Matrix metalloproteinase-9-mediated Syndecan-4 shedding correlates with Osteoarthritis severity

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Ce qui embellit le désert,
c'est qu'il cache un puits quelque part.

What makes the desert beautiful,
is that somewhere it hides a well.

-Antoine de Saint-Exupéry

Contents

Abstract

Osteoarthritis (OA) is a degenerative joint disease, characterized by the extracellular matrix (ECM) breakdown of articular cartilage, as well as by changes in the synovial membrane, subchondral bone and synovial fluid composition. The heparan sulphate proteoglycan Syndecan-4 (Sdc4) has been shown to play a crucial role in the progression of OA. In an animal model of OA, based on the destabilization of the medial meniscus (DMM), Sdc4 deficiency and antibody-mediated blocking of Sdc4 ameliorated clinical signs of OA. Interleukin-1 (IL-1) signaling is repeatedly correlated with OA pathology. It was shown that IL-1β, binds directly to Sdc4 and that Sdc4 regulates the IL-1 receptor type I (IL-1R1) presentation at the membrane surface and thereby modulates IL-1 signaling. Moreover Sdc4 is a target for extracellular domain shedding. Several studies show that Sdc4 shedding is crucial in physiological, as well as pathophysiological, processes. Thus, I investigated the involvement of Sdc4 shedding in the development and progression of OA.

Shed Sdc4 levels were analyzed in knee synovial fluid of OA patients using a specific ELISA. The severity of OA in the individual patients was assessed by the radiographic Kellgren-Lawrence score (KL 1-4). The levels of shed Sdc4 correlated with the KL score, and the receiver operating characteristic (ROC) curve presented shed Sdc4 as a suitable biomarker for discriminating early (KL 1-2) and late (KL 3-4) OA. Levels of the matrix metalloproteinases (MMP)-2 and -9 as potential sheddases, analyzed by specific ELISAs, showed an increase depending on the KL score for MMP-9, whereas levels of MMP-2 did not change. However, assessment with the ROC curve showed that MMP-9 was less suitable to discriminate between early and late OA. Further, levels of MMP-9 significantly correlated with levels of shed Sdc4, suggesting MMP-9 to be a potential sheddase for Sdc4 in OA. MMP-2 levels did not correlate with shed Sdc4 levels. Expression levels of Sdc4 and MMP-2 mRNA in articular cartilage and synovial membrane did not change depending on the KL score, whereas MMP-9 mRNA levels were upregulated with increasing KL score in the synovial membrane. MMP-9 mRNA expression in cartilage, however, did not change in context of OA severity. To elucidate the role of Sdc4 shedding in OA, Sdc4 shedding was induced in chondrocytes by 4-Aminophenylmercuric acetate (APMA), an organomercurial activator of MMPs, and IL-1β stimulation. The addition of an MMP-9 inhibitor reduced basal, as well as APMA- and IL-1β-induced, Sdc4 shedding. An inhibitor for MMP-2 did not influence Sdc4 shedding, whether basal or induced. In IL-1β-induced Sdc4 shedding the inhibition of MMP-9

had more prominent effects with higher concentrations of IL-1β. The same was apparent when MMP-9 was knocked down by siRNA. APMA-induced increase of Sdc4 shedding attenuated IL-1 signaling. The addition of the soluble ectodomain of Sdc4 had no effect on IL-1 signaling. Thereby, the possibility of shed Sdc4 being a decoy receptor for IL-1 was ruled out.

In conclusion, this study presents shed Sdc4 as a possible biomarker for knee OA severity for the first time, while MMP-9 was shown to be a sheddases in Sdc4 shedding in OA. Moreover, it is shown that IL-1 signaling can be modulated by Sdc4 shedding, displaying a possible role for the increased shedding in OA.

1 Introduction

1.1 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis. It is a degenerative joint disease, whose main feature is the progressive degradation of the articular cartilage, which is also accompanied by structural changes in the synovium, synovial fluid, and subchondral bone. As all these structures are affected during OA, it is considered a "whole joint" disease. According to the World Health Organization [1], it is one of the "chronic rheumatic conditions" with the most significant impact on society and one of the most disabling diseases in developed countries.

1.1.1 Risk factors and prevalence

In principle, all joints can develop OA, but most commonly the knee, hip, hands, and feet are affected. Patients suffering from OA mostly report of functional disability and pain of the affected joint. Multiple risk factors are known for OA, with the main risk factors being age, obesity, female gender, genetics joint injury, repetitive use of the joint, and traumatic injuries [2]. A study showed that the global prevalence of radiographically confirmed symptomatic knee OA was estimated to be 3.8 % in 2010. With that, the knee joint is one of the joints most commonly affected by OA [3].

1.1.2 Changes in synovial tissues during knee OA

As mentioned earlier, OA pathological changes involve the whole joint. Therefore, different joint tissues contribute differently to the overall "OA knee". Fig. 1.1 schematically displays the differences between a healthy joint and its OA changes. Strongly simplified, the knee joint

consists of two opposing bones, the femur and the tibia, which are covered with articular cartilage. The joint is held in place by several ligaments as with, for example, the anterior and posterior cruciate ligaments. The meniscus can be found medial as well as lateral between the articulating surfaces. In the joint space, synovial fluid works as a lubricating fluid. The joint is lined by the synovial membrane, and by the capsule. Figure 1.1 shows the main structural changes that occur during OA: bone remodeling and sclerosis, cartilage break down, meniscal damage, synovial hypertrophy, and the formation of osteophytes [4].

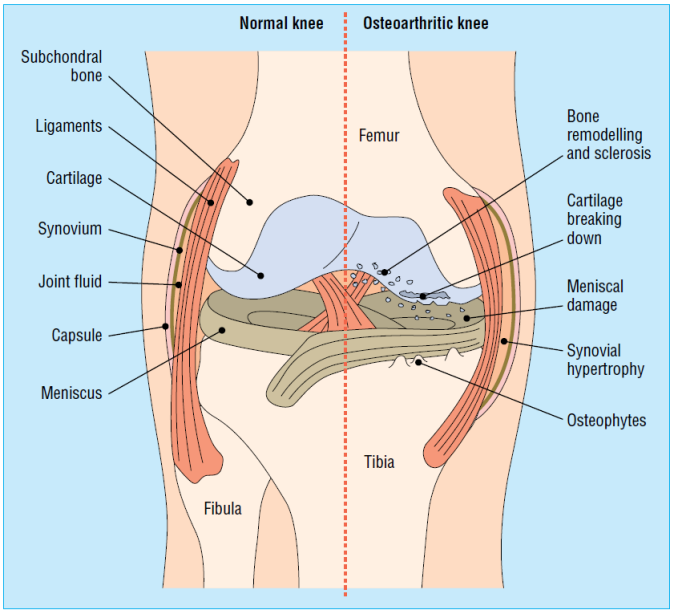

Figure 1.1: *Changes in the OA knee joint*

A schematically representation the different synovial joint tissues: Subchondral bone, ligaments, cartilage, synovium (synovial membrane), joint fluid (synovial fluid), capsule, meniscus (left panel). And it displays most characteristic changes of the OA knee: bone remodelling and sclerosis, cartilage breakdown, meniscal damage, synovial membrane hypertrophy, osteophytes (right panel), adapted from [4].

Articular cartilage

Articular cartilage is a key component of the synovial joint and extremely important for its physiological function. By building smooth and low friction surfaces it facilitates the unrestrained sliding of opposing articular surfaces.Healthy articular cartilage is 2 to 4mm thick and does not contain blood vessels, nerves or lymphatics [5]. The dense extracellular matrix (ECM)

is composed mainly of collagens and proteoglycans and smaller amounts of non-collagenous proteins and glycoproteins. The high amount of proteoglycans contributes to the resilience towards mechanical loads of the cartilage. They bind water via their negatively charged glycosaminoglycans (GAG) chains, which leads to the hydration of the cartilage with elevated tissue osmolarity and generation of swelling pressure [6]. The ratio between proteoglycans and collagens in healthy cartilage is 2:1. The collagen deposition varies between the different zones in the cartilage and comprises large fibril forming and fibril-associated collagens. Collagen type II (Coll2) comprises around 90 % of all collagen in the cartilage, while type IX and XI make up 1 % and 3 % respectively [7, 8].

Chondrocytes, the only cell type in articular cartilage, are highly specialized and metabolically active. They differentiate from mesenchymal stem cells, make up around 2 % of the articular cartilage and are needed in the development, maintenance, and repair of the cartilage ECM [9]. The shape, size, and number of chondrocytes varies dependent on their location within the cartilage. Chondrocytes create their specific microenvironment, which is important for the turnover of the ECM. The adjacent pericellular matrix and the chondrocyte together form the so called chondron. Chondrocytes react to stimuli such as growth factors, mechanical loads, piezoelectric forces, and hydrostatic pressure. Due to a low replication rate they are highly limited in their capability to heal injuries in the tissue. Chondrocytes need well-balanced chemical and mechanical conditions for their survival [9, 10].

OA pathology is characterized by the progressive breakdown of the articular cartilage. In early OA, altered chondrocyte metabolism leads to an expression of ECM degrading enzymes including collagenases and aggrecanases. The breakdown of Coll2 and the proteoglycan aggrecan are thereby hallmarks in OA progression. Coll2 is mainly degraded by the collagenases Matrix Metalloproteinase (MMP)-1 and MMP-13, whereas aggrecan is a key target for members of the aggrecanases of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family and especially ADAMTS-4 and -5. The loss of aggrecan from the cartilage leads to a decreased osmotic swelling pressure and thereby to a decreased ability of the cartilage to withstand compression and mechanical loads [11, 12, 13]. Fibrillation and erosion of the cartilage surface leads to the release of ECM breakdown products such as proteoglycan and collagen fragments into the synovial fluid [14]. The impact of these fragments on the synovial tissue will be described in the following section about the synovial membrane. During the progression of OA chondrocytes undergo a phenotypic shift towards hypertrophy. The chon-

drocytes get hypertrophic, similar to chondrocytes in the growth plate during endochondral bone formation, which facilitate bone mineral deposition. Chondrocyte hypertrophy is one of the hallmarks of OA. Increased expression of type X collagen and MMP-13 are the main markers of chondrocyte hypertrophy [15, 16, 17]. The final step in the hypertrophic differentiation results in chondrocyte cell death. Apoptotic chondrocytes have been associated with OA and cartilage degradation [18]. Beside the described changes in articular cartilage during OA, changes in the synovial membrane also contribute to the OA pathogenesis.

Synovial membrane

The synovial membrane is a specialized, connective tissue that encloses diarthrodial joints. It surrounds tendons and forms a barrier between bursae and fat pads, which separates the synovial cavity and the synovial fluid from its surroundings. Further, it contributes to the composition of the synovial fluid, mainly by the production of lubricin and hyaluronic acid [19, 20]. The healthy synovial membrane consists of two layers; the outer layer (subintima) and the inner layer (intima). Under OA conditions the synovial membrane changes and is characterized by synovial lining hyperplasia, sublining fibrosis and stromal vascularization [21]. Further, an invasion of leucocytes as a reaction to the secretion of cytokines and cell adhesion molecules can be seen, as well as recruitment of immune cells such as macrophages and T cells. Mast cells, B cells, and plasma cells can be found to a lesser extent in the synovial membrane during OA [20, 22, 23, 24].

Even though OA is not considered an inflammatory disease, a high prevalence of macroscopic inflammation has been reported, suggesting that synovitis is actively involved in the pathology of OA. Further, synovitis was associated with pain and structural progression of cartilage degradation [19]. Molecules from the degraded articular cartilage, such as aggrecan fragments, are released into the synovial cavity and initiate synovial inflammation. Further, it was suggested that in early knee OA, tissue debris from a damaged meniscus as well as molecules from the subchondral bone work similarly, resulting in the activation of synoviocytes to produce pro-inflammatory cytokines and chemokines, which attract immune cells, trigger angiogenesis and the above described phenotypic shift [25]. As these pro-inflammatory mediators cause the chondrocytes to initiate further cartilage damage, this leads to a positive feed-back loop which, in turn, initiates synovial inflammation [26]. For example, the aggrecan fragment (32-mer) is a

ligand for the Toll-like receptor 2 (TLR-2) and thereby accelerates cartilage destruction [27]. This shows that the synovial fluid is an important mediator in OA progression.

Synovial fluid

Synovial fluid is of clear straw color, has a viscous consistency and can be found in the joint cavity. It holds biomechanical, metabolic, and regulatory properties in the joint cavity. Biomechanically it works as a lubricant that enables a smooth movement of the joint with low friction and low wear articulation. Further, it supplies other structures in the joint, like cartilage, meniscus, and labrum with nutrients, which were e.g. secreted from the synovium into the synovial fluid [20, 28].

Synovial fluid is composed of blood plasma dialysate, so synovial fluid shares a similar protein composition with blood plasma. However, due to the permeability property of the synovium, high molecular plasma proteins are hindered to enter the joint cavity. Further, cells from the synovium as well as chondrocytes produce proteins that are secreted into the synovial fluid [20]. The primary lubricant proteins in synovial fluid are hyaluronic acid (HA) and lubricin (proteoglycan 4). HA is a non-sulfated glycosaminoglycan that can be present at different molecular weights, depending on the amount of repeated disaccharide units, but is mostly present at a high molecular weight. Lubricin is a glycoprotein that is secreted by synovial fibroblasts and superficial zone chondrocytes [29, 30, 31].

Changes in composition or volume of synovial fluid can occur due to trauma, inflammation, and bacterial, fungal, or viral penetrance. Also, during joint inflammation the total protein concentration of synovial fluid is elevated as the synovium loses its ability to selectively filter and retain proteins. At the same time, HA and lubricin levels are decreased, which reduces the lubrication property of synovial fluid [32, 33]. Synovial fluid reflects changes in cartilage and synovium metabolism, as different factors are secreted from cartilage and synovial membrane. This is interesting because it gives the opportunity to investigate cartilage metabolic processes in this fluid. Therefore, several studies have investigated the composition of synovial fluid aiming to determine biomarkers that either predict OA progression or cartilage regenerative capacity [14, 34, 35]. Cartilage metabolism, for example, can be monitored by the amount of pro-collagen type II C-terminal pro-peptide (PIIICP). During maturation of Coll2 this pro-peptide needs to get cleaved off and therefore reflects Coll2 synthesis [35]. On the contrary, during cartilage breakdown the C-telopeptide fragment of collagen type-II (CTX-II)

is created, which was shown to be upregulated in OA synovial fluid [14]. Similarly, synovial fluid also reflects changes in aggrecan synthesis and degradation, as well as increased levels of proteolytic enzymes, such as MMPs [36]. Changes in the metabolism of the synovium are for example represented by the amount of the glycoprotein YKL-40 (Chitinase-3-like protein 1), which increases proteoglycan synthesis. Levels of YKL-40 was positively correlated with OA severity [37]. Taken together these publications demonstrate that the synovial fluid is a good indicator for changes in the OA joint and therefore is well suited to establish biomarkers for OA. However, until now, no biomarker has been validated to be really specific for OA progression or cartilage regeneration in large clinical trials. Therefore there is a great need to establish one or more biomarkers to detect the therapeutic value of novel drugs, or identify fast progressing OA patients [38].

1.1.3 Interleukin-1 signaling in OA

Interleukin(IL)-1 is synthesized in the joint by chondrocytes, osteoblasts, synoviocytes, and mononuclear cells [22, 39, 40, 41, 42, 43]. It has been shown that different pro-inflammatory cytokines are upregulated in OA such as the tumor necrosis factor-α (TNF-α), IL-6, IL-15, IL-17 and IL-18 and in particular IL-1β [44]. Further, it has been reported that the Interleukin-1 receptor type I (IL-1R1) is upregulated in chondrocytes and fibroblast-like synoviocytes during OA [45, 46]. The loss of the articular cartilage is one of the most prominent features in OA and IL-1β has been implicated to be a key player in this process. IL-1β has been shown to hamper chondrocytes in producing important ECM proteins such as Coll2 and aggrecan [47, 48]. On the other hand IL-1β induced the upregulation of MMPs namely MMP-1, MMP-3 and MMP-13, which are well known to be involved in cartilage degradation [49, 50, 51]. Further, IL-1β induced ADAMTSs (esp. ADAMTS-4 and -5) which induced cartilage degradation in a human OA model [52]. Moreover, chondrocytes stimulated with IL-1β seem to age faster and undergo apoptosis earlier [53, 54, 55]. IL-1β can induce its own expression and its secretion in an autocrine manner to initiate the production of other pro-inflammatory cytokines like TNF-α, IL-6, IL-8, and Chemokine (C-C motif) ligand 5 (CCL5) [56, 57, 58, 59]. The IL-1 signaling pathway has been proposed as a main factor in the development of OA. But the therapeutic potential of targeting IL-1 is still widely discussed. In animal studies the inhibition of IL-1 signaling had positive effects on cartilage homeostasis [60, 61, 62, 63] and reduced surgically induced OA-like structural changes in the joint. However, clinical trials with IL-1R1 antagonists

[64, 65], or inhibiting IL-1α and IL-1β with antibodies [66], failed to improve OA symptoms compared to the placebo group. Therefore, further investigation of how IL-1 signaling is regulated in OA might help to determine which subset of OA patient might benefit from this treatment.

1.1.4 OA diagnostics

Multiple factors need to be taken into account in the diagnosis of OA. Movement-induced pain and functional limitations of the affected joint, as well as crepitus, bony enlargement and fixed flexion deformity, were all shown to be associated with OA. Meeting one or more risk factors, such as age over 60 years, female gender, a high body mass index or previous joint injury also increases the likelihood of developing knee OA. Imaging techniques are normally used to confirm the diagnosis based on described symptoms of the patient [67]. Radiography is the most common imaging technique in context with OA diagnosis. It can detect features such as marginal osteophytes, joint space narrowing, subchondral sclerosis, and cysts. Those are also the feature that are taken into account when scoring OA according to the Kellgren-Lawrence score. This score ranks the severity of OA from 0-4, as demonstrated in figure 1.2 where 0 (None) means the definite absence of structural changes in the joint; 1 (doubtful) refers to possible formation of osteophytes and doubtful joint space narrowing (JSN); 2 (minimal) shows definite osteophytes and possible JSN; 3 (moderate) multiple osteophytes, definite JSN, and subchondral sclerosis; 4 (severe) large osteophytes, marked JSN and severe bone sclerosis [68]. In Fig. 1.2 this scale is represented [69].

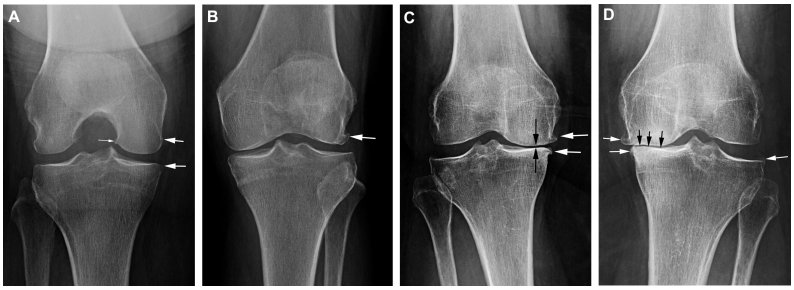

Figure 1.2: *The Kellgren-Lawrence classification scale*

(**A)**KL 1 (doubtful) refers to possible formation of osteophytes and doubtful joint space narrowing (JSN) (medial joint margins (large arrows)); **(B)** KL 2 (minimal) shows definite osteophytes and possible JSN (marginal osteophyte (arrow)); **(C)** KL 3 (moderate) multiple osteophytes, definite JSN, and subchondral sclerosis (joint space narrowing (black arrows) and marginal osteophytes (white arrows)); **(D)** KL 4 (severe) large osteophytes, marked JSN and severe bone sclerosis (bone-to-bone contact and complete obliteration of the joint space (black arrows), definite marginal osteophytes in addition (white arrows)); adapted from [69].

Recently, it has become evident that different phenotypes of OA exist [70]. Mobasheri et al. divided these phenotypes into four groups: intra-articular, extra-articular, secondary, and age-related and systemic phenotypes (see Fig. 1.3).

