GONÇALO BARRETO

Innate Immunity in Osteoarthritis:
The Role of Toll-Like Receptors and Cartilage Derived Mediators in the Disease Progression

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Doctoral Programme in Clinical Research (KLTO)

INNATE IMMUNITY IN OSTEOARTHRITIS: THE ROLE OF TOLL-LIKE RECEPTORS AND CARTILAGE DERIVED MEDIATORS IN THE DISEASE PROGRESSION

Gonçalo Barreto

Academic dissertation
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Helsinki 2016
“what you do today can improve all your tomorrows”

-Ralph Marston
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This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.


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In addition, currently, unpublished data is presented.

Gonçalo Barreto’s contribution to the articles:

I. The author participated in the conception and design of the experiments. Performed immunostainings of pellet cultures and articular cartilage. Analyzed data, interpreted results, and participated in writing and editing the manuscript.

II. The author participated in the conception and design of the experiments. Performed all experiments with the exception of cartilage sample collection and formalin-paraffin embedding. Analysed data, interpreted results, and participated in writing and editing the manuscript.

III. The author participated in the conception and design of the experiments. Performed all experiments with the exception of cartilage and synovial fluid collection, formalin-paraffin embedding, and some (50%) of nitric oxide and collagen measurements. Analysed data, interpreted results, and participated in writing and editing the manuscript.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADAMT</td>
<td>A disintegrin and metalloproteinase with thrombospondin type 1 motif</td>
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<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>ACAN</td>
<td>Aggrecan</td>
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<tr>
<td>ACL</td>
<td>Anterior cruciate ligament</td>
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<td>AP</td>
<td>Alkaline phosphatase</td>
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<td>APO</td>
<td>Apolipoprotein</td>
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<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
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<tr>
<td>ACTB</td>
<td>Beta-actin</td>
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<tr>
<td>BGN</td>
<td>Biglycan</td>
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<tr>
<td>sBGN</td>
<td>Soluble biglycan</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>CMC</td>
<td>Carpometacarpal</td>
</tr>
<tr>
<td>COMP</td>
<td>Cartilage oligomeric protein</td>
</tr>
<tr>
<td>COL2A1</td>
<td>Collagen type II</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CTSK</td>
<td>Cathepsin K</td>
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<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
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<tr>
<td>DCN</td>
<td>Decorin</td>
</tr>
<tr>
<td>sDCN</td>
<td>Soluble decorin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DIP</td>
<td>Distal interphalangeal</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<tr>
<td>EDN</td>
<td>Eosinophil-derived neurotoxin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid solution</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FLS</td>
<td>Fibroblast-like synoviocytes</td>
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<td>FFPE</td>
<td>Formalin-parafin embedding</td>
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SFA  French Arthroscopy Society
GWAS  Genome-wide association study
GXM  Glucuronoxylomannan
GAPDH  Glyceraldehyde 3-phosphate dehydrogenase
GAG  Glycosaminoglycan
HEK  Human embryonic kidney
HSV9  Herpes simplex virus
HMGB1  High-mobility group box 1
HRP  Horse radish peroxidase
HEK  Human embryonic kidney
HA  Hyaluronan; Hyaluronic acid
IκB  Inhibitor of kappa B
IKK  IκB kinase
IRAK1  IL-1R-associated kinase-1
ICAM  Intercellular adhesion molecule
IHC  Immunohistochemistry
IFN  Interferon
IL  Interleukin
IRF  Interferon regulatory transcription factor
KL  Kellgren-Lawrence
LAM  Lipoarabinomannan
LPS  Lipopolysaccharide
LBP  Lipopolysaccharide binding protein
LTA  Lipoteichoic acid or Lymphotoxin alpha
LMH-HA  Low-molecular-weight hyaluronan
LFA-1  Lymphocyte function-associated antigen-1
MRI  Magnetic resonance imaging
MMP  Matrix metalloproteinase
MCP  Metacarpophalangeal
MAPK  Mitogen-activated protein kinase
MMTV  Murine mammary tumor virus
MSC  Mesenchymal stem cell
MyD88  Myeloid differentiation factor 88
MD2  Myeloid-differentiation factor 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>NLR</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Nurr1</td>
<td>Nuclear receptor related 1 protein</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OLP</td>
<td>Oral lichen planus</td>
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<tr>
<td>OARSI</td>
<td>Osteoarthritis Research Society International</td>
</tr>
<tr>
<td>OSP</td>
<td>Outer surface protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline solution</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
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<tr>
<td>PG</td>
<td>Proteoglycans</td>
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<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SEAP</td>
<td>Secreted embryonic alkaline phosphatase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLRP</td>
<td>Small structural proteoglycan with leucine-rich repeats</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile alpha- and armadillo-motif-containing</td>
</tr>
<tr>
<td>SF</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>Pam3CSK4</td>
<td>Synthetic triacylated lipopeptide</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA Box Binding Protein</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF receptor-associated factor 6</td>
</tr>
<tr>
<td>TAG</td>
<td>TRAM adaptor with GOLD</td>
</tr>
<tr>
<td>TGF-β3</td>
<td>Transforming growth factor beta 3</td>
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<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TAB2</td>
<td>TGF-beta Activated Kinase 1</td>
</tr>
<tr>
<td>TN-C</td>
<td>Tenascin</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll IL-1 receptor</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TKA</td>
<td>Total knee arthroplasty</td>
</tr>
<tr>
<td>LP</td>
<td>Triacylated lipopeptide</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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Abstract

Osteoarthritis (OA), the most common form of arthritis, is estimated to be in the top 5 leading causes of disability worldwide. Yet OA incidence is estimated to keep growing partly due to the overall worldwide trend of increased obesity and ageing population. Cartilage erosion, a hallmark of OA, has its onset in the traumatic events caused by incorrect biomechanical loading of the joint and the consequent biological response. Currently we still poorly comprehend the molecular pathophysiology of preclinical and clinical symptomatic OA, which consequent results in no current available therapy to prevent OA progression.

We hypothesize that innate immunity and its receptor, in particularly toll-like receptors (TLRs), could be major drivers of OA disease progression and onset. The process could be initiated as a proinflammatory reaction against extracellular matrix (ECM)-derived damage-associated molecular patterns (DAMPs). DAMPs accumulate in avascular articular cartilage as a result of traumatization and degeneration, leading directly at their source to a reactive chondrocyte-mediated and TLR-dependent production of proinflammatory and algogenic secondary mediators, which then cause a secondary synovitis with consequent joint pain. For this propose, we collected cartilage and isolated primary chondrocytes from a total of 27 OA patients. Synovial fluid was obtained from knee meniscectomy, total knee arthroplasty (TKA) due to OA, and rheumatoid arthritis (RA) patients generating a total of 30 patient samples. HEK (human embryonic kidney)-blue TLR4 reporter cell line, primary OA chondrocytes, and cartilage explants were used for functional studies.

Our results confirmed that TLR1, TLR2 and TLR9 expression is present in healthy primary chondrocytes isolated from articular cartilage, and derived from chondroprogenitors. During our chondrogenesis differentiation studies initial high expression of TLR1, TLR2 and TLR9 was significantly reduced to baseline levels.

We demonstrated that proinflammatory cytokine tumour necrosis factor alpha (TNF-α) is able to increase the expression of TLR2 in both healthy primary chondrocytes and mesenchymal stem cells (MSC) derived chondrocytes cultured for 21 days. TNF-α stimulation was demonstrated to induce cartilage degradation in de novo ECM matrix from pellet cultures of MSC-derived chondrocytes cultured for 21 days. This implicates TNF-α as an inducer of matrix degradation, with wide implications in the use of MSCs strategies in cartilage repair strategies for OA. Our study also added further evidence of a role for TNF-α in TLR-innate immunity in the OA synovial joint.

TLRs protein expression in cartilage between knee and first carpometacarpal (CMC-I) joints from OA patients was shown to be strikingly different. Our study demonstrated for the first time all TLRs being expressed at protein levels in articular cartilage from knee OA patients. Moreover, we
demonstrated that their expression is up-regulated in a cartilage zone-dependent fashion according to the histological progression of knee OA. TLRs expression in cartilage from CMC-I OA patients was highly heterogeneous although it followed an expression pattern according to TLRs cellular organization. This indicates that TLR-mediated innate immune response between the two joints may be significantly different.

Decorin (DCN), a known small structural proteoglycan with leucine-rich repeats (SLRP) ligand able to activate TLR2 and TLR4, was discovered in knee synovial fluid from OA and RA patients. We confirmed the ability of soluble DCN (sDCN) to activate to TLR4 signaling. However, the observed low and stable concentration levels across the studied groups mean that this may not be of clinical relevance in OA pathogenesis and the associated TLR-mediated inflammatory events.

Biglycan (BGN), another known SLRP ligand able to activate TLR2 and TLR4, was discovered in knee synovial fluid from OA and RA patients. Interestingly, we discovered that soluble BGN (sBGN) is upregulated in synovial fluid from OA and RA patients. sBGN ability to activate TLR- innate immunity was confirmed to be essentially activated through TLR4 signaling by studies in articular chondrocytes and human HEK-blue TLR4 reporter cell line. The sBGN stimulation lead to the upregulation and release of proinflammatory cytokines, matrix-degrading enzymes and the release of ECM degradation products.

Overall, the results of this thesis demonstrate that TLRs are markedly present in articular cartilage from OA patients at different progression stages of the disease. The detection of BGN and DCN in synovial fluid, and their ability to activate TLR4-mediated proinflammatory cellular responses gives new knowledge of proinflammatory molecules present in the OA synovial joint. An enhanced molecular understanding of the triggering mechanisms by which TLRs are activated and regulated during OA progression stages may help find therapeutic options in the treatment of OA.
1. Introduction

Osteoarthritis (OA), the most common form of arthritis, is estimated to be in the top 5 leading causes of disability in Finland and worldwide. Furthermore, OA incidence is estimated to keep growing partly due to the overall trend of increased obesity and aging population. By 2003, the annual cost of just hip joint replacement procedures in Finland was nearly 70 million € without taking into account post-surgery rehabilitation (1). The current lack of treatment or prevention therapies coupled with an increasingly aging and overweight population lead to an estimated doubled number of people affected by OA disability at 2020. In Finland alone the incidence of total knee replacement surgeries among the baby boomer generation was increased by 130-fold during the short period of 1980-2006 (2).

OA has historically been considered as a simple “wear and tear” joint disease with cumulative events over time and hence considered as mere a disease of the “old” (3). Nowadays, however, OA is no longer considered as a simple wear and tear disease model, given that recent findings have clearly demonstrated that inflammatory molecular events are also major drivers of the disease progression. Cartilage erosion, the hallmark of OA, has its onset in the traumatic events caused by incorrect mechanical loading of the joint however its active degradation in mediated by proteolytic enzymes arising from “mild” inflammatory responses.

OA synovium is also known to have increased infiltrates of inflammatory cells as seen in the well recognized classical pattern of rheumatoid arthritis (RA). Several inflammatory cytokines such as interleukin 1 beta (IL-1β), tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are also increased in synovial fluid (SF), as a result of the crosstalk between synovium and cartilage tissues in OA patients (4).

Thus, the modern views of OA, consider OA as disease of the whole joint which affects the synovial joint structures, including articular and meniscal cartilage degeneration and loss, subchondral bone changes, synovitis and altered nervous system (5). Moreover, the new OA pathogenesis paradigms are based on the crosstalk between mechanical-transduction and injury responses with ensuing inflammatory responses. Activation of the innate immune system, in particularly by Toll-like receptors (TLRs and the complement system, have been shown to be intrinsically related to a mechanic induction but also to a recognition of mechanical injury products (6). Despite increased research efforts and attention, we are only now starting to poorly comprehend the molecular pathophysiology mechanisms of preclinical and clinically symptomatic OA. Hence, effective preventive therapy or treatment for established OA is still lacking.

This thesis studies TLRs at different differentiation stages of the chondroprogenitors to decipher the progenitor cells impact on OA cartilage repair strategies. TLRs are also studied during the
progression stages of OA and in different OA synovial joints, so that common and differential molecular players might be identified. We studied particularly the role of cartilage resident cells, the chondrocytes, and specifically the TLR-mediated inflammatory responses as measured by the production of cartilage breakdown enzymes, cytokines, and cartilage extracellular matrix (ECM) essential molecules.
2. Review of the Literature

2.1. Epidemiology of OA

The diagnosis of OA can be done clinically, radiographically and pathologically and therefore, the choice of diagnostic definition can significantly affect the prevalence estimates (7). Given the known incongruences in the descriptions of e.g. radiographic and clinical OA, other sensible and semi-quantitative methods e.g. magnetic resonance imaging (MRI) and biomarkers are starting to be employed in epidemiological studies. In this thesis, the epidemiology section data will be based on the gold standard namely the radiographic diagnosis of OA and epidemiological studies using the radiographic diagnosis of OA of three main joints with high incidences, the hand, knee and hip.

From as early as 1926 researchers have been studying the prevalence of OA. Their studies of pathological features of OA followed by systematic autopsy studies demonstrated an almost universal occurrence of cartilage damage in patients over 65 years of age (8).

In contrast, with the first research methods applied to OA epidemiology, the current methods differ substantially. Nowadays OA epidemiological data are generated from population-based radiographic surveys.

A common feature of all OA joints is the fact that radiographic OA prevalence rises progressively with age. To define radiographic knee and hand OA usually a radiographic Kellgren–Lawrence (KL) score of 2 or higher grade in specific joints is used (9). The distal interphalangeal (DIP) joints, the proximal interphalangeal (PIP) joints, and the first carpometacarpal (CMC) joints or thumb base are the most commonly studied hand joints in comparison to the often spared metacarpophalangeal (MCP) joints. Radiographic hand OA in any hand joint or a selected subset of hand joint sites is usually defined as the criteria for case definition of hand OA (10). Population-based studies such as the Zoetermeer survey indicated that 75% of women aged 60–70 years presented with hand OA of their distal interphalangeal joint (DIP) joints. As early as in the age of 40, 10 to 20% of subjects had evidence of severe radiographic disease in their hands or feet (11). The Rotterdam study of a population-based cohort of individuals with 55 years or older reported that 67% of women and 55% of men had radiographic OA, in at least one hand joint. Both hand and knee OA appear to be more frequent among women than men with a female-to-male ratio varying between 1.5 and 4.0 among studies.

For radiographic knee OA the Johnston County Osteoarthritis Project reported a prevalence of 28% in men over 45 years old, a slight decrease in comparison with 37% observed in the first National Health and Nutrition Examination Survey (NHANES III)(12,13). Both studies found significant differences in the prevalence of radiographic knee OA between Caucasian, Afro-American and
Mexican American races. Interestingly the Beijing Osteoarthritis Study reported that Chinese women presented with a higher radiographic knee OA prevalence in comparison with white US women studied in the Framingham OA Study, 46% and 34% respectively. However, Chinese and white men did have an equal prevalence of radiographic knee OA (14).

The overall prevalence of OA varies depending on the population studied. Nevertheless for hip OA the differences can be as small as 1% and up to 32% (15). Both NHANES III and Johnston County Osteoarthritis Project report a similar prevalence of 27-32% for radiographic hip OA among whites and blacks (16,17). In line with the knee OA prevalence, in hip OA Chinese women and men also have significantly lower rates, with a crude prevalence of ~1% (18).

In line with prevalence studies, the incidence of radiographic OA also rises progressively with increasing age. In hand, knee and hip OA it is also important to consider the incidence of radiographic grade progression in OA patients. Interestingly, several studies have shown that the progression rates from a healthy baseline score to a radiographic OA score are significantly lower when compared to summary radiographic grade progression of an OA patient (19,20).

2.2. Risk Factors for OA: systemic and local risk factors

Several risk factors for occurrence and progression of OA have been proposed, where many are in common to various OA joints while others may differ on the basis of the joint involved. Figure 1 resumes the known risk factors associated with the three main joint types affected by OA.

Figure 1. Risk factors of synovial joints radiographic OA. (Adapted from Felson et al. (21)).
2.2.1. Age and gender

As discussed in the epidemiological section, age is undeniably a risk factor for OA, with OA being mainly diagnosed in patients over 60 years of age. The aging of the joint environment undermines the joint integrity making it susceptible to the development of OA. During aging, cartilage gets thinner, and cartilage homeostasis is impaired due to changes in the cellular function of chondrocytes (22). Chondrocyte density is decreased, cells becomes senescent and alter the balance between anabolic and catabolic functions. This undermines the chondrocyte response to growth factors and leads to alterations of cartilage extracellular matrix structure, therefore, compromising cartilage integrity (22,23). Age usually also influences the degree of physical activity leading to reduced muscle strength, which itself is a risk factor for OA.

Gender also has a significant impact on the risk of developing OA. A recent systematic review of risk factors for OA analyzed 11 studies dealing with gender association to OA comprising more than 28,000 individuals. The study corroborated the original studies in calculating that females have an odd ratio of 1.7 to develop OA as compared with males (24). Several factors may influence this e.g. gender distribution by work type or gender differences by sports activities, which are risk factors themselves. However other physiological gender-dependent factors may also play a role. It is known that estrogen receptors are present in joint tissues and that estrogen influences cartilage metabolism and bone (25). It is assumed that after menopause the hormone related changes and estrogen loss may enhance the development of OA. Nevertheless, estrogen supplementation in the form of hormone replacement therapy has shown no benefit in preventing OA progression (24).

2.2.2. Obesity

An overwhelming number of studies have found associations between obesity and OA. Studies have shown that obesity and overweight causes malalignment as well as extra loading of the lower limb joints i.e. hip, knee, ankle and feet (26,27). However, the association between OA and obesity is beyond the improper loading of the joints. Interestingly several studies have shown that obesity is also associated with upper limb e.g. hand OA, suggesting that metabolic factors may also be driving cartilage degradation and OA progression (28-31). Obesity also has significant detrimental effects on the behavioral factors which could lead to diminished physical activity and ensuing loss of protective muscle strength (32,33). Several studies have shown that by targeting such behavioral factors in obese patients a significant improvement is seen in pain and activity scores (34,35).
2.2.3. Nutritional factors

Vitamins with their given biological activities have made them subjects of study in OA disease. That being said, it is also worth to note that there are a lot of conflicting reports in the literature about whether vitamins and other nutrient deficiencies are associated with OA disease. Nevertheless, vitamins role in OA pathogenesis therapy and treatment may one day be settled and prove to be important players. For this reasons, they are described in this section.

