# Synovial Tissue Research: State of the Art Review

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**Abstract**

Synovial tissue is the 'target organ' of inflammatory arthritides such as rheumatoid arthritis. The study of synovial tissue has advanced significantly over a number of decades, facilitated by arthroscopic and more recently ultrasonographic technology that allows easy visualisation and biopsy to be performed. The potential for study of pathogenesis, discovery of biomarkers and novel targets for therapy have all been progressed rapidly in the last decade with increasing analytical technology including molecular techniques and most recently systems biology. In this review we describe clinical and translational developments in the field of synovial tissue research outlining the new investigative and analytical technologies and how they can be applied to advance our understanding of the pathogenesis of disease, discover new biomarkers and identify novel targets for therapy.

# Introduction

## 1.1 General Considerations

Chronic inflammatory arthritides (IA) comprise a heterogeneous group of diseases characterised by inflammation of the synovium, leading in many cases to the destruction of adjacent tissues. Inflammation is characterised by synovial neovascularisation, proliferation and leukocyte extravasation.(1) For the propose of this review we will focus on Rheumatoid Arthritis (RA), due to its prevalence, and there being the most extensive body of research on this common cause of synovitis. RA is usually persistent and progressive, leading to joint damage, disability and deformity if left untreated. RA is associated with a reduction in quality of life as well as decreased longevity and constitutes an important burden on healthcare spending.(2-4)

Recent years have seen several advances in the treatment of IA in general, and particularly in RA. While it is true that the last two decades have witnessed an unprecedented, if qualified, success in treating RA, a substantial portion of patients still do not achieve low disease activity or remission.(5, 6)

The main challenges in biomedicine and translational research in RA focus on early diagnosis, personalised medicine, and the development of meaningful outcome assessments.(7) Each of these can be facilitated by the identification and development of appropriate biomarkers. While specific biomarkers such as ACPA have been shown to predict development of RA in asymptomatic individuals (8) Hensvold et al, ARD, 2016 (diagnostic accuracy of ACPA), our repertoire of biomarkers to assist with diagnosis, disease progression and response to therapy is currently limited.(9, 10)

Since the synovium is the principal target of inflammation in RA, one promising approach in the search for biomarkers may be found in the detailed analysis of the inflamed synovium. Using a combination of established methodologies, together with incorporating new high-throughput technologies with the capability of detailed examination of genes and their products on a scale never before possible, a new opportunity awaits in the search for these biomarkers.

## 1.2 Anatomy and Physiological Regulation of the Synovial Joint

The synovial joint comprises opposing bones, with the articular surface covered by cartilage. The main protein in bone is type I collagen, while cartilage, which is found on the articulating surfaces, comprises mainly type II collagen and proteoglycan molecules. The non-articulating

surfaces are lined by a thin adventitious layer known as synovium. Normal synovial tissue comprises 1 to 3 layers of specialized columnar cells called fibroblast-like synoviocytes (FLS) with interspersed macrophages.(15) The entire structure is housed by a fibrous capsule, and together with ligaments, muscles and tendons, this confers strength and stability to the joint.

Several factors contribute to the maintenance of normal homeostasis in the synovial joint. These include normal expression of the protective lubricin(16), FLS secretion of matrix metalloproteinases (MMPs), as well as local regulation by cytokines and growth factors.

Cytokines and growth factors are important regulators of synovial fibroblasts and chondrocytes.(17-19) Cytokines are categorized either as pro- or anti-inflammatory depending on their immediate effect on specific tissue, although there is considerable potential for pleiotropism depending on the cells targeted and the microenvironment. These regulators are ubiquitous in the synovium and synovial space and make their way either by filtration from plasma, or secretion by fibroblasts, chondrocytes and the surrounding tissues.(14)

The joint is a dynamic environment, and is necessarily the site of continuing evolution. The joint is subject to minor trauma continually, through movement, and in some joints, weight bearing. Therefore it is necessary for articular cartilage and adjacent bone to be continually replaced in a cycle of synthesis and degradation. This requires a balance of anabolic and catabolic enzyme activity on both cartilage and bone.

Carefully regulated proteolytic enzymes are responsible for the cartilage degradative processes within the joint.(20) Matrix degrading enzymes such as matrix metalloproteinase (MMPs) are present in normal synovial fluid, but are higher in concentration in RA, psoriatic arthritis (PsA) and osteoarthritis (OA).(20-22) The collagenases (MMP-1,-3?, -8,-13 and -18) are the most important of these enzymes as they are the only known enzymes that can directly cleave collagen at neutral pH(23), but other MMPs continue this breakdown when the triple helix collagen structure has become unravelled.(24)

Serine and cysteine proteinases are required to activate pro-MMPs (to MMPs) after they are secreted. Furthermore, inhibitors of these proteinases (e.g. tissue inhibitors of metalloproteinases (TIMPs) and inhibitors of serine proteinases (SERPINs)) are also present in the normal joint. Changes in the balance between anabolism and catabolism are determined by changes in the levels and activity of the matrix degrading proteinases. The activity of these enzymes can be monitored indirectly by measuring their degradation products in the synovial fluid.(25)

