with many samples and replicates (233), since it may overestimate the probabilities. Therefore, an additional software tool, Abacus, can be used that gives better estimates of the protein identifications in a large sample set. Abacus calculates the false discovery rate based on the target-decoy search strategy described above. One can then apply a filtering step to the identified proteins based on the false discovery rate. If one set the false discovery rate to 1%, one accept that 1% of the identified proteins are false positives (233).

Abacus also performs other useful computations. It calculates an adjusted spectral count of the identified proteins (233). Spectral count is the total number of peptide spectra that have been assigned to a protein, and gives a semi-quantitative measure of how much of that protein is present in the sample (234). The spectral count is adjusted in such a way that a peptide contributes less to the total spectral count, if the peptide is shared by several proteins (233).

**Selected reaction monitoring MS**

Selected reaction monitoring (SRM) is the main technique used for performing targeted MS. SRM is suitable when a limited number of proteins are going to be reproducibly quantified across multiple samples with complex composition (235). With SRM, one only monitor predetermined peptides and some of their fragment ions, which optimally represent the proteins of interest. Since the measurement time is focused on selected ions only, it results in better sensitivity (i.e. lower limit of detection and quantification) and repeatability compared to common shotgun tandem MS experiments (225,236). However, the SRM assay needs to be designed in advance and requires a lot of effort before it can be used for the biological samples.
SRM is most often performed on a triple quadrupole mass spectrometer, which consists of three quadrupoles as the name implies. The first quadrupole (Q1) works as a mass filter and selects the target peptide (or actually ions with m/z values centred around the m/z of the target peptide), which is transferred to the second quadrupole (Q2). There, the selected peptide is fragmented by collision-activated dissociation. In the third quadrupole (Q3), one or more of the produced fragment ions are sequentially let through to reach the detector that counts the number of ions. A fragment ion and its precursor ion are referred to as a transition. All the transitions included and targeted in the predesigned SRM assay, will be monitored over and over again during the whole chromatographic elution. The measured transitions will be presented as a chromatographic peak, with elution time and intensity displayed on the axes. The area under the transition curves corresponds to the amount of detected peptide (235).

**Figure 6. SRM performed in a triple quadrupole mass spectrometer.**

**SRM design and analysis**

It is important to carefully select target peptides for the SRM assay, which optimally represent the target proteins. The target peptides should be easily obtained by trypsin digestion, have properties suitable for mass spectrometric analysis, so that they are reproducibly detected with high intensity. Optimally, one can analyse purified proteins with shotgun tandem MS to find the most suitable peptides. One could also use information in databases from prior experiments to see which peptides of a protein are usually detected. Generally, peptides suitable for SRM have a length of around 10 amino acids and are obtained by trypsin cleavage at the ends. Preferably, the peptides should not contain amino acids that usually are subjected to post-translational modifications (235).

Not only the most suitable target peptides have to be selected. Also the best transitions have to be determined. One can predict the most intense SRM transitions by using data obtained from shotgun tandem MS experiments. However, which fragment ions are best detected can differ between different types of mass spectrometers. Therefore, the selected transitions have to be validated on
the triple quadrupole, by acquiring MS/MS spectra of the target peptides in the biological sample and then performing searches in a protein sequence database (235,237). It has it limitations since the triple quadrupole is slow in the spectra acquisition and the target peptides are commonly present with low abundance. To overcome those limitations, one can instead produce synthetic target peptides and analyse them on the triple quadrupole, to find the most intense SRM transitions to be used in the assay (237). The synthetic peptides can be analysed again just before the analysis of the biological samples, to work as a reference to indicate the expected retention times and relative intensities of the transitions in that particular instrumental setup (236).

In order to identify and relatively quantify peptides with high confidence in a biological sample, and to reduce the risk of false positives, certain criteria have to be fulfilled; all the monitored SRM transitions of a peptide elute at the same time (with overlapping elution curves), the transitions are detected clearly above the noise level, the relative intensities of the transitions and the elution time agree with that of the synthetic peptide. Preferably, several peptides and transitions per protein should be targeted. It is common practise that three or more transitions per protein are monitored (236,237).