A particular controversy lies around vitamin D, especially due to its wide range of implications including pain mechanisms and calcium uptake. Vitamin D deficiency has been implied as a risk and accelerating factor for OA (36). However, several studies reported no vitamin D effect (37,38). Vitamin D supplementation has also shown some positive effects in the prevention of progression of OA, albeit preventive effects have also been rejected in another study (39,40). Interestingly, Vitamin D receptor (VDR) gene polymorphisms in association with OA have been observed, albeit conflicting results for knee OA patients have also been reported (41-43). VDR gene polymorphisms cause a change in VDR receptor activity and, therefore, alters the response to vitamin D, which then has implications for numerous factors e.g. calcium uptake. A recently published meta-analysis review of the literature has shown a small but statistically significant association of VDR and OA susceptibility in the Asian population, albeit no association with the European population (44).

Vitamin E has also been a focus of interest. Low Vitamin E levels in SF of late OA patients were associated with severity of radiographic OA, while in the serum of late OA patients vitamin E was found to be increased (45,46). Nevertheless, in vitamin E supplementation studies no effect was observed regarding OA-based outcome of cartilage volume (47).

Vitamin K deficiency has also been shown to be associated with hand and knee OA (48-50). Nevertheless, a three-year randomized clinical trial of vitamin K supplementation demonstrated no overall improvement in radiographic hand OA albeit a slight benefit in joint space narrowing was observed (51).

Other antioxidants such as vitamin A, C and selenium, have been the focus of several OA studies. However, no significant associations with the levels of these antioxidants and OA have been found (52).

2.2.4. Physical activity

Physical activity of people varies immensely and as expected the effects on the joints are also variable. Therefore, conflicting and varied reports on the influence of exercise on the progression of OA are more than anticipated. If relations exist, these may depend on the type of activity and stage of OA
disease. However, extreme patterns of physical activity are known to be risk factors. While casual marathon senior runners may not be at high risk of developing OA, professional runners have a high predilection for knee and hip OA even at early ages (53,54). Moreover, professionals with intense and repetitive manual labor such as plumbers or miners have the predisposition to hand, knee and back OA at an early age (55,56). Further studies are still needed to decipher the influence of physical activity on the disease and on the risk of developing OA. However, current measurements of physical activity and its patterns are imprecise and self-reported which inherently pose a challenge for further accurate data from this field.

2.2.5. Joint abnormalities, malalignments, and injuries

A healthy joint with normal joint anatomy implies a correct physiological loading. However, when abnormalities occur the joint load can be significantly altered and this may have grave consequences for the joint health.

Abnormal joint alignment of the knee has been associated with accelerated structural deterioration, the risk of medial and lateral progression, bone marrow lesions and rapid cartilage loss (57-59).

Interestingly, abnormal muscle strength may also alter the joint alignment and, therefore, be a contributing factor for the development and progression of hand OA. High levels of grip strength can cause abnormal loading in hand joints leading to increased risk of OA in proximal hand joints (60). While the role of muscle strength for hand OA is clear, the same cannot be concluded for hip and knee OA. Albeit several and proper studies on the role of the muscle strength (e.g. quadriceps weakness) on hip and knee OA have been reported, findings are still discordant (59,61,62). The biggest limitations of these studies are whether muscle weakness is causal or the consequence of OA or caused by an OA-related risk factor e.g. obesity.

Joint abnormalities such as hip dysplasia-associated malformations or hip cam impingement are known risk factors associated with hip OA, and its progression (63-65). Interestingly femoroacetabular impingement (FAI) incidence is higher in white women in comparison with Chinese (66). This reported incidences also suggest that such anatomic abnormalities may help explain the observed low incidence of hip OA in Chinese people in China and abroad in comparison with whites (67).

2.2.6. Bone mineral density (BMD)

Bone mineral density (BMD) changes is a known risk factor for OA. However, reports are again conflicting. Increased BMD has been shown to be a risk factor for the incidence of radiographic knee,
hip and hand OA (68-70). However, numerous studies have also shown an inverse correlation between BMD loss associated with radiographic progression of OA (71,72). Additionally, high BMD concentration levels are observed in knee cartilage of healthy subjects (73,74) It is also worth to mention that in animal models the use of drugs targeting bone resorption (anti-resorptive) may retard OA progression. Interestingly, the same strategy has provided encouraging results in the study of strontium ranelate efficacy in knee osteoarthritis trial (SEKOIA) (75). Taken together the literature seems to suggest that BMD loss may be a risk factor for radiographic progression of OA. Nevertheless, contradictory results remain and will endure as long as the underlying mechanism of this observation remains unclear (76).

2.2.7. Previous joint injury

Significant damage to the structures of a joint is a risk factor for later development of OA in articular joints. Although some studies have demonstrated that Anterior cruciate ligament (ACL) surgical reconstruction may cause a moderate risk of developing knee OA, a recent systematic review showed that ACL reconstruction has no impact on radiographic progression of OA and, in fact, it “reduced” the risk of subsequent meniscus injuries (77). A more dramatic development was seen in patients with meniscal injury, who have a high risk of developing knee OA (78). Interestingly, surgical removal of torn meniscus seems to put patients at very high risk of developing knee OA (79). Therefore, indication of meniscectomy is a matter of concern in the research community with ongoing clinical trials addressing this issue. Furthermore, those who already have knee OA are at higher risk of radiographic progression of OA if meniscus tears occur (80).

It is also worth to mention that a joint with an inherently low incidence of OA, such as the ankle, may present with an inevitable development of OA after a significant injury e.g. fracture (81).

2.3. OA pathogenesis & pathology

2.3.1. The healthy synovial joint

The synovial joints are the main joint type found in the body, and they are of tremendous importance for mobility, due to their unique characteristics, such as freedom of movement in many directions, provided with almost frictionless contact, allowing limbs to act smoothly.

In order for the synovial joints to work effectively, they need stabilization mechanisms to avoid dislocations. Muscles surrounding the joint configuration are important stabilizing structures, and the efficient muscle action allows ligaments and fibrous capsule to guide the joint and limit the extension of motion. However, if timely concerted actions of the muscles are affected this can lead to excessive strain on the ligaments and capsule, leading at worst to their rupture (82).
Within the joint capsule, which defines the intra-articular space, is the synovial membrane. This membrane, composed of connective tissue and specialized cells, has two main functions: the provision of nutrients to the cells of the articular cartilage; and the production of lubricating fluid to ensure minimal friction that characterizes the synovial joints. The synovial membrane consists of two distinct layers: the synovial surface layer (also known as synovial lining), that secretes the SF, being in direct interface with the intra-articular cavity and the subintimal layer of connective tissue, which supports the synovial lining and the blood vessels that supply the membrane. The SF is, therefore, an essential element in maintaining joint integrity, as it provides nutrients necessary for cartilage matrix cells, and also lubricates the joint. The SF composition is crudely similar to plasma, but, in addition, it also contains hyaluronic acid (HA), a glycosaminoglycan, and lubricin, a mucinous glycoprotein. Together, these provide crucial contributions to the SF behaviour, due to their viscous and lubrication properties, respectively (83).

The synovial joint is known to be innervated, to respond to several stimuli, such as chemical, mechanical and pain stimuli. The synovium, initially thought to be aneural as the articular cartilage, is actually innervated by efferent sympathetic nerves and by primary afferent nociceptors, that are specialized free nerve endings of primary afferent nerves (A-delta fibers and C fibers)(84). Together these are known to respond to stimuli such as to chemical and mechanical and consequently mediate the vasculature response. There is also evidence of pain response (84).

In the meniscus, nociceptors and mechanoreceptors are widely distributed, to immediately signal mechanical realignment when extreme pressure or tension are sensed, as a consequence of misalignment over the tissue (of the meniscus)(85). Moreover, the innervation at the meniscus also generates prociceptive information for correct coordinated movement, velocity and direction. Furthermore, the meniscus high level of innervation, angiogenesis and nerve growth has led some to propose its contribution to the triggering of threshold levels of pain sensitivity in OA knee (86). Given the fact that articular cartilage plays a significant role in the synovial joint and is also intrinsically related to OA, detailed analysis of its characteristics will be discussed in the next section.

2.3.1.1. Articular cartilage biology

Different types of cartilage tissue are present at various sites throughout the body. Cartilage can be classified by histological analysis of its molecular composition into: elastic, hyaline and fibrocartilaginous. Of these cartilage types, hyaline cartilage is the most common cartilage and is associated with the skeletal system. Articular cartilage is attributed as hyaline cartilage, and can be divided into three individual zones: the superficial zone, the middle zone (can be also divided into subgroups: the transitional zone, the radial zone), the deep zone, and the calcified cartilage zone.
where the interface with the subchondral bone lies (87) (Figure 2). These individual zones are characterized as having different organized structures of collagen network and as well as different molecular types and levels of proteoglycans, and, therefore, distinct anisotropy and polarity can be observed. Table 1 resumes the features mainly observed in the different zones.

**Table 1 - Definition of structures composing the different articular cartilage zones.**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial zone</td>
<td>Cartilage zone at joint surface. Collagen fibers are aligned parallel to</td>
</tr>
<tr>
<td></td>
<td>surface. Chondrocytes, elongated and flattened, are aligned parallel</td>
</tr>
<tr>
<td></td>
<td>to collagen fibres and to joint surface</td>
</tr>
<tr>
<td></td>
<td>Zone subjacent to superficial zone. Collagen fibers are aligned</td>
</tr>
<tr>
<td></td>
<td>intermediately between superficial and deep zone alignments.</td>
</tr>
<tr>
<td></td>
<td>Chondrocytes present in groups (chondrons) aligned parallel to collagen</td>
</tr>
<tr>
<td></td>
<td>fibers.</td>
</tr>
<tr>
<td>Middle Zone</td>
<td>Zone subjacent to mid zone and above calcified cartilage. Collagen</td>
</tr>
<tr>
<td></td>
<td>fibres are aligned predominantly perpendicular to joint surface.</td>
</tr>
<tr>
<td></td>
<td>Chondrocytes within chondrons are aligned parallel to collagen fibers and</td>
</tr>
<tr>
<td></td>
<td>perpendicular to joint surface.</td>
</tr>
<tr>
<td>Deep zone</td>
<td>Zone of increased calcification at border of uncalcified and calcified</td>
</tr>
<tr>
<td></td>
<td>cartilage.</td>
</tr>
<tr>
<td>Tidemark</td>
<td>Calcified cartilage matrix. Collagen fibres and chondrocytes are</td>
</tr>
<tr>
<td></td>
<td>aligned similar to deep zone cartilage.</td>
</tr>
<tr>
<td>Calcified cartilage</td>
<td>Bone subjacent to articular cartilage. Collagen fibres aligned</td>
</tr>
<tr>
<td>Articular bone plate</td>
<td>predominantly parallel to articular surface.</td>
</tr>
</tbody>
</table>

Adapted from Pritzker et al. (87).

As previously described, throughout the cartilage zones there are different collagen molecular types. Of these different types of collagens, type II is the main component in healthy articular cartilage. However, collagens III, VI, IX, XI, XII and XIV also contribute, although with a minor proportion, to the mature cartilage matrix (88).

Among the proteoglycans present in cartilage, aggrecan is the most abundant. Aggrecan is a large chondroitin sulfate proteoglycan, which due to its molecular structure produces a rigid,
reversibly deformable gel that resists compression. Besides aggrecan and HA other proteoglycans are also found in cartilage, examples are: fibromodulin, lumican, epiphycan, decorin, biglycan, perlecan, syndecans and glypicans (89). Proteoglycans are composed of a core protein synthesized by endoplasmic reticulum, attached to several glycosaminoglycans (GAGs) abundant in sulfate and carboxyl groups. These groups, due to their negative charges (hydrophilic), drive the attraction of water molecules in large amounts, which then accounts for the resilient nature of cartilage (89).

The chondrocytes present in cartilage are disposed in zonal stratification and enclosed in the arcade-like network of collagens fibrils, to which proteoglycans are attached. This specialized spatial organization of the articular cartilage and the enclosed chondrocytes results from the complex endochondral ossification process that occurs during embryogenesis (90). The endochondral ossification has two centers of ossification; the primary center ossification that occurs at the diaphysis and the later secondary ossification center occurring at the epiphysis. Figure 2 presents schematically the cartilage structure across the different cartilage zones.

Figure 2. Histological Illustration is showing the features of healthy human articular cartilage and subchondral bone. At the superficial zone chondrocytes are small and flattened, which are orientated parallel to the joint surface. Small and medium-sized collagen fibrils are aligned parallel to the cartilage surface. In the middle zone chondrocytes become more rounded and are randomly oriented. The collagen fibrils start to become orientated towards a vertical direction relative to the cartilage surface. In the deep zone, chondrocytes are clustered in columns know as complex chondrons where they share pericellular matrix. At this point, the large collagen fibrils are aligned almost vertically to the cartilage surface.
As earlier stated, chondrogenesis is at the onset of the endochondral ossification process. It relies on synchronized events that allow mesenchymal cell recruitment and migration, proliferation and later condensation of mesenchymal chondroprogenitors cells, allowing them to establish the formation of pre-cartilaginous condensations. During chondroprogenitors differentiation into chondrocyte cells, the ECM composition undergoes changes. During chondroprogenitors differentiation, initial expression of collagen type I is reduced giving its place for chondrocytes to start producing collagen II, IX and XI, proteoglycans such as aggregan, and link protein and Gla protein (88). This composition will be then a remnant of the initial cartilage formation, which will be largely retained in adult articular cartilage. In the sites where embryonic cartilage is replaced by bone, chondrocytes differentiate further in a process called hypertrophic differentiation during which they start secreting collagen X. As bone formation starts to occur, cartilage is vascularized from the perichondrium. As a result ECM mineralization occurs, due not only to the hypertrophic chondrocytes but also later, as the formation of the bone matrix progresses, to the coordinated action of mineralizing osteoblasts and bone resorbing osteoclasts. These migrate to help remodel cartilage into mature bone (91).

In summary, articular cartilage is an avascular and aneural connective tissue made of chondrocytes entrapped in lacunas by an arcade-like network of fibrils of collagens and proteoglycans. Together this organized network of molecules will be passed to the orientation and shape of chondrons (basic cellular structure consisting of one or more chondrocytes surrounded by pericellular matrix). Moreover, since it is avascular, cartilage nutrition occurs through diffusion. The predominant source of diffusion is the SF. To nutrients reach the chondrocytes residing in the cartilage, they must pass through a double diffusion system, the synovial membrane and the cartilage matrix (92). This diffusion mechanism is helped by the pumping action of its compression or by the flexion of the elastic cartilage (93).

2.3.2. Preclinical OA

By the time OA is clinically established with observed radiographic joint changes, the joint tissue level of structural and degenerative changes as seen in cartilage, imply almost irreversible damage. Since OA treatment is symptomatically driven, this may help to explain why current treatments are not delaying OA arthroplasties. Currently, many researchers in the field believe that if any OA treatment is going to be successful, it should be started in the earliest phase of the disease before the clinical diagnosis, in other words, in preclinical OA. Until nowadays OA clinical diagnostics has relied on observed radiographic changes. However, OA joint abnormalities undetectable by X-ray can be observed using more sensitive imaging techniques such as MRI or ultrasounds (94,95).
While the use of more advanced imaging techniques may one day change the clinical threshold for OA diagnosis, OA associated symptoms cannot be used as diagnostic tool given that symptoms increase, decrease, and disappear during preclinical and clinical phases of OA, and may also be related to multiple causes. Therefore, the focus of this thesis section is on preclinical OA, the early stages of the disease, which precede OA joint-related structural changes being detectable by imaging techniques.

As discussed earlier, joint trauma is a known risk factor for OA, hence the categorization of post-traumatic OA given the apparent causes for its development (96). Therefore, studying the disease development from trauma to clinical diagnostic offers an exceptional insight into the mechanism of the disease and apparently corroborates the existence of preclinical OA phase within a defined time. Several longitudinal studies following a traumatic event of a joint have undeniably shown a persistent molecular preclinical disease phase portrayed by several protein biomarkers and RNAs at increased concentrations (97-102). Many of these protein biomarkers are fragment products of essential cartilage macromolecules e.g. aggrecan and collagen type II and are critical to the biomechanical properties of the unique structure of articular cartilage (103). Such damaging alteration of native cartilage structure is thought to be naturally irreparable and precursor of the development of post-traumatic radiographic OA. Moreover, many of these released fragments e.g. tenascin-c can also activate wound healing mechanisms which if left unbalanced can cause prolonged inflammation in resident chondrocytes and synovial cells (104,105). This in turn also make such biomarkers potential molecular players in OA pathogenesis.

In comparison with post-traumatic OA, tracking primary idiopathic OA development within a certain time frame is much more challenging. However, some studies have strikingly demonstrated that biomarkers, such as a combination of cartilage oligomeric protein (COMP) and hyaluronic acid (HA), could predict the development of joint radiographic abnormalities in knee and hip joints a few years earlier (106-108). Interestingly, preclinical OA existence seems to be further corroborated by cadaveric studies which have shown an incidence of 69% and 86% of articular cartilage lesions (radiographically undetected and asymptomatic) in cadaveric knees and hands, respectively (109,110). Together these studies have shown that molecular alterations do occur before radiographic OA, and thus, further support the existence of a preclinical phase of OA.

2.3.3. OA pathology

The progressive histological changes in articular cartilage in OA are well recognized. Macroscopically, normal hyaline articular cartilage is sleek and pale cream to yellow in colour. In OA, the cartilage seems discoloured, soft, and cracked with the underlying subchondral bone exposed at end stages. Such
changes may be observed and graded radiographically as well as further studied in detail, histologically. In early OA, cartilage physical or proteolytic disruption of the type II collagen network results in increased water content and proteoglycan swelling of articular cartilage leading to increased cartilage volume (111). As OA progresses and the expression and activity of cartilage matrix-degrading enzymes increase, the cartilage proteoglycan content is reduced substantially. Later on, direct physical forces on the weakened cartilage cause surface matrix fibrillation, cracks within the superficial layer of the articular cartilage that run parallel to the surface. These cracks expand, following the collagen fibers orientation within the middle and deep cartilage zones. The ongoing mechanical traumas cause fissure branches to propagate, and this, together with the continued proteolytic activity, results in continued cartilage loss. In contrast to the reduction in the volume of noncalcified articular cartilage, the thickness of the calcified cartilage zone will increase. At this stage, microcracks appear in calcified cartilage which may extend into subchondral bone lining causing bone remodelling. The interface zone between non-calcified and calcified cartilage, known as tidemark is seen as duplicated or multiplied reflecting the process of the cartilage thinning (87). The intimately related subchondral bone structure is altered, thickened, and bone sclerosis occurs. The failed remodelling of subchondral bone microfractures leads to the microfracture space to be filled with non-native fibrocartilaginous tissue derived from local joint stem cells. In OA late stages, cartilage-naked subchondral bone is covered with a fibrocartilaginous tissue arising from stem cell differentiation (87).

