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Enheten för reumatologi

Studies of the Synovial Membrane in Chronic Rheumatic Joint Disease

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Studies of the synovial membrane in chronic rheumatic joint disease

Background: The synovial membrane (SM) outlines the inner cavity of synovial joints except for cartilage surfaces. The SM is the target organ of immune-mediated responses in chronic arthritis, including rheumatoid arthritis (RA). RA is a common joint disease (prevalence of 0.5-1.0%) which is characterized by recurrent joint inflammation, with increasing joint damage and disability. The study of the SM in vivo provides important information about disease pathogenesis and response to treatment.

The aim of this thesis was to study a method for biopsy retrieval, arthroscopy, SM variation by gene and protein expression and SM response to anti-rheumatic therapy.

Results:
- In these studies we have added to existing evidence that arthroscopy is a safe and reliable method for synovial tissue retrieval.
- We have constructed and validated an easy to use macroscopic score for arthroscopy. This score needs validation against other methodologies.
- We have studied variability of synovial gene expression, where we could see that samples close to one another were more “related” than samples further apart, especially from other patients. Arthroscopic biopsies had less intrinsic variation than had orthopaedic material.
- We have shown that injection of intra articular glucocorticosteroids (GCs) reduces protein expression of synovial proinflammatory molecules (TNF, IL-1β, extra-nuclear HMGB-1, ICAM-1 and VEGF) and T cells. mRNA expression was reduced for IL-1α and IL-1β, but not for TNF or HMGB-1. Unexpectedly, no changes were evident in macrophage infiltration and the vascular compartment. Vascular proinflammatory cytokine expression persisted.
- We have shown that mPGES-1 (an enzyme in the prostaglandin E2 synthesis pathway) is strongly expressed in RA lining cells, and also in sublining macrophages, synovial fibroblasts and endothelial cells. We also saw that mPGES-1 and COX-2 were down-regulated by local GC treatment, but not by systemic TNF antagonists.
- We have shown that markers of destruction were positively affected by both GCs and TNF antagonists, although differently. RANKL was down-regulated by GCs and OPG was up-regulated by TNF-blockade, leading to a favourable reduction of RANKL/OPG ratio by both treatments.
- We have provided evidence that TNF-antagonists induce apoptosis in RA SM macrophages, but not lymphocytes.

Conclusion: Arthroscopy is a well tolerated, safe and reliable tool that will continue to be important for synovial tissue sampling. Synovial variation has to be considered when sampling the SM. By local GC treatment signs of inflammation persist, perhaps contributing to later disease relapse. We provided evidence for two inducible enzymes in the prostaglandin synthesis pathway, and showed that inhibition depended on therapy. mPGES-1 inhibitors would be an interesting future therapy possibly without the adverse effects of the cyclooxygenase inhibitors. Interestingly both GC and anti-TNF therapy reduced the RANKL/OPG ratio, an indirect sign of reduced osteoclast activity, although by different path ways. These findings provide support for a biological background for the slowing of joint destructions by these treatments. The increase in apoptosis of macrophages indicate that TNF antagonists make the local environment more normal, as reduced apoptosis is a classical feature in active RA SM.

Two important questions remain: Is it possible to predict treatment response, and thereby minimise patient suffering? Is it possible to understand disease pathogenesis, and develop a cure? These are questions that need answering, and using SM as a source for research will possibly provide with important leads. Studying old and especially the new targeted therapies represent important opportunities to these questions.

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Erik af Klint 2006
To all patients
with inflammatory joint disease

Wise words by king Solomon, second son of king David, probably born about 1035 B.C. He also studied life (Ecc 11:5-6), and towards the end of his own he concluded what was the most important after all had been considered (Ecc 12:12-14).

“Just as you do not know the path of the wind or how the bones are made of a child yet to be born, so you do not know the work of God Who makes all things. Plant your seeds in the morning, and do not be lazy in the evening. You do not know which will grow well, the morning or evening planting, or if both of them alike will do well.”
Ecclesiastes chapter 11:5-6

“But more than this, my son, be careful. There is no end to the writing of many books and reading many of them makes the body tired.”
Ecclesiastes chapter 12:12
Studies of the synovial membrane in chronic rheumatic joint disease

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2. Abstract

*Studies of the synovial membrane in chronic rheumatic joint disease*

**Background:** The synovial membrane (SM) outlines the inner cavity of synovial joints except for cartilage surfaces. The SM is the target organ of immune-mediated responses in chronic arthritis, including rheumatoid arthritis (RA). RA is a common joint disease (prevalence of 0.5-1.0%) which is characterized by recurrent joint inflammation, with increasing joint damage and disability. The study of the SM *in vivo* provides important information about disease pathogenesis and response to treatment.

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**Results:**

- In these studies we have added to existing evidence that arthroscopy is a safe and reliable method for synovial tissue retrieval.
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- We have shown that markers of destruction were positively affected by both GCs and TNF antagonists, although differently. RANKL was down-regulated by GCs and OPG was up-regulated by TNF-blockade, leading to a favourable reduction of RANKL/OPG ratio by both treatments.
- We have provided evidence that TNF-antagonists induce apoptosis in RA SM macrophages, but not lymphocytes.

**Conclusion:** Arthroscopy is a well tolerated, safe and reliable tool that will continue to be important for synovial tissue sampling. Synovial variation has to be considered when sampling the SM. By local GC treatment signs of inflammation persist, perhaps contributing to later disease relapse. We provided evidence for two inducible enzymes in the prostaglandin synthesis pathway, and showed that inhibition depended on therapy. mPGES-1 inhibitors would be an interesting future therapy possibly without the adverse effects of the cyclooxygenase inhibitors. Interestingly both GC and anti-TNF therapy reduced the RANKL/OPG ratio, an indirect sign of reduced osteoclast activity, although by different path ways. These findings provide support for a biological background for the slowing of joint destructions by these treatments. The increase in apoptosis of macrophages indicate that TNF antagonists make the local environment more normal, as reduced apoptosis is a classical feature in active RA SM.

Two important questions remain: Is it possible to predict treatment response, and thereby minimise patient suffering? Is it possible to understand disease pathogenesis, and develop a cure? These are questions that need answering, and using SM as a source for research will possibly provide with important leads. Studying old and especially the new targeted therapies represent important opportunities to these questions.
3. List of publications

This thesis is based on the following eight papers, which will be referred to in the text by their Roman numerals:

I. Evaluation of arthroscopy and macroscopic scoring
E af Klint, A Catrina, P Matt, P Neregård, J Lampa, A Ulfgren, L Klareskog, S Lindblad
Manuscript

II. Variability in synovial inflammation in rheumatoid arthritis investigated by microarray technology.
J Lindberg, E af Klint, AK Ulfgren, A Stark, T Andersson, P Nilsson, L Klareskog and J Lundeberg
Arthritis Res Ther. 2006 Feb 16;8(2):R47

III. Intraarticular glucocorticoid treatment reduces inflammation in synovial cell infiltrations more efficiently than in synovial blood vessels
E af Klint, C Grundtman, M Engstrom, A Catrina, D Makrygiannakis, L Klareskog, U Andersson, A Ulfgren

IV. Expression of microsomal prostaglandin E synthase 1 in rheumatoid arthritis synovium

V. Effects of antirheumatic treatments on the prostaglandin E2 biosynthetic pathway.
M Korotkova, M Westman, K Gheorghe, E af Klint, C Trollmo, A Ulfgren, L Klareskog, P Jakobsson

VI. Intraarticular corticosteroids decrease synovial receptor activator of nuclear factor-κB ligand expression in inflammatory arthritis
D Makrygiannakis, E af Klint, S Catrina, I Botusan, E Klareskog, L Klareskog, A Ulfgren, A Catrina
Arthritis Rheum. Accepted for publication

VII. Anti-tumor necrosis factor therapy increases synovial osteoprotegerin expression in rheumatoid arthritis.
A Catrina, E af Klint, S Ernestam, S Catrina, D Makrygiannakis, I Botusan, L Klareskog, A Ulfgren

VIII. Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report.
A Catrina, C Trollmo, E af Klint, M Engström, J Lampa, Y Hermansson, L Klareskog, A Ulfgren

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4. Abbreviations

ACR  American College of Rheumatology
AP-1  Activator protein 1
AS  Arthroscopy
B cell  Lymphocyte derived from the bone marrow
CD  Cluster of differentiation
COX  Cyclo-oxygenase
CRP  C-reactive protein
DC  Dendritic cell
DMARD  Disease modifying anti-rheumatic drug
ESR  Erythrocyte sedimentation rate
FLS  Fibroblast-like synoviocyte
GC  Glucocorticosteroids
HLA  Human leucocyte antigen
HMGB-1  High mobility group box chromosomal protein 1
IFN  Interferon
IHC  Immunohistochemistry
IL  Interleukin
MΦ  Macrophage
MA  Microarray
MMP  Matrix metalloproteinases
NFκB  Nuclear factor kappa B
NSAID  Non-steroidal anti-inflammatory drug
OPG  Osteoprotegerin
PG  Prostaglandin
RA  Rheumatoid arthritis
ra  Receptor antagonist
RAGE  Receptor for advanced glycation end products
RANKL  Receptor activator of NFκB ligand
RF  Rheumatoid factor
SM  Synovial membrane
SF  Synovial fluid
T cell  Lymphocyte derived from the thymus
TGFβ  Transforming growth factor beta
Th  T helper
TLR  Toll-like receptor
TNF  Tumor necrosis factor
TNFR  Tumor necrosis factor receptor
Studies of the synovial membrane in chronic rheumatic joint disease

5. BACKGROUND

5.1. Reasons for the thesis

Although significant progress has been made for rheumatic patients during recent years (e.g. teams, surgical methods, rehabilitation and medical treatments) it is important to state that there is still no cure for the majority of these diseases. It is the hypothesis of this thesis that studies of the target organ, the synovial membrane, are the key to understanding of the pathological processes and that such understanding is key for the development of new drugs in these diseases. It is my hope that these studies will contribute to such understanding.

Below follows a short background and summary of important aspects relevant for the studies on which this thesis is based.

5.2. Chronic arthritides

Arthritis is derived from the greek word for joint, “arthrein”, and the end “-itis” meaning inflammation. Joint inflammation is characterized by all the classical signs of inflammation – dolor (lat. pain), calor (lat. heat), rubor (lat. redness), tumor (lat. swelling) and functio laesa (lat. decreased function). The term chronic is defined as arthritis lasting more than six weeks, as most other causes of arthritis are resolved within this time frame.

Chronic arthritides can be divided into primary (without known cause) and secondary (where the cause is known). Examples of the latter are crystal deposition in the joint in gout, and infectious agents in septic/bacterial arthritis (e.g. Borrelia bugdorferi and Mycobacterium tuberculosis). The primary immune-mediated arthritides can be further defined as being self-limiting, such as reactive arthritis, and chronic destructive, such as rheumatoid arthritis.

5.3. Rheumatoid Arthritis (RA)

5.3.1. Clinical features and classification

RA is a common (prevalence 0.5-1.0%)[1] chronic polyarthritis with unknown cause. The word rheumatoid is derived from the greek word “rheumos” meaning fluid, indicating the swelling of joints. Two thirds of patients are women, and the incidence peaks at about 60 years of age. No single diagnostic or pathognomonic symptom, sign or test exists; patients
rather being classified according to a selection of criteria. The most recent are the ACR classification criteria from 1987[2] (see Table 1), that were originally created for research rather than diagnostic purposes. However, the sensitivity of the ACR criteria is low during early disease[3]. Characteristic is the involvement of almost any synovial joint, typically the small joints of the hands and feet. Recurrent inflammation leads to different degrees of joint destruction and disability. In a study by Dixey et al[4] 32% of patients had destructive changes when first examined, and by three years 70% had erosive disease despite conventional treatment. Both for the individual patient and for society costs are significant, including medication, orthopaedic surgery and reduced working capacity[5]. About 10% have extra-articular disease[6], that may affect exocrinal glands, skin, nervous system, lung tissue and blood vessels to name some. RA is also associated with premature mortality, especially cardiovascular related[7]. Osteoporosis is also increased, irrespective of treatment.

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>1. Morning stiffness</td>
<td>Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement.</td>
</tr>
<tr>
<td>2. Arthritis of three or more joint areas</td>
<td>At least three joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth) observed by a physician. The 14 possible areas are the right or left proximal interphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle and metatarsophalangeal (MTP) joints.</td>
</tr>
<tr>
<td>3. Arthritis of hand joints</td>
<td>At least one area swollen in a wrist, MCP or PIP joint.</td>
</tr>
<tr>
<td>4. Symmetry of arthritis</td>
<td>Simultaneous involvement of the same joint areas (as defined in 2.) on both sides of the body (bilateral involvement of PIP, MCP or MTP joints is acceptable without absolute symmetry).</td>
</tr>
<tr>
<td>5. Rheumatoid nodules</td>
<td>Subcutaneous nodules over bony prominences, extensor surfaces, or in juxta-articular regions, observed by a physician.</td>
</tr>
<tr>
<td>6. Rheumatoid Factor (RF)</td>
<td>Detected by a method positive in less than 5% of normal controls.</td>
</tr>
<tr>
<td>7. Radiographic changes</td>
<td>Radiographic changes typical of RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify).</td>
</tr>
</tbody>
</table>

Patients fulfilling at least four of seven of these criteria are classified as having RA. Criteria 1 through 4 must have been present for at least six weeks. Patients with two clinical diagnoses are not excluded.