Even though all phenotypes share similarities in terms of cartilage degradation, differences regarding the involvement of inflammation are evident. Secondary phenotypes like the post-traumatic and crystal disease phenotype are particularly connected with inflammation [71, 72, 73]. After a traumatic injury, such as anterior cruciate ligament (ACL) or meniscal tear, inflammatory markers and biomarkers for cartilage degeneration were reported to be upregulated [74]; total MMP levels were also increased [75]. Higher levels of pro-inflammatory cytokines were also found in the inflammatory phenotype (IL-1β, IL-8, cyclooxygenase-2 (Cox-2)). Higher inflammatory markers are correlated with worse pain and decreased physical function [76]. Depending on the phenotype, different treatment options might be useful. The inflammation driven phenotype might be well treatable with anti-inflammatory medication; subtypes that belong to the extra-articular phenotype might show better responses to physiotherapy or orthotics. Therefore, different biomarkers are important to generate a better profile of the OA disease and to improve treatment.

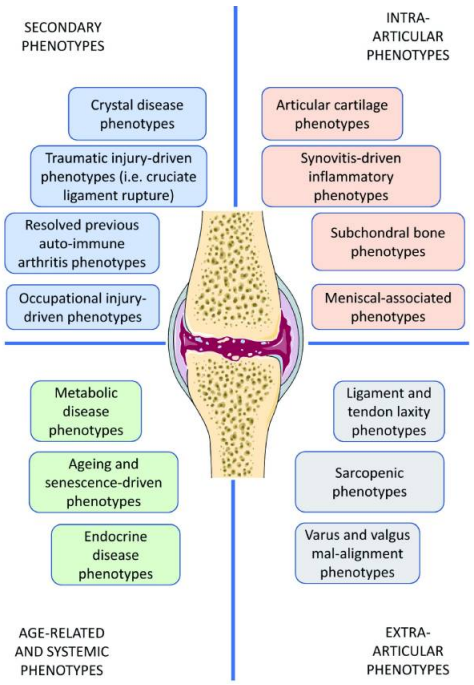

Figure 1.3: *Classification of different phenotypes in OA*

Classification of different phenotypes into: secondary, intra-articular, extra-articular, and age-related and systemic phenotypes; adapted from [70].

Biomarkers for OA

Biomarkers are an important tool in medicine to diagnose patients and to make the correct treatment decisions. They can be subgrouped into diagnostic, predictive, and prognostic biomarkers. A diagnostic biomarker detects the presence of a disease or condition or identifies a subtype of a disease. A predictive biomarker, on the contrary, provides information about the possible effect of a specific treatment on the patient, while a prognostic biomarker predicts the disease progression for the specific patient, regardless of the therapy [77].

Several biomarkers for OA have been proposed in recent years. Most studies have focused on knee OA. In particular they focused on systemic inflammation and tissue related inflammation and cartilage degradation markers [71, 78]. Examples of biomarkers currently in use to evaluate efficacy of novel treatment approaches are Coll2-1 (the peptide Coll2-1 (^{108}HRGYPGLDG^{116}), Coll2-1NO2 (nitrated form of Coll2-1), CTX-II (C-terminal cross-linked telopeptide of type II collagen), CS-846 (chondroitin sulfate 846 epitope), COMP (cartilage oligomeric matrix protein), MMP-3, and IL-6, which are measured in serum and urine [79].

Coll2-1 and Coll2-1NO2 are markers for collagenous ECM turnover. The specific Coll2-1 epitope of Coll2 is only detectable after its degradation. Coll-2-NO2 is the nitrated form of Coll2-1 and is associated with oxidative-related Coll2 degradation. Both have been shown to be relevant biomarkers in animal models of OA and to be predictive for the progression of OA. The Coll2-1 fragment is proposed to be a robust marker for the efficacy of therapeutic interventions, but also Coll2-1 could be a potential therapeutic target [80]. CTX-II is the cross-linked C-terminal telopeptide of Coll2, which is a byproduct of the breakdown of Coll2. CTX-II has been suggested to be a urinary biomarker and has been shown to elevate in patients with knee OA compared to healthy people. While differences in the levels of CTX-II in urine were found between early OA (KL 2) and late OA (KL 3 and 4), these changes were not statistically significant [81, 82].

Moreover, the cartilage oligomeric matrix protein (COMP) has been shown to be elevated in synovial fluid as well as serum from OA patients in comparison to the healthy control group [83], but further validation has yet to be done. In a post-traumatic model of OA in rats, COMP in combination with CS846, a CS synthetic marker and byproduct of proteoglycan metabolism, has been reported as a suitable biomarker for knee OA [84]. Both markers were upregulated 10 weeks after surgery and also correlated with observed OARSI score, which is a histological score for cartilage degradation [85].

Most proposed biomarkers in the literature represent the destruction of cartilage, which might be a good marker for the cartilage OA phenotype, but may be less sensitive for other phenotypes. In order to identify new potential biomarkers the pathogenesis of OA needs to be fully understood. For example, Syndecan-4 was shown to be crucial in the development of OA [86]. Therefore, in the next chapter I will focus on the involvement of this proteoglycan in OA progression.

1.2 Syndecans

Syndecans (Sdcs) were first described in the 1980s. Particularly close attention was paid to this protein family by the lab of Merton Bernfield. Sdcs were initially described as heparan sulfate-rich proteoglycans, which they found on the surface of mouse mammary epithelial cells. It was seen that these proteoglycans can bind to collagen I, and thereby link the cell to the ECM [87]. Sdcs owe their name to this early observation. As it was believed that linking the cytoskeleton of the cells to the ECM was the primary function of this proteoglycan, it was named after the Greek word 'syndein', which means 'to bind together' [88]. Nowadays it is know that Sdcs are a family of type I transmembrane heparan sulfate proteoglycans (HSPGs), which include four members in mammals (Sdc1, Sdc2, Sdc3 and Sdc4) (see Fig. 1.4). In general proteoglycans are made of a core protein to which glycosaminoglycans (GAGs) are covalently bound. These GAGs are linear chains of repeating disaccharides. Proteoglycans can be sub-grouped by the predominant type of GAG chains bound to the core protein, as with chondroitin sulfate, keratan sulfate, dermatan sulfate or, as mentioned in the case of syndecans, heparan sulfate. In addition to the heparan sulfate chains, Sdc1 and Sdc3 also exhibit chondroitin sulfate chains proximal to the cell membrane [89, 90]. The four Sdcs have a homology of 60-70% in the amino acid sequence. However, in contrast to the rest of the protein the ectodomain has only little resemblance among the four family members [91]. The transmembrane domain includes a GXXXG motif, which is believed to be responsible for the capability of the syndecans to form strong SDS-resistant dimers [92]. The cytoplasmic domain is highly preserved and consists of two conserved regions (C1 and C2), which flank a variable region (V). C1 is located proximal to the membrane and presents a 90 % homology among all Sdcs, while C2 is even higher conserved (100 % homology). As the name suggests, the V region has only a small homology (15 %) among the Sdcs, while the V region of Sdc1 and Sdc3 as well as Sdc2 and Sdc4 exhibit more resemblance respectively, which, in addition to the similarities in GAG chains, leads to the assumption that Sdc1 and Sdc3 as well as Sdc2 and Sdc4 are more closely related [91, 93]. Although Sdcs have different tissue-specific and developmentally regulated expression patterns [94], it has been seen that Sdcs can compensate for each other to a certain amount, but to what extent and how this functions is still not very well understood [95, 96].

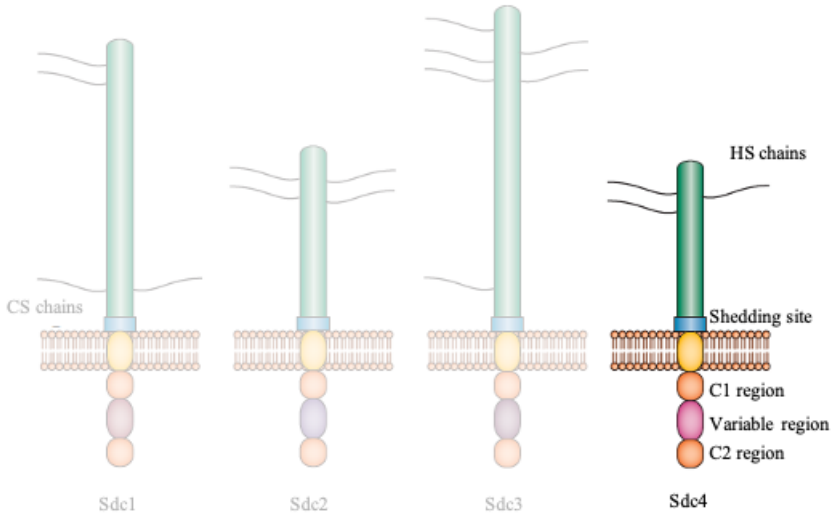

Figure 1.4: *The members and structure of the Syndecan family*

Representing the four members of the Syndecan familiy (Sdc1, Sdc2, Sdc3, and Sdc4). The extracellular domain is displayed in green, to which HS and CS chains are connected. In blue the juxtamembranous shedding region is depicted. The yellow oval displays the transmembrane region, embedded in the cell membrane. The intracellular domain is separated in the two conserved regions (C1 and C2) that flank the variable region; adapted and modified from [89].

1.2.1 Syndecan-4

Sdc4 signaling is involved in many processes. It is known that Sdc4 is involved in various physiological processes such as angiogenesis [97], blood pressure regulation [98], and wound healing [99]. Sdc4 mediates these different processes by various interactions. On the one hand, Sdc4 can interact with different extracellular factors by binding them to the heparan sulfate (HS)-side chains. Through the binding of growth factors or other ECM molecules to the heparan sulfate chains, Sdc4 can generate variable spatial distributions of these factors [100]. Upon ligand binding (e.g. growth factors), Sdc4 forms dimers or even higher oligomers [101, 102]. The dimerization was shown to be essential for Sdc4 dependent signal transduction [103, 104]. Further, Sdc4 contributes to the stabilization of the interaction between growth factors and their cell receptors [105]. It also forms a direct link between the ECM and intra-

cellular proteins, such as fibronectin and the actin-associated protein α-actin [106]. On the other hand, Sdc4 itself can initiate intracellular signaling cascades or recruit proteins like the dyoplamsic signaling protein syndesmos or PKCα (Protein kinase C α) to the site of focal adhesion [107, 108]. These proteins can in turn initiate further intracellular signaling. Another important function of Sdc4 is the mediation of receptor recycling, such as integrin. The direct phosphorylation of Sdc4 by the c-Scr functions as a molecular switch, which regulated in further steps, depending on RhoG (Ras homology Growth-related) and caveolin, whether α5beta1 or $\alpha 5\beta 3$ integrins are recycled to the membrane [109, 110]. Similarly the recycling of IL-1R1 was also shown to be dependent of Sdc4 [111]. Since IL-1β is a key cytokine in OA, the connection between Sdc4 and OA is highly interesting.

1.2.2 Syndecan-4 in OA

The expression of Sdc4 was repeatedly correlated with OA pathology. It was shown that the amount of Sdc4 positive cells increased in humans with OA severity, as well as in exercised induced OA in rats and surgically induced OA in mice [86]. The group of Risbud studied the connection of IL-1 and Sdc4 in the intervertebral disc. They could show in nucleus pulposus cells that IL-1β induced Sdc4 expression in a NF-κB dependent manner. They confirmed that Sdc4 is required for ADAMTS-5 activation [112]. Further they showed that, again in nucleus pulpous cells, IL-1β induces MMP-3 via the Mitogen-activated protein kinase (MAPK) extracellular kinase 1/2 (ERK1/2) and NF-κB pathways, and that the IL-1β induced MMP-3 expression requires Sdc4 [113]. It was shown that the Sdc4 promotor in cardiomyocytes and cardiac fibroblasts contains a functional NF-κB site and consequently IL-1β induced Sdc4 transcription [114]. A study by Bertrand et al. showed Sdc4 promoter activity at all stages of chondrocytes differentiation, while the deficiency of Sdc4 reduced chondrocyte proliferation [96]. Damaged OA cartilage exhibited more Sdc4 on mRNA level than normal looking cartilage [115]. Also Sdc4 mRNA was increased in hypertrophic chondrocytes compared to non-hypertrophic ones. Interestingly, this was only seen in chondrocytes obtained from the knee, but not in chondrocytes originating from the hip, indicating that the role of Sdc4 in OA might be joint specific. Besides the expression and protein content of Sdc4 being upregulated in knee OA [116], Sdc4 was shown to be involved in several pathways that are involved in OA development. One of these pathways is Wnt signaling, which has been correlated with OA progression [117]. Sdc4 was shown to regulate IL-1β induced phosphorylation of ERK1/2 in reaction to Wnt-3a

stimulation [118]. The prominent study that proves the involvement of Sdc4 in OA onset was conducted by Echtermeyer et al. Sdc4-deficient mice were protected from the proteoglycan loss in cartilage associated with surgically induced OA and showed decrease levels aggrecan neoepitopes. Similar effects were obtained when using a specific antibody to block Sdc4 in mice with surgically induced OA. Further, Sdc4 deficient mice showed less MMP-3 expression on mRNA levels as well as protein level and reduces IL-1 signaling and aggrecanase activity after IL-1β stimulation. Additionally, Sdc4 was shown to regulate the activation of ADAMTS5 through MMP-3 expression [86]. Therefore studying the interaction of Sdc4 and IL-1 in OA is of high interest.

1.2.3 Syndecan-4 and Interleukin-1

Sdc4 has frequently been shown to be upregulated in different cell types upon IL-1β stimulation in a dose dependent manner [114, 119, 120]. In a TNF-overexpresssing RA mice model, it was shown that deficiency of IL-1α and IL-1β resulted in less proteoglycan loss in those mice compared to WT mice. The same results were obtained in Sdc4 deficient mice, indicating a correlation between IL-1 and Sdc4, especially in cartilage destruction [121]. However, Sdc4 has not only been shown to be upregulated by pro-inflammatory cytokines, but has been proposed to be actively involved in IL-1β signaling. Godmann et al. showed that IL-1β can directly bind to the heparan sulfate chains of Sdc4, which induced the dimerization of Sdc4 independently of the presence of the IL-1R1. Upon dimerization Sdc4 regulates sensitivity towards IL-1β by regulating the caveolin vesicle-mediated trafficking of the IL-1R1. Upon binding of IL-1β to its receptor IL-1R1, ERK1/2 is activated, which is characterized by the phosphorylation of ERK. The studies by Echtermeyer et al. and Godmann et al. showed that Sdc4 deficiency leads to a reduced phosphorylation of ERK (p-ERK) and thereby less IL-1 signaling [86, 111]. As reduced responsiveness towards IL-1 signaling is obtained by the deficiency of Sdc4, as well the blocking of Sdc4 dimerization through specific antibodies [111], the question emerged whether the shedding of Sdc4 has a similar effect. Further, stimulation with IL-1β was reported to lead to an increased amount of Sdc4 shedding [120].

1.3 Syndecan-4 shedding

The shedding of Sdcs occurs constitutively to a small degree, but increases especially under pathological conditions such as inflammation [94, 122]. There are different functions proposed for this mechanism. It has been shown that the soluble form of the syndecan ectodomain is still intact and therefore able of binding growth factors, chemokines and cytokines [123, 124]. Thus, it was proposed that soluble syndecans might work as paracrine or autocrine effectors [100]. Further, it was also described that shed Sdcs work as an agonist [125] or create a morphogen gradient [126]. The shedding of Sdcs is a protease-dependent cleavage by so-called sheddases, which cleave syndecans at a sheddase-specific shedding site. Sdc4 shedding was shown to be induced by different stimuli such as mechanical strain [126, 127] as well as insulin [128] or inflammatory cytokines [120, 129]. In a cell free experimental setup, where a Sdc4 fusion protein was incubated with different MMPs, shedding sites were identified for MMP-2 (K105, Y37, R36, D31), MMP-9 (K105), and MMP-14 (N139, S130, D31), as well as plasmin (K128) and thrombin (K114, R36) [130], represented in Fig. 1.5. A study by Schmidt et al. also found shedding sites for plasmin (K114, K129) and thrombin (K114) [131]. Shedding of Sdc4 has been shown in various diseases and cell types. For example, shed Sdc4 has been proposed to be involved in promoting immune cell recruitment, ECM remodeling and reducing cardiac dysfunction triggered in mice, which were challenged with LPS [132]. This study suggested MMP-9, ADAMTS1, and ADAMTS4 to be possible sheddases as they were upregulated in parallel with Sdc4 shedding, whereas MMP-2 expression was downregulated. In an experimental setup, where Sdc4 shedding was induced by processed eggshell membrane powder (PEP), MMP-2 has been suggested as possible sheddase, as its activity and expression after PEP incubation was in increased, while MMP-9 and MMP-14 were less regulated. PEP is used in wound healing products and possesses anti-inflammatory capacities, which suggests Sdc4 shedding to be important in wound healing [133]. This is in line with the findings that Sdc4 -/- mice have impaired wound healing [99].

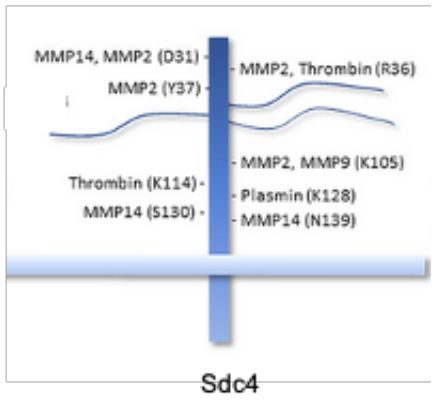

Figure 1.5: *Reported shedding sites in Sdc4*

Shedding sites in the extracellular domain of Sdc4 for MMP-2 (K105, Y37, R36, D31), MMP-9 (K105), MMP-14 (N139, S130, D31), plasmin (K128), and thrombin (K104) have been established in an *in vitro* experimental setup [130]. Adapted and modified from [91]

Moreover, Sdc4 shedding from glomerular endothelial cells was suggested to be responsible for glycocalyx dysfunction in diabetes. In this study Sdc4 shedding was attenuated with an MMP inhibitor specific for MMP-2 and MMP-9. Even though MMP-14 was shown to be increased on an mRNA level similar to MMP-2 and MMP-9, its influence on Sdc4 shedding was not further investigated [134]. Interestingly, these sheddases were also reported to be involved in OA pathology and are discussed later on in the study. In airway smooth muscle cells of asthma patients, Sdc4 shedding was shown to be increased through cytokines such as IL-1β and TNFα. Even though Sdc4 has been studied in the context of OA and has been proven to be important in OA development, the shedding of Sdc4 has been hardly looked at. One very recent study by Sanchez et al. showed that shedding in hypertrophic chondrocytes *in vitro* was increased. However, this observation. was not based on an increased shedding rate, but on increased Sdc4 expression. Further, this increase in Sdc4 expression was only observed in OA knee chondrocytes, but not for chondrocytes obtained from hip or shoulder OA cartilage [116].

1.3.1 Shed Syndecan-4 as biomarker

As Sdc shedding has been shown to be upregulated under certain pathological conditions, as for example inflammation or cancer, soluble forms of the Sdcs have been detected in different fluids and tissues such as blood (plasma and serum), bronchoalveolar lavage fluids, subrenital fluid, vitreous fluid, and pleural effusions. [91]. Shed Sdc4 so far has only been reported as a biomarker in serum and urine samples. Especially in the context of pneumonia, shed Sdc4 was proposed as a biomarker. In acute bacterial pneumonia an upregulation with disease progression was seen [135], while in idiopathic intestinal pneumonia, patients with higher baseline levels of shed Sdc4 had a worse prognosis [136]. In severe community acquired pneumonia serum, Sdc4 levels below 6.68 ng/mL prognosticated a higher mortality [137]. Moreover, shed Sdc4 was connected to heart failure and adverse left ventricular remodeling. In patients with hypertension serum, Sdc4 levels above 2.3 ng/mL was a predictor for heart failure [138]. Patients with cardiomyopathy showed a positive correlation between Sdc4 levels and adverse left ventricular remodeling [139, 140]. In atopic dermatitis shed Sdc4 levels were increased compared to the control group and also correlated with disease severity [141]. Serum Sdc4 levels in patients undergoing haemodialisis correlated with echocardiographic parameters that predict cardiovascular mortality [142]. Another study showed that, amongst others, post-transplant levels of Sdc4 in urine might be a possible biomarker for ubulointerstitial inflammation and interstitial fibrosis/tubular atrophy (IFTA) in kidney allografts [143]. These studies showed that shed Sdc4 can serve as a disease-specific serum marker in patients with different diseases. Chondrocytes were shown to shed Sdc4 and hypertrophic chondrocytes displayed increased Sdc4 expression on mRNA and protein level [116, 144]. Therefore, shed Sdc4 in synovial fluid might also be a possible biomarker for OA.