2.3.4. Grading of osteoarthritic cartilage alterations

Traditional diagnostic criteria commonly used in many other diseases are difficult to apply for OA classification, mostly due to the heterogeneity of the causes of disease and its symptoms. This task is even more difficult to implement for the classification of disease progression. Along with the progression of OA cartilage degeneration is increased. Although other joint tissues are also involved in OA, cartilage has traditionally been used to score OA severity.

Several scoring systems exist to estimate the progression of the disease process of OA, based on imaging techniques commonly applied to OA, i.e. radiological, MRI and ultrasound (112). These systems such as the Kellgren-Lawrence system, and others, enable the relative description of visible features through OA stage progression. However, these systems use radiographic evidence and other macroscopic observations. As tissues changes occur prior to visible lesions, this leads to the necessity of clearly identifying underlying molecular events in order to better understand the role of cartilage pathological features, characteristics of OA biological activity and progression. Histological evaluation enables researchers to observe at the microscopic scale the cellular/molecular events intrinsically related to the cartilage alterations that occur in OA progression.
For the histopathological assessment of OA, there are currently two dominating approaches: the one proposed by Mankin et al. and the one from Pritzker et al. which is endorsed by the Osteoarthritis Research Society International (OARSI) (87,113). Both grading systems have high reliability, reproducibility and variability (114). However, the OARSI grading arose from the need to address specific issues not covered by the Mankin based systems, such as severity of cartilage damage and percentage of area affected.

The OARSI grading system defines grade as the OA depth progression into cartilage, assuming that OA involvement of deeper cartilage layers reflects a more advanced disease and, therefore, is in line with the pathological cartilage features. The grading methodology of the OARSI system is summarized in Table 2. Description of cartilage zones natural architecture can be found in Table 1. Histological staining features used in the OARSI system to grade the OA cartilage degradation are represented in Figure 3.
### Table 2. The OARSI grading methodology for OA cartilage histopathology grade assessment.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Associated feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 0:</td>
<td>Surface and morphology</td>
</tr>
<tr>
<td></td>
<td>Intact, uninvolved cartilage</td>
</tr>
<tr>
<td>Grade 1:</td>
<td>Surface Intact</td>
</tr>
<tr>
<td></td>
<td>Matrix: superficial zone intact, edema and/or fibrillation</td>
</tr>
<tr>
<td></td>
<td>Cells: proliferation (clusters), hypertrophy, Reaction must be more than superficial fibrillation only</td>
</tr>
<tr>
<td>Grade 2:</td>
<td>Surface discontinuity</td>
</tr>
<tr>
<td></td>
<td>As previous</td>
</tr>
<tr>
<td></td>
<td>+/- Discontinuity at superficial zone</td>
</tr>
<tr>
<td></td>
<td>+/- Cationic stain matrix depletion (Safranin-O or Toluidine Blue) upper 1/3rd of cartilage (mid zone)</td>
</tr>
<tr>
<td></td>
<td>+/- Disorientation of chondron columns</td>
</tr>
<tr>
<td>Grade 3:</td>
<td>Vertical fissures</td>
</tr>
<tr>
<td></td>
<td>As previous</td>
</tr>
<tr>
<td></td>
<td>+/- Cationic stain depletion (Safranin-O or Toluidine Blue) into lower 2/3rd of cartilage (deep zone)</td>
</tr>
<tr>
<td></td>
<td>+/- New collagen formation (polarized light microscopy, Picro Sirius Red stain)</td>
</tr>
<tr>
<td>Grade 4:</td>
<td>Erosion</td>
</tr>
<tr>
<td></td>
<td>As previous</td>
</tr>
<tr>
<td></td>
<td>Cartilage matrix loss, cyst formation within cartilage matrix</td>
</tr>
<tr>
<td></td>
<td>Excavation: matrix loss superficial layer and mid zone</td>
</tr>
<tr>
<td>Grade 5:</td>
<td>Denudation</td>
</tr>
<tr>
<td></td>
<td>Surface is sclerotic bone or reparative tissue including fibrocartilage</td>
</tr>
<tr>
<td>Grade 6:</td>
<td>Deformation</td>
</tr>
<tr>
<td></td>
<td>Bone remodeling. Deformation of articular surface contour</td>
</tr>
<tr>
<td></td>
<td>(more than one osteophyte formation only)</td>
</tr>
<tr>
<td></td>
<td>Includes: microfracture and repair</td>
</tr>
</tbody>
</table>

Adapted from Pritzker et al. (87)
2.3.5. Articular cartilage and chondrocytes: the OA phenotype

As described elsewhere articular cartilage is a unique highly specialized tissue with one of a kind biomechanical properties, and solely populated by one cell type in an avascular, alymphatic and aneural microenvironment. These unique characteristics make it challenging for the cartilage to be naturally regenerated and also reconstructed or engineered. Disruption of the collagen network and proteoglycan by matrix-degrading proteases is a major threat to cartilage matrix integrity. Such cleavage and degradation of matrix molecular components is harmful per se but also compromises the structure of the residing supramolecular proteins which give cartilage so unique properties. This enzymatical degradation of articular cartilage leads to the erosion of pericellular matrix and eventually the interterritorial matrix which will ultimately compromise and alter cartilage biomechanical properties leading to the destruction of articular cartilage (115,116).

To date, we have extensive knowledge about the degradation processes of the two major components of articular cartilage: the collagen network and the rooted proteoglycans. Loss of aggrecan and its bounded cationic proteoglycans is one of the most striking features of early stages of cartilage degeneration (87). While collagen content is not as heavily reduced, its network, in turn, is highly disrupted making it also a fundamental feature of cartilage osteoarthritic changes. Yet the two have reciprocal effects i.e. collagen network degradation leads to loss of GAGs and GAGs loss alter biomechanical properties leading to joint overload which will inflict further damage to the collagen network structure.

As mentioned before the cartilage degradation process is prominent in the pericellular matrix and superficial zone. At this zone and throughout the osteoarthritic cartilage abnormal levels of many

![Figure 3. Toluidine blue staining of OARSI-graded osteoarthritis (OA) samples, grades G1-G4.5. Surface (tangential, gliding), middle (transient), deep (radial) zones, tidemark (between cartilage and calcified cartilage) and subchondral bone are marked.](image)
metalloproteinases including matrix metalloproteinases (MMPs), as well as members of the ADAM (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin type 1 motif) families are observed and undeniably contribute to the increased matrix degradation in OA cartilage (117). These enzymes play a significant role in cartilage degradation. However, attempts to identify the most crucial protease have been unsuccessful. This may imply that all of them are vital and that treatment strategies should target upstream regulators.

Collagen type II is the primary collagen type present in articular cartilage, which forms the collagen network necessary for cartilage stiffness. Therefore, proteases degrading such important molecule deserve significant attention. MMP-1 degrades collagen type II efficiently. It is upregulated in cartilage and SF from OA patients, its gene polymorphism has also been shown to be associated with OA in a population study, and MMP1 expression is typically up-regulated in chondrocyte induced-inflammation (118-120).

With the strongest OA cartilage staining features of all MMPs, MMP-3 is another important protease (121). MMP-3 also mediates the activation of other collagenases e.g. MMP-1 and MMP-13 (122). Mice models have shown that MMP-3 KO mice are protected against site-specific cleavage of collagen and aggrecan, but at the same time may produce a severe OA type when surgically induced (123,124). Another MMP shown to be associated with OA is MMP-9, a collagenase, which has been proved to be protective in OA mice models (125).

Among MMPs, MMP-13 has been the subject of most intensive attention given its extensive collagenase activity. MMP-13 is expressed by chondrocytes, and it hydrolyses type-II collagen more efficiently than others (126). MMP-13 has been shown to be upregulated in OA cartilage (127).

Together MMP-1,-2,-3,-7,-9, and -13 also are able to cleave aggrecan at the Asn341~Phe342 bond, however it has been shown that the majority of aggrecan neoepitopes present in OA are made at Glu373~Ala374 bond specific for ADAMTS-4 and ADAMTS-5 activity (128,129). The significant presence of Glu373~Ala374 neoepitopes gives further importance to the role of ADAMTS-4 and ADAMTS-5. These aggrecanases are typically upregulated in chondrocyte induced-inflammation as well as in human OA cartilage (119,130). Mice models have also shown that ADAMTS-4 and -5 mice KO with prevented cleavage of Glu373~Ala374 neoepitope are protective for cartilage destruction in surgically induced OA and antigen-induced arthritis (129,131,132).

Another class of major “players” are cathepsins which originate from the lysosomal compartment. Of the existing ones, cathepsin K (CTSK) deserve the most attention. It has been shown to be upregulated in cartilage, SF and serum from OA patients. Cathepsin-k activity is pH dependent, and it seems to give it further importance since OA cartilage becomes progressively acidic as the
disease progresses (133). Therefore it is not surprising that recent studies have been able to
demonstrate increased activity of cathepsin-k in OA cartilage and in chondrocytes (134,135).

The majority of these proteases localized in articular cartilage are produced by local resident
chondrocytes, the only cell type present in articular cartilage. They are, therefore, central “players” in
tissue homeostasis, acting in concert to preserve the structural integrity of ECM in articular cartilage.
Not only these cells play a role in matrix catabolism, but they do also actively regulate the matrix
anabolism. In healthy cartilage, chondrocytes synthesize low amounts of new ECM molecules to
replace damaged molecules, meanwhile, in OA their anabolic activity is clearly altered.

In chondrocyte-induced inflammation and OA chondrocytes phenotypic studies, the catabolic
events are not sufficiently counterbalanced by the anabolic events given the insufficient synthesis of
cartilage matrix molecules e.g. collagen type II, V and aggrecan (119,136). Such imbalance leads to
failure to compensate the total matrix cartilage damage induced in the local synovial joint.

2.3.6. OA pathogenesis: a modern view

As earlier described, clinical OA is preceded by a preclinical stage which together with the presence of
risk factors and/or other pathological processes cause the disease to enter in a state of radiographic
OA. Several of these risk factors are considered by some to be the main single pathological event
which may explain OA pathogenesis. However, along the research history in this field, neither a single
nor two risk factors could alone explain OA pathogenesis. The emerging current view in the field is
that such risk factors and triggering mechanisms act together in driving the disease into the
radiographic stage.

As discussed in previous sections, the presence of preclinical stage OA demonstrates that OA
is an apparently active disease process. As cartilage is subject to considerable mechanical impact,
compression, tensile and shear loading this may cause micro and or major trauma to the cartilage.
Moreover, such mechanical insults can be mechanically transduced by mechanosensors present in
resident chondrocytes which then may alter their normal phenotype and activate certain matrix
degrading enzymes (137). The generated fragments may then accumulate to increase levels during
time, given the cartilage alymphatic, avascular and aneural characteristics and the nonlinear and size
dependent molecular clearance rate of the synovial membrane (138,139). As the cartilage
degenerates, matrix molecules and fragments are released and accumulated leading to the
propagation of inflammation through the activation of innate immune response by damage associated
molecular patterns (DAMPs) (140). Such occurrence of inflammatory and immune reactions, clinically
presenting as inflammatory flares, are unmistakable points for OA diagnosis. While inflammation as
the principal triggering mechanisms or a primary driver is still very much debated, inflammatory pathways are undoubtedly involved in the pathogenesis of OA (6,141,142).

Metabolism and associated cascades of chondrocyte and fibroblast-like synoviocytes (FLS) can be altered by inflammatory factors such IL-1 and IL-6 which can lead to a phenotypic change of chondrocytes towards an inflammatory type i.e. becoming hypertrophic, destructing cartilage and initiate bone remodelling (143). Interestingly, chondrocyte can also be sensitized to inflammatory signals. It was shown in mice that upregulation of syndecan-4 during induced OA progression mediates the response to IL-1, by altering the secretion of several collagenases and aggrecanases (144). Not only inflammatory signalling cascades are triggered by cytokines, but inflammatory pathways mediated by complement, metabolic and innate immune system are also present.

As we know today, the innate immune system is an intrinsic part in the inflammatory cycle of OA (145,146). The key point of innate immune systems role in OA lies in how it reacts to the mechanical, physiological and biological changes in the joint over time. In contrast to the adaptive immune system, innate immunity plays an essential role not only in host defense against microbial agents but also in modulation of tissue homeostasis by recognizing distinct pathogen-associated molecular patterns (PAMPs) and DAMPs, respectively, by pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and NOD-like receptors (NLR) (147). Therefore, cartilage matrix degradation products derived either from trauma, microtrauma (from repetitive overuse), or normal aging degeneration lead to the release of DAMPs that may then activate a local innate immune system reaction (148,149). The activation of these inflammatory pathways induces chondrocytes signalling changes which lead to the upregulation of cartilage matrix degrading proteases such as MMP-1, MMP-3, MMP-13, and ADAMTS aggrecanases while also downregulating aggrecan and collagen type II, a pattern generally seen in the OA chondrocytic phenotype. Such gene expression patterns within articular chondrocytes are mediated via multiple intracellular pathways including also the macrophage adhesion molecule (MAC) and Mitogen-activated protein (MAP) kinases and nuclear factor-κB pathways (NF-κB) (150,151).

As mentioned earlier, inflammation occurs in local synovial joint during OA, and, therefore, cartilage and local chondrocytes are not solo “players” in OA pathogenesis. Besides clinical relevance, synovial inflammation is also part of the OA pathogenic mechanisms. In contrast to RA, synovial inflammation in OA is thought to be secondary to the release of cartilage degradation products including DAMPs (140). OA synovium has increased FLS activation, proliferation and infiltration of inflammatory cells consisting of macrophages and lymphocytes (152). Such events may, in fact, represent reactive changes as a response to the overwhelming need of synovial fluid clearance flooded in cartilage debris and DAMPs. Studies have also shown that altered molecular composition does also
change the SF viscosity among other mechanical properties essential for synovial joint mechanical function (153,154). Therefore, when synovial alterations occur such as in inflammation and hyperplasia, the permeability of the membrane is also altered (139). Different permeability may help explain why systemic markers, e.g. HA, which are increased in local OA joint, may then be found in serum from OA patients (155). Activated FLS are able to secrete not only matrix-degrading proteases such as collagenases and aggrecanases but also catabolic cytokines and growth factors (e.g., IL-1, TNF-α) as well as ROS/NOS which will further cross-talk with ongoing inflammatory and catabolic signaling pathways active in chondrocytes. The interplay of mechanical traumas, environmental factors, potentiated by risk factors and genetics, ensued by an inflammation perhaps driven by innate immune response and impaired cartilage repair, is one of the latest modern views of the OA pathogenesis theory (145). Figure 4 represents the unifying model of OA pathogenesis schematically.

![Figure 4. Unifying model of OA pathogenesis.](image-url)

2.4. Toll-like receptors

As previously mentioned, innate immunity may play a significant role in OA pathogenesis, therefore also PRRs and, in particular, TLRs may have an important role given recent findings of their presence in both chondrocytes and chondroprogenitor cells (156-158).

2.4.1. Toll receptor

After 10 years of the proposed theory of pattern recognition of pathogens, the first human PRR was discovered. This work had roots in earlier discovery in the fruit fly Drosophila (159). Although Drosophila had provided ground breaking research for human genetic developmental studies when it came to human immunity, drosophila was considered less significant for immunological studies given its lack of adaptive immune response (160). Interestingly in one of those gene mutations developmental studies eventually a gene for TLR was mutated, at the time baptized Toll, TLR was mutated leading to strange looking drosophilas. Cloning efforts of such gene demonstrated that it encoded a membrane receptor (161).

Later on, functional studies demonstrated that loss-of-function Toll mutations in Drosophila increase the susceptibility for fungal infection while gain-of-function mutations may have been more resistant to fungal infection given the increase production of antifungal peptide “drosomycin” (162). By comparing Toll mutation to other gene mutations, researchers realized the importance of Toll receptor in fungal infections and as part of innate immunity response in Drosophila. Later on by using the amino acid sequence of Toll, researcher were able to find the first human TLRs (163,164).

2.4.2. Structure and function

TLRs are typical type I integral membrane receptors composed of a ligand recognition ectodomain, a single transmembrane helix, and a cytoplasmic signaling domain projecting from the inside part of the membrane (165).Toll IL-1 Receptor (TIR) domains are the signalling domain of TLR. The name was given since they are homologues to the IL-1R family members signalling domains (166). Interestingly TIR domains homologs are also present in plant proteins and nematode C. Elegans conferring them therefore also pathogen resistance. Such phylogenetic relations indicate that the TIR domain and TLR have an ancient evolutionary origin in host defense mechanisms (167-169).

As previously mentioned TLRs play a critical role in the activation of innate host defense, especially against infections by recognizing PAMPs. PAMP-TLR ligand recognition leads to the initiation of cellular activation. Currently, 11 TLR gene members have been discovered, numbered from 1 to 11, of which the first 10 are also functional in humans, albeit to date no natural ligand to TLR10 is known.
TLR1, TRL2, TLR4, TLR5, TLR6 and TLR10 recognize microbial surface patterns and are therefore located on the cell membrane surface to enable an immediate response. Some other TLRs, such as TLR3, TLR7, TLR8 and TLR9, are expressed in the endosomes or phagosomes inner membranes to allow contact with internal microbial structures revealed upon microbial degradation/lyses, such as double and single stranded RNA and DNA (170).

TLR-mediated recognition leads to host responses in the form of *de novo* expression of genes, such as inflammatory cytokines tumour necrosis factor (TNF)-α, IL-1, IL-6, IL-8 and IL-12 and cell-membrane bound co-stimulatory molecules, such as intercellular adhesion molecule-1 (ICAM-1) and its counterpart lymphocyte function-associated antigen-1 (LFA-1) (171).