Table 1. The 1987 American College of Rheumatology classification criteria for RA.[2]

5.3.2. Etiology and outcome

RA is considered to be a multifactorial disease, resulting from the interaction of many different factors which contribute to its occurrence and expression. The main risk factors for the disease include genetic susceptibility, sex and age, environmental exposures such as smoking, infectious agents, hormonal, dietary, socioeconomic and ethnic factors. Most of
these factors are likely to be associated with both disease occurrence and severity. Although infectious diseases have been implicated (including parvovirus, rubella virus, Epstein-Barr virus, *Borrelia burgdorferi* and others) the role of these agents remain unclear. Furthermore, RA cases do not present any time or space clustering as would be the case if an infectious disease were causative[8].

In support of the importance of genetic factors is the fact that disease concordance is greater in monozygotic twins (12-15%) as compared to in dizygotic twins (3-4%)[9, 10]. Familial clustering of RA also occurs. The strongest genetic association is to the HLA-DR gene loci, and in particular to those allelic forms that code for the so-called “shared epitope”[11] (SE). These genes are associated not only with incidence, but also with severity and outcome[12, 13]. Other, non-HLA-genes, have also recently gained interest, such as PTPN22, CTLA4 and PADI4[14].

An individual prognosis is difficult to make, although prognostic risk factors have been recognized[15]; presence of shared epitope, early destructive changes visible by X-ray, early involvement of many joints, low functional score early in the disease, evidence of inflammation in serum (high erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP), presence of certain autoantibodies (rheumatoid factor (RF) and antibodies against citrullinated peptides).

### 5.3.3. Pathogenesis

Although RA is thought of as being immune-mediated disease targeting the synovium, the precise molecular mechanisms that contribute to the pathogenesis are yet incompletely known. It is also not known to what extent different molecular mechanisms are active in different patients with similar clinical features, and it is likely that what we consider as synovitis really represents a common pathway for many different pathologies. The common target organ of inflammation is the synovial membrane.

RA was initially considered to be an autoimmune disease based on the discovery of autoantibodies against immunoglobulin G (RF) in sera from RA patients[16]. It was later proposed that RA was an RF-mediated immune complex disease[17], but this hypothesis did not explain disease in RF negative patients nor the presence of RF in normal individuals. Later on association with HLA-DR was established, the first evidence for a genetic component. As the function of this molecule is to present antigens to T cells, this was a compelling evidence for the involvement of these cells in the pathogenesis. Further support of this view arose from in the characterization of the HLA molecules and their association with
disease susceptibility and severity, which resulted in the shared epitope hypothesis[11]. However, T cell cytokines (such as IFNγ and IL-2) are relatively low in RA synovial membrane[18]. The predominance of macrophage and fibroblast derived cytokines in the RA joint has shifted focus to these cells and their effector mechanisms. Strong support for the importance of the cytokine network was provided through the efficiency of treatments antagonizing TNF and IL-1, producing not only symptomatic relief but also slowing of radiographic progression. However, not all patients have benefit, and disease activity rises when these treatments are stopped. Interest has now returned to the lymphocyte population. Autoantibodies produced by B cells may precede disease presentation by several years[19], and B cells also function as antigen presenting cells involved in T cell activation. The efficiency of treatment targeting the interaction of T cells and antigen presenting cells by blocking costimulatory molecules[20] have also reimplicated T cells in the pathogenesis of RA.

In summary, interest and hypothesizes have changed over the years, from autoimmune lymphocyte-mediated theories to effector mechanisms by macrophages (MΦ) and fibroblasts and back again. The difficulty to understand important immune mechanisms in individual patients from the analyses of blood and synovial tissue is interesting. The new efficient therapies, targeting immunological molecules, present new opportunities to not only treat patients more effectively, but also to better understand the molecular pathogenesis involved. By studying alterations in molecular and cellular patterns in the inflamed joint before and after treatment with compounds specifically targeting immune molecules, we have the opportunity to understand what role these molecules have in the disease process in different RA patients. Through understanding these mechanisms it might be possible to individually tailor treatment, and maybe even reverse the disease process, thus providing a cure.

Below is given a brief summary of important characteristics of the immune system, how it functions normally in joint inflammation, and thereafter a presentation of the knowledge we have retrieved so far of how the immune system participates in disease initiation and progression and finally how it is affected by different therapies.

5.4. The immune defence system

5.4.1. Basic aspects

The immune system is part of the body’s defence and performs several important tasks; 1) defence against microbes, 2) clearing of transformed cells, 3) clearing of dead cells and 4) aid in healing after tissue injury. These tasks often coincide. The immune system is often divided
into innate and adaptive immunity. In RA, evidence exists for pathology in both systems[21, 22].

The innate immune system is phylogenetically the oldest. Innate responses are specific through pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs), which are common in many microorganisms. However, they are not adaptive or associated with immunological memory. Cells that contribute to innate responses are dendritic cells, \( \text{M} \), neutrophils, mast cells and natural killer cells.

The T and B cells of the adaptive immune system are able to recognize specific antigens through cell specific receptors on these cells. After activation they undergo clonal proliferation and maturation. These features allow the adaptive immune response to be directed towards specific pathogens. Subpopulations of these cells later become memory cells that can quickly mount an immune response to the same microbe when reactivated.

5.4.2. Triggering the immune response

The innate immune system is the first to respond to microbes. Resident \( \text{M} \) become activated, through e.g. activation of PRRs (such as toll-like receptors (TLRs)), and release cytokines activating other cells including endothelial cells of nearby vessels. The vessels upregulate adhesion molecules on their surface, inviting other immune cells, including neutrophils and monocytes, to the site of inflammation/infection. The cells of the innate immune system thus try to rapidly clear the infection. In vertebrates, a second line of defence (the adaptive immune system) is activated through DCs. Resting resident DCs survey their surroundings endlessly for pathogens. After activation, the DCs differentiate, start migrating to the lymph node where they present antigen to naïve T cells (T cells that have not encountered their specific antigen) through HLA-TCR interaction (signal 1). If properly activated, the DCs also provide a second activating signal through costimulation (signal 2), activating the antigen-specific T cell which starts to proliferate and mature into effector T cells. The T cells leave the lymph node and together with memory T cells (T cells that have encountered their specific antigen earlier) they search the vasculature for the site of inflammation. The T cells can respond differently to different pathogens, thereby directing the immune system to optimal defence actions in the tissue. CD4\(^{+}\) T cells differentiate into subclasses; in the presence of IL-12 T helper (Th) 1 cells develop (characterized by IFN\( \gamma \) and IL-2 secretion) activating cellular immunity stimulating e.g. \( \text{M} \). Th2 cells are characterized by IL-4, IL-5 and IL-10 production, activating humoral immunity, i.e. antibody secretion from
B cells and plasma cells. During these processes all signs of inflammation may occur (pain, heat, swelling, redness and decreased function). When the pathogen is cleared, the immune system shuts down and many cells go into apoptosis (self destruct and die). A small proportion of lymphocytes become memory cells for future encounters with the same pathogen. Memory cells are more easily activated then are naïve cells and do not require DCs, as MΦ and B cells can also present antigen and activate them in the tissue.

As these cells and products of the immune response are very potent, their tight control is highly important. When these mechanisms are out of order immune-mediated disease might be the outcome. An analogy for the signal 1 and 2 for T cell activation, the “director” of the late immune response, would be the car and the car key. Not any key can start the car, only the one that fits the lock (signal 1). However, this is not enough. You need to actively turn the key to start the car (signal 2). Now you have an activated car (T cell) that will take you where you need to go – to the shopping mall (or the site of infection). Several other intrinsic factors exert control of the immune system, some of which will be discussed in following sections.

5.5. Immune reactions in the inflamed synovial membrane

5.5.1. Basic aspects of the synovial membrane (SM)

The normal synovium outlines the inner cavity of synovial joints except for cartilage surfaces. It consists of a thin lining (intima) with macrophage-like and fibroblast-like cells, no more than 2-3 cells thick, and a sublining, normally quite acellular containing blood vessels, fat cells, fibroblasts and extracellular matrix. No basement membrane is present. The SM comprises the border between vessels and the joint cavity. It produces synovial fluid which carries nutrients to and lubricates the cartilage. The SM also takes care of joint waste products. Beneath lies subsynovial tissues; fat, joint capsule, tendons and muscles.

During inflammation synoviocytes proliferate and inflammatory cells infiltrate from the vasculature. The lining layer is thickened to 3-10 cell layers, as is the sublining layer, both with heavily increased cellularity. The edematous and hypertrophied SM forms granulations and villi, visible by arthroscopy. Due to the high energy demand of the activated and proliferating cells local ischemia is present, driving angiogenesis, a hallmark of RA (see fig 1). A pannus of cells is formed at the synovial-cartilage-bone junction, invading bone and cartilage, the most typical sign of RA.
5.5.2. **Cells of the inflamed synovium in RA**

5.5.2.1. **Fibroblast-like synoviocytes (FLS)**

FLS are non-phagocytic cells that may attract, retain and stimulate lymphocytes and promote angiogenesis. In RA FLS, especially FLS of the lining layer, are highly activated and significant producers of proinflammatory cytokines (e.g. IL-6, IL-8, IL-15) and proteolytic enzymes capable of destroying tissue (e.g. cathepsins and metalloproteinases (MMPs), such as MMP3). Furthermore, they stimulate (together with T cells) the differentiation of pre-osteoclasts to osteoclasts – the main cell type involved in bone destruction – via the expression of RANKL. FLS also have the capacity to invade matrix and cartilage themselves[24]. *In vitro*, loss of contact inhibition and anchorage dependence has been shown[25], which is also a feature of transformed, malignant cells. Evidence indicates that the high number of FLS is due to both proliferation and reduced apoptosis, many FLSs in fact being resistant to Fas-induced apoptosis[24]. Activated FLS also contribute to the defective apoptosis of T[26] and B[27] cells. FLS can express HLA II on their surface[28]. It has also been suggested that FLS can differentiate into follicular DCs, thereby contributing to
germinal center formation and activation of lymphocytes on site[27]. Conversely, inflammatory cells stimulate the FLS.

5.5.2.2. Macrophages (Mφ)

Mφ are cells with great phagocytic capacity which are abundant in the inflamed synovium and pannus. They are highly activated, expressing HLA class II[29] and costimulatory molecules[30] (both important for T cell activation through presentation of antigens) and produce high amounts of proinflammatory cytokines[31] such as IL-1[32], IL-6, IL-12, IL-15, IL-18, TNF[32] and granulocyte-macrophage colony-stimulating factor (GM-CSF) as well as chemokines and MMPs, although their matrix degrading capacity is modest compared to FLS. Mφ have been associated with erosive disease, high levels of macrophage cytokines being present locally, and the good evidence for their importance in RA pathogenesis is the efficiency of IL-1 and TNF antagonists, reducing inflammation and preventing destruction.

5.5.2.3. Dendritic cells (DCs)

Immature DCs are present in the synovial lining layer whereas mature DCs are detected in lymphocyte infiltrates. They are capable of, as Mφ and B cells, presenting antigen to T cells and are high producers of proinflammatory cytokines. DCs, together with Mφ and B cells, are termed “professional” antigen presenting cells (APCs), due to their ability to process antigens and present them to T cells in lymphoid tissues, such as lymph nodes, thereby stimulating cellular immunity. DCs are the most potent APCs, as they are able to recruit also naïve T cells, not only memory T cells.

DCs have been proposed to play a role in the initiation and perpetuation of RA by presentation of arthritogenic antigens to autoreactive T cells[33]. Differentiated DCs are enriched in the ST of active RA joints, in perivascular lymphoid aggregates and are likely to be efficient APCs *in vivo*[34], adding to the suggestion that the RA SM acts as an ectopic lymphoid organ activating and stimulating immune cells in the synovium itself[35]. Immature DCs are found in the lining, and mature DCs in lymphocyte infiltrates exclusively[36]. DCs are also found in the SF, although in a less differentiated state. Of interest, not only DCs in the synovial joint but also DCs obtained from peripheral blood of patients with RA showed phenotypic and functional differences in comparison with those from healthy controls[37, 38]. DCs in the SM can produce large amounts of pro-inflammatory cytokines[39].
There are several types of DCs, of which the myeloid and lymphoid/plasmocytoid DCs are thought to be of importance in RA. They may be responsible for generating different T cell responses as is proposed in different models; myeloid DCs stimulate Th2 responses and lymphoid DCs Th1, respectively. Both are seen in RA SM.

5.5.2.4. Lymphocytes

Lymphocytes are not present in the normal joint lining layer, they may be present, but are rare in the inflamed joint lining layer, but gather in the sublining layer, in three basic patterns[35]; 1) diffusely scattered or as 2) perivascular aggregates forming 3) germinal centers if follicular DCs are present. The latter contains all the necessary components of antigen presentation, implying that the synovium may function as an ectopic secondary lymphoid organ, activating cells on site.

