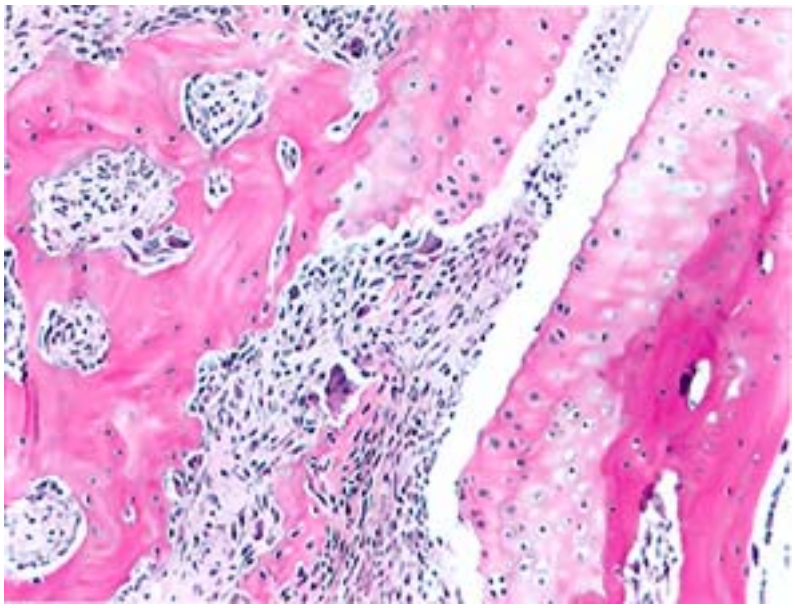
Tissue destruction in arthritis: Experimental studies

By

Esbjörn Larsson



Stockholm 2003

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Cover illustration: Collagen-induced Arthritis; section of inflamed joint at day 21 post immunization. The articular cartilage is partly destroyed.

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Too slow?

This thesis is based on the following articles, which are referred to in the text by their roman numbers

I

Larsson, E., Müssener, Å., Heinegård, D., Klareskog, L., Saxne, T. "Increased serum levels of cartilage oligomeric matrix protein and bone sialoprotein in rats with collagen arthritis". Br J Rheumatol 1997 Dec; 36(12): 1258-1261

II

Larsson, E., Erlandsson Harris, H., Lorentzen, J. C., Larsson, A., Månsson, B., Klareskog, L., Saxne T. "Serum concentrations of cartilage oligomeric matrix protein, fibrinogen and hyaluronan distinguish inflammation and cartilage destruction in experimental arthritis in rats". Rheumatology 2002 Sep; 41(9): 996-1000

III

Larsson, E., Erlandsson Harris, H., Larsson, A., Månsson, B., Saxne, T., Klareskog, L. "Corticosteroid treatment of experimental arthritis retards cartilage destruction as determined by histology and serum COMP". In press, Rheumatology

IV

Larsson, E., Erlandsson Harris, H., Palmblad, K., Månsson, B., Saxne, T., Klareskog, L. "CNI-1493, an inhibitor of proinflammatory cytokines, retards cartilage destruction in rats with collagen-induced arthritis". Submitted to Rheumatology

Table of contents

Abbreviations

ACR	American college of rheumatology
AD	Anno Domini
BC	Before Christ
BSP	Bone sialoprotein
CD	Cluster of differention
CIA	Collagen-induced arthritis
COMP	Cartilage oligomeric matrix protein
CRP	C-reactive protein
Cs	Corticosteroid
DA	Dark Agouti
DMARD	Disease modifying antirheumatic drug
EAE	Experimental autoimmune encephalomyelitis
ESR	Erytrocyte sedimentation rate
Fc	Fragment cristallic of immunoglobulin
FGF	Fibroblast growth factor
HA	Hyaluronan
HPA	Hypothalamo-pituitary-adrenal
IGF	Insulin growth factor
IL	Interleukin
MAPK	Mitogen activated protein kinase
MBP	Myelin basic protein
MHC	Major histocompatibilty complex
MIP	Macrophage inhibitory peptide
MMP	Matrix metalloproteinases
MOG	Myelin oligodendrocyte
OIA	Oil-induced arthritis
PDGF	Platelet derived growth factor
PLP	Proteolipoprotein
RA	Rheumatoid arthritis
TGF	Transforming growth factor
TNF	Tumor necrosis factor

Abstract

Destruction of joint cartilage and bone is one of the most serious consequences of chronic arthritis. Clinically, the standard assessment of joint destruction is performed by radiography. The sensitivity of radiographic analysis is rather poor, however, and destructive reactions have usually been ongoing for 3-6 months before erosions become radiologically visible. This thesis addresses the potential use of biological molecules, i.e. COMP (cartilage oligomeric matrix protein) and BSP (bone sialoprotein), and their presence in serum as assessment tools for joint destruction, which would allow an earlier and more sensitive verification of destructive joint events. We have established that serum markers used in clinical trials and clinical practice are also applicable in experimental arthritis models for the evaluation of joint destruction and of new therapies aimed at preventing destruction

We initially demonstrated that serum levels of COMP, BSP, hyaluronan and fibrinogen could be used as markers of cartilage destruction, bone destruction, synovitis and systemic inflammation, respectively in collagen-induced arthritis and oil-induced arthritis. Secondly, we confirmed the usefulness of COMP analysis in the evaluation of the cartilage-saving effects of therapies by comparing serum COMP levels with histological examination of joints. We could demonstrate that corticosteroid treatment, as well as treatment with the novel proinflammatory inhibitor CNI-1493, had cartilage-saving effects. In these studies we could observe a correlation between serum levels of COMP and the degree of cartilage destruction as evaluated by histology.

Taken together, these results demonstrate that biological markers for inflammation, synovitis, bone and cartilage destruction can be used in experimental models, both in order to evaluate disease course and severity and in order to evaluate the effects of anti-rheumatic drugs.

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Introduction

Prevention of tissue destruction is one of the aims of modern anti-rheumatic therapy. Evaluation of the tissue protective properties of drugs is, however, hampered by the fact that radiography, the golden standard for quantification of joint destruction, is insensitive. At least 6 months of treatment is needed in order to allow evaluation of the possible tissue-saving effects of the chosen therapy by radiography [1]. Pre-clinical evaluation of anti-rheumatic drugs is often performed in experimental arthritis models, where collagen-induced arthritis (CIA) is one of the most frequently used models. CIA presents a destructive polyarthritis with many clinical similarities to rheumatoid arthritis (RA) [2].

In order to improve early evaluation of anti-rheumatic drugs for their tissue protective capacity, there is an obvious need for biomarkers which can be used in both experimental models and in human arthritis, and which have the ability to rapidly mirror changes in cartilage as well as bone destruction. This thesis addresses the potential use of biomarkers for cartilage and bone degradation in experimental models for arthritis. Such biomarkers have recently been used in efforts to measure cartilage and bone destruction during the course of arthritis in human [3], but had prior to the work in this thesis not been used in experimental models of arthritis. We therefore thought it should be of value to investigate the usefulness of these markers for cartilage and bone destruction in relevant experimental models for arthritis, and also to test their usefulness in evaluation of cartilage-sparing effects of anti-rheumatic drugs tested in these experimental systems. The thesis work has therefore been devoted both to the validation of the markers in experimental arthritis models, and with efforts to use the biomarkers in evaluating the tissue-saving effects of anti-rheumatic therapies, i.e. one classical anti-rheumatic therapy (corticosteroids) and one new potential anti-rheumatic therapy (CNI-1493).

