UNIVERSITEIT GENT

FACULTEIT FARMACEUTISCHE WETENSCHAPPEN

Proteomics: A close encounter with rheumatology

Een ontmoeting tussen proteomics en reumatologie

Kelly Tilleman

November 2006

Ghent University

Faculty of Pharmaceutical Sciences

Laboratory for Pharmaceutical Biotechnology

Promotoren: Prof. D. Deforce – Prof. D. Elewaut

Thesis submitted in fulfilment of the requirements for the degree of Doctor in Pharmaceutical Sciences
Proefschrift voorgelegd tot het bekomen van de graad van Doctor in de Farmaceutische Wetenschappen

Opgedragen aan Maria Van Houtte,
M'n klein bonbontje
die content was
met een appelflap
en een tas koffie

Most of the luxuries, and many of the so-called comforts of life, are not only not indispensable, but positive hindrances to the elevation of mankind. With respect to luxuries and comforts, the wisest have even lived a more simple and meagre life than the poor.

(H.D. Thoreau)

TABLE OF CONTENTS

ABBREVIATIONS

2-DE	two-dimensional gel electrophoresis
α-MEM	alpha-minimum essential medium
anti-CCP	antibodies against citrulline containing proteins
ACR	American College of Rheumatology
ACPA	antibodies against citrullinated proteins
AMC	anti-modified citrulline
AS	akylosing spondylitis
CAPS	3-(cyclohexylamino)-1-propanesulfonic acid
CCL2	monocyte chemotactic protein-1 (MCP-1)
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate
CD44	hyaluronan receptor
Cp	ceruloplasmin
Ct	cycle threshold
DAF	decay accelerating factor
DIGE	difference gel electrophoresis
DTT	dithiothreitol
DMARD	disease-modifying anti-rheumatic drug
DMEM	Dulbecco's modified eagle's medium
ESI	electrospray ionization
FBS	fetal bovine serum
FGF	fibroblast growing factor
FLS	fibroblast-like synoviocytes
HA	hyaluronan
HIF	hypoxia inducible factor
HLA	human leukocyte antigen
HRE	hypoxia regulating element
HC gp-39	human cartilage glycoprotein 39
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ICAT	isotope-coded affinity tag
IEF	isoelectric focussing
Ig	immunoglobin
IL	interleukin
INF-β	interferon beta
INT x mm	intensity x millimeter
IPG	immobilized pH gradient
LC	liquid chromatography
MAP kinase	mitogen activated protein kinase
MALDI	matrix assisted laser desorption ionization
MLS	macrophage-like synoviocytes
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
MRP-8	migration inhibitory factor-related protein-8 = calgranulin A
MS	mass spectrometry
MSMS	tandem mass spectrometry
MW	Molecular weight
NF-κB	nuclear factor kappa B
NK	natural killer
NSAID	non-steroidal anti-inflammatory drug

OA	osteoarthritis
OPG	osteoprotegerin
PAD	peptidylarginine deiminase
PDGF	platelet derived growth factor
pI	isoelectric point
pO_2	partial oxygen pressure
PsA	psoriatic arthritis
PMF	peptide mass fingerprinting
PTM	post-translational modifications
Q	quadrupole
RA	rheumatoid arthritis
RANKL	receptor activator of NF-κB ligand
ReA	reactive arthritis
RFLF	rheumafactor latex fixation
SCID	severe combined immunodeficient
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SE	shared epitope
SpA	spondyloarthropathy
TNF-α	tumor necrosis factor alpha
TM	tetrathiomolybdate
TOF	time-of-flight
TPI	triosephosphate isomerise
U/µg	unit per microgram
UDPGD	uridine diphosphoglucose dehydrogenase
USpA	undifferentiated spondyloarthropathy
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

General introduction

Part I:

Rheumatoid arthritis and Spondyloarthropathy: two frequent forms of inflammatory arthritis

Kelly Tilleman

ABSTRACT

Rheumatoid arthritis (RA) and spondyloarthropathies (SpA) are two frequent forms of chronic arthritis. During these pathologies, similar joints can be affected; however, the clinical presentation of these inflammatory arthritides is very different. RA is characterized by symmetric polyarticular joint inflammation and destruction as well as by extra-articular manifestations such as rheumatoid nodules or vasculitis. The hallmark symptoms of SpA, on the contrary, are quite distinct. They include sacroiliitis, spondylitis, pauciarticular synovitis and enthesitis. Extra-articular manifestations such as uveitis or subclinical bowel inflammation may also occur in SpA. There are several entities that belong to the concept of SpA such as ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA) and undifferentiated SpA (USpA).

Despite these different clinical presentations a major-shared feature between both diseases is the presence of synovitis, the chronic inflammation of the synovial tissue.

This overview is intended to explain the process of synovitis. It guides the reader through the different characteristics of the normal synovium and gives a representation of the successive steps that this tissue undergoes upon inflammation. Additionally, a summary of the known histopathological synovial differences between RA and SpA will be discussed.

INTRODUCTION

Arthritis actually means joint (~arthro) inflammation (~itis). When elderly people experience pain in the joints, they often refer to it as being arthritis. In reality, there are many different types of rheumatic diseases which are not only related to the joint, but which can also affect kidneys, lungs, eyes, the skin and the central nervous system, not only in elderly persons but in people of all ages. They can largely be divided into non-inflammatory and inflammatory pathologies.

For a better understanding of the pathology of these two forms of arthritis, it is of interest to first take a good look at the organ that is most affected by arthritis; the joint.

A joint, or articulation, is the place where two bones come together. There are three types of joints depending on the amount of movement that they allow; immovable joints or synarthroses, slightly movable joints or amphiarthroses and the freely movable joints or diarthroses. This last group is subject to arthritis and is also called synovial joints [1].

THE SYNOVIAL JOINT

The synovial joint can be seen as a capsule that covers the articulating surfaces (fig. 1). The end of each bone is made up of smooth articular cartilage making the movements of the joint as lithe as possible. The joint cavity is filled with an aqueous solution of hyaluronan (HA) which serves as a lubricant of the articular surfaces during movement. It also functions as a

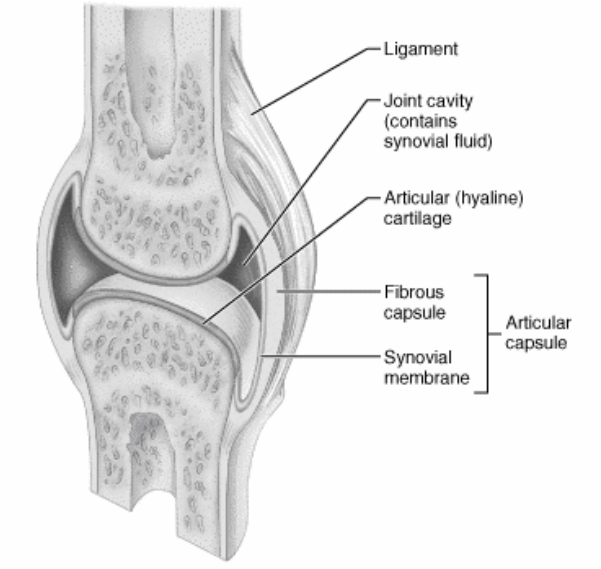


Figure 1: A simplified representation of the synovial joint (obtained from http://www.med.howard.edu; copyright © 2004 Pearson Education Inc. publishing as Benjamin Cummings).

carrier of nutrients to the chondrocytes or cartilage cells and transports waste products away from the cartilage. In pathological conditions, this synovial fluid is actually an ultra filtrate of plasma with the addition of locally synthesized HA. The regulation of the amount of synovial fluid in the joint cavity and its content is regulated by the synovial membrane [1].

The synovial membrane can be identified macroscopically as a connective tissue layer lining the joint cavity [2]. Normal synovial tissue is composed of two distinct layers: a surface layer (intima or synovial lining) and an underlying layer (subintima) (fig. 2) [3, 4]. The intima, in direct contact with the intra-articular space, is 1 to 3 cell layers deep, loosely organized, does not contain any blood vessels and is not supported by a basement membrane. The subintima consists of a network of loosely organized connective tissue with cells and blood vessels [3].

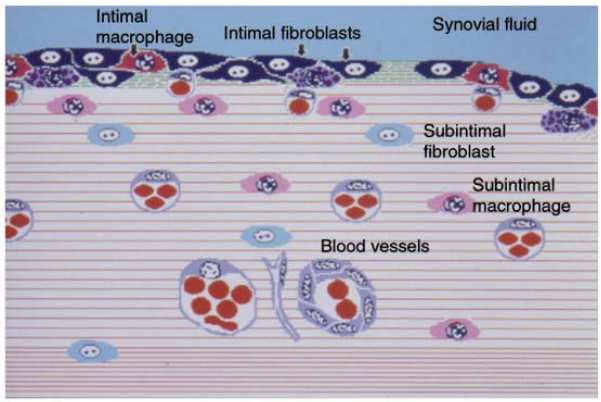


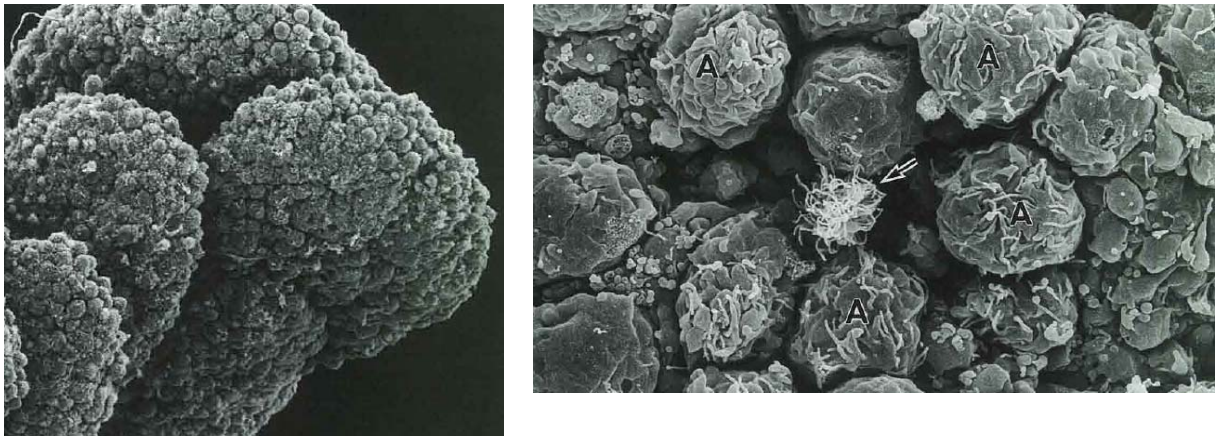
Figure 2: Schematic representation of a normal synovium.
The intima contains specialized fibroblasts expressing vascular cell adhesion molecule-1 (VCAM-1), decay accelerating factor (DAF) and uridine diphosphoglucose dehydrogenase (UDPGD) (dark blue), and the specialized macrophages expressing FcγRIIIa (dark red). The deeper subintima contains relatively unspecialized counterparts (pale colors) (obtained from Edwards, J.C. [4]_© Current Science Ltd).

The intima and subintima are populated with two cell types: cells that are similar to macrophages (type A synoviocytes) and fibroblast-like cells (type B synoviocytes) [5, 6].

Macrophage-like synoviocytes

Macrophage-like synoviocytes (MLS) have been shown to originate from bone-marrow-derived monocytes and they express a number of macrophage markers including CD68, CD14 and CD45 [7]. Type A-synoviocytes can phagocytise cell debris and waste in the joint cavity and have an antigen-presenting role. They show non-specific esterase activity and express the immunoglobin receptor FCγRIIIa [7]. MLS are spherical in shape and densely covered with

filo- and lamellipodia; characteristics unique to macrophages, hence their name (fig. 3) [8]. MLS make up a minority of cells in the normal synovial intima, however, in diseased tissue the proportion of macrophages may rise up to 80% [9].



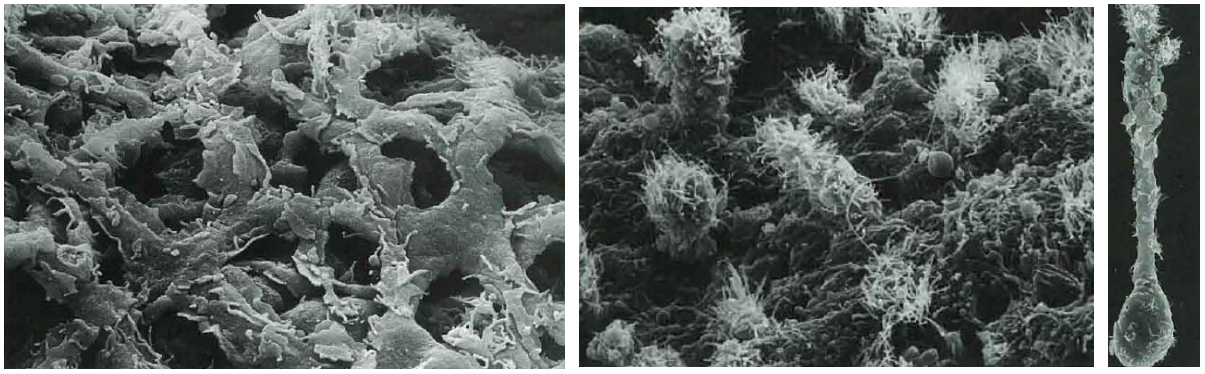
(1) (2)

Figure 3: Scanning electron microscopy of synovial villus in a healthy horse carpal joint.
The round cells are identified as type A synoviocytes (x 430) (1). Higher magnification (x 3800) of the luminal surface at the villus tip (2). The MLS are indicated by (A), the arrow shows long microvilli extending from a B type cell (obtained from Shikichi M. *et al.* [8]).

Fibroblast-like synoviocytes

The majority of native synoviocytes are fibroblast-like cells or FLS. FLS have a rather irregular dendritic shape. Through scanning electron microscopy, type B synoviocytes were visualized containing a round cell body and several long rod-shaped cytoplasmic branches along the surface of the synovial intima (fig. 4.1). These rod-shaped cytoplasmic 'arms' overlapped and crossed each other forming a network. FLS can also be directed apical towards the joint cavity (fig. 4.2). They penetrate the joint space like a kind of antenna covered with long microvilli (fig. 4.3) [8].

The function of intimal FLS can largely be divided into playing a role in joint homeostasis, on the one hand, and interaction with leukocytes, on the other hand [3]. Type B synoviocytes have a high activity of the enzyme Uridine Diphosphoglucose Dehydrogenase (UDPGD) producing and secreting extensive amounts of hyaluronan (HA) into the joint space [10]. HA plays an important role in lubricating the joint. Also lubricin and plasminogen activator are produced by FLS, contributing to the viscosity of the joint space and allowing normal movement by preventing fibrous adhesions in the joint, respectively [11].



(1) (2) (3)

Figure 4: Scanning electron microscopy of FLS in synovial villus in a healthy horse carpal joint.
The type B cells have a dendritic shape and form a network along the luminal surface (x 3900) (1). FLS that extend vertically whose tips contain microvilli (x 2600) (2). An isolated type B-synoviocyte projecting towards the joint cavity (x 1800) (3) (obtained from Shikichi M. *et al*. [8]).

Synovial fibroblasts can interact with leukocytes through Vascular Cell Adhesion Molecule-1 (VCAM-1) and Decay Accelerating Factor (DAF) [12, 13]. VCAM-1 interacts with $\alpha 4\beta 1$ integrin present on mononuclear leukocytes. Since polynuclear leukocytes do not express this molecule, granulocytes are not retained in the synovial intima and are released into the joint space traveling trough the synovial fluid [14].

Expression of DAF is associated with the local macrophage expression of FCγRIIIa through its co-ligand CD97. However, its primary role is regulating a tissue response to the alternative complement pathway through degrading complement on the cell surface [13, 15].

Several other connective tissue components like fibronectin, type IV and type VI collagen, laminin, chondroitin proteoglycans and fibrillin are also secreted by FLS [16]. They participate in the maintenance of the joint capsule.

The fibroblasts in the sublining are less specialized, as they lack the expression of UDPGD, DAF or VCAM-I. However, upon synovial inflammation, type-B synoviocytes resident in the subintima start to obtain the specific characteristics of synovial lining fibroblasts.

SYNOVITIS

Synovitis or inflammation of the synovium, is believed to be the result of a combination of synovial hyperplasia, extensive angiogenesis, infiltration of cells from the circulation and a disturbance in apoptosis (fig. 5) [2].

In inflammatory arthritis, the phenotype of the synovial tissue changes into an activated, proliferative, invasive tissue. This uncontrolled process of tissue growth leads to narrowing of the joint space and subsequent increased efflux of synovial fluid, which results in swollen

joints. This tissue proliferation is accompanied by the production of certain destructive enzymes, eventually leading to cartilage and bone loss, especially in RA. As for SpA, the synovitis results in less joint destruction, it is characterized by bone formation in addition to bone erosions [17].

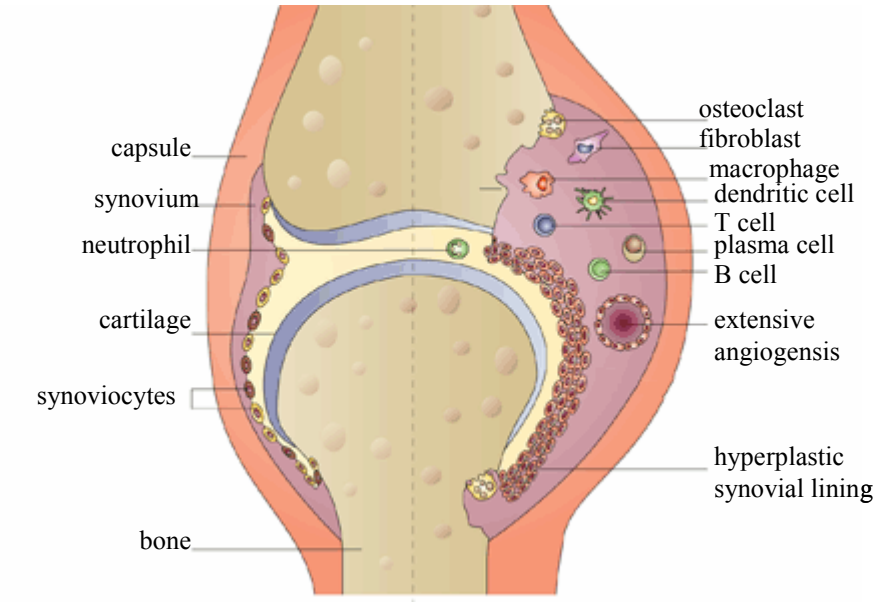


Figure 5: Representation of normal synovial tissue (left) and synovitis (right) (obtained from http://www.rheumatoid-arthritis.net).

Histological, synovitis is characterized by the thickening of the synovial lining, often called synovial hyperplasia, which can result in a cell depth of more than 8 cells [18]. This proliferation is probably the result of infiltration of circulating macrophages and monocytes and is also driven by local proliferation of synoviocytes by catabolic cytokines. The thickened lining cells are comprised, up to 80%, of CD68 positive macrophages [9, 18]. These macrophages produce Tumor Necrosis Factor-α (TNF-α), interleukin (IL)-1 and IL-6, which activate the FLS and stimulate them to proliferate *in vitro* [19] .

Impairment of apoptosis of the type B synoviocytes also contributes to the thickening of the lining [20, 21]. Apoptosis, or programmed cell death, is tightly regulated and ensures the elimination of cells from tissue. A wide variety of so-called death signals exists, and a lower expression or deregulation of these signals leads to a reduction of apoptosis. Reduction of apoptosis can lead to the accumulation of cells and could, in part, play a role in the hyperplasia of the synovium. Especially, the Fas-mediated apoptosis pathway has been shown to be impaired in arthritis [22]. In addition, mutations in the p53 tumor suppressor gene [23]

and the higher expression in FLS of anti-apoptotic proteins like Bcl-2 [24] and sentrin-1 are responsible for impairement of apoptosis in the synovial tissue. Sentrin-1, also called SUMO-1, is a small ubiquitin-like modifier that alters the binding of target molecules to subsequent molecules, instead of preparing them for degradation as ubiquitin does. In its specialized way, it 'sumolates' the correct form of Fas associated death domain and hereby interferes with Fas-associated apoptosis in FLS [25]. Recently, synoviolin, an E3 ubiquitin ligase, has been found to be over expressed in RA synovium and this enzyme exerts both anti-appototic and pro-proliferative characteristics. It is considered very important in the process leading to synovial hyperplasia as mice expressing lower amount of synoviolin, are free of synovial lining thickening due to enhanced apoptosis of synovial cells [26, 27].

The thickened synovium requires nutrients and adequate oxygenation and because of the synovial hyperplasia, the distance between the synovial lining cells and the blood vessels, present in the sublining, increases. These alterations in the microenvironment of the joint, lead to regional hypoxia, which itself is a strong trigger for angiogenesis, the formation of new blood vessels [28]. The principal regulator of gene expression in a hypoxic environment is Hypoxia Inducible Factor (HIF) [29]. This transcription factor is stabilized in hypoxic areas and binds to Hypoxic Regulating Elements (HRE) in the promoter region of certain genes, hereby regulating gene expression. In addition, pro-angiogenic factors are produced locally by the FLS and by the infiltrating macrophages. These include Vascular Endothelial Growth Factor (VEGF), Fibroblast Growing Factor (FGF), Platelet Derived Growth Factor (PDGF) and IL-8 [30, 31]. Angiogenesis can be visualized upon microscopic investigation of the synovial tissue and is observed as a fine network of vessels [32]. Vascularization does not only permit the supply of nutrients to the augmented cell mass, but it also promotes leukocyte infiltration and thereby stimulates the continuation of the synovial inflammation [31, 32].

Inflamed synovial tissue is also characterized by the infiltration of leukocytes, mainly lymphocytes and monocytes. Infiltrating lymphocytes comprising mainly of $CD4^+$ memory T cells, also small amounts of $CD8^+$ T cells, NK cells and B cells are organized in three kinds of synovial infiltration patterns: diffuse; where there is a lack of organisation (~50% of synovia), perivascular; around the endothelial venules (~20%) and organized in lymphoid follicles with or without germinal centres (~20%) [33].
Infiltration of macrophages is also observed in the process of synovitis [7, 34]. These CD68+ monocytes, produce increased amounts of pro-inflammatory cytokines like TNF-α and IL-1

[7] and there is a correlation of the quantity of macrophage infiltrates and the degree of joint damage [35]. Moreover, they are present in the synovium prior to clinical synovitis [36, 37].