The majority of T cells are memory cells[40], suggesting previous exposure to antigen. T cells are of an activated phenotype[40], but they also show signs of anergy[41, 42] and do not express high levels of cytokines. It is proposed that of CD4+ T cells, the Th1 subset predominates over Th2, although this is based in large on indirect evidence[43]. Only low amounts of Th1 cytokines (IFNγ and IL-2) are seen in RA, and Th2 cytokines are virtually absent[44, 45]. RA T cells are hyporesponsive to antigen stimulation, and resistant to proliferation[46] and apoptosis[26] possibly due to long exposure to high levels of TNF. In fact, it has been shown that chronic exposure to TNF suppressed T cell activity[47], as opposed to acute[48].

B cells and plasma cells comprise less than 5%[49] of mononuclear cells in RA SM, accumulating in the sublining layer. B cells and plasma cells were first recognized in RA as contributors to inflammation by producing autoantibodies (such as RF[50], anti-citrullinated peptide antibodies and anti-collagen type II antibodies[51], the two former being well established serological markers in RA). However, indications are that B cells also have other important properties.,They are suggested to be absolutely vital components for the formation of germinal centers and activation of T cells in the synovial tissue[52]. Through which mechanism B cells drive T cell activation is still under debate, although antigen presentation and providing survival signals have been suggested[53]. Similar to T cells, B cells also resist apoptosis in RA[54].
5.5.2.5. Neutrophils

Neutrophils are cells with high phagocytic capacity that comprise the most abundant cell type in the inflamed synovial fluid (70-95%), but only small numbers are present in the SM. In the joint cavity they are preferentially located at the cartilage surface, contributing to cartilage damage[55] and to the inflammatory milieu by releasing proteolytic enzymes (e.g. MMPs), various degrading molecules (such as oxygen metabolites), chemokines and cytokines (e.g. IL-1α and IL-1β[56]). Increased survival is proposed to be due to reduced ability to undergo apoptosis[57].

5.5.3. Cytokines

Local cell communication, as in the synovium, occurs principally by cell to cell contact through cell surface molecules or by soluble molecules called cytokines. Cytokines are proteins or peptides mainly produced by inflammatory cells, but any cell can produce them. They are soluble products either secreted or cleaved from the surface of the producing cell, and engage with a receptor molecule on the target cell, thereby stimulating intracellular pathways. Cytokines exert potent control of a number of physiologically important functions; inflammation, cell differentiation and cell proliferation to name some. Therefore tight regulatory mechanisms are at work. Examples of these are decoy receptors, soluble receptors, receptor antagonists, transcriptional and translational control mechanisms. If the balance between pro- and anti-inflammatory cytokine pathways is disturbed, inflammatory disease can be the outcome.

As previously mentioned CD4+ T cell cytokines can be divided into Th1 and Th2 type cytokines, promoting cell mediated and humoral immunity, respectively, although this is an oversimplification. Th1 cells produce IL-2, IFNγ and LTα, whereas Th2 cells produce IL-4, IL-5 and IL-10 among others. It has been suggested that there is a dysregulation in RA synovium promoting a Th1 type response with almost no Th2 cytokines being expressed, in turn inducing abundant production of proinflammatory cytokines by Mϕ, fibroblasts and endothelial cells, e.g. TNF and IL-1. The Th1 type response is further enhanced by the fact that Th1 and Th2 cytokines inhibit each other mutually.

Another T cell cytokine is IL-17, reviewed by Lubberts et al[58], secreted by predominantly activated memory T cells. IL-15 produced by synoviocytes and IL-23 produced by activated DCs and Mϕ are potent inducers of IL-17. From in vitro studies evidence shows that IL-17 is a proinflammatory cytokine. IL-17 synergizes with IL-1 and TNF and induces T cell proliferation, RANKL expression and promotes tissue destruction.
High levels have been found in SF and IL-17 producing cells were evident in T cell-rich areas in RA SM, suggesting that this cytokine is involved in RA pathogenesis.

Besides IL-23, activated MΦ are high producers of cytokines such as IL-1, IL-6, IL-15 and TNF. Of these TNF seems to be of particular importance. TNF can induce the production of almost any other cytokine, including IL-1 and IL-6. As TNF has a special role in this thesis, the following chapter is focused on this cytokine.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>mRNA</th>
<th>Protein</th>
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<tbody>
<tr>
<td><strong>Proinflammatory Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1αβ (interleukin 1)</td>
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<td>Yes</td>
</tr>
<tr>
<td>TNF (tumour necrosis factor alpha)</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LT (lymphotoxin)</td>
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<td>±</td>
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<td>GM-CSF (granulocyte macrophage colony stimulating factor)</td>
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</tr>
<tr>
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<td>MCP-1 (monocyte chemoattractant protein 1)</td>
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<td>ENA-78 (epithelial neutrophil activating peptide 78)</td>
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</tr>
<tr>
<td>RANTES (regulated upon activation T cell expressed &amp; secreted)</td>
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<tr>
<td><strong>Mitogenic Cytokines</strong></td>
<td></td>
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<tr>
<td>FGF (Fibroblast growth factor)</td>
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<td>Yes</td>
</tr>
<tr>
<td>PDGF (Platelet-derived growth factor)</td>
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<td>Yes</td>
</tr>
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Table 2. Cytokine expression in RA Synovial Tissue. Adapted from Feldmann et al[23]
5.5.3.1. Tumor necrosis factor (TNF)

TNF is a member of the TNF superfamily of cytokines. The members of this family all exert important functions in control of cell proliferation, apoptosis or inflammation. TNF is no exception, being able to promote all of these activities[62]. TNF stimulates the expression of other pro-inflammatory cytokines, chemokines and adhesion molecules, increasing cell infiltration. It promotes vascular endothelial growth factors (VEGF) inducing angiogenesis. It stimulates the acute phase response in the liver, with increased levels of CRP, and it promotes MMP production contributing to the destruction of cartilage and bone. Many of the features of RA inflammation can thus at least be partially attributed to this cytokine.

TNF is expressed as a 26 kDa protein on the cell surface. It is cleaved from the surface by a matrix metalloprotease called TNFα converting enzyme (TACE), after which it trimerises. Both membrane bound and soluble forms are biologically active. The effect is mediated by either of two receptors; TNFRI (p55 or p60) or TNFRII (p75 or p80)[63]. TNFRII requires membrane bound TNF for activation, whereas TNFRI can be triggered by either soluble or bound TNF. TNFRI is constitutively expressed, whereas TNFRII is induced. The membrane half-life of TNFRI is 33 minutes, whereas for TNFRII has a half-life of 1 minute, as they too are cleaved from the surface upon cell activation. Both receptors have high affinity for TNF but the dissociation rate is faster for TNFRII. TNFRs may act as decoy receptors or possibly as ligand passers (in the case of TNFRII).

Upon ligation TNF induces trimerisation of the receptor, which is essential for intracellular signaling. Interestingly, TNFRI stimulation can produce almost opposite results, depending on which intra-cellular signaling pathway is activated. One leads to activation of multiple genes by activating proinflammatory transcription factors such as NFκB and AP-1. The other leads to cell inactivation and death through apoptosis. TNFR-I is mainly responsible for these signals, although TNFR-II also is capable but through somewhat different intra cellular signaling pathways. It is interesting to note that soluble TNFR-II can induce a signal on cells with membrane bound TNF[64], which is also seen in treatment with anti-TNF antibodies[65].

TNF stimulates the production of several proinflammatory cytokines; IL-1, IL-6, IL-15, IL-18, GM-CSF and prostaglandin E2 (PGE2). In RA TNF and TNF receptors are present in the SM and SF, Mϕ being the principle source of TNF. Activation of Mϕ results in a 10,000-fold increase in TNF biosynthesis with only a 3-fold increase in transcription[66, 67].
5.5.3.2. Interleukin 1 (IL-1)

IL-1, also a proinflammatory cytokine[68], exists in two isoforms, IL-1 alpha (IL-1α) and beta (IL-1β). They are produced as pro-forms and are activated by proteases such as IL-1 converting enzyme (ICE). There are two receptors for IL-1; IL-1RI and IL-1RII. IL-1RII is a decoy receptor. To promote activation of IL-1RI, a receptor accessory protein (IL-1Racc) must bind. A natural antagonist, IL-1R antagonist (IL-1Ra), exists which binds to the receptor without activating it. IL-1β is secreted whereas IL-1α, due to lack of leader sequence, is not. IL-1 upregulates other proinflammatory cytokines such as IL-6, but only to a minor extent TNF. The main source of IL-1 is Mφ after activation by T cells.

The importance of IL-1 has been confirmed. Injection of either IL-1 isoform resulted in arthritis within hours, which could be blocked by IL-1Ra. IL-1Ra knockout mice spontaneously develop an erosive arthritis. High concentrations of IL-1 have been detected in RA SM and SF. IL-1 also induces the formation of osteoclasts, responsible for bone resorption, through upregulation of receptor activator of NFκB ligand (RANKL), an osteoclast differentiation factor. IL-1 and RANKL also stimulate mature osteoclasts directly (Dayer, -02)[69]. In the clinic, IL-1Ra is in use as an agent retarding both joint inflammation and destruction. In light of this evidence, it has been suggested that IL-1 is more of a destructive cytokine, whereas TNF is more of an inflammatory cytokine.

5.5.3.3. High mobility group box chromosomal protein 1 (HMGB-1)

HMGB-1 is a highly conserved intranuclear DNA binding protein with dual functions. The primary function is in gene transcription; however, it also carries properties of a proinflammatory cytokine. There are two ways that HMGB-1 leaves the cell; 1) through disruption of the cell membrane during cell necrosis (not present in apoptosis) and 2) active secretion by immune cells in inflammation. In case of the second mechanism the immune system mimics the tissue damage of necrosis, and HMGB-1 acts as a mediator, activating tissue repair responses, partly by binding to the receptors RAGE, TLR2 and TLR4.

HMGB-1 is present in serum, SF and SM in patients with RA[70]. When administered to murine joints it causes destructive arthritis. It is capable of inducing proinflammatory cytokine synthesis in monocytes, including TNF and both isoforms of IL-1, although not in lymphocytes. Extracellular HMGB-1 is present in RA but not normal synovium, and aberrant expression precedes clinical arthritis in a murine model. HMGB-1 antagonistic treatment ameliorates disease in mice[71]. It has thus been suggested that HMGB-1 antagonists could
have great impact in chronic inflammatory diseases such as RA. However, so far no trial has been performed in humans.

5.5.4. **Prostaglandin E2 (PGE2)**

PGs were discovered in human semen in 1935 by Ulf von Euler, who named them thinking that they were secreted by the prostate gland[72]. PGs are twenty carbon fatty acids synthesized from arachidonic acid in the cell membrane by different enzymes; phospholipase A2, cyclooxygenase 1 or 2, and several specific PG synthases downstream. They are very potent molecules which have auto- or paracrine effects.

PGE2 is the most common and most biologically active of the PGs. It is produced by a variety of cells and tissues and has both physiological and pathological functions. It is involved in the regulation of vascular and kidney homeostasis, tension of smooth muscle, sleep, bone metabolism, respiration, reproduction and displays a protective effect in the intestinal mucosa[73, 74]. It is also involved in pathological conditions and inflammatory states. All the signs typical of inflammation (pain, swelling, heat, redness) can be mediated by PGE2, by inducing hyperalgesia, vasodilation and increased vascular permeability (Larsen Henson 1983). It may also induce fever[75] and exert effects on the cells of the immune system[76].

In RA patients high levels of PGE2 have been reported in the SF[77]. Several cells within the joint have the capacity of producing PGE2, such as chondrocytes[78] and synoviocytes[79]. In a CIA model EP4 (one of the four receptors for PGE2) deletion resulted in reduced incidence and severity of arthritis[80]. The enzymes responsible for the formation of PGE2 are also upregulated in the inflamed synovium. PGE2, directly or indirectly, is involved in the destruction typical of RA, e.g. regulation of MMPs, collagen synthesis and induction of chondrocyte apoptosis[81]. Furthermore, PGE2 together with IL-1 and TNF stimulates bone resorption[82] in a mouse coculture system of osteoblasts and bone marrow cells. By induction of VEGF in FLSs PGE2 is also involved in angiogenesis[83]. It suppresses B cell proliferation and differentiation[84], promotes Ig class switching[85], suppresses T cell activity and proliferation[86] and regulates T cell apoptosis [87, 88]. PGE2 has also been implicated in the induction of FOXP3 expression[89], a specific transcription factor for regulatory T cells. PGE2 also enhances the production of cytokines by human FLSs[83] and leukocytes[90].

Enzymes in the PGE2 pathway are also upregulated in the SM[91-95] of RA patients, and their levels correlate with disease activity. IL-1, TNF and IL-6 may induce expression of
COX-2 in human articular chondrocytes[96]. Arthritis susceptible mPGES-1 knockout mice demonstrate a reduction in incidence and severity of arthritis[97]. Some of these enzymes co-localize intracellularly. Important enzymes for this thesis are cyclooxygenase 1 and 2 (COX-1 and COX-2), and microsomal PGE synthase 1 (mPGES-1).

These data support that PGE2 is actively involved in arthritis and joint destruction, and that targeting this molecule, its synthesis or receptors might have important clinical implications.