1.4 Matrix Metalloproteinases

MMPs comprise a family of 23 zinc-dependent endoproteases. They belong to the metzincis superfamiliy of proteases. MMPs are normally involved in physiological processes such as cell apotosis, embryogenesis, immune response, morphogenesis, tissue remodeling, tooth enamel formation, reproduction and menstruation, wound healing, angiogenesis, and axonal growth [145]. Many diseases are based on the dysregulation of these processes such as arthritis, nephritis, cancer, encephalomyelitis, chronic ulcers, and fibrosis. [146, 147, 148]. They are synthesized as pre-proenzymes and during translation the signal peptide is removed, resulting in an inactive MMP form. These inactive proMMPs are further activated by cleaving of the propetide by tissue and plasma proteinases [149]. It has also been shown that MMPs can be activated by different chemical compounds such as mercurial compounds, SH-reagents include sulfanyl group, and chaotropic agents. 4-Aminophenylmercuric acetate (APMA) is an organomercurial activator of MMPs. It chemically disrupts the bond of a conserved cysteine residues in the propeptide with the zinc atom located at the active site of the catalytic domain. Moreover, it was reported to interact with the protein and additionally disrupts salt bridges within the protein [150]. APMA can activate several MMPs, which also includes MMP-2 and -9 [149, 151]. MMPs can be divided into subgroups base on their favored substrate; collganesases, gelatinases, stromelysins, matrilysin, membrane-type-MMPs, and other non-classified MMPs. In healthy articular cartilage MMP-1, -2, -3, -8, -9, -13, -14 are expressed. Of those, the collagenases (MMP-1, -8, and -13) degrade the interstitial collagens (type I, II and III). MMP-13 is described as a major catabolic factor in OA, as it effectively cleaves collagen type II, the main collagen in healthy cartilage. Stromelysins (MMP-3) target non-collagen matrix proteins. High levels of MMP-3 were reported in OA, therefore MMP-3 has previously been proposed as a marker for OA [152]. MMP-3 can degrade a broad range of matrix molecules, such as collagens type II, III, IV, IX and X, as well as fibronectin, laminin, elastin, and several proteoglycans [153]. Further, MMP-3 was shown to be involved in the activation of ADAMTS-5, a major enzyme in aggrecan degradation [86, 154]. MMP-14 is bound to the membrane by a transmembrane domain [155] and has been shown to be able to degrade aggrecan. Further, MMP14 is upregulated in OA [156, 157] and has been shown to activate, MMP-2 [158] and MMP-9 [159, 160], for example.

1.4.1 The gelatinases - MMP-2 and MMP-9 - in OA

Both gelatinases have been associated with OA. MMP-2 has a variety of substrates such as collagen type I, IV, V, VII, and X, laminin, elastin, fibronectin, and proteoglycans. It has been reported that normal phenotype chondrocytes produce MMP-2 constitutively, leading to the assumption, that MMP-2 is responsible for normal cartilage homeostasis [161, 162, 163, 164]. MMP-9, is hardly expressed by normal phenotype chondrocytes, giving reason to the presumption that MMP-9 is not necessary for normal cartilage turnover [165]. The expression of MMP-9 can be stimulated by growth factors, chemokines, and other signals [166]. Native collagen type IV, V, VII, and X, elastin and fragments of collagen type I, II, and III have been reported to be substrates of MMP-9 [167]. Further, Kimm et al. showed that MMP-9 is involved in the activation of MMP-13, which is a major collagenase in OA [168]. A study by Dreier et al. presented pro-MMP-9 as a specific macrophage product, that is activated by OA chondrocytes through MMP-3 or a MMP-14/MMP-13 cascade [159]. Even though MMP-2 and -9 are both in general connected to OA pathology, previous published research showed very contradictory results in for the regulation of their expression. MMP-9 was reported to be upregulated in the plasma, serum and on mRNA level in peripheral blood mononuclear cells (PBMCs) of OA patients, in comparison the healthy individuals [126, 169], while other serum samples for MMP-9 showed no differences between OA patients and healthy individuals. MMP-immunostaining for MMP-2 and MMP-9 showed higher frequency and distribution of positive chondrocytes in OA cartilage compared to normal cartilage [144]. MMP-9 was reported to be not present in OA effusions, while MMP-2 was shown to be mainly present in an inactive form in OA effusions [170, 171]. In synovial fluid of OA patients MMP-9 was seen to be increased in comparison to healthy controls (patients with non-inflammatory, nonhemorrhagic traumatic sports related injuries) [172], while Rübenhagen et al. saw no upregulation of MMP-9 in OA patients compared the control group (cruciate ligament, meniscal reconstructive surgery patients without OA-like changes). They also saw no difference between synovial fluid from patients with OA KL1-2 vs. 3-4 [173]. Furthermore, MMP-9 was also shown to be decreased in synovial fluid of OA patients with KL 4 in comparison to KL 2 and KL 3. MMP-2 levels did not change in serum. MMP-2 in synovial fluid was, however, reported to be increased in OA patients compared to normal (traffic casualties) synovial fluid. Another study showed no differences in synovial fluid of OA patients to a control group (cruciate ligament, meniscal

reconstructive surgery patients without OA-like changes) and also no changes between OA patients with KL 1-2 vs. KL 3-4. [173]. These contradictory results could be explained by the different detection techniques (e.g. zympograhy vs. ELISA) for MMP-2 and the different sources of samples. Interestingly, cultured chondrocytes increase expression of MMP-9 upon IL-1β stimulation, which was inhibited by NF-κB65 specific siRNA [174]. As described before, IL-1β is a key cytokine in OA MMP-2 and -9 could act as possible sheddases for Sdc4 in OA pathology.

1.5 Aim of the thesis

The aim of this thesis was to investigate whether shed Sdc4 is a promising biomarker for OA progression. Furthermore, I aimed to elucidate the regulation and function of Sdc4 shedding in OA. In order to understand Sdc4 shedding in the context of the OA I focused on knee OA and investigated synovial fluid, serum, articular cartilage, and synovial membrane from patients that underwent total knee arthroplasty.

The specific questions that were answered during this thesis are:

- Is there a correlation between Sdc4 shedding and knee OA severity and could shed Sdc4 serve as a biomarker for OA?
- What is the source of the shed Sdc4 in synovial fluid and what are the potential sheddases?
- What is the relevant sheddase for Sdc4 in OA, and what function does the shedding have on chondrocyte signal transduction?

2

Materials and Methods

2.1 Materials

2.1.1 Reagents

All chemicals that were used during experiments were obtained from these main companies: AppliChem (Darmstadt), Merk (Darmstadt), Sigma Aldrich (München), Roche (Mannheim) and Roth (Karlsruhe). If reagents were used that were obtained from another company it will be stated.

2.1.2 Consumables

Laboratory consumables for cell culture, including flasks, plates and cell scrapers were obtained from Greiner (Frickenhausen). 15 and 50 mL tubes as well as disposable pipettes of various volumes were retrieved from BD Biosciences (Heidelberg). Small tubes, specifically 0.2, 1 and 2 mL, were obtained from Eppendorf (Hamburg). Sterile filters were ordered from Sartorius (Göttingen), while pipette tips (10, 200, 1000 and 5000 μL) were purchased from Sarstedt (Nürnbrecht).

2.1.3 Equipment

All equipment used to conduct the experiments are listed below (see Table 2.1).

Table 2.1: Equipment

Equipment	**Manufacturer**
Agarose gel imager Infinity-1000/26M	PeqLab, Erlangen, Germany
Biological Safety Cabinets S2020 1.5	Thermo Scientific, Waltham, USA
Biological Safety Cabinets KS 18	Thermo Scientific,Waltham, USA
Binder CO_2 incubator	Binder Labortechnik GmbH, Herbertshaus, Germany
Centrifuge Mikro 200R	Hettich, Tuttlingen, Germany
Centrifuge Heraeus, Fresco 17	Thermo Scientific , Waltham, USA
Centrifuge Heraeus, Fresco 17	Thermo Scientific ,Waltham, USA
Centrifuge Heraeus, Megafuge 16R	Thermo Scientific , Waltham, USA
ChemieDoc MP Imaging Systems	Bio-Rad Laboratories,Herculas, USA
Embedding Center Shandon HistoCentre 2	GMI, Ramsey, Minnesota, USA
Inverse microscope Axio Observer.Z1	Zeiss, Oberkochen, Germany
Inverse microscope BX51	Olympus, Hamburg, Germany
Inverse microscope Eclipse TS100	Nikon, Alzenau, Germany
Horizontal Electrophoresis Systems	Bio-Rad Laboratories, Herculas, USA
Micro scale AW-224	Sartorius, Göttingen, Germany
Microplate reader Infinite F200 Pro	Tecan, Männedorf, Switzerland
Microtome Hyrax M55	Zeiss, Oberkochen, Germany
QuantStudio 6 Flex	Applied Biosystems, Foster City, USA
Perfect Spin Plate Spinner C1000-PEQ-230EU	PeqLab, Erlangen, Germany
Platform rocker SSL4	Stuart, Staffordshire, UK
Homogenisator, Precellys® 24	Bertin, Montingy-le-Bretonneux, France
Real-Time PCR Detection System	Applied Biosystems, Foster City, USA
ABI PRISM 7900HT and 7500	
Roll-Mixer RM 5.40	Hecht Assistant, Sondheim
Roll mixer RS-TR05	Phoenix Instrument
Rotor Fa-45-30-11	Eppendorf, Hamburg, Germany
Rotor F-35-6-30	Eppendorf, Hamburg, Germany
Scale ALC-810.2	Sartorius, Göttingen, Germany
Scale Kern EWB 620-2M	Sartorius, Göttingen, Germany
Scale A 120 S	Sartorius, Göttingen, Germany
Shaking Incubator Thermo Mixer C	Eppendorf, Hamburg, Germany
Standard Power Pack P25	Biometra, Göttingen, Germany

Equipment	Manufacturer
Tissue processor TP1020	Leica Biosystems, Nussloch, Germany
Tube Revolver/Rotator	Thermo Scientific, Waltham, USA
T100 Thermal Cycler for PCR	Bio-Rad Laboratories, Herculas, USA
Thermocycler iCycler thermal cycler	Bio-Rad Laboratories, Herculas, USA
Thermal shaker Thermomixer compact	Eppendorf, Hamburg, Germany
Vortex Genie 2 Mixer	Bohemia, USA
Western blot (SDS-PAGE) and equipment	Bio-Rad Laboratories, Herculas, USA
Tank blot system inclusive	

2.1.4 Buffers and solutions

Buffers or solutions used to conduct the experiments are listed below (see Table 2.2).

Table 2.2: Buffers and solutions

Buffer or solution	Composition	Manufacturer
1 % acetic acid	1 % acetic acid in water	Roth, Karlsruhe
Blocking solution	5 % BSA in 1x TBS	AppliChem, Darmstadt
Blotting buffer	48 mM Tris base	Roth, Karlsruhe
	39 mM Glycine	Roth, Karlsruhe
	0.1 % SDS	Roth, Karlsruhe
	20 % Ethanol	Roth, Karlsruhe
Comassie Blue	0.5 % (w/v) Coomassie Blue	Sigma Aldrich, München
	50 % (v/v) Methanol	Roth, Karlsruhe
	7 % (v/v) acetic acid	Roth, Karlsruhe
	42.5 % (v/v) distilled water	
Collagenase IV solution	1 mg/mL Collagenase IV	AppliChem, Darmstadt
	in chondrocyte culture medium	
1x Electrophoresis Buffer	25 mM Tris base	Roth, Karlsruhe
	192 mM Glycine	Roth, Karlsruhe
	0.1 % (w/v) SDS	Roth, Karlsruhe
	2.5 % Glycerol	Roth, Karlsruhe
NP-40 lysis buffer	50 mM Tris (pH 8)	Roth, Karlsruhe
	150 mM NaCl	Roth, Karlsruhe
	1% (v/v) NP-40	Sigma Aldrich, München

Buffer or solution	**Composition**	**Manufacturer**
1 x PBS	ready to use (#D8537)	Sigma Aldrich, München
Pronase solution	1 mg/mL Pronase	Sigma Aldrich, München
	DMEM (ready to use #D5796)	Sigma Aldrich, München
2 % Safranin-Orange solution	2 % (w/v) Safranin-Orange in water	ApplChem, Darmstadt
5 x SDS-PAGE sample buffer	5 % (v/v) β-Mercaptoethanol	Roth, Karlsruhe
	0.02 % (w/v) Bromophenol blue	AppliChem, Darmstadt
	30 % (v/v) Glycerol	Roth, Karlsruhe
	10 % (w/v) SDS	Roth, Karlsruhe
	250 mM Tris/HCl, pH 6.8	Roth, Karlsruhe
Stripping buffer	62.5 mM Tris/HCl, pH 6.7	Roth, Karlsruhe
	100 mM β-Mercaptoethanol	Roth, Karlsruhe
	2 % (w/v) SDS	Roth, Karlsruhe
1 x TAE Buffer	40 mM Tris base	Roth, Karlsruhe
	17.5 % (v/v)Acetic acid	Roth, Karlsruhe
	50 mM EDTA pH 8.6	Roth, Karlsruhe
1 x TBS buffer	1 % (v/v) 20 x TAE (ready to use #sc-362305)	Santa Cruz, Dallas, USA
	49 % (v/v) distilled water	
1 x TBS-T	0.1 % (v/v) Tween 20	AppliChem, Darmstadt
	99.9 % (v/v) 1 x TBS	
Zymography Developing buffer	50mM Tris-base pH 7	Roth, Karlsruhe
	0.2 M NaCl	AppliChem, Darmstadt
	5 mM $CaCl_2$	Sigma Aldrich, München
	0.02 % (v/v) NP-40	Sigma Aldrich, München
	1 μ M $ZnCl_2$	Sigma Aldrich, München
Zymography Destaining solution	50 %(v/v) methanol	Roth, Karlsruhe
	10 % (v/v) acetic acid	Roth Karlsruhe
	40 % (v/v) distilled water	
Zymography Wash buffer	2.5 % (v/v) Triton-X100	Roth, Karlsruhe
	97.5 % (v/v) distilled water	

2.1.5 Primers

All primers used in this study were obtained dissolved at a concentration of 100 μM from Metabion (Martinsried) and are listed below (see Table 2.3).

Table 2.3: Human primers used for Real-Time RT-PCR

Gene	product length	sequence (5'-3')	
Sdc4	203 bp	forward	ATT CGA GAC ACA GAG GTC ATC G
		reverse	TGG TTA TCC AGT GGC ACC AAG
MMP-2	102 bp	forward	TCA CTC CTG AGA TCT GCA AAC AG
		reverse	TCA CAG TCC GCC AAA TGA AC
MMP-9.1	68 bp	forward	AGA CGC CCA TTT CGA CGA TGA C
		reverse	CAA ACC GAG TTG GAA CCA CGA C
MMP-9.2	273 bp	forward	GTA CTC GAC CTG TAC CAG CG
		reverse	TCA GGG CGA GGA CCA TAG AG
GAPDH	90 bp	forward	CCC ACT CCT CCA CCT TTG AC
		reverse	AGC CAA ATT CGT TGT CAT ACC AG

2.1.6 Antibodies

All primary antibodies for western blot analysis can be found in table 2.4 and all secondary antibodies in table 2.5. All antibodies were diluted in TBS-T containing 5 % BSA.

Table 2.4: Primary antibodies for western blot

Antibody	IgG	MW [kDa]	Dilution	Manufacturer
Phospho-p44/42 MAPK (Erk1/2)	rabbit	42, 44	1:1000	Cell signaling #9101
p44/42 MAPK (Erk1/2)	rabbit	42, 44	1:1000	Cell signaling #9102
GAPDH	rabbit	37	1:1000	Cell signaling #2118
Sdc4 intracellular	rabbit	22	1:100	Biovision #3644
Sdc4 extracellular	mouse	24	1:100	Santa cruz #sc-12766

Table 2.5: Secondary antibodies

Antibody	Reporter	Analysis	Dilution	Manufacturer
Rabbit IgG	HRP	WB	1:10000	Santa Cruz #sc-2030
Mouse IgG	HRP	WB	1:10000	Santa Cruz#sc-2005

2.1.7 Recombinant Proteins

All recombinant proteins that were used in this study are listed in table 2.6.

Table 2.6: Recombinant Proteins

Protein	Manufacturer	Number
human IL-1β	R&D Systems	201-LB
human Sdc4	R&D Systems	2918-SD-050

2.1.8 Cell lines

C28 chondrocytes The C28/I2, in short C28, is an immortalized chondrocyte cell line which is commonly used to study chondrocyte cell behaviour under normal and pathological conditions. C28 cells are a clonal line, which were immortalized by using retroviral vetor-mediated simian virus SV40 large T antigen (Tag) expression of the nonclonal T/C-28a4 cells.

2.2 Molecular biological methods

2.2.1 RNA isolation from soft tissue and cells

Total RNA was isolated using QIAzol® Lysis Reagent. Soft tissue was disrupted using a Precellys® 24 homogeniser (Bertin, Montingy-le-Bretonneux, France). Therefore, 1 mL of QIAzol® Lysis Reagent and a piece of the soft tissue was put into a Precellys Lysing Kit (P000918-LYSK0-A, Bertin, Montingy-le-Bretonneux, France) tube and incubated for 2 x 20 s at 6,500 Hz. This was repeated once, in between the steps the samples were kept on ice. The supernatant was transferred into a new 1.5 mL tube. Cells were disrupted by simply incubating them in QIAzol® Lysis Reagent for 5 min at room temperature and then repeatedly pipetting the solution up and down. Again the supernatant was transferred into a new 1.5 mL tube. Resulting supernantant was centrifuged at 12,000 rpm for 10 min to remove cell debris, extracellular matrix and other impurities. Per 1 mL QIAzol® Lysis Reagent 200 μL chloroform were added to the supernatant and vigorously mixed. After the samples rested for 15 min on ice the aqueous and the organic phases were separated by centrifuging for 15 min at 12,000 g and 4 °C. The aqueous phase was taken off and transferred into a new 1.5 mL tube. Then the aqueous phase was mixed with the same amount of ice-cold isopropanol. The solution was mixed well on the vortexer and incubated on ice for 20 min to allow the RNA to precipitate. In order to pellet the RNA, the solution was centrifuged for 40 min at 20,000 g at 4 °C. Afterwards the supernatant was aspirated, the pellet was washed with 1 mL of 70 % ethanol and again centrifuged at 4 °C and 20,000 g for 10 min. After the washing step, the ethanol was carefully removed and the RNA pellet was dried at room temperature for approximately 15 min. When dry, the RNA was resuspended in RNase free water. Finally, RNA content as well as quality was determined by measuring the absorbance at 260 and 280 nm using the Tecan Infinite® Pro 200 spectrophotometer (Tecan, Männedorf, Switzerland).

2.2.2 RNA isolation from articular cartilage specimen

Articular cartilage was crushed under liquid nitrogen with pestle and mortar. The resulting powder was given into a 1.5 mL tube and covered with 1 mL of QIAzol® Lysis Reagent. To disrupt the cells the mixture was incubated rotating for at least 24 h at 4 °C. Due to the high amount of ECM the quality of RNA from articular cartilage using the QIAzol® Lysis Reagent

method as described above was insufficient for Real-Time RT-PCR. Therefore the isolation was performed as described in the previous section until the separation of the organic and the aqueous phase. But subsequently RNA was extracted from the aqueous phase using the RNeasy® Plus Micro Kit (QIAGEN, Hilden). Therefore the aqueous phase was mixed with an equal amount of 70 % ethanol and was loaded onto the supplied column. Further the column was washed with 350 μL of the buffer RW1 and centrifuge for 15 s at 8,000 g. Then the column was washed with 500 μL Buffer RPE and centrifuged as described before. A last washing step was done by adding 500 μL of 80 % ethanol to the column and centrifuge it at 8,000 g for 2 min. To erase all residual fluid the column was centrifuged at full speed with open lid for 5 min. The RNA was eluted by adding 14 μL of RNase free water to the column and centrifuging at full speed for 1 min. Again RNA content as well as quality was determined by measuring the absorbance at 260 and 280 nm using the Tecan spectrophotometer.