Rheumatology

The word Rheumatology can be derived from the Greek word *Rheuma,* which is a term for a substance that flows. *Rheuma* was conceived to be derived from phlegm, one of the four primary humors. Various ailments were caused depending on where the flow of *Rheuma* stopped. If the *Rheuma* stopped in a joint, it resulted in swelling and redness of the joint.

Today rheumatology is a speciality of medicine concerned with the diagnosis and management of diseases and of painful and

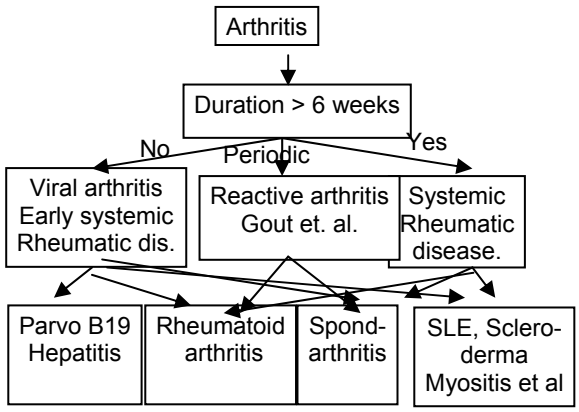
functional disorders of the musculo-skeletal apparatus that is, of the locomotor and connective tissue systems as well as of the adjoining soft tissues [4].

Despite the long-term awareness of rheumatic diseases (Hippocrates described rheumatic diseases already in the 4th century BC), their pathogenesis is not completely revealed. There is no general cure available for many rheumatic diseases and current therapies need improvement.

Arthritis

Arthritis is inflammation of the joint including inflammation of synovial membrane (synovitis), increased volume of synovial fluid and often engagement of cartilage and bone. Arthritis affects several percents of the population and results in loss of motion and pain in the affected and destroyed joints. In severe cases arthritis leads to disability and increased mortality.

The aetiology of arthritis differs; infectious arthritis (septic arthritis) being caused by viral or bacterial infections. Reactive arthritis can follow a bacterial infection targeting another site of the body, e.g. gut and urinary tract. Crystal deposition can also be the cause of arthritis, e.g. gout. For many rheumatic diseases, however, the aetiology is unknown. Polyarthritic diseases, including the subgroups of rheumatoid arthritis, spondylo-artropathies (pelvo-spondylitis, Reiter and psoriatic arthritis) and enteropathic arthritis, have a prevalence of several percent in the population with rheumatoid arthritis being the most common with a prevalence of 0,5-1%. Systemic rheumatic diseases such as vasculitis, myositis, scleroderma and systemic lupus erythematosus have arthritis as one feature of the disease.



Simplified guide in evaluation of polyarthritis.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common chronic polyarthritis in human, predominately affecting women. The prevalence is about 0.5-1% and the annual incidence is 25/100.000 persons in Western countries [5]. RA is a systemic disease, not only affecting the joints but also the serosa, vasculae, skin, kidneys, bowels and the haematopoietic system. As

for other arthritides, RA can result in disability and increased mortality [6].

The diagnosis of RA is performed by clinical evaluation, based on the patient's history, on physical examination and laboratory tests. Typically, RA is a symmetric arthritis affecting the small joints in hands and feet, with presence of rheumatic factors, morning stiffness and, if the symptoms have lasted for more than 6 months, often erosions in the periarticular bone.

For scientific use the American College of Rheumatology has published the so-called ACR criteria for the diagnosis and classification of RA [7].

1. Morning stiffness at least 1 hour
2. Arthritis of three or more joint areas
3. Arthritis of hand joints
4. Symmetric arthritis
5. Rheumatoid nodules
6. Serum rheumatoid factor
7. Radiographic changes

The ACR Criteria for diagnosis and classification of RA
**For classification purposes, at least 4 of the above 7 criteria must be fulfilled for the diagnosis rheumatoid arthritis. Criteria 1 through 4 must have been present for at least 6 weeks.*

The ACR criteria were originally intended for classification of groups of patients participating in clinical investigations and were not intended for diagnosis of individual patients. However, the criteria have been used as guidelines for patient diagnosis as well as for research classification but they might not always be appropriate in clinical practice.

As for many other rheumatic diseases, the aetiology of RA is still unknown. Many theories have been presented over the years, e.g. viruses, bacteria, myco-bacterium and mycoplasma inducing immune responses which might crossreact with autoantigens, although nothing has yet reached acceptance as the pathogenic agent inducing RA.

If RA is an autoimmune disease, there should be an immune response targeting an autoantigen. Several autoantigens have been suggested as being the disease-driving autoantigen; immunoglobulins, heat shock proteins and cartilage antigens are among those listed. Both genetic and environmental factors have been demonstrated to influence the development and severity of RA. Twin studies have demonstrated that the genetic influence may be as large as 60% [8], leaving 40% to environmental factors. As the strongest genetic factor influencing disease, the susceptibility to RA is demonstrated to be linked to the MHC class II haplotypes DR4 [9]. As an environmental risk factor smoking has been identified [10].

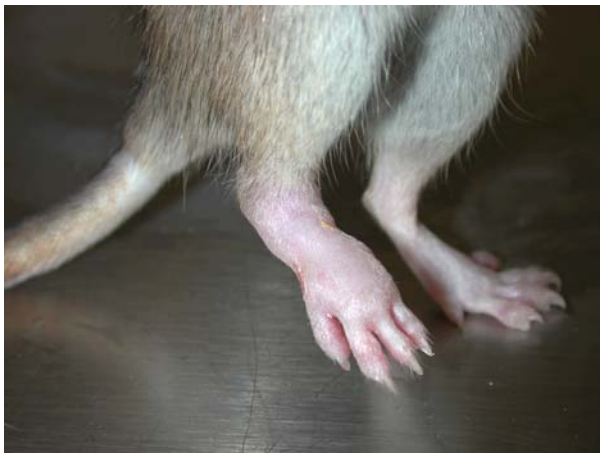
A high proportion of RA patients are positive for rheumatoid factor (RF) a marker associated with a more erosive disease progress (Rheumatoid factor is an autoantibody directed towards the Fc region of IgG [11]).

Despite the aetiology of RA being unclear, an increased knowledge of the cellular and molecular inflammatory reactions involved in arthritis pathogenesis has contributed to new and improved treatments.

The immune cells involved in arthritic inflammation are primarily lymphocytes, monocytes and neutrophils. During recruitment of inflammatory cells to the joint, a number of chemotactic substances are involved e.g. leukotrienes, chemokines and platelet activating factor. Recruited neutrophils release proteinases, prostaglandins, leukotrienes and reactive oxygen species, that all contribute to the inflammatory reaction. Recruited monocytes differentiate into tissue macrophages which apart from performing phagocytosis also release inflammatory mediators including the cytokines IL-1 (interleukin-1) and TNF (tumor necrosis factor). IL-1 and TNF interact with other cells of the joint, such as fibroblasts, which upon activation produce matrix metallo-proteinases (MMPs), serine proteases and cathepsins. These are enzymes that play important roles in the degradation of bone and cartilage.

Experimental models

In this thesis work we have utilized Dark Agouti (DA) rats, which develop arthritis after subcutaneous immunization with either collagen type II in adjuvant (collagen-induced arthritis, CIA) [12] or pure mineral oil (oil-induced arthritis OIA) [13].



A DA rat with maximal arthritis in the right hind paw

Collagen-induced arthritis (CIA)

CIA was first described by Trentham *et al* in 1977 [12]. They screened components from joints for their arthritogenic capacity and could detect that with Freunds adjuvant as a general enhancer of the immune system, immunization with collagen type II (the main collagen type in cartilage) produced a severe, destructive polyarthritis. Larsson *et al* reported that when using rat collagen type II for immunization of DA rats, a chronic

arthritis developed which had a true autoimmune origin [14]. CIA has many similarities to RA and the model has been utilized in many preclinical trials of anti-rheumatic drugs [15-23].