Fig. 1. Biosynthetic pathways of prostanoids. Formation of series 2 prostaglandins (PG), PGD₂, PGE₂, PGF₂α, PGG₂, PGH₂, and PGI₂, and a thromboxane (Tx), TxA₂, from arachidonic acid is depicted. The initial step of releasing arachidonic acid from the cell membrane by phospholipase A₂ is not shown. The following two steps of the pathway, i.e. conversion of arachidonic acid to PGG₂ and then to PGH₂, are catalyzed by cyclooxygenase, and subsequent conversion of PGH₂ to each PG is catalyzed by respective synthases as indicated. Image adapted from Narumiya et al[73].

5.5.5. **Apoptosis**

There are basically two ways for a cell to die, either uncontrolled through necrosis as a result of injury where cell contents are released extracellularly, or quietly through programmed cell death; apoptosis. Apoptotic cells undergo characteristic changes including 1) shrinking and condensation of cytoplasm and nucleus, 2) aggregation of chromatin, 3) fragmentation of DNA and 4) membrane blebbing leading to small particles called apoptotic bodies which are phagocytised by Mϕ. As a consequence apoptosis, in contrast to necrosis,
does not result in an inflammatory response. The term was coined from a Greek word meaning “falling of”, as leaves do in the autumn, to describe this natural timely death of cells.

Apoptosis can be induced in a variety of ways, but mainly through the activation of so-called death receptors via cell-cell contact. Several members of the TNF superfamily of cytokines and receptors are potent inducers of apoptosis, perhaps Fas and FasL being the most recognized couple, but also TNFR stimulation can be involved. Different receptors have in common the activation of proteases called caspases. These enzymes are synthesized as inactive precursors that are converted to the active form by proteolytic cleavage, catalyzed by other caspases. The initial activation of a caspase therefore starts off a chain reaction leading to activation of additional caspases and death of the cell by cleaving more than 40 different cell target proteins, which leads to fragmentation of DNA and the nucleus and disruption of the cytoskeleton, membrane blebbing and cell fragmentation.

In the synovium of RA this process is disturbed[98]. Low levels of apoptosis have been recorded in RA SM (1-3% of cells) by the use of different techniques, TUNEL staining[99] (labeling DNA fragments) and electron microscopy[100] (recognizing apoptotic morphology. Several NFkB-regulated proteins that can suppress apoptosis have been identified. These include tumour-necrosis factor receptor (TNFR)-associated factor 1 (TRAF1), inhibitor of apoptosis 1 (IAP1) and IAP2, survivin, Fas-associated death domain-like interleukin-1 converting enzyme inhibitory protein (FLIP), X-chromosome linked inhibitor of apoptosis protein (XIAP), decoy receptor (DCR) and BCL-XL. These proteins inhibit different steps in the apoptotic pathways. In a study from our group we could see that apoptosis was more pronounced in late as compared to early RA SM[101] and that FLIP, an inhibitor of caspase 8 (part of the intracellular Fas receptor) was upregulated and correlated to \(\text{M}^{\Phi}\) numbers. Both T and B cells are protected from apoptosis by FLS[26, 27], particularly through direct cellular interactions. FLS also show stable activation that includes alterations in apoptosis. Despite high expression of Fas, FLS seem to be relatively resistant to FasL-induced apoptosis[100]. Another explanation could be the elevated level of soluble Fas in SF of RA patients[102], acting as a decoy receptor for FasL.

5.5.6. Destruction of cartilage and bone

RA results in extensive cartilage and bone resorption as evidenced by joint space narrowing on X-ray (a proxy for reduced cartilage thickness), focal bone erosions, juxta-articular osteopenia and systemic osteoporosis. About 80% of patients show erosive signs on X-ray (a marker of poor prognosis) already within the first two years of disease, and <50%
within six months 80% of which is explained by inflammation. Bone loss is an inherent feature of RA that necessitates further understanding of synovial pathogenic processes, since increasing joint destruction and bone loss leads to progressive disability.

The synovium itself contains activated cells such as neutrophils, Mϕ, FLS, endothelial cells (ECs) and osteoclasts that secrete a series of degrading molecules and enzymes capable of destroying tissue. Both matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), have raised concentrations in serum, SF and SM, correlate with disease activity and joint damage RA patients, and proinflammatory cytokines such as IL-1 and TNF stimulate production and secretion.

MMPs are able to degrade non-mineralized tissues but for bone degradation osteoclasts are required. Osteoblasts, FLS, Mϕ and T cells in RA SM induce differentiation and activation of osteoclast precursors by RANK-RANKL interaction. The process can be inhibited by OPG, a decoy receptor for RANKL whose expression is also increased in RA synovium.

It is interesting to note that RANKL is upregulated directly or indirectly by TNF via IL-1. As IL-1 has long been thought of as a destructive rather than inflammatory cytokine, the RANK system could serve as a link connecting destruction and inflammation.

5.6. Treatment

Treatment can be divided into medical, surgical and supportive measurements. For the purpose of this thesis focus will be on the medical treatments. Treatment strategies for RA have changed radically recently, in particular during the last decade. Outlined below are described the main groups of drugs used in RA today.

5.6.1. Aspirin and non steroidal anti-inflammatory drugs (NSAIDs), or coxibs

The mode of action of this group of compounds is through the inhibition of either of the two COX isoenzymes, thereby reducing prostaglandin (PG) synthesis. This provides efficient symptomatic relief of pain and stiffness in many patients.

Salicylic acid is said to have been used for the redemption of pain since the times of ancient Egypt, 1500 BC. It was not until the mid 19th century that it was chemically modified, and in 1899 Aspirin (acetyl salicylic acid) was introduced by Dr Heinrich Dreser at the Bayer company. It is still one of the worlds most used drugs. Because of its symptomatic relief Aspirin was the basic treatment for patients with RA until the 1960’s, but adverse effects were a problem. Already in 1953 perforated ulcers were reported in 1.5% of RA patients[103].
Aspirin was later replaced by aspirin-like drugs, the NSAIDs, the name given from their distinction from corticosteroids which at the time had just recently been introduced. NSAIDs shared many of the effects with Aspirin but carried less adverse effects.

NSAIDs are still widely used in RA patients for symptomatic relief today. NSAIDs have not been shown to affect disease progression or destruction in RA and should therefore be used as an adjuvant therapy. However, adverse effects are still a problem; gastrointestinal ulcers/perforations/bleeds, renal and cardiovascular effects, liver toxicity and so forth. Risk factors for peptic ulcer disease in RA patients are old age, concomitant use of GCs, and history of ulcers. In a Finish study of RA patients, 30 (1.7%) of 1666 deaths were related to NSAIDs[104], and 28 of these were related to peptic ulcers, bleeds or perforations. This and similar studies have inspired the development of COX-2 specific inhibitors, carrying less GI-toxicity. However, COX-2 inhibitors have recently been associated with increased risk of cardiovascular disease that led to the much noted withdrawal of rofecoxib in September 2004REF, possibly through a disturbed balance in blood homeostasis resulting in increased thrombus formation[105].

5.6.2. Glucocorticosteroids (GCs)

GCs are small steroid stress hormones whose secretion is controlled by the hypothalamus – pituitary – adrenal (HPA) axis. Circulating GCs enter cells passively and bind to GC receptors, bound to soluble large protein complexes in the cytosol. Upon ligation, the hormone receptor complex is released. After dimerisation the complex is transported into the nucleus of the cell where it acts as a transcription factor, both enhancing anti-inflammatory (IL-10) and reducing proinflammatory (IL-1, IL-2, IL-6, IL-8, TNF and IFNγ) cytokine gene expression, e.g. by downregulating the activity of other transcription factors, such as NFkB and AP-1. GCs also reduce PG biosynthesis, and affect survival and activity of several cell types; numbers of circulating neutrophils are increased, and numbers of peripheral lymphocytes, eosinophils and monocytes are decreased by inhibition of proliferation and stimulation of apoptosis.

When first introduced in rheumatology by Hench and Kendall in 1948, GCs produced remarkable results as patients bedridden by RA could suddenly “jump out of bed”. Serious disease complications (e.g. pericarditis) could be treated effectively. However, long-term treatment with high doses of GCs was later discovered to lead to such complications as osteoporosis, osteonecrosis, hypertension, diabetes mellitus, glaucoma, cataract, skin bruises and infections. Due to their potent anti-inflammatory effect GCs are still very much in use,
but care must be taken to keep them at low doses. This can be achieved either by adding other anti-inflammatory compounds, like DMARDs or biologics, or by giving GCs locally, allowing smaller doses.

Studies from the 1950’s already suggested that GCs also had protective properties regarding destructions[106]. Other recent studies support this concept, implying that low dose GCs have a proper role in RA as a disease modifying drug[107] and not only as an anti-inflammatory agent.

5.6.3. Disease-modifying anti-rheumatic drugs (DMARDs)

DMARDs are chemically different compounds with the following common characteristics; slow onset of action, affect acute phase response, improve functional status and retard the destructive process in bone and cartilage. Several of these drugs have been developed in other disciplines and then transferred to rheumatology when effect in rheumatic disease also became evident or theoretically attractive.

<table>
<thead>
<tr>
<th>DMARD</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimalarials</td>
<td>Stabilise lysosomal membranes, inhibit metabolism of nucleotides[108]</td>
</tr>
<tr>
<td>Gold compounds</td>
<td>Prolongate neutrophil apoptosis</td>
</tr>
<tr>
<td></td>
<td>Inhibit NFκB activation</td>
</tr>
<tr>
<td></td>
<td>Inhibit B cell activation</td>
</tr>
<tr>
<td></td>
<td>Inhibit osteoclastic bone resorption</td>
</tr>
<tr>
<td></td>
<td>Increase IL-1ra production</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>Affects free radical scavenging</td>
</tr>
<tr>
<td></td>
<td>Catalyzes removal of metal ions from metalloproteinases</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>In monocytes; inhibit TNF, IL-1 and IL-6, possibly by NFκB inhibition.</td>
</tr>
<tr>
<td></td>
<td>In T cells; inhibits IL-2 production dependant proliferation; inhibits IFNγ production; increases apoptosis.</td>
</tr>
<tr>
<td></td>
<td>In B cells; reduces gammaglobulin production (including rheumatoid factor).</td>
</tr>
<tr>
<td></td>
<td>In fibroblasts; inhibits proliferation</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>Adenosine-mediated anti-inflammatory effects</td>
</tr>
<tr>
<td></td>
<td>Increases apoptosis</td>
</tr>
<tr>
<td></td>
<td>Decrease production of IL-1 and IL-6</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>Inhibits T cell proliferation by inhibition of pyrimidine synthesis</td>
</tr>
<tr>
<td></td>
<td>Inhibits NFκB activation</td>
</tr>
<tr>
<td></td>
<td>Inhibits IL-1 and MMP-1 production</td>
</tr>
</tbody>
</table>

Table 3. Disease-modifying anti-rheumatic drugs[109].

During the last 15 years major changes have occurred in the practical use of DMARDs in RA. Methotrexate (MTX) has replaced earlier favourites as the DMARD of choice due to its high efficiency/tolerability ratio[110, 111]. Sulfasalazine has seen a revival after its original
introduction more than 50 years ago, and Leflunomide, a new DMARD, was introduced at the end of the last millennium[112]. More aggressive use of existing DMARDs than the earlier conventional pyramid approach (starting with symptomatic treatment and adding DMARDs only if response is inadequate) has become the norm. Examples are step-up approach (starting with one DMARD early, and adding a second if response is inadequate), step-down approach (starting with combination of DMARDs allowing for cessation of drugs when response is stable) and other variants.

Combination of DMARDs with a new class of drugs, the biologics, has ultimately changed the goals of treatment today. Combining drugs from all classes described have allowed physicians to talk about remission as a treatment goal, which was unthinkable before. Early diagnosis has become increasingly important as erosions can be prevented or at least reduced by early treatment.

5.6.4. Biologics

Biologics is the collective name for a new class of biochemically engineered drugs that are similar to natural endogenous molecules. Examples are antibodies and soluble receptors that target immune molecules and cells such as TNF, IL-1, CD20 on B-cells, receptors for T cell co-stimulation and others. A number of new biologics are being tested in clinical trials or e.g. antibodies targeting IL-6, IL-15 and others. This evolution has changed the treatment protocols as well as treatment outcomes in rheumatology and given this field of medicine a newborn interest also outside the rheumatology speciality. However, as with existing treatments and treatment concepts, many patients still have a high inflammatory activity and there is no cure. Therefore, there is still a mandate for research of new drugs, by the study of disease pathogenesis and the effect of new and old compounds.

A word of caution must be added as is evident from what happened in London the 14th of March this year, when a new biological agent (agonistic anti-CD28) was tested with immediate serious illness for the six healthy volunteers. Without much knowledge about what really happened, it is evident that we are tampering with important functions of the immune system. Going from interesting targets in cells or even animals is not necessarily predictive of what is going to happen in humans. Great care and effort has to be made when new drugs are taken to the clinic.

As focus is on TNF antagonizing agents in this thesis, these agents will be discussed below.
5.6.4.1. TNF antagonists

Three different TNF antagonists are currently available:

Infliximab; a chimeric monoclonal anti-TNF human IgG1κ coupled to a variable region of a murine anti-human TNF antibody. It binds with high affinity to both soluble and membrane bound TNF, and is able both to neutralise the biological action of soluble TNF and to induce antibody-dependent lysis of cells with membrane bound TNF. It is normally given as an infusion of 3mg/kg every eight weeks, after the three initial doses. Indication is RA patients failing DMARDs, including MTX. In RA it is given together with MTX. Other indications are inflammatory bowel disease, ankylosing spondylitis, psoriasis and psoriatic arthritis (Swedish FASS).