## 1.3 The Inflamed Joint

The inflamed synovium has been studied at several levels; macroscopic, microscopic and molecular. Synovium is the primary target of disturbed immunomodulatory pathways in RA. Rheumatoid synovial tissue is hyperplastic and oedematous and is characterized by marked intimal lining hyperplasia, and by accumulation of T and B lymphocytes, plasma cells, macrophages, neutrophils, mast cells, natural killer and dendritic cells in the synovial sublining.(26) Most of this cell accumulation comprises synovial fibroblasts and macrophages,

Angiogenesis accompanies this cell accumulation, but it occurs in an abnormal manner resulting in a significant number of immature vessels.(27) Additionally, patterns of blood vessels have been recognised to be associated with particular types of inflammatory arthropathies.(28) These new vessels allow for increased leucocyte migration and the synovial tissue becomes an invading 'pannus', causing cartilage and bone destruction.(29, 30) Despite increased vascular supply, profound hypoxia in inflamed synovial membrane *in vivo* has been demonstrated.(27, 31)

Many of the pathological changes manifest in the inflamed synovial tissue are observable in the synovial fluid (SF), which has also been studied intensively. Inflammation alters the permeability of synovial tissue. In relation to RA synovium, the permeability to large molecules is increased, but that of small molecules is decreased (urea and glucose). This is due to a combination of increased permeability of the vessels, cellular infiltration and synovial hyperplasia.(32) The total protein content in SF is higher in inflammation and synovial inflammation inhibits the ability of the synovium to selectively filter proteins from entering and leaving the joint space.(32)

The molecular weight distribution of the lubrication macromolecule hyaluronic acid (HA) is also altered, with a shift towards lower molecular weight forms in RA.(33) There is increased loss of HA from the joint and the mean HA concentration is lower in OA and RA synovial fluid.(34, 35)

Pathological SF samples have markedly raised cytokine concentrations.(36) The role of cytokines in initiating and perpetuating the inflammatory response is being studied intensively.(19) This has already assisted in the development of useful therapeutic targets, and the identification of further potential targets.

# Synovial biopsy

## 2.2 Retrieving Synovial Tissue Samples

The utility of synovial biopsy has already been demonstrated in increasing our understanding of the pathogenesis of RA, as well as in identifying potential therapeutic targets and

evaluating current and new treatments.(37-53) It has also been proposed that synovial biopsies may give insight into the mechanism of action of a given agent.(49)

There are three ways to biopsy synovium: using arthroscopy, ultrasound or blind needle biopsy.(54) Arthroscopic biopsy allows direct visualisation of the synovium and the operator can select an area of macroscopic inflammation to biopsy. Ultrasound depicts synovial thickness in greyscale and with Doppler synovitis and therefore assists in selecting a suitable biopsy site. While blind biopsy has been validated, arthroscopic and ultrasound guided biopsy are favoured for proof of concept experiments.(55)

Arthroscopic and ultrasound guided biopsy are safe and well tolerated. Data from 15,682 arthroscopies performed by rheumatologists revealed a complication rate for haemarthrosis of 0.9%, for deep vein thrombosis of 0.2% and wound and joint infection both of 0.1%.(56) This incidence is reproducible at other centres where the overall complication rate was shown to be less than 0.3%.(57) Similarly, an overall major complication rate is reported as 0.4% for ultrasound guided biopsy procedures.(58)

However retrieved, biopsies from one large joint (usually knee) in studying IA, assumes that the area biopsied is representative of the process in all other joints. In fact, a study taking biopsies from 9 RA patients in both an inflamed knee and an inflamed small joint, did not demonstrate differences in mean cell numbers for all markers investigated in both sample sources.(59) Of further note, patients with clinically evident disease manifest at small joints have been shown to have similar abnormalities in clinically uninvolved knee joints.(60, 61)

Questions remain about where to biopsy within a given joint. In particular there have been concerns that mediators of inflammation may be differentially expressed in different parts of the same joint, especially between the cartilage pannus junction (CPJ) and non-CPJ sites which are known to behave differently. However for T-cells(62, 63), plasma cells(63),several metalloproteinases(63, 64) and granzymes(63), there have been similar results for biopsies from CPJ and non-CPJ sources. One study did find a difference for macrophages(65), but two others did not.(62, 63)

Despite the clinical role of synovial histology in the differential diagnosis of arthritis (such as infectious, granulomatous, infiltrative diseases, and crystal arthropathies), there are still profound unmet needs regarding predictors for diagnosis, disease progression and response to treatment. Therefore much effort has been made to identify biomarkers in this field. The two methods of searching for biomarkers in the joint itself involve studies of the synovial tissue and studies of the synovial fluid. It is worth noting however, that there are definite differences in the cellular, and likely cytokine and molecular, make-up of the synovium and the synovial fluid. Therefore examining synovial fluid or blood may give an indication of the production of soluble mediators and an understanding of the dynamics of migration of inflammatory cells into different compartments, but studying these alone only provides indirect information about what is happening in the primary target of RA; the synovium.(66)

Various prognostic biomarkers for RA have been identified first in SF and then later validated in serum.(67) One study performed using this strategy first identified proteins that may be of interest in the synovial fluid (SF), and then searched for antibodies to these proteins in the plasma.(68) It is possible that this methodology may be useful in future experiments, and the results of such research may be more easily translated into clinical practice.