Oil-induced arthritis (OIA)

OIA was recognized in an experiment in which DA rats were immunized with pure mineral oil as a control to immunization with oil together with collagen II [24]. OIA has a less severe disease course than CIA and is not chronic in DA rats. The genetic factors influencing disease are partly different from those influencing CIA, as OIA cannot for example be induced in Lewis rat, which is a rat strain susceptibly to CIA.

Experimental arthritis is quantified using a clinical scoring system, ranging from 0-16. A classification of the joints in the paw was made. The interphalangeal joints of the digits, metacarpohalangeal and the wrist in the forepaw, metatarsophalangeal and anklejoint in the hindpaw were each categorized as one type of joint. Each paw is scored as follows: 0 = no arthritis, 1 = swelling in one type of joint, 2 = swelling in two types of joints, 3 = swelling in three types of joints and 4 = swelling of the entire paw. A total score for each rat is calculated by summarizing the scores for each of the 4 paws, giving a maximum score of 16 for each rat [24, 25]. This clinical scoring system is quite similar that used in humans, and is therefore preferred to other methods such as measuring increase in volume of the paws or thickness of paws.

Experimental autoimmune encephalo-myelitis (EAE)

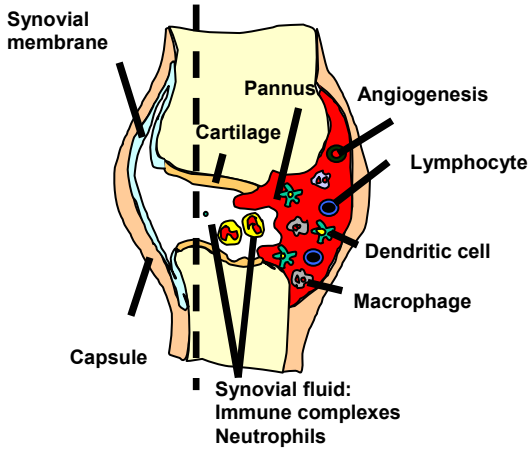
EAE is an experimental disease used as a model for human multiple sclerosis. EAE develops after immunization with mineral oil together with spinal cord extract (or purified myelin antigens such as MBP, PLP and MOG) and gives rise to a very severe MS-like disease [26]. This experimental disease was utilized as a non-arthritic inflammatory control to OIA and CIA in paper II, for evaluating the molecular markers for inflammation, synovitis, cartilage and bone destruction.

The normal joint

The function of a joint is to allow movement between adjacent bones. There are two types of joints, synovial (or diarthrodial) joints and synarthrodial joints. Examples of synovial joints are hip, knee and ankle joints, as well as the small joints of the hands and feet. Synovial joints have a synovial lining and are highly mobile,

whereas synarthrodial joints do not have a synovial lining and are much less mobile. Examples of the latter joint type are symphysis and intervertebral joints. A synovial joint is the joint most prone to arthritis development.

A normal synovial joint consists of opposing bones with surfaces of articular cartilage. The joint is surrounded by a capsule, which can vary in thickness. The internal side of the capsule is covered with a thin synovial lining which is comprised of macrophage-like cells and fibroblast-like cells.



Schematic drawing of normal and inflamed (to the right) joint adapted from Feldmann [27]

The synovial membrane functions as an ultrafilter for plasma and produces synovial fluid. Synovial fluid conveys nutrients for the articular cartilage and also functions as a joint lubricant. Fibroblast-like synovial cells produce hyaluronan and lubricin, the two major lubricating constituents of synovial fluid. Clearance of joint fluid is made by lymphatic drainage [28].

The inflamed joint

In the inflamed joint, cells of the immune system have mounted an attack on joint tissue. The inflamed synovium is infiltrated by activated macrophages [29] differentiated from blood monocytes. The macrophages produce inflammatory mediators such as TNF and IL-1. Activated macrophages are also potent phagocytes and antigen-presenting cells. Their expression of Fc receptors renders them efficient in the binding of immune complexes, which can precipitate further inflammation.

Apart from macrophages, the synovium is also invaded by fibroblasts, which will develop into protease-producing cells. Activated fibroblasts are thus involved in the direct degradation of cartilage and bone. The inflamed synovium is also infiltrated by newly formed blood vessels, which supports the synovial hyperplasia. This angiogenesis is driven by the production of the growth factors FGF-1 and FGF-2 as well as other growth factors such as VEGF (Vascular endothelial growth factor) [30, 31].

Cells of the adaptive immune system, T- and B-cells, also accumulate in the

inflamed synovium where they can form germinal center-like structures [32, 33].

The volume of synovial fluid is increased in an arthritic joint and the synovial cavity contains large amounts of macromolecules such as hyaluronan. Synovial fluid from a non-inflamed joint is acellular, while inflammatory cells accumulate in the inflamed synovial fluid during arthritis. Neutrophils are the dominant cell type, but all above-mentioned inflammatory cell types are also found in inflamed synovial fluid. Activated neutrophils produce proteinases, prostaglandins, leukotrienes and reactive oxygen species, thereby contributing to both inflammation and destruction.

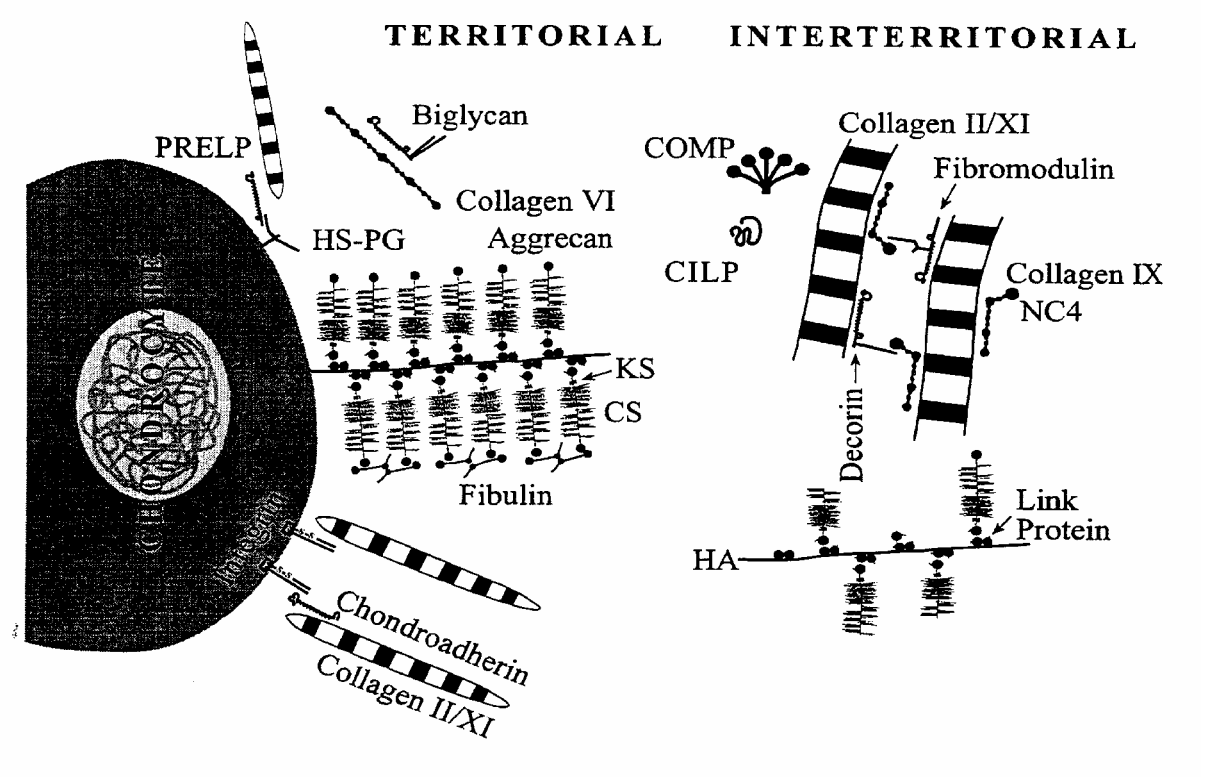
In the subchondral bone, osteoclasts are stimulated to increase their phagocytosing activity, leading to increased bone degradation. In the cartilage, chondrocytes are prematurely killed in response to the inflammation, leading to a loss of extracellular matrix. The end result is degradation of cartilage and bone, which is visualized as erosions on radiographic films.