The SRM assay can be used without or with isotope-labeled internal standards. The label free method is semi-quantitative and is useful for comparative measurements of proteins between different samples and gives an estimation of changes in abundance (236). If isotope-labeled versions of every analyte are included, it results in increased repeatability as well as confidence in that the right peptides are detected. The repeatability is increased, since the labeled control can be used to compensate for ion suppression and fluctuations in the spray (in ESI). Ion suppression refers to the phenomenon that certain components of the sample matrix, such as lipids and salts, can compete with the target ion to get ionized in the ESI. Since both an analyte and its corresponding internal standard are subjected to the same suppressive effects, one can relate the quantity of the analyte to the labeled standard, which is present in a known amount (236).

In paper IV, we constructed a SRM assay that targets human complement proteins with the help of the software Skyline 6 (MacCoss Lab Software, University of Washington). The target proteins were digested in silico by trypsin and peptides that could be detected in other human proteins were excluded. Peptides in intracellular domains of transmembrane proteins were avoided and peptides in the most interesting and relevant parts of complement activation products were selected to the extent that was possible. Those peptides that could also be identified in in-house shotgun tandem MS data (Orbitrap) passed the final selection. The selected peptides were synthetically produced and the seven best transitions of each peptide according to the shotgun data were analysed on a triple quadrupole (TSQ Quantiva, Thermo Scientific). The results were used to refine the assay to contain the best 3-5 transitions per peptide. The final assay contained 92
peptides that represented 40 complement proteins. The mixture of synthetic peptides was always analysed prior to the biological samples, to give the expected retention times and relative intensities of the transitions. Several empty injections were done after the synthetic peptides to minimize carry-over into the biological samples. Regularly, between injections of samples, control samples (yeast-digests) were injected to monitor the overall instrument performance. The data of the biological samples were analysed in Skyline. The software automatically suggests which identified transitions correspond to each peptide. However, these suggestions need to be further validated by manual inspection. The software calculates the area under the transitions, which can be used for quantification of the identified peptides.
The present investigation

Since there are many proofs of complement involvement in arthritis patients as well as in mouse models, the general belief is that complement activation contributes to the inflammation seen in arthritis. In previous studies it has been shown that several proteins of the ECM, i.e. SLRPs and COMP can interact with the complement system and affect its activity in various ways. The SLRP proteins fibromodulin and osteoadherin strongly activate the classical pathway of complement by binding to the heads of C1q (14,193). Chondroadherin has the same but weaker effect on the classical pathway. These three proteins cause only limited activation of the terminal pathway, since they also bind C4BP and FH (14,193). Two other SLRPs, decorin and biglycan can on the other hand inhibit complement by binding to the stalk region of C1q (238,239). COMP has both activating and inhibiting effects: it inhibits the initiation of the classical and lectin pathways while activating the alternative pathway (240).

In paper I-III we studied the interactions between complement and three additional ECM proteins to determine if they regulate complement activation in a positive or negative direction, which could then affect the inflammatory process in the joint. In paper IV we tried a more holistic approach by studying the interactions between complement and degraded cartilage explants and their released components, in order to see the outcome when there is a complex mixture of ECM molecules and complement proteins.

PRELP is an inhibitor of the membrane attack complex

Since several proteins of the SLRP family have been shown to interact with the complement system and affect its activity in various ways, we ought to investigate if also the PRELP had any complement-modulating properties. It has previously been shown that PRELP can bind to the complement inhibitor C4BP (241). Importantly, this interaction did not prevent C4BP from acting as a cofactor for the factor I-mediated cleavage of C4b. This implies that PRELP could localize C4BP-mediated complement inhibition.