Etanercept; a dimer of p75 TNF receptors linked to an Fc portion of human IgG. It can bind two TNF molecules, soluble or membrane bound, as well as lymphotoxin alpha. It does not, however, mediate lysis of the cell. 50mg is given subcutaneously (sc) per week. A better effect is obtained when given together with MTX, but the combination is not required. Indications are active RA patients failing DMARDs (including MTX), severe, progressive RA without prior DMARD trial and psoriasis (Swedish FASS).

Adalimumab; a human anti-TNF IgG monoclonal antibody. It is given as sc injections of 40mg once every two weeks. Indications are RA in patients failing DMARDs (including MTX), severe, progressive RA without prior DMARD trial and psoriatic arthritis (Swedish FASS).

Before TNF antagonists were tried in RA, drugs of this class were studied in an acute inflammatory disorder, sepsis, albeit with disappointing results[113]. Several explanations were proposed, but most likely treatment was given too late, and the cytokine storm had already exerted its effects. When the same compounds were tried in RA, a chronic inflammatory disease, outcome was much improved. The first clinical trial used infliximab and was published in 1993[114] by investigators from the Kennedy Institute in London, with impressive results. However, etanercept was the first TNF antagonist to be approved by the Food and Drug Administration (FDA) in USA in 1998. Infliximab followed suit, and we now have a third compound accepted; adalimumab.

The introduction of TNF antagonists has revolutionised the treatment strategy and goals in RA. They efficiently reduce disease activity and halt radiographic progression. The best effect is achieved in combination with MTX. They are generally well tolerated, and safety is so far similar to that of conventional DMARDs. However, increase in tuberculosis incidence has
been seen, typically reactivation of latent infections. Therefore patients must be screened for infection and started on tuberculostatic medication before commencing anti-TNF therapy. All three drugs have reported autoimmune events, such as development of anti-nuclear antibodies (ANA), anti-DNA antibodies, lupus-like disease and exacerbation of demyelinating disease. Caution also needs to be taken in cardiac insufficiency. Concern has been raised for increase in malignant disease, theoretically due to reduced immune surveillance on these treatments. This has, however not been seen so far, and larger groups of patients have now been receiving these drugs for more than 8 years.

5.7. Reasons for studying the effect of therapies in the synovial membrane

The pathogenesis of RA still remains elusive. Although new risk factors (antibodies, genes, and combinations of environmental and genetic factors[115]) and possible auto antigens (e.g. citrullinated alpha-enolase[116]) have been recognized over the last years we still cannot cure disease or predict treatment response.

The introduction of biological therapies of recent years has fundamentally changed the life of many patients to the better. However, some patients do not respond to therapy, others lose effect after some time, and when treatment is stopped disease often flares. As these treatments have specific targets, an interesting opportunity arises to study disease pathogenesis.

Obviously, examination of peripheral blood and synovial fluid may provide insights into the production of soluble factors and migration of cells. However, these kinds of studies can only yield indirect information of the events in the synovium, the target organ of RA. The relevance of examining the SM was emphasised in a study with the monoclonal antibody Campath-1. Despite indications for the importance of T cells in disease pathogenesis (e.g. HLA-II association and high numbers of active T cells in the synovium) targeting these cells failed to produce amelioration of disease activity[117]. Although marked depletion of circulating lymphocytes did occur, synovial infiltrates of lymphocytes persisted, indicating that peripheral blood studies may not accurately reflect synovial tissue response[117]. Studies of the synovial membrane before and after specific treatment intervention may reveal information about the mechanism of action of drugs, and contribute to the understanding of disease pathogenesis.

A number of studies have described changes in the rheumatoid synovium after anti-rheumatic treatment[118, 119]. SM studies of depleting anti-CD4 antibodies[120], targeting Th cells, revealed not only decreased numbers of CD4+ T cells, but also reduced numbers of
MΦ, plasma cells and FLSs, indicating that T cells are important for the activation and organisation of inflammatory cells in RA SM. Alas, again no clinical improvement was evident and thus interest in T cells dropped.

As anti-MΦ cytokine therapies were introduced with great results, improving both clinical inflammation and almost abolishing the destruction rate of RA, interest shifted to these cells. Indeed, synovial studies of anti-TNF therapy showed decreased cellularity, in part due to reduced expression of adhesion molecules[121] and chemokines[122]. In a small study of eight patients number of synovial TNF producing cells were reduced and numbers prior to treatment corresponded to effect[123], the latter being supported in another study of nine patients[124]. Reduced numbers of MΦ was recorded, as was also the case for other treatments. MΦ correlate with effect[125, 126], erosive disease[127, 128] and local inflammation[129], leading to the suggestion to use these cells as an early predictor of effect of therapy[125]. In fact, both prednisolone[130], gold[131], methotrexate[132] and leflunomide[132] all inhibit macrophage infiltration, and it has been proposed that there are no effective anti-rheumatic therapies that leaves the macrophage population unaffected[119].

One study showed changes in the synovium in patients with only a modest decrease in serum levels of acute phase reactants[133]. This suggests that synovial studies can be used as a screening method to test new compounds, requiring only small numbers of patients. In fact this concept have been used as a “proof-of-concept” early on in clinical trials[134, 135].

The lymphocyte populations of cells have regained interest as treatments targeting B cells[136] and costimulatory molecules on DCs thus inhibiting T cell activation[20], have been successful.

Combining knowledge from each treatment will provide further understanding of RA disease pathogenesis. In summary, it seems as though all cells contribute to joint inflammation, albeit in different ways. It is through understanding these processes that we may be able to individually target different molecules or pathways leading to remission (and cure) of disease. It is our hypothesis that investigations of the synovium are important for the success of these attempts.

5.8. Arthroscopy (AS)

Historically, SM samples have been retrieved from end stage destructive joints after joint replacement surgery. The advantage of this method is the amount of tissue possible to collect; however, this method is limited to late, destructive and often inactive disease. Later blind
needle biopsy was introduced which could also sample the SM in early, active disease. This method was easy to perform and safe, but not reliable in getting accurate samples of SM[137, 138] especially in inactive joints which may be the case in follow-up sessions evaluating treatment. With the introduction of arthroscopy performed by rheumatologists it was possible to sample the synovium under direct vision, and reliably retrieve accurate samples. Recently biopsy under imaging (e.g. ultrasound) guidance has also been introduced. The development of this procedure, including local or regional anaesthesia, has allowed the procedure to be performed in the out-patient clinic[139].

Several methodological issues have been addressed. Samples obtained by needle biopsy had similar cell composition as samples obtained by AS at the same occasion from the same joint[140]. Many immunohistochemistry (IHC) marks were equally expressed in small (wrist and metacarpophalangeal) and large (knee) joints[141]. The AS procedure itself has not shown effect on follow up samplings in several studies; treatment with IL-10 produced no clinical benefit or synovial changes four weeks later[142]; a subtherapeutic dose of anakinra (30mg/day) failed to alter synovial morphology at 24 weeks[143]; IFNβ in RA revealed no changes in clinical data or synovial MΦ, fibroblasts or lymphocytes [144] at 24 weeks. Intra-articular bupivacaine or adrenaline did not change synovial characteristics[145] which is concordant with our own unpublished results of prilocaine. No significant changes were seen in studies using placebo in 4 to 12 patients[130, 135, 142, 146]. Furthermore, a recent study proposed that synovial markers are more reliable than clinical markers, as they are less prone to placebo effects[147], although the authors conclude that “synovial histopathology is certainly not aimed to be a surrogate marker replacing clinical outcomes”. The safety of arthroscopy in the hands of rheumatologists has also been adressed[148].

Several studies have addressed the issue of intra-articular variation of synovitis[149]. It has been dealt with either by sampling predefined anatomical sites[145, 150], sampling according to visual inflammation[151, 152], using a combination of these two methods[149] or by sampling several sites and calculating an average[153, 154]. In our studies we have developed the method proposed by Lindblad et al[149], taking samples from macroscopically active synovitis both distant and close to cartilage.
6. **AIMS OF THIS THESIS**

6.2. **General aim**

To evaluate arthroscopy as a method of tissue acquisition, contribute to the understanding of disease pathology and drug mechanisms in the synovium in patients with chronic arthritis.

6.3. **Specific aims**

1) Study safety of arthroscopy (study I)

2) Study yield of synovial tissue adequate for histologic studies sampled by arthroscopy (study I)

3) Evaluate a method for macroscopic scoring (study I).

4) Study variation of gene expression within and between joints in RA patients (study II).

5) Study effect of anti-rheumatic therapy on
   a. cells and cytokines
      i. Intra articular steroids (study V)
   b. enzymes involved in PGE2 synthesis
      i. Protein expression in RA SM (study III)
      ii. Intra articular steroids (study IV)
      iii. Systemic TNF antagonists (study IV)
   c. molecules involved in destruction
      i. Intra articular steroids (study VI)
      ii. Systemic TNF antagonists (study VII)
   d. apoptosis
      i. Systemic TNF antagonists (study VIII)
7. RESULTS

7.2. Safety of and yield of adequate synovial samples by arthroscopy (study I)

AS has gained renewed interest among rheumatologists during the last 15-20 years after the introduction of the needle AS using local or regional anaesthesia. AS allows physicians to directly visualise joint components for diagnostic and grading purposes, loose body removal, synovectomy, synovial biopsy and therapeutic lavage. During the last twenty years interest increased as the relationship between synovial features and clinical and radiological parameters became evident[149, 155]. In a survey by Kane et al[148] from 2002 AS was used in 36 rheumatology centres in Europe, USA and Australia as compared to five centres in the early 1980s. The number of ASs performed by each center varied from 20 to 5000.

It is important to regularly validate routines for safety and results whatever the field. This is of special importance in operative procedures, as risks are evident and undue suffering must be minimized. AS has been validated for safety by both orthopaedic surgeons[156-159] and rheumatologists[138, 148, 160-165]. The numbers of evaluated procedures from individual rheumatological centres vary between over 100 and under 350. Here we present our own experience of 408 procedures.

During seven years (1998-2005) we performed 408 procedures in 237 patients. 2/3 of patients were classified as RA, mean age was 52 years (range 20-85), 170 were women and 67 were men. Mean disease duration was 91 months (range 1-623) and mean arthritis duration was 43,5 weeks (range 0-564). 148 patients received NSAIDs (which was not required to be stopped before AS) and 99 patients had DMARDs. The majority of procedures were performed for research purposes (96.3%) (all by approval of ethical committees), and only 26 (6.4%) for clinical indications.

Only 2 patients presented with major complications (haemarthrosis, 0.5%). One minor complication was a wound infection in a patient that never removed the surgical strips (0.2%). Minor bleeding occurred during some procedures affecting vision, especially in small joints, which were all stopped during or immediately after the procedure. Pain in the investigated or other joint occurred in a smaller number of procedures, restricting the synovial sampling procedure in 12 (2.9%). In one patient pain was considered severe two weeks after the procedure (0.2%), after given proper treatment pain slowly subsided. No patients had to stay at the hospital more than one hour after the procedure, and no long term complications were encountered.
We also evaluated the yield of synovial biopsies adequate for histology per joint examined—97% of knee AS were conducted as planned, with proper samples retrieved in 95%, resulting in a total tissue yield of 92% in studied samples. For smaller joints this figure was much smaller (34%).

<table>
<thead>
<tr>
<th>study</th>
<th>N of AS</th>
<th>major (%)</th>
<th>minor (%)</th>
<th>haemarthrosis (%)</th>
<th>joint infection (%)</th>
<th>operative †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sherman[156] ‡</td>
<td>2640</td>
<td>126 (4.8)</td>
<td>97 (3.7)</td>
<td>(2.0)</td>
<td>(&lt;0.5)</td>
<td>majority</td>
</tr>
<tr>
<td>Small[157] ‡</td>
<td>10262</td>
<td>(1.7)</td>
<td>104 (1.0)</td>
<td>21 (0.2)</td>
<td></td>
<td>majority</td>
</tr>
<tr>
<td>Szachnowski[160]</td>
<td>335</td>
<td>(1.2)</td>
<td>(12.8)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>majority</td>
</tr>
<tr>
<td>Kuzmanova[162]</td>
<td>206</td>
<td>4 (2.0)</td>
<td>15 (7.5)*</td>
<td>7 (3.5)</td>
<td>1 (0.5)</td>
<td>all</td>
</tr>
<tr>
<td>Reece[165]</td>
<td>278</td>
<td>1 (0.4)</td>
<td>13 (4.7)</td>
<td>0</td>
<td>1</td>
<td>none</td>
</tr>
<tr>
<td>Wollaston[163]</td>
<td>342</td>
<td>1 (0.3)</td>
<td>5 (1.5)</td>
<td>0</td>
<td>0</td>
<td>minority</td>
</tr>
<tr>
<td>Smith[138]</td>
<td>128</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>minority if any</td>
</tr>
<tr>
<td>Baeten[161]</td>
<td>150</td>
<td>0 (&lt;10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>minority if any</td>
</tr>
<tr>
<td>Kane[148] ☼</td>
<td>16532</td>
<td>237 (1.5)</td>
<td>141 (0.9)</td>
<td>16 (0.1)</td>
<td></td>
<td>minority</td>
</tr>
<tr>
<td>af Klint</td>
<td>408</td>
<td>2 (0.5)</td>
<td>1 (0.2)</td>
<td>2 (0.5)</td>
<td>0</td>
<td>none</td>
</tr>
</tbody>
</table>

Table 4. Complications of arthroscopic procedures. † Type of procedure. ‡ Orthopedic surveys. *Haemarthrosis was included in minor complications in this study. ☼ Rheumtologic survey. N number; AS arthroscopy; n.d. not determined.