Although inflammatory cells play an important role in synovitis, the persistence of the inflammation and the destructive feature of the synovitis is largely attributed to FLS [38-40].
Type B synoviocytes can produce a variety of pro-inflammatory cytokines, adhesion molecules and chemokines such as IL-6, INF-β, CCL2, IL-8, which attract and retain large numbers of leukocytes in the synovial tissue [41-43]. Studies indicate that the NF-κB transcription factor is of high importance in this so-called stromal cell contribution to the inflammation [44].
Moreover, the pronounced role of FLS in the detrimental outcome of the synovitis was demonstrated when activated inflamed synovial tissue was implanted in Severe Combined Immunodeficient (SCID) mice. Within these implants, lymphocytes infiltrates disappeared, however, the synovial lining cells survived and their biological characteristics were maintained [45]. In the SCID mouse model, implanted activated fibroblasts were able to destroy cartilage and bone [46]. Studies have shown that Matrix Metalloproteinases (MMPs), which are secreted by FLS, are responsible for eroding the cartilage in the joint [47]. The expression of these zinc-dependent proteases is regulated by a variety of mechanisms including cell-cell interactions [48], upon stimulation by adhesion molecules and cytokines [49], MAP kinases have been reported to stimulate the secretion of MMPs [50] and the hyaluronan receptor (CD44) is apparently also implicated in the joint destruction by inducing the secretion of MMPs [51].
Other studies also indicate that the chondrocytes, the cellular components of the cartilage, participate in the joint destructive process. They do not only respond to the catabolic cytokines released from the synoviocytes, but they contribute to the pathology by being a source of cytokines themselves [52, 53].
It has been speculated that synovial fibroblasts are mesenchymal stem cell look-a-likes. This could allow these cells to take on certain characteristics of other cell types that are of mesenchymal origin like osteoclasts, myocytes, chondroblasts and adipocytes, which are residents of the joint space [54]. This could in part explain the diversity of functional properties that these synoviocytes posses upon activation and this could contribute to their ability to proliferate themselves, activate and hold on to cell infiltrates and eventually transform into a devastating population of cells.

The different stages in synovitis are difficult to describe, because it is still largely unknown what the correct order of the different steps involved are. Also, the characterization of the different players taking part in the inflammatory process and especially who stimulates who and maybe more importantly when, is still not fully elucidated. Further studies will undoubtedly reveal more details of this complex interplay of cells leading to such a dramatic phenotypic change inside the joint tissue.

NON-INFLAMMATORY JOINT DISORDERS

Non-inflammatory rheumatic pathologies also known as osteoarthritis (OA), are often referred to as degenerative arthritis or degenerative joint disease, and are viewed as an age-related dynamic reaction pattern of a joint in response to insult or injury.

The pathology is diverse and characterized by progressive destruction of the articular cartilage eventually affecting the entire joint. The most striking feature is loss of articular cartilage and changes in the adjacent bone [55]. These changes, consisting of bone growth at the joint margins, are called osteophytes and may be the result of an attempted repair process in the affected joint [56].

Although severe injury can be sufficient to cause OA, there are many risk factors both systemic (age, sex, bone density, ….) and local (obesity, sport participation, muscle weakness…) that contribute to the severity of the pathology [57]. Osteoarthritis is the most common form of joint diseases worldwide e.g. OA of the knee occurs in 1% among people aged 25-34 (based on radiographic information) and mounts up to 80% in those aged 75 and above [58]. Since the prevalence of this degenerative joint disease increases with age, its occurrence will become even more dramatic in the future because of the aging population, with high socio-economic impact on our society.

OA is not considered a classical inflammatory arthropathy, due to the absence of neutrophils in the synovial fluid and systemic manifestations of inflammation. The primary target in OA is the articular cartilage, in contrast with inflammatory joint disorder where the synovial tissue is primary involved. Studies indicate the participation of the chondrocytes, the cellular component of the articular cartilage, in the joint destructive process by the production of catabolic cytokines, like IL-1, and destructive enzymes like MMP's [59].

We included OA synovial tissue samples as non-inflammatory controls throughout this study.

INFLAMMATORY JOINT DISORDERS

Rheumatoid Arthritis (RA) and Spondyloarthropathy (SpA) are two frequent forms of chronic inflammatory arthritis. The prevalence of both pathologies is similar. For RA, the prevalence worldwide is around 1-2%, as for SpA 1 up to 2.5% of the population worldwide is affected [60, 61]. Both inflammatory arthritides are multi-factorial diseases that occur in genetically predisposed persons. Although they have a different clinical presentation, they both are characterized by synovitis (fig. 6).

Rheumatoid arthritis		**Spondyloarthropathy**
1-2%	*Prevalence*	1-2.5%
1:3	*Male:Female ratio*	3:1 (AS)
	Clinical manifestations	
Peripheral Symmetric Polyarthritis Prominent in hands Prominent in feet Rows of joint involved		Axial and/or peripheral Asymmetric Oligoarthritis Moslty weight-bearing joint, lower limbs Dactylitis (‘sausage digits’) Enthesitis
	Extra- articular manifestations	
Rheumatoid nodules (picture) Serositis Vasculitis 		Psoriasis Mucositis No vasculitis Uveitis (picture)  Inflammatory bowel disease
	Radiology	
Joint space narrowing Erosions		Sacroiliitis Bone formation and spinal ankylosis (bamboo spine) Erosions
	Genetic association	
HLA-DR – shared epitope		HLA-B27

Figure 6: Characteristics of the two frequent forms of inflammatory arthritis; RA and SpA.

Rheumatoid arthritis

Rheumatoid arthritis is a symmetric polyarticular inflammatory arthritis that affects the small diarthrodial joints. The primary site of inflammation is the synovial membrane. As described

above, this inflammation is accompanied by infiltration of lymphocytes that produce pro-inflammatory mediators that attract other immune cells to the site. This process activates resident cells to proliferate. In addition, an excess of synovial fluid is produced which eventually results in a swollen, inflamed joint. Because of immunological disturbances in the joint, there is a perpetuation of the inflammation which results in the chronic nature of the joint inflammation in RA. Destructive enzymes are produced in the joint that lead to cartilage destruction. Activated synovial tissue transforms, invades and destroys the bone. Severe bone erosions can be seen radiographically and severe joint deformations can be the result of longstanding RA (fig. 7).

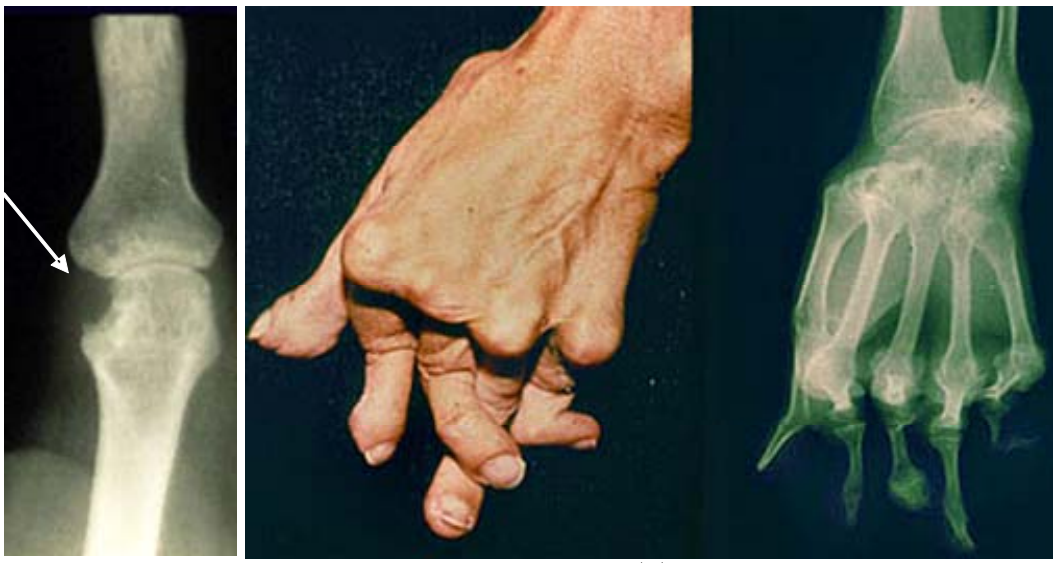


Figure 7: Joint erosions and deformations in RA.
Erosion seen in X-ray, occur rapidly in the disease event (A), visible changes in joint architecture resulting in joint deformation with function loss associated with severe RA (B) (Obtained form John Hopkins arthritis center :http://www.hopkins-arthritis.som.jhmi.edu/; Clinical slide collection on the rheumatic diseases © 1991, 1995, 1997).

Symmetric joint swelling and inflammation are hallmarks in RA. In addition, extra-articular manifestations can occur of which the rheumatoid nodules are the most well known (fig. 6).
The most common serological marker for RA is the presence of rheumatoid factor. This factor is an immunoglobin (Ig) M antibody response directed against the Fc portion of the IgG; forming complexes [62, 63]. However, this antibody is not very specific for RA (present in 70-75% of patients with RA), and can be found in many inflammatory diseases and in healthy elderly individuals [64].

Recently, antibodies directed against citrulline containing proteins (anti-CCP) have gained much attention [65, 66]. During citrullination of proteins, arginine residues are converted into citrulline residues (fig. 8). The enzyme responsible for this conversion is peptidylarginine deiminase (PAD). Anti-CCP antibodies are highly specific for RA and can be detected very early in the course of the disease, even before clinical onset [67, 68]. With a very sensitive assay called CCP2, anti-citrullinated protein antibodies can be detected in almost 80% of RA sera with a specificity of 98% [69-71]. Anti-CCP producing plasma cells have been shown in the inflamed synovial RA tissue [72] suggesting the presence of citrullinated proteins in the synovial membrane causing an antigen driven maturation of CCP-specific B cells at the site of inflammation.

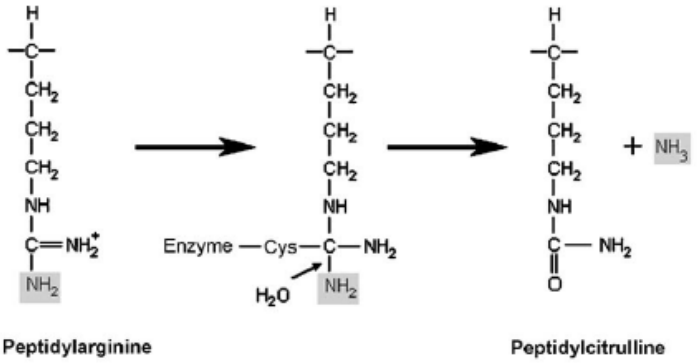


Figure 8: The citrullination process involves enzymatic conversion from arginine to citrulline. The enzyme that catalyzes this reaction is peptidylarginine deiminase (PAD). During the reaction, the arginine is attacked by the Cys residue of the enzyme establishing a tetrahedral adduct while ammonia is released. The adduct is then cleaved by the nucleophilic attack of a water molecule that regenerates the Cys residue and forms the keto-group (obtained from van György B. *et al*. [73]).

Indeed, the presence of citrullinated proteins, like fibrinogen α and fibrinogen β, have been shown in the RA synovium [74]. However, these forms of fibrin are not specific for RA but are also observed in non-RA synovium [75]. Another citrullinated protein associated with RA is citrullinated vimentin, better known as the Sa-antigen. However, information of the presence of this form of vimentin *in vivo* in the synovial tissue is limited [76]. It is still largely unknown, what the exact role is of protein citrullination in arthritis, as PAD expression and citrullination of proteins are found in other tissue and pathologies [77, 78]. This process of arginine transformation could be a more universal characteristic of inflammation, although very little is known about the function of protein citrullination in general.

Recently, there have been reports that show a link between protein citrullination and the genetic predisposition of RA patients.

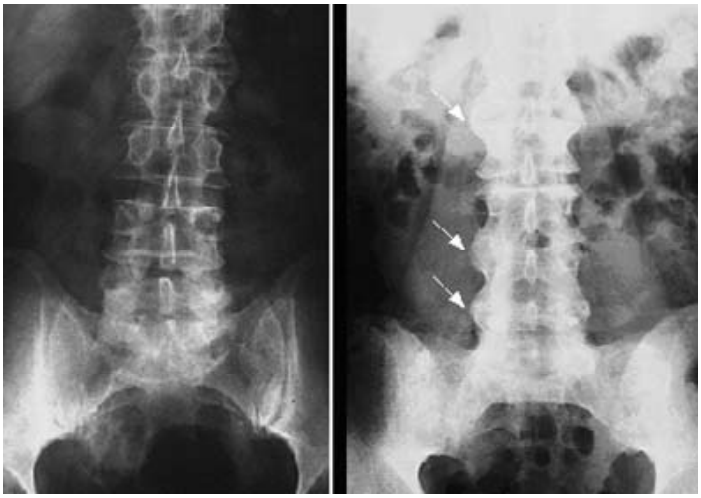
The strongest genetic link with RA is its relationship with the specific human leukocyte (HLA)-DR genes. Already in the late seventies, Stastny found that 70% of white patients with RA, compared to 28% of patients with non-RA, expressed HLA-DR4 [79]. HLA-DR4 genes are part of the MHC class II molecules that contain the shared epitope (SE). This SE is a conserved amino acid motif (QK/RAAA) found within the third variable region of the DRβ chains of DRB1*0101, DRB1*0404 and DRB1*0401 [80]. This SE hypothesis postulates that the SE motif is directly involved in the pathogenesis of RA by allowing the presentation of an arthritogenic peptide to T cells [81]. It has been shown that the peptide-MHC interaction of peptides containing citrulline in stead of arginine is dramatically increased and leads to the activation of $CD4^+$ T cells in HLA-DRB1*0401 transgenic mice [82]. A recent study of van der Helm-van Mil A. H. *et al*. showed that SE alleles are primarily associated with the presence of anti-CCP antibodies [83]. These recent findings indicate that SE alleles are not associated with RA as such, but rather with a distinct phenotype of the disease, namely the anti-CCP-positive RA. Moreover, it suggests that the SE alleles act as a classic immune response gene for the development of anti-CCP antibodies.

Spondyloarthropathy

SpA reflects a group of interrelated and overlapping chronic inflammatory diseases including ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA), arthritis associated with inflammatory bowel disease, and undifferentiated SpA (USpA). SpA is characterized as a chronic inflammatory rheumatoid factor negative arthritis of the spinal and peripheral joints (fig. 9). The observed synovitis is pauciarticular and is frequently associated with inflammation of the entheses. Hallmarks of these types of chronic arthritis are that the destructive properties are simultaneously combined with the formation of new bone. Some groups have therefore termed these types of disorders as an example of destructive and remodeling arthritis. Extra-articular manifestations are observed and include inflammation of gut, skin, and the eye (fig. 7).

The diseases are not associated with rheumatoid factor, but they show a strong association with HLA-B27, an allele of the major histocompatibility complex class I [84, 85]. Misfolding of HLA-B27 molecules, homodimerisation of the heavy chains could lead to a pro-inflammatory unfolded protein response [86, 87]. Also, the activation of T-lymphocytes by presenting bacterial peptides or self-derived peptides could, in part, play a role in the inflammatory nature of SpA. In addition, peptides derived from HLA-B27 itself can be

recognized by $CD4^+$ T cells [88]. The true nature of the underlying mechanism of HLA-B27 susceptibility is not fully understood. The link with HLA-B27 is not absolute, as there in no association of HLA-B27 in African patients with SpA [89], indicating that other genes may also contribute to the disease susceptibility.



(A) (B)

Figure 9: The radiographic hallmark of SpA is the presence of erosions of the sacroiliac joint (A). In patients with advanced and severe SpA, the entire spine becomes fused into a single structure termed the bamboo spine (B) (obtained from UCLA Rheumatology department: http://www.cdaarthritis.com/; Craig W. Wiesenhutter ©2005).

HLA-B27 transgenic rats do not develop SpA in germ-free conditions, which indicate the importance for bacterial infections [90]. Indeed, bacterial strains that infect the gut or the urogenital tract, especially in ReA, can play a role in triggering inflammation. Also, there are indications that the increased intestinal permeability of patients with SpA could lead to the starting or continuing events of chronic inflammation [91, 92].

Synovial histopathology

The study of the synovium is important in understanding the nature of inflammatory synovial diseases. Histological studies have thus far provided insight into the pathofysiology of chronic joint inflammations. The introduction of needle arthroscopy provided a tool with which both visualization and sampling of the synovium became possible. Previous studies concentrated largely on the histological description of RA synovitis. However, as it became clear that the prevalence of SpA could be as high as, or even higher than RA [60], the investigation of SpA

synovium was stimulated. This was partly due to the use of needle arthroscopy which is a safe and well-tolerated technique.

As previously indicated, SpA consists of an interrelated group of inflammatory pathologies. It is difficult to say if there are specific differences in synovial histopathology in SpA subtypes since they were never systematically compared. As the synovial heterogeneity observed in SpA was found to be independent between SpA phenotypes [93], the section below will not discriminate between the different subtypes.

A first major characteristic of synovitis is the hyperplasia of the synovial lining. There is a significant difference in lining-layer thickness between RA and SpA synovitis. SpA is characterized by a lower score in maximum and mean lining thickness measured semi-quantitatively [93, 94]. This difference in hyperplasia could be related to higher infiltration of immune regulatory cells as it has been reported that infiltration of lymphocytes and especially lymphoid aggregation appears to be more prominent in RA than SpA. There is an increase in $CD3^+$, $CD4^+$ and $CD20^+$ T cells in RA although lymphoid aggregates are only seen in about 10% of the SpA synovia [94, 95]. Although the number of $CD68^+$ macrophages is similar between the synovitis of the two forms of inflammatory arthritis, a specific subset of $CD163^+$ macrophages is increased in SpA [93-96]. This scavenger receptor has the unique function of internalizing the haemoglobin/haptoglobin complex upon rupture of red blood cells. This prevents oxidative stress caused by the free heme group. The CD163 receptor, thereby, is considered to play a role in an anti-inflammatory response [97].

Angiogenesis, the formation of new blood vessels has also been indicated as an important feature in the process of synovitis. Apparently, the vascularity is increased in SpA as opposed to RA [93, 95, 98] and the vascular morphology seems to be related to the form of inflammatory arthritis; straight vessels are predominantly seen in RA, whereas tortuous patterns are observed in SpA. These data were confirmed independently in a larger patient cohort containing 100 cases [99].

The proliferative vascularised inflamed synovial tissue in RA eventually transforms into an invasive tissue destroying cartilage and bone by production of matrix metalloproteinases (MMPs). Although the detrimental nature of synovitis is more pronounced in RA, there is no difference in MMPs staining pattern of diseased RA and SpA synovial tissue [100]. Also, similar expression of the tissue inhibitors of MMPs was found. The authors suggested that the destructive progression in SpA and RA was probably not related to the MMP system [100]. In addition to enzymatic destruction of cartilage, the role of osteoclast formation in destruction

of the bone matrix has gained interest [101, 102]. The primary mediator of osteoclast formation is the receptor activator of NF-κB ligand or RANKL and is present both in SpA and RA synovial tissue, although there is on average a lower expression of RANKL in the SpA synovial tissue in comparison to RA [103]. This down regulation in SpA could be explained by the fact that the osteoprotegerin (OPG) which blocks the interaction between RANK and RANKL is significantly more active in SpA than in RA synovium [104]. The authors concluded that a deficiency in OPG could have a role in the pathogenesis of bone erosion which characterizes RA.

It is of interest to notice that there has only been one histopathology study on healthy normal synovium. This study indicated the presence of macrophages and T lymphocytes; T helper and T suppressor/cytotoxic cells in the normal synovium and HLA-DR+ cells were confirmed. The major difference in contrast to diseased synovium is the absence of B lymphocytes [105]. These findings, however, need to be confirmed by other investigators.

Besides the differences indicated above, there are currently only two specific immunohistochemical features that discriminate between RA and SpA. The presence of intracellular citrullinated proteins has been specifically associated with RA [71, 93, 94, 106]. Also the presence of HLA-DR4-positive cells presenting human cartilage glycoprotein 39 (HC gp-39) was found to be highly specific for RA [94, 107, 108].

REFERENCES

[1.] Harris, E., *Rheumatoid arthritis*, W.B. Saunders Company, Philadelphia 1997.

[2.] Tarner, I. H., Harle, P., Muller-Ladner, U., Gay, R. E., *et al.*, *Best Pract Res Clin Rheumatol* 2005, 19, 19-35.

[3.] Firestein, G. S., *Rheumatoid synovitis and pannus*, Mosby, London 1997, pp. 13.11 - 13.15.

[4.] Edwards, J. C., *Arthritis Res* 2000, 2, 344-347.

[5.] Wilkinson, L. S., Pitsillides, A. A., Worrall, J. G. and Edwards, J. C., *Arthritis Rheum* 1992, 35, 1179-1184.

[6.] Barland, P., Novikoff, A. B. and Hamerman, D., *J Cell Biol* 1962, 14, 207-220.

[7.] Athanasou, N. A., *Ann Rheum Dis* 1995, 54, 392-394.

[8.] Shikichi, M., Kitamura, H. P., Yanase, H., Konno, A., *et al.*, *Arch Histol Cytol* 1999, 62, 219-229.

[9.] Edwards, J. C., *Structure of synovial lining*, Chapman & Hall, London 1987, pp. 17-40.

[10.] Yielding, K. L., Tomkins, G. M. and Bunim, J. J., *Science* 1957, 125, 1300.

[11.] Brommer, E. J., Dooijewaard, G., Dijkmans, B. A. and Breedveld, F. C., *Ann Rheum Dis* 1992, 51, 965-968.

[12.] Rice, G. E., Munro, J. M., Corless, C. and Bevilacqua, M. P., *Am J Pathol* 1991, 138, 385-393.

[13.] Medof, M. E., Walter, E. I., Rutgers, J. L., Knowles, D. M., *et al.*, *J Exp Med* 1987, 165, 848-864.

[14.] Muller-Ladner, U., Gay, R. E. and Gay, S., *Structure and function of synoviocytes*, Lippincott-Williams and wilkins, Philidelphis 2001, pp. 243-251.

[15.] Hamann, J., Wishaupt, J. O., van Lier, R. A., Smeets, T. J., *et al.*, *Arthritis Rheum* 1999, 42, 650-658.

[16.] Iwanaga, T., Shikichi, M., Kitamura, H., Yanase, H., *et al.*, *Arch Histol Cytol* 2000, 63, 17-31.

[17.] Baeten, D. and De Keyser, F., *Curr Mol Med* 2004, 4, 1-12.