In the current study, we showed that PRELP could inhibit the assembly of the C3 convertase of the alternative pathway, via a direct binding to C3. This would
inhibit the amplification loop of complement, decreasing also the effect of any activation of the classical or lectin pathways. Further, PRELP was found to bind C9, but also to other proteins of the MAC. PRELP did not prevent the formation of the C5b-8 complex or the binding of one C9 molecule to the C5b-8 complex. Rather, PRELP inhibited the C9 polymerisation and therefore the formation of a functional MAC.

Further, several PRELP fragments were found in synovial fluids of RA patients. It has not been determined what fragments of PRELP are released and when this occurs during the process of cartilage degradation. It would be interesting to know whether any of the fragments retained in cartilage or released would inhibit complement in the same way as the full-length protein. The biological importance of any inhibitory effect on complement would probably depend on the local concentration of the fragments, in relation to the presence of other complement-interacting ECM-molecules. Other questions to answer are whether the same PRELP fragments are present during normal turnover of cartilage, and therefore can be found in the synovial fluid of healthy joints.

The formation of MAC seems to contribute to the pathology of RA and OA, since CD59 deficient mice develop more severe disease in an antigen-induced arthritis (RA) model (242) as well as in a surgical OA model (5). Whether endogenous PRELP has a significant and physiologically relevant protective role during cartilage degradation is not known, however, use of PRELP might be envisioned as therapy. In a mouse model, PRELP seems promising as a complement inhibitor useful in the treatment of age-related macular degeneration of the eye (243). Non-sufficient complement regulation and excess MAC-formation in the choroid and retinal pigment epithelium are believed to contribute to the disease. When PRELP was delivered through as an adeno-associated virus vector to the retina of mice, the deposition of MAC was reduced, as well as the injurious choroidal neovascularization (243). Similarly, PRELP could perhaps be used as an inhibitor for MAC formation in RA or OA patients, which might cease the disease progression.

NC4 domain of Collagen IX inhibits complement

Almost the whole NC4 domain of collagen IX can be found released during cartilage degradation; in vitro (114) and in arthritic synovial fluid (67). In the present study, we investigated whether it could modulate the complement system.

We showed that NC4 could inhibit the classical pathway of complement and the MAC-mediated lysis of sheep erythrocytes. NC4 could not inhibit the alternative pathway-mediated lysis of rabbit erythrocytes, but it might be due to that more
serum was used, requiring more of the inhibitor as well. We investigated further what mechanism lay behind the inhibition of the classical pathway.

We showed that NC4 directly inhibited the polymerisation of purified C9 in solution by a direct binding to C9. NC4 could also inhibit the formation of MAC on cell membranes of antibody-sensitized sheep-erythrocytes using whole human serum as the source of complement proteins.

NC4 also bound to the complement inhibitors FH and C4BP, and to C3, C3b, C4 and C4b. Binding of the complement inhibitors did not alter their function as cofactors for the factor I-mediated degradation of C3b and C4b, and NC4 could not by itself work as a cofactor. Unexpectedly, NC4 could instead enhance the cofactor activity of FH as well as of C4BP in the cleavage of C3b/C4b by factor I. This implies that NC4 has the capacity to localise and enhance the inhibitory capacity of FH and C4BP.

Further, the interactions between NC4 and C4BP was investigated in detail using C4BP mutants lacking individual domains. We showed that binding of NC4 probably simultaneously engages several alpha chains of C4BP, and that NC4 has several binding sites along the alpha-chain, with the main binding region at CCP1-3. Further, the interaction between NC4 and C4b, rather than between NC4 and C4BP, was suggested to be responsible for the enhanced cofactor-activity of C4BP in the presence of NC4.

Since NC4 can bind several of the ECM molecules that are known to interact with complement as well, we investigated how these interactions could influence one another. NC4 was shown to compete with C1q for binding to fibromodulin and osteoadherin, which resulted in less C1q binding and complement activation by these proteins. NC4 could also partially block the binding of C1q to COMP. During cartilage degradation, when NC4 is released to the synovial fluid, these interactions might limit the activation initiated by fibromodulin and osteoadherin, or limit the inhibitory effect that COMP imposes on the classical pathway activation.