In table 4 is presented complication rates from different studies is presented. The studies by Sherman \textit{et al} and Small \textit{et al} are surveys of orthopaedic AS, the remainder were performed by rheumatologists. The numbers are not completely comparable as study procedures differ in nature and purpose, as well as in definition of complications. Some are retrospective and some are prospective studies. However, this table gives an indication of the complication rate in arthroscopy, especially in the hands of rheumatologists. Importantly, our study did not reveal more complications, major or minor, than the orthopaedic studies or the survey of rheumatologists by Kane \textit{et al}. Of importance no joint infection, deep vein thrombosis or other major complications were apparent in our study. Haemarthrosis was over-represented in our hands, although we saw only two cases, which could be random. Haemarthrosis has been associated with operative procedures such as cartilage biopsy[148], and type of joint (knee)[157]. Joint infection has been positively correlated with irrigation volume[148], perhaps a proxy for operating time. Routinely we keep irrigation fluids at a minimal, only rarely exceeding 1 liter (due to leakage from joint portals). We also abstain
from the use of a tourniquet (associated with complications if used for >1 hour[156]), as this hampers visualisation of synovial vascularity, an important feature of inflammation.

Evaluating the yield of synovial material adequate for analysis, including immunohistochemistry, is important. In studies using the blind needle biopsy technique the yield is not satisfactory, only 60-85%. By arthroscopy the yield can be much higher, as indicated in our study of knee joints with a yield of 92%. However, the yield in smaller joints was disappointing, only 34%. This is in contrast to other studies[164, 166], suggesting that sampling these joints may need more training than do knee joints.

Thus we conclude that AS is a safe and reliable method for retrieving adequate samples of synovial tissue, especially in large joints. This is important in successive samplings of joints in follow up studies, e.g. to evaluate effects of drugs.
7.3. Variation in the inflamed synovium. Evaluation of a method for macroscopic scoring (study I) and study of gene expression variation by microarray technology (study II)

Variation is a common problem in biological studies. In RA variation also exists between patients, between joints within patients and also within joints. The latter is especially obvious in large joints such as the knee, and may have pathophysiological implications. Firstly, as destruction of cartilage and bone occurs in RA it is likely that synovial features are different close to rather than distant to these structures. Secondly, macroscopic changes within the joint vary, and possibly different inflammatory activity will tell us different things. Thus it is vital to sample the synovium in a representative way. Intra-articular variation has been confirmed macroscopically[149] and microscopically for cells[167], protein expression[32] and gene expression[154]. Correlations between macroscopic and microscopic data have resulted in conflicting results[140, 145, 149, 151, 152, 168, 169]. For this reason attempts were made to examine a limited area of tissue with encouraging results[170-172]. As different cells and molecules have different patterns of mRNA and protein expression, sample methods may have to vary accordingly, depending on the purpose of investigation. For T cells[153] and gene expression[154] of certain genes, six biopsies have been proposed as being an optimal number to represent the joint as a whole. Some studied have indicated no difference between samples taken at the cartilage-pannus junction to those taken more distant to it[145, 173, 174]. Other studies have indeed reported differences[140, 175, 176] although the absolute distance for detecting a difference may be very small, perhaps only a few cells[177].

Several scoring systems have been suggested for macroscopic synovial changes in inflammation[149, 151, 152, 168, 178]. However, intra- and inter-observer variation for macroscopic features/scores has not been properly addressed.

With the development of new techniques, variation within the synovium can be further investigated. By microarray (MA) technology it is possible to study the gene expression of thousands of genes at a time. This technique has been used in RA before to investigate gene expression profiles in early[179] and long-standing RA[180]. Interestingly, both studies generated hypothesis of different gene expression profiles in high versus low inflammatory status. Gene expression variability in RA SM has to our knowledge only been addressed in one study using real time- polymerase chain reaction (RT-PCR)[154]. Four genes were analysed (TNF, IL-1β, IL-6 and MMP-1) against GADPH as a control house-keeping gene, and the conclusion was that at least six biopsies were required to limit sampling error due to variation.
Macroscopic scoring

In order to address the issue of correlation between macro- and microscopic features of synovitis, we undertook to create and validate a macroscopic score. During individual and joint sessions seven raters scored a number of arthroscopic video-printer images of synovitis from AS procedures in chronic arthritis patients. A “Macro-score Instruction” paper, including a set of jointly scored images, was prepared. After calibration to the instructions, 50 images were scored by each rater separately for hypertrophy, vascularity and synovitis, producing 2100 individual scores. Intra- and inter-rater variation was calculated.

Intra-rater variation was low; 99% of scores were scored within +/-1 scoring step at the second scoring session. A perfect match (same score twice by the same rater) was 71% for hypertrophy, 69% for vascularity and 71% for synovitis. No significant difference was evident between parameters that would indicate anyone harder to score. Inter-rater variation was also low; 1036 (98.7%) of 1050 individual scores had a deviation of less than +/-1 point from the median score. We also analysed inter-rater variation by calculating the range of scores for each image parameter, given by the seven raters. Range was 2 or less for 139 of 150 image scores (93%).

Furthermore, the “Macro-score” instructions were tested in a second group of five raters, that had no previous experience of arthroscopy. They received the written instructions without any further instruction whatsoever, and used it to score the same set of 50 images scored by the first group.

Scoring biological phenomena are intrinsically difficult. Regarding macroscopic evaluations or scoring methods this is indicated by the existence of many different scoring systems. Although many studies mention roughly the same parameters to measure, there is no consensus. Also different scales have been used for the same parameters. In this study we aimed at scoring the most simple and biologically important parameters by a semiquantitative scale score. We chose to evaluate only hypertrophy and vascularity as these are obvious features of synovitis. We abstained from scoring synovial fluid and synovial hyperemia as these features have intrinsic difficulties. For instance, hyperemia is very sensitive to intra-articular pressure, distance and strength of the light source, and perhaps is influenced by drugs and the emotional status of the individual. We also scored hyperemia as such. We showed that by defining each parameter closely, we could reach a consensus of how to score, with very encouraging results. From this experience we constructed a set of “Macro-score” instructions,
with scored and testing images provided. This instruction was used by the first group as a calibrating instrument to score 50 images, with small intra- and inter-rater variation.

**Variation of gene expression by microarray technology**

We also investigated variation in gene expression by MA in RA SM, within and between joints. Several SM samples from three distinct areas of the synovium of seven RA patients with end-stage destructive disease were retrieved at joint replacement surgery. We also retrieved SM tissue from different areas in six RA patients with active synovitis by arthroscopy. In all patients variation between biopsy sites and between patients were measured, and in three patients variation within the biopsy site was measured.

The average number of differentially expressed genes was 143 between biopsy sites from arthroscopic samples. The corresponding number for the orthopaedic tissues was 455, revealing a much higher gene expression variation. IHC studies showed that some of these samples largely consisted of fat tissue (common subsynovially). When corresponding samples were removed from the gene expression profile, differentially expressed genes were reduced to 171. In the sub-analysis of DE genes within a biopsy site, the result was 173 genes on average. Between patients the number of differentially expressed genes was much higher, between 1-2000. When we performed cluster analysis on the arthroscopic and orthopaedic groups of samples individual samples clustered almost perfectly to biopsy site and patient, especially when fat tissue samples were removed from the analysis.

In this study we demonstrated that variation in gene expression is larger between patients than within patients. Variations within patients and within sites were of the same order of magnitude. Single samples showed almost perfect relations to biopsy site and patient. We also showed that cell-composition is highly important, and that macroscopically retrieved samples varied less, most likely due to more precise sampling. As has been suggested above the issue of biological variation has been dealt with differently. It has been proposed that six samples from different sites within a large joint like the knee should be pooled and analysed together[154], thus representing the joint as a whole. Using this method perhaps important differences will be reduced. Instead we suggest that in longitudinal studies single samples are taken in the same region of the joint. If reproduced in other independent studies, it is conceivable that MA technology could be performed on single biopsies which might provide information that allows for predicting disease course and response to treatment in diseases such as RA.
7.4. Effect of anti-rheumatic therapy on cells and cytokines; intra-articular GCs (study III)

The potent anti-inflammatory effect of intra-articular treatment with GCs has been known since 1951, when Hollander et al [181] first injected hydrocortisone acetate intrasynovially into patients with RA and found that symptoms of pain and swelling were reduced in 90% of treated joints. Intra-articular GC-injections became standard treatment for rheumatologists, and is still commonly used as an adjuvant treatment to NSAIDs and/or DMARDs in RA patients upon flare of disease or individual joints for maximal quick response and bridging therapy. Although the effect varies from patient to patient, it may last for years [182, 183]. As a local treatment it reduces systemic effects to a minimum as compared to systemic treatment, though they may be evident in some patients [184].

The effects of GCs on different cells and tissues are significant and manifold. This largely depends on their effect on gene expression, directly or indirectly. Although many studies have tried to elucidate cellular effects, only few studies have been performed in arthritis patients examining the synovium in vivo.

Treatment with steroids has been shown to reduce synovial volume [182, 185], influx of cells [186-188], MΦ [130], cytokines [130, 189], adhesion molecules [130], but not MMPs or TIMPs in osteoarthritis [187] although reduced in RA [190]. Only few studies have looked into the intra-articular effect of GCs on synovial histology in RA [190, 191]. When this study was initiated in 1998 not much was known about the in vivo effects of intra-articular GCs on the RA SM. We set out to study the effect of GCs on cells, cytokines and vessels of the SM.

We studied the effect of triamcinolone hexacetonide (a synthetic GC) on synovitis in chronic arthritis patients. We determined that T cells, but not MΦ, were reduced after 1½ weeks. Immunohistochemical analysis revealed that the presence of proinflammatory cytokines such as TNF, IL-1β and HMGB-1 was diminished after GC treatment. IL-1α levels did not change. Gene expression changes did not follow the same pattern; IL-1α and IL-1β levels were reduced, whereas levels of TNF and HMGB-1 did not change. HMGB-1 pattern of expression changed from an extracellular to a cytosolic/nuclear pattern. Interestingly, vascularity did not change and several markers, although reduced, were still present in synovial vessels.
Our results are partly in contrast with those from another study of systemic GC therapy of RA patients which recorded a reduction in Mφ after two weeks[130]. They could show a reduction of CD68+ cells, mainly attributable to a decrease in the number of Mφ localized in the synovial sublining. There were also trends toward decreased infiltration by T cells, plasma cells, fibroblast-like synoviocytes and CD163+ Mφ. The cytokine patterns were basically the same in both studies. In one study of the intra-articular effects of GCs in osteoarthritis patients, minor but significant reductions of Mφ were evident in the lining layer and no changes in the sublining layer after four weeks[187]. Although lymphocytes have been shown to be sensitive to GC-induced apoptosis, this effect differs depending on tissue, cell-type and concentration of GCs[192]. Among other effects GCs have been shown to reduce proliferative responses and infiltration by down-regulating adhesion molecules. In effect, there are many possible explanations for the reduction of T cells after GC-therapy. In an ongoing study at our lab it seems that the inherent resistance to apoptosis also in RA SM T cells is kept even after treatment with GCs, suggesting other modes of action.

Although compelling evidence exists for the importance of Mφ as a sensitive marker for response to therapy, these studies have been evaluating DMARDs[125] and biologics[193] with great differences in drug action as compared to GCs. Although Mφ were not reduced in our study, macrophage derived cytokines were, indicating the pleiotrophic actions of GCs. As is the clinical experience, these drugs complement each other, probably by different mode of actions.

We also evaluated the effect of intra-articular GC treatment on HMGB-1 expression. HMGB-1 is a nuclear protein under normal conditions, with potent pro-inflammatory properties when released outside the cell such as during necrosis. Inflammatory cells have the ability to secrete this protein when stimulated. In our study the staining pattern of HMGB-1 changed from being extracellular to intracellular following steroid therapy. As no change was evident in HMGB-1 transcription, it is likely that the reduction in positive area in IHC represents a redistribution of HMGB-1; HMGB-1 is no longer secreted and the proinflammatory cytokine properties of this molecule are kept inactive, inside the cells. As for IL-1α and VEGF, expression of HMGB-1 in the vascular compartment did not change significantly. Although GC therapy is efficient, it does not cure, and for most patients disease flares later. One possible explanation for this could be the partially resistant response of the vascular compartment to this treatment. Although adhesion molecules are also rapidly down
regulated in vessels, it seems likely that they might be upregulated again due to proinflammatory molecules still present.
7.5. Effect of anti-rheumatic therapy on enzymes involved in PGE2 synthesis; intra-articular GCs and TNF antagonists (studies IV + V)

Many of the features of RA can be directly or indirectly associated with effects of prostaglandins, especially PGE2. PGE2 acts as a mediator of pain and inflammation and promotes bone destruction. PGE2 is synthesized by the specific actions of a chain of enzymes, including COX-1/COX-2 and terminal PGE synthases (PGES). COX-1, cytosolic PGES (cPGES) and microsomal PGES (mPGES)-2 are more constitutive in character, as opposed to COX-2 and mPGES-1 which are inducible. Interestingly, one study reported co-expression of COX-2 and mPGES-1 in RA synoviocytes *ex vivo* after stimulation with proinflammatory cytokines[194]. mPGES-1 expression has also been detected in RA synovial lining cells[195]. In animal experiments, mPGES-1 was evident in inflamed paws of adjuvant induced arthritic rats[196], and in mPGES-1 knockout mice the incidence, severity and destruction of joints in arthritis was significantly reduced[97, 197]. Taken together, studies of mPGES-1 support the importance of mPGES-1 in RA pathogenesis.