Since 2002, cohorts of patients with early arthritis have been gathered. Having a cohort of early arthritis patients with clinical data, histological data, DNA arrays and mRNA arrays and proteomics is an instrumental resource for investigating differences in synovial tissue, comparing several inflammatory joint diseases with persistent self-limiting, persistent active disease as well as erosive and non-erosive disease.(55)

Most research with synovial biopsies has been done on RA samples but some results suggest that synovial tissue sampling may be used in other inflammatory arthropathies.(69) As mentioned above, given that the principal target of inflammation in IA is the synovium, it makes both intuitive and practical sense that this tissue should be the principle target of research. This research should aim at seeking a fuller understanding of the disease pathogenesis, investigating mechanisms of action of current treatments, and in the identification of novel targets and biomarkers. Indeed, the effects of various treatments for RA on the synovium has been studied, and the principle results of these data are presented in table 1.

## 2.2 Early Arthritis and Early Diagnosis

Despite our progress in diagnosing RA much earlier than before, signs of joint destruction might still be present already at the diagnosis time (89) We know today that aggressive and early treatment is more successful than later or less aggressive treatment (11-13). Therefore the utility of biomarkers in securing a diagnosis as early as possible will allow treatment in the most timely manner, securing the best outcomes.(1, 90) Those with undifferentiated arthritis may benefit most from this. Although anti-citrullinated peptide antibodies (ACPA) are reasonably specific (90%),(91) the diagnostic sensitivity in early arthritis is between 40-70%.(92, 93) and up to 30% of RA patients never develop ACPA.(91) Delay in diagnosis of RA may arise from either a lack of a definitive biomarker, or a failure to meet current diagnostic criteria(55), and these criteria have a significant reliance on biomarkers. Therefore, despite the advent of ACPA, there remains a need to identify further and specific susceptibility biomarkers.

The so-called ‘at-risk of arthritis’ cohort have been the subject of much research in recent years. It has been proposed that studying this cohort could lead to important clues in understanding the timeline and evolution of rheumatoid arthritis, as well as in potentially identifying better biomarkers that allow accurate predictions to be made before onset of established arthritis. One potential corollary of this is the promise of developing a cure as

distinct from a treatment for RA, where the initial break in self-tolerance is identified and targeted therapeutically.(94, 95)

Identifying those at risk of developing RA remains a problem. Although a positive ACPA status is associated in those with arthralgia with the subsequent development of arthritis, only about 20-30% of these people had developed RA after a follow up of up to 30 months (94, 96, 97). The synovial tissue of patients who are at risk of arthritis has been studied. Whether there is any evidence of sub-clinical synovitis during this phase of the disease appears to be conclusively answered in the negative. In one study examining the knee synovial biopsies of 13 patients with arthralgia who were auto-antibody positive (to enrich the true pre-RA recruitment), no differences were seen in their synovium compared with healthy controls (98). In a follow up larger study, only subtle infiltration of T-cells in the synovium of patients at-risk of developing arthritis was noted (94). Some research is now focussing on alternative tissues that may be important in the very early stages of arthritis- usually as sites of first antigen presentation, such as the lung and lymph node.

Very interestingly, despite the realisation that there is a 'window of opportunity' during which RA can be most successfully treated in the earliest phase, there are no differences in cytokine, chemokines, matrix metalloproteinases or adhesion molecule expression comparing early disease with late disease, controlling for disease activity and the use of anti-rheumatic drugs (98-101). Only more recently has a cellular infiltration difference been identified. A highly expanded, specific T-cell clone present in early stages, but not later stages of RA, has been identified, which serves to underline the importance of T-cells in the early stage of the disease.(102)

The use of synovial biopsy as a diagnostic and prognostic tool in undifferentiated peripheral inflammatory arthritis was evaluated in a meta-analysis from 2011 including 6 publications(103). Here in contrast to serological ACPA testing, ACPA staining was shown not specific for the diagnosis of RA. Synovial CD22 and CD38 positivity seemed to differentiate between RA and non-RA, while synovial CD38 and CD68 positivity could differentiate among RA, spondyloarthritis (SpA), and other diagnoses. The value of the selected diagnostic and prognostic markers described in these studies were limited; the research in the field of using synovial biopsy markers to establish an early diagnosis is still evolving.