### 2.4.3. TLR signalling

The surface membrane and endosomal TLRs share similar ectodomain sequences, which is in sharp contrast to the different ligands they bind. By having distinct residues present in the ectodomains of the various TLRs is a way of discriminating between ligands. At the ectodomains of TLRs lie the leucine-rich repeat modules each of them composed of 20–30 amino acids with the consensus sequence LxxLxLxxN. By having different amino acids inserts within the modules, this leads to variations which allow TLR-ligand interaction (172). Nevertheless, such amino acids differences and TLR heterodimerization are not able to fully recognize all the existing TLR ligands. In order for this to occur specific accessory proteins and co-factors are necessary. Co-factors lipopolysaccharide (LPS)-binding protein (LBP), cluster of differentiation 14 and 36 (CD-14; CD-36), myeloid differentiation factor-2 (MD-2), TRIL are some of the currently known existing TLR homo- and heterodimers helping to recognize the broad range of TLR ligands (173). A classic example of co-factor importance is LPS recognition by TLR4 homodimer, where LPS is initially bound to the LBP. This is followed by LBP-LPS complex delivery to CD14, either to its soluble form or cell surface form, which splits LPS into monomeric molecules and presents them to the TLR4 in complex with MD2 (174). Ligand-induced receptor homo- or heterodimerization, leads to the dimerization of the cytoplasmic signaling domains of TLRs. The majority of TLRs form homodimers, however, TLR2 is able to form heterophilic dimers with TLR1 or TLR6, and TLR4 to form and heterodimer with TLR6 (175,176). Such TIR-TIR dimer complexes trigger the signaling downstream, through recruitment of specific adaptor molecules. To date five distinct adaptors are known: myeloid differentiation factor 88 (MyD88), MyD88-adaptor like (Mal), TIR domain-containing adaptor inducing IFN-beta (TRIF), TRIF-related adaptor molecule (TRAM), and sterile alpha and HEAT-Armadillo motifs (SARM) (172). With the exception of TLR3, which is exclusively TRIF dependent, all TLRs depend at least in part on the MyD88-pathway for their full signalling activity. IL-1R-associated kinase-1 (IRAK1), IRAK-2, TNF receptor-associated factor 6 (TRAF6)
and mitogen-associated protein kinase (MAPK) are some of the known accessory molecules to be involved that ultimately will lead to triggering of the transcription factor NF-kB. By being transported via the IkB kinase (IKK) complex it will ultimately activate the production of proinflammatory cytokines. As mentioned earlier TLR3 does not signal through the MyD88 (due to mutation in amino acid chain in position 712 to histidine), instead it uses the TRIF pathway. TRIF associates with TRAF3 and TRAF6, but also with receptor-interacting proteins, that will activate downstream the NF-kB and MAPKs, resulting in the production of interferon (IFN). TLR4 can also signal through this pathway, but requires the adaptor TRAM under some conditions (177). Table 3 resumes some of the currently well described existent PAMPs and DAMP triggering TLRs activation.
Table 3. PAMPs, DAMPs and danger signals able to trigger TLRs signalling activation. (Data adapted from (178,179)).

<table>
<thead>
<tr>
<th>TLRs</th>
<th>Exogenous ligands</th>
<th>Endogenous ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>TLR1/2 bacterial triacyl lipopeptides, OSP of borrelia spp.</td>
<td>—</td>
</tr>
<tr>
<td>TLR2</td>
<td>TLR1/2 bacterial triacyl lipopeptides bacterial peptidoglycan, LAM, LTA, GXM, Zymosan, TLR1/2 Pam3CSK4, viral envelope glycoproteins, APO CIII</td>
<td>HSPs, Biglycan, Hyaluronan, Monosodium urate crystals, Versican</td>
</tr>
<tr>
<td>TLR3</td>
<td>ssRNA, dsRNA, TLR3/9 CMV, Stahmin</td>
<td>ssRNA</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, MMTV, RSV fusion protein, LPA, Fibronectin, viral envelope glycoproteins, Heparin sulphate, Paclitaxel, BCG</td>
<td>Biglycan, B-defensin, decorin, Fibrinogen, Fibronectin isoforms, Heparan, HSPs, Hyaluronan sulphate, Tenascin-C, S100- A4, S100-A8/A9, Resistin, Surfactant protein A, Uric acid</td>
</tr>
<tr>
<td>TLR5</td>
<td>Bacterial Flagellin</td>
<td>—</td>
</tr>
<tr>
<td>TLR6</td>
<td>TLR2/6 di-acyl-lipopeptides, peptidoglycan from gram positive bacteria, Zymozan</td>
<td>—</td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA, imidazoquinoline, bropririme, loxoribine</td>
<td>Self RNA, siRNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>Bacterial ssRNA</td>
<td>Self RNA, siRNA human myosin</td>
</tr>
<tr>
<td>TLR9</td>
<td>Hypo and unmethylated CpG motifs in microbial DNA, HSV-2</td>
<td>Self DNA</td>
</tr>
<tr>
<td>TLR10</td>
<td>unknown</td>
<td>—</td>
</tr>
</tbody>
</table>

CMV - cytomegalovirus; dsRNA - double-stranded; siRNA – small interfering RNA; ssRNA - single stranded; LPS -lipopolysaccharide; MMTV - murine mammary tumor virus; RSV- respiratory syncytial virus; LTA - lipoteichoic acid; HSV - herpes simplex virus; HA -hyaluronan; OSP - outer surface protein; BCG - Bacillus Calmette-Guerin, LAM- lipoarabinomannan; EDN - eosinophil-derived neurotoxin; Pam3CSK4- synthetic triacylated lipopeptide; LTA - lymphotoxin alpha; APO - Apolipoprotein; GXM - glucuronoxylomannan

2.4.4. Negative regulation of TLR signalling

Uncontrolled inflammation responses are also dangerous and sometimes lethal as seen in autoimmune diseases and septic shock. TLRs together with PRRs are the first line of defence, they recognize danger signals and activate innate and adaptive immune system. It is, therefore, of primary importance to strictly control this response. Several mechanisms exist to control TLR-signalling. These regulatory mechanisms may act by restricting TLR activation or by inhibiting the ongoing TLR-
signalling. TLR-signalling regulatory mechanisms can be categorized into three major groups: dissociation of adaptor complexes; degradation of signal proteins; and transcriptional regulation (180). So far several TLR signaling negative regulating molecules have been discovered. However, only a few will be exemplified in this thesis section.

As for dissociation of adaptor complexes, several molecules are known to exist. TRAM adaptor with GOLD domain (TAG) and sterile alpha- and armadillo-motif-containing protein (SARM) are known to bind directly to TRIF, preventing this way the TRIF accessory molecules to bind and activate the TLR4 TRIF-dependent pathway (181,182). As for MyD88 dependent pathway interferon regulatory transcription factor 4 (IRF4), which is induced by TLR activation, is known to compete with IRF5 for MyD88 TIR domain binding, and, therefore, altering the IRF5-dependent gene induction (183).

At the TLR-transcriptional regulation level, different classes of molecules are known to exist. Cyclic AMP-dependent transcription factor (ATF3), TLR-inducible inhibitor of kappa B (IκB) protein, nuclear receptor related 1 protein (Nurr1) are some of the known transcriptional TLR regulators. However, miRNAs have now emerged as fine tuners of TLR signaling by targeting TLR-mRNA and have been the subject of many studies in innate immunity relevant diseases and also in OA.

MicroRNA miR-155 is a TLR-inducible miRNA with both positive and negative regulatory effects on immune responses (184-187). On one hand, TLR signaling is suppressed by miR-155 through targeting of MyD88, TGF-beta Activated Kinase 1 (TAB2) and IκB kinase (IKK) (184). On the other hand, miR-155 leads to increased TLR-signalling e.g. by targeting the anti-inflammatory lipid phosphatase SHIP (186,187). Together this suggests that miR-155 can fine tune TLR signalling depending on the cells and tissue TLRs are expressed.

MicroRNA miR-146a was initially identified as a TLR4-induced miRNA that can target the signalling proteins TRAF6 and IRAK1 (188). Moreover, miR-146a upregulation leads to repression of IRAK1 expression levels which seem beneficial for healing of bacteria-induced tissue damage (189). Interestingly in cartilage retrieved from TKA patients, miR-146a expression appears to be upregulated in cartilage with low degradation further decreasing while cartilage degradation score increases (190). As it is also known receptor decoys are a general natural strategy to regulate the action of proinflammatory cytokines and chemokines (191). Interestingly, naturally present receptor decoy strategy for TLR-signalling receptor regulation has been also reported. Initially decoy receptor of TLR2 was found in the bacteria-rich saliva. By then researchers were able to detect soluble TLR2 in human plasma and breast milk. Moreover, they were able to demonstrate monocyte production of sTLR2 and its negative regulatory properties of TLR2-mediated signalling. Interestingly sTLR2 presence in breast milk was speculated to have a role in avoiding an excessive local inflammation of the neonatal gut.
following bacterial colonization (192). Such ground-breaking work led to a biomimetic strategy of the construction of a recombinant soluble form of TLR4. This led to the demonstration in vitro that soluble TLR4 together with accessory molecule MD-2 is able to bind to LPS outside cell surface and attenuated LPS-mediated production of IL-8 (193). This was followed by in vivo proof of concept study which demonstrated the ability of recombinant sTLR4 together with sMD2 to damp LPS-induced pulmonary inflammation in mice (194). sTLR4 has then been found in several body fluids and species such as, in urine during acute renal failure in a mouse model, in human bacteria-rich saliva in oral lichen planus (OLP) saliva, and in human plasma of patients with inflammatory and autoimmune conditions (195-197). Interestingly, sTLR2 present in breast milk was shown to mediate HIV infection and inflammation (198). Other body fluids reported to contain sTLR2 were saliva from oral lichen planus disease, parotid saliva, and human plasma of several inflammatory conditions (197,199,200). Given the role of innate immunity and TLR-mediated signalling in OA pathogenesis, it remains, therefore, to be seen if TLR decoy receptors are present e.g. synovial fluid from joint pathology conditions such as OA or RA.

2.4.5. TLR and their role in OA

2.4.5.1. Synovium

OA is a disease affecting the entire joint. Although the main feature of OA is cartilage degeneration, synovium and subchondral bone are also affected. Synovitis is present in about half of the patients with OA and has been shown to correlate with cartilage damage severity (201). Therefore, TLR expression in the synovium is of interest. TLR 1-7 and TLR9 have been shown to be present in the synovium of OA patients, although not to the same extent as in those with RA (202,203). Moreover, functional studies in isolated primary FLS from normal, OA and RA patients have shown that expressed TLRs do actively respond to several DAMP ligands, such as tenasin-C or S100A8/A9 (104,204). TLR activation and the ensuing NF-κB activation followed by production of chemokines (e.g. IL-8 and CCL5) and cytokines (e.g. IL-1, IL-6 and TNF) are able to recruit macrophages, granulocytes and lymphocytes which indeed have been shown in the synovium of OA patients (152). Moreover, such secreted cytokines and chemokine can also cross talk with resident chondrocytes.

Several DAMPs are present at increased concentration levels in the synovial joint fluid and tissues in the setting of joint injury and OA. The majority of them are cartilage degradation products, albeit some arise from cell apoptosis e.g. high mobility group box protein 1 (HMGB1). These include danger signals such as S100/calgranulin family (e.g., S100A4, the S100A8/A9 heterodimer, S100A11, S100A12), tenasin-C (TN-C), high and low molecular weight hyaluronan, fibronectin isoforms, and SLRPs such as biglycan and decorin (104,204-212). In this context, the observation that large majority of this DAMPs are TLR4 ligands, and TLR4 itself is a highly expressed TLR in synovial fibroblasts is of
interest (203). Moreover in TLR4 KO mice, synovitis is less pronounced as compared with wild-type mice when IL-1β is administered, indicating that perhaps local IL-1-driven joint pathology is dependent on TLR4 activation and the agonist expression (213). Interestingly, transient plasma proteins in SF were reported to induce TLR4-mediated inflammatory responses in macrophages, which as mentioned earlier infiltrate the OA synovium (152,214).

### 2.4.5.2. Subchondral bone

Subchondral bone alterations are known to occur in OA and events such as the development of osteoporotic and sclerotic bone under the cartilage degradation zones are fine examples of this intimate correlation (215). Mechanical loading and cross-talk between cartilage and intimately connected subchondral bone enable that the chondrocytes secrete catabolic, inflammatory and anabolic factors diffused thorough cartilage to influence the subchondral bone microenvironment and vice versa (68,216). It is, therefore, interesting to note that also TLR2 and TLR4 are expressed in osteoblasts. Moreover, osteoblasts have the same ability as macrophages to down-modulate cytokine secretion during chronic TLR4 challenge (217). TLR signalling alters not only inflammatory properties of osteoblasts but also their osteogenic potential. Studies have shown that by activating TLR2 while TLR4 is inhibited, leads to increasing levels of osteogenic markers. On the other hand, if TLR4 is strongly activated this will downregulate osteogenic markers (218). In vivo studies using TLR2 KO mice showed an increased bone loss which in contrast was not observed in TLR4 KO. However, careful interpretation is necessary given that the observed bone loss effect was dependent on mice strains used (219,220). In addition, TLR4 activation was shown to inhibit mouse osteoblast differentiation albeit in the co-presence of bone morphogenetic protein 2 (BMP-2) (221,222). TLR4 also has effects on another important bone remodelling cell, the osteoclast by modulating their formation, survival and activity (223,224). These findings are of particular importance given the known implication of bone mineral density, albeit conflicting reports, as a risk factor for OA development. There seems to be a lack of studies reporting TLR expression in human OA subchondral bone tissue. However, the known baseline expression of TLRs in human osteoblasts and osteoclasts, together with the known increased upregulation of TLR4 expression in subchondral bone in surgically induced OA mouse model, makes TLR-mediated inflammation of subchondral bone an important component of OA pathogenesis (225).

### 2.4.5.3. Articular Cartilage

As cartilage degradation is a central feature of OA, the joint innate immunity regulation by local chondrocytes is, therefore, of particular interest. Despite the generalized consensus of TLR expression in the synovial membrane, the same cannot be said of this finding in articular cartilage. Most probably
this is due to the fact that up until recently only a few consider this tissue as a primary mediator of
inflammation in OA. Conflicting TLR expression profiles in chondrocytes and OA cartilage have been
reported, particularly for TLR1 and TLR9, with only concordant results for TLR2 (156,157,226).

Currently, OA progression is graded according to the degradation severity of OA cartilage. However, less is known about OA pathogenetic mechanisms and its key players such as TLRs during the disease progression. Interestingly, so far only one study has in fact shown that TLR4 seems to increase as the Mankin- histological score severity is increased during OA progression (227). Nevertheless, more studies would be beneficial to the field, by applying the same strategy to the other TLRs expressed in chondrocytes and their ligands, using updated and more consensual histological score such as the OARSI score (87).

Cartilage matrix-mediated mechanotransduction mechanism and contribution to OA development has been gaining further support and evidence as one of the limitation factors of OA pathogenesis. It is interesting to note that TLR4 expression seems to be regulated by the applied shear stress to chondrocytes during time, with high shear stress causing TLR4 upregulation. In contrast, during prolonged shear stress TLR4 is downregulated. The ensuing inflammatory response and IL-6 production was dependent on TLR4 upregulation (228).

Several DAMPs such as low molecular weight hyaluronan, s100A8/9, fibronectin fragments and others, all with cartilage matrix origins have been shown to be recognized by TLRs expressed in chondrocytes.

In line with cartilage ageing contribution to OA pathogenesis, advanced glycation end product (AGE) which are part of the existing DAMPs family, seem to accumulate at a higher rate in articular cartilage than in skin during natural aging. This increased presence of AGE was shown to accumulate particularly in the collagen network which in turn undermined its mechanical properties (229). Such increase of accumulation has been shown to be recognized by PRRs such as the receptor for advanced glycation end-products (RAGE) and TLR4 expressed in human chondrocytes leading to the response of secretion of catabolic factors such as IL-6, COX-2, HMGB1 and MMP-13. Interestingly AGE was not only recognized by TLR but also upregulated TLR4 expression levels (230). Together this suggests PRRs, and, in particular, TLR4 to be involved in age-related OA.

Present in cartilage matrix, fibronectin is a glycoprotein which has been shown to be upregulated in synovial fluid and cartilage of OA patients (231,232). Initially, it was also shown that some fibronectin domains induce catabolic responses on cartilage (233). Later, it was shown that upregulated proteases participating in cartilage degradation of OA were able to cleave fibronectin into different fragments lengths similar to those found in OA cartilage (234).
It took the discovery of human TLRs to decipher further how fibronectin fragments were being recognized. While some fibronectin fragments have been shown to elicit catabolic responses through TLR2 recognition, the domain 13-14 of 29 kDa fibronectin fragment are also recognized by TLR4 (226,235).

Serum amyloid A, a TLR4 ligand, is also found at increased concentrations as OA severity increases. Serum amyloid A was found to be upregulated in the serum and synovium of OA patients and serum amyloid A levels correlated with radiographic progression of OA. In vitro studies further demonstrated serum amyloid A recognition by TLR4 in human chondrocytes and synovial fibroblasts which resulted in increased production of cytokines and collagenases (236).

Other known TLR ligands present at increased levels in the OA synovial joints are a member of the calcium-binding proteins family, specifically, S100A8 and S100A9. Initially, S100A8 and S100A9 were found to be increased at cartilage lesions of RA patients. However, following studies from the same lab demonstrated that this particular feature was also true in OA cartilage lesions. S100A8 and S100A9 levels were correlated with expression of MMPs and proteoglycan depletion (237,238). Interestingly the initial studies demonstrated that chondrocytes were able to synthesize S100A8 and S100A9, and their synthesis was upregulated by known OA inflammatory factors such as IL-1β, TNFα, IL-17, and IFN (237). Further studies demonstrated that S100A8 and S100A9 were able to be recognized by TLR4 expressing chondrocytes, which induced catabolic responses leading to upregulated secretion of collagenases and aggrecanases while collagen type II and aggrecan were inhibited (238). The findings of this study were replicated in OA mouse models, where S100A8 and S100A9 stimulations caused a similar increase of known OA catabolic factors (239). Interestingly, S100A9 KO in experimental arthritis mouse model caused a decrease in osteophyte formation compared to the wild type. Moreover, S100A8 stimulation during murine mesenchymal stem cells (MSC) chondrogenesis leads to upregulation of collagenases, which indicate that local MSC actively participating in osteophytosis may be primed with the s100A8 ligand to continue osteophyte formation (240). Finally, S100A8 and S100A9 concentration levels at baseline of early symptomatic OA patients predicted osteophyte formation after 2 or 5 years (240).

Besides the above-mentioned proteins, two other proteins with TLR activation properties and present in cartilage matrix have yet to be studied in OA regarding their inflammatory potential. Biglycan (BGN) and decorin (DCN) are two small structural proteoglycans with leucine-rich repeats (small leucine-rich proteoglycan, SLRP) composing cartilage matrix structure (89). However, if BGN or DCN are released from the cartilage matrix, possibly when the collagen network is disrupted and during tissue injury, their soluble forms could act as an endogenous danger signals (241). Several studies have shown that sBGN can trigger TLR2 and TLR4 activation in aortic valve cells, activate the
NLRP3 inflammasome, and to trigger acute ischemic kidney failure in mouse models (242-244). DCN, on the other hand, has been less studied. However, recent studies have shown it can activate TLR2 and TLR4 leading to the production of proinflammatory mediators able to modulate and control tumour growth in a cancer mouse model (212). Several studies seemed to point to the direction of BGN and DCN having a role in OA, given their upregulation in OA cartilage and increased forms of BGN and presence of DCN auto-antibodies in SF from OA (245,246). Table 4 resumes the known receptors expressed in the synovial joints and their know DAMPS and modulators present in OA joint.