[18.] Youssef, P. P., Kraan, M., Breedveld, F., Bresnihan, B., *et al.*, *Arthritis Rheum* 1998, 41, 663-669.

[19.] Tak, P. P. and Bresnihan, B., *Arthritis Rheum* 2000, 43, 2619-2633.

[20.] Nishioka, K., Hasunuma, T., Kato, T., Sumida, T., *et al.*, *Arthritis Rheum* 1998, 41, 1-9.

[21.] Baier, A., Meineckel, I., Gay, S. and Pap, T., *Curr Opin Rheumatol* 2003, 15, 274-279.

[22.] Peng, S. L., *Rheumatology (Oxford)* 2006, 45, 26-30.

[23.] Sun, Y. and Cheung, H. S., *Semin Arthritis Rheum* 2002, 31, 299-310.

[24.] Perlman, H., Georganas, C., Pagliari, L. J., Koch, A. E., *et al.*, *J Immunol* 2000, 164, 5227-5235.

[25.] Pap, T., Cinski, A., Baier, A., Gay, S., *et al.*, *Joint Bone Spine* 2003, 70, 477-479.

[26.] Amano, T., Yamasaki, S., Yagishita, N., Tsuchimochi, K., *et al.*, *Genes Dev* 2003, 17, 2436-2449.

[27.] Yamasaki, S., Yagishita, N., Tsuchimochi, K., Nishioka, K., *et al.*, *Arthritis Res Ther* 2005, 7, 181-186.

[28.] FitzGerald, O. and Bresnihan, B., *Ann Rheum Dis* 1995, 54, 511-515.

[29.] Wang, G. L. and Semenza, G. L., *J Biol Chem* 1993, 268, 21513-21518.

[30.] Paleolog, E. M., *Br J Rheumatol* 1996, 35, 917-919.

[31.] Taylor, P. C. and Sivakumar, B., *Curr Opin Rheumatol* 2005, 17, 293-298.

[32.] Hirohata, S. and Sakakibara, J., *Lancet* 1999, 353, 1331.

[33.] Takemura, S., Braun, A., Crowson, C., Kurtin, P. J., *et al.*, *J Immunol* 2001, 167, 1072-1080.

[34.] Sack, U., Stiehl, P. and Geiler, G., *Rheumatol Int* 1994, 13, 181-186.

[35.] Yanni, G., Whelan, A., Feighery, C. and Bresnihan, B., *Ann Rheum Dis* 1994, 53, 39-44.

[36.] Pando, J. A., Duray, P., Yarboro, C., Gourley, M. F., *et al.*, *J Rheumatol* 2000, 27, 1848-1854.

[37.] Soden, M., Rooney, M., Cullen, A., Whelan, A., *et al.*, *Br J Rheumatol* 1989, 28, 287-292.

[38.] Pap, T., Meinecke, I., Muller-Ladner, U. and Gay, S., *Ann Rheum Dis* 2005, 64 Suppl 4, iv52-54.

[39.] Mor, A., Abramson, S. B. and Pillinger, M. H., *Clin Immunol* 2005, 115, 118-128.

[40.] Burman, A., Haworth, O., Bradfield, P., Parsonage, G., *et al.*, *Joint Bone Spine* 2005, 72, 10-16.

[41.] Bombara, M. P., Webb, D. L., Conrad, P., Marlor, C. W., *et al.*, *J Leukoc Biol* 1993, 54, 399-406.

[42.] Smith, R. S., Smith, T. J., Blieden, T. M. and Phipps, R. P., *Am J Pathol* 1997, 151, 317-322.

[43.] Georganas, C., Liu, H., Perlman, H., Hoffmann, A., *et al.*, *J Immunol* 2000, 165, 7199-7206.

[44.] Bondeson, J., Brennan, F., Foxwell, B. and Feldmann, M., *J Rheumatol* 2000, 27, 2078-2089.

[45.] Geiler, T., Kriegsmann, J., Keyszer, G. M., Gay, R. E., *et al.*, *Arthritis Rheum* 1994, 37, 1664-1671.

[46.] Pap, T., Nawrath, M., Heinrich, J., Bosse, M., *et al.*, *Arthritis Rheum* 2004, 50, 2794-2802.

[47.] Pap, T., Claus, A., Ohtsu, S., Hummel, K. M., *et al.*, *Arthritis Res Ther* 2003, 5, R163-173.

[48.] Wernicke, D., Schulze-Westhoff, C., Brauer, R., Petrow, P., *et al.*, *Arthritis Rheum* 2002, 46, 64-74.

[49.] Dayer, J. M. and Burger, D., *Eur Cytokine Netw* 1994, 5, 563-571.

[50.] Han, Z., Boyle, D. L., Chang, L., Bennett, B., *et al.*, *J Clin Invest* 2001, 108, 73-81.

[51.] Tolboom, T. C., Huidekoper, A. L., Kramer, I. M., Pieterman, E., *et al.*, *Clin Exp Rheumatol* 2004, 22, 158-164.

[52.] Goldring, S. R. and Goldring, M. B., *Clin Orthop Relat Res* 2004, S27-36.

[53.] Tetlow, L. C., Adlam, D. J. and Woolley, D. E., *Arthritis Rheum* 2001, 44, 585-594.

[54.] De Bari, C., Dell'Accio, F., Tylzanowski, P. and Luyten, F. P., *Arthritis Rheum* 2001, 44, 1928-1942.

[55.] Schouten, J. S., van den Ouweland, F. A. and Valkenburg, H. A., *Ann Rheum Dis* 1992, 51, 932-937.

[56.] Dieppe, P., *Osteoarthritis Cartilage* 1999, 7, 325-326.

[57.] Felson, D. T., Lawrence, R. C., Dieppe, P. A., Hirsch, R., *et al.*, *Ann Intern Med* 2000, 133, 635-646.

[58.] Arden, N. and Nevitt, M. C., *Best Pract Res Clin Rheumatol* 2006, 20, 3-25.

[59.] Goldring, M. B., *Best Pract Res Clin Rheumatol* 2006, 20, 1003-1025.

[60.] Khan, M. A., *Ann Intern Med* 2002, 136, 896-907.

[61.] Smith, J. B. and Haynes, M. K., *Ann Intern Med* 2002, 136, 908-922.

[62.] Masi, A. T., Maldonado-Cocco, J. A., Kaplan, S. B., Feigenbaum, S. L., *et al.*, *Semin Arthritis Rheum* 1976, 4, 299-326.

[63.] Neumann, E., Barnum, S. R., Tarner, I. H., Echols, J., *et al.*, *Arthritis Rheum* 2002, 46, 934-945.

[64.] Lisse, J. R., *Postgrad Med* 1993, 94, 133-134, 139.

[65.] Schellekens, G. A., de Jong, B. A., van den Hoogen, F. H., van de Putte, L. B., *et al.*, *J Clin Invest* 1998, 101, 273-281.

[66.] Yamada, R., *Autoimmun Rev* 2005, 4, 201-206.

[67.] Nielen, M. M., van Schaardenburg, D., Reesink, H. W., van de Stadt, R. J., *et al.*, *Arthritis Rheum* 2004, 50, 380-386.

[68.] Rantapaa-Dahlqvist, S., de Jong, B. A., Berglin, E., Hallmans, G., *et al.*, *Arthritis Rheum* 2003, 48, 2741-2749.

[69.] van Boekel, M. A., Vossenaar, E. R., van den Hoogen, F. H. and van Venrooij, W. J., *Arthritis Res* 2002, 4, 87-93.

[70.] Bizzaro, N., Mazzanti, G., Tonutti, E., Villalta, D., *et al.*, *Clin Chem* 2001, 47, 1089-1093.

[71.] Zendman, A. J., van Venrooij, W. J. and Pruijn, G. J., *Rheumatology (Oxford)* 2006, 45, 20-25.

[72.] Masson-Bessiere, C., Sebbag, M., Durieux, J. J., Nogueira, L., *et al.*, *Clin Exp Immunol* 2000, 119, 544-552.

[73.] Gyorgy, B., Toth, E., Tarcsa, E., Falus, A., *et al.*, *Int J Biochem Cell Biol* 2006, 38, 1662-1677.

[74.] Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., *et al.*, *J Immunol* 2001, 166, 4177-4184.

[75.] Chapuy-Regaud, S., Sebbag, M., Baeten, D., Clavel, C., *et al.*, *J Immunol* 2005, 174, 5057-5064.

[76.] Vossenaar, E. R., Despres, N., Lapointe, E., van der Heijden, A., *et al.*, *Arthritis Res Ther* 2004, 6, R142-150.

[77.] Chang, X. and Han, J., *Mol Carcinog* 2005.

[78.] Ishigami, A., Ohsawa, T., Hiratsuka, M., Taguchi, H., *et al.*, *J Neurosci Res* 2005, 80, 120-128.

[79.] Stastny, P., *N Engl J Med* 1978, 298, 869-871.

[80.] Nepom, G. T., *Adv Immunol* 1998, 68, 315-332.

[81.] Gregersen, P. K., Silver, J. and Winchester, R. J., *Arthritis Rheum* 1987, 30, 1205-1213.

[82.] Hill, J. A., Southwood, S., Sette, A., Jevnikar, A. M., *et al.*, *J Immunol* 2003, 171, 538-541.

[83.] van der Helm-van Mil, A. H., Verpoort, K. N., Breedveld, F. C., Huizinga, T. W., *et al.*, *Arthritis Rheum* 2006, 54, 1117-1121.

[84.] Brewerton, D. A., Hart, F. D., Nicholls, A., Caffrey, M., *et al.*, *Lancet* 1973, 1, 904-907.

[85.] Khan, M. A., *Curr Opin Rheumatol* 1995, 7, 263-269.

[86.] Bird, L. A., Peh, C. A., Kollnberger, S., Elliott, T., *et al.*, *Eur J Immunol* 2003, 33, 748-759.

[87.] Dangoria, N. S., DeLay, M. L., Kingsbury, D. J., Mear, J. P., *et al.*, *J Biol Chem* 2002, 277, 23459-23468.

[88.] Boyle, L. H., Goodall, J. C., Opat, S. S. and Gaston, J. S., *J Immunol* 2001, 167, 2619-2624.

[89.] Reveille, J. D., Ball, E. J. and Khan, M. A., *Curr Opin Rheumatol* 2001, 13, 265-272.

[90.] Taurog, J. D., Maika, S. D., Satumtira, N., Dorris, M. L., *et al.*, *Immunol Rev* 1999, 169, 209-223.

[91.] De Vos, M., Mielants, H., Cuvelier, C., Elewaut, A., *et al.*, *Gastroenterology* 1996, 110, 1696-1703.

[92.] Martinez-Gonzalez, O., Cantero-Hinojosa, J., Paule-Sastre, P., Gomez-Magan, J. C., *et al.*, *Br J Rheumatol* 1994, 33, 644-647.

[93.] Baeten, D., Kruithof, E., De Rycke, L., Boots, A. M., *et al.*, *Arthritis Res Ther* 2005, 7, R359-369.

[94.] Kruithof, E., Baeten, D., De Rycke, L., Vandooren, B., *et al.*, *Arthritis Res Ther* 2005, 7, R569-580.

[95.] Baeten, D., Demetter, P., Cuvelier, C., Van Den Bosch, F., *et al.*, *Ann Rheum Dis* 2000, 59, 945-953.

[96.] Baeten, D., Moller, H. J., Delanghe, J., Veys, E. M., *et al.*, *Arthritis Rheum* 2004, 50, 1611-1623.

[97.] Moestrup, S. K. and Moller, H. J., *Ann Med* 2004, 36, 347-354.

[98.] Reece, R. J., Canete, J. D., Parsons, W. J., Emery, P., *et al.*, *Arthritis Rheum* 1999, 42, 1481-1484.

[99.] Canete, J. D., Rodriguez, J. R., Salvador, G., Gomez-Centeno, A., *et al.*, *Semin Arthritis Rheum* 2003, 32, 378-387.

[100.] Vandooren, B., Kruithof, E., Yu, D. T., Rihl, M., *et al.*, *Arthritis Rheum* 2004, 50, 2942-2953.

[101.] Takayanagi, H., Iizuka, H., Juji, T., Nakagawa, T., *et al.*, *Arthritis Rheum* 2000, 43, 259-269.

[102.] Haynes, D. R., Crotti, T. N., Loric, M., Bain, G. I., *et al.*, *Rheumatology (Oxford)* 2001, 40, 623-630.

[103.] Crotti, T. N., Smith, M. D., Weedon, H., Ahern, M. J., *et al.*, *Ann Rheum Dis* 2002, 61, 1047-1054.

[104.] Haynes, D. R., Barg, E., Crotti, T. N., Holding, C., *et al.*, *Rheumatology (Oxford)* 2003, 42, 123-134.

[105.] Singh, J. A., Arayssi, T., Duray, P. and Schumacher, H. R., *Ann Rheum Dis* 2004, 63, 785-790.

[106.] Baeten, D., Peene, I., Union, A., Meheus, L., *et al.*, *Arthritis Rheum* 2001, 44, 2255-2262.

[107.] Baeten, D., Steenbakkers, P. G., Rijnders, A. M., Boots, A. M., *et al.*, *Arthritis Rheum* 2004, 50, 444-451.

[108.] Steenbakkers, P. G., Baeten, D., Rovers, E., Veys, E. M., *et al.*, *J Immunol* 2003, 170, 5719-5727.

General introduction

Part II:
Proteomics and its use in the field of rheumatology

Kelly Tilleman[1]
Dieter Deforce[1]
Dirk Elewaut[2]

[1] Laboratory for Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium
[2] Department of Rheumatology, University Hospital Ghent, Ghent, Belgium

Rheumatology 2005, 44, 1217-1226 as 'Rheumatology: a close encounter with proteomics'

ABSTRACT

Proteomics is a fast growing discipline in biomedicine that can be defined as the large-scale characterization of the entire protein complement of a cell, tissue or organism. Because protein levels and function may be critically dependent upon posttranscriptional mechanisms (e.g. posttranslational modifications) there has been significant interest in directly examining protein structure and function. It is now clear that proteomics studies may unmask previously unknown functions of proteins or protein interactions. However, proteomics in the field of rheumatology is still in its infancy.

This review guides the reader through the consecutive steps of a proteomics study and provides an outline of the applications in the field of rheumatology which may range from proteome analyses of biological fluids of rheumatic diseases to identify possible new diagnostic tools towards more pathophysiological studies on target tissues such as synovial tissue or articular cartilage.

Proteomics has great potential in the field of rheumatology and will no doubt have a great significant impact on our molecular understanding of these complex diseases.

INTRODUCTION

1. *Definition of proteomics*

The term 'proteome' was first coined by Marc Wilkins, an Australian postdoctoral fellow and was originally defined as the complete protein complement expressed by a genome [1]. This definition, however, does not take into account that the proteome is highly dynamic and that protein expression is influenced by environmental conditions of the cell, tissue or organism. Therefore, the definition of a proteome should specify that it is the protein complement of a given cell at a specified time, including the set of all protein isoforms and protein modifications [2]. Proteomics is then defined as the large-scale characterization of the entire protein complement of a cell, tissue or organism.

2. *From genomics to proteomics*

The Human Genome Initiative provided us with a blueprint of the human genome [3]. By studying the genome, it became clear that the behaviour of genome products is difficult to predict from the gene sequence alone. This realization has led to a variety of new large-scale disciplines analyzing down stream of the genome sequence. The introduction of these new techniques came along with a variety of 'omics'-terminologies (fig. 1).

First of all, genes are transcribed into mRNA. Since cells use alternative splicing, there is no one-to-one relationship between the genome sequence and the transcript [2]. Determining the level of mRNA in cells or tissues and the relationship between these levels in different conditions (e.g. diseased versus healthy) is the main goal of functional genomics also called 'transcriptomics'.

Transcripts are further translated into proteins, which are considered to be the main carriers of biological activity. One gene, can lead to different mRNA molecules due to alternative splicing. These mRNA species are further translated into proteins. These proteins can become fully active by adding post-translational modifications (PTM) or by interaction with other proteins. Due to all these processes, one gene can result in many different protein isoforms. Protein function is highly dynamic and influenced by environmental factors. It not only depends on the amino acid sequence, but also on PTM, degradation and/or compartmentalization of proteins in protein complexes. Proteomics aims at determining protein expression levels, but also protein structure, modifications, localization and interaction with other proteins.

Proteomics is one area among the various 'omics' disciplines, which also includes new disciplines such as metabolomics. Thus, a total integration of all these areas, called systems biology, will lead to a comprehensive understanding of cellular biology.

For evident reasons, we will only further discuss proteomics and its applications in the field of rheumatology.

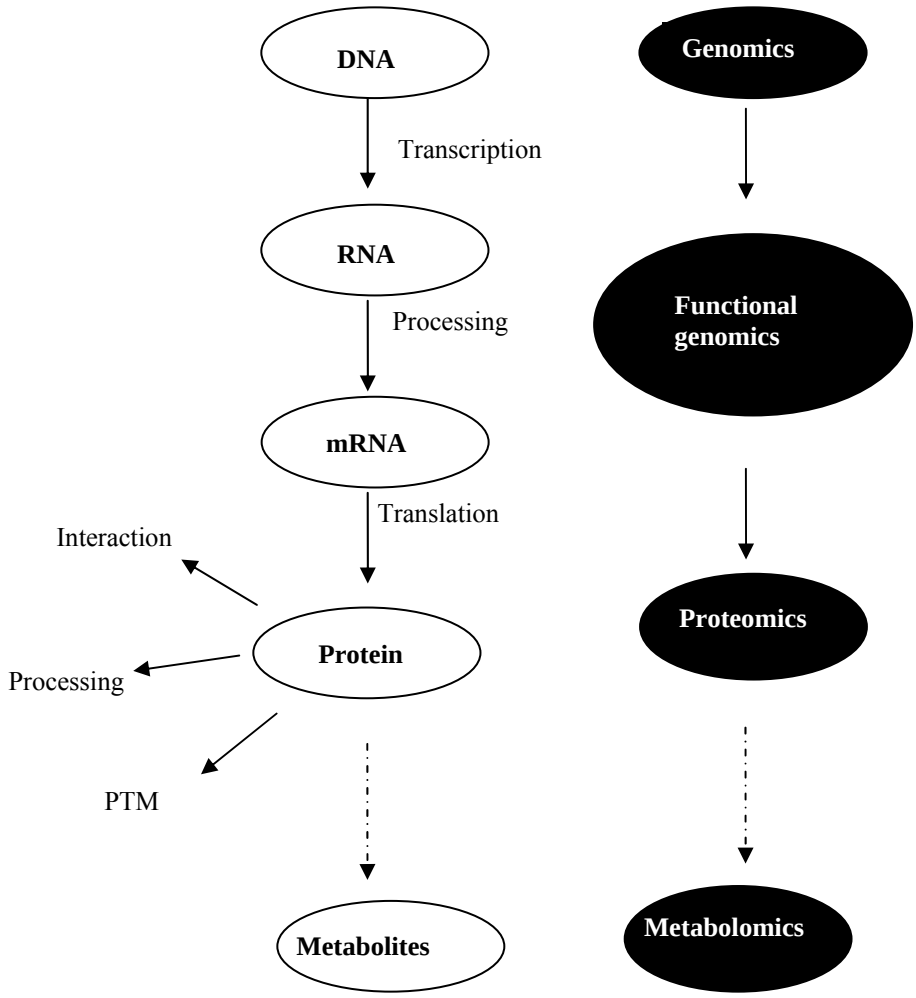


Figure 1: Overview of the currently available disciplines for large-scale analyses of genes, transcripts, proteins and metabolites.

3. Types of proteomics

Roughly, there are three types of proteomics: expression proteomics, structural proteomics and functional proteomics.

Expression proteomics seeks to identify all the protein species present in a proteome of a cell, tissue or organism at a certain time. *Structural proteomics*, by contrast, aims at identifying the molecular structure, i.e. the amino acid sequence of the protein entities involved in a given

process and to relate this information to the database of identified genes [4]. De Hoog C.L. *et al*. defined it as a comprehensive coverage of a single protein or domain structure [2]. Finally, *functional proteomics* describes the changes in protein abundance and modification during differentiation, proliferation and signalling of cells, both in qualitative and quantitative terms. It also includes studies of co-ordinated expression of proteins, as well as elucidation of the sequence of regulatory events during all stages that a cell or an organism undergoes during its entire life span [4].

PROTEOMIC APPROACHES

1. *Classic approach: gel-based proteomics*

a) Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE), the prototype of the classic proteomics has been around since the mid 1970s [5] [6], but has undergone major technological improvements over the past decade. Especially, in terms of reproducibility, handling, resolution, separation of extremely basic or acidic proteins and data analysis [7].

2-DE is a high resolution method for separating proteins in two dimensions; according to their isoelectric point (pI) in the first dimension and according to their size (molecular weight) in the second dimension. Firstly, proteins are separated by isoelectric focussing (IEF). Therefore, proteins are brought into a small gel strip that contains an immobilized pH gradient (IPG). This gel strip is applied on a plastic backing for easier handling and is further referred to as the IPG strip. When an electric field is applied over this IPG strip, the proteins migrate along the pH gradient in the strip until they reach the pH at which their overall charge is neutral; the isoelectric point (pI) of the protein. The result of this separation is a gel strip containing discrete protein bands at the pH position of their pI. This IPG strip is then applied onto a polyacrylamide gel. An electric current drives the focussed proteins out of the strip into the gel and separates the proteins according to their size. (for reviews on 2-DE and sample preparation see [7-9]).

One of the greatest strengths of 2-DE is its ability to resolve proteins differing in a single charge and consequently, *in vivo* modifications of proteins can be visualized. Multiple protein isoforms can be analyzed, by determining factors as solubilization conditions and pH range of the IPG strip. Currently, a wide range of pH gradients are available on the market, varying in different pH gradients [9, 10].

b) 2-D gel visualization

In order to visualize the separated proteins, 2-D gels can be stained with a variety of different stains. Detection methods in 2-DE can be divided in two large groups: general protein staining and specific detection of PTM. A limited overview of the most commonly used detection methods will shortly be discussed.

Coomassie Blue staining is probably the most widely used general protein stain. Major advantages are its low cost, ease of use and its compatibility with identification by mass spectrometry. Because of its limited sensitivity (10ng per protein spot), Coomassie Blue is mostly used for detection of proteins in preparative gels from which spots are cut to be identified (see section 2.2 'Mass spectrometry and protein identification). Although *silver stains* are more sensitive (0.5-1ng per protein spot), they have a very small linear range and are time-consuming because of the numerous steps involved in the protocol. Moreover, they are less compatible with mass spectrometry based identification of gel separated proteins.