A more recent study included 50 patients with early arthritis, who had undergone synovial biopsy at inclusion and were followed for two years (104). The focus was on the angiogenic processes in the initiation and perpetuation of synovial inflammation, in particular vascular endothelial growth factor (VEGF) and angiopoietins 1 and 2 (Ang-1 and Ang-2) and their tyrosine kinase receptors VEGFR and TIE-2. Expression of TIE-2 was significantly increased in the group with erosive disease as compared to the group with a self-limiting disease, and plasma–TIE-2 was significantly increased in the groups with persistent non-erosive disease and persistent erosive disease as compared to the group with self-limiting disease.

Although more research is needed, the studies suggest that a synovial biopsy at disease presentation could be of important help for both patients and physician, for early disease stratification into short self-limiting course versus severe persistent inflammatory course, and in the former between erosive versus non erosive disease, and thereby informing the most appropriate treatment strategy.

## 2.3 'Personalised Medicine' and Disease Stratification

In addition to problems with diagnosis, predicting the course of established disease is imprecise. Identification of the subgroup of patients with early arthritis who will go on to develop destructive disease is important in selecting effective treatments.(105-107)

Much has been made of so-called 'tailor-made' treatment, which is informed by biomarkers. Biomarkers may be used to assess what Lindstrom has referred to as 'disease signatures'.(108) Lindstrom and Trusheim contend that a more accurate description of this process would be disease 'stratification'.(108, 109) In this concept, a disease can be stratified into distinct subsets that exhibit differential outcomes and responses, each subset labelled by a biomarker or, more likely, a combination of biomarkers.

Selection of therapies are commonly made on a trial and error basis, but less than 50% of RA patients experience a 50% improvement in their arthritis in response to any single biological therapy.(110-112) During the time that an ineffective treatment is being given, the disease is being allowed to progress and patients are potentially exposed to unnecessary adverse events. Therefore biomarkers that predict response to a given treatment will be of great clinical utility.

## 2.4 Recent advances

Synovial tissue biopsy procedures and analysis are becoming more widely available throughout the world.(55) This will inevitably enable a targeted approach to identifying biomarkers in synovial tissue.

With respect to disease stratification, for the purpose of diagnosis and predicting response to treatments, sensitivity and specificity can be theoretically improved by combined use of biomarkers. Examples of this are already apparent. Positive clinical response of RA to anti-tumour necrosis factor (TNF) treatment with etanercept has been demonstrated using a biomarker signature generated by 13 autoantibodies and 11 cytokines. The study included three ethnically distinct populations, and for North Americans it demonstrated a positive predictive value of 71%.(10)

The advent of new proteomic, transcriptomic and genomic technologies, and the ability to combine clinical and radiological markers with these technologies, will likely make stratifying disease the norm in the future. It is possible that disease stratification will become so sub-categorised such that it is truly approaching personalised medicine. The –omics approach have been usefully applied to identify key players and protein interactions in several diseases. Studying the genome, the RNA or the protein will each have different sets of bias and variance, and it has been argued that combining approaches may lead to a more accurate assessment of important protagonists.(14)

Proteomics offer the advantage that the functional units of the cell are being studied directly, likely most accurately representing what is actually happening in the synovium. The development of technologies such as SomaLogics that have the power to measure thousands of protein in small tissue volumes has the potential to allow a far more complete characterisation of the disease network of RA.(14) Thus far, in investigating RA, the proteomics approach has focussed on peripheral blood mononuclear cells, serum and synovial fluid (113-116); our possibility that the synovial tissue itself may hold the key to unlocking the disease network has yet to be fully exploited. Furthermore, new technologies in protein separation, processing and identification are expected to increase proteome coverage

In relation to transcriptomic analysis, one of the most frequently employed strategies in the field of biomarker research concerns the use of microarrays. This facilitates the identification of candidate genes in the pathophysiological processes. However, gene expression levels do not always predict protein levels, due to transcription and translational regulatory mechanisms and the activity of the protein degradation processes.(14) A good example of the use of transcriptomic data, was demonstrated by Woetzel *et al* while determining a rule-based classification that allows differentiation between RA and osteoarthritis.(117)

Currently, microarrays contain probes for thousands of different genes so that it is not necessary to hypothesise in advance what the candidate genes are.(55) Another advantage of the high throughput techniques used in transcriptomics is the ability to detect significant gene expression differences with relatively modestly sized cohorts compared with the size required to identify genetic variants.(118)

There have been a number of studies where DNA array technology used to study gene expression in RA has been shown to be a useful and practical methodology. Different subtypes of affected synovium in RA have been characterised by gene expression analysis.(119-121) Huber *et al*., identified gene-expression variance among RA patients impacting several pathways involved in cell proliferation, cell survival, angiogenesis and regulation of inflammation.(122)

# Biomarkers

## 3.1 General Considerations

Synovial biomarkers can be categorised either according to their properties or according to their demonstrated clinical utility. A great deal of work has concentrated on comparing features of inflamed synovium in known RA patients with samples taken after clinical improvement following treatment. More recently, a number of studies have analysed the predictive capabilities of synovial tissue biomarkers for disease course and response to therapy.