We speculated that in healthy cartilage, NC4 might be accessible for serum proteins at the cartilage surface and could recruit FH and C4BP to protect the collagen fibrils from complement attack. However, in paper IV, we could not detect any complement inhibitors on healthy cartilage. Instead, when NC4 is released in disease, it might have a function as a local inhibitor, by accelerating the cofactor activities of C4BP and FH, and therefore limiting C5a release, and by inhibiting the C9-polymerisation and MAC formation. The net outcome of the interactions of NC4 with complement proteins, as well as with fibromodulin, osteoadherin and COMP would likely depend of the local accessibility of these proteins.
In addition to the release of NC4 during degradation of cartilage explants, another fragment of collagen IX, containing NC4 and COL3 domains, is also released (114). Whether this fragment has the same ability to interact with complement is not known.

The aggrecan G3 domain activates complement

Aggrecan fragments are released from cartilage during pathological degradation, including the C-terminal region where the globular G3 domain is located. We showed that the C-type lectin domain within the G3 domain can activate the complement system, via the classical pathway and to a lesser extent via the alternative pathway. Up to the stage of C3b and C4b deposition, the activation was as pronounced as the activation initiated by positive controls, while C9 deposition was limited, especially for the alternative pathway. Complement activation on G3 was confirmed by using serum depleted of C1q or FB, which resulted in abolished activation via the classical or the alternative pathway, respectively.

We used electron microscopy to visualize the interactions between full-length aggrecan and C1q. It revealed that C1q bound preferably via its heads to the G3 domain (79 %), but also to the aggrecan core protein. Whether the latter interaction caused activation as well was not investigated.

The complement inhibitor FH was also shown to bind to the G3 domain, via a binding site that likely did not overlap the binding site of C1q. Binding of FH probably caused the inhibition of the alternative pathway convertases, since there was a limited generation of MAC. Inhibition of the alternative pathway would also affect the later stages of the classical pathway, since the alternative pathway also amplifies the activation initiated by the classical pathway. The simultaneous binding of C1q and complement inhibitors FH and C4BP have been observed for several other endogenous ligands, for example fibromodulin and osteoadherin (14), prions (13) and dying cells (191). This might have a biological function, where a limited opsonisation with C3b promotes efficient clearance, while preventing a vast release of C5a and formation of MAC, which could trigger excessive inflammation.

Whether aggrecan can activate complement when released into synovial fluid, would probably depend on the presence and availability of other ECM binding partners as well as of complement proteins. Binding of e.g. fibulins or tenascins to G3, might mask the complement activation binding sites. Complement proteins could also bind to other ECM molecules, possibly causing other effects on complement, which would also decrease the availability of complement factors.
Complement is activated on degraded cartilage explants

Complement is a crucial contributor to inflammatory processes in joints, but it is not clear which complement pathways are mainly involved in these processes and how the degraded cartilage is involved in the regulation of complement activity. To address these questions we investigated what complement proteins interacted with bovine cartilage explants that had been subjected to prior inflammation-induced proteolytic degradation. We also investigated changes in protein composition of the cartilage, as well as what proteins were released during cartilage degradation. We used tandem MS for the identification and approximate quantification of proteins and targeted MS (SRM) to specifically quantify human complement proteins bound to cartilage. Additionally, we used several more “standard” complement assays to support the SRM data.

In short, cartilage explants were cultured for various numbers of days (0-15) with or without the pro-inflammatory cytokine IL-1α in serum-free media. Culture media were collected and changed regularly. After culture, cartilage explants were incubated with NHS as a source of complement proteins. After thorough washing, cartilage were incubated with urea and reducing and alkylating agents, and lastly with trypsin to obtain peptides of bound proteins and cartilage matrix.