Table 4. Known DAMPS and molecular modulators of TLRs present in the osteoarthritic joint.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Receptor expression in OA synovial joint cell</th>
<th>DAMP present in OA synovial joint</th>
<th>Modulator of receptors in the synovial joint</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1/2</td>
<td>FLS, chondrocytes</td>
<td>_______</td>
<td>_______</td>
<td>(202)</td>
</tr>
<tr>
<td>TLR2</td>
<td>FLS, chondrocytes</td>
<td>Fibronectin fragments,</td>
<td>Mechanical strain, TNF-α</td>
<td>(247,248)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMGB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR3</td>
<td>FLS, chondrocytes</td>
<td>dsRNA</td>
<td>_______</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(202,249)</td>
</tr>
<tr>
<td>TLR4</td>
<td>FLS, chondrocytes</td>
<td>Fibronectin fragments,</td>
<td>Mechanical stress, TNF-α, SF plasma proteins</td>
<td>(248)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S100A8-A9, TN-C, LMW-HA, HMGB1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td>FLS, chondrocytes, bone</td>
<td>Functionally proofed but ligand unidentified</td>
<td>TNF-α, IL-8</td>
<td>(250)</td>
</tr>
<tr>
<td>TLR6/2</td>
<td>FLS, chondrocytes</td>
<td>Versican</td>
<td>_______</td>
<td>(251,252)</td>
</tr>
<tr>
<td>TLR7</td>
<td>FLS, chondrocytes</td>
<td>ssRNA</td>
<td>_______</td>
<td>(202,249)</td>
</tr>
<tr>
<td>TLR8</td>
<td>FLS, chondrocytes</td>
<td>ssRNA</td>
<td>_______</td>
<td>(202)</td>
</tr>
<tr>
<td>TLR9</td>
<td>FLS, chondrocytes</td>
<td>dsRNA</td>
<td>_______</td>
<td>(202)</td>
</tr>
</tbody>
</table>

ssRNA: single-stranded RNA; dsRNA: double-stranded RNA; FLS: Fibroblast-like synoviocytes; TN-C: tenascin c; SF: synovial fluid; LMH-HA: low-molecular-weight hyaluronan; HMGB1: high-mobility group box 1; TNF-α: tumour necrosis factor alpha; IL-6: interleukin 6; IL-8: Interleukin 8; S100A9: S100 calcium-binding protein A9.
3. Aims of the study

The principal aim of this study was to further extend the knowledge of how TLRs are regulated in OA with a particular focus on the role of chondrocytes.

In order to achieve the principal aim of this study, we focused on addressing the following specific issues:

A. To study the regulation of TLRs during the differentiation of chondroprogenitors, and the response of mature chondrocytes to inflammatory mediators present in OA.

B. To study the regulation of TLRs in articular cartilage during the OA development in defined disease severity stages.

C. To compare the expression of TLRs in articular cartilage from different osteoarthritic joints.

D. To study synovial fluid for the presence of never reported endogenous ligands able to trigger TLR activation and study their possible impact on inflammatory mechanisms of OA and cartilage degradation.
4. Material and Methods

4.1. Patients and samples

4.1.1. Ethical aspects

All patient samples used in studies of this thesis were approved by the ethical committees of Helsinki University Central Hospital (I, II, III), ORTON Invalid Foundation (I, II, III), and Biobanco-IMM (III).

4.1.2. Patients with hyaline cartilage collected (Studies I, II, and III); tissue samples collection

Twelve samples (10 women, 2 men) from distal trapezium were obtained during partial or complete trapeziectomy and arthroplasty performed for thumb basal joint OA. Patients mean age was 58.2 (range 43-80). All patients had no pain relief or functional improvement after treatment with non-steroidal anti-inflammatory drugs and a thumb spica splint. Eaton-Glickel grading system was used to assess the severity of the CMC-I OA (253). Non-erosive OA of the CMC-I was confirmed in all patients, after examining radiological features (9,254). Complete or partially excision (trapeziectomy) of the arthritic trapezium bone was performed depending on the presence of OA in the scaphotrapezial joint. Eaton-Glickel grade I OA patients were not included since it is a contraindication for arthroplasty. For healthy controls, cartilage-subchondral bone samples were obtained from the non-affected CMC-III joint, resected from patients undergoing total wrist arthrodesis for post-traumatic OA of the wrist. CMC-III joint was included in the wrist fusion to enable plate fixation from the third metacarpal bone to the distal radius. Healthy controls CMC-III were obtained from two male patients, 45 and 53 years, respectively.

Fifteen osteochondral cylinder samples (11 women, 4 men) were prepared from tibial plateaus obtained from total knee replacements due to knee OA. Patients mean age was 67.5 (range 52-87). The tibial plateau was fixed to an in-house purpose designed sample holder and samples were extracted from different areas of the joint surface using a hollow 9 mm bore attached to a power drill. Samples were graded for disease severity using the OARSI grading system (87).

All samples were fixed in neutral buffered 10% formalin for 2 weeks and decalcified in 10% Ethylenediaminetetraacetic acid (EDTA), pH 7.4, for 5 weeks at 4 °C (until the bone tissue was pliable) before dehydration in ethanol series, clearing in xylene and embedding in paraffin. Samples were cut using a Leica RM 255 microtome (Leica, Wetzlar, Germany) to 3 μm thick sections and placed in microscope slides.
4.1.3. Patients whose cartilage was used for explant and primary chondrocytes cell culture (Studies I and III)

Hyaline cartilage was collected from OA patients undergoing total knee arthroplasty (TKA) (N=12) at ORTON Hospital. Half of the patients were female with a mean age of 63.5 (range from 53-73). Primary chondrocytes were also isolated from femoral heads from 4 femoral neck fracture arthroplasty patients, with no clinical history of inflammation or any macroscopically visible chondropathy. Tibial plateau containing cartilage and subchondral bone was cut off to provide space for the tibial component of the TKA implant. Samples were collected into sterile sample containers containing cold phosphate-buffered saline solution (PBS) and processed within four hours.

4.1.4. Patients with synovial fluid collected (Study III)

Samples with blood contamination were excluded. SF maintained at +4 °C was within two hours aliquoted into sterile Eppendorf tubes, snap frozen in liquid nitrogen and stored at −80 °C. After first thawing, SF was incubated with protease inhibitor cocktail (Roche Diagnostics, Meylan, France) and centrifuged at 1200 x g for 5 min at room temperature in order to separate solid debris and cells from the fluid phase.

4.2. RNA extraction and cDNA synthesis (Studies II and III)

Total RNA was isolated from cartilage explants by using the RNAqueous®kit (Thermo Fisher Scientific, MA USA) and from primary chondrocytes by using the RNeasy® mini kit (Qiagen, CA, USA). For MSc pellets total RNA isolation was done using TRIzol reagent (Invitrogen) followed by mRNA isolation using magnetic Oligo(dT)25 polystyrene beads (Dynal, Oslo, Norway). Isolated RNA quantity, purity and integrity were controlled with spectrophotometric analysis using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) synthesis for an equal amount of total RNA from each sample was performed using SuperScript® First-Strand Synthesis System (Invitrogen, CA, USA) for study II or iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) for study III and IV all accordingly to the manufacturers protocol.

4.3. RT-PCR (Studies II and III)

Quantitative RT-PCR was run in a LightCycler PCR machine using LightCyclerFastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany), twice for all samples. Primers were designed with Primer3 (SourceForge, CA, USA), the sequences were searched with the NCBI Entrez search system, and sequence similarity search was done using the NCBI Blast program. The mRNA copy numbers were determined from each sample in duplicates.
Relative quantification of the gene levels was performed by comparing the Ct values of the different genes, correcting for beta-actin (ACTB) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TBP content (∆Ct) and for non-stimulated conditions (ΔΔCt) and then expressed as fold of changes.

**4.4. Cell and Tissue culture**

**4.4.1. Cartilage explant culture, primary chondrocytes isolation, MSC isolation, chondrogenic cultures and culture stimulations (Studies I and III)**

Hyaline cartilage was extracted from TKA OA patients for cartilage and primary chondrocyte isolation and culture. Hyaline cartilage macroscopic appearance of the visible pathological changes in different sampling areas was collected following the arthroscopic grading scale of Société Française d’Arthroscopie (SFA) during harvesting (255). For study design purposes, samples were extracted from only two different types of areas: areas with no macroscopically visible lesions grade 1 OA (representing early OA cartilage degradation stages) or areas with full-thickness osteochondral lesions as the grade 4 OA (256). Such collection strategy was designed for stratified analysis of hyaline cartilage cultures based on the degree of OA cartilage, very mild vs. severe. Using a 4-mm circular punch biopsy blade (Kai Medical, Oyana, Japan) cylindrical full-thickness cartilage explants were obtained from graded tibial plateau areas and, subsequently, cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Life Technologies, Carlsbad, USA) supplemented with 50-100 U/ml penicillin, 50-100 U/ml streptomycin and 0.25-0.3 μg/ml amphotericin B (Thermo Fisher Scientific) and 10% fetal calf serum (FCS) in cell culture chamber incubator for 24 h prior to stimulations.

For chondrocyte isolation hyaline cartilage tissue was also used. Since chondrocytes account to only 1-3% of the volume of the cartilage, it is necessary to use the whole cartilage in order to obtain enough cells. Such process implies that the isolated chondrocytes represent a heterogeneous population of OA chondrocytes, therefore not allowing a stratified cartilage zone analysis. Cartilage specimens were then minced into 5 mg pieces and washed in sterile phosphate-buffered saline (PBS) followed by a sequential digestion in 2.5 mg/ml pronase and 250 mg/ml collagenase-P (Roche, Basel, Switzerland), with a PBS wash in between, for 60 min and overnight, respectively, under slow agitation at +37°C. The obtained cell suspension was filtered through a 70 μm nylon cell strainer, centrifuged, washed twice in sterile PBS, and seeded at 1.5x10^5/cm² in DMEM/F12 supplemented with 10% FCS, 100 units/ml penicillin, 100 units/ml streptomycin and 0.25 μg/ml amphotericin B (Thermo Fisher Scientific). Cells were maintained in cell culture chamber incubator for 3-4 days prior to stimulations.

All steps were performed in sterile conditions under a cell culture chamber incubator. For
cryopreservation, cells were suspended in FCS containing 10% (v/v) dimethyl sulfoxide (DMSO) and frozen in an isopropanol container for 24 hours at -80°C before storage in liquid nitrogen.

Primary MSC lines were established from healthy adult donors, who had given their informed consent and following a protocol approved by an institutional review board. Mononuclear cells were isolated from 10 ml bone marrow aspirate using a density gradient (Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden) and plated at 4x10^6/cm² in low glucose DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% FCS (StemCell Technologies, Vancouver, Canada), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Euroclone, Siziano, Italy). Non-adherent cells were removed by washing after 72 hours. Culture medium was changed twice weekly, and the passage 0 cells were harvested using TrypZean (Sigma) from subconfluent cultures, usually fourteen days after plating. The cells were re-plated at 1000/cm² in complete culture medium and passaged when subconfluent, as in agreement with earlier methods (257). Multipotency of the established cell-lines was tested with standard osteogenic and adipogenic protocols. For chondrogenesis experiments, MSC from passages three and four were used. MSC cells were washed twice in basal chondrogenic MSC Medium (Lonza, Basel, Switzerland) using resuspension and centrifugation at 150 x g for 5 min. Washed cells were transferred to complete Chondrogenic Medium (Lonza, Basel, Switzerland; supplemented with 10 ng/ml of transforming growth factor beta 3 (TGF-β3), R&D Systems, MN, USA) divided into different tubes each containing 2.5 x 10^5 cells and centrifuged again at 150xg for 5 min. Formed non-adherent pellets were kept in polypropylene tubes in cell culture chamber incubator. The medium was replaced every 3 days. Differentiation in the pellets was studied at days 0, 7, 14 and 21.

Cartilage explants, primary chondrocytes, and chondrogenic pellets were stimulated with different concentrations and combinations of probe molecules at relevant time points. Table 5 summarizes the cells exposure to the different proteins stimulations and stimulation duration.
Table 5. Molecular concentrations and duration of stimulations used in this thesis.

<table>
<thead>
<tr>
<th>Molecules/stimulus</th>
<th>Concentration</th>
<th>Duration (hours)</th>
<th>Company</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biglycan (sBGN)</td>
<td>5 µg/ml</td>
<td>24, 48</td>
<td>Sigma</td>
<td>III</td>
</tr>
<tr>
<td>CLI-095</td>
<td>1 µM</td>
<td>24, 48</td>
<td>Invivogen</td>
<td>III</td>
</tr>
<tr>
<td>Decorin (sDCN)</td>
<td>8 µg/ml</td>
<td>24</td>
<td>R&amp;D Systems</td>
<td>III</td>
</tr>
<tr>
<td>LPS</td>
<td>1 µg/ml</td>
<td>24, 48</td>
<td>Sigma</td>
<td>III</td>
</tr>
<tr>
<td>Pam 3CSK4</td>
<td>0.5 ng/ml</td>
<td>12</td>
<td>Invivogen</td>
<td>I</td>
</tr>
<tr>
<td>Polymyxin B (PL-B)</td>
<td>25 mg/ml</td>
<td>24, 48</td>
<td>Invivogen</td>
<td>III</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>50 mg/ml</td>
<td>24, 48</td>
<td>Thermo Scientific</td>
<td>III</td>
</tr>
<tr>
<td>TLR2 antibody</td>
<td>20 µg/ml</td>
<td>24, 48</td>
<td>Santa Cruz</td>
<td>III</td>
</tr>
<tr>
<td>TLR4 antibody</td>
<td>20 µg/ml</td>
<td>24, 48</td>
<td>Santa Cruz</td>
<td>III</td>
</tr>
<tr>
<td>rhTNF-α</td>
<td>5 and 100 ng/ml</td>
<td>12</td>
<td>R&amp;D Systems</td>
<td>I</td>
</tr>
</tbody>
</table>

4.4.2. TLR4 reporter assay (Study III)

Human HEK-blue TLR4 reporter cells (Invivogen) were seeded at 2.5 × 10⁴ cells per well in a 96-well plate according to the manufacturer’s instructions. Human HEK-blue TLR4 reporter cells are genetically-modified to contain a TLR4/NF-κB/secreted embryonic alkaline phosphatase (SEAP) reporter construct to monitor the activation of NF-κB induced by TLR4 stimulation. To validate specificity stimulations were performed with ultra-pure lipopolysaccharide (LPS) preparation (Invivogen) (Table 5). Cells were stimulated with different molecules and solutions listed in Table 5.

The conditioned medium of human HEK-blue TLR4 reporter cells culture stimulations in study III, was assed to measure the level AP activity engaged by the SEAP protein, using the commercial assay QUANTI-Blue™ (Invivogen). Controls, blanks and samples were mixed QUANTI-Blue™ medium preheated to +37°C, were incubated in wells of 96-well microplates for 1 hour at +37°C. After incubation, absorbance was measured at 655 nm in triplicates readings using a microplate reader (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany). Samples relative AP activity was calculated from OD of samples relative to OD of controls.

4.5. Biochemical and protein assays

4.5.1. Soluble collagen assay (Study III)

From cell culture stimulations of study III, the conditioned medium was used for measurements of concentrations levels of soluble collagen using the commercial assay Sircol™(Biocolor Ltd,
Soluble Collagen II standards, blanks and samples were mixed with collagen-binding Sircol Dye Reagent® in a mechanical shaker for 30 min at room temperature. Next samples were centrifuged at 12,000 rpm for 10 min. Samples were then drained, and the remaining collagen-dye pellet was washed with a salt-wash reagent followed by centrifugation at 12,000 rpm for 10 min. Next the collagen-dye complex was dissociated using an alkali reagent vortexing-wash (Biocolor Ltd.). After samples absorbances were measured at 540 nm, using a plate reader (Chameleon-Hidex, Turku, Finland). Samples collagen concentrations levels were retrieved from the standard curve equation. All steps were performed at room temperature.

4.5.2. Glycosaminoglycan Assay (Study III)

The conditioned medium of cell culture stimulations of study III, was assed for the concentrations levels of sulfated glycosaminoglycan (sGAG) using the commercial assay Blyscan™ (Biocolor Ltd, Carrickfergus, UK). sGAG standards, blanks and samples were mixed with sGAG-binding Blyscan Dye Reagent® in a mechanical shaker for 30 min at room temperature. Next samples were centrifuged at 12,000 rpm for 10 min. Samples were then drained, and the remaining sGAG-dye pellet was washed with dissociation reagent (Biocolor Ltd.), followed by centrifugation at 12,000 rpm for 5 min. Next samples were transferred into 96 well microplate and absorbances measured at 595nm using a plate reader (Chameleon-Hidex). Samples sGAG concentration levels were retrieved from the standard curve equation. All samples and standards were run in duplicates. All steps were performed at room temperature if not otherwise stated.

4.5.3. Nitric oxide measurement (Study III)

Cell culture and tissue culture medium from study III were assessed for the presence and quantification of Nitric Oxide species (NO). NO was measured using a commercial nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, USA). Initially, cell and tissue culture medium samples were diluted with assay buffer in order to assure NO concentrations levels were within the detection range of the assay kit. This was followed by an addition of nitrate reductase and its co-factor for an incubation period of one hour and two hours for cell and tissue culture medium, respectively, at room temperature. This incubation time is essential to assure complete nitrate conversion to nitrite. Subsequently, samples were incubated with Griess reagents for 10 min at room temperature, which will cause nitrite to convert into a deep-purple colour compound. The light absorbance of this azo chromophore accurately determines NO concentration. Absorbance was measured at 544 nm using a plate reader (Chameleon-Hidex). NO concentration levels were calculated from standard curve equation. All samples and standards were run in duplicates.
4.5.3.1. **Safranin-O staining (I,II,III)**

Formalin-fixed paraffin-embedded (FFPE) tissue sections from OA cartilage were stained with Safranin-O for evaluation of cartilage proteoglycan (PG) content. Sections were deparaffinised in xylene and rehydrated in ethanol series using an automated immunohistochemical robot (Varistain XY, Shandon, Thermo Scientific). This was followed by a rinsing during 10 min in Weigert’s Iron Hematoxylin working solution (Merck, Darmstadt, Germany), followed by a wash in H$_2$O for 10 min with a 15 seconds rinsing intermediate step in a differentiation solution (1% HCl in 70% alcohol ). This was followed by a 5 min rinsing in a Fast Green solution (SIGMA, C.I 42053, MO, USA), and a rinse in 1% Acetic Acid solution for 15 seconds. The sections were then rinsed for 5 min in 0.1% Safranin-O solution (Fluka-SIGMA). Sections were then manual dehydrated in a series of ethanol, cleared in xylene and mounted. All steps were performed at room temperature if not otherwise stated.