## 3.2 Cellular Composition

Simple cell counts densities (or cellular infiltration) were appreciated, more than 20 years ago, to be associated with RA. In a study published in 1989, a group showed that there was a decrease in T cell numbers after at least 6 months of gold treatment. They also reported a reduction in the ratio of T-helper cells to T-suppressor/cytotoxic cells in those who were treated successfully. Macrophages were not reported in this study. Furthermore, the number of biopsy samples where B cells could be identified decreased from 36% before successful treatment to 7% after treatment. (37)

More recently it was shown that patients taking prednisolone had a reduction in CD68 staining cells after two weeks treatment compared with controls. The reduction in CD68 cells was mostly attributable to a decrease in the number of macrophages localising to the synovial sublining. There was also a decrease in the CD4 and CD5 (T and B cells) and CD38 (plasma cells) and CD55 (fibroblast like synoviocyte) cells.(123)

Another study showed a reduction in macrophages in synovium, 12 weeks after gold therapy. CD68 cells were most commonly identified in all layers independently of where the synovial biopsies were taken from. There was no significant change in the numbers of lymphocytes following treatment in this study.(124)

Again, a significant reduction in macrophages in the sublining region following treatment with DMARDs (Disease Modifying Anti-Rheumatic Drugs), mostly methotrexate and gold, was demonstrated in another study. This was particularly pronounced in those who were responding clinically by ACR criteria. Although a significant reduction in memory T cells was seen (CCR7), (and this was interestingly associated with CRP reduction), memory T-cells could still be found in the synovium of patients who attained remission.(125)

Macrophages were also significantly reduced after 16 weeks of treatment with leflunomide in the synovial sublining, as well as with 16 weeks of monotreatment with methotrexate in the intimal lining layer. T cell numbers were decreased with both treatments but did not reach statistical significance in this study.(42)

In a similar study, after 16 weeks of methotrexate, a decrease in CD3, CD8, CD38, CD68 and Ki67 were demonstrated to be statistically significant. Notably CD4 infiltration was not reduced in this study.(126)

Treatment with infliximab has been shown to reduce CD3 (T cell) in repeat biopsies taken 4 weeks after treatment, and this finding was associated with clinical response.(40) In ten patients with longstanding RA who received infliximab, reduced numbers of synovial CD3 (T cells), CD22 (B cells) and CD68 macrophages, 2 weeks after treatment were demonstrated in a separate study.(43) In a study of infliximab versus placebo in which 24 patients with active RA underwent arthroscopy and biopsy before, and 48 hours after infliximab, revealed a significant reduction in CD68 intimal macrophages, as well as a non-statistically significant reduction in CD68 macrophages, T cells and plasma cells in the sublining. (127).

In a large prospective study in 143 RA patients the clinical response to infliximab was predicted by the synovial expression level of TNFα and the number of TNFα producing cells and macrophage subsets.(128) In a follow-up study, the number of lymphocyte aggregates was also predictive of the clinical response.(129) Positivity for lymphocyte aggregates increased the power to predict the clinical response, when analyzed in a prediction model that included baseline disease activity evaluated by the Disease Activity Score in 28 joints, anti-cyclic citrullinated peptide antibody positivity, and synovial TNFα expression.

Anakinara, a specific Il-1 antagonist, has also been shown to significantly reduce the intimal macrophage population.(44) Responders were also demonstrated to have a significant decrease in the intimal lining of CD68 macrophages at both 4 weeks and 16 weeks following rituximab infusion when compared with non-responders.(130)

In a cross sectional study using stepwise multiple regression analysis, it was revealed that these same biomarkers were associated with scores for local disease activity in RA.(26)

A double-blind, randomised, placebo controlled trial, including 16 patients with active RA, examined synovial biopsies on days 1 and 15 of treatment with an oral CCR-1 antagonist or placebo. The 12 patients who received the CCR-1 antagonist had significantly fewer CD68 cells both overall and in the intimal lining, as well as significantly fewer CCR1 positive cells when compared with the four controls after 14 days treatment. The changes in overall cellularity as well as in CD4, CD8, CD22 (B-cell), CD55 (fibroblast-like synoviocyte) and CD138 (plasma cell) populations were not statistically significant. In this (small) study, none of the placebo group met the ACR20 criteria for response while 33% of the treated group did.(131) Furthermore, a study of a CCL-2/MCP-1 monoclonal antibody antagonist demonstrated no change in CD86

sublining macrophages, and this correlated with no change in disease activity.(51) The synovial cell infiltrate was not affected by a C5aR antagonist, and there was no improvement in disease activity.(132)