Radio-active labelling is a very sensitive method of protein visualization; however it's hazardous and expensive. It is only applied for very specific analyses like protein synthesis (^{35}S) or protein phosphorylation (^{32}P). To date, *fluorescent dyes like Sypro Ruby* or *Tangerin dyes* are becoming more and more the standard for general protein staining. They are sensitive (1ng per protein spot), have a large linear range (over three orders of magnitude), are extremely easy to use and are compatible with mass spectrometry. Fluorescent dyes can also be used to label protein complexes prior to 2-DE. This so-called 'difference gel electrophoresis' or 'DIGE' approach is gaining interest. The method allows to fluorescently label (with cyanine dyes Cy2, Cy3, Cy4) as many as 3 different complex protein populations prior to mixing them together and running them on the same 2-D gel [11].

As the role of post-translational modifications became more prominent, specific detection methods of protein modifications were developed in which specifically glycoproteins, phosphoproteins and other modified proteins can be visualized in the gel. For detailed information on detection in proteomic analysis we refer to [12].

c) 2-D gel data analysis

Although, 2-D gel data analysis is a complex and laborious work, state-of-the art software programs have made this process easier and more straightforward. After acquiring 2-D images, protein spots have to be detected. Such a protein spot has three characteristics: the x and y-axis of the spot represented by the pI of the protein and its molecular mass, respectively and the z-axis which is a measure for the intensity of the spot and is demonstrative for the

amount of protein present in the spot. A spot on a 2-D gel can therefore correspond to a single protein, an isoform of a protein or a modified protein.

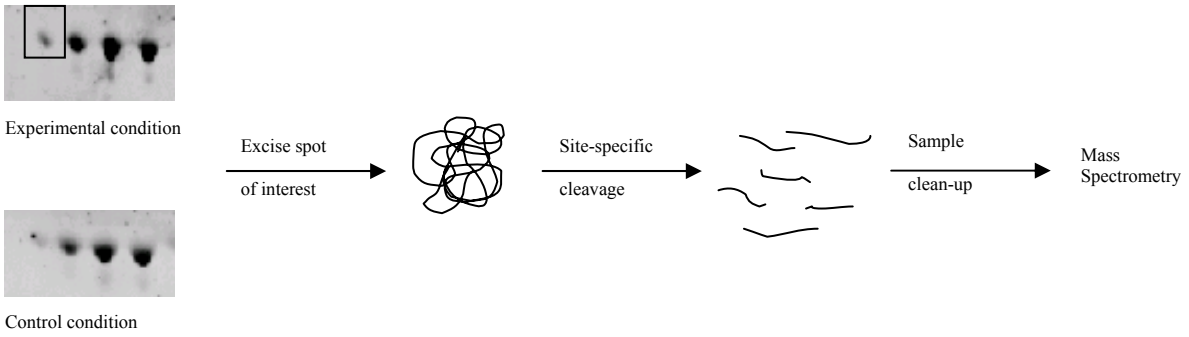


Figure 2: In-gel digest of differentially expressed proteins. Spots of interest are excised from the gel and digested by site-specific proteases. The resulting peptides are eluted from the gel, desalted, concentrated and analyzed by mass spectrometry.

In order to compare spots across several gels, spots have to be matched to each other. This is generally done by highlighting spots that are clearly present in all the gels as marker points. Using these markers, other spots present in the gels are matched to one another. Exploring differences in spot intensity or tracking protein spots that appear or disappear on gels derived from experimental and control conditions is the main goal of functional proteomics.

Spots of interest are further processed and identified by mass spectrometry (MS) (fig. 2). Briefly, protein spots are cut from the gels and digested by using a sequence-specific protease (e.g. trypsin, chymotrypsin, Arg-C, Asp-N). The resulting mixture of peptides is desalted and analyzed by MS.

2. Mass spectrometry

Mass spectrometry (MS) is an analytical technique that measures an intrinsic property of a molecule, its mass, with very high sensitivity. Although this technique dates back to the early days of the last century, it's only in the beginning of the 1980s with the development of new ionization techniques that mass spectrometry has found its applications in biological sciences. Mass spectrometers require charged gaseous ions and this was initially achieved by heating the sample. Since high temperatures are detrimental for proteins and peptides, this method could not be applied to vaporize these large biomolecules. Therefore, soft ionization methods

had to be developed in order to analyze biomolecules by MS. Almost simultaneously two soft ionization methods were developed: matrix assisted laser desorption ionization (MALDI) [13] and electrospray ionization (ESI) [14]. (For extended reading on analysis of proteins and peptides by mass spectrometry see review [15]).

Although both proteins and peptides can be analyzed, we only further discuss peptide analysis by MS since it is the way to identify proteins derived from different proteomic approaches.

Ionization

a) MALDI

MALDI is a soft ionization technique that uses matrix material in order to get the peptides into the gaseous phase. Protonated peptides are co-precipitated with matrix material on a metal surface and air dried. The resulting dried spots are irradiated by laser pulses, usually from small nitrogen lasers. The matrix contains small organic molecules with absorbance at the wavelength of the laser (e.g. α-cyano-4-hydroxycinnamic acid or dihydrobenzoic acid).

The exact nature of the ionization process in MALDI is largely unknown. During ionization and desorption, matrix molecules pass energy absorbed from the laser light to the charged peptides which are dispersed into gaseous phase (Fig. 3). Ionized peptides are further analyzed in the mass spectrometer.

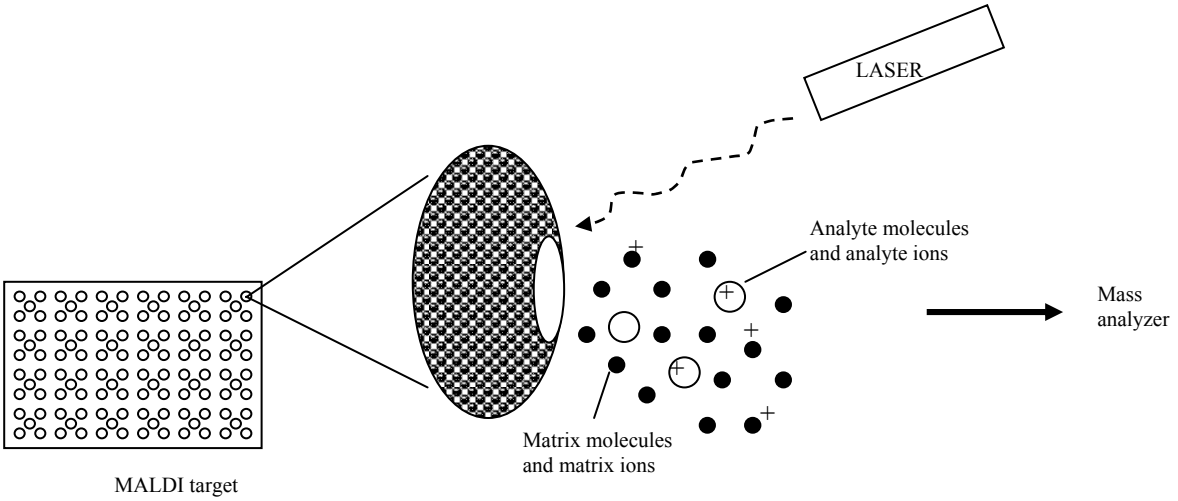


Figure 3: The MALDI ionization process. Charged peptides are co-precipitated with matrix molecules on a MALDI target plate and are irradiated by laser pulses. The gaseous charged peptides are further analyzed by mass spectrometry.

b) ESI

In contrast with MALDI where ionization takes places from a dried sample, ESI ionizes the peptides from solution. The liquid sample flows through a micro capillary tube into the mass analyzer. A high electrical potential is applied between the capillary and the inlet of the mass spectrometer. The result is a mist of small highly charged droplets which evaporate rapidly, either by field desorption or solvent evaporation. The ionized peptides are consequently released into to the gaseous phase and further analyzed by MS [16] (Fig. 4).

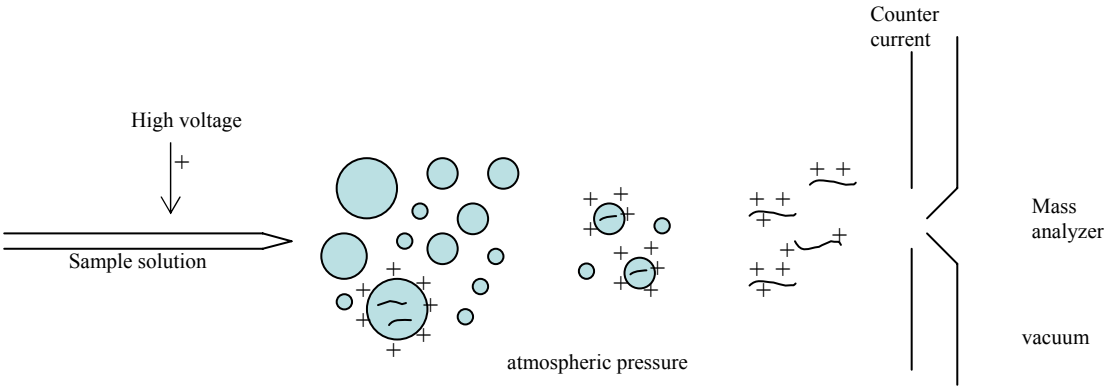


Figure 4: The ESI ionization process. Charged peptides in solution are brought into a capillary on which a high electrical voltage is applied. Charged droplets are evaporated and gaseous ionized peptides are released into the mass spectrometer.

There are several ways in which the sample can be delivered to this micro capillary tube. The simplest method is to load each sample in an individual capillary. Although cross-contamination is avoided, the method is slow and tedious. As an alternative approach, ESI sources can be coupled to liquid chromatography (LC) systems. The benefit of this on-line coupling is that the sample clean-up, concentration and analysis becomes semi-automated.

2.2 Mass analyzers and protein identification

a) Mass analyzers

Every mass spectrometer consists of 3 basic elements: an ionization source, one or more mass analyzers and a detector. The names of the mass spectrometers are simply a compilation of their ionization source and the mass analyzer. There is a wide range of different types of mass spectrometers, commonly divided in two groups: single MS machines, when only one mass analyzer is present and tandem MS or MSMS machines, if more mass analyzers are present.

The most commonly used single MS analyzers are Time-Of-Flight (TOF), Quadrupole (Q) and iontrap. The most generally used MSMS analyzers are Q-TOF and TOF-TOF.

The list of mass analyzers cited above is far from complete, however they can be viewed as those most routinely used in protein identification. (for extended reading on mass analyzers we refer to specific sections of [15, 17])

The great difference between single MS and MSMS analyzers is that the latter can not only separate the ions according to their mass, but they are able to select and fragment ions. The benefit of this will become evident in the next section.

b) Identification of proteins by peptide mass fingerprinting

When ions are passed into a mass spectrometer, they are separated according to their mass. This analysis results in a "mass fingerprint" of the peptides present in the mixture. These peptides are the result of the cleavage of a particular protein using a sequence-specific protease such as trypsin. The set of masses obtained by the mass spectrometer is compared to the theoretically expected tryptic peptide masses for each protein present in a certain database (Fig. 5).

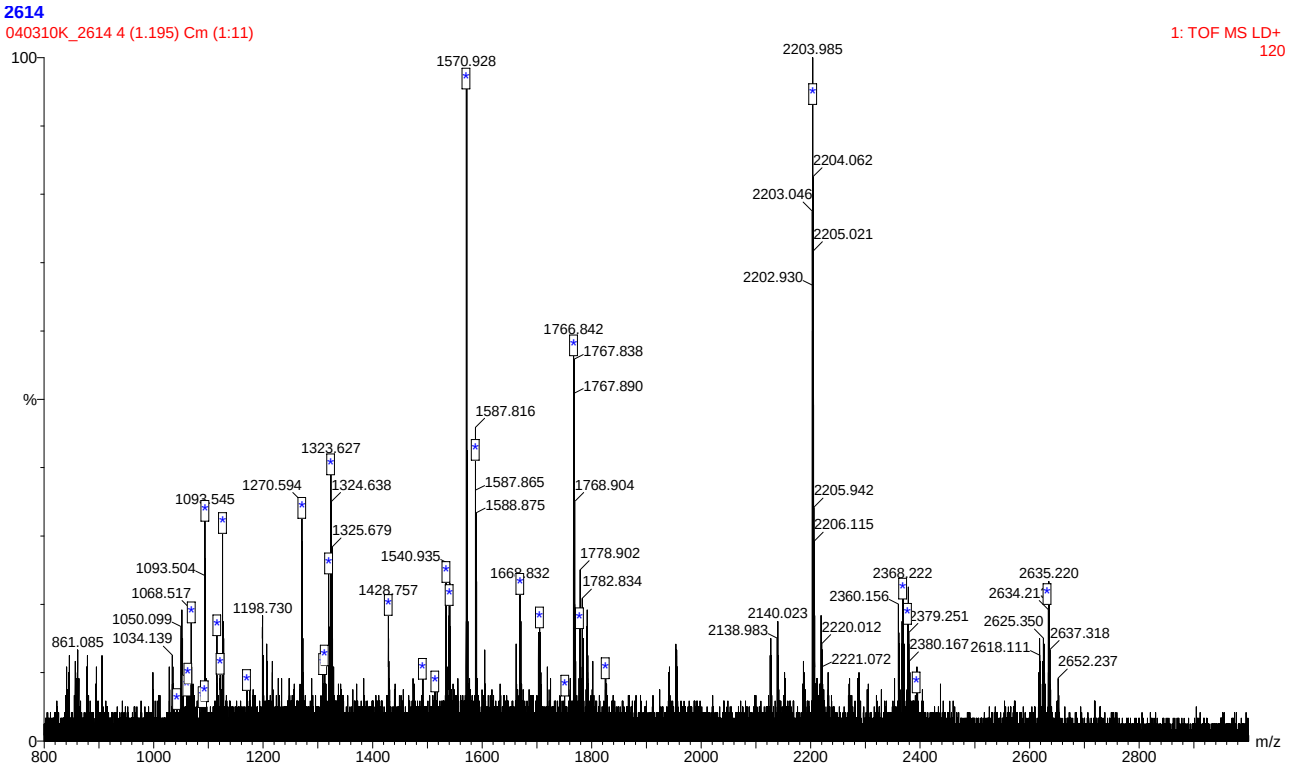
There are numerous databases on the World Wide Web which are publicly accessible. The ExPASy biological server (http://www.expasy.org) has an extensive collection of proteomics tools and links to other sites for identification of proteins by peptide mass fingerprints (PMF) like mascot, PepSea, PeptideSearch and many more.

These search engines will subsequently rank the positively identified proteins according to the number of peptide matches. Positively identified proteins identified by PMF have, on average, minimum peptide coverage of 20%. Identification of proteins by PMF is often the result of MALDI-TOF analysis.

c) Identification by tandem mass spectrometry

Mass spectrometers can not only determine the mass of the peptide, but also its amino acid sequence which is typical for MSMS mass analyzers. A particular peptide is selected out of a mixture of peptides in the first mass analyzer and is subsequently dissociated by collision with an inert gas, such as nitrogen or argon in a 'collision' cell. During these energetic collisions of the selected peptide and the collision gas, bonds are broken along the peptide backbone. In most applications, this leads to so called b and y ions, which indicate fragmentation at the amide bond with charge retention on the N or C terminus, respectively [18]. The resulting fragments are analyzed by the second mass analyzer, producing a tandem mass spectrum also called fragmentation spectrum or MSMS spectrum. Each peptide fragment in a series differs

from its neighbour by only one amino acid. It is therefore possible to determine the amino acid sequence by considering the mass difference between the neighbouring peaks in a series [18]. The experimental MSMS spectrum is matched against a calculated spectrum for all peptides in the database (e.g. Mascot, SEQUEST).

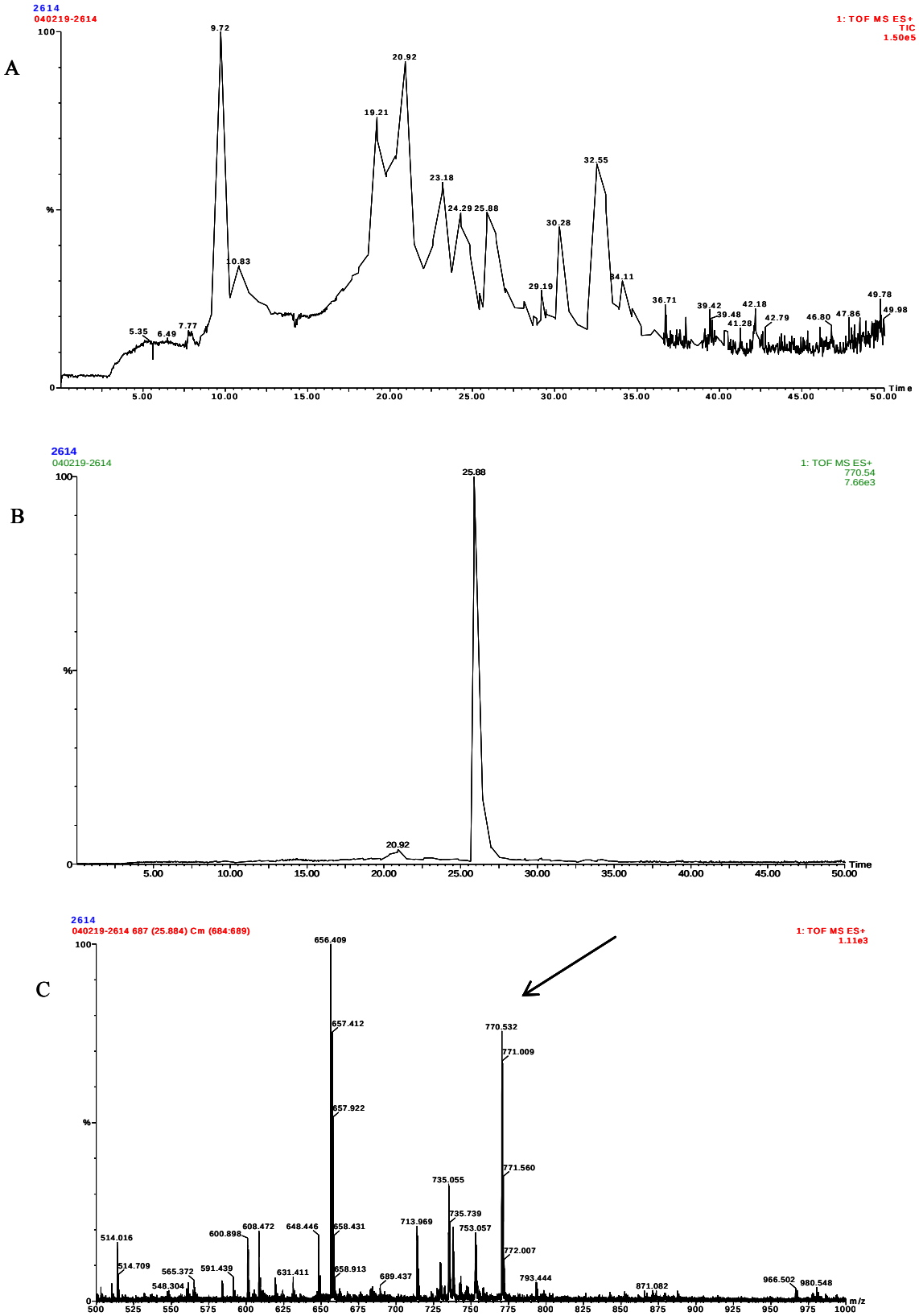


STRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSALRPSTSRSLYASSPGGVY
ATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTNEKVELQELNDRFANYIDKV
RFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQVDQLTNDKARVEVERDNLAED
IMRLREKLQEEMLQREEAENTLQSFRQDVDNASLARLDLERKVESLQEEIAFLKKLHEEE
IQELQAQIQEQHVQIDVDVSKPDLTAALRDVRQQYESVAAKNLQEAEEWYKSKFADLSEA
ANRNNDALRQAKQESTEYRRQVQSLTCEVDALKGTNESLERQMREMEENFAVEAANYQDT
IGRLQDEIQNMKEEMARHLREYQDLLNVKMALDIEIATYRKLLEGEESRISLPLPNFSSL
NLRETNLDSLPLVDTHSKRTLLIKTVETRDGQVINETSQHHDDLE

VIMENTIN_HUMAN

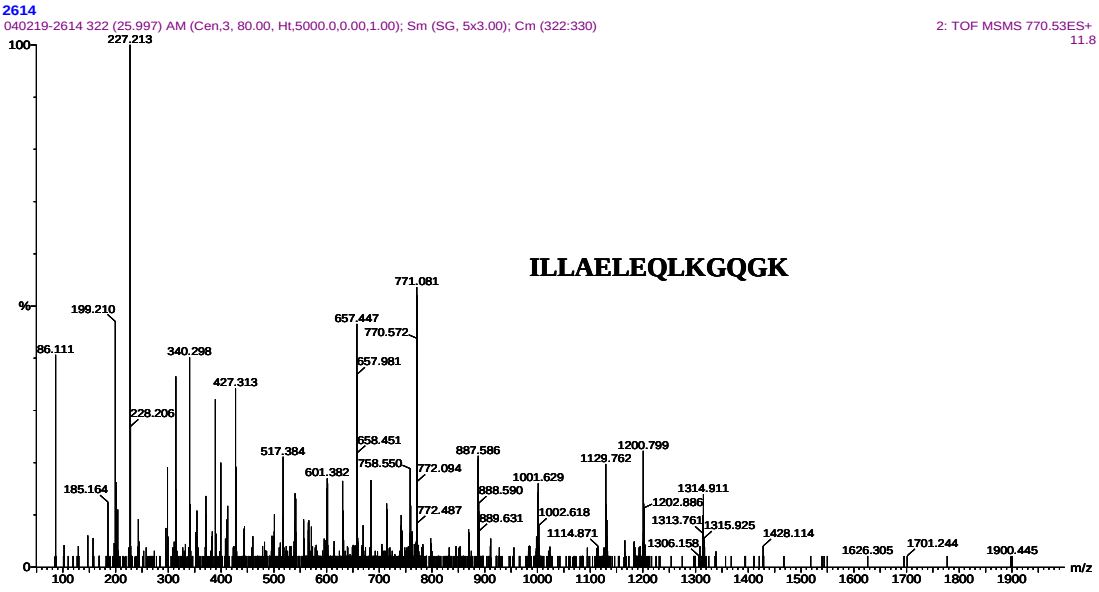
Figure 5: MALDI peptide map and identification of a protein. A spot was excised from a 2-D gel and in-gel digested with trypsin. 10% of the peptide solution was applied on a MALDI target plate in combination with 1mg/ml α-cyano matrix material. The samples were acquired on a MALDI-Q-TOF (Waters, Milford, USA). The peptide mass fingerprint was identified as vimentin_human. A total of 34 peptides were derived from vimentin and are indicated by a squared asterix. Matched peptides are highlighted and indicate protein coverage of 59.6%. Please note that the sequence of several identified peptides can overlap.