Articular cartilage

Articular cartilage is an avascular connective tissue that covers the weight-bearing surfaces in synovial joints. The principal roles of articular cartilage in joints are reduction of friction and absorption of the shock associated with movement. The sole cell type in adult synovial cartilage is the chondrocyte, which produces proteins making up the cartilage matrix. Collagen type II is the most abundant protein in cartilage and forms a fibrillar network. This network is interspersed by aggrecan molecules, large proteoglycans with the ability to retain water. The charged groups of the aggrecan molecule attract ions and create an osmotic pressure which facilitates the retention of water in cartilage. Up to 80% of the wet weight of cartilage is water [34, 35]. Aggrecan also contributes to the loadbearing capacity of cartilage. Up to 100 aggrecan molecules bind to hyaluronan and form large complexes [36]. At the protein level, aggrecan consists of a central core protein with glycosamino-glucans radiating from the core. The glycosaminoglucans are long chains of polydimeric saccharides (e.g. chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate).

Furthermore, several other proteins such as collagen type VI, IX and XI, as well as thrombospondins (one being COMP) are important contributors to the cartilage extracellular network [36-38]. Being an avascular tissue, the nutrition distribution is mediated by diffusion from the synovial fluid [39]. Cartilage is continuously

renewed and old molecules are degraded by proteolytic enzymes such as metalloproteinases. Matrix turnover is controlled by growth factors (TGF, IGF-1) [40] and cytokines (IL-l, TNF) [41], the latter being catabolic. However, in comparison to many other connective tissues, the turnover of adult articular cartilage is slow and the capacity of repair is limited.



Articular cartilage and its major constituents, CS (chondroitin sulphate), KS (keratan sulphate), NC4 (domain in collagen IX), PRELP (Proline/arginine-rich end leucine-rich repeat protein), HS-PG (Heparan sulphate proteoglycan), CILP (Collagen I lysylpyridinoline).

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Bone

Bone is a connective tissue with several functions. It is a supportive tissue that enables motion, protects internal organs, contains the blood-forming organ, and has a central role in maintaining Ca^{2+} homeostasis. Bone comprises three celltypes: osteoblasts, osteocytes and osteoclasts. Osteoblasts and osteocytes are derived from mesenchymal stem cells and are closely related. Osteoblasts synthesize and mineralize the bone by producing collagen type I, osteonectin, sialo-protein and osteocalcin. Glucocorticoid treatment increases osteoblast apoptosis and thereby leads to decreased bone formation [42]. Osteocytes are important in conveying nutrition throughout the bone. Osteoclasts, as opposed to osteoblasts and osteocytes, are of hematopoetic origin [43] and have an important role as macrophages in bone remodeling and in fracture healing.The bone-remodelling activities of osteoblasts and osteoclasts are tightly coupled to each-other, as both cell types produce mediators regulating the function of each other [44].
The strength and rigidity of bone is created by its mineral compartment. About two thirds of bone consists of hydroxyapatite crystals.

Molecular markers

Molecular markers in the meaning that is discussed in the current thesis reflect either catabolic or anabolic (or sometimes both) processes in a particular tissue. An ideal molecular marker would be specific for the studied tissue, easy obtainable from the blood stream with no diurnal changes, only reflect pure catabolism (or anabolism) depending what process you will follow and would be possible to analyze at low cost. There would be small inter- and intra-individual level variations and high sensitivity and specificity. Regrettably such markers are rare. In most tissues there is remodeling ongoing, and catabolic and anabolic processes are occurring simultaneously. Many proposed markers are not ideal. There is thus a great need for further development and evaluation of molecular markers, which fulfill the above mentioned criteria.

Cartilage markers

Molecular markers obtained from serum, urine or synovial fluid and their use as diagnostic tools for assessing joint damage in arthritis have been a research topic for some time. Antibodies that recognize products of collagen or proteoglycan degradation or recognize synthesis of newly synthesized matrix components that

represent attempts to repair the damaged matrix have been developed. An example of a degradation product of collagen II is collagen type II C-telopeptide [45], and examples of degradation products of aggrecan are coreprotein fragments and keratansulphate [46]. These have also been suggested as potential biomarkers of cartilage destruction.

Proteoglycans measured in synovial fluid [47] and in the circulation [48] were early candidate markers for cartilage destruction. The levels of proteoglycan fragments were high in early RA and in osteoarthritis, but low in late RA [47].

Markers for formation of collagen II e.g. N and C pro-peptides have been investigated in osteoarthritis and RA [49-51] and can prognosticate cartilage destruction.

Serum markers are useful in animal experiments and in this thesis I have established and used COMP (Cartilage Oligomeric Matrix Protein) as a serum marker of cartilage degradation. As is discussed below, COMP is, however, not only a marker for cartilage degradation; the synthesis of COMP increases during cartilage destruction and it is thus possible that increased serum levels of COMP also reflect an increased syntesis of this molecule

COMP

COMP is a pentameric protein and a member of the thrombospondin family. COMP has a molecular weight of 524 kDa and is comprised of five identical subunits which are connected by their N-terminal ends [52]. COMP was originally identified as a constituent of cartilage [28] and has later also been found in tendons [53], ligaments [54] and in synovial membranes [55].

COMP interacts with Collagen I and II in cartilage and is important for the formation of the fibrillar collagen network [56]. Patients with mutations in their COMP gene develop multiple epiphyseal dysplasia and psuedoachondroplasia. COMP is a candidate biomarker for the monitoring of cartilage destruction [57].

The origin of increased serum COMP levels in osteoarthritis and arthritis has been debated, and it has been demonstrated that COMP is produced not only in the cartilage, but can also be produced in tendons and in the synovial tissue [53, 54] Increased levels of COMP in serum have been detected in marathon runners [58] and studies of osteoarthritis patients have revealed increased levels of serum COMP [59]. COMP has also been demonstrated to reflect cartilage destruction in early RA patients [60]. In patients with longstanding RA, high levels of serum COMP correlate

with future joint destruction [61]. COMP measurements have further been utilized in therapeutical intervention studies in mice [62] and in humans e.g. TNF treatment has been demonstrated to retard cartilage destruction in RA [63] and decreased levels of serum COMP has also been reported as a result of TNF modifying treatment [64]. Serum COMP levels do not correlate with serum levels of C-reactive protein during inflammation, implicating that COMP is not a marker of inflammation in general but rather for cartilage destruction.