SRM analysis of the peptide samples clearly revealed that complement proteins of the classical, alternative and terminal pathways, as well as complement inhibitors interacted with degraded cartilage, whereas almost no complement proteins were detected on healthy cartilage. Already at day 6 of IL-1α stimulation, the cartilage structure was altered in such a manner that complement was activated on the surface, and the activation was only slightly increased after prolonged culture. Further, the tandem MS data revealed that clear alterations in proteoglycan and collagen content had already occurred before day 6. It seems likely that new epitopes in the ECM network become accessible when the structures are starting to become degraded, which triggers the complement activation. C1q has several possible, known ligands in cartilage, which could activate (fibromodulin (193), aggrecan G3-domain (244)) or inhibit (biglycan, decorin, COMP (14,239,240)) the classical pathway upon binding. Even if some of them (such as fibromodulin, COMP) were found in decreased amounts in cartilage due to degradation, residual fragments could still bear important neo-epitopes capable to interact with complement. Further, there was a trend of an increased level of Annexin A2 and A5 in IL-1stimulated cartilage. These proteins are known to interact with C1q on apoptotic cells, and might serve as complement activators in cartilage (245). In addition to these mentioned ligands, there are likely several other, unknown C1q-ligands present that could contribute to the activation.
Apart from the direct binding of C1q to ECM ligands, C1q might also bind autoantibodies and activate the cascade (186). Whether these autoantibodies were present in the serum that we used, and whether these could cross-react with bovine ECM, was not studied. Further, the detection of alternative pathway proteins could be a result of direct activation of that pathway (e.g. by COMP (240)), as well as a result of the pathway serving as an amplification loop of the classical pathway.

Complement activation on degraded cartilage was confirmed by the detection of C3 cleavage products in the urea-sample using western blot, and of soluble C4d in the serum after incubation with cartilage, confirming alternative and classical pathway involvement. Remarkably, there seems to be a rapid processing of deposited C3b on cartilage, since mainly C3, iC3b and C3d fragments were detected. Whether this processing was accelerated by any ECM components (similar to the effect of NC4 of collagen type IX), or if it only reflects the normal work by present complement inhibitors, remains unanswered.

In vivo, complement activation on damaged cartilage and the release of C3a and C5a would contribute to inflammation by e.g. mediating chemotaxis and activation of macrophages and neutrophils (246) that infiltrate the synovium and synovial fluid. C3aR and C5aR are expressed on human chondrocytes (202,203), but the functional effects of the interactions with their ligands have not yet been determined. However, the formation of MAC on chondrocytes induces the gene expression of matrix-degrading proteases, which would then worsen the catabolic processes (5).

Interestingly, in RA the inflamed synovial tissue attaches to and invades cartilage after the proteoglycan loss (164). An interesting thought is whether complement could be involved in this process, since complement activation on cartilage also occurs after the proteoglycan loss. Receptors for C3a and C5a have been detected in RA synovium (177). C5aR are also found expressed by synoviocytes isolated from OA and RA patients, and binding of C5a enhanced the LPS induces TNF-α release (201). It could be interesting to study if the interaction between C3a/C5a and their receptors could promote the actual invasion of these cells.

We observed that also molecules released from degraded cartilage interacted with complement, but apparently causing inhibition and not the expected activation of complement. This effect was however a little bit delayed compared to the activating effect of cartilage (day 12 compared to day 6). The most pronounced loss of aggrecan from stimulated cartilage into the culture medium occurred at day 3, and those samples might contain more of the complement-activating G3 domain of aggrecan than later collected samples. However, day 3 culture medium samples have not been tested for any complement activation properties. It would be interesting to investigate if the molecules released at this early time point actually could activate and not inhibit complement, and to look into which aggrecan peptides are present then.
The apparent complement inhibitory effect of the molecules released from cartilage upon stimulation with IL-1α, seen by their ability to prevent C3b deposition on IgG in microtiter plates, needs to be investigated further. Could MMPs or other proteases inhibit complement by cleaving complement proteins? Or do released molecules strongly bind complement proteins preventing them from interacting with coated IgG to initiate complement activation. Or could it be due to complement consumption: that released molecules initiate such a strong activation that less is available for activation in the plate?