A score is calculated which reflects the quality of the match between the experimental spectrum and the theoretical one (fig.6).



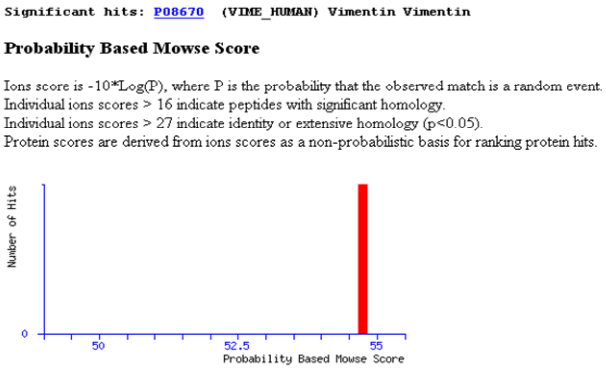
Parent Ion selection

D



Fragmentation spectrum

E



Database searching

Figure 6: LC/MSMS analysis of a mixture of peptides derived from a protein spot. The remainder of the peptide solution of the sample analyzed by MALDI-Q-TOF (fig. 5), was loaded onto a nanoLC column and eluted with a gradient of acetonitrile and 0.1% formic acid. The nanoLC system was online coupled to an ESI-Q-TOF (Water, Milford, USA) and spectra were acquired of only doubly charged peptides over a mass range from 500Da to 1000Da every 1 sec. The resulting chromatogram is displayed in which the total ion current (TIC) is plotted against the retention time of the eluting peptides (A). The chromatogram of the ion with m/z= 770.54 is replotted and this is called a single ion current chromatogram (SIC) (B). Several other peptides co-eluted with ion m/z= 770.54 at time 25 min. and are displayed in a single mass spectrum (C). When the ion current (IC) of certain ions reaches a predefined IC threshold, the mass spectrometer selects these ions for fragmentation. This figure shows the subsequent fragmentations spectrum of peptide m/z= 770.54 (D). This MSMS spectrum reveals the amino acid sequence of the peptide and is used to identify which protein the peptide is derived from (E). The protein was identified as vimentin_human by the mascot search engine.

Since tandem mass spectra data contain information on the sequence of the peptides, these searches are generally more specific and discriminating than peptide mass fingerprints.

d) Identification by sequence-tag analysis

It is not necessary to obtain a complete amino acid sequence of a peptide in order to be able to identify the protein. A short sequence tag is often sufficient to identify the corresponding protein from which the peptide(s) was (were) derived. A small stretch of amino acids can be combined with the start mass and the end mass of the series, which specifies the exact location of the sequence in the peptide and the known cleavage specificity of the enzyme. Such a peptide sequence will then retrieve from the database (e.g. MS-Tag, mascot, dbEST) one or a few sequences whose theoretical fragmentation pattern is matched against the experimental one.

Tandem mass spectrometry and sequence-tag analysis are routinely obtained by ESI-Q-TOF, MALDI-Q-TOF or MALDI-TOF-TOF mass spectrometers.

3. Alternative approach: gel-free proteomics

2-DE is still widely used as separation and quantitative analysis of complex protein mixtures. It is by far the best technique to visualize and analyze PTM of proteins; however, the technique also has its limitations. These limitations include the low-throughput of samples, problems in detecting low abundant, extremely basic and acidic proteins and proteins with very high or very low molecular weights. Because of these restrictions, several groups explored alternative separation techniques for proteins using MS [19-26]. Many of these alternative separation techniques rely on the ability of tandem mass spectrometers to collect sequence information from a specific peptide even if several other peptides, derived from other proteins, are in the sample at the same time. In order to be able to perform quantitative analysis, stable isotope labelling of the samples is used. This is achieved in different ways: proteins can be labeled metabolically by culturing cells in media that are enriched (e.g. containing ^{15}N salts or ^{13}C-labeled amino acids) or by enzymatic digestion of proteins in ^{18}O containing water [19, 26]. Proteins can also be labeled at specific sites with an isotopically encoded reagent. One of the best known examples of this technique is called ICAT (isotope-coded affinity tag). This method stably labels peptides of two populations of proteins using reactive probes that differ in isotope composition [24]. Proteins from the experimental mixture are labeled with either the heavy reagent composed of eight deuterium atoms (d8-

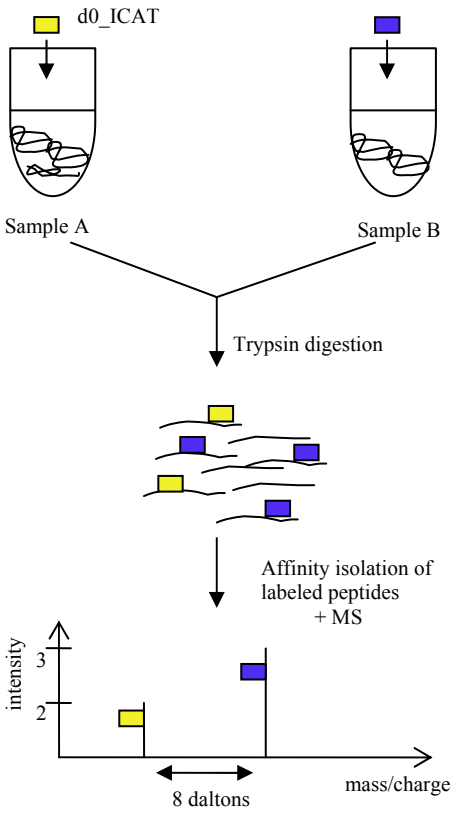


Figure 7: Differential gel-free proteomics by ICAT labelling of the samples. Proteins are extracted from two different populations or cell states and are labeled on cysteine residues with either the light (d0_ICAT) reagent or the heavy (d8_ICAT) reagent. Next, the samples are mixed together and digested with a protease e.g. trypsin. Peptides that were labeled with the ICAT reagents are isolated by affinity chromatography because of the biotin tag present in the reagent. After purification, the peptides are analyzed by MS and the peak ratios of the differently labelled peptides, which differ by 8 Da, are quantified. Identification of the peptides is done by sequencing the peptides by MSMS.

ICAT) or the light reagent composed of eight hydrogen atoms (d0-ICAT) and then mixed together. The mixed proteins are digested by a site-specific protease e.g. trypsin. ICAT-labeled peptides are isolated from the complex mixture utilizing the biotin tag present in the ICAT reagent. Selected peptides are further separated and analyzed by liquid chromatography on-line coupled to electrospray tandem mass spectrometry (LC-ESI/MSMS). Mass spectrometry is used to reveal the ratio of the isotopic molecular weight peaks that differ by 8 Da, and this gives a measure of the relative amount of each protein present in the original samples [20, 21] (fig. 7). Other gel-free proteomic approaches are chip-based techniques based on different technologies [27, 28].

It is important to notice that both gel-based and gel-free proteomic approaches have there strengths and drawbacks. It is therefore crucial to find out which method is best suited for achieving the goals that were set out.

PRESENT STATUS OF PROTEOME RESEARCH IN THE FIELD OF RHEUMATOLOGY

Searching the NCBI database, mid-march 2005, using keywords like <arthritis> and <proteomics> revealed 22 hits. For comparison; using the keyword <cancer> in combination with <proteomics> revealed 758 hits. Notwithstanding the fact that the amount of reports is rather small, the content is extremely interesting, and the methods vary from the classic 2-DE approach to gel-free isotopic labelling of samples.

Proteomic papers in the field of rheumatology can largely be divided in three groups: protein target identification by differential screening of biological fluids, biomarker search in rheumatic tissues and proteomic surveillance of autoantigens.

1. Protein discovery by differential screening of biological fluids

The comparison of protein patterns in body fluids of diseased and healthy individuals has the potential to identify new diagnostic tools and could subsequently lead to new disease specific therapies. Analysis of serum and synovial fluids is of great importance in the diagnosis of rheumatic diseases but also in monitoring a patient's response to certain medication. Currently available biomarkers are related to the degree of inflammation, but lack straightforward correlation with disease severity or do not change dramatically in response to treatment [29]. Moreover, it is known that the joint damage may progress in spite of decreased inflammatory activity and erosions may develop in patients who have few clinical signs of inflammation [30, 31]. In addition, there is a need for serum biomarkers in other rheumatic pathologies such as spondyloarthropathy (SpA).

Large-scale analysis of synovial fluid and serum provides information on disease specific differences due to local processes in the inflamed joint, compared to systemic disease symptoms. Several discriminating acute-phase proteins have been identified in serum, plasma and synovial fluid of different rheumatic pathologies using classic proteomic approaches [32-36]. Serum amyloid A protein is present in synovial fluid and plasma of RA patients, but is undetectable in plasma or synovial fluid of osteoarthritis (OA) patients. This acute-phase protein has a crucial role in the very early organization of host-defence, but could have a destructive effect in chronic inflammation [33]. Certain fibrinogen and the calgranulin protein isoforms show diseases associated expression and processing in biological fluid matrices of RA and OA patients [33, 36].

Although the classic proteome approach is able to identify new discriminating targets, it is not applicable for fast screening. Several groups have therefore attempted to directly analyze pathologic fluids by mass spectrometry [30, 37, 38]. Liao H. *et al*. identified at least 33 possible protein biomarkers in synovial fluid of RA patients by applying a semi-quantitative gel-free proteomics approach using internal ^{13}C labeled peptide standards [30]. Besides members of the S100 proteins, other proteins like osteopontin (plays a role in attachment and invasion of synovial fibroblasts [39]), cyclophilin (proinflammatory mediator in arthritis [40], cathepsin B and many others were highly increased in synovial fluid of RA patients with erosions [30]. These newly identified proteins need further validation in larger patient cohorts, to evaluate their specificity and sensitivity.

Large-scale proteomic analysis of biological fluids has great potential, however, one must realize that these samples constitute of highly abundant proteins (e.g. albumin, IgG's). These proteins are detrimental for the detection of other species in the sample. A good sample clean-up, or efficient enrichment techniques are therefore pivotal for the investigation of proteins present in smaller concentrations.

2. Biomarker discovery in rheumatic tissue samples

Potentially interesting samples are not only synovial fluid and serum but may also include analysis of joint components such as synovial tissue and cartilage. The molecular understanding of the process leading to joint destruction is not complete yet. Therefore, large-scale proteome and genome analysis of joint tissue could reveal important information about mechanisms leading to joint damage.

Joint destruction in non-inflammatory arthritis or OA is characterized by degradation of articular cartilage. It is thought that this is due to an imbalance between anabolism and catabolism of the extracellular matrix. Therefore, several groups have been studying the regulation of the protein synthesis and protein secretion by cartilage using proteomic approaches [41, 42].

Proteome analysis of the phenotype of articular cartilage in OA and normal patients could be of great help in understanding the endogenous control mechanisms of matrix turnover in cartilage. Moreover, this information could be of help in tissue engineering during stem cell differentiation.

Joint destruction in inflammatory arthritis is very different from OA and is initiated by chronic inflammation of the synovial tissue. To unravel the molecular and cellular mechanism of chronic synovitis, synovial proteomes of RA and OA patients have been investigated. The group of Thiessen HJ, has used a multi-Western blot Powerblot^{TM} (BD Biosciences), for differential protein screening of synovium obtained from OA and RA patients [43]. This commercially available method offers the possibility to analyze over 700 protein species simultaneously. Some differentially expressed proteins were further validated in a larger patient cohort, namely Stat 1, p47phox and MnSOD which were up regulated in RA and cathepsin D which showed a lower expression in RA in comparison to OA. All of these proteins are related to inflammation, cytokine induced activation or have been described in tissue damage and joint destruction in arthritic joints [43].

Chronic synovitis is not only a characteristic of RA, but also of SpA. These two frequent forms of inflammatory arthritis have a different clinical outcome, but have some shared features such as chronic synovitis. Our group analyzed the synovial proteome of RA, SpA and OA patients and came to the conclusion that the synovial proteome of RA patients consists of a unique protein expression pattern in contrast to the synovial proteome of SpA patients [44]. We identified several proteins related to either RA or SpA inflammation amongst them MRP-8 (calgranulin A), a well known biomarker for inflammatory arthritis. MRP-8 was highly up regulated in the inflammatory arthritides in comparison with OA patients, in which this protein spot was completely absent from the 2-D images [44] (Chapter 1).

Proteome studies of fibroblast-like cells, derived from the synovium of patients with inflammatory arthritis can aid in resolving the mechanism(s) behind the synovial hyperplasia and the associated tissue damage [45]. Likewise, tissue derived from inflamed joints of animals can also be helpful in gaining access to the complex pathology of joint inflammation [46].

3. Proteomic surveillance of autoimmunity

The majority of rheumatic diseases are characterized by autoimmune processes. Identification of autoantigens playing a role in these complex pathologies is extremely important. Also, the presence of autoantibodies in otherwise healthy individuals may perhaps predict the development of autoimmune disease. Furthermore, the appearance of certain autoantibodies might predict the clinical course of a patient with established disease [47].

Using 2-DE to separate tissue proteins, autoantibodies against these proteins can be detected with serum or synovial fluid of patients by Western Blot. This technique has already been

used in rheumatic pathologies in the late 80s [48, 49], and is currently widely applied. A recent example of this technology is described by Xiang Y. *et al.* [50], in which they investigated autoantigens present in chondrocytes in both RA and OA patients. Triosephosphate isomerase (TPI) was identified as an OA specific autoantigen. The autoantibodies recognized multiple epitopes on TPI and titres were both high in serum and synovial fluid [50].

Also alpha-enolase, a well known autoantigen, has been investigated by proteomics in several autoimmune diseases [51, 52]. Autoantibodies against glycolytic proteins like glucose-6-phosphate isomerase [53] and Aldolase A [54] have been identified in RA sera. Moreover, dysregulation of glucose metabolism could play a role in the hyperplasia of synoviocytes in RA. Autoantibodies against these proteins might be implicated in the pathology of RA.

Not only the discovery of new autoantigens, but also analysis of specific post-translational modifications of these autoantigens, has great potential in proteomic applications in rheumatic pathologies. Immunoreactive spots can clearly be identified using 2-DE. These spots can represent a specific isoform of a protein or particular modification of a protein. Some of these PTM, particularly citrullination of certain proteins, elicit autoimmune reactivity in RA [55, 56]. Other post-translational modifications which may play a key role in the acquisition of autoantigenicity in rheumatic pathologies may be unmasked by proteomics [57].

A LOOK TOWARDS THE FUTURE

Proteomics is a technique with great promise in the field of rheumatology. Since both tissue and body fluids can easily be obtained from patients suffering from rheumatic pathologies, the *in vivo* situation can readily be examined.

There is no doubt that fast screening methods like array-based protein chips and quantitative mass spectrometry will become very important in the future. Using these high-throughput techniques, the potential application for diagnosing or to monitor prognostic markers of patients with rheumatic diseases, is almost unlimited.

There is, however, still a need for classic proteomic approaches. As the role of post-translational modifications will increase in the future, 2-DE could play a part in elucidating specific protein modifications.

Synovial tissue proteomics could help us understand the complex nature of rheumatic pathologies. Techniques like laser-capture micro dissection in combination with 2-DE and

mass spectrometry allow *in situ* analyses [58]. Specific cell types can be isolated and the diseases associated proteins subsequently determined.

As biomarkers, protein interactions and signalling pathways become apparent during proteome analyses, disease mechanisms may be unravelled that eventually could open new avenues for drug targets in the field op rheumatology.

ACKNOWLEDGEMENTS

We thank all our colleagues for their help and stimulating discussions. We apologize for omissions of important work due to length restrictions. This work was supported by the Fund for Scientific Research-Flanders, the Research-Fund of Ghent University and the Marató Foundation.

REFERENCES

[1.] Wilkins, M. R., Sanchez, J. C., Gooley, A. A., Appel, R. D., *et al.*, *Biotechnol Genet Eng Rev* 1996, 13, 19-50.

[2.] de Hoog, C. L. and Mann, M., *Annu Rev Genomics Hum Genet* 2004, 5, 267-293.

[3.] Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., *et al.*, *Science* 2001, 291, 1304-1351.

[4.] Righetti, P. G., Castagna, A., Antonucci, F., Piubelli, C., *et al.*, *Clin Chem Lab Med* 2003, 41, 425-438.

[5.] Klose, J., *Humangenetik* 1975, 26, 231-243.

[6.] O'Farrell, P. H., *J Biol Chem* 1975, 250, 4007-4021.

[7.] Gorg, A., Weiss, W. and Dunn, M. J., *Proteomics* 2004, 4, 3665-3685.

[8.] Shaw, M. M. and Riederer, B. M., *Proteomics* 2003, 3, 1408-1417.

[9.] Gorg, A., Obermaier, C., Boguth, G., Harder, A., *et al.*, *Electrophoresis* 2000, 21, 1037-1053.

[10.] Westbrook, J. A., Yan, J. X., Wait, R., Welson, S. Y., *et al.*, *Electrophoresis* 2001, 22, 2865-2871.

[11.] Unlu, M., Morgan, M. E. and Minden, J. S., *Electrophoresis* 1997, 18, 2071-2077.

[12.] Patton, W. F., *J Chromatogr B Analyt Technol Biomed Life Sci* 2002, 771, 3-31.

[13.] Karas, M. and Hillenkamp, F., *Anal Chem* 1988, 60, 2299-2301.

[14.] Fenn, J. B., Mann, M., Meng, C. K., Wong, S. F., *et al.*, *Science* 1989, 246, 64-71.

[15.] Mann, M., Hendrickson, R. C. and Pandey, A., *Annu Rev Biochem* 2001, 70, 437-473.

[16.] Mann, M. and Wilm, M., *Trends Biochem Sci* 1995, 20, 219-224.

[17.] Graves, P. R. and Haystead, T. A., *Microbiol Mol Biol Rev* 2002, 66, 39-63; table of contents.

[18.] Steen, H. and Mann, M., *Nat Rev Mol Cell Biol* 2004, 5, 699-711.

[19.] Zappacosta, F. and Annan, R. S., *Anal Chem* 2004, 76, 6618-6627.

[20.] Patton, W. F., Schulenberg, B. and Steinberg, T. H., *Curr Opin Biotechnol* 2002, 13, 321-328.

[21.] Peng, J. and Gygi, S. P., *J Mass Spectrom* 2001, 36, 1083-1091.

[22.] Aebersold, R., *J Am Soc Mass Spectrom* 2003, 14, 685-695.

[23.] Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., *et al.*, *Proc Natl Acad Sci U S A* 2000, 97, 9390-9395.

[24.] Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., *et al.*, *Nat Biotechnol* 1999, 17, 994-999.

[25.] Gevaert, K., Van Damme, J., Goethals, M., Thomas, G. R., *et al.*, *Mol Cell Proteomics* 2002, 1, 896-903.

[26.] Heller, M., Mattou, H., Menzel, C. and Yao, X., *J Am Soc Mass Spectrom* 2003, 14, 704-718.

[27.] Zhu, H. and Snyder, M., *Curr Opin Chem Biol* 2003, 7, 55-63.

[28.] Marko-Varga, G., Nilsson, J. and Laurell, T., *Electrophoresis* 2003, 24, 3521-3532.

[29.] Ward, M. M., *J Rheumatol* 2004, 31, 884-895.

[30.] Liao, H., Wu, J., Kuhn, E., Chin, W., *et al.*, *Arthritis Rheum* 2004, 50, 3792-3803.

[31.] Lindqvist, E., Eberhardt, K., Bendtzen, K., Heinegard, D., *et al.*, *Ann Rheum Dis* 2005, 64, 196-201.

[32.] Doherty, N. S., Littman, B. H., Reilly, K., Swindell, A. C., *et al.*, *Electrophoresis* 1998, 19, 355-363.

[33.] Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B., *et al.*, *Electrophoresis* 2002, 23, 3445-3456.

[34.] Yamagiwa, H., Sarkar, G., Charlesworth, M. C., McCormick, D. J., *et al.*, *J Orthop Sci* 2003, 8, 482-490.

[35.] Drynda, S., Ringel, B., Kekow, M., Kuhne, C., *et al.*, *Pathol Res Pract* 2004, 200, 165-171.

[36.] Dotzlaw, H., Schulz, M., Eggert, M. and Neeck, G., *Biochim Biophys Acta* 2004, 1696, 121-129.

[37.] Kuhn, E., Wu, J., Karl, J., Liao, H., *et al.*, *Proteomics* 2004, 4, 1175-1186.

[38.] Uchida, T., Fukawa, A., Uchida, M., Fujita, K., *et al.*, *J Proteome Res* 2002, 1, 495-499.

[39.] Petrow, P. K., Hummel, K. M., Schedel, J., Franz, J. K., *et al.*, *Arthritis Rheum* 2000, 43, 1597-1605.

[40.] Harigai, M., Hara, M., Takahashi, N., Kitani, A., *et al.*, *Clin Immunol Immunopathol* 1992, 63, 58-65.

[41.] Hermansson, M., Sawaji, Y., Bolton, M., Alexander, S., *et al.*, *J Biol Chem* 2004, 279, 43514-43521.

[42.] Freyria, A. M. and Becchi, M., *Methods Mol Med* 2004, 100, 165-182.

[43.] Lorenz, P., Ruschpler, P., Koczan, D., Stiehl, P., *et al.*, *Proteomics* 2003, 3, 991-1002.

[44.] Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I., *et al.*, *Proteomics*, in press.

[45.] Dasuri, K., Antonovici, M., Chen, K., Wong, K., *et al.*, *Arthritis Res Ther* 2004, 6, R161-168.

[46.] Lorenz, P., Bantscheff, M., Ibrahim, S. M., Thiesen, H. J., *et al.*, *Clin Chem Lab Med* 2003, 41, 1622-1632.

[47.] Scofield, R. H., *Lancet* 2004, 363, 1544-1546.

[48.] Herbert, K. E., Coppock, J. S., Griffiths, A. M., Williams, A., *et al.*, *Ann Rheum Dis* 1987, 46, 734-740.

[49.] Williams, J. M., Gorevic, P. D., Looney, R. J. and Abraham, G. N., *Immunology* 1987, 62, 529-536.

[50.] Xiang, Y., Sekine, T., Nakamura, H., Imajoh-Ohmi, S., *et al.*, *Arthritis Rheum* 2004, 50, 1511-1521.