Interestingly, an arthritogenic effect of COMP has also been described [65, 66]. Rats immunized with either bovine or rat COMP developed an MHC-associated, rather mild, arthritis

The analyses of COMP in previous studies (as well as in the present thesis as presented later in this text) have mainly been carried out with the help of ELISA techniques [67]. In most cases, ELISA plates have been coated with an antibody directed against COMP, incubated with samples of interest and the bound COMP detected by the addition of a secondary COMP-specific antibody. In these systems, sample values are calculated using comparisons with standard curves made from known dilutions of COMP.

Bone markers

As for cartilage, several molecules have been suggested as markers of bone destruction. Osteocalcin [68] and skeletal alkaline phosphatase [69] are markers for bone synthesis and increased levels can therefore be correlated to bone repair as a consequence of destruction. Markers suggested as direct indicators of destruction are degradation products from collagen type I such as deoxypyridinoline and amino- and carboxy- terminal crosslinked telopeptide [70].

The non-collagenous protein bone sialoprotein is highly expressed in subchondral bone which is destroyed during arthritis development. Thus serum bone sialoprotein is a potential marker of bone destruction.

Conversely to cartilage, bone is a tissue that undergoes continuous remodelling influenced by several conditions such as osteoporosis, menopause and aging, and not only during arthritis. This complicates the use of bone markers in evaluation of joint destruction during arthritis, and the therefore, cartilage markers are often considered to be more useful for the analysis of tissue destruction during arthritis.

Bone sialoprotein

(BSP) is a bone-specific protein synthesized by osteoblasts. It has a molecular weight of 70kDa and is preferentially expressed in subchondral bone. The fact that it is mainly localized near the cartilage makes it particularly interesting as a marker of bone loss during destructive arthritis [71]. The function of BSP is unclear, although it has been demonstrated that it can promote formation of hydroxyapatite [72].

BSP has been investigated as marker of bone loss in studies of osteoarthritis and arthritis. Increased serum concentrations of BSP have been correlated to radiologically verified destruction of bone in patients with RA/OA [73, 74].

Serum BSP is usually measured by ELISA in both the human and animal experimental contexts. In the case of the rat assay, used in the present thesis, a modification of the assay for human BSP was utilised. Rat BSP was prepared from powdered bone, and the purified BSP was used for coating ELISA plates and for preparing the standard curve. The antiserum was raised against rat BSP in a rabbit in the same way as previously described for antibodies to human BSP [68, 75].

Synovial markers

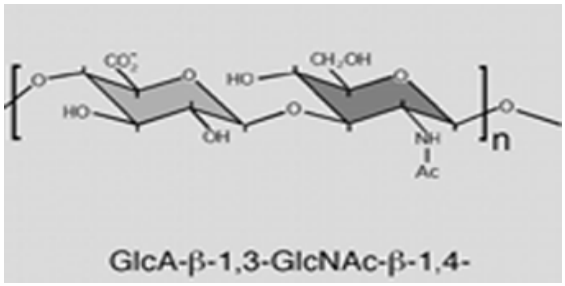
Several biomolecules have been suggested as systemically measurable markers of synovial inflammation (synovitis). In this thesis work we have utilized the detection of hyaluronan in serum building on the experience from previous studies that have suggested HA as a good marker for synovitis, and where it was reported that levels of HA in serum increase during both CIA and RA [76, 77]. Other proposed markers include the N-propeptide of type III procollagen, the noncollagenous protein YKL-40 [78] and the pyridinoline derivative Glc-Gal-PYD [79-81]. At the point when our studies were performed, of the above mentioned markers, only HA was available for use.

Hyaluronan

Hyaluronic acid (HA), is a macro-polysaccharide with a molecular weight varying between 1000-3000kD. More specifically, HA is a glycosaminoglucan, meaning that it consists of linear anionic polysaccharides containing a hexosamine residue. HA was first isolated from the eye and later from cartilage and synovial fluid and now HA is demonstrated in all connective tissue. HA is present in inflamed joints in humans [82] and rats [76]. It is synthesized by fibroblasts and other cell types (e.g. smooth muscle cells

[83], liver parenchymal cells [84], glial cells [85]). HA is also produced by malignant mesothelioma and has been used in diagnosing mesothelioma in pleura [86]. The production of HA is influenced by hormones (Parathormone increases [87, 88], cortisone decreases [89, 90] and IL-1 increases [91]. HA has different functions throughout the body; in joints it has a lubricant function and in eyes a supportive effect. HA regulates water balance. One study reported an interaction between fibrinogen and HA and suggested that increased levels of HA in sera could interfere with blood haemostasis and result in both bleeding and thrombosis [92]. HA is eliminated via the liver [93] and the kidneys [94]. Patients with liver diseases have an increased risk of thrombosis events and one possible explanation could be high systemic levels of HA. Similarly, patients with RA have an increased risk of developing thrombosis, which could possibly be related to the increased levels of HA detected in serum [95]. HA has a receptor (CD44), an integrin that has been identified on several cells and is involved in cell-matrix interactions [96]. The levels of HA in blood is known to have a diurnal variation [97]. Care was therefore taken in our studies to collect all blood samples around 9 a.m. to avoid such diurnal changes.

HA was analysed using a previously described radiometric technique according to the manufacturer's instructions (Pharmacia HA test, Pharmacia Diagnostica, Uppsala, Sweden) [98]. The feasibility of the assay technique for rat serum samples has previously been documented [76].



Sequence of HA, a linear polymer built from repeating disaccharide units that consist of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA)

Inflammation markers

In measuring inflammation, the erythrocyte sedimentation rate (ESR) is a widespread method which was already described in 1920 [99]. Acute phase proteins mainly produced by the liver are other markers and include fibrinogen, C-reactive protein (CRP), orosomucoid and serum amyloid protein. For measurement of inflammation in rats, ESR is less convenient as it demands large blood samples. CRP does not increase substantially during inflammation in rats and serum amyloid protein can not be

detected at all. Fibrinogen levels, on the other hand, increase 3-5 times during inflammatory conditions such as arthritis in both rats and humans.

Fibrinogen

Fibrinogen is a protein of 340kDa and consists of 3 polypeptide chains. Fibrinogen is produced in the liver both normally and in response to inflammation somewhere in the body. Fibrinogen plays an important role in coagulation, when it is cleaved to fibrin, which causes clot formation. Cleavage products of fibrinogen increase vascular permeability. During arthritis, fibrin is deposited in the joint. These depositions can be detected in both synovial membrane, in the synovial fluid and on the cartilage surface, and it has recently been reported that fibrinogen induces synovial fibrinoblast cell to produce the adhesion molecule ICAM 1 [100]. Fibrinogen seems to promote inflammation in this setting.

The analyses of fibrinogen performed in this thesis work were made by nephelometry [101]. In this assay the levels of fibrinogen in plasma are presented as percentage of the value from healthy nonarthritic DA rats.

	Degradation	**Synthesis**
Cartilage		
Noncollagenous protein	COMP	Human cartilage glycoprotein 39
Collagen type II	PYD, Crosslinks hydroxylysyl-pyridinoline (CTX-II)	N and C propeptides (PIICP, PIINP),
Bone		
Noncollagenous protein	BSP, coreprotein fragments, keratansulphate	Osteocalcin, Bone alkaline phosphatase,
Collagen type I	Pyridinoline (PYD), CTX-I	N and C propeptides (PICP, PINP),
Synovium		
Collagen type III	PYD, CTX-III	N-propeptid type III procollagen
Noncollagenous protein	Glucosyl-galactospyridinoline (Glc-Gal-Pyd)	HA, COMP, YKL 40 (=HC gp-39)

Example of molecular markers

Anti-rheumatic treatment

Joint-inflammation, arthritis, alters the structure and function of joints. This causes pain, reduced mobility, decreased strength and loss of endurance for motor tasks. During the early stages of disease, the patient can adapt and through altered physical performance compensate for and reduce the above mentioned disease features. Rather soon, however, the need for rehabilitating treatment is obvious. Such treatment can be of several kinds; most common is medicational treatment but also physiotherapy, surgical treatment and practise of coping strategies are important.