Conventional inhibitors of PG actions used in RA are COX-inhibitors, GCs, DMARDs and TNF-antagonists. GCs inhibit enzymes in the PGE2 biosynthetic pathway; COX-2 expression[93] in RA synovial explants and phospholipase A2 activity[198]. The synthetic GC dexamethasone efficiently suppresses mPGES-1 mRNA and protein expression and enzyme activity in synovial fibroblasts *in vitro*[194]. However, systemic GCs in RA patients showed no reduction of PGE2 in RA SM by 24 hours[189].

TNF antagonists are effective inhibitors of inflammation and bone destruction in RA.

Neither mPGES-1 in RA SM expression *in vivo* in the RA synovium, nor the effect of treatment by intra articular GCs or systemic TNF inhibitors was fully known at the start of this study.

We demonstrated mPGES-1 protein expression in SM from RA patients. Both intra- and extracellular staining was evident in all tissues, especially prominent in synovial lining cells. Expression was also observed in sublining macrophage- and fibroblast-like cells, although less pronounced. In 25% of patients positive staining was also seen in endothelial cells. Using double immunofluorescence staining, expression was recorded in MΦ and fibroblasts, but not in lymphocytes (T and B cells). cPGES and COX 2 was detected with the same cellular pattern as for mPGES-1. Co-expression of mPGES-1 and COX-2 was seen in a considerable number of cells. In contrast, no increased expression was evident for COX-1 in cells expressing mPGES-1.
Ten days after treatment with intra-articular GCs (triamcinolone hexacetonide) mPGES-1 expression was significantly reduced in 16 chronic arthritis patients, seven of which had RA, while the expression of housekeeping cPGES was not reduced. Expression of both COX-1 and COX-2 was significantly reduced by GC treatment. After systemic therapy with infliximab (ten RA patients) or etanercept (eighteen RA patients) for eight weeks, neither mPGES-1 nor COX-2 SM staining changed significantly.

Synovial fluid mononuclear cells from RA patients were isolated and cultured in the presence of LPS. Double immunofluorescence staining revealed co-expression of mPGES-1 and CD163 (indicating alternatively activated Mφ as a source), and mPGES-1 and COX-2. After induction with LPS for 42 hours expression of mPGES-1 and COX-2 was greatly enhanced, as opposed to COX-1 expression which was reduced. After treatment with either GCs, infliximab or etanercept both mPGES-1 and COX-2 expression was reduced.

This study demonstrated that mPGES-1 expression is evident in RA SM. It is likely that this enzyme is involved in PGE2 biosynthesis as it co-localises with COX-2 in the same cell types in RA synovium, and both enzymes are induced upon inflammation. mPGES-1 has recently been linked to the increase in PGE2 production in synoviocytes in response to inflammatory stimuli, whereas mPGES-2 and cPGES may be responsible for the basal synthesis of PGE2. It is likely that PGE2 has a homeostatic role in low concentrations under normal conditions, but at high concentrations PGE2 may contribute to important symptoms of RA such as pain and inflammation and, possibly, bone destruction. The terminal PGE2 enzyme mPGES-1 is likely the mediator of this effect.

Intra-articular treatment with GCs efficiently reduces inflammation clinically. In our study GCs consistently down-regulated mPGES-1 expression, suggesting that this molecule is also a target of GCs in RA. Interestingly, COX-1 and COX-2 levels were also reduced. Although COX-1 is generally considered a constitutively expressed enzyme, evidence shows that it may be upregulated by IL-1α[199] and VEGF[200], both of which are up-regulated in RA SM.

Neither of the two TNF antagonists studied showed any reduction of mPGES-1 after eight weeks in vivo. However, in ex vivo SF cells both COX-2 and mPGES-1 were reduced by etanercept, infliximab and GCs. The difference in effect of TNF blocking treatment in vivo and in vitro is probably due to the difference in cell composition in the SM and in the SF, the latter not having any FLS present. The lack of effect of enzymes in the PGE2 pathway could contribute to the lack of effect in some patients by this treatment.
7.6. Effect of anti-rheumatic therapy on molecules involved in destruction; intra-articular GCs and TNF antagonists (studies VI + VII)

Bony erosions, a hallmark of RA and a marker of poor prognosis, occur at the bone synovium junction and are visible by radiography in <50% within six months of disease duration, and in 70% using magnetic resonance imaging. It was only recently that interest turned to osteoclasts as mediators of this feature.

Pathological bone resorption depends on numbers of osteoclasts, which in turn depend on development (osteoclastogenesis) and death (apoptosis) of these cells. OPG[201] and RANKL[202] are vital regulators of both osteoclast differentiation, survival and bone resorption. RANKL binds to RANK on osteoclast progenitors and induces intra-cellular signaling, OPG is a soluble decoy receptor for RANKL. M-CSF and RANKL, two cytokines essential for osteoclast formation[202, 203], are produced by activated synoviocytes and T cells. Besides preosteoclasts, MΦ, dendritic cells and a B cell precursor can also differentiate into osteoclasts[204].

In animal experiments mice lacking osteoclasts (RANKL–/–) were protected from bone erosions[205], and treatment with RANKL antagonists (OPG) prevented bone erosions in adjuvant-[206] and collagen-[207]induced arthritis and in TNF transgenic mice[208]. In all studies reduction of progression of erosions was associated with reduced numbers of osteoclasts.

In RA SM RANKL expression is increased in the SM, including T cells[209]. The RANKL/OPG ratio is higher in RA compared to other inflammatory joint diseases and osteoarthritis[210]. A high RANKL/OPG ratio is associated with increased bone resorption[211] and with active synovitis[212]. In a recent study RANKL expression in RA SM was relatively restricted to the pannus-bone interface and at the site of subchondral bone erosion. At the same sites RANK-expressing preosteoclasts were present. OPG expression was observed in multiple cells remote from the bone, but was limited at sites of erosions, especially in areas of high RANK expression[213].

Interestingly, chronic exposure to TNF in vivo increases osteoclast formation, directly or indirectly; TNF can prime bone marrow cells to differentiate into osteoclast progenitors via a RANK/RANKL independent mechanism; TNF can induce RANKL expression of various cells, including synovial cells and T cells; TNF may also bind to its receptor on preosteoclasts and directly induce their differentiation to mature osteoclasts[214]. TNF antagonists are potent inhibitors of bone damage in RA[215].
GCs are known inducers of bone loss through multiple mechanisms such as impaired calcium intestinal absorption, suppressed osteoblast formation and increased osteocyte apoptosis[216], and in vitro treatment of osteoblasts induces upregulation of RANKL and inhibition of OPG expression[217]. However, low dose steroid treatment has been implicated in a number of studies as a disease-modifying drug with bone sparing effects early in disease[106, 107].

As both TNF antagonists and GCs target inflammatory cytokines, including TNF, and both are suggested to have bone protective properties in arthritis, we set out to study the effects of these compounds on the RANKL-OPG system.

In vitro treatment with infliximab induced a significant increase in RA synovial OPG after eight weeks, but only a trend in etanercept treated patients. Non-significant trends were determined for both treatments in reducing RANKL expression. Treatment with intra-articular GCs (triamcinolone) induced a significant reduction of RANKL expression after ten days, but hardly any change in OPG. The RANKL/OPG ratio was significantly reduced by all three compounds. To determine whether these changes are clinically important we evaluated the effect of TNF antagonists in responders and non-responders. For intra-articular GCs this was not possible due to small numbers in the non-responder group. Both for infliximab (significant) and etanercept (trend) groups there was more pronounced decrease in the RANKL/OPG ratio in responders versus non-responders. By double staining we could detect RANKL expression in CD3+ T cells but not in CD163+ MΦ.

We also conducted cell culture experiments using etanercept, infliximab and a synthetic steroid, dexamethasone. Unprimed endothelial cells did not significantly change the RANKL/OPG ratio after treatment with either infliximab or etanercept. However, after priming with TNF – creating a milieu similar to that in the inflamed joint – both infliximab and etanercept decreased the RANKL/OPG ratio, primarily by increasing OPG expression. In both primed and unprimed osteoblasts the effect was similar as for endothelial cells.

Both RANKL and OPG are affected by GCs and TNF antagonists, lowering the RANKL/OPG ratio, although by different means. TNF antagonists primarily induce OPG expression, whereas GCs act through a reduction of RANKL. It is plausible that both treatments complement each other when used together, as they are in the clinic. RANKL/OPG ratio not only correlates with destruction, but also with synovial inflammation as also indicated by our studies (study VII). In clinical studies of TNF antagonists these agents also
have anti-bone resorptive properties in clinical responders as well as non responders[215], but this effect is greater in responders.

Dexamethasone induced RANKL and reduced OPG expression in unprimed osteoblasts, as shown earlier, thus promoting osteoclastogenesis. After priming with TNF, however, dexamethasone decreased expression of RANKL, resulting in a diminished RANKL/OPG ratio. Thus depending on the local environment GCs exert opposing effects on the RANKL/OPG ratio. In RA synovial fluid lymphocytic cells, RANKL was also significantly reduced by dexamethasone, mimicking the effect on SM *in vivo*. These studies emphasize the importance of performing mechanistic studies of the target organ in RA, as cells taken from serum behave differently from those obtained from the SM and the local cell environment of the arthritic joint has fundamental effects on treatment responses.
7.7. Effect of anti-rheumatic therapy on synovial apoptosis; TNF antagonists (study VIII)

Cellularity in RA SM is highly increased but is significantly reduced by anti-TNF treatment[121]. The increased cellularity in RA synovium can be explained by several factors; 1) increased inflow of cells to the synovium, 2) increased survival of cells in the synovium, 3) increased proliferation on site and 4) decreased exit of cells from the synovium. Evidence exists for 1) in RA; adhesion molecules expression is increased in vessels and tissue, cells in the SM have bone marrow origin, studies of radio labeled neutrophils show increased ingress in joint inflammation reduced by GC treatment[186]. Evidence for 2) was provided in our group showing decreased apoptosis in synovial tissue of RA patients[98]. This feature was more pronounced in early as compared to late RA, which inversely paralleled cellularity findings[101]. It was further suggested that this could be explained by apoptosis of Mϕ. It is interesting to note that cells in the synovium are resistant to TNF- and FasL-induced apoptosis, possibly due to NFκB-induced apoptosis inhibitory proteins. Levels of one of these, FLIP, was indeed raised in SM in this study[101]. 3) Proliferation is increased in RA SM. The possibility of 4) has to my knowledge not been studied. Anti-TNF treatment in RA patients can reduce inflammation and, interestingly, delay joint destruction and even stop it[215, 218]. SM cellularity is decreased[121], partly explained by downregulating adhesion molecules[121] and chemokines[122]. Decreased proliferation does not in itself explain why cellularity is reduced. Decreased survival through apoptosis is therefore an interesting mechanism to consider.

We report that both etanercept and infliximab induce an increase in RA SM apoptosis evidenced by TUNEL and caspase 3 staining after eight weeks of treatment. A significant decrease in synovial Mϕ numbers, but not T cells (trend only) were seen. These changes were paralleled by in vitro studies of SF and peripheral blood derived cells. Both treatments induced apoptosis in RA SF-derived Mϕ, but not in lymphocytes. In peripheral blood monocytes from RA patients receiving either drug increased the number of apoptotic cells, albeit to a lesser degree. No change was seen in peripheral T cells.

Our findings are partly in contrast to a study by Tak et al[121], who reported a decrease in lymphocytes and not Mϕ at four weeks, although they used a higher dose of infliximab (10 or 50mg/kg). In a study by Smeets et al[146], decrease in cellularity by infliximab treatment was
reported, including Mφ. However, increase in apoptosis was not seen at either of the two time points (48 hours and 4 weeks), as measured by TUNEL and electron microscopy. Our time point of evaluation was later, eight weeks. It is conceivable that although apoptosis as such is a rapid event, it takes time for the inflammatory milieu to normalize. This is supported by a study in Crohn’s disease demonstrating increased levels of apoptosis after ten weeks of infliximab therapy[219]. A suggestion would therefore be that early changes after TNF treatment are due to a reduced influx of cells, whereas later apoptosis also contributes to the effect of reduced cellularity.

The lack of effect on lymphocytes is interesting. In Crohn’s disease patients treated with infliximab, but not etanercept, apoptosis is induced in activated lymphocytes, but not in resting. Gut-derived lymphocytes are known to have a highly activated phenotype and are able to produce high levels of cytokines. Joint-derived lymphocytes in RA are low producers of cytokines and exhibit signs of anergy, and in vitro TNF suppresses these T cells. It therefore seems that T cells from these different organs and disease states encompass different characteristics. This could be due to the local environment of the arthritic joint. In fact in the same study we could demonstrate that another growth factor for T cells, IL-15, was not affected by TNF antagonists[124]. It is likely that T cells develop intrinsic changes, like FLS, in the rheumatoid joint that allows them to escape apoptosis.