[51.] Lee, K. H., Chung, H. S., Kim, H. S., Oh, S. H., *et al.*, *Arthritis Rheum* 2003, 48, 2025-2035.

[52.] Saulot, V., Vittecoq, O., Charlionet, R., Fardellone, P., *et al.*, *Arthritis Rheum* 2002, 46, 1196-1201.

[53.] Schaller, M., Burton, D. R. and Ditzel, H. J., *Nat Immunol* 2001, 2, 746-753.

[54.] Ukaji, F., Kitajima, I., Kubo, T., Shimizu, C., *et al.*, *Ann Rheum Dis* 1999, 58, 169-174.

[55.] Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., *et al.*, *J Immunol* 2001, 166, 4177-4184.

[56.] Vossenaar, E. R., Despres, N., Lapointe, E., van der Heijden, A., *et al.*, *Arthritis Res Ther* 2004, 6, R142-150.

[57.] Zhou, Z. and Menard, H. A., *Curr Opin Rheumatol* 2002, 14, 250-253.

[58.] Marko-Varga, G., Berglund, M., Malmstrom, J., Lindberg, H., *et al.*, *Electrophoresis* 2003, 24, 3800-3805.

Purpose and outline

Proteomics: A close encounter with rheumatology

Kelly Tilleman

Inflammatory arthritides include different forms of joint inflammation which lead to joint deformations, loss of joints, and disability. These pathologies have a tremendous socio-economic impact, not only on the patient, but on society as a whole.
We distinguish two frequent forms of chronic inflammatory arthritis; RA as an aggressively eroding joint disease and SpA as a less erosive disease with extensive remodelling and repair processes. As described in the general introduction, there are clearly distinct clinical and pathological features that are specific for the different forms of arthritis. However, RA and SpA have one characteristic in common; synovitis, the chronic inflammation of the synovial tissue.
The synovial membrane, more specific its inflammation, has been investigated from an immunological point of view. Numerous reports indicate that similar immune cells and cytokines play a role in both pathologies. Therefore it is possible that certain molecular differences between RA and SpA synovitis are responsible for the differences in clinical outcome. The knowledge of the molecular pathophysiology of chronic arthritis and synovitis is still far from complete, especially in SpA. In part this is due to the fact that the prevalence of SpA was thought to be rather low. It is only recently, that the interest in this related group of inflammatory arthritides has grown, since it is now known that the prevalence of SpA is of the same magnitude as RA. Several research groups are including SpA in their studies whereas before, the emphasis was exclusively on RA and OA.

The primary aim of this project was to learn more about the molecular nature of synovitis in chronic inflammatory arthritides. Especially the expression of proteins in the inflamed synovial tissue was investigated. This was accomplished by using a traditional proteomics approach. 2-DE patterns of synovial tissue extracts of RA, SpA and OA patients were analyzed and differentially expressed proteins were identified by mass spectrometry (**chapter 1**).

Looking at the synovial proteome of inflammatory arthritides, we observed different isoforms of vimentin. Citrullinated vimentin is known as the Sa-antigen in RA; however, citrullinated vimentin has not been identified in protein extracts of inflamed synovial tissue. We therefore further characterized these different isoforms of vimentin and investigated the presence of antibodies against processed citrullinated forms of vimentin in RA and SpA (**chapter 2**).

Differential proteome analysis also revealed differentially expressed proteins of which their expression is hypoxia-induced. It is known that the RA joint is hypoxic. Since both RA and SpA are characterized by synovitis, it is plausible that the environmental condition in the RA and SpA joint are alike. Angiogenesis, which can be observed in RA and SpA inflamed synovial tissue, is triggered by hypoxia. Moreover, there is a significant higher vascularity in SpA than in RA. We investigated whether this dissimilarity could be the result of a different molecular response to the hypoxic environment. We therefore analyzed the stabilisation of HIF, the transcription factor responsible for the gene expression under hypoxia, in RA and SpA FLS. In addition, the possibility of hypoxia induced expression of VEGF and Ceruloplasmin by FLS, two proteins involved in angiogenesis, were examined (**chapter 3**).

The majority of rheumatic diseases are characterized by autoimmunity. Identification of autoantigens playing a role in these pathologies is important. The study presented in chapter 4 was conducted in order to register the spectrum of synovial autoantigens that induce an autoimmune humoral response. 2-D immunoblotting with RA and control patients' sera to an RA synovial protein extract was performed, followed by identification by mass spectrometry (**chapter 4**).

In summary, this thesis describes a close encounter of rheumatology and proteomics. The latter was the central technique during this research project. Because of its global approach, results of proteome analyses are often descriptive. It is therefore a good starting point to learn about the molecular pathophysiology of a certain diseased tissue, in our case, the synovial tissue. In addition, by using a classic proteomics approach and thereby using 2-DE as protein separation technique, we clearly show that it is possible to visualize PTM, like citrullination, which are important in rheumatic pathologies.

Chapter 1

Chronically inflamed synovium from spondyloarthropathy and rheumatoid arthritis investigated by protein expression profiling followed by tandem mass spectrometry

Kelly Tilleman[1]
Katrien Van Beneden[2]
Aline Dhondt[1]
Ilse Hoffman[2]
Filip De Keyser[2]
Eric Veys[2]
Dirk Elewaut[2]
Dieter Deforce[1]

[1] Laboratory for Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium
[2] Department of Rheumatology, University Hospital Ghent, Ghent, Belgium

Proteomics 2005; 5 (8): 2247-2257

ABSTRACT

We investigated the cytosolic proteome of inflamed synovial tissue by hierarchical clustering analysis and validated the feasibility of this proteome analysis by identifying proteins that were differentially expressed between rheumatoid arthritis (RA), spondyloarthropathy (SpA) and osteoarthritis (OA).

Synovial biopsy samples were obtained from 18 patients undergoing needle arthroscopy for knee synovitis associated with RA (n=6) and SpA (n=6), and for joint effusion of the knee associated with OA (n=6). Cytosolic proteins were extracted from the tissue and subjected to two-dimensional gel electrophoresis. Protein expression patterns were statistically analyzed and used for hierarchical cluster analysis. Proteins of interest were independently identified by MALDI and ESI mass spectrometry.

Hierarchical cluster analysis of the complete match set, containing 640 spots, remarkably segregated SpA from RA and OA. Next, we used a subset of spots that were statistically differentially expressed ($p<0.01$), between RA and SpA, SpA and OA or RA and OA, in both a Student T-test and Mann Whitney U test. The dendrograms revealed distinct clustering of RA versus SpA and RA versus OA.

Spots that were differentially expressed between the groups were identified by tandem mass spectrometry. Fructose bisphosphate aldolase A and alpha-enolase showed higher expression levels in SpA than in OA ($p<0.01$). Calgranulin A (MRP8) was markedly upregulated in RA and SpA patients in comparison with OA patients where this spot was below detection limit.

The analysis of the cytosolic proteome of synovial tissue is a useful approach to identify disease-associated proteins in chronic inflammatory arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) and spondyloarthropathies (SpA) are two frequent forms of chronic arthritis with an important morbidity and socio-economic impact in our society. Although similar joints can be affected in both diseases, their clinical presentation is different. RA is characterized by symmetric polyarticular joint inflammation and destruction as well as by extra-articular manifestations such as rheumatoid nodules or vasculitis. The hallmark symptoms of SpA, by contrast, are quite distinct. They include sacroiliitis, spondylitis, pauciarticular synovitis and enthesitis. Extra-articular manifestations such as uveitis or subclinical bowel inflammation may also occur in SpA. There are several entities that belong to the concept of SpA such as ankylosing spondylitis (AS), reactive arthritis (ReA), psoriatic arthritis (PsA) and undifferentiated SpA (USpA) [1,2]. Despite these different clinical presentations a major-shared feature between both diseases is the presence of synovitis. Joint effusion may also occur in patients suffering from osteoarthritis (OA). However, this occurs as a secondary symptom induced by biomechanical stress on cartilage and subchondral bone, in contrast to inflammatory joint diseases such as RA and SpA where primary inflammation occurs within the synovial membrane.

In both RA and SpA, the synovial membrane of affected joints undergoes several profound changes in synovial architecture with thickening of the synovial lining layer, neovascularization and lymphocyte infiltration [3-5]. Besides differences in general histological characteristics between RA and SpA (lining thickness, vascularization, and cellular infiltration) [6], two specific immunohistochemical features that recently have been described, discriminate RA from SpA. The presence of intracellular citrullinated proteins has been specifically associated with RA [7]. Also, synovial immunohistochemical staining for a combination epitope of complexes of the RA associated autoantigen epitope, HC gp-39 263-275, and the RA associated DRB1*0401 HLA class II molecule was found to be restricted to HLA-DRB1*0401 positive RA patients [8].

Despite intensive investigations into the nature of chronic synovitis, the molecular differences of RA versus SpA synovial inflammation are poorly understood. While several groups have therefore initiated gene expression profile studies using cDNA microarrays in order to identify disease relevant genes and proteins, it became apparent that these techniques have several limitations. There are situations where RNA expression does not match protein expression and function [9], for example in the event that mRNA encodes for 2 or more alternative splice variants of a given protein with distinctive functions [10]. Furthermore, several regulatory elements exist such as 3' mRNA AU-rich elements that regulate translation

of mRNA into protein [11-13]. Finally, many posttranslational modifications by enzymes such as kinases or proteinases regulate protein function. One of the best examples is the cascade of phosphorylation events upon crosslinking of T cell receptors. Proteomics refers to the large-scale study of protein expression and function in a tissue or organism [14]. Several different technologies exist to study the proteome of autoimmune diseases [15] and it is clear that this area of investigation is still immature. While proteomics has become increasingly popular in several areas of research particularly in oncology [16-19], no detailed analysis of the human proteome in chronically inflamed synovium exists to date.

Therefore, the aim of this study was to use and compare protein expression profiles of cytosolic proteins in inflamed synovial tissue from RA, SpA and OA. The data presented here indicate that a differential screening approach of the synovial cytosolic proteome by 2-DE is a feasible approach that may be useful to identify proteins implied in the pathogenesis of RA and SpA.

PATIENTS AND METHODS

Patients

Synovial biopsy samples were obtained from 18 patients undergoing needle arthroscopy of the knee for diagnostic work-up or for therapeutic reasons. Six patients with RA fulfilling the American College of Rheumatology (ACR) criteria [20], 6 patients with SpA fulfilling the European Spondyloarthropathy Study Group criteria [21] and 6 patients with osteoarthritis (OA) fulfilling the ACR criteria [22] were included in this study. All patients undergoing needle arthroscopy had active synovitis (RA and SpA) or joint effusion (OA) of the knee.

Preferably, patients did not receive DMARD at the time of tissue sampling. However, some patients received DMARD in the past but were not treated with DMARD at the time of or up to one month before tissue sampling. Reasons for discontinuation of previous DMARD were intolerance and non-compliance. Disease duration at the moment of tissue sampling varied from one month to more than 10 years. No significant differences existed in disease duration between the various groups studied.

The clinical characteristics of these patients are summarized in table 1. Within the SpA group, the precise clinical entities are indicated as well.

The study was conducted after approval by the local ethics committee. Written informed consent was obtained from all participating patients.

Table 1: Clinical data on the patients with RA, OA and SpA.*

Diagnosis	Patientn°	age (years)	sex	NSAID's	DMARD's	SJC	CRP (mg/dl)	ESR (mm/hour)	RFLF	Anti-CCP	Disease duration (years)
RA	25	70	M	-	-	5	8.9	77	10240	>1600	1
RA	9	51	F	+	-	9	2.2	41	160	143	10
RA	17	65	M	+	-	19	10.7	87	1280	>1600	5
RA	5	74	M	+	-	14	3.6	42	20480	>1600	3
RA	36	62	M	+	-	7	9.6	56	0	2	0.3
RA	29	29	F	+	-	8	3.3	45	160	1600	0.3
OA	20	54	M	+	-	2	0.1	3	0	2	0.1
OA	47	76	F	+	-	0	0.2	9	0	3	0.2
OA	64	62	F	-	-	1	1.4	25	0	3	6
OA	7	73	F	-	-	1	0.5	13	0	2	4
OA	19	60	M	+	-	0	0.2	3	0	1	35
OA	58	89	M	-	-	1	ND	ND	ND	1	20
USpA	55	45	F	+	-	1	0.9	18	0	3	0.3
USpA	23	21	M	+	-	4	4.2	39	0	5	0.1
USpA	27	48	M	-	+(a)	1	2.6	42	0	4	20
AS	41	59	M	+	-	2	4.0	30	0	4	25
USpA	50	57	M	+	-	2	0.6	4	ND	3	10
USpA	60	48	M	-	+(b)	1	1.7	8	0	0	2

*Except where mentioned otherwise: M = male; F = female; (+) = receiving; (-) = not receiving; NSAID's = nonsteroidal antiinflammatory drugs; DMARD's = disease-modifying antirheumatic drugs where (a) = Sulfasalazine, (b) = Imuran (Azathioprine). Treatment characteristics were determined at the time of tissue sampling. Patients were chosen preferably DMARD negative at the time of tissue sampling; RA = rheumatoid arthritis; SpA = spondyloarthropathy; OA = osteoarthritis; USpA = undifferentiated spondyloarthropathy; AS = ankylosis spondylitis; ND = no data available; SJC = swollen joint count; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; RFLF = rheumafactor latex fixation; anti-CCP = antibody against cyclic citrullinated protein determined by ELISA (Immunoscan RA, mark 2, Euro-diagnostica AB (Arnhem, The Netherlands)) with a cut-off of 25 U/ml; the disease duration displayed was determined at the time of tissue sampling.

Synovial biopsies

Synovial biopsy samples were obtained by needle arthroscopy [23]. Briefly, needle arthroscopy of the knee was performed under local anesthesia, using a 2.7mm Hopkins rodlens telescope (Storz, Tuttlingen, Germany). The joint cavity was carefully inspected and synovial membrane biopsy samples (8-10 specimens/patient) were obtained using a 2.7mm biopsy forceps (Storz, Tuttlingen, Germany). The biopsy samples were immediately snap frozen in liquid nitrogen and stored in liquid nitrogen until further analysis. In addition, 8 biopsy samples from each patient were stored in formaldehyde and embedded en bloc in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin for histological analysis.

Histological assessment

Stained sections were coded and analyzed by 2 independent observers who were blinded to the patient's clinical data. A validated semiquantitative scoring system for synovial tissue was used [4, 6, 24]. The analysis included all areas of the biopsy samples and a global semiquantitive score was given for each parameter (0-3 scale where 0=lowest expression and 3=highest). In cases of discordant scores, which differed by a maximum of 1 point, the mean of the 2 scores was used.

Histological evaluation of the paraffin sections included the mean synovial lining layer thickness (0 = 1-2 cell layers, 1 = 3-4 cell layers, 2 = 5-6 cell layers, and 3 = ≥ 7 cell layers), degree of vascularity of the sublining layer, degree of infiltration of the sublining layer, number of plasma cells and neutrophils, and number of lymphoid aggregates. Comparison of histological semi-quantitative scores was performed using the non-parametric Mann-Whitney U test. P values less than 0.05 were considered to be statistically significant.

Protein extraction

The protein content of the pooled synovial biopsy samples was extracted using the ReadyPrep Sequential Extraction Kit from Biorad (Hercules, CA, USA) according to the manufacturer's instructions. A cocktail of phosphatase inhibitors containing cantharidin, bromotetramisole, microcystin LR, sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole (Sigma, Steinheim, Germany) was added to each sample. Additional to endonucleases (Sigma, Steinheim, Germany), protease inhibitors for a broad range of serine, cysteine and metalloproteases, as well as calpain (Roche diagnostics, Mannheim, Germany) were also

added to each sample. Before protein extraction, synovial biopsy samples were transferred to an eppendorf and homogenized during 2 minutes using a Turrax homogenizer (Ika-Werke, Stanfer, Germany) in buffer I of the ReadyPrep Sequential Extraction kit (Biorad, Hercules, CA, USA) containing 40mM Trisbase. Protein concentrations were measured using the Coomassie Protean Reagent assay from Pierce (Rockford, USA) according to the guidelines provided by the manufacturer.

Two-dimensional gel electrophoresis

Fifty µg of total protein extract was precipitated with cold acetone overnight at –20°C. After centrifugation at 20000g for 5 minutes, the pellet was airdried. 2-DE was performed according to Görg A. *et al*. [25] with minor adjustments. Briefly, 50µg of the soluble protein extract of the synovial biopsy pool of each patient was dissolved in 360µl of rehydration buffer solution containing 7M urea, 2M thiourea, 4% CHAPS, 20mM DTT and 0.2% Carrier Ampholyte solution (Amersham Biosciences, Uppsala, Sweden). Once the pellet was completely dissolved, each sample was incorporated in a linear immobilized pH gradient strip (IPG) with a pH gradient of 3 up to 10 and rehydrated overnight [26]. After in-gel rehydration, the strips were iso-electrically focused on the Protean IEF cell (Biorad, Hercules, CA, USA) at 18°C, using 250V for 30 min (linear ramping), 500V for 1h (linear ramping), 1 kV for 1h (linear ramping), 3kV for 1h (linear ramping), rapid ramping to 10 kV in 3h and steady state at 10 kV for 45 kVh. After iso-electric focusing, the strips were equilibrated in two steps by gently shaking for 15 minutes in a solution containing TrisHCl buffer (50mM, pH 8.8), 6M urea, 20% v/w glycerol, 2% SDS. DTT (1.5% v/w) was added to the first, and 4% iodoacetamide to the second step. After equilibration, the IPG strips were placed on a polyacrylamide gel (12% T; 2.6% C) and run in sets of 6 gels in the vertical Protean II xi Multi Cell (Biorad, Hercules, CA, USA) at 16mA/gel for 30 minutes and 32mA/gel for approximately 4 hours and 30 minutes at 10°C. The gels were stained with Sypro Ruby (Biorad, Hercules, CA, USA) for 1 hour, according to manufacturer's guidelines.

Gel scanning and image analysis

2-D gels stained with Sypro Ruby were scanned and digitized by the ProXpress Imager from Perkin Elmer (Wellesley, MA, USA) using top illumination, maximum scanning area and a resolution of 100µm. Image exposure was set to 15 seconds. A yellow flatfield was used to correct the data for uneven illumination.

Digitized images were analyzed with PDQuest 2D-analysis Software v7.1 (Biorad, Hercules, CA, USA). Spots were detected by using the spot detection wizard. Background was removed by applying the floater background subtraction and horizontal and vertical streaks were also removed from the gels in this step. All gels were matched to each other creating a match set standard image containing the match information of all the gels.

Data analysis and statistical analysis

In order to compare protein expression levels across the 18 gels, the gels were corrected for small pipetting errors or minor staining differences. This was realized by normalizing the protein expression data of each gel for its total density in the gel image. Statistical analysis of the data was done according to Aliya A. A. *et al.* [27] and Tan F. L. *et al*. [28] by using SPSS v.11.5 software (SPSS inc., Chicago, Illinois, USA). Tests for normality and equal variances were performed in order to examine whether the data qualified for parametric (Student T-test) or non-parametric statistical tests (Mann Whitney U test). The majority of our data was not normally distributed (Shapiro-Wilk $p<0.05$) with equal variances (equal variance test, $p>0.05$). However, some protein expression levels showed a normal distribution (Shapiro-Wilk $p>0.05$) (data not shown). Therefore, data that were significantly different in both Mann Whitney U test ($p<0.01$) and Student T-test ($p<0.01$, after Log-transformation) were used as variables to perform hierarchical cluster analysis. The between-group linkage method and the Pearson correlation similarity measure were utilized for clustering analysis and the result of the cluster analysis was displayed in a dendrogram.

Protein identification by mass spectrometry

In-gel digestion with trypsin

Spots of interest were excised, from semi-preparative 2-D gels containing 200µg protein material, and digested with modified sequence grade trypsin (Porcine) (Promega, Madison WI, USA). Briefly, spots were cut into small pieces of +/- 1mm^3 and washed with 400µl 50%ACN/50mM NH_4HCO_3 for 15 minutes. After the buffer was removed, the gel pieces were dehydrated by adding 100µl of 100% ACN and rehydrated in 15µl 25mM NH_4HCO_3 containing 10ng/µl sequence grade modified trypsin (Promega, Madison WI, USA) for 30 minutes on ice. In-gel digestion with trypsin was continued over night at 37°C. Peptides were extracted in 60%ACN/5%TFA, completely dried and resuspended in 10µl 0.1% TFA.

Identification by Maldi Q-Tof

A peptide solution of 1.5µl was mixed 1:1 with matrix solution (2mg/ml recrystilized α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN/50% 0.1%TFA) and 1µl was spotted onto the Maldi target plate. On-target desalting was performed by adding 4µl of Milli-Q purified water to each spot. The data were acquired on Maldi Q-Tof (Waters, Milford, USA) using Data Dependent Analysis software. Processing and database searching of the data against the Swiss-prot database was performed by ProteinLynx Global Server v2.0 software (PLGS) (Waters, Milford, USA).

Identification by ESI mass spectrometry

The remaining peptide solution (8.5µl) was injected on a on-line RP-HPLC system using column switching (LC Packings, Sunnyvale, CA, USA) coupled to a Q-Tof I mass spectrometer (Waters, Milford, USA) fitted with an orthogonal Z-spray [29]. The data were acquired using the Automatic Function Switching software. Fragmentation spectra, resulting from tandem mass spectrometry were searched against the mascot search engine (http://www.matrixscience.com).

Real-time RT-PCR

TRIzol (Life Technologies, Paisley, U.K.) was added to synovial biopsy samples of OA (n=10), RA (n=9) and SpA (n=10) patients. The total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Before reverse transcription, digestion of DNA was performed using the RNase-Free DNase Set (QIAGEN). cDNA was synthesized with oligo(dT) as primer using the Superscript II kit (Life Technologies). Primers for the housekeeping enzymes GAPDH and cyclophylin A were designed using the Primer Express software (Applied Biosystems, Foster City, CA). For GAPDH, primers were TCC TCT GAC TTC AAC AGC GAC A (sense) and GTG GTC GTT GAG GGC AAT G (antisense). For cyclophylin A, primers were AAG CAT GTG GTG TTT GGC AA (sense) and CCA TTC CTG GAC CCA AAG C (antisense). Amplification of cDNA with housekeeping enzymes primer pairs was monitored using the SYBRGreen I chemistry. Assays on Demand (Applied Biosystems, Foster City, CA) were used for the amplification of Calgranulin A (Hs000374264_g1). Amplification and on-line detection of PCR products were carried out with optical 96-well plates (Applied Biosystems, Foster City, CA) in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City,

CA). mRNA expression levels were calculated according to Vandesompele J. *et al* . [30]. Statistical analysis was performed using Student T-test.