4.5.3.2. **Alcian blue staining (Study II)**

OA cartilage FFPE sections were stained with Alcian blue for evaluation of cartilage proteoglycan content and as an alternative comparison with Safranin-O staining. Sections were deparaffinised in xylene and rehydrated in ethanol series using an automated immunohistochemical robot (Varistain XY). This was followed by a rinsing during 30 min in Alcian blue solution (Sigma), followed by a wash in H$_2$O for 2 min. This was followed by a 5 min rinsing in a nuclear fast red solution (Sigma), and a rinse in H$_2$O for 1 min. Sections were then manual dehydrated in a series of ethanol, cleared in xylene and mounted. All steps were performed at room temperature if not otherwise stated.

4.5.4. **Protein expression**

4.5.5. **Immunohistochemistry (Studies I, II, and III)**

Cartilage samples and MSC pellets paraffin sections were used for immunohistochemistry (IHC) studies. Sections were deparaffinised in xylene and rehydrated in ethanol series using an automated immunohistochemical robot (Varistain XY). This was followed by antigen retrieval in 10 mM citrate buffer, pH 6.0, for 1 hour at 70°C and 10 mM sodium citrate buffer, pH 6, followed by AR 98°C-S30M program for 24 min in MicroMed T/T Mega Laboratory Microwave Systems (Milestone, Sorisole, Italy), for cartilage and MSC pellets respectively. After quenching of endogenous peroxidase in 0.3% H$_2$O$_2$ in deionized water for 25 min, sections were blocked with normal goat serum or normal horse serum (Vectorlabs, CA, USA) diluted in 0.1% bovine serum albumin-phosphate buffered saline (BSA-PBS) for 1 h. Sample sections were then incubated overnight at 4°C with TLRs, CD31, CD34, CD105, CD166, COL2A-3/4M and TNF-α at specific concentrations (Table 6). This was followed by a 1h incubation period in biotinylated goat anti-rabbit or horse anti-mouse IgG (Vectorlabs). Next avidin-biotin-
peroxidase complex were developed during 1h followed by the addition of colour developer solution H$_2$O$_2$-3,3’-diaminobenzidine (DAKO A/S, Glostrup, Denmark) during 10 min, succeed by washing step in distilled water and counterstaining in Mayer hematoxylin (Merck, Darmstadt, Germany) for 1 min. After that the slides were dehydrated in ethanol series, cleared in xylene and mounted. For negative staining controls non-immune IgG was used instead of and at the same concentration as the primary specific antibodies. All incubations were performed at 22°C in humidified chambers, with 3 times PBS washes in between the steps if not otherwise stated.

Table 6. Concentrations of primary antibodies used for IHC in the studies. (*) primary antibody concentration used for MSC pellets immunostainings

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Polyclonal rabbit IgG</td>
<td>2/0.8* μg/mL</td>
<td>Santa Cruz</td>
<td>Study I, II</td>
</tr>
<tr>
<td>TLR2</td>
<td>Polyclonal rabbit IgG</td>
<td>1/2.6* μg/mL</td>
<td>Santa Cruz</td>
<td>Study I, II</td>
</tr>
<tr>
<td>TLR3</td>
<td>Polyclonal rabbit IgG</td>
<td>2 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I</td>
</tr>
<tr>
<td>TLR4</td>
<td>Polyclonal rabbit IgG</td>
<td>1.3-1 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I, II</td>
</tr>
<tr>
<td>TLR5</td>
<td>Polyclonal rabbit IgG</td>
<td>1.3 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I</td>
</tr>
<tr>
<td>TLR6</td>
<td>Polyclonal rabbit IgG</td>
<td>1 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I</td>
</tr>
<tr>
<td>TLR7</td>
<td>Polyclonal rabbit IgG</td>
<td>0.8 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I</td>
</tr>
<tr>
<td>TLR8</td>
<td>Polyclonal rabbit IgG</td>
<td>1 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I</td>
</tr>
<tr>
<td>TLR9</td>
<td>Polyclonal rabbit IgG</td>
<td>1/2.5* μg/mL</td>
<td>Santa Cruz</td>
<td>Study I, II</td>
</tr>
<tr>
<td>TLR10</td>
<td>Polyclonal rabbit IgG</td>
<td>1.6 μg/mL</td>
<td>Santa Cruz</td>
<td>Study I</td>
</tr>
<tr>
<td>CD31</td>
<td>Polyclonal rabbit IgG</td>
<td>18 μg/mL</td>
<td>Santa Cruz</td>
<td>Study II</td>
</tr>
<tr>
<td>CD34</td>
<td>Polyclonal rabbit IgG</td>
<td>2 μg/mL</td>
<td>Santa Cruz</td>
<td>Study II</td>
</tr>
<tr>
<td>CD105</td>
<td>Monoclonal mouse IgG2a</td>
<td>10 μg/mL</td>
<td>Abcam</td>
<td>Study II</td>
</tr>
<tr>
<td>CD166</td>
<td>Polyclonal rabbit IgG</td>
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</tr>
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<td>Collagen II</td>
<td>Mouse IgG1</td>
<td>6.65 μg/mL</td>
<td>Chemicon</td>
<td>Study II</td>
</tr>
<tr>
<td>COL2A-3/4M</td>
<td>Polyclonal rabbit IgG</td>
<td>1:1000 μg/mL</td>
<td>Ibex</td>
<td>Study I, II</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Mouse IgG</td>
<td>10 μg/mL</td>
<td>Research Diagnostics Inc.</td>
<td>Study I</td>
</tr>
</tbody>
</table>

4.5.6. Enzyme-linked Immunosorbent Assay (ELISA) (Study III)

Stored synovial fluid samples were thawed until they were at room temperature. SF samples were used to measure the concentration levels of intact- only sBGN and sDCN using specific sandwich Enzyme-linked Immunosorbent Assay (ELISA) (all purchased from Uscn Life Science Inc., Hubei, PRC).
Initially, SF samples were diluted to the optimum working range after initial pilot runs were performed. Samples were incubated in 96 well strip plates coated with the respective antibody. Next samples were washed and incubated with avidin conjugated to horse radish peroxidase (HRP) followed by a second wash step and incubation in biotin. Colour was then developed by an incubation period in substrate solution. Absorbance was measured at 450 nm using a plate reader (Chameleon-Hidex). SF samples concentrations of sBGN and sDCN were determined by comparing the OD of the samples to the standard curves were exact concentrations of the respective recombinant proteins were used. All samples and standards were run in duplicates. All incubation periods were performed at 37 °C.

4.5.7. Luminex xMAP® technology (Study III)

Protein concentration levels secretion into cell culture supernatant was done using xMAP® technology (Luminex, TX, USA). A custom detection multiplex kit was designed to detect the following targets: MMP-1, MMP-3, MMP-9 and MMP-13, IL-6, and IL-8. The detection-multiplex kit (ProcartaPlex-eBioscience, CA, USA) was run in combination with xMAP® technology on the Bio-Plex 200® system (Bio-Rad Laboratories, Hercules, CA, USA). Cell culture supernatant was diluted (diluted 1:2) before 25 µl of supernatant per sample was used for the assay.

4.6. Grading of OA patients and OA samples

4.6.1. Radiographic grading of CMC-I OA using the Eaton-Glickel grading system (unpublished data)

The radiological Eaton-Glickel scale was used to evaluate the disease severity of the CMC-I OA (253). It would have been preferable to use a histological grading as OARSI grading. However trapeziectomy was sometimes only partial which lead to non-uniform sampling zones. Sampling uniformity is an essential feature for OARSI grading. Still, radiological grading of hand OA correlates positively with the histological assessment in post-mortem samples (258). Therefore, both scales reflect the severity (grade) of OA.

4.6.2. Histopathological grading of osteoarthritic cartilage (Study I and II)

The histopathological OARSI grading was carried out blindly by 3 independent researchers from coded samples using light microscopy. Researchers were familiar with OARSI grading methodology and used OARSI templates as guidance (87). Associated features to OARSI grade of OA cartilage can be seen in Table 2.
Stained samples were evaluated under a Nikon LV-DIA-BASE microscope (Nikon, Tokyo, Japan) provided with a motorized XY-stage (Scan Prior III, Prior, Rockland, MA, USA), connected to a DS-Fi1 digital camera using Nikon Imaging Software (NIS) - Element Basic Research (BR) analysis (Nikon).

4.7. Image analysis

4.7.1. Histomorphometry (Study II and in unpublished data)

The TLR-positive cells percentage was calculated from at least 5 high-magnification (200×) fields separately for the surface, middle, and deep zones of the hyaline articular cartilage.

4.8. Statistical analysis (Study I, II, and III)

Student’s t-test was used for comparison of paired samples. The non-parametric methods One-way ANOVA, Mann-Whitney and Wilcoxon sign-rank test were used for multiple group comparisons, as appropriate. Relevant post-hoc tests were performed for all statistical hypothesis tests. Effects of covariates were analysed by multiple linear regression. All statistical analysis were performed using SPSS v. 21 (IBM Corp, NY, USA). Data is presented as mean and standard deviation (SD). P-values < 0.05 were considered significant.
5. Results

5.1. TLR1, 2 and 9 expression in healthy articular chondrocytes (Study I)

Given the conflicting reports on the expression of TLR1, 2 and 9 in human articular chondrocytes we decided to study the expression of these TLRs in chondrocytes to settle the apparent literature discrepancies. Moreover, as these TLRs recognize several DAMPS, which could arise from cartilage ECM breakdown and biological processes, and they contribute to the pathogenic mechanism in the osteoarthritic synovial joint it would be of utmost importance to elucidate their expression in synovial joints.

TLR1, TLR2 and TLR9 mRNA expression was studied in isolated chondrocytes obtained from trauma patients with no clinical history of inflammation or any macroscopically visible chondropathy. Primary chondrocytes were expanded in monolayer cultures (2D culture) during one passage to obtain sufficient amounts of isolated RNA. As the results in Figure 5 disclose, TLR1, TLR2 and TLR9 were detected in healthy primary chondrocytes, albeit at different relative mRNA expression levels.

![Figure 5](image_url)

**Figure 5.** Toll-like receptors TLR1, TLR2, and TLR9, are expressed in primary chondrocytes. TLR1 and TLR2 mRNA levels were higher than TLR9 mRNA levels relative to a housekeeping gene († p < 0.001). Cells were isolated from four different donors (N=4).
5.2. TLR1, 2 and 9 expression during mesenchymal stem cells chondrogenesis

During traumatic events, cartilage matrix products are released and they can initiate a danger signal driven inflammation response by local resident cells. By using MSCs which are directed towards chondrocyte differentiation we believed we could study a human mature chondrocytes in microenvironment free of danger signals or inflammatory mediators and their products which can potentially alter TLR expression levels. Moreover, MSCs are widely being studied to be used as for cartilage repair and also for blocking ongoing inflammation given their anti-inflammatory properties. Studying TLR1, 2 and 9 expression during chondrogenesis and comparing differentiated mature chondrocytes (21 days) with phenotypically healthy chondrocytes seemed, therefore, a necessary analysis.

As demonstrated in Figure 6 and Figure 11 collagen type II and Safranin-O PG staining confirm the differentiation of MSC into mature chondrocyte cells (21 days). Collagen type II and PG are essential components of cartilage matrix and the main molecules compromised during osteoarthritic cartilage degradation and chondrocyte phenotypic changes. Furthermore SOX9 and COL2A1 gene expression levels, two gene markers of articular chondrocyte, were upregulated by 9 (±7) and 570 (±92) folds respectively, when compared to the negative control undifferentiated MSCs kept for 21 days in regular medium.

As for TLR1, 2 and 9 expression during chondrogenesis their mRNA expression was reduced significantly from the progenitor stage until the mature differentiated chondrocytes, as Figure 7 shows. However, TLR1, 2 and 9 expression levels did not follow exactly the same expression patterns during the conventional differentiation stages. TLR1 and TLR2 proteins were also detected by immunostaining, as Figure 8 shows.
Figure 6. Toll-like receptors immunostainings of chondrocyte pellets at day 21 produced from human bone marrow-derived mesenchymal stem cells. A-C. TLR1, TLR2 and TLR9 immunostaining, respectively, D, compared to a negative staining control. Original magnification 200x. Insets, lower magnification general view of the chondrogenic pellets. Original magnification 100x.

Figure 7. Toll-like receptors expression during chondrogenesis of human bone marrow-derived mesenchymal stem cells. Toll-like receptors TLR1, TLR2 and TLR9 in mesenchymal stem cells (day 0) differentiating via progenitor stage (day 7 and 14) to chondrocytes (day 21). Quantitative real time-polymerase chain reaction was used to measure the mRNA copy numbers per $10^6$ β-actin copies. In primary chondrocyte isolates TLR1 and TLR2 mRNA relative expression was higher than TLR9 (*p<0.05). Overall gene expression changes of TLR2 and TLR9 were statistically significant during chondrogenesis period. Each dot represents one sample. Shown are mean values (± the standard deviation [SD]) from 4 independent experiments (using samples obtained from 4 different biological donors). Samples were measured as technical duplicates and averaged.
Figure 8. Chondrocyte pellets at day 21 produced from human bone marrow-derived mesenchymal stem cells. A, Collagen type II immunostaining. B, negative control. Original magnification 200x. Insets, Lower magnification general view of the chondrocytes pellets at day 21. Original magnification 50x.

5.3. Chondrocyte TLR1-TLR2 heterodimer activation

After TLR1 and TLR2 expression was confirmed by gene expression analysis, we then tested their functionality. We used Pam3CSK4 a known synthetic triacylated lipopeptide (LP) that mimics the acylated amino terminus of bacterial LPS. Pam3CSK4 activates innate immunity through the NF-κB pathway and is recognized by the TLR1-TLR2 heterodimer.

Both primary chondrocytes from trauma patients and MSC-derived chondrocytes cultured for 21 days were stimulated with Pam3CSK4. As one of the measurements of innate immune inflammatory activation we decided to measure the TNF-α gene expression, given it's known proinflammatory properties and increased levels in OA synovial joints and tissues. Pam3CSK4 caused upregulation of TNF-α mRNA expression levels in both chondrocyte cell types, however this was only statistically significant in MSC-derived chondrocytes cultured for 21 days (Figure 9). Interestingly TLR1 and TLR2 mRNA expression levels were also upregulated in both chondrocyte cell types upon addition of Pam3CSK4 demonstrating TLR1-TLR2 heterodimer activation. In contrast, as expected TLR9
expression levels remained unchanged (Figure 9). Together the results also show that MSC-derived chondrocytes cultured for 21 days are able to regulate TLR and TNF-α mRNA expression with a similar pattern as observed in primary chondrocytes.

![Figure 9](image)

**Figure 9.** Pam 3CSK4 stimulation of primary chondrocytes and MSC-derived chondrocytes cultured for 21 days. (A) In primary chondrocytes stimulated with Pam 3CSK4, TLR1, TLR2 and TNF-α mRNA levels are significantly upregulated. (B) In MSC-derived chondrocytes stimulated with a Toll-like receptor 1/2 specific ligand Pam 3CSK4, lead to a significantly upregulation of Toll-like receptors TLR2 and TLR9. Mean ± standard deviation, compared to unstimulated cultures. *p<0.05

5.4. TNF-α stimulation of chondrocytes

As shown in the previous results, a ligand such as Pam3CSK4 which is recognized by the TLR1-TLR2 heterodimer can cause the upregulation of TNF-α. However, TNF-α can also arise from other innate immune inflammatory pathways involved in OA pathogenesis, such as the complement system. It is, therefore, important to know how this inflammatory cytokine influences TLR expression and activation.

As shown in Figure 10, recombinant human TNF-α at 5 ng/ml and the supraphysiological concentration of 100 ng/ml caused the upregulation of TLR1 and TLR2, in a dose-dependent manner. Interestingly, TLR9 expression remained fairly unchanged in both TNF-α concentrations. TNF-α stimulations also caused an increase in TNF-α mRNA expression levels in MSC-derived chondrocytes cultured for 21 days at both TNF-α concentration levels.
Figure 10. TNF-alpha stimulation of MSC-derived chondrocytes cultured for 21 days. Folds of changes of tumour necrosis factor-α (TNF-α), Toll-like receptor 1 (TLR1), TLR2 and TLR9 upon stimulation of MSC-derived chondrocytes with 5 and 100 ng/ml rhTNF-α. Mean ± standard deviation, compared to unstimulated cultures. *p<0.05

Stimulation of 21 day MSC chondrocyte pellets with TNF-α at 0, 5 and 100 ng/ml also caused a dose-dependent loss of GAG deposited in the formed ECM matrix surrounding the mature chondrocytes (Figure 11). Safranin-O staining is a cationic-based dye which can stain the negatively charged GAGs. Depletion of GAGs implies that TNF-α stimulation lead to activation of aggrecanases which can cleave the aggrecans supramolecular structure as well of the core proteins of aggrecan attached GAGs.
Figure 11. Safranin-O staining of MSC-derived chondrocytes cultured for 21 days. A, untreated (control), B, C, treated with 5 and 100 ng/ml of TNF-α, respectively. Safranin-O staining is markedly decreased from pellet borders towards the pellet centrum. Original magnification 50x.

Since the loss of GAGs is associated with disruption of collagen network structure, we then studied whether the main cartilage collagen type, collagen type II, was damaged. Staining of 21 days MSC chondrocyte pellets with COL2A-3/4M neoeptope antibody (currently known as C2C) specific for collagen type II (cleavage by MMP-1, MMP-8 and MMP-13), demonstrated increased staining after TNF-α stimulations (Figure 12). Immunostaining of COL2A-3/4M was observed in chondrocytes and surrounding ECM matrix.

Figure 12. Immunostaining with the COL2A-3/4M antibody of MSC-derived chondrocytes cultured for 21 days.
(A) unstimulated control and (B) negative staining control. (C) MSC-derived chondrocytes cultured for 21 days stimulated with 5 ng/mL TNF-α and (D) stimulated with 100 ng/mL TNF-α. Note the marked increase of COL2A-3/4M immunostaining in TNF-α stimulations. Magnification 100×.

5.5. TLR expression is dependent on the severity of OA (Study II and unpublished data)

TLR have been shown to be present in chondrocytes obtained from knee and hip joints of healthy and OA patients. However, TLR expression during OA progression has not been studied, despite it could offer clues of their involvement in the disease development. Moreover, no study so far has addressed TLRs expression in other OA joints besides the knee joint. Therefore, we decided to study by IHC the expression patterns of all human TLRs in cartilage obtained from knee and CMC-I joints of OA patients accordingly to a well validated histological and radiographical scores of OA severity. For the CMC-I joint OA severity we used a validated OA radiographic score, due to the incompatibility of a histological score to the non-uniform sampling of cartilage from the CMC-I joint.