2.2.3 Synthesis of cDNA and Real-Time RT-PCR

For the synthesis of cDNA from the extracted total RNA the High capacity cDNA reverse transcription kit was used (Thermo Scientific, Langenselbold). 500 μg of RNA in 10 μL RNase free water was mixed with 10 μL reaction mix (Table 2.7) and the reverse transcription (RT) was performed using the following protocol (Table 2.8).

Table 2.7: Reagents Reverse Transcription

RT Buffer	2.0 μL
25x dNTPS (100 mM each)	0.8 μL
10x RT-Random Hexamer Primer	2.0 μL
Multi Scribe Reverse Transcriptase (50 U/μL)	1.0 μL
RNase Inhibitor	1.0 μL
RNase free water	3.2 μL
total	10.0 μL

Table 2.8: Protocol Reverse Transcription

step	temperature	time
step 1	25 °C	10 min
step 2	37 °C	120 min
step 3	85 °C	5 min
step 4	4 °C	hold

After the RT the resulting cDNA mix was diluted 1:5 by adding 80 μL of RNase free water. For the Real-Time RT-PCR a SYBR® Green PCR Master Mix (ABI life technologies, Waltham, USA) which included ROX as a loading control was used. For specific PCR this Master mix was mixed with suitable primers to investigate the mRNA of interest (see Table 2.3).

Table 2.9: Reagents Real-Time PCR

forward primer (10 μM)	0.5 μL
forward primer (10 μM)	0.5 μL
SYBR Green Master Mix	5.0 μL
RNase free water	1.5 μL
total	7.5 μL

For the Real Time RT-PCR a 384 well plate was used. Per well 7.5 μL of the primer containing master mix was mixed with 2.5 μL diluted cDNA. The PCR protocol is displayed in Table 2.10. For the analysis a relative standard curve was run on every plate for each gene of interest.

Table 2.10: Protocol PCR

step		temp.	time
initial denaturation		95 °C	15 min
denaturation		95 °C	30 s
annealing	40x	60 °C	30 s
elongation		72 °C	30 s
dissociation stage		95 °C	15 s
		60 °C	15 s
		95 °C	15 s

2.2.4 Semiquantitative PCR

For the semiquantitative PCR, the cDNA was synthesized as described in 2.2.3 before. The cDNA was diluted 1:5 with RNase free water. 5 μL of cDNA was mixed with 15 μL of Master MiX (Table 2.11)

Table 2.11: Reagents semiquantitative PCR

forward primer (10 μM)	1 μL
forward primer (10 μM)	1 μL
SYBR Green Master Mix	10 μL
RNase free water	3 μL
total	15 μL

The same PCR protocol was executed as for the Real-Time RT-PCR (Table 2.10) in a regular ThermoCycler. The amount of cDNA was analyzed by agarose gel electrophoresis.

2.2.5 Agarose gel electrophoresis

A 1 % agarose gel has been used for DNA separation. Therefore, 1 g of agarose was melted in 1 x TAE buffer. Per 100 mL 5 μL of ethidium bromide was added. The mixture was poured into a casting chamber and after polymerization the resulting gel was transferred into the electrophoresis chamber. The samples were mixed 1:6 with 6x Gel loading dye (B7025S, New England Biolabs, Ibswich, USA) and applied to the gel. To determine the size of the separated DNA fragments a 100 bp DNA ladder (N0467, New England Biolabs, Ibswich, USA) was included. The chamber was filled with 1x TAE buffer and run at 150 V for 30 -60 min. After size separation the gel was documented by UV light using the Imager Infinity-1000/26M (PeqLab).

2.3 Cell culture methods

2.3.1 Cultivation of primary and C28 chondrocytes

Primary and C28 chondrocytes were cultivated in chondrocyte specific culture medium, which was composed of Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 1 mM sodium pyruvate and penicillin (10.000 U/ml)/streptomycin (10 mg/ml), at 37°C in a humidified atmosphere containing 5% CO_2. The cells were splitted every 2-3 days. Therefore the cells were washed twice with PBS and trypsinized with 0.25% Trypsin/EDTA (Biochrom GmbH, Berlin) for 7 min. Trypsin was inactivated by adding 10 mL culture medium. The cell suspension was centrifuged at 700 g for 5 min at room temperature. The pellet was dissolved in fresh chondrocyte culture medium and one third of the cell suspension was transferred into a new culture flask.

2.3.2 Isolation of human primary chondrocytes

Cartilage from the most destructed part of the tibial plateau from patients who underwent total knee replacement surgery was cut into small pieces and digested in a pronase solution (1 mg/mL in DMEM without supplements) for 30 min at 37 °C. Afterwards the digestion solution was removed and the cartilage pieces were washed once with PBS. Then, the pieces were digested over night at at 37 °C using 1 mg/mL collagenase IV (Worthington Biochemical Corporation, Lakewood, USA), diluted in DMEM, 10 % FCS, 1 % penicillin/streptomycin, 1 % Na-Pyruvat. The next day, the remaining pieces were broken up through repeated pipetting. The solution was given through a strainer (40 μm) and centrifuges for 5 min at 700 g. The resulting cell pellet was resuspended in chondrocyte culture medium and plated in a T25 culture flask.

2.3.3 Cryopreservation and recovery of cells

For long term storage of cell lines such as C28/I2, a confluent 75 cm^2 flask was washed twice with PBS and trypsinized with 0.25% Trypsin/EDTA (Biochrom GmbH, Berlin). Trypsin was inactivated by adding 10 mL culture medium. The cell suspension was centrifuged at 700 g for 5 min at RT. The pellet was dissolved in DMEM containing 20% FCS, 1 mM sodium pyruvate, penicillin (10.000 U/mL)/streptomycin (10 mg/ml) (Biochrom, GmbH, Berlin), 20% DMSO and

transferred into a cryo tube. At first the cells were frozen overnight in a freezing container at -80°C, afterwards the cells were stored in liquid nitrogen. When needed, frozen cell suspension was carefully thawed in a 37 °C water bath. Afterwards the cells were suspended in 10 mL culture medium and centrifuged at 700 g for 5 min at RT. The cell pellet was dissolved in chondrocyte culture medium and plated in T25 culture flask.

2.3.4 Cell treatment with recombinant Sdc4 protein and IL-1β

C28 chondrocytes were seeded 24 h before the experiment at a density of 1.5x10^5 cells/well (12 well plate). For the treatment with the Sdc4 ectodomain (Table 2.6), the recombinant human Sdc4 was added to the cells with a final concentration of 1μg/mL and incubated for 1 h. Afterwards, IL-1ß was added with a final concentration of 10 ng/mL for various time points (15, 30, 60 min). Subsequently the cells were harvested as explained in 2.4.1 and kept at -20 °C until western blot analysis (see 2.4.3).

2.3.5 Chondrocyte experiments with inhibitors for MMP-2 and -9

C28 chondrocytes were seeded 24 h before the experiment at a density of 1.5x10^5 cells/well (12 well plate). Before the treatment with the inhibitors the culture medium was replaced with 0.5 mL of fresh chondrocyte culture medium. The stock solutions of the inhibitors were dissolved in DMSO and diluted to the working solution with culture medium. As control DMSO was diluted with culture medium according to the inhibitors before. The C28 chondrocytes were treated with a final concentration of either 5 nM, 113 nM, 1.05 μM, or 5 μM of MMP-9 Inhibitor I (Merk Millipore, Burlington, USA), or with 12 nM, 200 nM, 4.5 μM, or 50 μM of the MMP-2 inhibitor ARP-100 (Santa Cruz, Dallas, USA). Additionally, C28 chondrocytes were treated with either 4-Aminophenylmercuric acetate (APMA, A9563, Sigma Aldrich, St. Louis, Missouri, USA) or IL-1β. Previous to the experiment, APMA was dissolved in water and afterwards the solution was incubated in a 37 °C water bath until APMA was completely solved. Subsequently, the solution was sterile filtered using a 0.22 μL filter and the cells were treated with a final concentration of 1 mM or 5 μM APMA for 5 h. Or additionally to the treatment with the inhibitors, the cell were treated with a final concentration of 10 ng/mL IL-1β for 5h. Subsequently the culture medium was taken off and stored at -20 °C until further ELISA analysis 2.4.5.

2.3.6 Knock down of MMP-9 with siRNA

Primary chondrocytes were seeded 24 h before the experiment at a density of 0.5x10^5 cells/well (24 well plate). The transfection was carried out by using the jetPRIME® transfection reagent (Illkirch-Graffenstaden, France). Per well a mixture of 50 μL jetPRIME® buffer and 2 μL of either 50 μM siMMP-9 (Silencer® Select, s8862, AMBION GmbH, Kaufungen, Germany) or 50 μM siScrambled (Silencer® Negative Control 1, AMBION GmbH, Kaufungen, Germany) was vortexed for 10 s. Afterwards 2 μL of jetPRIME® reagent were added, vortexed and incubated for 10 min at room temperature. The 50 μL of the transfection mix was added to 450 μL of culture medium per well. The cells were incubated for 24 h. Afterwards the transfection medium was replaced by fresh culture medium and the cells were incubated with 10 ng/mL IL-1β for 5 h. Subsequently the supernatant was transferred into a 1.5 mL reaction tube and kept at -20 °C until ELISA analysis was performed (see 2.4.5). RNA was isolated from the cells as described in 2.2.1 and stored at -80 °C until a semiquantitative PCR was performed (see 2.2.4).

2.4 Protein biochemical methods

2.4.1 Cell lysis and protein extraction

Cells were harvested by taking off the culture medium and washing the cells once with cold PBS. Afterwards fresh PBS was applied to the well and the cells were detached by using a cell scraper. The cell solution was transferred into a 1.5 reaction tube and centrifuged at 700 g and 4 °C for 5 min. The supernantant was taken off and the cell pellet was resuspendent in 50 μL of NP40 lysis buffer. Cells were incubated for 60 min, rotating at 4 °C. Afterwards they were centrifuged at 12,000 g and 4 °C for 10 min. The resulting protein solution was taken off and transferred into a new 1.5 reaction tube and store at -20 °C until further analysis.

2.4.2 Quantitative determination of protein concentration

The amount of protein, obtained in 2.4.1 was determined using the Pierce^{TM} BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, USA). Therfore a BCA standard was prepared from a stock solution using distilled water. The samples were diluted 1:2.5 in PBS. 25 μL of BCA standard and diluted samples were applied to a 96-well plate and covered with 200 μL of working solution (Reagent A: Reagent B (50:1)). The plate was incubated for 30 min at 37 °C and then measured at 560 nm with the Tecan spectrophotometer

2.4.3 SDS-PAGE and Western blot analysis

For the casting of a 10 % SDS-PAGE the following reagents were mixed:

Table 2.12: Composition of 10 % SDS PAGE; separating gel

reagent	amount [mL]
H_2O dest.	1.900
30 % Acrylamide Mix	1.700
Tris/HCl (1.5 M; pH 8.8)	1.300
10 % SDS	0.050
10 % APS	0.050
TEMED	0.002

Per gel, 4 mL of the separating gel (Table 2.13) were pipetted between two glass plates with a 1.0 mm spacing, fixed in the BioRad gel casting system and covered with isopropanol. After polymerization 1 mL of stacking gel was added and either a 10 or 15 well comb was put into the stacking gel.

Table 2.13: Composition stacking gel

reagent	amount [mL]
H2O dest.	0.680
30 % Acrylamide Mix	0.170
Tris/HCl (1.0 M; pH 6.8)	0.130
10 % SDS	0.010
10 % APS	0.010
TEMED	0.001

The resulting SDS-PAGE was fixed into the BioRad Protein Gel Electrophoresis Chamber System. The system was filled with 1x electrophoresis buffer. Cell protein lysates were diluted with PBS to obtain equal protein concentrations in all samples. Afterwards the lysates were mixed 1:5 with 5x protein loading buffer. The mixture was heated at 95 °C for 5 min, shortly centrifuged and then loaded into the wells of the SDS-PAGE. A prestained protein ladder (Pager Ruler^{TM} Plus (10-250 kDa)) was included for later size discrimination of the proteins. The SDS-PAGE was run at 130 V for ~ 90 min. The gel was transferred to a nitrocellulose membrane and fixed into the BioRad wet blotting chamber systems. The chamber was filled with 1x blotting buffer and run at 300 mA for 90 min. Afterwards the free binding sites on the nitrocellulose membrane were blocked block with 5 % BSA in TBS for 1 h. The primary antibody was diluted in TBS-T as indicated in Table 2.4. The membrane was incubated with the primary antibody over night at 4 °C on an orbital shaker. Afterwards the membrane was washed three times with TBS-T for 10 min. The secondary antibody was diluted in TBS-T as indicated in Table 2.5 and added to the membrane for 1 h at room temperature on an orbital shaker. Following the antibody was washed off two times with TBS-T and the third time with TBS for 10 min for each washing step. Finally, antibody binding was detected through proton detection of the HRP reaction with the ECL solution. The chemiluminescence signals were detected with the ChemiDoc MP Imgaing System (BioRad, Hercules, USA).

2.4.4 Stripping of membranes

Membranes were stripped of their binding antibody by washing the membrane twice with stripping buffer for 5 min each. Afterwards the membrane was washed three times with TBS-T and afterwards blocked again with 5 % BSA in TBS for 1 h at room temperature.

2.4.5 Sdc4, MMP-2 and -9 Enzyme-linked Immunosorbent Assay

Levels of different proteins in the synovial fluid, serum and cell culture supernatant were measured using Enzyme-linked Immunosorbent Assays (ELISAs). The assays were carried out as described in the manufacturers' manuals. Samples were used undiluted or diluted as indicated in Table 2.14.

Table 2.14: ELISAs

protein	sample	dilution	manufacturer
MMP-2	synovial fluid	1:100	R&D Systems #MMP200
MMP-9	synovial fluid	undiluted	R&D Systems #DMP900
Sdc4	synovial fluid	undiluted	IBL international
	serum	1:1000	JP27188
	supernatant	undiluted	

2.4.6 Gelatin zymography analysis

For the gelatin zymography a 8 % separating gel, including gelatin, was prepared as described in Table 2.15 (1 mm gel).

Table 2.15: Gelatin SDS PAGE

reagent	amount [mL]
H_2O dest.	2.30
Acrylamide	1.60
Tris/HCl (1.5 M; pH 8.8)	1.50
1 % gelatine (in H20 dest.)	0.50
10 % SDS	0.06
APS	0.06
TEMED	0.005

The casting and the running of the SDS PAGE was performed as describe in 2.4.3 with little differences. After applying the separating gel between the glass slides the solution was covered with water instead of isopropanol. The samples were mixed with 2 x Laemli buffer, which did not contain β-mercaptoethanol. The mixture was incubated for 30 min at room temperature and not heated. After the electrophoresis was finished the gel was washed 30 min with zymography wash buffer to remove the SDS from the gel. Afterwards, the gel was kept in developing buffer for 30 min at room temperature while shaking. Subsequently the developing buffer was replaced by fresh developing buffer and the gel was incubated for 18 h at 37 °C. The next day the gel was incubated for 30 min in Comassie blue staining solution and afterwards destained in destaining solution until clear, sharp bands were visible. Only the parts in the gel where the gelatinases digested the gelatin destained. Images of the gel was taken with the BioRad ChemiDoc MP Imaging System.

2.5 Histological methods

2.5.1 Paraffin sections

Cartilage samples were fixed in 4 % formaldehyde (#27279, Fischer, Zurich, Switzerland) for at least 24 h. The samples were subsequently dehydrated using a tissue processor and afterwards embedded in paraffin. For following stainings the embedded samples were cut into 4 μm thick section with a microtome, picked up on microscope slides and dried overnight at 37 °C.

2.5.2 Safranin-Orange/Fast Green staining

Cartilage specimens were pretreated twice for 10 min with Xylol. Afterwards the slides were hydrated by washing them in 100 %, 96 %, 80 %, 70 % ethanol and afterwards pure water for 5 min each. Afterwards the slide were incubated in Fast Green solution for 1 min, then 30 sec in 1 % acetic acid and subsequently 30 min in 2 % Safranin-O staining solution. After that the specimens were rinsed with 96 % ethanol and then washed three times in 96 % ethanol and two times in 100 % ethanol for 1 min each. Finally, the specimens were washed two times in Xylol for 3 min and covered with Canada balsam and a glass cover slide.

2.6 Statistical analysis

All statistical analysis was performed with GraphPad Prism (Version 7.01). Values were either presented as scatter blots, showing individual values and the median of those values. Or as bar charts or dot plots displaying the mean ± SEM. The chosen representation method is indicated in the figure legends of the respective figure. The used statistical test for each graph was also indicated in the respective figure legend.

3 Results

3.1 Shed Sdc4 as potential marker for OA severity

For the investigation of Sdc4 shedding in the progression of OA, cartilage, synovial membrane, synovial fluid, and blood samples were collected from patients who underwent total knee replacement arthroplasty. The OA severity in these patients was assessed using the Kellgren-Lawrence (KL) score, a radiographic score considering the formation of osteophytes, joint space narrowing, sclerosis and bone deformation [68]. Fig. 3.1 shows representative X-ray pictures, photographs of the explanted tibial plateau, and the corresponding Safranin-O stained microscopy pictures of the most damaged part of the tibial plateau's cartilage. The three pictures presented per KL group belong to the same patient. For all following experiments, which were conducted with human cartilage, the specimens were taken from the most loaded site of the tibial plateau of each patient. This was determined by the highest macroscopically visible degree of cartilage destruction. In the Safranin-O stainings the different stages of cartilage destruction were microscopically noticeable. KL 1-2 showed some surface discontinuity and superficial fibrillation. The amount of proteoglycan, indicated by the Safranin-O staining, was decreased in the superficial-mid zone. Further, the perpendicular orientation of the chondrocytes in the superficial layer was lost (Fig. 3.1 KL 1-2). KL 3 showed increased surface fissures that went down into the deep cartilage layers. The proteoglycan content further decreased and increased cell proliferation – hypercellularity and complex chondrons - of disoriented chondrocytes were visible (Fig. 3.1 KL 3). KL 4 showed advanced cartilage erosion. Fissures were longer and went down into the calcified cartilage zone. The cartilage texture became heterogeneous and the amount of chondrocytes was deceased. The remaining chondrocytes formed clusters and showed signs of hypertrophy (Fig. 3.1 KL 4).

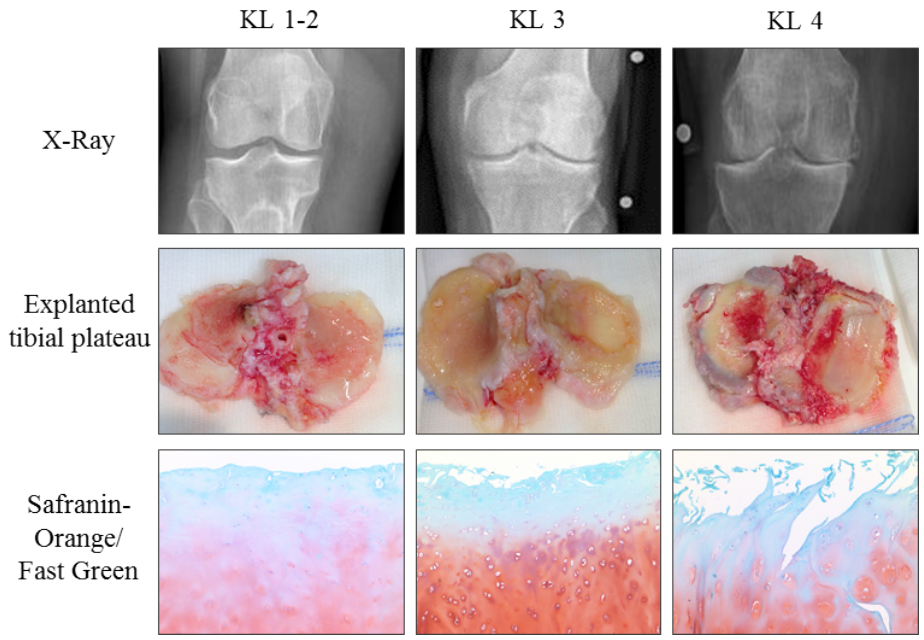

Figure 3.1: *Representative photographs of different Kellgren-Lawrence scores*

Representative X-Rays, photographs and Safranin-0 stainings of explanted tibial plateaus of patients with different severity levels. All three picture (X-Ray, explanted tibial plateau, Safranin-O/Fast Green staining) per KL group belong to the same OA patient.