Since there is still no cure for arthritis, all existing treatments aim at relieving pain and retarding the inflammatory process. Below, classical and newly introduced pharmaceutical therapies will be discussed.

Aspirin and Nonsteroidal anti-inflammatory drugs (NSAID)

Already 1500 years B.C. the Egyptian Ebers advised the usage of myrtle leaf concoctions for relief of rheumatic pain. 400 B.C. Hippocrates recommended sap from willow trees as a treatment of fever and pain. The active substance in willow trees is salicin and in 1860 Hoffman was able to synthesize acetylsalicylate. In 1899 this compound was introduced to the market as Aspirin [102] and has been used ever since. The NSAID Phenylbutazone was introduced in 1949 and since then several NSAID have been developed. NSAIDs, including aspirin, inhibit cyclooxygenase (COX), which affects the production of inflammatory mediators such as prostaglandins [103], aspirin and NSAID reduce, but do not eliminate, the signs and symptoms of inflammation. They are not considered to have any major impact on the underlying disease process and will thus probably not prevent tissue destruction.

Colchicine

Colchicine-containing extracts from the plant autumn crocus were already used for acute gout in the sixth century A.D [104] and pure colchicine was isolated in 1820. Colchicine interacts with several steps in the inflammatory cascade, leading to decreased migration of neutrophils into areas of inflammation [105], and is considered as an anti-inflammatory drug of its own kind.

Disease-modifying anti-rheumatic drugs (DMARDs)

Another group of anti-rheumatic treatments is the so-called DMARDs. DMARDs should by definition be able to delay bone and cartilage destruction. Examples of DMARDs with this effect are methotrexate [106], sulphasalazine [107, 108], gold [109], cyclosporine [110, 111] and leflunomide [112]. All DMARDs have complex and various mechanisms of action, and all have multiple effects.

Anti-rheumatic biological agents

Anti-rheumatic biological agents constitute a broadly defined group of large protein molecules targeting immune reactions. The group includes monoclonal antibodies directed against cell surface structures or soluble mediators as well as soluble receptor-fusion proteins which compete with cell-bound receptors [113, 114].

Etanercept is a fusion protein of TNF receptor type II and an immunoglobulin Fc part. Thereby, Etanercept competes with cell-bound TNF receptors for the binding of TNF and diminishes TNF-induced inflammatory events [115]. Infliximab [63] and lately adalimumab [116] are monoclonal antibodies directed against TNF which also block the action of TNF. Etanercept, infliximab as well as adalimumab have been demonstrated to suppress both inflammation and destruction in arthritis patients [117].

Anakinra is a recombinantly produced IL-1 receptor antagonist which competes with the IL-1 receptor type I for binding of IL-1 [118]. As with the TNF-targeting therapies, Anakinra has been demonstrated to have beneficial effects on both inflammation and tissue destruction [118].

Corticosteroids

Corticosteroids have also been associated with symptomatic effects, but there are studies proving also tissue-sparing effects (see below). The Nobel Prize for Medicine for the discovery of corticosteroids was awarded in 1950 to Philip Showalter Hench, Edward Calvin Kendall and Tadeus Reichstein.

In 1948, Hench treated the first RA patient with cortisone with dramatic effects. Cortisone treatment soon demonstrated side-effects that were not neglectable, such as psychosis, diabetes and osteoporosis and this gave Corticosteroids (Cs) a bad reputation. The drugs lifesaving effects in vasculitis, asthma, anaphylaxis, nephritis and increased intracranial pressure are well described and established. In recent years Cs have gained more popularity and it is quite normal to include low dose of Cs in treatment of RA. Local Cs are frequently used and considered to improve the prognosis for the treated joint [119].

Dr Hench was asked, "How do you go about winning a Nobel Prize?"

As a part of the answer the following story was told: A fifty-year-old man announced that he had $100000 in the bank and was retiring: A friend asked him, "In these days of high taxes, how did you manage to save $ 100.000?" The man replied: "Well, I've worked hard very hard for thirty years; I've rarely taken a vacation; I've saved every dollar and I've just received from my uncle's estate $95,000." Hench then emphasised that it is important to have the right relationships, select a loyal wife and then one of the worlds prominent chemists at the time; Nick Kendall".

From Hench PS. "A reminiscence of certain events before, during and after the discovery of cortisone". [120]

Corticosteroid action

CS binds to a cytoplasmatic receptor, then the Cs-receptor complex modulates protein synthesis [121]. Cs have many different effects throughout the body. They are catabolic steroids providing the body with energy for fight and flight. They increase hepatic gluconeogenesis, inhibit insulin action, stimulate lipolysis and contribute to peripheral catabolism. Cs have effects on connective tissue, especially bone, by decreasing collagen synthesis, inhibiting bone formation, elevating parathyroid hormone levels and lowering levels of calcium.

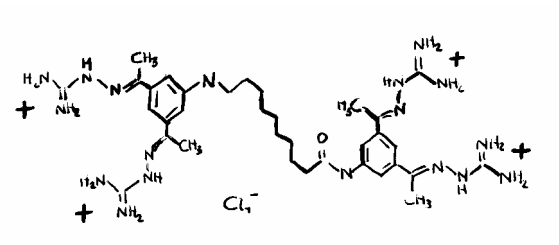
Cs immune modulating effects are not fully known. Effects on antigen opsonization, cell adhesion, leukotriene and prostaglandin synthesis, on cytokine production or direct antagonism of proinflammatory cytokines, have all been demonstrated [122-124]. Abnormal function in the HPA axis may be a factor in susceptibility to inflammatory diseases [125]. In a more recent study [126], however, RA patients showed no difference in plasma concentrations of the adreno-corticotrophic hormones: cortisol, prolactin and thyroid-stimulating hormone when compared to healthy controls, making it uncertain whether abnormal functions of the HPA axis are of importance also for arthritis in humans.

CNI-1493

CNI-1493 is a synthetically produced tetravalent guanylhydrazone [127]. It was originally developed as an inhibitor of macrophage activation. One of the major effects of CNI-1493 on macrophages was demonstrated to be the suppression of TNF production. This effect is mediated via a

dose-dependent inhibition of the translation of TNF mRNA via interaction with p38 MAPK. Furthermore, CNI-1493 inhibits the production of NO in macrophages but does not inhibit the production of the anti-inflammatory cytokine TGF-beta [128, 129]. Recently, it has also been demonstrated that CNI-1493 can exert anti-inflammatory effects by stimulating parasympathetic actions of the vagus nerve, and thereby prevents inflammation through a previously un recognised pathway [130]. The effects of CNI-1493 are transient and repeated treatment is necessary.

CNI-1493 has previously been demonstrated to diminish inflammation in rats with CIA, when delivered either in conjunction with immunization or as a therapeutical treatment [25]. The effect of CNI-1493 treatment on tissue destruction was not evaluated in that study.



The chemical structure of CNI-1493

A further evaluation of the effects of CNI-1493 also on tissue destruction in arthritis was considered to be of importance, since this drug has lately been used successfully not only in animal models of inflammatory disease, but also in Crohn's disease in humans. Twelve patients received CNI-1493 daily for 12 days. A significant clinical improvement was detected in 67% of the patients after 4 weeks and in 58% of the patients after 8 weeks. There were no observed sideeffects [131].