Interestingly, low doses (1 and 10 µg/ml) of both drugs were more effective than higher doses (100 µg/ml) in vitro in our hands. In earlier studies high doses of etanercept did not induce cytokine suppression, and high doses of infliximab did not induce apoptosis in the presence of very low doses of TNF. These findings suggest an escape route in the presence of high concentrations of these drugs.

There are several ways that TNF antagonists could induce apoptosis. Both infliximab and etanercept can bind to cell membrane bound TNF, and induce intracellular signalling, perhaps resulting in apoptosis. TNF protects monocytes from death receptor-mediated apoptosis through upregulation of intracellular anti-apoptotic molecules (such as FLIP) through NFκB activation. Both treatments are able to inhibit NFκB and increase apoptosis, possibly through down-regulation of the same molecules.
8. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The studies included in this thesis are all clinical studies of patients with chronic arthritides, the majority having rheumatoid arthritis. Patients were recruited from the outpatient clinic of the Rheumatology department at the Karolinska University Hospital. Ethical approval for studies was given by the local ethical committees, and all patients approved before study entry. Only study I also includes patients studied for exclusively clinical reasons.

Study I is a methodological study, concerning safety, yield of samples and scoring of synovial features. Studies II and IV are exploratory studies, and studies III and V-VIII are open label effect studies with internal controls. The latter studies aimed to investigate drug mechanisms of the target organ, the synovium of arthritis patients.

8.2. Major findings

8.2.1. In these studies we have added to existing evidence that arthroscopy is a safe and reliable method for synovial tissue retrieval.

8.2.2. We have constructed and validated an easy to use macroscopic score for arthroscopy. This score needs validation against other methodologies.

8.2.3. We have studied variability of synovial gene expression, where we could see that samples close to one another were more “related” than samples further apart, especially from other patients. Arthroscopic biopsies had less intrinsic variation than had orthopaedic material.

8.2.4. We have shown that injection of intra articular steroids reduces protein expression of synovial proinflammatory molecules (TNF, IL-1β, extra-nuclear HMGB-1, ICAM-1 and VEGF) and T cells. mRNA expression was reduced for IL-1α and IL-1β, but not for TNF or HMGB-1. Unexpectedly, no changes were evident in Mφ infiltration and the vascular compartment. Vascular proinflammatory cytokine expression persisted.

8.2.5. We have shown that mPGES-1 is strongly expressed in RA lining cells, and also in sublining Mφ, FLS and endothelial cells. We also saw that mPGES-1 and COX-2 co-localised in cells from synovial fluid and tissue. Finally mPGES-1 and COX-2 were down-regulated by GC treatment, but not by anti-TNF treatment.

8.2.6. We have shown that markers of destruction were positively affected by both GCs and TNF-antagonists, although differently. RANKL was down-regulated by
GCs and OPG was up-regulated by TNF-blockade, leading to a favourable reduction of RANKL/OPG ratio by both treatments.

8.2.7. We have provided evidence that TNF-antagonists induce apoptosis in RA SM MØ, but not lymphocytes.

8.3. Methodological issues and internal critique

When we first started this project clinical parameters were considered of minor importance and the collection of tissue for studies of pathology was the main focus. This led to poor clinical characterisation of patients. During the last couple of years this has changed and all patients included in arthroscopic studies are now properly characterised.

A problem with clinical invasive studies is the usually small number of patients possible to collect, as was the case also in our hands. Because of this we have chosen to make use of internal controls where the patients constitute their own control. This could be a problem in studies collecting patients when disease activity is high. As chronic arthritis, including RA, is not a stable condition but often a relapsing-remitting disease, it is likely that simply by nature patients will be better in time – “reduction to the mean” to use a statistical term. This cannot be overcome easily without external controls. This is why one has to consider if studied molecular events are likely to be effect of treatment or not.

Expectation bias and placebo effect have been considered as possible draw-backs. Expectation bias was not considered a problem as all investigations of tissue were blinded as to identity and order of samples. Placebo effects are not likely to affect results of tissue studies, which have been confirmed in studies using consecutive sampling from patients receiving placebo or ineffective therapy. A recent study also suggests that synovial markers do not change in the absence of effective treatment, although placebo effect could still be seen[147]. Moreover, where measured, clinical results in our studies were of the same magnitude as in double blinded placebo controlled clinical trials. Taken together, these studies support the hypothesis that changes seen in serial biopsy studies of synovium is indeed a consequence of therapy, and not a placebo effect.

Arthroscopy was the main method used for tissue acquisition in these studies, although some samples also arose from orthopaedic joint replacement surgery. The advantage of arthroscopy to other methods of synovial tissue sampling is the possibility to sample under direct vision. We decided early on not to sample the whole joint and to calculate a mean for different markers. Instead we developed the method suggested by Lindblad et al[149]. Today we chose biopsy site according to macroscopic inflammation and distance to cartilage,
sampling three sites with different characteristics. In follow-up studies we resampled in close proximity to the first sites by the aid of a drawn map and photographs of each site. An obvious critique against this technique is that some markers will have an intrinsic variation within the joint that is greater than the change seen. For other markers this is not as important. However, when calculating a mean important changes might not be observed and differences within the joint may not be studied. Both methods have associated pros and cons. We can thus state that calculating a mean from several biopsies is more representative for a joint, whereas our method evaluates local sites within a joint. By this method mapping and photographing each site becomes very important, as we only compare corresponding sites. We do not compare samples from different sites in consecutive or cross-sectional studies. Perhaps the biopsy procedure itself changes synovial parameters? This has not been shown to be the case in other studies, although they might not have tried to sample as close to previous sites as we did. This question is being addressed in an ongoing study by us.

8.4. Key contributions and future perspectives

8.4.1. Intra-articular variation

There are still methodological issues to be addressed by rheumatological arthroscopists and synovial researchers. Intra-articular variation is an obvious problem that has not been finally studied. Macroscopically the synovial membrane can have very different appearances within the same joint. By developing and evaluating a set of easy to use “Macro-score” instructions we have contributed in so that it is possible to use these and correlate macroscopic scores with other studies, such as histological studies. Some correlation does exist between macroscopic scores and synovial volume/activity by imaging methods and some synovial markers or cells in some studies. The “Macro-score” can be easily introduced without previous training.

The issue of biological variation was further addressed in the study using microarray analysis of thousands of genes (study II). Other methods for gene expression, such as PCR, often make use of one (most often GADPH) house-keeping gene as a standard for the studied genes to be compared against. By MA many genes can be used as a background control set, and when individual genes in this set don’t behave as expected they may be withdrawn from the analyses, thus being less vulnerable to biologic activities and changes. By MA we could show that by using this method, single samples could be used to represent single patients.

There are biological reasons for the variation within a joint. Several studies point to the synovial pannus – bone and cartilage interface where the cellular composition and protein
expression is different from further away. It is therefore likely that biological questions will
be answered not by pooling samples, but by diligently characterizing each sample including
location. In a study evaluating treatment (10 early RA-patients with methotrexate or
leflunomide) by MRI disclosed smaller changes close to cartilage than further away[176],
implying SM features close to cartilage more difficult to normalise. This is interesting as
destructive changes occur close to cartilage and are to some extent disconnected from
inflammation. Pooling samples would make it harder to note these differences. What method
to use depends on what marker is analysed and purpose of study.

8.4.2. Response to treatment

In response to intra-articular GCs we could noted a decrease in T cells, thereby re-
emphasizing the role of lymphocytes also as a response marker, as much interest have been
focused on MΦ recently. This is supported by the effect of rituximab[136], targeting B cells,
and CTLA4Ig[20], targeting costimulatory molecules on DCs abrogating T cell stimulation
turning them anergic. Although macrophages have been implicated in several studies as
important mediators of destruction and correlate to response to therapy, other cell types are
clearly involved. B cells are necessary for the forming of ectopic lymphocyte organs and
indications are that activation of T cells is B cell dependant in RA[53]. T cells are thought to
contribute to the effectiveness of other immune cells, and when they are turned anergic,
patients are becoming better. It seems therefore logic that different treatments will have
different early response markers, although late markers of response may coincide.

Even more important would be to investigate markers for effect. Most likely the
mentioned array technique will provide with interesting ideas in this field. The advantage of
this technique is obvious; to investigate the gene expression of thousands of genes at a time.
However, it is quite costly, and gene expression is not equal to protein expression due to post-
transcriptional regulation. Also protein and tissue arrays are becoming more and more used.
These techniques will also provide important information, driving research from hypothesis
testing to hypothesis generating type of research. If technical and financial issues used to be a
problem, the availability of well characterized patient tissue samples is now the rate-limiting
step. Possibly the study of the target organ, together with genetic and epidemiological studies,
will provide information that allows us to, in the future, take a simple blood sample and from
this preclude about prognosis and response to different treatments.
We could see that the vascular compartment was more resistant to the effect of GCs. As vascularity has been associated with active, erosive disease and response to DMARDs[220] and TNF antagonists, it is interesting to further investigate this compartment in disease pathogenesis.

Another important finding in this study was the effect on HMGB-1 by GCs, reducing and relocating this molecule. As HMGB-1 induces inflammation extra-cellularly, the study supports that GCs may act also by this novel route, possibly by inhibition of NFκB or phospholipase A2. It was recently shown that HMGB-1 also has great proangiogenic potential[221], in vitro and in vivo (chick embryo chorioallantoic membrane), as angiogenesis is a hallmark of RA synovitis. This molecule carries both pro- and anti-inflammatory properties, the latter being tested in rodents with encouraging results[71]. We are still waiting for this treatment principle to enter the human field.

PGE2 exerts almost all the typical effects attributed to PGs. We showed (in study IV+V) that synthesising enzymes mPGES-1 and COX-2 were coexpressed in RA SM, and even colocalised in certain cells, making successful PGE2 synthesis a likely following event. Intra articular GC therapy, as opposed to TNF blocking treatment, down regulated mPGES-1 and both COX enzymes. This was not expected, especially since PGE2 is associated to destruction and this is halted by anti TNF treatment.

An interesting finding is that human embryonic kidney cells transfected with both COX-2 and mPGES-1 led to cellular transformation, characterized by proliferation and morphological changes[222]. Thus it is possible that elevated co-expression of both enzymes in synovial fibroblasts contribute to cellular transformation resulting in fibroblast activation and destructive behaviour characteristic of RA.

Blocking mPGES-1 has great joint protective potential, possibly without the adverse effects associated with COX inhibition. Furthermore, specific inhibition of mPGES-1 might allow shunting of PGH2 into anti-inflammatory PGs such as PGD2 and its metabolites, providing even better efficacy in the treatment of arthritis.

Osteoclasts are vital for bone destruction, and a proxy for their formation and activity is the RANKL/OPG ratio. We studied the effects of intra-articular GCs (study VI) and two systemic TNF antagonists (study VII) on this ratio. It became clear that both treatment principles affected the ratio in a positive direction, reducing it, however by different means; GCs decreased RANK and the TNF antagonists upregulated OPG. These findings are
interesting as both treatments have been shown to reduce or even halt destructive disease. The destructive process in the joint can be mediated by several different players; e.g. SF cells such as neutrophils; SM cells such as FLS and Mφ; soluble destructive enzymes and; specialised osteolytic cells as the osteoclasts. Earlier studies have shown effects of the former pathways, here we also indicate effect of the latter. TNF antagonists report the best clinical response and retardation of destruction when used in combination with MTX[215, 218]. It has also been implicated that even in clinical non-responders destructions are not progressing with the above combination (Klareskog, personal communication), indicating that it is possible to uncouple inflammation from destruction in the disease process. As for GCs, we showed a decrease in RANKL instead of an increase as suggested previously[217]. We propose that this is due to the inflammatory environment in RA SM, as it has been shown that GCs have both pro- and anti-inflammatory effects, depending on the environment and concentration of GCs[192]. This would implicate that GCs should only be used in an inflammatory setting, indicating that the use of intra-articular treatment in active joints actually takes hold of the most advantageous characteristics of GC treatment; maximising anti-inflammatory at the same time minimizing systemic and destructive adverse effects of this drug.

Apoptosis is decreased in RA SM. Increase in apoptosis could be one way that effective treatment works by. We could show, contrary to an earlier study[146], that TNF blockade by both infliximab and etanercept induced apoptosis in RA SM macrophages, however not in lymphocytes by eight weeks. This was supported by in vitro studies of cells derived from RA SF. The inflammatory milieu of the arthritic joint has been shown to induce defective apoptosis in several cell types. This is in part due to high expression of anti-apoptotic molecules, making cells resistant to Fas-induced killing. NFκB activation, a strong feature in RA SM, seems to mediate this effect. By blocking TNF NFκB activation will subside, allowing for apoptosis to occur. It seems as though this is a cell specific effect, affecting activated Mφ but not hypo responsive T cells. Studying apoptosis induction in other targeting therapies (like rituximab and CTLA4Ig) will likely prove very interesting. By continue sampling the RA SM in vivo important contributions will likely be made to disease pathogenesis.
10. REFERENCES


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