3.1.1 Shed Sdc4 is present in synovial fluid

First, whether Sdc4 shedding takes place in OA was validated. Synovial fluid, which is often tested for biomarkers and structural changes in OA [78], was withdrawn from knee joints of OA patients during endoprosthesis implantation. Proteins of the synovial fluid were TCA-precipitated and analyzed using western blot. Indeed, the extracellular domain of Sdc4 was present in the synovial fluid, while no intracellular part was detected. The Sdc4 ectodomain was present, in monomeric and dimeric forms (Fig. 3.2A). Next, I checked whether differences in shed Sdc4 levels in synovial fluids of patients with different OA grades were detectable. Patients with higher KL scores showed increased levels of shed Sdc4 compared to patients with lower KL scores (Fig. 3.2B).

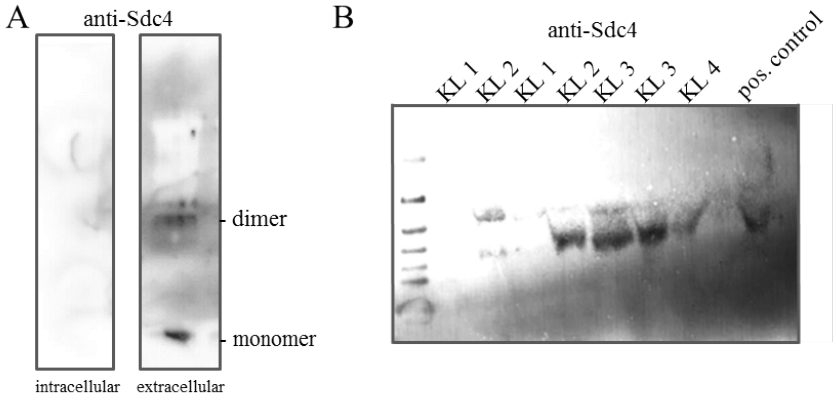

Figure 3.2: *Shed ectodomain of Sdc4 is present in the synovial fluid of OA arthroplasty patients*
Total protein of the synovial fluid of OA patients was TCA precipitated and analyzed by using western blot analysis. **(A)** Antibodies against the intracellular and the extracellular domain of Sdc4 showed no contamination with the membrane bound part of Sdc4, but the presence of the extracellular domain as a monomer and dimer. **(B)** The extracellular domain Sdc4 antibody showed increased shed Sdc4 with increased Kellgren-Lawrence score (KL). As positive control rhSdc4 was applied.

3.1.2 Shed Sdc4 levels correlate with OA severity independently of the gender

For a more precise and higher throughput analysis, levels of shed Sdc4 in synovial fluid were quantified with a commercially available ELISA Kit. The ELISA included an antibody raised against the extracellular domain of Sdc4. In total 103 synovial fluid samples of OA patients with KL 1-2 (N = 23), KL 3 (N = 50) and KL 4 (N = 30) were investigated. Levels of shed Sdc4 in synovial fluid were significantly higher (KL 1-2 vs. KL 3 = 85 %; KL 1-2 vs. KL 4 = 174 %) with increasing KL score, as shown in figure 3.3A. The acquisition of the samples was performed consecutively, and both genders were included in the study. Patients that came for knee endoprosthesis implantation to the department of Orthopaedic Surgery in Magdeburg, Münster, and Tartu were included in this study after written consent. It was repeatedly reported that the gender might influence the development and progression of OA [175]. Therefore, I tested whether gender had an influence on the amount of shed Sdc4 in the synovial fluid of patients in my knee OA cohort. Between both genders, a slightly higher amount of shed Sdc4 was visible in female patients, which was not statistically significant (Fig. 3.3B). Interestingly, only 25 % of the tested synovial fluid samples came from male knee OA patients. Further, I examined whether both gender groups - males and females - showed the same KL score dependent increase in

shed Sdc4 in synovial fluid. Indeed, both groups separately showed significantly increased levels of shed Sdc4 in accordance with the KL grade. In the male group, based on the lack of patient samples with KL 4, it was only possible to compare KL 1-2 with KL 3. The median value of shed Sdc4 in the synovial fluid of KL 3 in the male cohort increased by 107 % compared to the KL 1-2 group (Fig. 3.3C). In the female group the median value of shed Sdc4 in synovial fluid increased by 59 % compared to female KL 1-2 patients. Between median levels of KL 1-2 and KL 4 in the female group, a difference of 124 % was detected (Fig. 3.3D). Since women and men showed comparable total levels of shed Sdc4 in synovial fluid and presented both an increase of shed Sdc4 in relation to OA severity, I disregarded the gender in the following analysis.

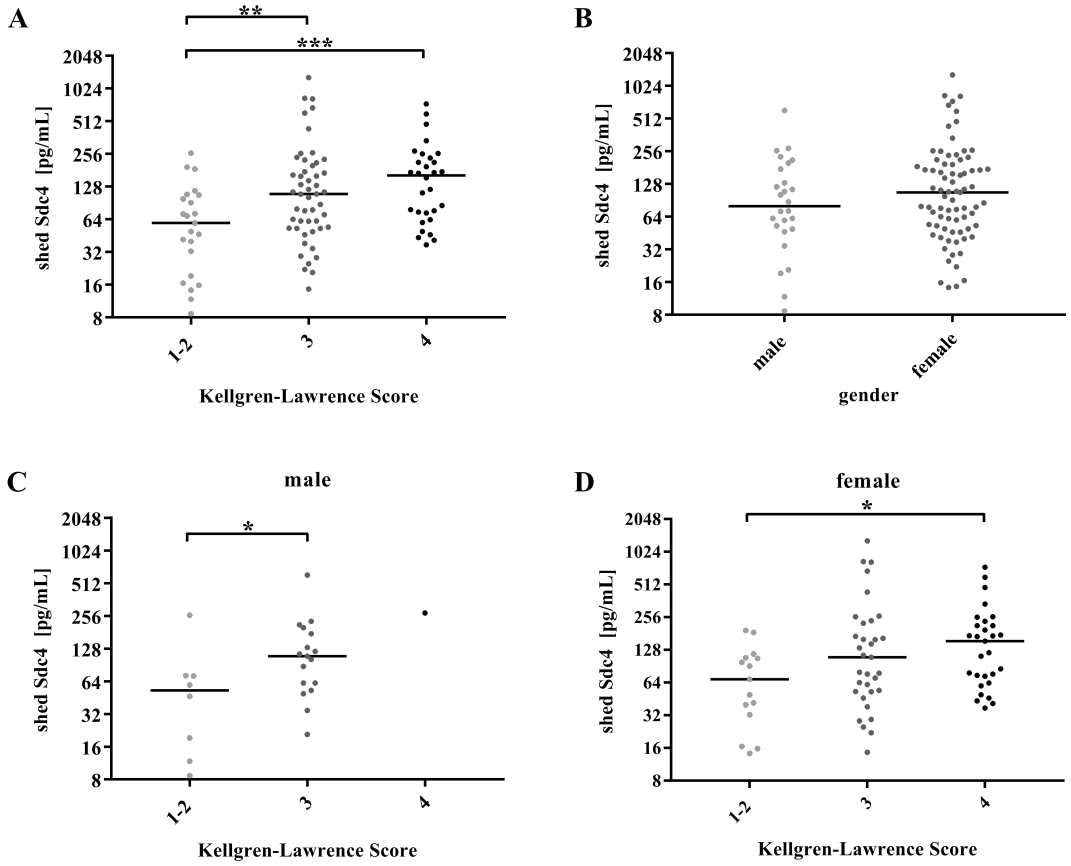

Figure 3.3: *Shed Sdc4 is upregulated depending on the OA degree, but independent of the gender*
Levels of shed Sdc4 in synovial fluids with different Kellgren-Lawrence (KL) scores were analyzed by using an ELISA that recognizes the extracellular part of Sdc4. **(A)** Total levels of shed Sdc4 were upregulated with the KL score (KL 1-2 = 59.25 pg/mL (N = 23), KL 3 = 109.40 pg/mL (N = 50, p = 0.009), KL 4 = 162.20 pg/mL (N = 30, p = 0.001). **(B)** Total shed Sdc4 separated by gender showed no relevant differences (male = 80.04 pg/mL (N = 26), female = 107.30 pg/mL (N = 77); p = 0.3820). **(C)** Male shed Sdc4 levels separated by KL score showed a significant increase (KL 1-2 = 52.89 pg/mL (N = 8), KL 3 = 109.40 pg/mL (N = 17); p = 0.0495). **(D)** Female shed Sdc4 levels separated by KL score showed a significant increase (KL 1-2 = 68.62 pg/mL (N = 15), KL 3 = 109.50 pg/mL (N = 33, p = 0.064), KL 4 = 154.50 pg/mL (N = 29, p = 0.012). Values are presented as median. A Kruskal-Wallis test with following uncorrected Dunn's test was performed for A + D; a Mann-Whitney-U test was performed for B + C; * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.1.3 The level of shed Sdc4 is a possible marker for OA severity in synovial fluid but not in serum

In the next step, the potential of shed Sdc4 in synovial fluid as a biomarker for OA severity was assessed. To investigate whether Sdc4 shedding can distinguish between early and late OA, KL 1-2 was defined as "early OA" and KL 3-4 as "late OA". A significant upregulation of the median shed Sdc4 levels of about 93 % was detected between those two groups (Fig. 3.4A). When the true positive rate (sensitivity) was plotted against the false positive rate (100 % - selectivity) of shed Sdc4, the resulting receiver operating characteristic (ROC) curve presented a suitable threshold of 72.9 pg/mL (indicated by red dotted lines in Fig. 3.4 A+B). The threshold was chosen by maximizing sensitivity and selectivity, resulting a sensitivity of 67.5 % and specificity of 65.2 % (Fig. 3.4B). The area under the curve (AUC) describes the suitability of a biomarker. For shed Sdc4 in synovial fluid the AUC was 0.72. Since synovial fluid is drawn during an arthroscopy, which must be performed by a specialist and poses risks, such as micro-injuries and bacterial infection, it was analyzed whether shed Sdc4 showed OA severity dependent changes in serum samples. No differences between shed Sdc4 levels of healthy individuals and patients with knee OA were detected (Fig. 3.4C). To investigate whether the same correlation of shed Sdc4 with KL score, which was seen in synovial fluid, was also visible in serum, shed Sdc4 levels were plotted against the KL scores. No correlation between median shed Sdc4 levels and KL score was detected (Fig. 3.4D).

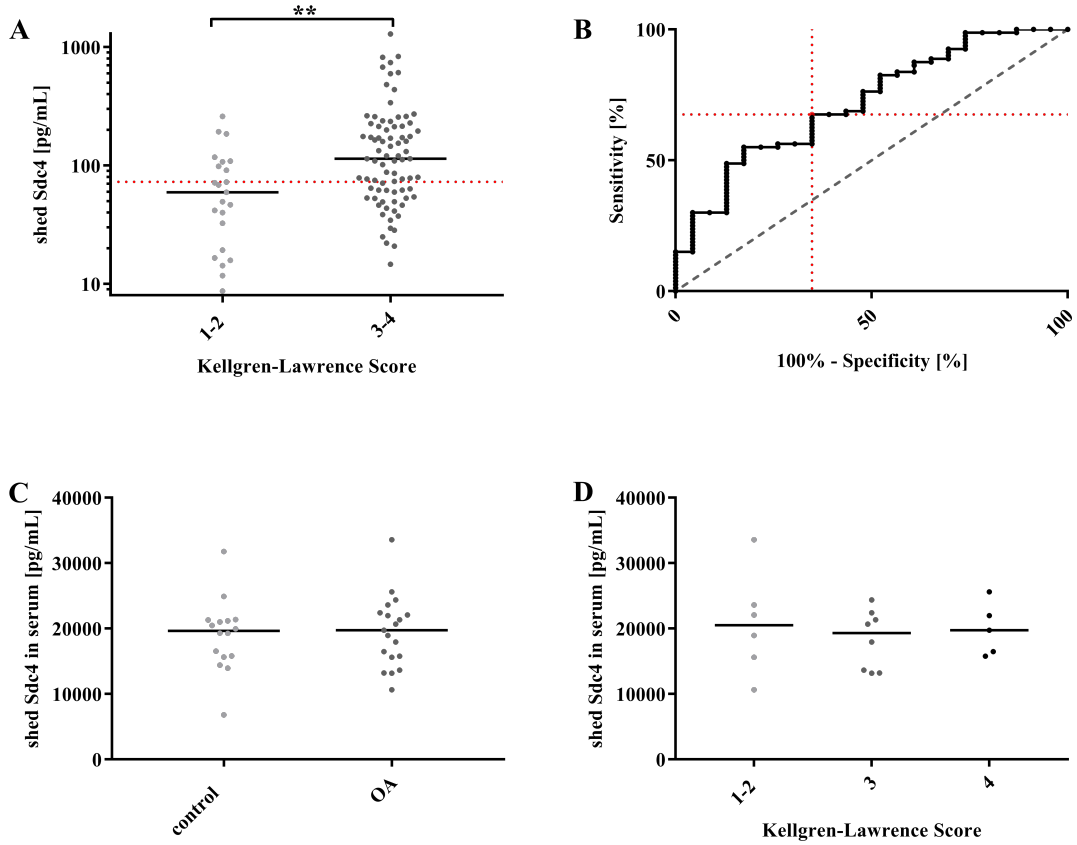

Figure 3.4: *Shed Sdc4 is a marker for distinguishing between early and late OA in synovial fluid, but not serum*

(A) Shed Sdc4 levels as determined in Fig. 3.3A were separated in early (KL 1-2) and late (KL 3-4) OA. Between both groups a significant increase was detected (KL 1-2 = 59.25 pg/mL (N = 23), KL 3-4 = 114.20 pg/mL (N = 80); p = 0.001). The red dotted line indicates the proposed threshold (72.9 pg/mL) to distinguish between early and late OA as calculated with the ROC curve. **(B)** The receiver operating characteristic (ROC) curve blotted the true positive rate (sensitivity) of all possible thresholds (early vs. late OA) against the respective false positive rate (100 % – specificity). The point with the best ratio of sensitivity to specificity was selected. The selected threshold of 72.9 pg/mL had a sensitivity of 67.50 % (95 % CI = 56.11 % to 77.55 %) and a specificity of 65.22 % (95 % CI = 42.73 % to 83.62 %). The area under the curve (AUC) is 0.72 (95 % CI = 0.60 to 0.84). **(C)** Levels of shed Sdc4 were measured in the serum of patients with OA (OA) and healthy individuals (control) by using an Sdc4 ELISA. No relevant differences were seen between the two groups (control = 19635 pg/mL (N = 16), OA = 19188 pg/mL (N = 29); p > 0.99). **(D)** Serum levels of shed Sdc4 did not change with the KL score (KL 1-2 = 20510 pg/mL (N = 6), KL 3 = 19323 pg/mL (N = 8), KL 4 = 19754 pg/mL (N = 5); p = 0.816). Values of A, C, and D are presented as median. A Kruskal-Wallis test was performed for D; a Mann-Whitney-U test was performed for A + C; ** = p≤0.01. Serum samples of the healthy control group were collected and measured for shed Sdc4 in context of another ongoing study by Karsten Pinno and were kindly provided for illustrative and statistical purposes.

3.2 MMP-9 - a possible sheddase for Sdc4 in OA

As mentioned earlier, OA is a disease of the whole joint. Therefore, I examined cartilage and synovial membrane as tissues that are likely to contribute to the amount of shed Sdc4 found in the synovial fluid. RNA was isolated from both tissues and gene expression was analyzed by real time RT-PCR. The expression levels of Sdc4 demonstrated no changes among the KL scores in either cartilage or synovial membrane (Fig. 3.5A+B). This data suggests that elevated amounts of shed Sdc4 in synovial fluid might not originated from increased Sdc4 expression but an increased shedding rate of Sdc4. In cartilage Sdc4 was consistently expressed in comparison to the synovial membrane, where some samples only showed minimal expression of Sdc4 (Fig. 3.5A+B).

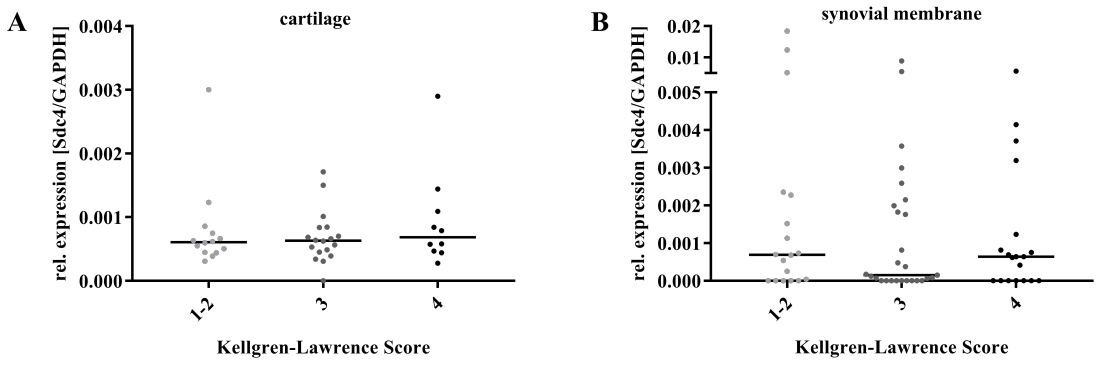

Figure 3.5: *No differences in Sdc4 mRNA expression level*

RNA was isolated from cartilage taken from the most loaded part of the tibial plateau or synovial membrane from patients with different KL scores (KL 1-2, KL 3, and KL 4). **(A)** Analysis of Sdc4 mRNA revealed no changes in cartilage (KL 1-2 = 0.0006 (N = 14), KL 3 = 0.0006 (N = 18), KL 4 = 0.0007 (N = 10); p = 0.8159), **(B)** as well as no relevant changes in synovial membrane (KL 1-2 = 0.0007 (N = 18), KL 3 = 0.00001 (N = 27), KL 4 = 0.0006 (N = 19); p = 0.7414). Values are presented as median. The Kruskal-Wallis test was performed for all figures.

3.2.1 MMP-9, but not MMP-2, increased in synovial fluid

As the shedding rate seemed to be regulated, I looked at potential MMPs that might be responsible for the increase in Sdc4 shedding. The work by Manon-Jensen et al. reported shedding sites for MMP-2, -9, and -14 within the Sdc4 ectodomain [130]. In contrast to MMP-2 and -9, MMP-14 is a membrane-bound MMP. Therefore, I focused on MMP-2 and -9, which are detectable in the synovial fluid. The total content of MMP-2 and -9 in the synovial fluids of knee OA patients were analyzed by using specific ELISAs. The overall median amount of total MMP-2 in synovial fluid was about 500x higher than the amount of total MMP-9 (Fig. 3.6A+B). Levels of total MMP-9 correlated with the KL score (Fig. 3.6A). MMP-9 synovial fluid levels were increased by 15 % between KL 1-2 and KL 3, and 68 % between KL 1-2 and KL 4. The levels of MMP-2 showed no relevant changes with the increasing KL-Score (Fig. 3.6B). In order to investigate whether MMP-2 and MMP-9 are activated in OA synovial fluid a gelatin zymography was performed. This assay is based on the ability of MMP-2 and MMP-9 to digest gelatin. Due to the SDS in the zymography gel also the pro-forms of MMP-2 and MMP-9 were able to digest gelatin. The differentiation between the pro and active forms of MMPs was therefore made by size discrimination. The zymography proved the presence of pro- and activated MMP-9 as well as pro- and activated MMP-2 in the synovial fluid of OA patients (Fig. 3.6C). As levels of shed Sdc4 and MMP-9 in synovial fluid were elevated with increasing OA severity, I assumed that MMP-9 might be a potential sheddase for Sdc4 during OA pathogenesis. Thus the amount of shed Sdc4 was correlated with the levels of total MMP-9 in synovial fluid. Indeed, a significant positive correlation was apparent (Fig. 3.6D). I also correlated MMP-2 levels with shed Sdc4 levels, but did not observe any significant correlation between both proteins (Fig. 3.6E).