Synovial response to rituximab has also been assessed by serial biopsy. A series of 6 patients with seropositive RA who had synovial biopsy before treatment with rituximab, and then 4 were re-biopsied after 8 weeks has been reported. The authors assert that although it is known that rituximab depletes circulating B cells, as well as B cells in salivary glands, little is known of its effect on synovial tissue. 4/6 agreed to follow up biopsy. 2 had complete depletion of CD20, 1 had no change and 1 biopsy was insufficient to analyse.(133)

In other studies rituximab induced a similar variable depletion in synovial B cells with an indirect decrease in macrophages, T cells and plasma cells at the time of the clinical response.(80, 81, 130) Of interest, the change in plasma cells was associated with the clinical response.(81, 130)

A study designed to determine if the correlation between the change in number of sublining CD68 cells and the change in DAS28 could be confirmed at more than one centre demonstrated excellent inter-centre agreement.(134) It has been shown that the quantity of CD68 macrophages decreases with a reduction in disease activity as measured by the DAS, thus demonstrating that CD68 numbers could be used as a biomarker of therapeutic response.(135)

Furthermore, CD68 expression is superior to clinical evaluation as it is less susceptible to both the placebo effect and expectation bias (59% of delegates at OMERACT agreed). It could therefore be used to assess the therapeutic efficacy of novel treatments.(134) In conclusion, CD68 expression presents itself as a clear marker of activity above other cell populations in RA.

A number of studies addressed if markers of synovitis are associated with clinical phenotype or development of a persistent, erosive disease course. In two large studies in established RA, large lymphocyte aggregates were found in around 30% of patients but did not associate with a clinical phenotype.(136, 137) In early arthritis, the presence of lymphocyte aggregates did not predict an aggressive disease course and aggregates were rapidly diminished by several anti-rheumatic treatments.(129, 130, 138) Taken together, these studies suggest that lymphocyte aggregates are a pro-inflammatory phenomenon and not a persistent primary driver of synovitis. In contrast, as described above, biomarkers of angiogenesis, namely the activation of the tyrosine kinase receptor TIE-2, predicted an aggressive disease course in early arthritis patients.(104)

## 3.3 Cytokines

The increased expression of several cytokines in the inflamed synovial tissue is well established, and for TNFα and IL-6 concentrations correlation with disease activity,

independent of disease duration, has been demonstrated.(26) The levels of CCL-2/MCP-1 were also found to be increased in RA serum and synovial tissue.(139)

With regards to treatment effect, the expression of IL-1ß and TNF was 40% (95% CI 18–56%) and 52% (95% CI 10–74%) respectively lower following prednisolone therapy compared with placebo. Notably, this effect was mainly attributable to changes in the synovial sublining, and appeared to correlate with clinical improvement.(123) Significant cytokine expression changes after 12 weeks of gold treatment, in three areas of synovium; lining, perivascular and connective tissue, have also been reported. In the lining layer, IL-1α, IL-1ß, IL-6 were statistically significantly reduced after treatment, and this seems to correlate with clinical response. TNFα was also reduced in all three areas, but did not reach statistical significance in the lining.(124)

TNFα was only slightly reduced in synovial samples after 16 weeks treatment with either methotrexate or leflunomide. IL-1ß was only moderately reduced in the leflunomide-treated patients while reductions in the methotrexate patients were significant, which highlights potential different mechanism of action between DMARDs.(42) In a separate study IL-1ß, but not IL-1α was shown to have a statistically significant reduction in expression after 16 weeks of treatment with methotrexate, and this again seemed to correlate with clinical response.(126)

In biopsy samples of ten active RA patients taken 2 weeks after infliximab, IL-8 and MCP-1 were shown to be reduced in both the lining and sublining, and, despite a downward trend in synovial expression of Groa, RANTES, and MIP-1b this was not significant.(43)

Acute-Serum Amyloid A (A-SAA) production in RA synovial tissue has been demonstrated and shown that this induces angiogenesis, leukocyte recruitment, and chemokine and MMP expression in RA.(140) A–SAA has a significant role in the inflamed joint increasing expression of MMP-1, MMP-3, MMP-13, and MMP/TIMP expression in RA FLS and synovial explants. Furthermore, blockade of its receptor (scavenger receptor class B type 1 (SR-B1)) inhibited MMP-2, MMP-3 and MMP-9 expression. Importantly, A-SAA has the ability to induce TNFα expression in RA synovial explant cultures. The baseline serum A-SAA level but not the ESR or the CRP level correlated with the 28-joint swollen joint count and was independently associated with 1-year radiographic progression. Therefore, A-SAA is a promising biomarker of disease activity both in the synovium and in the serum.(141)

## 3.4 Growth Factors/Adhesion Molecules

ICAM-1 expression was significantly reduced in patients treated with both leflunomide and methotrexate. Notably, a decrease in ICAM-1 was seen in those that responded to leflunomide and to methotrexate, while non-responders did not experience a statistically

significant decrease. VCAM-1 was reduced in both groups, but this difference was significant only in the leflunomide-treated patients.(42)