The existing similarities between RA and Crohn's disease in cytokine expression and in treatment results [132] suggest that CNI-1493 may be utilised as an anti-rheumatic treatment in RA.

Aims of this thesis

To analyze whether connective tissue markers for cartilage and bone destruction (COMP and to some extent BSP) can be used to monitor joint destruction in experimental arthritis in rats, and, if so, to further evaluate the usefulness of these markers in determining the eventual effects of antirheumatic drugs on tissue destruction in experimental arthritis. If successful, such experiments may point to new ways of using the tissue markers for rapid evaluation of effects of new and old drugs on tissue destruction also in human arthritis.

Papers included in this thesis

Paper I

The aim of this study was to investigate whether serum levels of cartilage and bone macromolecules can be used to monitor tissue destruction in experimental arthritis in rats. We evaluated BSP and COMP as markers for bone and cartilage destruction in experimental arthritis (CIA induced in DA rats) since BSP and COMP have previously been employed in investigations of potential anti-rheumatic drugs in humans. A clinical scoring grading the redness and swelling of joints in the four paws was used to evaluate the severity of joint inflammation.

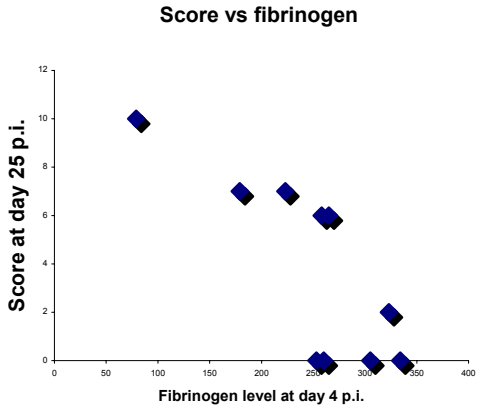
Rats were immunized with collagen type II dissolved in Freund's incomplete adjuvant. After approximately 14 days the first clinical signs of arthritis were noticed and thereafter the arthritis severity increased during the observation period of 14 days. Increased serum levels of COMP and BSP were detected on day 21 after immunization and were further increased on day 28 p.i., at the termination of the experiment. These increased serum levels of COMP and BSP correlated with increases in the clinical joint score and histopathological signs of cartilage and bone erosions. This study thus indicates that serum analyses of COMP and BSP may be a useful, non-invasive way of monitoring tissue destruction in experimental arthritis.

Paper II

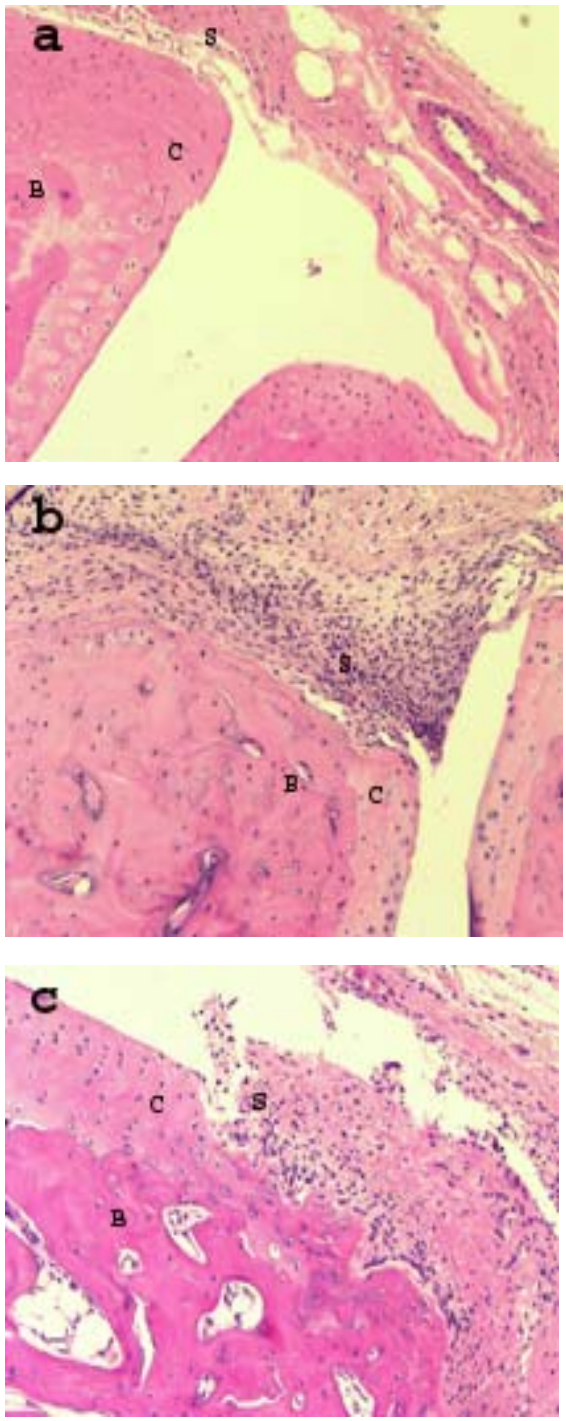
Since in paper I we had established that serum COMP and serum BSP levels to mirror cartilage and bone destruction, we sought to establish serum markers that could reflect also synovitis (the local inflammation in joints) and systemic signs of inflammation. In the literature, serum hyaluronan (HA) and serum fibrinogen had previously been suggested as markers for synovitis and systemic inflammation, respectively. We thus, decided to investigate these markers and their usefulness in experimental arthritis. In parallel, we also wanted to further test the sensitivity of serum COMP as a cartilage destruction marker. We utilised three different experimental disease models of chronic inflammation with known differences in cartilage destruction. The tree disease models were Collagen-induced Arthritis (CIA), Oil-induced Arthritis (OIA) and Experimental Autoimmune Encephalitis (EAE). CIA and OIA differ in clinical disease severity and chronicity with CIA being more severe and chronic. EAE was used as a control, being a chronic inflammatory disease without joint involvement. Our results confirm that serum fibrinogen is a marker of systemic

inflammation since it increased both during arthritis and during EAE. Serum hyaluronan levels correlated with the degree of synovitis (verified by histological scoring) and COMP levels correlated with the different degrees of cartilage involvement in the two arthritis models, also verified by histological scoring.

Interestingly, in one of our experiments the arthritis incidence only reached 60% as compared to the normal incidence of near 100 %. We had in this study obtained plasma every 4th day and when analyzing the fibrinogen levels of the samples we noticed that rats with the highest levels of fibrinogen at day 4 p.i. did not develop arthritis. One explanation to this finding could be that an acute phase response high plasma fibrinogen levels triggered by the immunization may have induced an arthritis-protective immune response.



Plasma fibrinogen levels in rats immunized with mineral oil 4 days previously plotted against arthritis score at day 25 post immunization. At day 25 p.i., the maximal arthritis incidence and severity is reached



a. Paraffin-section of a normal rat paw.
b. Paraffin-section of a hind paw joint from a rat with oil-induced arthritis, 28 days p.i.
c. Paraffin-section of a hind paw joint from a rat with collagen–induced arthritis, 28 days p.i.
B =Bone, C = Cartilage, S = synovia

Paper III

After having validated COMP, HA and fibrinogen as serum markers for joint destruction, synovitis and systemic inflammation in papers I and II, we went further by investigating if these markers could be useful in evaluating the effects of an anti-rheumatic treatment in experimental arthritis. In order to do this we set out to compare changes in serum COMP levels with histological examinations of joint inflammation in CIA. A corticosteroid betamethasone, was chosen as the anti-rheumatic treatment to investigate. Betamethasone has pre-dominately a glucocorticoid effect, whereas prednisolone has more of a mineral corticosteroid effect. We preferred to use betamethasone as the model corticosteroid as as we wanted to minimise the mineralocorticosteroid effects in the present experiments. In order to define the optimal dose to utilize in the study we made a titration study and chose a dose of 0.1mg/kg/bodyweight. This dose had a clear effect on the arthritis but did not completely abolish the disease, a situation that is often encountered in the treatment of patients with inflammatory joint disease. Corticosteroids are known to be very effective in suppressing synovitis but whether corticosteroids have a retarding effect on bone and cartilage destruction has been debated.