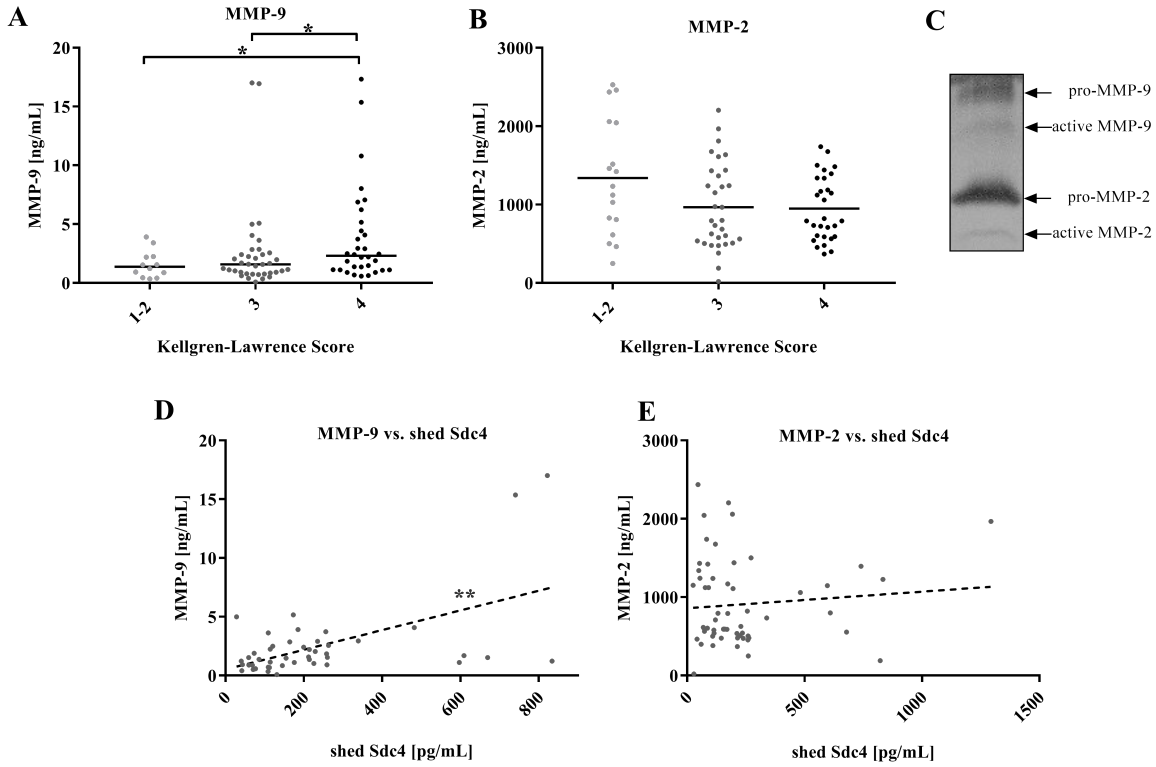

Figure 3.6: *MMP-9, but not MMP-2, is increased in synovial fluid*

Levels of total MMP-2 and MMP-9 in synovial fluids from patients with different Kellgren-Lawrence (KL) scores were analyzed by using an ELISA. **(A)** The amount of total MMP-9 was increased with increasing KL score (KL 1-2 = 1.37 ng/mL (N = 12), KL 3 = 1.58 ng/mL (N = 37), KL 4 = 2.31 ng/mL (N = 32); p(KL 1-2 vs. 4) = 0.040, p(KL 3 vs. 4) = 0.040). **(B)** The amount of total MMP-2 was decreased with increasing KL score (KL 1-2 = 1235.0 ng/mL (N = 17), KL 3 = 796.1 ng/mL (N = 32), KL 4 = 791.3 ng/mL (N = 29); p = 0.1776). **(C)** A gelatin zymography of synovial fluid showed the presence of pro- and active MMP-2 and -9 **(D)** In order to test to relation between increased MMP-9 and increased shed Sdc4 the respective values were plotted against each other. Both groups positively correlate with a Spearman r of 0.437 (95 % CI = 0.155 to 0.652; p = 0.0027; N = 45). **(E)** The same was carried out for MMP-2; shed Sdc4 and MMP-2 did not show any correlation (Spearman r = -0.090; 95 % CI = -0.346 to 0.177; p = 0.500; N = 59). A Kruskal-Wallis test with following uncorrected Dunn's test was performed for A + B; * = $p \leq 0.05$.

Next, the usefulness of MMP-9 levels to discriminate between early (KL 1-2) and late OA (KL 3-4) was analyzed. The median MMP-9 level in late OA was increased by 34 % compared to the median level of MMP-9 in early OA. However, this difference was not statistically significant (Fig. 3.7A). In line with the analysis of shed Sdc4, I performed an ROC curve analysis for MMP-9 synovial fluid values. The AUC of MMP-9 presented a value of 0.62 (Fig. 3.7B), which was considerably lower than of shed Sdc4 (see Fig. 3.4B).

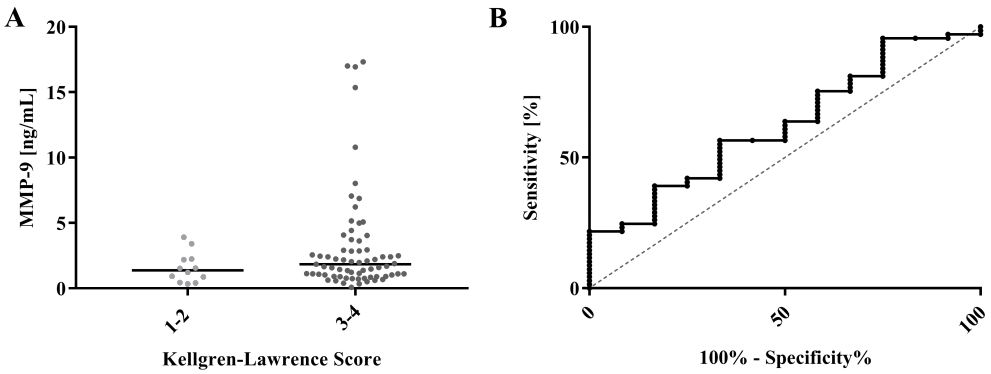

Figure 3.7: *Levels of total MMP-9 cannot discriminate early from late OA*

The potential of MMP-9 levels as determined in Fig. 3.6A to distinguish between early (KL 1-2) and late (KL 3-4) OA was tested. **(A)** When regrouping all measurements into this two groups only a slight upregulation was seen (KL 1-2 = 1.374 ng/mL (N = 12), KL 3-4 = 1.846 ng/mL (N = 69); p = 0.175). **(B)** The resulting ROC curve had an AUC of 0.62. Values are presented as median. Mann-Whitney-U test was performed for A.

3.2.2 Increased MMP-9 mRNA expression in OA synovial membrane

After showing that MMP-9 was increased in the synovial fluid of OA patients with an increasing KL score, the mRNA expression in the surrounding tissues, synovial membrane and cartilage, was examined. MMP-9 mRNA levels in cartilage showed a KL score dependent upregulation, which, however, did not reach statistical significance (Fig. 3.8A). In synovial membrane the median value for KL 4 OA patients was 150 % higher than the median value for KL 1-2 (Fig. 3.8B).

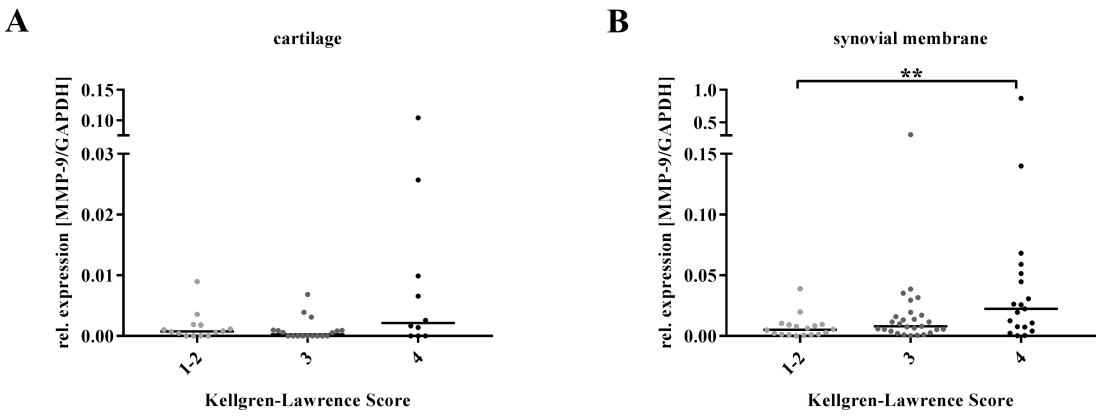

Figure 3.8: *Increased MMP-9 mRNA expression in synovial membrane*

RNA was isolated from cartilage taken from the most loaded part of the tibial plateau or synovial membrane from patients with different KL scores (KL 1-2, KL 3, and KL 4). **(A)** Analysis of MMP-9 mRNA revealed no significant changes (KL 1-2 = 0.0008 (N = 14), KL 3 = 0.0003 (N = 18), KL 4 = 0.002 (N = 10); p = 0.103). **(B)** In synovial membrane mRNA was significantly upregulated (KL 1-2 = 0.0052 (N = 18), KL 3 = 0.0079 (N = 27), KL 4 = 0.0223 (N = 19, p = 0.008)). Values are presented as median. A Kruskal-Wallis test with following uncorrected Dunn's test was performed for all figures, ** = $p \leq 0.01$.

Similar to the analysis of mRNA expression levels of MMP-9, I also examined MMP-2 mRNA expression. MMP-2 mRNA levels in cartilage showed an upregulation between KL 1-2 and KL 4 of 590 %. However, this increase in expression was not statistically significant (Fig. 3.9A). The MMP-2 mRNA analysis of the synovial membrane showed no relevant changes (Fig. 3.9B).

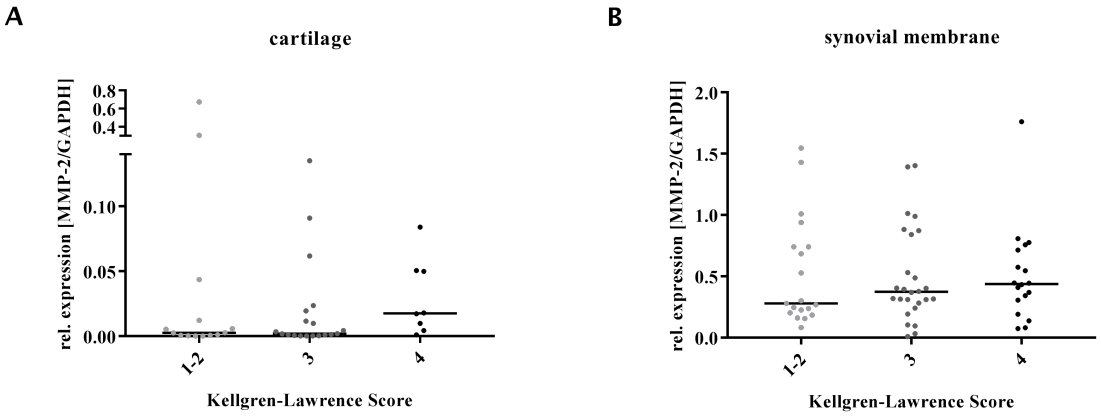

Figure 3.9: *MMP-2 expression on mRNA level did not change in cartilage nor synovial membrane* RNA was isolated from cartilage taken from the most loaded part of the tibial plateau or synovial membrane from patients with different KL scores (KL 1-2, KL 3, and KL 4). **(A)** Analysis of MMP-2 mRNA revealed no significant changes in the gene expression level in cartilage (KL 1-2 = 0.0026 (N = 14), KL 3 = 0.0019 (N = 19), KL 4 = 0.018 (N = 18); p = 0.195). **(B)** as well as no changes in synovial membrane (KL 1-2 = 0.2789 (N = 19), KL 3 = 0.3734 (N = 26), KL 4 = 0.438 (N = 18); p = 0.852). Values are presented as median. A Kruskal-Wallis test was performed for all figure.

3.3 MMP-9-mediated Sdc4 shedding regulates IL-1 signaling

Even though OA is not considered a high inflammatory disease like rheumatoid arthritis, inflammation is a reported factor in OA. The pro-inflammatory cytokine IL-1 β has been intensively studied in the context of OA and was reported to be involved in OA pathophysiology. So far, this study showed that Sdc4 shedding was upregulated during OA, and most likely, MMP-9 is a sheddase involved in this process. In the final part, MMP-9 involvement in Sdc4 shedding was verified and the function of Sdc4 shedding in OA, especially in IL-1 signaling, was examined.

3.3.1 Inhibitor for MMP-9, but not MMP-2, reduces Sdc4 shedding

I further investigated the role of MMP-2 and MMP-9 in Sdc4 shedding by suppressing their enzymatic function with specific inhibitors. The chondrocyte cell line C28 was treated with different concentrations of the inhibitors. As readout for their inhibitory capacity, the amount of shed Sdc4 in the culture medium after 5 h was compared to the control. The tested concentrations were chosen based on the manufacturers' datasheets and previous studies [176]. The MMP-9 inhibitor showed a significant 35 % decrease of basal Sdc4 shedding at the highest concentration of 5 μM (Fig. 3.10A). There was no influence of the MMP-2 inhibitor on the basal Sdc4 shedding (Fig. 3.10B). The chemical compound 4-Aminophenylmercuric acetate (APMA), an organomercurial activator of MMPs, was used to induce Sdc4 shedding. The treatment of C28 chondrocytes with APMA significantly increased the shedding of Sdc4 by 70 %. The addition of the MMP-9 inhibitor (5 μM) attenuated this effect. The MMP-2 inhibitor, on the contrary, did not affect APMA-induced Sdc4 shedding (Fig. 3.10C). Another way to induce Sdc4 shedding was the stimulation with the pro-inflammatory cytokine IL-1β. Figure 3.10D shows that treatment of C28 chondrocytes with IL-1β upregulated Sdc4 shedding in a dose dependent manner. Shed Sdc4 was increased in the culture medium of C28 chondrocytes by 32 % for 0.1 ng/ml IL-1β, by 82 % for 1 ng/mL, and by 112 % for 10 ng/mL. Interestingly, between 0.1 and 1 ng/mL the difference in Sdc4 shedding induction is higher (0.1 vs. 1 ng/mL = 51 %) than between the other concentrations (w/o vs. 0.1 ng/mL = 31.8 %; 1 vs. 10 ng/mL = 28.2 %). Incubating the cells with IL-1β and MMP-9 inhibitor decreased Sdc4 shedding by 47 % (0.1 ng/mL), 69 % (1 ng/mL), and 72 % (10 ng/mL) compared to its respective control. Further, the increased induction of Sdc4 shedding between 0.1 and 1 ng/mL IL-1β was gone. Between all

concentrations a gradual increase of around 29 % was apparent (w/o vs. 0.1 ng/mL = 33.1; 0.1 vs 1 ng/mL = 27.4, 1 vs. 10 ng/mL = 28.2 %), indicating an IL-1β depending role of MMP-9. Adding the MMP-2 inhibitor had no influence on basal or IL-1β-induced Sdc4 shedding (Fig. 3.10D).

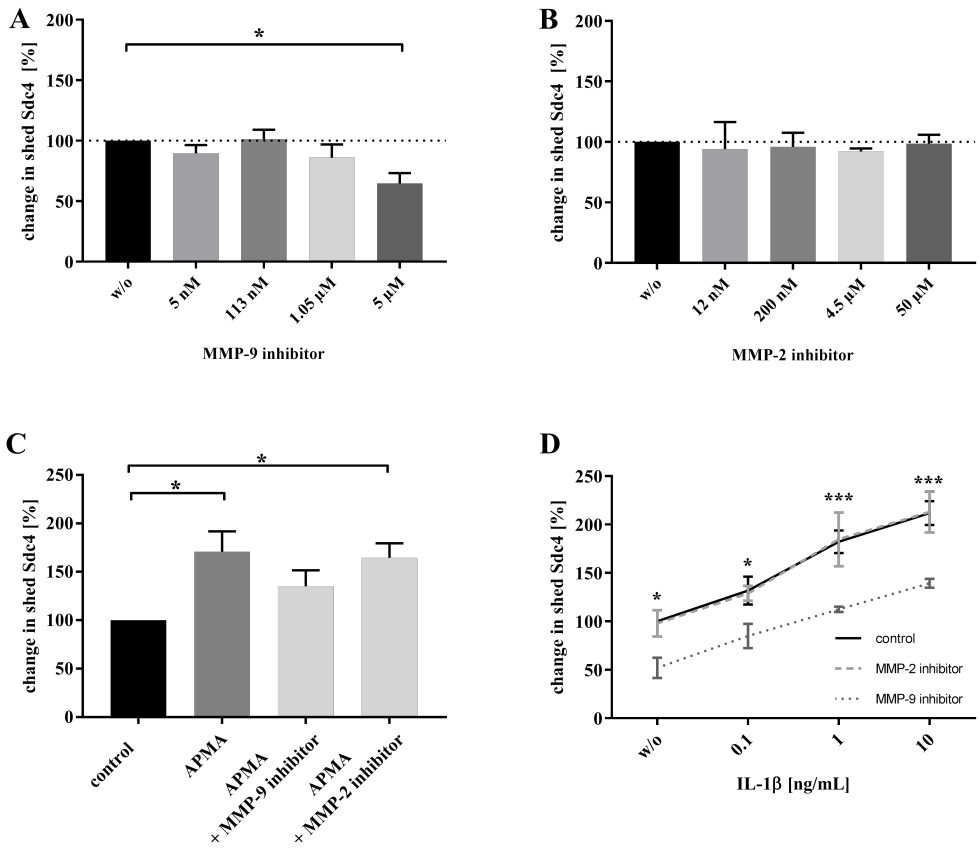

Figure 3.10: *MMP-9 inhibitor reduced APMA and IL-1β induced Sdc4 shedding*

C28 chondrocytes were treated with different concentrations of MMP-2 or -9 inhibitor for 5 h. The changes in shed Sdc4 levels are presented in percentage compared to the untreated control. **(A)** The MMP-9 inhibitor decreased the shedding of Sdc4 at a concentration of 5 μM (w/o = 100 %, 5 μM = 64.6± 8.71 %; N = 3; p = 0.038) **(B)** Even the highest concentration of 50 μM of MMP-2 inhibitor had no effect on Sdc4 shedding (w/o = 100 %, 50 μM = 64.6± 8.71 %; N = 3; p = 0.988). **(C)** Upon stimulation with APMA (5 μM, 5 h) shedding was increased (control = 100 %, APMA = 170.6± 21.03 %; N = 3; p = 0.022), 5 μM MMP-9 inhibitor attenuated the effect (control = 100 %, APMA + MMP-9 inhibitor = 126.1± 16.57 %; N = 3; p = 0.309), while 50 μM of MMP-2 inhibitor did not change Sdc4 shedding increase (control = 100 %, APMA + MMP-2 inhibitor = 164.4±.7 %; N = 3; p = 0.036) **(D)** C28 cells were treated with different concentrations of IL-1β and either MMP-2 (50 μM) or MMP-9 (5 μM) inhibitor for 5 h. The MMP-9 inhibitor decreased Sdc4 shedding at all IL-1β concentrations compared to the control, while the MMP-2 inhibitor again had no effect (w/o : (control = 100 %, MMP-9 inhibitor = 51.89± 10.51 % (p = 0.014), MMP-2 inhibitor = 98.1± 13.67 %), 0.1 ng/mL : (control = 131.8 ± 14.43 %, MMP-9 inhibitor = 84.98± 12.50 % (p = 0.017), MMP-2 inhibitor = 128.8± 7.74 %), 1 ng/mL : (control = 182.8 ± 11.77 %, MMP-9 inhibitor = 112.40± 2.81 % (p = 0.0006), MMP-2 inhibitor = 184.6± 27.87 %), 10 ng/mL : ((control = 211.9 ± 12.39 %, MMP-9 inhibitor = 139.3± 4.73 % (p = 0.0004), MMP-2 inhibitor = 212.7± 21.3 %)). Values are presented as mean ± SEM. Ordinary One-way ANOVAs, followed by Sidak's multiple comparisons test were performed for A - C; a two-way ANOVA with Sidak's multiple comparison was performed for D; * = p ≤ 0.05, *** = p≤0.001.