RESULTS

Protein extraction of synovial biopsy tissue and 2-DE

Extraction of the cytosolic proteome from synovial biopsy tissue (wet weight: 41,001 ± 21,84mg) yielded an average cytosolic protein concentration of 1116,43 ± 468,93µg/ml x 1ml. The cytosolic proteome of synovial biopsy tissue of 18 patients was analyzed. A total amount of 50µg of cytosolic proteins was subjected to 2-DE using IPG strips pH 3-10.

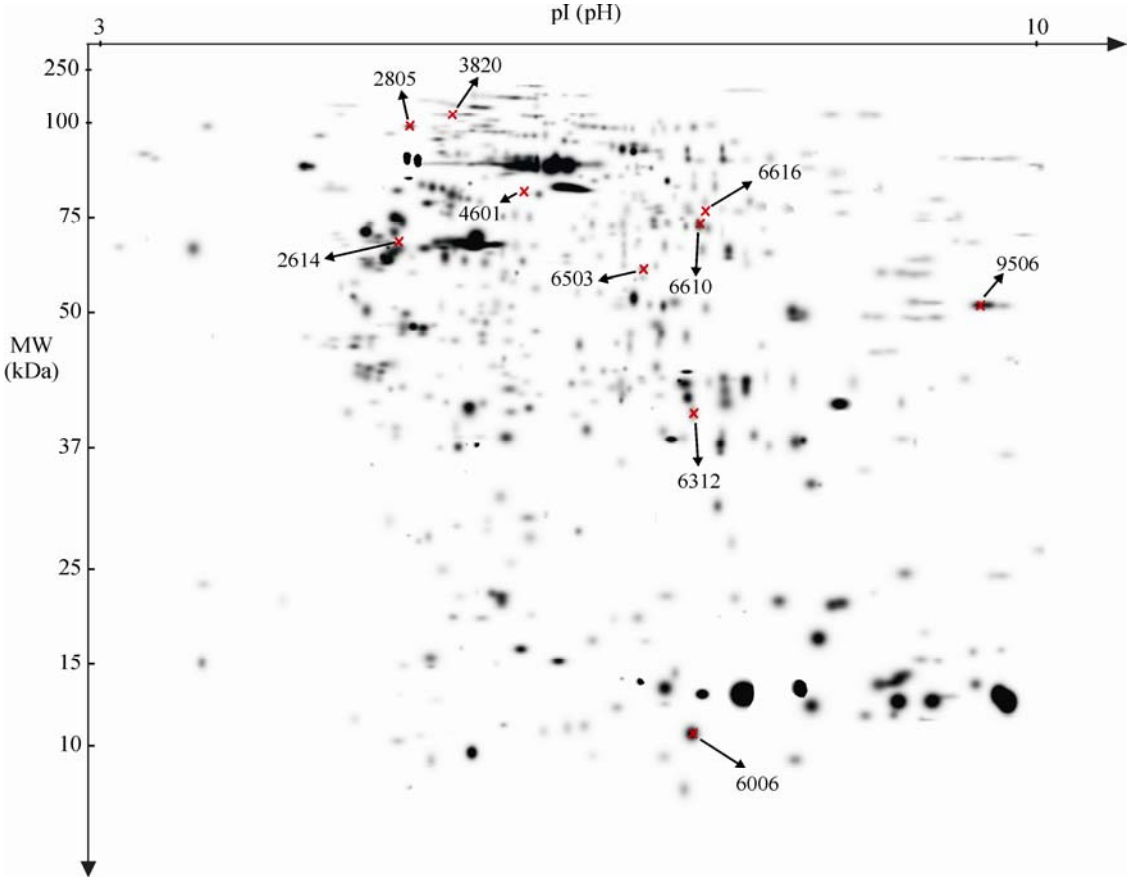


Figure 1: Match set standard of the cytosolic proteome of synovial tissue in RA, OA and SpA.
Spots that were identified are indicated by their corresponding spot number.

Gels were visualized, scanned and digitized as described in the section patients and methods. A data set of 18 gels was analyzed. Six gels originated from RA patients, 6 gels from patients with SpA and 6 gels were obtained from OA patients. Spot detection and matching of these

gels was done using the automated detection and matching dialog in PDQuest v.7.1 (Biorad, Hercules, CA, USA). After manual editing, a match set was created. In a match set, the protein spots from different gels are matched to each other and are included in a synthetic image, called a match set standard. This match set standard is originally based on a real raw data gel and this gel is arbitrarily chosen. It is preferably the gel with the most spots and the least streaking on it, which in our case was the gel of patient SPA55. Although the standard is based on a real raw data gel, it was extended to a complete artificial composite image containing all the matched information of the gels in the analysis. Spots that were matched to at least two other members of the match set, but were absent from the initial standard, were also added to the match set standard. This artificial composite image contains all the information the software needs to analyze the gels quantitatively, qualitatively or statistically. A total of 640 spots were present in the artificial composite image (Fig.1). The way, in which the match set standard is chosen, extended and transformed to a complete artificial image, implies that the initial selection of another gel as match set standard eventually leads to the same results.

Analysis of protein expression levels by hierarchical clustering analysis

We evaluated whether the expression levels of all the spots matched in this 2-D analysis could be used for hierarchical cluster analysis. A total of 640 spots were separated by 2-DE and matched between the gels. This entire match set was used for clustering analysis. The resulting dendrogram is displayed in figure 2.

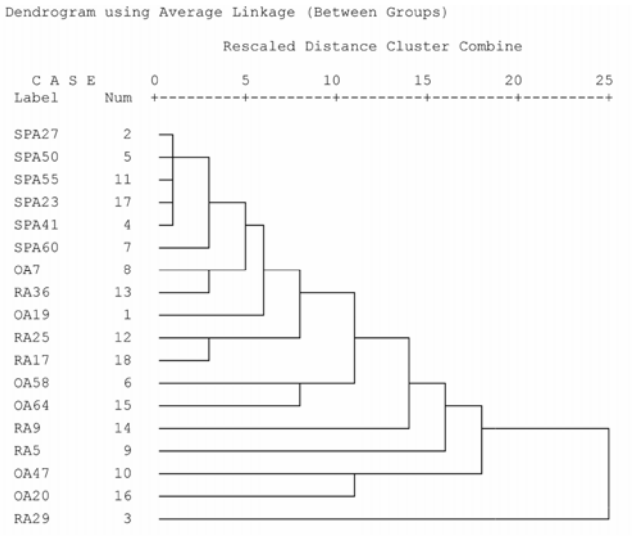


Figure 2: Hierarchical cluster analysis of entire match set using 640 variables.
The dendrogram revealed a complete segregation of SpA from RA and OA.

All patients diagnosed with SpA clustered together. According to the clustering, patient SPA60 is less related to the rest of the SpA patients, but still joins the cluster early. Clustering was also observed between patient RA25 and RA17, both RA patients and less strongly between patient OA58 and OA64, both OA patients. Also patients OA47 and OA20, patients both diagnosed with OA, clustered together.

Thus, hierarchical clustering on the entire match set remarkably segregated the SpA as a group from the patients diagnosed with RA or OA. However, no distinctive clustering was seen between OA and RA as a group.

If an unselected data set of spots does not result in accurate clustering, the use of a defined subset of differentially expressed proteins between the groups could improve the clustering of these groups [27]. Therefore, we next evaluated the use of statistically differentially expressed protein expression levels for hierarchical cluster analysis. We analyzed our match set statistically, using both a parametric (Student T-test after Log-transformation, $p<0.01$) and a non-parametric test (Mann Whitney U test, $p<0.01$) for statistical analysis of our data since our data set was comprised of both normally and not normally distributed data. In addition, by requiring significance for both tests, this approach minimizes the likelihood of false positives [27,28]. Using this statistical approach 24, 16 and 5 spots were significantly ($p<0.01$) differentially expressed between RA and SpA, SpA and OA, and RA and OA, respectively. A flowchart demonstrating the experimental and statistical approach is displayed in figure 3.

In order to explore the possibility to segregate between RA and OA, the 5 spots that were statistically differentially expressed ($p<0.01$) between both diseases were used as variables for hierarchical cluster analysis. The dendrogram represented in figure 4(a) showed 6 very closely related RA patients segregating from the 6 OA patients that were divided in two sub clusters.

Subsequently, we investigated whether using the spots that were differentially expressed between SpA and RA (n=24) as variables would allow to obtain improved clustering features upon hierarchic cluster analysis. Interestingly, this analysis revealed complete segregation between RA and SpA. The resulting dendrogram represented in figure 4(b) displayed two major branches (a) and (b). Branch (a) clusters 6 closely related RA patients, whereas branch (b) combines 3 related SpA patients with 3 SpA patients that are more distant.

Likewise, statistical analysis between SpA and OA revealed 16 spots to be expressed differentially. Hierarchical cluster analysis of these expression levels resulted in almost a complete segregation between SpA and OA (data not shown).

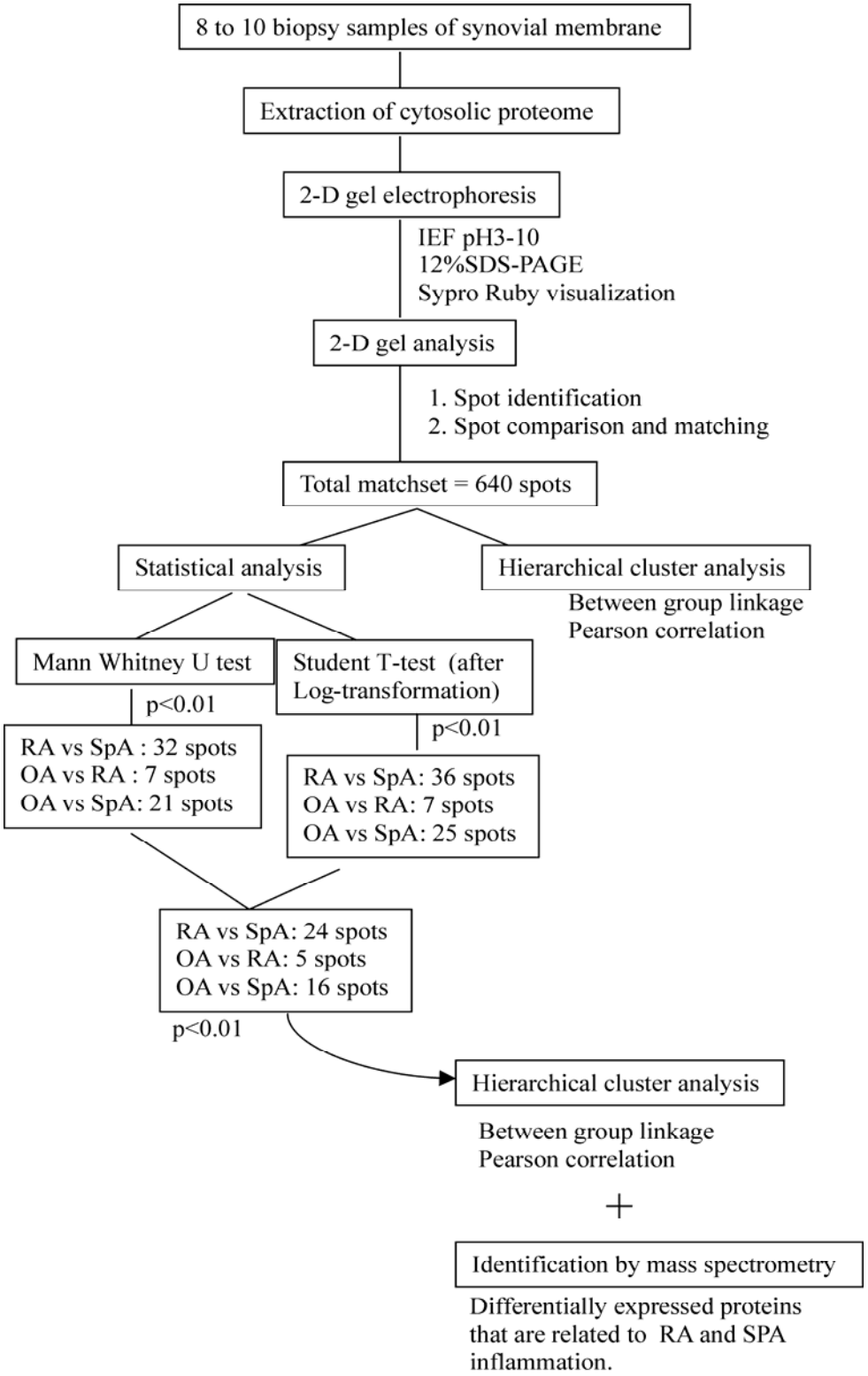


Figure 3: Flowchart of experimental and statistical design. Five, 16 and 24 spots are the subsets of differentially expressed proteins in the cytosolic synovial tissue proteome between OA and RA, OA and SpA and RA and SpA, respectively, which were further subjected to hierarchical cluster analysis. Some of these spots were selected for identification by mass spectrometry. These subset of selected spots were obtained by determining protein spots that significantly differed in both the non-parametric Mann Whitney U test and the parametric Student T-test (after Log transformation) ($p<0.01$).

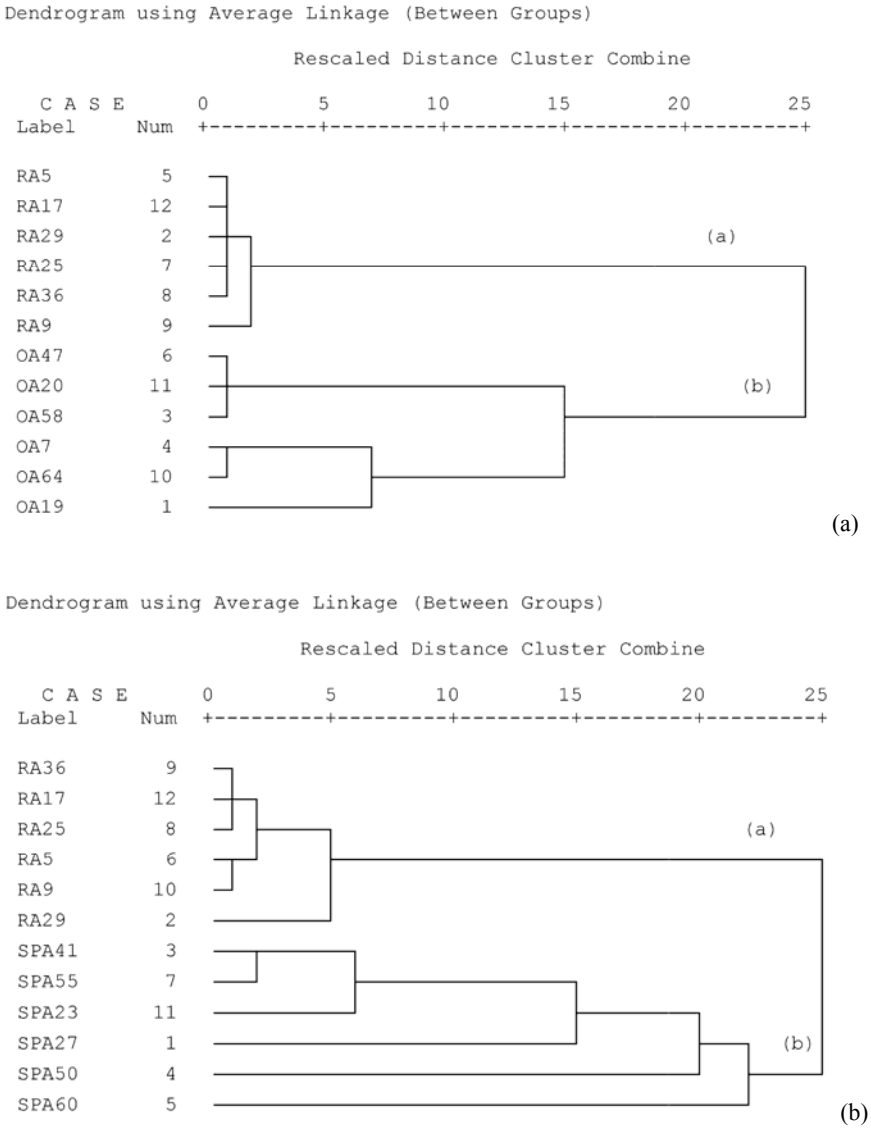


Figure 4: Hierarchical cluster analysis on a subset of protein expression levels that are statistically differentially expressed (both in Mann Whitney U test and Student T-test, after Log-transformation, $p<0.01$) between two groups studied.

(a) Hierarchical cluster analysis of protein spots which are significantly differentially expressed between RA and OA (n= 5 spots). The dendrogram separated into two clusters segregating 6 closely related RA patients from 6 OA patients.

(b) The dendrogram of the hierarchical cluster analysis of protein expression levels statistically differently expressed between RA and SpA (n=24 spots), revealed complete segregation of RA from SpA

Histological evaluation and protein expression pattern

While the above mentioned results indicated that cytosolic protein profiles from RA synovial tissue could be segregated from OA and SpA, it remained possible that these differences could be attributed to differences in the synovial microarchitecture. To examine this, we performed a histological assessment with hematoxylin-eosin staining of synovial tissue using

semiquantitative scoring methods as described earlier. All RA and SpA patients suffered from active synovitis for which they underwent needle arthroscopy.

As previously described, synovial tissue of both patient groups showed increased thickness of the synovial lining layer and infiltration of inflammatory cells. Whereas in large patient cohorts some significant differences were observed in synovial tissue from RA versus SpA patients in routine histological assessment (6) by hematoxylin-eosin staining, no statistical significant differences were observed in the presently studied patient cohort (data not shown). Nevertheless, marked differences in protein expression patterns were observed as described above. These findings clearly indicate that the observed differences in protein expression in this group of patients can not be solely attributed to changes in synovial histology. As a non-inflammatory control, a group of OA patients suffering from joint effusion in the knee was also included. No signs of inflammation could be observed in OA synovial tissue. This resulted in some significant differences between RA and SpA synovial tissue in this patient cohort (data not shown).

Identification of differentially expressed proteins

We identified proteins that were related to either RA or SpA inflammation in the synovial tissue. Spots that fulfilled this criterion were spots that were statistically differentially expressed either between RA and OA or SpA and OA. In addition the spots should show a higher expression level in the inflammatory arthritis in comparison to OA. Five spots were statistically different between RA and OA (fig. 3). However, only one spot (6006) showed a higher expression level (>10-fold) in RA than in OA. In the group of spots that were statistically differentially expressed between SpA and OA (n=16), 15 spots were overexpressed in SpA in comparison to OA.

In summary, 16 spots of which 1 spot was related to RA inflammation and 15 spots were related to SpA inflammation, were subjected to tandem mass spectrometry. We unambiguously identified 10 proteins as these were the most abundantly expressed and yielded the most peptides for protein identification by ESI and MALDI mass spectrometry (table 2 – fig. 1).

We investigated whether these proteins were previously described being related to inflammation in general or inflammatory rheumatoid diseases, like RA and SpA. Literature searches revealed several papers in which these proteins were related to inflammation. The references are indicated in table 2.

Table 2: Identification of spots of which the protein expression level is statistically significantly different between RA and OA or SpA and OA*.

spots upregulated in RA in comparison with OA

SSP	Identification	OA av	RA av	Maldi MS	Maldi MSMS	ESI MSMS
6006	Calgranulin A[33-39]	0,46 (0/6)	860,96 (6/6)	12	1	1

spots upregulated in SpA in comparison with OA

SSP	Identification	OA av	SPA av	Maldi MS	Maldi MSMS	ESI MSMS
2614	Vimentin[45-47]	0,46 (0/6)	506,88 (6/6)	49	0	26
4601	Protein disulfide isomerase A3 prec.	0,46 (0/6)	151,78 (5/6)	18	1	3
6312	Triosephosphate isomerase[48,49]	0,46 (0/6)	549,36 (5/6)	12	1	4
6610	Alpha-enolase[41]	133,61(1/6)	1253,38 (6/6)	10	2	2
6616	Glutamate dehydrogenase 1	0,46 (0/6)	248,08 (4/6)	25	0	1
9506	Fructose bisphosphate aldolase A[40, 42-44]	86,78 (1/6)	1072,21 (5/6)	12	1	1
2805	Endoplasmin precursor[50-54]	0,46 (0/6)	526,95 (6/6)	39	1	5
3820	Ceruloplasmin[55,56]	0,46 (0/6)	118,05 (5/6)	18	0	1
6503	Creatine kinase M chain[57-58]	0,46 (0/6)	499,55 (6/6)	24	2	2

*'SSP' shows the number of the spot on fig.1; 'av' is the average expression level of the protein spot in OA or SpA; The number of patients in which a particular spot was detected is shown between brackets; 'Maldi MS' indicates the number of peptides used to identify the protein by peptide mass fingerprinting; 'Maldi MSMS' describes the number of amino acid sequences obtained from Maldi-Q-TOF used for identification; 'ESI MSMS' refers to the amount of amino acid sequences obtained from ESI-Q-TOF used for independent identification of the proteins by Mascot database searching; the references displayed between brackets next to the identification of the protein show papers found in the literature describing a role for the identified protein in inflammation or more specific in inflammatory arthritis like RA or SpA.

Real-time RT-PCR

To further establish de significance of the protein expression level profiles of calgranulin A, better known as MRP-8, a real-time RT-PCR experiment was conducted on a separate cohort of 29 patients (see section patients and methods). Results showed statistically higher mRNA expression level of MRP-8 in inflamed synovial tissue of RA (average mRNA expression of

MRP-8 (normalized to the geometric means of 2 household genes; GAPDH and CyclophilinA) in RA patients ± SD = 1,04 ± 0,99) in comparison with mRNA expression levels of MRP-8 in OA patients (0,31 ± 0,29) on p=0.04 using Student T-test. The average mRNA expression level of MRP-8 normalized to the geometric means of GAPDH and Cyclophilin A in SpA patients is 0,37 ± 0,39 (fig.5).

These data are in line with the protein expression profiles of MRP-8 and indicate local synthesis of MRP-8 in inflamed synovial tissue.