Betamethasone was delivered intra-peritoneally once daily after the onset of arthritis. The treatment reduced the clinical signs of arthritis as compared to placebo treatment (arthritis score reduced $p<0.001$ at day 25 p.i.). Fibrinogen levels were also reduced compared to placebo treatment. The clinical improvement was paralleled by decreased serum levels of fibrinogen as compared to placebo treated rats ($p<0.01$). The levels of serum COMP remained unchanged in the betamethasone-treated arthritic animals, whereas an increase in serum levels of COMP was observed in rats treated with placebo ($p<0.001$). The histopathological changes in the joint were less severe in the betamethasone-treated animals (median cartilage destruction score 4 in placebo group compared to 0 in the betamethasone-treated group ($p<0.01$)). There was a significant correlation between serum COMP levels and the degree of cartilage destruction at day 25 p.i. ($r=0.77$, $p<0.001$). Our results demonstrate that moderate dosages of corticosteroids given therapeutically to arthritic rats diminish joint destruction as measured by histology. This effect was reflected by stable normal serum COMP values. The study thus supports the concept that serum COMP measurements can be used to evaluate the cartilage-protective

effects of corticosteroids, and possibly anti-rheumatic therapies in general.

Paper IV

In the last paper of this thesis work, our aim was to elucidate if blockade of proinflammatory cytokines through treatment with the synthetic substance CNI-1493 would affect the destructive events ongoing in arthritic joints, and to investigate the usefulness of serum COMP levels in this context. CNI-1493 is a tetravalent guanylhydrazone, which was originally developed for inhibition of macrophage activation. Cytokine-inducible NO production was targeted, but it was demonstrated that CNI-1493 was more effective in inhibiting TNF synthesis. By suppressing the production of TNF, interleukin 1 (IL-1), IL-6, MIP-1α and MIP-1β syntheses are reduced.

DA rats with early clinical arthritis were treated daily with intraperitoneal injections of CNI-1493. The clinical development of arthritis was followed and both serum and tissue samples (for histology) were obtained at different time points.

In the CNI-1493-treated group, clinical arthritis was significantly reduced. The histological examinations demonstrated a clear cartilage-saving effect. Measurement of serum COMP levels demonstrated increased levels in the placebo group whereas the CNI-1493-treated group had normal levels of serum COMP.

In this study, we confirmed previous data that CNI-1493 is a potential therapy for arthritis with the ability to reduce clinical joint inflammation. Furthermore, we could demonstrate that CNI-1493 therapy could reduce or stop cartilage destruction.

Discussion and conclusions

During the disease course of arthritis, a chronic inflammation is ongoing in affected joints. This inflammation leads to two things; one is an invasive growth of the synovial membrane with an overproduction of proinflammatory mediators. The second is production of cartilage and bone degrading proteins, such as MMPs, which will ultimately lead to joint destruction and loss of joint function. Thus, one of the major aims of anti-rheumatic treatment is to inhibit, or at least to suppress, destructive immune reactions.

Up until recently, in order to evaluate the anti-destructive effects of the chosen therapy, clinicians have been restricted to utilise either radiological examinations of subchondral bone, which will reflect advanced structural damage on bone but is insensitive for cartilage damage detection, or to utilise clinical assessments of joint motility in combination with laboratory tests of the inflammatory status of the

patients. In the best cases, all these parameters have been used together.

A drawback with radiological analyses is that, as previously mentioned, they primarily provide information of bone destruction and only indirect information regarding cartilage destruction. Radiography also has a limited sensitivity, and progression of destruction can only be detected after 6-12 months. With clinical assessments, it is mostly the inflammatory synovitis which is revealed, and not the destructive process destroying cartilage and bone.

As discussed previously in this thesis, a number of molecular markers have been suggested as appropriate tools for the evaluation of cartilage and bone destruction. Most attention has been focused on the measurements of COMP in sera, as a reliable way to follow cartilage destruction during arthritis. Changes in serum COMP levels have been demonstrated to reflect cartilage damage with kinetics faster than that of radiography.

It has also, become increasingly clear that the two processes leading to synovitis or to cartilage destruction do not always occur simultaneously or with similar kinetics. Some patients might have a severe synovitis but no ongoing destruction while others may have a minor synovitis but severe destruction. It is therefore of major importance to monitor both inflammation and destruction.

As a consequence of these partly uncoupled mechanisms of inflammation and destruction, it is not always the case that anti-rheumatic therapies targeting inflammatory events have as good suppressive effects on destructive events, or vice-versa. The effect of the chosen therapy therefore needs to be monitored both regarding inflammation and destruction with markers able to distinguish between the two.

The use of experimental arthritis models has proven useful both for the understanding of the pathogenesis of arthritis and for the evaluation of potential new anti-rheumatic therapies. When performing such therapy evaluation, it is of importance to monitor both the effect on synovitis and on destruction. Preferentially, the same methodology utilised in humans should be utilised in the experimental models.

In this thesis work, we have established for the first time that quantification of serum COMP levels can be utilised in experimental arthritis in a manner similar to its usage as a marker for cartilage destruction in humans. We have demonstrated that serum levels of COMP correlate with cartilage destruction as evaluated by histological examination of joints from rats with experimental arthritis.

We could also demonstrate that in different experimental models of arthritis, with similar degrees of synovitis, there were differences in serum COMP levels correlating to differences in cartilage and bone destruction, as verified by histological observations. Furthermore, we evaluated a molecular marker of bone destruction, bone sialoprotein (BSP). As with COMP, BSP has been demonstrated in humans to be a possible molecular marker for monitoring the progression of arthritis. In our studies, serum BSP levels correlated well with bone destruction occurring in experimental arthritis.

To test whether the investigated molecular markers could reflect the efficacy of anti-rheumatic therapies to suppress destruction, we performed two studies in which we investigated changes in serum COMP levels during therapy with either a corticosteroid (betamethasone) or with a potential new anti-rheumatic therapy, CNI-1493. The obtained serum COMP values were compared with histological scoring of the destruction. By these studies (paper III and paper IV), we could demonstrate that both therapies are efficient in preventing destruction as measured by serum COMP levels and that changes in serum COMP levels reflect destructive events even after short duration of therapy.

As corticosteroids are classic anti-rheumatic drug with chondroprotective effects reported as early as 1959 [133], this study supports the usefulness of COMP as a marker of destruction in experimental arthritis. Our report that CNI-1493, apart from being anti-inflammatory, also suppresses cartilage destruction increases the suitability of this synthetic substance as an anti-rheumatic treatment. CNI-1493 is presently undergoing clinical trials as a therapy for Crohn's disease.

A final possible implication of our studies is also that COMP might be a useful marker for the titration of correct corticosteroid doses in arthritis patients. By measuring serum COMP levels, it may be possible to quickly (as compared to radiography), determine the chondroprotective dose having minimal adverse effects.

It is my hope that through the work presented in this thesis, we have contributed to the knowledge of COMP as a marker of cartilage destruction during arthritis in both humans and in experimental animals. I also hope that we have established COMP as a convenient parameter for analysis during evaluation of potential anti-rheumatic drugs in experimental animal models.

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