3.3.2 MMP-9 knockdown by siRNA decreases IL-1β induced Sdc4 shedding

The specificity of MMP inhibitors is repeatedly discussed. Therefore MMP-9 was knocked down to verify its role in Sdc4 shedding. Primary chondrocytes were treated for 24 h with siMMP-9 or a control siRNA. Afterwards the cells were incubated with different concentrations of IL-1β for 5 h. The levels of shed Sdc4 in the culture medium were analyzed by the Sdc4 ELISA. The efficiency of the knockdown was confirmed by semiquantitative RT-PCR (Fig. 3.11A). Figure 3.10A shows that cells treated with siMMP-9 expressed 66 % less MMP-9 mRNA than cells treated with the control siRNA. GAPDH served as loading control. The measurements of shed Sdc4 in the culture medium that were treated with either siMMP-9 or a control and different concentrations of IL-1β to induce Sdc4 shedding confirmed the results obtained by the inhibitor previous experiments. The inhibition of Sdc4 shedding by MMP-9 inhibition was again more prominent with a higher concentration of IL-1β. Cells pre-treated with siMMP-9 and stimulated with 0.1 ng/mL IL-1β shed 22.5 % less Sdc4 compared to the respective control. For stimulation with 1 ng/mL, shedding of Sdc4 was reduced by 39.4 %, and for 10 ng/mL 41.4 % less shed Sdc4 was detectable (Fig. 3.11B).

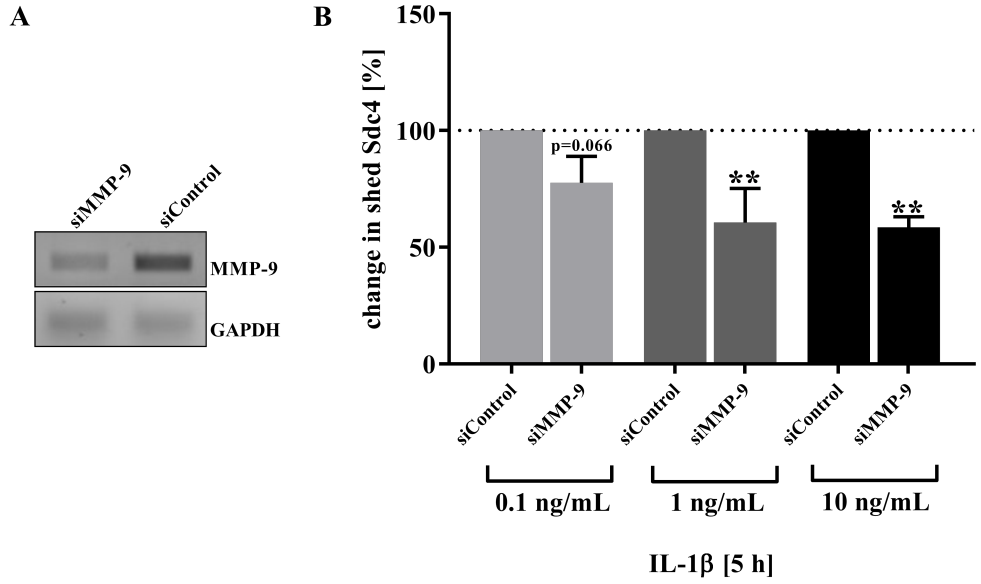

Figure 3.11: *MMP-9 knockdown with siRNA decreases Sdc4 shedding*

Primary chondrocytes were treated for 24 h with siMMP-9 or a control siRNA. Afterwards cells were stimulated for 5 h with different concentrations of IL-1β. **(A)** Semi-quantitative PCR analysis showed a MMP-9 knockdown of 66 %. GAPDH was run to ensure similar cDNA levels. **(B)** Changes in Sdc4 shedding per IL-1β concentration (0.1, 1, and 10 ng/mL) were normed to the respective control, which as set to 100 %. The percentage of shed Sdc4 in comparison to its control decreased with increasing IL-1β levels (0.1 ng/mL: (siControl = 100 %, siMMP-9 = 77.52 ± 11.57 % ($p = 0.066$), 1 ng/mL: (siControl = 100 %, siMMP-9 = 60.60 ± 8.39 % ($p = 0.002$), 10 ng/mL: (siControl = 100 %, siMMP-9 = 58.61 ± 4.42 % ($p = 0.001$). Values are presented as mean ± SEM. Ordinary One-way ANOVA, followed by Sidak's multiple comparison was performed for B; ** = $p \leq 0.01$.

3.3.3 Decreased IL-1 signaling through Sdc4 ectodomain shedding

As mentioned before, APMA was used to activate MMPs. To ensure that APMA indeed activated MMP-2 and MMP-9, I performed a gelatin zymography. Pro-MMP-2, as well as pro-MMP-9, was present in the culture medium (Fig. 3.12A). Similar to the MMP levels in synovial fluid (see Fig. 3.6A+B), more MMP-2 was present than MMP-9. However, more MMP-9 was active on a basal level. Upon stimulation with APMA, MMP-2 and -9 got activated (Fig. 3.12A). The amount of activated MMP-2 and -9 upon APMA stimulation was similar in all samples, showing that the inhibitors did not interfere with the proteolysis of the pro-MMPs. The absence, as well as blocking Sdc4 with antibodies, was reported to result in decreased IL-1 signaling [86, 111] Thus, I investigated whether an increased Sdc4 shedding could have a similar effect on IL-1 signaling. Therefore, chondrocytes were treated with APMA to induce Sdc4 shedding. The amount of IL-β induced ERK phosphorylation (p-ERK) was determined using western blot analysis. Without treatment, the stimulation with IL-1β showed an increase of 100 % (15 min) to 110 % (30 min) in p-ERK/ERK. The pre-treatment with APMA reduced the time dependent phosphorylation of ERK. The most visible difference was at 30 min with 75 % reduction in p-ERK in the APMA pretreated cells compared to the respective control (Fig. 3.11B). Shed syndecans were also proposed to work as decoy receptors. To evaluate the function of shed Sdc4 in IL-1 signaling, I pre-treated C28 chondrocytes with 1 μg/mL recombinant Sdc4 ectodomain or PBS as a control and subsequently stimulated the cells with 10 ng/mL IL-1β for different time points (Fig. 3.11C). Again, an IL-1β induced increase of p-ERK using western blot analysis was visible by 117.5 % (15 min) and 97.8 % (30 min). Interestingly, the presence of the Sdc4 ectodomain in the culture medium had no effect on the levels of p-ERK at 15min (101.5 %) or 30 min (92.8 %).

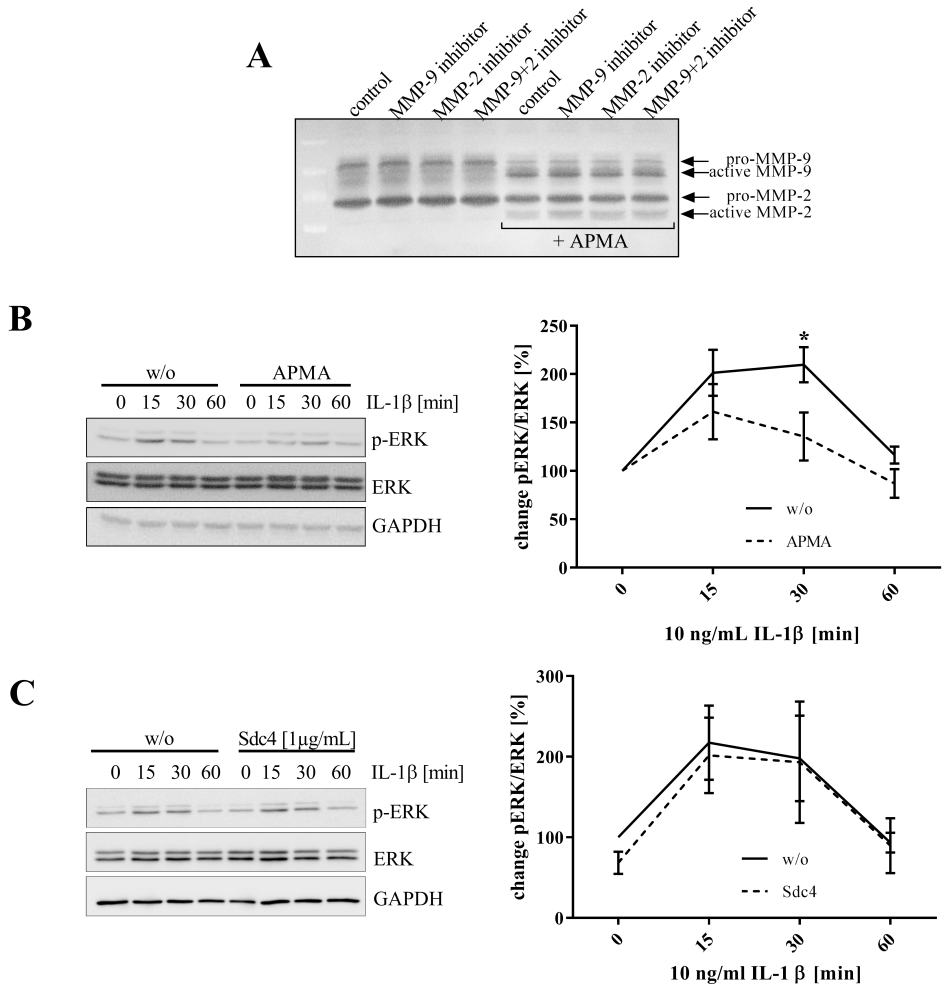

Figure 3.12: *Decreased IL-1 signaling through Sdc4 deficiency by increased shedding*

(A) Cell culture medium from C28 chondrocytes treated with APMA (1 mM) or water as control for 5 h was run on a gelatin SDS gel. Additionally, inhibitors for MMP-2 (50 μM) and MMP-9 (5 μM) or the combination of both was added. APMA increased the amount of active MMP-2 and -9 after treatment independent from MMP-inhibitors.**(B)** C28 chondrocytes were treated with APMA or water as control (w/o). Afterwards cells were stimulated with 10 ng/mL IL-1β for 0, 15, 30, and 60 min. IL-1β signaling was analysed by the phosphorylation of ERR (p-ERK) to total amount of ERK (ERK). The left panel shows a representative western blot. The right panel shows the respective quantitative analysis (N = 3). At 30 min a significant decrease of p-ERK was visible (w/o = 209.60 ±18.15 %, APMA = 135.60 ± 24.88 %, p = 0.013). **(C)** C28 chondrocytes were treated with Sdc4 (1 μg/mL) or PBS as control (w/o). Afterwards cells were stimulated with 10 ng/mL IL-1β for 0, 15, 30, and 60 min. IL-1β signaling was analysed by the phosphorylation of ERR (p-ERK) to total amount of ERK (ERK). The left panel shows a representative western blot. The right panel shows the respective quantitative analysis (N = 4). Soluble Sdc4 had no effect on IL-1 signaling.

4 Discussion

A role of the heparan sulfate proteoglycan Sdc4 has been well established in the pathophysiology of OA. It was shown to be expressed under inflammatory conditions and in hypertrophic chondrocytes [86, 116]. Further, the deficiency, as well as blocking Sdc4 using a polyclonal-antibody, decreased OA-like cartilage changes in a murine surgically induced OA model. The ectodomain of Sdc4 can be cleaved off, resulting in a soluble form of Sdc4. This process is called shedding. Shedding of Sdc4 is an important process that is involved in several diseases [91]. However, the shedding of Sdc4 in OA has hardly been looked at. Therefore, the main goal of this thesis was to investigate the shedding of Sdc4 in the pathogenesis of OA.

4.1 There is no gender specific difference in Sdc4 shedding in OA

In the first part of the study, the presence and levels of shed Sdc4 in the synovial fluid of knee OA patients was investigated. The results indicated that Sdc4 shedding indeed occurred in the knee joint, and the shed ectodomain of Sdc4 was detectable in the synovial fluid (Fig. 3.2). So far, no studies have been conducted that analyzed the amount of shed Sdc4 in the synovial fluid of OA patients or synovial fluid. However, the presence of shed Sdc4 in synovial fluid was expected as both articular chondrocytes [115], and synovial fibroblasts [111] express Sdc4, and the shedding of Sdc4 happens constitutively [177].

Western blot analysis proved that only the extracellular domain of Sdc4 was present in the synovial fluid in OA patients, thereby excluding contamination of cells that express Sdc4 leading to false-positive results (Fig. 3.2A). Using an ELISA against the extracellular domain of Sdc4 revealed that shed Sdc4 was increased with increasing OA severity (Fig. 3.3A). OA severity was assessed by using the radiographic KL score (Fig. 1.1+3.1).

Gender is one of the risk factors in the development of OA, with women having a higher risk of

developing knee OA than men [178]. Further, Sdc4 shedding was gender-specific regulated in patients with myocardial infarction [179]. In that study, women had a stronger association of serum Sdc4 levels and incident myocardial infarction than men. Hence, in the beginning, I checked whether shed Sdc4 level in knee OA patients' synovial fluid showed gender-specific differences (Fig. 3.3B). Only 25 percent of the included patients were men, corroborating the fact that knee OA is more prevalent in women than in men [180]. The samples analyzed in the study were collected consecutively without regarding the sex of the donor. In comparison to other publications the percentage of affected men is relatively low in my cohort [181]. Nevertheless, taking into account the gender of the donors, the overall amounts of shed Sdc4 showed very similar distributions in both populations (Fig. 3.3B). The distribution of shed Sdc4 levels in the female cohort showed a slightly higher median value compared to the male samples. However, this gender specific difference is not significant. This difference might be due to the limited amount of male samples in the KL 4 group. Unfortunately, the male KL 4 subgroup only had one patient (Fig. 3.3C). The reason for the low number of male patients in the KL4 group might be explained by the review by Hame and Alexander indicating that even though women have a higher prevalence of developing OA, they underutilize total knee arthroplasty compared to men [182]. Further, the study showed that women have a lower functional and higher disease score at the timepoint of surgery. The authors explained those differences between the two genders by an unconscious bias of physicians to recommend knee arthroplasty more frequently to men than to women. They presented that the chances of a male patient to get a recommendation for total knee arthroplasty by an orthopedic surgeon are 22 times higher than for a woman [182, 183]. The data presented in this thesis might reflect this discrepancy. Comparing the levels of shed Sdc4 regarding the KL score of the male and female subgroups, both groups showed a similar upregulation within increasing KL score (Fig. 3C+D), therefore the gender was disregarded in further analyses.

4.2 Assessing the state of OA progression by levels of shed Sdc4

Shed Sdc4 has previously been proposed to be a possible biomarker for other diseases [91], such as different forms of pneumonia [135, 137, 184] or for heart failure in patients with hypertension [138, 139]. Further, the synovial fluid of OA patients was already a frequent object of investigation in the quest for new biomarkers [78]. Therefore, the hypothesis was put

forward that shed Sdc4 in synovial fluid might be a suitable marker to assess the state of OA progression in patients with OA symptoms.

When assessing the suitability of shed Sdc4 in the synovial fluid to mirror cartilage changes of OA patients, the ability to distinguish between early (KL 1-2) and late (KL 3-4) OA was evaluated (Fig. 3.4A+B). The late OA group showed a significant increase in shed Sdc4 (Fig. 3.4A). The ROC curve plots the sensitivity of every possible cut off of the two groups against the respective inverted specificity. The AUC thereby serves as the performance analysis of the marker. In general, the higher the value under the curve, the better the marker. Shed Sdc4 in synovial fluid had an AUC of 0.72 (Fig. 3.4B). Several biomarkers were already proposed for the diagnosis of OA. The cleavage products of Coll2 and aggrecan were investigated as they mirror cartilage degradation. The collagen type II C-telopeptide (CTX-II) fragment presented as a suitable biomarker in urine and serum. In urine, CTX-II (uCTX-II) had an AUC of 0.82 when comparing healthy individuals with OA knee diagnosed patients [185]. In the terms of OA progression CTX-II was less suitable as a biomarker. Again, in urine, CTX-II presented an AUC of 0.63 in women when comparing its levels in patients with KL 0-1 to those with KL 2-5 [186]. CTX-II levels in serum of in patients with diagnosed OA compared to an age-matched healthy control group exhibited an AUC of 0.89. However, in this study they did not differentiate between the joint affected by OA [187]. Jung et al. saw differences in the usefulness of CTX-II as a biomarker for OA between hip OA and knee OA. The AUC of uCTX-II for hip OA patients was higher than for knee OA (0.92 vs. 0.82, respectively). In the synovial fluid of OA knee joints the collagenase-generated neoepitope of Coll2 failed to detect injury-mediated OA onset [188]. The same study also investigated aggrecan-based biomarkers. In synovial fluid of knee OA patients, decreased levels of the glycosaminoglycan keratan sulfate side chain (KS) were detectable (AUC = 0.67) [188] compared to the control. Another approach was to measure circular RNA (circRNA) in the synovial fluid of OA patients compared to healthy controls. The cirRNA hascirc0102061 showed the most promising results with an AUC of 0.75 [189]. Hence, in synovial fluid, shed Sdc4 (AUC = 0.72) showed a similar performance compared to previously described potential biomarkers. In order to enhance the performance of shed Sdc4, it could be combined with other possible markers and thereby increase specificity and sensitivity.

However, the need of an arthroscopy to draw synovial fluid poses the risks of a microbial infection or microdamage and has to be performed by a specialist. Thus, a blood-based biomarker would be beneficial. Healthy serum samples, as well as serum samples from OA

patients, were analyzed for differences in their shed Sdc4 levels. No differences between healthy and OA were detectable (Fig. 3.4C). Also, among the different KL scores, no differences in serum shed Sdc4 were measurable (Fig. 3.4D). The levels of Sdc4 in the serum have been reported to be influenced by several processes in the body, such as physical exercise and glycogen levels [190] or different types of pneumonia [135, 136], as well as others, summarized in the review by Bertrand and Bollmann [91]. The mean values of healthy individuals were reported to be around 15-16 ng/mL, which were increased upon physical exercise (up to 30 ng/mL in some individuals) and in patients with pneumonia (20.3 and 25.2 ng/mL) [135, 184, 190]. In this study Sdc4 levels in serum were around 19-20 ng/mL, being a bit higher than the described baseline values of 15-16 ng/ml. However, the levels were still below the proposed pneumonia cutoff of 20.3 - 25.2. Therefore, OA-dependent changes of serum shed Sdc4 levels might either be masked by other tissue-specific Sdc4 shedding processes. Or it is also possible that OA dependent Sdc4 shedding is a local process, restricted to the affected joint and is not detectable systemically in the serum.

4.3 Increased Sdc4 shedding by MMP-9, derived from the synovial membrane

In my study I saw no increase of Sdc4 mRNA expression in cartilage or synovial membrane, indicating that the shedding rate is increased instead of the overall expression (Fig. 3.5). A recent publication by Sanchez et al. showed that, compared to non-hypertrophic chondrocytes, hypertrophy in chondrocytes leads to an increased expression of Sdc4, whereas the shedding rate was not increased by hypertrophy [116]. However, by looking at the synovial fluid, I assessed the contribution of all synovial tissues to Sdc4 shedding in the joint. Therefore, sheddases or Sdc4 from other tissues, such as the synovial membrane, might have contributed to the increased Sdc4 shedding in synovial fluid. Furthermore, Sanchez et al. only compared non-hypertrophic chondrocytes with hypertrophic chondrocytes from the same donors and the same KL scores [116], while I compared the Sdc4 expression among different KL scores to map the course of Sdc4 expression during OA progression. Therefore, the two studies do not necessarily contradict each other.

Another interesting fact that was visible during this study was that Sdc4 seemed to be more constitutively expressed in cartilage than in the synovial membrane (Fig. 3.5). Sdc4 was not detectable in several samples in the synovial membrane, while all samples of cartilage showed Sdc4 expression. Sanchez et al. also saw a higher variation of Sdc4 in inflamed synoviocytes, but the expression levels were, in accordance with this study, not significantly different from normal-reactive synoviocytes [116]. Other studies showed that inflammation increased Sdc4 in synovial fibroblast, [111, 121] indicating a more prominent role for Sdc4 expression in cartilage than was previously described, though contradictory results were reported. Some studies showed that Sdc4 was increased in OA [86, 115, 116], while others claimed reduced Sdc4 in OA cartilage [191].