As Figure 13 demonstrates, we were able to detect all human TLRs in cartilage from knee OA at the OARSI histological grade 2, with TLR positive chondrocytes already detected in the middle zone of knee articular cartilage. TLRs immunostainings were specifically localized to chondrocytes with very weak levels of ECM background stainings.
Figure 13. Immunostainings of Toll-like receptors (1 to 9) in knee OA cartilage with OARSI grade II. All TLRs stained positive in OA cartilage sample with OARSI grade II. TLRs positive stainings are observed in cartilage surface and middle zones. Negative staining control, rabbit IgG, confirmed the specificity of the immunostainings. Representative pictures of N=15 had similar results. Original magnification ×100.

Interestingly, in contrast to TLRs staining pattern in knee OA, TLR immunostaining were substantially different in cartilage from CMC-I OA of grade II. Instead, we observed very heterogeneous TLRs immunostainings across samples of the same equivalent grade (Figure 14). CMC-I joint samples were graded accordingly to the Eaton-Glickel grading system which is based on radiological observations. Figure exemplifies the Eaton-Glickel grading of patients whose samples were collected, with the exception of stage I samples which were not collected since trapeziectomy is a clinical contraindication.
Figure 14. Immunostainings of Toll-like receptors (1 to 9) in CMC-I OA cartilage with Eaton-Glickel grade 2. Immunostainings clearly indicates the expression of all TLR1, TLR2, TLR3 and TLR6 in the Eaton-Glickel stage 2 patient sample. Negative staining control, rabbit IgG, confirmed the specificity of the stainings. Representative pictures of N=15 were similar results were observed. Original magnification ×100. The data is unpublished.
Figure 15. Eaton-Glickel radiological grading of OA lesions of the CMC-I joint. **Grade I**: Normal or only slightly widened joint space (due to joint effusion or synovitis). **Grade II**: Mild CMC-I cartilage space narrowing and small osteophytes or loose bodies < 2 mm in diameter. **Grade III**: Significant narrowing or complete obliteration of the CMC-I joint space with osteophytes or loose bodies > 2 mm in diameter. **Grade IV**: Same as grade III, but also the scaphotrapezotrapezoidal (STT) joint is affected. The data is unpublished.

Histomorphometric analysis of immunostainings of TLRs in cartilage samples from knee OA across OARSI grades revealed a rather similar and homogeneous increase of the proportion of TLR⁺ chondrocytes as the OARSI score increased (Figure 16).

Figure 16. Toll-like receptors expression in chondrocytes resident in knee OA cartilage zones and during OARSI grade progression. Knee OA cartilage degradation stages were graded according to the Osteoarthritis Research Society International (OARSI) histological score. Knee cartilage zones evaluated were the surface (gliding, tangential), middle (transient), and deep (vertical) zones. TLR10 was not found in any of the studied cartilage samples from knee OA. *p < 0.05, **p < 0.01 indicate statistical significant differences for pairwise comparisons of histomorphometric levels in cartilage zone differences of TLR expression in different OARSI grades (G),
separately for the surface zone (first asterisk) and for the middle zone (second asterisk). N=3 per each OARSI grade.

In comparison, immunostainings of TLRs in CMC-I OA samples across Eaton-Glickel grade revealed a rather heterogeneous pattern of the proportion of TLR+ chondrocytes (Figure 17). As can be observed, there was a great variation within same grade further stressing that TLRs expression in cartilage from CMC-I OA is patient-dependent. Interestingly the only striking pattern was the homogenous relatively low or absent expression of TLR4 immunostaining across all studied samples.

![Figure 17](image.png)

Figure 17. Toll-like receptors expression in chondrocytes resident in CMC-I OA cartilage zones and during SFA stage progression. CMC-I OA cartilage degradation stage were graded according to the French Arthroscopy Society (SFA) radiological score. CMC-I cartilage is particularly small hence no zone stratification analysis is possible. TLRs expression was heterogeneous with no statistical significance during stage progression. N=4 per each SFA score. This data has not been published.

PCA was performed over histomorphometric data to assess the relationships of individual TLRs variables across all dataset. PCA identified no significant difference between TLR expression profiles from samples of knee OA. On the other hand, PCA revealed significant differences in CMC-I OA. PC1 and PC2 disclosed two clusters separated by 95% confidence ellipsoids, one cluster being composed
of TLR1-3, TLR5 and TLR6 while the other of TLR7-9 (Figure 18). PC1 (56%) and PC2 (20.7%) represented 76.7% of the data. TLR4 did not belong to these two TLR clusters and correlated negatively to the higher expression of all the other TLRs.

**Figure 18.** Principal component analysis (PCA) of the histomorphometric TLR data in OA cartilage of the CMC-I joint. Score plot of PCA with PC1 and PC2 component extraction in a rotated space. 95% Confidence ellipsoids drawn by PCA show a clear separation of the TLR7, TLR8 and TLR9 cluster from the TLR1, TLR2, TLR3 and TLR5 cluster and that TLR4 has no association with either. The eigenvalue 1 criterion was used to select the PCs. Loading values vary from -1 to 1 and represent correlation of the variable with the principal components. Missing data cases were excluded list wise. Oblique promar and orthogonal varimax rotations produced similar results. This data has not been published.

**5.6. Identifying clinically relevant endogenous ligands able to activate TLR4 in OA joints (Study III)**

SLRPs are essential matrix components of articular cartilage. We hypothesized that class I SLPRs BGN and DCN, could be released from the matrix and become available in their known soluble proinflammatory forms. The detection and study of their biological role is of significant relevance to the knowledge of the pathomolecular mechanisms of OA.

We, therefore, started by analysing synovial fluid, which is in direct contact with articular cartilage, for the presence of sBGN and sDCN using specific sandwich ELISA assays. The sBGN and sDCN
ELISAs were performed in SF from knee meniscectomy, OA and RA patients. Results clearly show higher sBGN levels in OA (582 ± 307 ng/ml) in comparison to meniscectomy patients (14 ± 2 ng/ml). SF from RA patients had the highest levels of soluble BGN, with levels up to 80 folds higher than those in meniscectomy patients. SF-sBGN concentration levels were significantly different between all groups (Figure 19 A). In sharp contrast to sGBN results, sDCN concentrations levels were up to 10 fold lower and remained constant across all arthritic groups (Figure 19 B). Covariates (age, gender and body mass index) tested with multiple linear regression models showed no association with SF-sBGN and sDCN levels.

![Figure 19](image)

**Figure 19.** Soluble biglycan and decorin concentrations levels measured by ELISA assay in synovial fluid from different arthritic conditions groups. A, sBGN concentration levels were significantly increased in knee SF from TKA OA and RA patients when compared to meniscectomy patients. B, sDCN concentration levels remain unchanged in knee SF from meniscectomy, TKA OA and RA patients. Each dot represents one sample. Shown are mean values (± the standard deviation [SD]) from ten independent samples. **p < 0.01, ****p < 0.001 indicate statistical significant differences between pairwise comparisons groups. Knee SF samples were obtained from 10 patients (N=10) to each arthritic group.

The fact that sBGN was increased in synovial fluid from OA patients was a strong indicator of its potential pathophysiological role. We then decided to study whether sBGN could influence the cartilage metabolism, due to its known proinflammatory role in other diseases. Given the knowledge about the role of markers of joint destruction in OA, we studied whether sBGN stimulation had any effect on their expression. In OA primary monolayer chondrocytes sBGN significantly increased gene expression of ADAMTS4, ADAMTS5, MMP13, CTSK and MMP9, almost as efficiently as LPS, which was used as positive control for TLR4 activation (Figure 20A). A similar response was observed in cartilage explant tissues obtained from patients with different severity of OA. However, the degree of response
to sBGN was dependent on the grade of cartilage degradation. The mRNA expression of markers of joint destruction were more upregulated in grade I OA cartilage in comparison to grade IV OA cartilage (Figure 20B).

**Figure 20.** Effect of soluble biglycan (sBGN) on the gene expression of proteinases associated with matrix remodelling and cartilage degeneration in osteoarthritis. (A) In OA primary chondrocytes sBGN stimulation led to upregulation of proteinases examined. Lipopolysaccharide (LPS), the positive control, caused a similar increase although at different scale reflecting it is known potent activation of TLR4. (B) In OA cartilage explants sBGN induced the upregulation of MMP9, MMP13, ADAMT5 and CTSK in grade I OA, while in severe grade IV OA sBGN did not alter their expression levels. Results are expressed as fold changes (Mean and SD) relative to TATA Box Binding Protein (TBP) housekeeper and compared to non-stimulated controls. Samples were run as technical duplicates, and each experiment was done using samples obtained from at least 6 different donors (biological replicates). *p<0.05, **p<0.01, ***p<0.001 vs. non-stimulated controls or pairwise comparisons. ADAMTS = a disintegrin and metalloproteinase with thrombospondin type 1 motif; MMP = matrix metalloproteinase; CTSK = cathepsin K.

The presence of joint destruction markers were also confirmed on the protein level by Luminex Xmap technology. Stimulation with sBGN caused increased release of the markers into cell culture supernatant of OA primary chondrocytes (Figure 21). In addition proinflammatory cytokines IL-6 and IL-8, both mediators of joint destruction, were also upregulated by sBGN stimulation. Due to the limited number of samples statistical analysis could not be applied.
Figure 21. Effect of soluble biglycan (sBGN) on the expression MMP-1, -3, -9 and -13 and IL-6 and IL-8 proteins in OA primary chondrocytes. (A-E) Cell culture supernatant concentration levels of MMP-1,-3,-9 and -13 and IL-6 and IL-8 proteins, respectively. sBGN lead to the upregulation of several markers of joint destruction known to play a role in OA. sBGN-induced concentration levels compared to controls did not reach the statistical significance p-value <0.05. Results are expressed as ng/ml or pg/ml concentration levels. Each dot represents one sample. Shown are mean values from 3 independent experiments (using samples from 3 different biological donors). Samples we measured as technical duplicates and averaged.

Altered cartilage turnover is a prominent feature of OA. Therefore we studied whether sBGN also had influence on the anabolic functions of OA chondrocytes. In primary OA chondrocytes sBGN significantly inhibited aggrecan and Col-II mRNA expression (Figure 22). On cartilage explants the response was dependent on the OA grade of cartilage severity, with grade I OA explants able to keep maintaining the expression of Col-II much in contrast with down-regulation observed in explant from grade IV OA (Figure 22).
Figure 22. Effects of soluble biglycan (sBGN) on the expression of aggrecan (ACAN) and collagen type II (COL2A1). (A) In primary chondrocytes sBGN stimulation lead to down-regulation of ACAN and COL2A1. Lipopolysaccharide (LPS), the positive control, lead to a similar response. (B) In OA cartilage explants sBGN stimulation leads to a down-regulation of COL2A1 levels in grade I OA which was significantly different from the observed response in grade IV OA. ACAN levels were not altered significantly after sBGN stimulation. Results are expressed as fold changes (Mean and SD) relative to TATA Box Binding Protein (TBP) housekeeper and compared to non-stimulated controls. Samples were run as technical duplicates, and each experiment was done using samples obtained from at least 6 different donors (biological replicates). *p<0.05, **p<0.01 vs. non-stimulated controls or pairwise comparisons.

In situ cartilage explant degradation by sBGN-catabolic chondrocytes was confirmed by analysis of cell culture media for the release of PGs and soluble collagen. Release of sGAG was more notable in grade I OA explants, whereas sBGN-induced increased collagen release was observed in grade IV OA cartilage (Figure 23).

Figure 239. Effects of soluble biglycan (sBGN) on the release of glycosaminoglycans and soluble collagen from cartilage explants compared to non-stimulated controls. (A) In grade I OA, the sBGN stimulation lead to an
increased release of glycosaminoglycans. (B) In grade IV OA, sBGN stimulation increased collagen release. Each dot represents one sample. Shown are mean values (± the standard deviation [SD]) from 6 independent experiments (using samples obtained from 6 different biological donors). Samples were measured as technical duplicates and averaged. *p<0.05 vs. non-stimulated controls. #p<0.05 for pairwise comparisons between mild grade I OA and severe grade IV OA.

As demonstrated by the results presented in Figure 24, the TLR4 expression is increased in OA cartilage as disease severity increases. Most likely this is due to the continuous release and accumulation of endogenous ligands emanating from articular cartilage degradation such as fibronectin, hyaluronan as well as sBGN. Therefore, we studied the effect of sBGN on the mRNA expression of TLR2 and TLR4, find out whether sBGN has the ability to regulate TLR2 and 4 expressions. In primary OA chondrocytes sBGN increased TLR4 mRNA expression significantly while TLR2 mRNA expression remained much unchanged. In line with previous results, cartilage explant TLR-response to sBGN was dependent on the grade of cartilage degradation. TLR4 expression was upregulated by about 3 folds in sBGN-stimulated grade I OA explants in comparison with the response observed in grade IV OA explants (Figure 24). Blocking of TLR4 by using a small molecular weight inhibitor of TLR4 signaling, CLI095, abrogated both sBGN and LPS induced upregulation of TLR4 mRNA expression in primary chondrocytes (Figure 24).

Figure 24. Effects of soluble biglycan (sBGN) on the TLR4 mRNA expression levels in primary chondrocytes and cartilage explants. (A) In primary chondrocytes, sBGN caused a significant increase on TLR4 expression to the same extent as LPS. The TLR4 upregulation was inhibited by CLI095, a synthetic inhibitor of TLR4 signalling. (B) In cartilage explants, sBGN stimulation caused a significant increase of TLR4 in grade I OA explants. Samples were run as technical duplicates, and each experiment was done using samples obtained from at least 6 different donors (biological replicates). All results are expressed as mean and SD *p<0.05, **p<0.01 vs. non-stimulated controls or pairwise comparisons.
The same effect was observed in a HEK-blue TLR4 reporter cell line used as control, where pre-incubation of CLI095 before sBGN lead to a much lower level of SEAP, the reporter gene of NF-kB activity mediated by TLR4 activation (Figure 25). Further blocking studies demonstrated that sBGN proinflammatory effects, measure by the synthesis of NO species were mainly mediated in primary chondrocytes via TLR4 (Figure 26).

Figure 25. Effects of soluble biglycan (sBGN) on HEK-blue TLR4 NF-kB reporter gene SEAP production. SEAP assay was used to determine NF-kB activity following sBGN, sDCN or LPS stimulation of human HEK-blue TLR4 reporter cells. Lipopolysaccharide (LPS) was used as a positive control. The addition of polymyxin B had no effect on the sBGN-induced SEAP production while proteinase K restored SEAP production to levels seen in controls, further confirming no endotoxin contamination of sBGN. Results are from 3 independent experiments. All results are expressed as mean and SD. ***p<0.001 vs. non-stimulated controls.
Figure 26. Effects of soluble biglycan (sBGN) on chondrocyte production of nitric oxide (NO) compared to control groups. (A) In primary chondrocytes, sBGN stimulation caused an increase in NO production release to cell culture supernatant. Lipopolysaccharide (LPS), used as a positive control, induced a similar effect on NO production albeit the production levels were higher than during sBGN stimulation. (B) In cartilage explants sBGN stimulation led to a marked increase NO production in grade I osteoarthritis (OA), while in grade IV OA the differences were not substantial. (C) The addition of polymyxin B had no effect on the sBGN-induced NO production while proteinase K restored NO production to levels observed in control groups, strongly suggesting endotoxin contamination of sBGN was not present. The addition of TLR2 neutralizing antibody caused no significant alteration of sBGN-induced NO production levels while TLR4 neutralization impaired NO production. Samples were run as technical duplicates, and each experiment was done using samples obtained from at least six different donors (biological replicates). All results are expressed as mean and SD. ***p<0.005 vs. non-stimulated controls or pairwise comparisons.
6. Discussion

6.1. Primary vs. differentiated MSC-derived chondrocytes: TLR1/2 heterodimer response

Our results demonstrated that TLR1, TLR2 and TLR9 were expressed in mature chondrocytes but also in MSC differentiated for 21 days to chondrocytes at mRNA and protein level. Together these findings may settle the apparent discrepancies in some previous studies, which did not report TLR1 and TLR9 protein expression and contradictory results of their mRNA expression levels (156,226,227).

In this study, we used primary chondrocytes and MSC-derived chondrocytes cultured for 21 days to study the innate immune response capacity of MSC-derived chondrocytes, given their active use and potential for cell therapy applications of cartilage regeneration in OA joints. Interestingly our study revealed that TLR-profile is altered during the activation and differentiation stage of chondroprogenitors. As results show TLR1, TLR2 and TLR9 were mostly upregulated in primary OA chondrocytes and chondroprogenitors (day O) but were weakly expressed in healthy MSC-derived chondrocytes cultured for 21 days.

TLR1/2 heterodimer activation by Pam3CSK4 was studied given that TLR1 and TLR2 had high expression levels in trauma-derived primary chondrocytes. Heterodimer activation was studied instead of TLR2 homodimer given that the latter has been already studied in chondrocytes (259). The results demonstrated that TLR1 and TLR2 are functionally active in human chondrocytes (Pam3CSK4 ligand stimulation) in heterodimer TLR1/2 form. Importantly, danger signal-TLR1/2 interaction led to a significant up-regulation of the proinflammatory cytokine TNF-α, but also of TLR1 and TLR2 expression.

Because Pam3CSK4 caused significant increase in levels of TNF-α in trauma-derived primary chondrocytes, its local effects were studied, since a local production by chondrocytes could act in an autocrine manner and, therefore, might have a pathophysiological role in OA, as proposed by others (260,261). Chondrons contain more than one cell and thus chondrocyte production of TNF-α could have juxta and paracrine effects on other chondrocytes (262). Such stimulation revealed interesting results. TNF-α lead to upregulation of TLR2 levels in a dose-dependent manner. TLR1 and TLR9 mRNA levels were mostly unchanged and not statistically significant. This effect was surprising and unexpected with only similar reports in inflammatory cells such as macrophages, microglia or fist-line defense pleural mesothelial cells (PMCs) (263-265). Also, we demonstrated that TNF-α stimulation also caused self-upregulation of mRNA levels in primary OA chondrocytes.

By studying proteoglycan deposition with Safranin-O staining, we could observe the effects TNF-α-stimulation caused on newly formed ECM in pellets from MSC-derived chondrocytes cultured for 21 days. TNF-α-stimulation caused a major depletion of PGs in a dose-dependent manner. In line
with observed proteoglycan depletion, TNF-α-stimulation caused a marked increase of COL2A-3/4M immunostaining, hence an increase in collagen type-II cleavage in the ECM of pellets from MSC-derived chondrocytes cultured for 21 days. The observation of proteoglycan depletion and collagen cleavage is typical. Proteolytic enzymes degrade cartilage ECM cartilage by disrupting ECM PGs to which may follow collagen cleavage and degradation (266).