In the present study, we cultured the cartilage in the absence of serum. Since the cartilage was frozen at the time of harvest, and was incubated with serum at a later time-point, we might have missed important information on how serum affects the live chondrocytes and the degradation process. It could be worth investigating what happens when new serum is added, every hour, during a shorter culture time, to see if there are differences in the degradation process, expression profile of chondrocytes as well as in the complement activation on cartilage.

Since the IL-1 stimulated cartilage explant initiates complement activation, and the released molecules might inhibit complement, it would be interesting to incubate the harvested cartilage with serum in the presence of the culture medium for one hour, and then determine the deposited complement on cartilage. Imagine if the released components are able to partially counter-balance the activation occurring on the cartilage. In the present study, we could see more inhibition from the culture medium at day 12, but also slightly more activation on cartilage, than at earlier time-points, so the question remains about what the result is when all complement interactions are summarized. It would be of biological importance, if the activation is kept in check during minor cartilage damage, and complement supports a silent clearance of degraded proteins, while upon severe damage, the complement activation is exaggerated, leading to harmful tissue damage and formed MAC stimulates chondrocytes to produce more MMPs.

Additional interesting experiments to perform, would be to culture healthy human articular cartilage explants in the presence of cytokines, followed by incubation with sera of RA patients, known to contain little or a lot of autoantibodies. It could reveal if the presence of autoantibodies increase the complement activation.
Major findings

- The G3-domain of aggrecan activates the classical pathway of complement, and simultaneously binds FH that limits C9 deposition.

- PRELP inhibits the assembly of the alternative pathway C3 convertase and the formation of MAC. PRELP fragments are detected in the synovial fluid of RA patients.

- NC4 enhances the cofactor activity of C4BP and FH in the factor I-mediated cleavage of C4b/C3b and inhibits the formation of MAC. NC4 might also interfere with the complement activating potential of fibromodulin and osteoadherin, but also with the inhibitory potential of COMP.

- Complement activation occurs on cartilage explants, subjected to inflammation-induced degradation, via the classical and alternative pathways. Components that are released from cartilage during degradation appear to inhibit complement activation.
As highlighted in the thesis, complex interactions occur between several ECM proteins and complement proteins, each protein having several possible binding partners. Some interactions cause activation of the complement cascade, while others have an inhibitory effect on complement. How important these interactions are for the homeostasis and pathology remains to be elucidated. It seems that non-damaged cartilage is quite indifferent to interactions with complement, while damaged cartilage exposes new binding sites to which complement proteins can bind and cause activation. A certain degree of complement activation by altered proteins might be beneficial, as it serves to enhance the phagocytosis of them. One can imagine that a little degradation is manageable, which could occur during normal conditions. The question is how much cartilage damage is needed to evoke an excessive and pathological response by complement? Would complement activation be a help or make the degradation process worse and irreversible?

There seems to be a complex interplay between different actors that all can contribute to the pathology of joint disease; apart from complement, also the adaptive immunity, TLRs, cells of the joint tissues (synoviocytes, chondrocytes) and the ECM. A lot of work is still needed to elucidate these complex interactions, in order to understand if some particular events or conditions cause the final shift from health to disease.

More studies would be needed to clarify if the released fragments from degraded cartilage have an inhibitory effect on complement. It would be interesting to culture cartilage, obtained from patients with early OA and RA, with IL-1α and see if the released fragments have the same effect as those obtained from bovine cartilage. It might be possible that the patient material carries citrullinations or has other structural differences that would influence the outcome of the complement interactions. Further, one could take cartilage from patients with early or late OA and RA, and investigate at which stages of the disease process that complement proteins can be detected on cartilage. It would also be interesting to study if serum from RA patients, containing a well characterized set of autoantibodies, can cause an increased complement activation on IL-1α-stimulated cartilage explants, compared to normal sera.
Människans immunförsvar ska hjälpa oss att bekämpa infektioner från bakterier och virus. Immunförsvaret kan delas in i två delar; det adaptiva immunförsvaret, som utvecklas hela tiden under livets gång, samt det medfödda immunförsvaret.