In order to unravel the underlying mechanisms of Sdc4 shedding in OA, I looked at possible sheddases for Sdc4. The study by Manon-Jensen reported MMP-2, MMP-9, and MMP-14 as possible MMPs, mediating Sdc4 shedding. I decided to focus on MMP-2 and MMP-9, which both comprise the subfamily of gelatinases, as MMP-14 is a membrane-bound MMP [192], which makes it difficult to detect in synovial fluid. When looking at the levels of total MMP-2 and -9 in synovial fluid of OA, it was apparent that MMP-2 levels were around 500 times higher than total levels of MMP-9 (Fig. 3.6A+B). Moreover, the levels of MMP-9 were regulated in

the context of OA severity (Fig. 3.6A), while no relevant changes were seen in the levels of MMP-2 (Fig. 3.6B). These results might indicate that MMP-2 is needed for the healthy cartilage turnover, while MMP-9 is only upregulated under pathological conditions. Moreover, a correlation between the levels of shed Sdc4 and MMP-9 was found (Fig. 3.6 D), which indicates that MMP-9 might be partly responsible for Sdc4 shedding during knee OA. In other cell types and diseases, MMP-9 was previously suggested to be involved in Sdc4 shedding [124, 129, 193], which supports my hypothesis that MMP-9 is a relevant sheddase for Sdc4 in OA. Further, the KL score-dependent increases in MMP-9 mRNA in synovial membrane also supports this connection (Fig. 3.7B). Like Sdc4, the regulation of MMP-9 in OA was contradictorily described in previous publications. Most of these measured levels of total MMP-9 in synovial fluid of OA patients and compared them to different types of arthritis, such as RA [169, 194, 195, 196]. From these studies, it became clear that MMP-9 has much higher expression in inflammatory rheumatoid arthritis, measured with zymography [195, 196] or ELISA [169, 194]. The studies of Tchetverikov et al. and Kim et al. detected MMP-9 in OA synovial fluid, while Makowski et al. and Hsieh et al. did not detect any MMP-9 in synovial fluid. This contradiction is most likely based on the use of zymography, which is a less sensitive detection method, which may not have been able to detect small amounts of MMP-9. Tchetverikov et al. also showed that MMP-9 levels were increased in OA synovial fluid compared to controls obtained from traffic casualties [169]. This was confirmed by the study of Iwaki-Egawa, where OA synovial fluid was compared to synovial fluid aspirated from patients with non-inflammatory, non-hemorrhagic traumatic sport related injuries [172]. However, it has to be considered that joint trauma and injuries also induce inflammation and do not necessarily reflect the healthy synovial fluid composition. Two publications compared synovial fluid MMP-9 levels among different KL scores. A study by Sachdeva et al. showed decreased MMP-9 levels in the synovial fluid of KL 4 OA patients, compared to KL 2 and KL 3, which was contradictory to my findings (Fig. 3.3A)[197]. Another study by Rübenhagen et al. described an increase in MMP-9 in synovial fluid when comparing KL1-2 with KL 3-4, which, however, was not significant [173]. These studies show that there might be a variation in MMP-9 synovial fluid levels of OA patients, depending on the affected joint, the control used, and maybe also the assessment technique. As the MMP-9 protein levels were upregulated in my cohort of knee OA patients with increasing OA severity (Fig. 3.6A), I assessed the ability of MMP-9 to discriminate between early and late OA. The resulting ROC curve gave an AUC of 0.62, which was considerably worse than shed

Sdc4 (AUC = 0.72) (Fig. 3.7B+3.4B). This result is more or less in line with the previous studies on MMP-9 synovial fluid levels, as MMP-9 alone does not seem to be a potent biomarker for the prediction of OA severity.

In contrast to MMP-9 levels in synovial fluid, mRNA levels [165, 198] and protein expression [144, 165] are almost exclusively reported to be upregulated in OA compared to control groups. OA cartilage was compared with non-OA controls (patients with femoral neck fracture or post-mortem) and the regulation between different stages of OA was barely looked at. One publication looked at different OA severities and found the highest amounts of MMP-9 mRNA in early to intermediate OA [199], while the study by Söder et al. compared early to severe OA and saw the highest expression of MMP-9 in severe OA [165]. On the other hand, I observed no differences between mRNA expression in cartilage between patients with KL 1-2 and KL 3 or KL 4 (Fig. 3.8A). However, MMP-9 mRNA expression was increased in synovial membrane. These results show that MMP-9 seems to be disease dependently upregulated in OA joint tissues; however, a variation depending on the exact disease entity (affected joint, subtype of OA or used control) needs to be considered. MMP-2 levels in synovial fluid are, similar to MMP-9, inconsistent. Even though MMP-2 was always present, MMP-2 levels were reported not to change [173], while others saw an upregulation by MMP-2 in OA [169], both measured with an ELISA . The previously mentioned study by Rübenhagen et al., which compared MMP-2 synovial fluid levels with different KL scores, showed similar results to those obtained in this study (Fig. 3.6B). They showed a much higher amount of MMP-2 compared to MMP-9 levels that did not change during OA progression [173]. Again, these data indicate that the use of MMP-2 as marker for OA severity is not the best choice. There seems to be a marked interindividual variance in MMP-2, but also MMP-9 levels, which cannot be explained simply by the grade of radiological OA severity. Recently, a great effort was made in determining subtypes of OA in order to improve personalized treatment for each patient [72, 200].

It was shown that MMP-9 is highly increased in the synovial fluid of RA patients compared to OA patients [194]. The same study showed that MMP-2, for example, is also increased in RA, but considerably less than MMP-9. This might indicate that MMP-9 upregulation, and thereby Sdc4 shedding could be dependent on inflammatory stimuli. Further, Sdc4 is also described to be NF-κB-dependent upregulated upon IL-1β stimulation [112]. Although OA is not a primarily an inflammatory condition, an intensive database search (spanning 6085 patients) by Marty et al. revealed inflammatory flare ups in 52.3 % of knee OA patients [201].

These results might suggest that MMP-9 levels, as well as shed Sdc4, could help to diagnose an inflammatory subtype. Moreover, ongoing work by Sonia Nasi [202] could connect chondrocalcinosis (CC), which is an inflammatory form of cristallopathy, with increased Sdc4 and MMP-9 expression. Further, it was shown that CC is strongly associated with knee OA [203]. This could also be an interesting subtype of OA that might be detectable via levels of shed Sdc4.

4.4 Decreased IL-1 signaling via MMP-9-mediated Sdc4 shedding

In the first two parts, Sdc4 shedding, as well as MMP-9 expression was associated with OA pathology. Therefore, the last part of this study verified MMP-9 as sheddase for Sdc4 in OA, and possible functions of Sdc4 shedding in OA were addressed. It is known that Sdc4 is involved in various physiological processes such as angiogenesis [97], blood pressure regulation [98], and wound healing [99], but also pathophysiological processes such as tumor progression [204, 205] or OA [86]. The same applies to the shedding of Sdc4. For example, a very recent study presented a protective role for Sdc4 in fibrosis development in the heart. The study proposed that Sdc4 protects osteopontin from being cleaved by thrombin, which is a profibrotic mechanism, as the cleaved osteopontin was shown to induce collagen expression in cardiac fibroblasts. This protective function was lost when the ectodomain of Sdc4 was shed [206]. Similarly, Ramnath et al. showed that in early diabetic kidney disease blocking the shedding of Sdc4 restores the endothelial glycocalyx and glomerular filtration barrier function [134]. On the other side, the presence of Sdc4 can also aggravate cellular responses. It was proposed that the upregulation of Sdc4 in the eutopic endometrium of endometriosis patients is connected to promoting invasive cell growth [114]. Moreover, Sdc4 upregulation is repeatedly connected with inflammation. Recently it was shown that Sdc4 dimerization is crucial for trafficking and signaling of the IL-1R1 [111]. As the functional roles of Sdc4 can be so varied, it is of importance to fully understand the role of Sdc4 in OA. Therefore, possible functions of Sdc4 shedding in chondrocytes were investigated.

In order to do so, MMP-9-mediated Sdc4 shedding was verified by inhibiting shedding through a MMP-9 inhibitor and siRNA for MMP-9. Partial inhibition of Sdc4 shedding was achieved using the MMP-9 inhibitor. However, a visible effect was only reached with a concentration of 5 μM (Fig. 3.10A), while the IC50 value of the inhibitor, as stated by the manufacturer's datasheet, was 5 nM. The MMP-2 inhibitor did not affect the Sdc4 shedding, not even with a concentration of 50 μM, which is 25,000 times its half maximal inhibitory concentration (IC50 = 12 nM, according to datasheet)(Fig. 3.10B). The MMP-9 inhibitor reduced the amount of shed Sdc4 in the supernatant of cultured C28 chondrocytes (Fig. 3.10A). Further, it attenuated APMA induced Sdc4 shedding (Fig. 3.10C). The results suggest that MMP-9 could be involved in the basal and inflammation-induced shedding of Sdc4. Interestingly, the influence of MMP-9 inhibition seems to be more prominent with a higher IL-1β concentration,

as the inhibitor mitigated the increase in Sdc4 shedding, especially at 1 ng/mL, compared to 0.1 ng/mL (Fig. 3.10D). This finding is supported by previous studies, which showed that MMP-9 was NF-κB dependently upregulated by IL-1β in rat articular chondrocytes [207]. Further, it was also previously described that IL-1β does increase Sdc4 shedding [120, 129]. The selectivity of MMP inhibitors is frequently questioned, especially when they are used in higher amounts as the indicated IC50 value. Therefore, in the next step, MMP-9 was knocked down using siRNA for MMP-9 (Fig. 3.11). Using primary chondrocytes, a knockdown of 66 % compared to cells treated with the control siRNA was achieved (Fig. 3.11A). In accordance with the results obtained with the MMP-9 inhibitor, the knockdown decreased IL-1β dependent Sdc4 shedding. Indicating that, indeed, MMP-9 is involved in Sdc4 shedding. As MMP-9 inhibition or knockout only inhibited the Sdc4 shedding partially, other sheddases are most likely involved.

Sheddases such as MMP-14 or plasmin and thrombin could play a role in basal, as well as inflammation induced Sdc4 shedding. To get the whole picture of Sdc4 shedding in OA, further experiments will be needed to investigate all potential sheddases [122, 131]. Moreover, MMP-14 was associated with OA [156, 157], and was shown to degrade ECM components [208]. But mostly it was described to be involved in the activation of other MMPs, such as MMP-2 [158] and MMP-9 [159, 160]. Plasmin [209] was likewise reported to be connected to OA. Plasmin was shown to be increased in immunohistological stainings of cartilage and synovial membrane in a surgically induced canine OA model [210]. Additionally, the plasminogen activators were reported to be upregulated in OA [211]. Further, plasmin has been reported to activate MMP-2 and MMP-9 [212]. Also thrombin has been connected to cartilage degradation in OA. It was shown to induce proteoglycan release in the degradation of bovine and human OA cartilage [213]. Moreover, it was shown to promote MMP-13 expression in human chondrocytes [214]. Those above mentioned sheddases could also play a role in mediating Sdc4 shedding in OA, as all of them have been associated with OA disease progression.

In this study I used two methods to increase Sdc4 shedding, and thereby study the involvement of MMP-2 and -9, as well as possible functions of Sdc4 shedding. One method is the addition of APMA, an organomercurial activator of MMPs, which activates MMPs by the disrupting bonds inside the 3D structure of the enzymes. APMA is not capable of activating all MMPs, but MMP-2, as well as MMP-9, is activated by APMA, which was confirmed in this study by gelatin zymography (Fig. 3.12A). This technique provides information about the ratio of inactive to

active MMP-2 and MMP-9. Interestingly, the zymography reflected the results obtained from the ELISAs discussed before. It showed that the chondrocyte cell line produced more MMP-2 than MMP-9. However, on a basal level, more MMP-9 was active compared to MMP-2, which showed no active form. Nevertheless, some MMP-2 might have been active, but the levels were below the detection limit of the zymography. It could be seen that upon addition of APMA, MMP-2, as well as MMP-9, were proteolytically processed, resulting in a smaller and potentially active protein. Moreover, the zymography showed that neither the MMP-2 inhibitor, nor the MMP-9 inhibitor, nor the combination of both interferes with this proteolytic process (Fig. 3.12A). Therefore APMA was proven to be a suitable approach to induced Sdc4 shedding.
The study by Godman et al. showed that IL-1β directly binds to Sdc4. Sdc4 regulates its receptor (IL-1R1) presentation at the membrane surface and through that modulates IL-signaling. Sdc4 deficiency or inhibition by a specific antibody reduced IL-1 signaling in fibroblasts and chondrocytes. Therefore, I investigated whether Sdc4 shedding might have similar effects on IL-1 signaling. Hence, shedding was induced in C28 chondrocytes, and its impact on IL-1β signaling was investigated. The results of this experiment showed less phosphorylation of ERK, thus less IL-1 signaling, after APMA-induced Sdc4 shedding (Fig. 3.12B). A similar effect on ERK phosphorylation was seen in chondrocytes and fibroblasts from Sdc4 deficient mice [86, 111], showing that Sdc4 shedding has a similar effect on IL-1 signaling to its complete absence. Additionally, less IL-1 signaling based on Sdc4 deficiency resulted in less MMP-3 and ADAMTS-5 activation, which are both important mediators of cartilage destruction [86, 111].
Combining the results from this study with the data presented by Godmann et al. and Echtermeyer et al., IL-1β-dependent increased MMP-9 levels seem to regulate the responsiveness of chondrocytes towards IL-1β by mediating Sdc4 shedding. Previously in this study, I presented that MMP-9 expression was increased in the synovial membrane, but not in cartilage samples in my knee OA cohort. A study by Dreier et al. suggested that especially macrophages produced pro-MMP9, while proteases (MMP-3, MMP-13) expressed by OA chondrocytes were necessary for the activation of MMP-9 during OA. These data suggest that increased production of MMP-3 and MMP-13 in OA chondrocytes can activate MMP-9. This would desensitize chondrocytes towards IL-1β signaling by an increased shedding of Sdc4, which in turn would again result in less MMP-3 expression, as MMP-3 is downstream of IL-1 [86].
As it was shown that IL-1β can directly bind to the extracellular domain of Sdc4 [111], the final part investigated whether the shed Sdc4 might work as a decoy receptor for IL-1, and through

that reduces the binding of IL-1β to the cell bound Sdc4 protein and the IL-1R1. The addition of the extracellular domain of Sdc4, however, had no effect on IL-1β signal transduction. This showed that indeed the absence of the extracellular domain of Sdc4 through increased shedding regulated the responsiveness of chondrocytes towards IL-1β, but not the shed form of Sdc4.

One limitation of this study was that no synovial fluid of healthy probands was available; therefore the basal amount of shed Sdc4 in the synovial fluid could not be determined, and it was not possible to conclude whether Sdc4 is already upregulated in the early stages of OA in comparison to a healthy cohort. The same was true for the mRNA analyses of synovial membrane and articular cartilage samples analysis. Obtaining healthy samples of synovial fluid, synovial membrane or cartilage is a major problem in OA studies in general. Samples that were analyzed in other studies and declared as “healthy controls” mainly originated from patients with traumatic injuries, which do not necessarily have to mirror healthy conditions. The healthy knee joint exhibits only small amounts of synovial fluid and the risk of infection is too high to draw synovial fluid from healthy probands. The use of animal models would be the only solution for this general problem. However, in this case the question would be how similar human OA would be to the animal model.

5

Conclusion and outlook

5.1 Conclusion and Outlook

This study demonstrates for the first time that levels of shed Sdc4 are increased in synovial fluid depending on the OA severity, and proposes shed Sdc4 as a suitable biomarker in synovial fluid for the assessment of the OA severity.

Nevertheless, three major questions remain to be answered that were not possible to be addressed by this study. First, healthy control samples are necessary to investigate whether the amount of shed Sdc4 can distinguish between healthy knee joints and early OA. Second, it would be interesting whether shed Sdc4 can represent OA progression in individual patients. Therefore, collecting several samples of synovial fluid at different time points during the disease process of individual patients would be necessary. Last, as it was reported that Sdc4 might only be increased in OA knee, but not in the hip or shoulder, synovial fluid of other OA joints needs to be examined.

Further, it would be interesting to see whether shed Sdc4 might correlate with other proposed biomarkers, and through combining different biomarkers increase the specificity and selectivity. Moreover, this study presents MMP-9 as one of the major sheddases in Sdc4 shedding in the OA knee joint. However, other sheddases must also be involved which need to be investigated (plasmin, thrombin, MMP-14). Also activity studies of MMP-2 and MMP-9 in human synovial fluid might give a better insight into Sdc4 shedding in OA, as well as immunohistological studies for the extracellular and intracellular part of Sdc4 in cartilage and synovial membrane to better assess the source of the shed Sdc4 in the synovial fluid.

In conclusion, in this study shed Sdc4 was presented for the first time as a possible biomarker for knee OA severity, while MMP-9 was shown to be a sheddase in Sdc4 shedding in OA.

Moreover, it was shown that IL-1 signaling can be modulated by Sdc4 shedding, displaying a possible role for the increased shedding in OA.

A

Abbreviations

Acronym	Meaning
°C	Degree Celcius
ACL	Anterior cruciate ligament
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
APMA	4-Aminophenylmercuric acetate
Aqua dest.	water
AUC	Area under the curve
bp	base pairs
BSA	Bovine serum albumin
CCL5	Chemokine (C-C motif) ligand 5
cDNA	Complementary deoxyribonucleic acid
CO2	Carbon dioxide
Col2	Collgagen type II
Col2-1	The peptide Coll2-1 (108HRGYPGLDG116)
Col2-1NO2	nitrated form of Coll2-1 (108HRGYPGLDG116)
COMP	cartilage oligomeric matrix protein
Cox-2	cyclooxygenase-2
CS-846	chondroitin sulfate 846 epitope
c-Scr	Proto-oncogene tyrosine-protein kinase Src
CTX-II	C-terminal cross-linked telopeptide of type II collagen
Da	Dalton
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DMM	destabilization of the medial meniscus

Acronym	Meaning
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extra cellular membrane
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ERK	Extracellular signal-regulated protein kinase
FCS	Fetal calf serum
FGF	Fibroblast Growth Factor
g	Gravity
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyd-3-phophate-dehydrogenase
h	Hours
H2O	Water
HA	Hyaluronic acid
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HSPGs	Heparan sulfate proteoglycan
Hz	Hertz
IFTA	interstitial fibrosis/tubular atrophy
IgG	Immunoglobulin G
IL-1	Interleukin
IL-1R1	Interleukin-1 receptor type I
JNK	C-Jun-N-terminale Kinase
JNS	Joint Space Narrowing
kb	Kilobase
KL	Kellgren-Lawrence
L	Liter
m	Milli
M	Molar
MAPK	Mitogen-activated protein kinase
MMP	Matrix Metalloproteinases
mRNA	messenger RNA

Acronym	Meaning
min	Minutes
n	Nano
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
N-terminal	Amino-terminal
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEP	processed eggshell membrane powder
pERK	phosphorylated Extracellular signal-regulated protein kinase
PFA	Paraformaldehyde
PKCα	Protein kinase C alpha
RhoG	Ras homology Growth-related
PIIICP	procollagen type II C-terminal propeptide
RNA	Ribonucleic acid
ROC	receiver operating characteristic
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
s	Seconds
Sdc	Syndecan
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
TBS	Tris-buffered saline
TLR	Toll-like Rezeptoren
TNF-α	tumor necrosis factor-α
U	Units
V	Volt
v/v	Volume by volume
w/v	Weight by volume
Wnt-3a	Wnt Family Member 3A
WT	Wild type
YKL-40	Chitinase-3-like protein 1
μ	Micro

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Publikationen

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Ehrenerklärung

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„Ich versichere hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; verwendete fremde und eigene Quellen sind als solche kenntlich gemacht.
Ich habe insbesondere nicht wissentlich:

- Ergebnisse erfunden oder widersprüchlich Ergebnisse verschwiegen,
- statistische Verfahren absichtlich missbraucht, um Daten in ungerechtfertigter Weise zu interpretieren,
- fremde Ergebnisse oder Veröffentlichungen plagiiert,
- fremde Forschungsergebnisse verzerrt wiedergegeben.

Mir ist bekannt, dass Verstöße gegen das Urheberrecht Unterlassungs- und Schadensersatzansprüche des Urhebers sowie eine strafrechtliche Ahndung durch die Strafverfolgungsbehörden begründen kann. Ich erkläre mich damit einverstanden, dass die Arbeit ggf. mit Mitteln der elektronischen Datenverarbeitung auf Plagiate überprüft werden kann. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form als Dissertation eingereicht und ist als Ganzes auch noch nicht veröffentlicht.

(Ort, Datum)

(Unterschrift)