As mentioned earlier TNF leads to the upregulation of TLR2, but not TLR1 or TLR9. It will be interesting to study further its effects on the remaining TLRs. The results open new questions about the role of other OA relevant cytokines in the innate immunity mediated inflammation in OA and their role in TLRs expression and signalling. Critical OA cytokines such as IL-6, GM-SF and IL-17 have already been shown in other cells to regulate the expression of TLR2 and TLR4, TLR3 and TLR7, and TLR3 respectively (267-269).

Taken together these results suggest that TLR1, TLR2 and TLR9 in OA chondrocytes not only play some homeostatic maintenance role but may be actively involved and differentially regulated during OA inflammation and chondroprogenitor differentiation. We also demonstrated that in chondrocytes TNF-α stimulation, a potent proinflammatory cytokine present in OA, also leads to chondrocyte TLR2 activation. Together TNF-α and TLR2 activation mounted an inflammatory response sufficient to compromise the integrity of native PGs and collagen in new cartilage ECM synthesised by MSC-derived chondrocytes.

Our results should be considered in the current strategies utilizing MSCs as novel treatment- and regenerative therapy efforts in the OA field. Our research demonstrated that if in the OA joint inflammatory environment elements such as cytokines or DAMPs are not neutralized, they can affect and degrade newly synthesized ECM by MSC, even despite MSCs known anti-inflammatory properties.

6.2. TLR expression during OA progression in knee and CMC-I joints

In our previous study, we demonstrated that TLR1, TLR2, and TLR9 were present in articular cartilage from trauma patients, and their ability to be activated and initiate a cartilage degrading response. However, in the OA joint several more other cytokines and DAMPs are present, and in higher concentrations, which can further activate other TLRs. Therefore, it is important also to expand our knowledge to include the full list of TLRs, and how their expression is regulated during progressing degradation of OA cartilage. Moreover, in the current study we were also interested in studying TLRs expression in smaller joints such as the CMC-I given its different size, biomechanical loading, and different risk factors.
Our results demonstrated that human articular cartilage resident chondrocytes in the surface zone were fully equipped with all TLRs even at early stages of OA (OARSI grade I). Our results also indicate that TLR expression in cartilage may start at the surface zone and that TLR signalling of chondrocytes in deeper layers become activated later. These findings seem to fit in the current conceptual mechanisms of OA pathogenesis since it is at the surface zone where initial wear and tear occurs and where the surface fibrillation and focal loss of cationic staining (proteoglycan loss) are first seen (87). Therefore, resident chondrocytes at this location are the first line responders to the released ECM degradation, also known as danger signals, able to trigger the danger signal receptors namely the TLRs. At OARSI grade 2, the proportion of TLR-positive cells further increases but also extends into the middle zone of cartilage. At OARSI grade 3 and 4, the TLRs expression reach highest proportions. Surprisingly, such level of expression corresponds with the peak of extensive cartilage degradation. At this levels cartilage fissure extends up to the deep zone, and 2/3 of cartilage is proteoglycan depleted. Interestingly, it is also at this level where one may expect the highest peak of cartilage degeneration and release of danger signal at highest concentration levels, due to accumulation as well. These results are in line with a similar pattern only studied for TLR4 using an equivalent histological scoring [11]. It is also tentative to speculate that this proportion of TLR positive chondrocytes is congruent with the severity and topology of cartilage damage, maybe being a way to adjust the degree of TLR-mediated proinflammatory response in chondrocytes during the disease development.

In contrast, with the consistent increase in the expression of TLRs as the disease process of knee OA progresses through cartilage layers (OARSI grade definition), the TLR expression pattern in cartilage from CMC-I OA was substantially different.

Given the heterogeneous CMC-I histomorphometric data, we applied principal component analysis (PCA) in order to discover if protein-expression patterns were present thereby allowing easier interpretation. PCA revealed a clear clustering of TLRs which can form heterodimeric complexes at the surface of the cell (TLR1, 2, 5 and 6), in parallel with the clustering of the endosomal TLRs (TLR7, 8 and 9). In general, all these MyD88-dependent TLRs co-operate with each other (170). The only exception was TLR3 clustering with the cell surface TLRs. Unlike the previously mentioned TLRs, TLR3 does not use MyD88 but uses a TRIF-dependent signaling pathway exclusively. Nevertheless, the association with surface TLRs such as TLR2 might have functional consequences because they have synergistic proinflammatory effects mediating a “two-hit” trigger. Interestingly TLR4 acted unequivocally differently compared to other TLRs. It is worth in this context to note that TLR4 is the only TLR using both MyD88 and TRIF signaling. TLR4 protein expression constantly remained low across all CMC-I samples in a striking contrast to the increased expression in knee OA disease progression.
Our study shows conclusively that all TLRs are actively expressed in knee OA cartilage which settles the debate about their expression in chondrocytes from knee cartilage origin (157,226,227,271). However, we observed differential expression of TLRs in OA cartilage which depended on the joint in question. Reasons contributing to such differences may relate to the common and independent factors known to be associated with hand and knee OA joints. Risk factors such as obesity, for example, may be involved. Obesity is known to increase the likelihood of hand OA by two-fold (272). However, given that mechanical overload does not affect non-weight-bearing joints such as hand OA, other obesity-related systemic factors such as adipokines may be involved (28,273,274). Adipokines are not only present systemically but are also present in OA joint SF in significant concentrations (275). Adipokines have been shown to have anti- and proinflammatory properties but also shown to regulate TLR expression and vice-versa (276,277).

Genetic and epigenetic differences between the two joints may also play a significant role in the substantially different TLR expression. As OA is a polygenic disease, recent genome-wide association study (GWAS) efforts have identified several risk-conferring loci for hip, knee and hand OA. This information also showed that certain loci only associate with certain joints, further stressing the need for joint stratification when studying OA (278-280). Interestingly TLR3 and TLR9 polymorphisms are associated with end-stage knee OA (281,282). TLR polymorphisms associated with other OA joints, such as hand OA remain to be studied.

Dilution of cartilage matrix-derived danger signals (damaged/solubilized ECM) may also differ significantly. It has been known for some time that knee joint SF clearance depends on the disease type and stage (139). However, it remains to be studied if clearance kinetics of SF in other joints, differ from the finding in the knee joint. Different SF clearance rates would clearly affect local concentrations of potent TLR-activators such as cytokines and DAMPS and could underline our current observation of differential TLR expressions between CMC-I stages and CMC-I and knee OA cartilage. Other intrinsic differences may also play a role such as biomechanical loading (macro vs. micro), anatomy and joint size.

Epidemiological associations between hand OA and other OA joint incidence have been intensely studied, and many associations have also been found. Nevertheless, to date, few studies have confirmed extensive common pathomechanisms occurring in OA joint (283,284). Our findings at this early stage suggest that the expression of TLRs is inherently different between knee and CMC-I OA joint, which also would suggest significant differences in the innate immunity mechanisms driving OA in different joints.
Our results add an extra piece of evidence to the current literature such as the epigenetic and transcriptomic studies stressing that the “disease process” of OA is unlikely to be uniform across the synovial joints of the body (283,284).

6.3. The role of SLRPs in OA

BGN and DCN are two major SLRPs embedded into the cartilage ECM matrix known to be maintained by articular chondrocytes (285). In the current study, we demonstrated, to our knowledge, for the first time, that sBGN and sDCN native forms are present in SF collected from patients with meniscectomy, TKA due to advanced OA stage or RA. Interestingly the highest concentration levels of sBGN are found in SF from advanced OA and RA patients. The concentrations of intact sDCN, in contrast to sBGN, were relatively low and constant across meniscectomy, in advanced OA-, and RA patients. The presence of sBGN in SF implies that chondrocytes, as well as other resident cells in joint tissues, may be in contact with the fluid phase of sBGN and sDCN.

The measured concentration levels of intact soluble sBGN are in congruence with the reported up-regulation of gene and protein expression levels of sBGN in human OA cartilage and as well with reported levels in sheep cartilage, obtained from a sheep meniscectomy model of OA. Although BGN and DCN are closely related and follow the same trend of increased gene and protein expression, the levels of DCN remain unchanged and in striking contrast with measured BGN levels in SF (245,286,287). Despite the close structural relationship shared by these two SLRPs, several studies have shown they compete for same bindings sites but also bind to different sites and are differentially cleaved by proteolytic enzymes. It is, therefore, tempting to speculate that these may well be explanatory factors for the different concentrations of intact BGN and DCN in SF (288,289).

Intact sBGN and sDCN, but not their fragment forms, have been shown to engage proinflammatory responses (244,290). BGN fragments have been shown to be increased in cartilage of OA patients, and to be released from cartilage explants in in vitro studies. Hence, the presence of sBGN fragments is a reflection of disease activity, without further molecular implications being mostly suited for diagnostic biomarker applications. Interestingly, SF from OA and RA patients is known to contain BGN, DCN, and other cartilage IgG autoantibodies of matrix molecules, indicating SLRPs release from cartilage matrix and subsequent local loss of immunological tolerance (291). Immune complex formation leading to complement and FcγR activation plays a role in RA, but according to recent studies also in OA (292-294). The present finding of intact sBGN in SF, together with the recognition of sBGN as a DAMP-type ligand for TLR2/4 and P2X7R, provides a third mechanism of relevance for autoinflammation, co-stimulation, and autoimmunity.
Elsewhere in this thesis, it was demonstrated that in knee OA the resident cartilage chondrocytes express all known TLRs, including sBGN ligand receptors TLR2 and TLR4, and that as the disease progresses also the proportion of TLR+ chondrocytes increases. In our most current study, we demonstrated that sBGN was able to regulate TLR4 expression in a similar fashion as the potent activator LPS used as a positive control. We further confirmed that such observation was not caused by the sBGN isolation process since endotoxin tests were negative and sBGN incubation with polymyxin B did not alter the measured response in OA primary chondrocytes. These results were further confirmed in a parallel experiments using HEK-blue TLR4 reporter cell line. Together these were strong indicators that sBGN, derived from articular cartilage, could in fact act as a DAMP. Moreover, we were also able to determine that the sBGN mechanism of action proceeded through TLR4 activation and the NF-κB pathway. Using a human HEK-blue TLR4 reporter cell line, which only expresses TLR4, we showed that sBGN and sDCN stimulations caused upregulation of AP production, as a result of increased activation of the reporter gene SEAP monitoring NF-κB activation. Also, AP production was halted by CLI095 incubation, which is a synthetic inhibitor of TLR4 signaling. NF-κB activation by sBGN was further confirmed in primary OA chondrocytes by studying NO production and release to cell culture medium. TLR4 signaling in chondrocytes is mediated through NF-κB, and NF-κB activation is the major inducer of iNOS (295,296). Moreover, NO is a proinflammatory molecule at increased levels in OA synovial fluid and is an active contributor to the inflammatory mechanism of OA (297-299). sBGN stimulation leads to increased NO concentration levels in supernatant medium from in primary chondrocyte cultures while sBGN incubation with CLI095 or TLR4 neutralizing antibody restored NO to baseline concentration levels. Interestingly TLR2 neutralizing antibody did not have any effect on the sBGN-induced increased production of NO. Taken together the observed increase of secreted AP and NO in our cell culture studies, strongly suggested that sBGN can mount a chondrocyte-mediated inflammatory response.

The sBGN concentrations used in our in vitro experiments were significantly higher than those measured in SF from OA patients, and may well be a limitation in our research. Nevertheless, in earlier in vitro experiments, sBGN stimulation exerted dose-dependent effect up to 20 fold concentration levels (300). In addition, BGN is mainly localized at cartilage pericellular matrix where it is most likely found at much higher concentrations than in SF (301). Moreover, sBGN was used at higher concentrations to assure outside-in access since in our in vitro experiments cartilage explants lacked native biomechanically cyclic compression which causes a decrease in interstitial fluid pressure. Thus, we expect the used sBGN concentrations of 10 fold higher than those found in SF, to be most likely of pathophysiological relevance.
sBGN stimulation of primary chondrocytes caused an upregulation of known OA-related proteolytic enzymes at the mRNA and protein level (302). The fact that sBGN caused upregulation of ADAMTS-4/5, MMP-9, MMP-13 and cathepsin-k means that all the main structural elements of cartilage matrix can be degraded indicating that cartilage integrity can be compromised by sBGN-TLR4 activation. Stimulation of chondrocytes with sBGN also caused an increase of production of proinflammatory cytokines IL-6 and IL-8. Their active release into the synovial joint could then lead to recruitment and inflow of neutrophils and macrophages (303,304). The sBGN induced induction of MMPs and proinflammatory cytokines could add further stress to the OA joint and might contribute tilting the balance towards continuous inflammation and perpetuating cartilage degradation.

Our current study also demonstrated that the response of OA cartilage explants to sBGN was clearly dependent on the macroscopic grade of OA. We observed a substantially higher sBGN-induced TLR4 upregulation in grade I OA in comparison to grade IV OA. Interestingly, BGN and TLR4 are more upregulated in advanced stages of OA when compared to early stages (245). This suggests that desensitization to BGN but also TLR4 impaired function in grade IV OA explants may have occurred due to long-term exposure to sBGN and other known DAMPs at this end stage. However, negative regulatory mechanisms and functio laesa due to long-term exposure and chronic inflammation should also not be excluded as possible reasons. Thus, in grade I OA, the sBGN induction of high TLR4 expression leads to significantly higher levels of MMPs and ADAMTS as well as NO release to supernatant.

Stimulation with sBGN also inhibited the synthesis of collagen type II and aggrecan which are two essential structural elements of cartilage matrix. The sBGN effect on this two matrix elements was also dependent on the grade of cartilage explants, with the highest inhibition occurring in grade IV OA explants. The resulting imbalance between de novo synthesis and matrix degradation will then lead to a net loss of cartilage matrix elements and compromise of cartilage integrity (305,306).

Given the high efficiency and consequences of enzymatic activity, the proteolytic activation of cartilage degrading proteases is highly regulated. It is, therefore, no surprise that at the core of known pathomolecular mechanism of OA lies the proteolytic activation of aggrecanases and collagenases to which we have shown that sBGN stimulation was able to contribute. In the current study, we demonstrated the increased production and released of ADAMTS, MMPs, and cathepsin-k, which are known for their ability to degrade structural cartilage molecules. In addition, we also demonstrated the final product of their activity, measured by the release of PGs and collagen degradation products. By measuring the release of PG using a dye-based glycosaminoglycan assay we determined that sBGN stimulation caused sharper increased release of PGs in grade I OA vs. grade IV OA much in line with the observed higher production and release of cartilage degrading proteases. Collagen release was
also determined by dye-based collagen assay, however, and in contrast to PG release results, collagen release to the supernatant medium was only significantly different in type IV OA. This may be due to an already advanced stage of PG depletion which, allows better access to collagenolytic enzymes to degrade collagen fibers (266).

7. Conclusions

This study focuses on the role of chondrocytes and chondroprogenitors in OA inflammation, especially in terms of cartilage degradation, TLR expression, the response of chondrocyte cells to proinflammatory cytokines and newly identified DAMPs, and the production of proinflammatory cytokines, cartilage degrading enzymes and ECM molecules. Our main findings can be summarized as follows:

1. TLR1, TLR2 and TLR9 expression was confirmed in trauma-derived primary chondrocytes, articular cartilage, and chondroprogenitors. We demonstrated that during chondrocyte differentiation the initial high expression of TLR1, 2 and 9 decreases towards baseline levels in fully differentiated chondrocytes.

2. We demonstrated that the proinflammatory cytokine TNF-α is able to increase the expression of TLR2 in both healthy primary chondrocytes and in MSC-derived chondrocytes for 21 days. TNF-α was demonstrated to induce cartilage degradation in *de novo* ECM matrix from pellet cultures of MSC-derived chondrocytes cultured for 21 days. This implicates TNF-α as an inducer of matrix degradation, with wide possible implications when using MSCs strategies in cartilage repair strategies for OA. Our study also added further evidence for a role of TNF-α in inducing TLR-innate immunity in the OA synovial joint.

3. TLR protein expression in cartilage from knee and CMC-I OA patients was shown to be strikingly different. Our study demonstrated, for the first time, all known TLRs being expressed at protein level in cartilage of knee OA patients. Moreover, we demonstrated that their expression is up-regulated in a cartilage zone-dependent fashion according to the histological progression of knee OA. Expression of TLRs in cartilage from CMC-I OA patients was highly heterogeneous, although it followed an close expression pattern according to the known TLR cell compartment localizations. This study implicates that TLR mediated innate immune response in the two joints may be significantly different.

4. Decorin, a known SLRP, and a ligand able to activate TLR2 and TLR4, was for the first time shown to be present in knee synovial fluid of both OA and RA patients. We
confirmed the ability of sDCN to activate TLR4 signaling. However, the observed low and stable concentration levels across the studied arthritis groups mean that these findings may not be of clinical relevance in OA pathogenesis and the associated TLR-mediated inflammatory events.

5. Biglycan, another known SLRP ligand able to activate TLR2 and TLR4, was for the first time found to be present in knee synovial fluid of OA and RA patients. Interestingly we discovered that sBGN is upregulated in synovial fluid from OA and RA patients. The ability of sBGN to activate TLR-mediated innate immunity was shown to be mediated through TLR4 signaling. The sBGN stimulation leads to upregulation and release of proinflammatory cytokines, matrix-degrading enzymes and the release of ECM degradation products.

As a final conclusion we believe TLRs to be potentially important drivers in the synthesis of proinflammatory and destructive enzymes associated with OA. Our results together with extensive evidence from the literature point towards a prominent role of TLRs in OA. TLR activation could be initiated as a proinflammatory reaction against cartilage matrix degradation products which may be accumulated into synovial fluid due to low synovial fluid clearance and the avascular and alymphatic nature of articular cartilage. As results of TLR activation in joint tissue cells, proinflammatory, proteolytic enzymes and algogenic secondary mediators are released, which then may further compromise cartilage structure, upregulate TLRs, and activate infiltration of inflammatory cells to the synovium leading to secondary synovitis and pain (307,308). Such close regulation may likely orchestrate the rate and severity of the OA joint tissue catabolism and degradation. Figure 27 illustrates the conceptualization of TLR innate immunity role in the perpetuating cycle of OA disease.
Figure 27. Conceptualization of the Toll-like receptors importance in the perpetuating circle of cartilage degradation and inflammation that characterize the pathogenesis model of osteoarthritis.
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Original publications I-III