Another study demonstrated that VCAM-1 and E-selectin both statistically significantly reduced in expression after 16 weeks of treatment with methotrexate, but in this study ICAM-1 did not reach significance.(126) Treatment with infliximab has been shown to reduce VCAM-1 and E-selectin in repeat biopsies taken 4 weeks after treatment.(40)

In relation to treatment with anakinra, a patient taking the dose of 150mg/day, was shown to have a 74% reduction in synovial membrane E-selectin, but the 5 patients taking the lower dose (30mg/day) did not demonstrate this. No change was seen in P-selectin. A significant decrease in ICAM-1 and VCAM-1 was seen in the high dose patients, and a small decrease in 2 of the 5 on the lower dose of anakinara.(44)

## 3.5 Mediators and Products of Bone, Cartilage and Tissue Degradation

Type II collagen is the main collagen of articular cartilage, and is excessively degraded in RA. It is known that collagen biomarker and matrix metalloproteinases (MMP) levels predict radiographic progression of RA, and therefore may act as a prognostic biomarker.(142-144) A study with the primary objective of attempting to understand more about the mechanism of action of methotrexate (19 subjects) and leflunomide (16 subjects) demonstrated that MMP-1 was significantly reduced by both the leflunomide and the methotrexate. The level of TIMP-1 was significantly reduced in the leflunomide-treated patients, but not the methotrexate-treated patients. Furthermore, both leflunomide and methotrexate reduced the overall expression of MMP-1 and the MMP-1: TIMP-1 ratio after 4 months of treatment. The changes were more pronounced in patients who fulfilled the ACR 20% response criteria.(42)

A number of studies analyzed the effect of immunomodulatory treatment on synovial mediators of bone destruction. Treatment with both infliximab and etanercept increased the expression of osteoprotegerin (OPG) in synovial tissue and had no effect on RANKL, resulting in an increased OPG:RANKL ratio.(50) In contrast, rituximab induced a 99% decrease in receptor activator of nuclear factor κB (RANK)-positive osteoclast precursors and a decrease of 37% in RANKL and a trend towards reduced synovial OPG expression. In serum, both OPG and RANKL levels were significantly reduced, but the OPG/RANKL ratio increased (157%).(88) Finally, abatacept did not exert a significant effect on synovial OPG, RANK or RANKL mRNA expression in a study in 16 patients.(82)

The family of S100 proteins are a closely related group of low-molecular weight (9–14 kDa) acidic calcium-binding proteins. Originally described in oesophageal epithelium, as well as neutrophils and macrophages, they are involved in calcium dependent cell activities such as

cytoskeleton regulation and cell migration and adhesion. The extracellular role of these proteins is of interest as they have been found to be overexpressed in inflammatory compartments. They are in effect pro-inflammatory cytokines. S100A12 has important activities in relation to innate and acquired immune responses.(145) While a study did demonstrate that S100A12 levels in SF (but not serum) were independently associated with measures of severity of OA(146), there has been a correlation demonstrated, using quantitative proteomics, between the severity of joint erosion in RA and the S100 proteins A8, A9 and A12 levels.(113)

## 3.6 Antigens and Antibodies

Expression analysis of five important proteins: vimentin, gelsolin, alpha 2 HS glycoprotein (AHSG), glial fibrillary acidic protein (GFAP), and a1Bglycoprotein (A1BG) by Western blot analysis, using their specific antibodies, revealed a higher expression in RA synovial fluid as compared to non-RA samples. This study aimed at identifying novel autoantigens involved in RA pathogenesis using an immune-proteomics strategy where antigens from RA SF were correlated with DAS score and clinical demographic characteristics. Recombinant proteins from 2 autoantigens (GFAP and A1GP), were further analysed to assess their utility for clinical diagnosis by serum sampling. For both GFAP and A1GP, the group observed an increase in autoantibodies in serum that were significantly higher than controls.(68)

Other than rheumatoid factors and anti-CCPs, several other autoantibodies have been described in RA. Examples include antibodies against heat shock proteins (Hsp65, Hsp90, DnaJ), immunoglobulin binding protein (BiP), heterogenous nuclear RNPs, annexin V, calpastatin, type II collagen, glucose 6-phosphate isomerase, elongation factor human cartilage gp39(92), and mammose binding lectin (MBL).(147)

It is known that some antigens such as such as citrullinated vimentin, type II collagen, fibrinogen and alpha enolase generate high antibodies in the sera of RA patients. While their presence is higher in SF than serum(113), it is less well characterised in SF, and cannot be used for early diagnosis(148).