Det medfödda immunförsvaret är minst lika viktigt eftersom det hela tiden är redo att aktiveras och gå till omedelbar attack när en invaderande bakterie upptäcks. Till det medfödda immunförsvaret hör flera olika sorters immunceller som bland annat har till uppgift att äta upp och oskadliggöra bakterier eller virusinfekterade celler. Hit hör även komplementsystemet, som bland annat känner igen generella strukturer på bakterier och döende celler, och som varnar resten av immunförsvaret att här är fara och färde.

Komplementsystemet består av bortåt 40 olika proteiner som cirkulerar runt i kroppen eller finns bundet på ytan av kroppens celler. En del av komplementproteiner är ordnade i en kaskad, och när komplementsystemet utsätts för aktiverande stimuli (t.ex. en bakterie) startas en kedjereaktion där en komponent i övre delen av kaskaden klyver och aktiverar nästa komponent i kaskaden. Det resulterar i att flertalet olika klyvningsprodukter bildas som har olika biologiska funktioner. En del lockar till sig immunceller till platsen där aktiveringen har skett och startar olika inflammatoriska reaktioner, medan andra märker in den komponent som orsakat aktiveringen med markörer som underlättar att den blir bortstädat. Proteiner i slutet av kaskaden kan även skapa porer i bakteriers cellmembran så att de går sönder.

Komplementsystemet kan aktiveras genom tre olika vägar beroende på vilket stimuli systemet stöter på. Den klassiska vägen aktiveras av att proteinet C1q känner igen och binder till antikroppar, döende celler, en del typer av bakterier
eller vissa kroppsegna proteiner. Lektinvägen aktiveras när proteinet MBL exempelvis binder till vissa typer av sockerkedjor som bara finns på bakterier. Den alternativa vägen aktiveras spontant i låg grad hela tiden på alla ytor, men tillåts bara ge en stark respons på främmande ytor. Komplementaktiveringen hålls under normala fall hela tiden under noga kontroll av komplementämmare, så att en lagom respons tillåts.

Det finns ett välutvecklat samspel och kommunikation mellan komplementsystemet och olika sorts immunceller för att ge ett så effektivt och balanserat försvar som möjligt. Responsen gentemot ett yttre hot, som en bakterie, ska inte vara densamma som mot skadade kroppsegna strukturer, även om många av de involverade cellorna och molekylera komponenterna är densamma. Är det obalans i responsen gentemot kroppsegna strukturer kan det bland annat uppstå överdriven inflammation, cellulära förändringar och skador. Obalans i immunförsvarvet ligger bakom flera olika sjukdomar, såsom reumatoid artrit (ledgångsreumatism), osteoartrit (artros) och systemisk lupus erythematosus. Komplementsystemets roll i just reumatoid artrit och osteoartrit diskuteras i denna avhandling.

Hos patienter med ledsjukdomarna reumatoid artrit och osteoartrit sker en gradvis försämring av de drabbade ledernas funktion, vilket orsakar stor smärta och begränsningar i rörelseförmågan. På benytorna i en led finns brosk som har som uppgift att underlätta benens rörelser mot varandra och fungera som stöddämpare. I de här sjukdomarna sker en onormal nedbrytning av brosk och ben i de drabbade ledena, och man ser även tydliga tecken på inflammation framförallt i ledgångsreumatism. I flertalet studier har man visat att komplementsystemet är extra aktiverat i ledena hos dessa patienter, jämfört med hos friska personer. Man tror att komplementaktiveringen bidrar starkt till det inflammatoriska tillstånd man ser hos de här patienterna.


Frågan är varför brosket inte alltid stimulerar komplementsystemet och ger inflammation. En anledning skulle kunna vara att molekylerna i brosket inte är tillgängliga förrän en viss brosknedbrytning redan har påbörjats. Brosknedbrytningen kan göra så att nya strukturer exponeras hos proteiner vid ytan av brosket, till vilka komplementproteiner från ledvätskan kan binda in. I

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