Increased levels of neoepitopes are seen in RA. They are formed by collagenase cleavage of type II collagen, type 1 collagen and type II procollagen, and may be of assistance in securing diagnosis.(144)

## 3.7 Genes and Transcripts

In a study in 18 RA patients treated with infliximab several biological processes, related to inflammation, were up-regulated in patients who responded to therapy in pre-treatment

synovial tissue biopsies.(149) In a larger follow-up study, Lindberg *et al* reported the results of RNA analysis of synovial biopsies of 62 patients with RA before treatment with infliximab. They found that the presence of lymphocyte aggregates dominated the expression profiles and that there was a significant overrepresentation of lymphocyte aggregates in patients who had a good response which confounded the analysis. Although, in those that were lymphocyte aggregate positive, 38 transcripts were associated with differences between good and non-responders. (150)

In a study of paired synovial biopsies (n=8) of RA patients, before and 12 weeks after adalimumab, 632 genes were found to be differentially expressed in the 6 responders between biopsies. These genes could be split onto two distinct families: genes involved in the regulation of immune responses and genes involved in the regulation of cell division. To confirm the microarray findings, synovial expression of selected molecules was assessed using specific antibodies. Synovial expression of IL-7R, CXCL11, IL-18, IL-18rap, and MKI67 was significantly higher in poor as compared with moderate and good responders, thereby serving as a potential biomarker of response to adalimumab.(151)

In another study of paired synovial biopsies of 20 RA patients before and 12 weeks after rituximab treatment clinical responders demonstrated higher expression of macrophage and T cell genes, while clinical poor responders showed higher expression of interferon-α and remodelling genes (109). In a study in 16 RA patients treated with abatacept gene expression analysis by quantitative PCR of selected cytokines and mediators of tissue and bone degradation only revealed a significant decrease in interferon γ. This may reflect a limited change in these mRNA transcripts after abatacept in the context of a limited number of patients in this study.(82)

## 3.8 Other

The properties of the cells in the inflamed synovium differ markedly from normal cells Profound hypoxia in the inflamed synovial membrane has been described *in vivo*.(27, 31) Low tissue partial oxygen pressure (tPO2) levels in the inflamed synovial joint tissue are significantly associated with increased markers of macroscopic and microscopic inflammation. There is an association of tPO2 with macroscopic synovitis as well as CD68 and CD3 cell infiltrate in the sublining, and various pro-inflammatory cytokines (TNFα, IL1β, IFNγ and the chemokine MIP3α). When primary synovial fluid cells (SFCs) were exposed *in vitro* to pO2 levels, similar to those in the inflamed joint, there was a significant increase in cell migration. (152)

# Conclusion and Current Limitations

Much of the work on serial synovial biopsies has been performed on patients with known diagnoses, and has been done to investigate responses to treatment. There remains a critical need for identifying biomarkers for diagnosis which can be applied in clinical practice.

Biomarkers may also make it possible to decrease the time it takes and the number of patients required to screen for the potential efficacy of new drugs.(53, 153) The number of patients with active disease eligible to participate in studies is limited. As with all trials, the number of patients who are to be put at risk by exposure to drugs at an early stage of development, as well as to be placed on placebo, are restricted by ethical considerations.(154)

Although finding biomarkers in peripheral blood is attractive because it is more feasible and less invasive than synovial biopsy, since inflamed synovium is the ultimate target of inflammation, it should be a potentially rich source of potential biomarkers.(49) Furthermore, many confounding factors might interfere with peripheral blood profiles. Some authorities have suggested that a more targeted approach to searching for serum markers should be to first identify potential biomarkers in the inflamed synovial joint, and later studying the plasma for the presence of the same biomarker.(10) Such an approach in RA patients has demonstrated clinical utility (Dennis G ART 2014) when candidate peripheral biomarkers of synovial pathotype predicted response to biologic therapy.

Although new technology has enabled faster and more complete analyses of proteins, because of the high complexity in protein and protein isoforms in the synovial joint, interpreting the results of 'shotgun' proteomics is a challenging endeavour.(14)

New technologies are experiencing difficulties. Expression levels from three widely used microarray platforms demonstrated poor reproducibility.(155) In addition, as there are high levels of background signals in array datasets, there is a decreased sensitivity to transcripts present in low numbers.(156)

The development of high quality immunoanalytical assays can be slow and expensive. This makes the verification of candidate biomarkers a challenging process, and at the moment, despite the increase in availability of means to biopsy synovial tissue, there remains a lack of diagnostic makers.(14)

While there are a great many genomic biomarkers that can predict response to treatment, or those at most risk of adverse events in many areas of medicine, rheumatology appears to have experienced only limited benefit from this emerging field. Many studies have attempted to identify biomarkers to predict response to anti-TNF treatment, but to our knowledge, only the three reported above have used synovial tissue to search for these.(128, 150, 151)

In addition, where data from control synovial tissue specimens is available, osteoarthritis (OA) is often used as controls, although it is increasingly recognised that OA has an underlying inflammatory response, even if more limited and less associated with specific autoimmunity than RA.

It is very much more likely that in the near future a reasonable goal will be to stratify disease before the phenotype is established, and this represents an early step toward eventual 'personalised medicine'. Identifying surrogate markers is therefore an aim for which synovial tissue is an indispensable research tool.

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