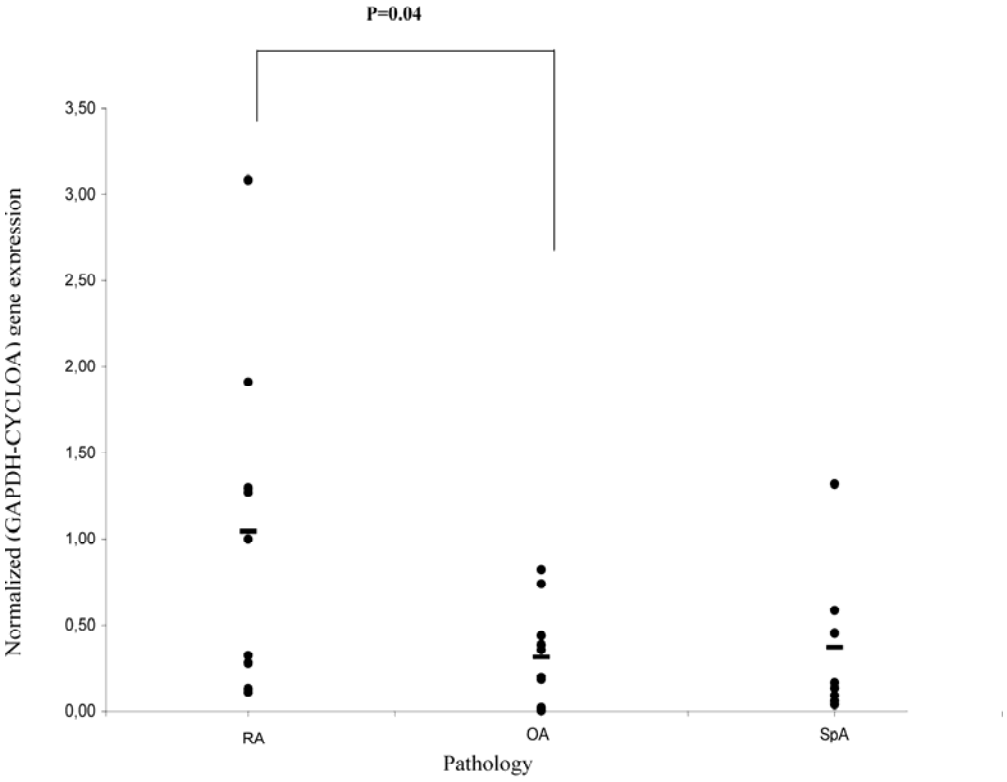


Figure 5: Gene expression of MRP8 in synovium of RA, OA and SpA patients.
Gene expression levels are normalized with GAPDH and Cyclophilin A. (°) represents the gene expression of MRP8 in the individual patients. (-) shows the average gene expression of MRP8 in the group. Gene expression levels of MRP8 in RA and OA patients showed a significant difference on $p<0.05$.

DISCUSSION

Proteomics has become increasingly popular to identify disease-associated proteins in a variety of human diseases, particularly in the study of human cancers [31, 32]. The identification of proteins with distinct expression in a diseased state may improve our

understanding of the pathogenesis of a given disease and may potentially result in the identification of novel therapeutic targets.

We performed a differential screening of the synovial protein expression of cytosolic proteins between RA, SpA and OA. High resolution 2-DE in combination with Sypro Ruby staining was used to separate, quantify and analyze the cytosolic proteome of the synovium obtained from synovial biopsy tissue of 18 patients, divided in 3 groups of 6 patients diagnosed with RA, SpA and OA respectively. Quantification of protein spots using Sypro Ruby was preferred over silver staining. Although it is as sensitive as silver staining, it is much more reliable to use for quantification given its large linear range of staining [33]. Silver staining by contrast is impeded by rapid saturation of the spots.

Utilizing synovial tissue biopsies for proteomics enabled us to examine the primary site of inflammation and to select a patient cohort of clinically good defined patient samples. In fact, all RA and SpA patients had clinical signs of synovitis, and the majority of them did not receive DMARD's (Table 1). We incorporated routine histological assessment to exclude potential differences attributed to alterations in synovial microarchitecture.

In a first approach, we used the complete match set of 640 spots to perform a hierarchical cluster analysis on all patient samples (Fig 2). Interestingly, all the patients of the SpA group clustered together, despite the presence of both USpA and AS in the patient group. This could indicate that the nature of inflammation is quite homogenous in SpA. In this initial approach, no clear segregation was observed between RA and OA. Therefore, we explored whether using a subset of differentially expressed proteins as variables for hierarchical cluster analysis would improve the obtained clustering features. We therefore analyzed our data set statistically (Fig. 3) and used spots that were significantly differentially expressed ($p<0.01$) between two groups studied as variables for cluster analysis, as previously described by Aliya A.A. *et al*. [27].

Five spots were significantly differentially expressed between RA and OA. The resulting dendrogram of the hierarchical clustering of the protein expression levels of these proteins revealed complete differentiation between RA and OA (fig. 4(a)). Likewise, using the 24 spots that were statistically different between RA and SpA, complete segregation between these two inflammatory arthritides was accomplished (fig. 4(b)). This approach was also applied to the 16 spots that were different between OA and SpA. Hierarchical cluster analysis of these protein expression levels resulted in a dendrogram that almost completely differentiated between the two groups studied (dendrogram not shown).

The obtained distinct clusters between RA and SpA do not necessarily imply that the use of 2-DE on synovial tissue extracts would be suitable for diagnostic purposes, which would require validation in a separate patient cohort. This however, was beyond the scope of the present study. Rather, the data indicate that this approach is useful to identify proteins implicated in the pathogenesis of chronic inflammatory rheumatic diseases such as RA and SpA.

Next, we identified proteins that were related to RA or SpA inflammation. These proteins, which were statistically differentially expressed between RA and OA or SpA and OA and demonstrated an up regulated expression level in inflammatory arthritides (table 2 – fig. 1), were subjected to identification by tandem mass spectrometry. Several of these proteins have clearly been implicated in chronic arthritis such as calgranulin A, fructose bisphosphate aldolase A and alpha-enolase.

Calgranulin A also known as myeloid related protein-8 or MRP-8, was found to be higher expressed in all RA patients and in 3 SpA patients. By contrast, this spot (6006) was below the detection limit in the OA group. The expression of this protein is specific for cells of myeloid origin, namely granulocytes, monocytes and macrophages. It is known that in chronic inflammation, the infiltrating macrophages express MRP-8 [34]. Moreover, MRP-8 is specifically released during the interaction of monocytes with inflammatory activated endothelium, probably at the site of inflammation [35]. MRP-8 levels are found increased in serum, synovial fluid and synovium of PsA, RA and SpA patients [36]. A detailed study on MRP8 expression between RA and SpA was conducted by De Rycke L. *et al*. [37], in which they revealed a local presence of MRP8 in the synovial sub lining layer of mononuclear cells in both RA and SpA patients. Calgranulin A was also identified as 'biomarker' in a cyphergen experiment using synovial fluid of RA and OA patients [38]. In addition, a microarray analysis of peripheral blood mononuclear cells of SpA, RA, PsA and OA patients shows that MRP-8 is higher expressed in SpA, RA and PsA patients in comparison with OA patients [39]. Real-time RT-PCR analysis of synovial tissue of 29 patients showed a statistically higher expression of MRP-8 in RA patients in comparison with OA patients on $p=0.04$. These mRNA expression profiles were in line with the observed protein expression profiles of MRP-8 in inflamed synovial tissue and indicate a local synthesis of this protein.

Alpha-enolase (6610) and fructose bisphosphate aldolase A (muscle type) (spot 9506) are two enzymes playing a major role in the glycolysis. Antibodies against these two proteins have been found in the sera of especially RA patients [40, 41]. Upregulation of aldolase A has been described in proliferating cells [42], which may suggest that higher expression of this protein in patients with inflammatory arthritides could be related to the proliferating synovial lining.

Aldolase A binds to actin-containing filaments of the cytoskeleton, more specifically to F-actin [43] and could play a role in the structure of the cytoplasm. It may even contribute to metabolic regulation and cell motility. Interestingly, F-actin plays an important role in joint fibroblasts determining the normal synovial microvascular resistance to fluid filtration. F-actin disruption has been described as a potential factor in the link between inflammatory mediators and the formation of arthritic joint effusions [45]. The expression of aldolase A is regulated by TNF-α in vitro and it is known that microbial infections or tissue invasion can induce in the host a systemic inflammatory response that is frequently associated with increased glucose metabolism [46].

Vimentin (2614), a major structural component of the intermediate filaments in many cell types, is found highly expressed in fibroblasts and it is stated that there is some expression in T and B lymphocytes [Swissprot]. It is shown that vimentin plays an important role in vital mechanism and biological functions such as cell contractility, migration and proliferation [45]. Vimentin is also a growth related gene and is often expressed when epithelial cells are stimulated to proliferate by serum or growth factors. In cancer, vimentin expression is associated with dedifferentiated malignant phenotype, increased motility and invasive ability, drug resistance and poor clinical prognosis [46]. It could be possible that a higher expression of vimentin contributes to the invasive character of the synvovial pannus like tissue in inflammatory arthritides.

Ceruloplasmin (3820), a copper-binding glycoprotein is higher expressed in synovial tissue of SpA patients in comparison with OA patients. This protein has been shown to be up regulated in serum and synovial fluids of patients with inflammatory arthritides [47]. The higher expression of ceruloplasmin in synovial tissue could be the result of active diffusion of ceruloplasmin from serum and synovial fluid into the synovial tissue. It is known that the synovial permeability in the knee joint of patients with inflammatory arthritis if significantly higher than in patients suffering of OA [48]. In contrast, ceruloplasmin mRNA expression has been found in invasive tumor tissue and has been discussed playing a role or being associated with tumor invasion and metastasis [49]. Moreover, the expression of ceruloplasmin is regulated by the pro-inflammatory IL1-β in rat glioma cells [50]. Although, there is no data on mRNA expression of ceruloplasmin in synovial tissue, it could be that this protein plays a larger role in invasiveness of synovial tissue in joint of inflammatory arthritides than is currently known.

In conclusion, the results of this study indicate that the use of expression levels of proteins derived from 2-DE is a useful approach to investigate the inflammation behind different complex rheumatic pathologies. The cytosolic synovial proteome of SpA as opposed to RA patients consists of a unique set of proteins with a defined expression pattern for each form of chronic synovitis.

Altogether, this proteomics analysis may significantly increase our understanding of the molecular biology of inflammation of these diseases.

ACKNOWLEGDMENTS:

We thank Dr. Leen De Rycke for performing the needle arthroscopy and Dr. Dominique Baeten and Dr. Elli Kruithof for the histological evaluation of the synovium biopsy tissue samples.

This work was supported by FWO and BOF grants from the University of Ghent.

REFERENCES

[1] Wright, V., *Arthritis Rheum*. 1978, *21*, 619-33.

[2] Zeidler, H., Mau, W., Khan, M. A., *Rheum. Dis. Clin. North Am*. 1992, *18*, 187-202.

[3] FitzGerald, O., Soden, M., Yanni, G., Robinson, R., Bresnihan, B., *Ann. Rheum. Dis.* 1991, *50*, 792-6.

[4] Smeets, T. J., Dolhain, R. J., Breedveld, F. C., Tak, P. P., *J. Pathol*. 1998, *186*, 75-81.

[5] Tak, P. P., Smeets, T. J., Daha, M. R., Kluin, P. M., *et al*. *Artritis Rheum.* 1997, *40*, 217-25.

[6] Baeten, D., Demetter, P., Cuvelier, C., Van-Den-Bosch F., *et al*. *Ann. Rheum. Dis.* 2000, *59*, 945-53.

[7] Baeten, D., Peene, I., Union, A., Meheus, L., *et al*. *Arthritis Rheum*. 2001, *44*, 2255-62.

[8] Baeten, D., Steenbakkers, P. G., Rijnders, A. M., Boots, A. M., *et al*. *Arthritis Rheum*. 2004, *50*, 444-51.

[9] Futcher, B., Latter, G. I., Monardo, P., McLaughlin, C. S., Garrels, J. I. *Mol. Cell Biol*. 1999, *19*, 7357-68.

[10] Jiang, Z. H., Wu, J. Y., *Proc. Soc. Exp. Biol. Med*. 1999, *220*, 64-72.

[11] Jackson, R. J., Standart, N., *Cell* 1990, *62*, 15-24.

[12] Sheets, M. D., Fox, C. A., Hunt, T., Vande Woude, G., Wickens, M., *Genes Dev*. 1994, *8*, 926-38.

[13] Kontoyiannis, D., PaSpArakis, M., Pizarro, T. T., Cominelli, F., Kollias, G., *Immunity* 1999, *10*, 387-98.

[14] Pandey, A., Mann, M., *Nature* 2000, *405*, 837-46.

[15] Robinson, W. H., Steinman, L., Utz, P. J., *Arthritis Rheum*. 2002, *46*, 885-93.

[16] Wang, H., Kachman, M. T., Schwartz, D. R., Cho, K. R. , Lubman, D. M., *Electrophoresis* 2002, *23*, 3168-81.

[17] Michener, C. M., Ardekani, A. M., Petricoin, E. F., 3rd, Liotta, L. A., Kohn, E. C., *Cancer Detect Prev.* 2002, *26*, 249-55.

[18] Daly, M. B., Ozols, R. F., *Cancer Cell* 2002, *1*, 111-2.

[19] Ardekani, A. M., Liotta, L. A., Petricoin, E. F. 3rd., *Expert Rev. Mol. Diagn.* 2002, *2*, 312-20.

[20] Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., *et al*., *Arthritis Rheum*. 1988, *31*, 315-24.

[21] Dougados, M., van-der-Linden, S., Juhlin, R., Huitfeldt, B., *et al*., *Arthritis Rheum*. 1991, *34*, 218-27.

[22] Altman, R., Asch, E., Bloch, D., Bole, G., *et al*., *Arthritis Rheum*. 1986, *29*, 1039-49.

[23] Baeten, D., Van-den-Bosch, F., Elewaut, D., Stuer, A., *et al*., *Clin. Rheumatology* 1999, *18*, 434-41.

[24] Kraan, M. C., Haringman, J. J., Ahern, M. J., Breedveld, F. C., *et al*. *Rheumatology* 2000, *39*, 43-9.

[25] Görg, A., Obermaier, C., Boguth, G., Harder, A., *et al*., *Electrophoresis* 2000, *21*, 1037-53.

[26] Rabilloud, T., Valette, C., Lawrence, J. J., *Electrophoresis* 1994, *15*, 1552-8.

[27] Alaiya, A. A., Franzen, B., Hagman, A., Dysvik, B., et al., *Int. J. Cancer* 2002, *98*, 895-9.

[28] Tan, F. L., Moravec, C. S., Li, J., Apperson-Hansen, C., et al., *PNAS* 2002, *99*, 11387-92.

[29] Vanhoutte, K., Van Dongen, W., Hoes, I., Lemiere, F., *et al*., *Anal. Chem*. 1997, 3161-8.

[30] Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., *et al*., *Genome biol.* 2003, RESEARCH0034.

[31] Petricoin, E. F., Zoon, K. C., Kohn, E. C., Barrett, J. C., Liotta, L.A., *Nat. Rev. Drug Discov*. 2002, *1*, 683-95.

[32] Bichsel, V. E., Liotta, L. A., Petricoin, E. F. 3rd., *Cancer J.* 2001, *7*, 69-78.

[33] Lopez, M. F., Berggren, K., Chernokalskaya, E., Lazarev, A., Robinson, M., Patton, W.F., *Electrophoresis* 2000, *21*, 3673-83.

[34] Odink, K., Cerletii, N., Bruggen, J., Clerc, R. G., *et al*., *Nature* 1987, *330*, 80-82.

[35] Frosch, M., Strey, A., Vogl, T., Wulffraat, N. M., *et al*., *Arthritis Rheum.* 2000, *43*, 638-37.

[36] Kane, D., Roth, J., Frosch, M., Vogl, T., *et al*., *Arthritis Rheum*. 2003, *48*, 1676-85.

[37] De Rycke, L., Baeten, D., Foell, D., Veys, E. M., *et al*., *Arthritis Rheum.* 2003, *48*, S1196.

[38] Uchida, T., Fukawa, A., Uchida, M., Fujita, K., Saito, K,. J. Proteome Res. 2002, *1*, 95-9.

[39] Gu, J., Märker-Herman, E., Baeten, D., Tsai, W. C., *et al*., *Rheumatol*. 2002, *41*, 759-766.

[40] Ukaji, F., Kitajima, I., Kubo, T., Shimizu, C., Nakajima, T., Maruyama, I., *Ann. Rheum. Dis.* 1999, *58*, 169-74.

[41] Saulot, V., Vittecoq, O., Charlionet, R., Fardellone, P., *et al*., *Arthritis Rheum*. 2002, *46*, 1196-201.

[42] Gautron, S., Maire, P., Hakim, V., Kahn, A., *Nucleic Acids Res*. 1991, *19*, 767-74.

[43] Poli, A., Scott, D., Bertin, K., Miserocchi, G., Mason, R. M., Levick, J. R., *Microvasc. Res.* 2001, *62*, 293-305.

[44] Benigni, F., Atsumi, T., Calandra, T., Metz, C., *et al*., *J. Clin. Invest*. 2000, *106*, 1291-300.

[45] Wang, N., Stamenovic, D., *Res. Cell Motil*. 2002, *23*, 535-40.

[46] Yoon, W. H., Song, I. S., Lee, B. H., Jung, Y. J., *et al*., *Cancer Lett*. 2004, *203*, 99-105.

[47] Cogalgil, S., Tayi, S., *Ann. Clin. Lab. Sci*. 2002, *32*, 264-70

[48] Pejovic, M., Stankovic, A., Miltrovic, D. R., *Br. J. Rheumatol*. 1995, *34*, 520-4

[49] Kluger, H. M., Kluger, Y., Gilmore-Hebert, M., DiVito, K., Chang, J. T., *et al*., *Lab. Invest*. 2004, *84*, 320-31

[50] Bonaccorsi di Patti, M. C., Persichini, T., Mazzone, V., Polticelli, F. Colasanti, M., Musci, G., *Neurosci. Lett*. 2004, *363*, 182-186

Chapter 2

Caspase cleaved fragments of vimentin are citrullinated in synovial tissue of inflammatory arthritides and show autoantibody reactivity rheumatoid arthritis.

Kelly Tilleman[1]
Dirk Elewaut[2]
Tineke Cantaert[2]
Filip De Keyser[2]
Dieter Deforce[1]

[1] Laboratory for Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium
[2] Department of Rheumatology, University Hospital Ghent, Ghent, Belgium

Sumitted to Journal of Immunology on 04/09/2006

ABSTRACT

The presence of autoantibodies in the sera of patients diagnosed with inflammatory arthritis, like rheumatoid arthritis (RA), reflects the humoral autoimmune processes occurring during this pathology. Many autoantibodies directed against a variety of autoantigens have been described in RA, amongst them the Sa antigen. Although this antigen has been elucidated as citrullinated vimentin, there is an apparent lack of information on these modified forms in synovial tissue.

In this present report we investigated the characteristics of citrullinated vimentin isoforms in cytosolic protein extracts obtained form inflamed synovial tissue. On two-dimensional gel electrophoresis of cytosolic synovial tissue extracts, vimentin was visualized as a specific cluster of spots. Interestingly, our results indicate that these isoforms are probably the result of caspase cleavage. In addition, these cleaved forms of vimentin were found to be citrullinated in synovial cytosolic protein extracts of RA patients, contrary to SpA where the presence of citrullinated vimentin appeared to be limited. Furthermore, the presence of autoantibodies against these citrullinated processed forms of vimentin was found to be highly specific for RA patients.

These findings indicate a novel concept in the development of autoantibodies in RA by which humoral autoimmunity is targeted against caspase cleaved and citrullinated fragments of an intermediate filament in synovial tissue, in a highly disease specific manner.

INTRODUCTION

Rheumatoid arthritis (RA) and Spondyloarthropathies (SpA) are two frequent forms of inflammatory arthritis which are both characterized by synovitis, the chronic inflammation of the synovial membrane. As a result, synovial hyperplasia, neovascularisation and the infiltration of lymphocytes and macrophages eventually leads to the transformation of the synovial membrane into an invasive and destructive tissue, called the pannus [1]. Although there are some differences in synovial histopathology between RA and SpA [2], the molecular differences between RA and SpA synovial inflammation remain poorly understood.

The presence of autoantibodies in the sera of patients diagnosed with inflammatory arthritis reflects the humoral autoimmune processes occurring during these pathologies. Many autoantibodies directed against a variety of autoantigens have been described [3], amongst them the Sa antigen (named after the patients' name who began with 'Sa' where specific autoimmunity was described using this patients' serum) [4]. Although, Vossenaar E.R. *et al*. showed that anti-Sa antibodies targeted citrullinated epitopes of vimentin [5], information on in vivo citrullinated vimentin, in particular, in synovial tissue extracts is scarce.

Vimentin, an intermediate filament which is abundantly expressed in synovial fibroblasts [6], has long been thought to solely play a role in the stability of the cytoplasmic architecture [7]. It is now known, that vimentin is a highly dynamic protein whose assembly and disassembly is regulated by phosphorylation [8, 9]. In addition, its function stretches far beyond being part of the cytoskeleton. This was elucidated by Eckes B. *et al*. who observed impaired wound healing in vimentin$^{-/-}$ mice by a failure of mesenchymal contraction [10].

More recently, studies indicate a possible role for vimentin in inflammation, since fragments of this protein can be secreted by activated macrophages during inflammation [11]. Secretion of vimentin was induced by TNF-α and this extracellular vimentin seemed necessary for efficient killing of bacteria [11]. Cell surface expression of vimentin peptides has also been observed by neutrophils undergoing spontaneous apoptosis [12]. The possible consequence of secreting or presenting vimentin or fragments of this protein is the development of auto-antibodies against this intermediate filament. Indeed, the presence of anti-vimentin autoantibodies in autoimmune diseases like RA has been demonstrated. Citrullinated vimentin has been identified to be the Sa antigen in RA [5]. Although the presence of citrullinated vimentin has been reported in monocytes, macrophages and synovial fluid mononuclear cells [13], there are no reports that indicate that the antigen itself is present in protein extracts of synovial tissue.

In the present study, we further explore the characteristics of vimentin isoforms in 2-D images of inflamed synovial tissue. Our data show that the isoforms could be the result of caspase-3 cleavage of vimentin. In addition, evidence is shown for the presence of citrullinated vimentin fragments in cytosolic protein extracts obtained from synovial tissue biopsy samples derived from patients with inflammatory arthritis. Finally, development of autoantibodies against these modified vimentin fragments was found to be highly specific for RA.

PATIENTS AND METHODS

Patients

Synovial tissue biopsy samples were obtained from patients undergoing needle arthroscopy of the knee [14] for diagnostic work-up or for therapeutic reasons. Patients with RA fulfilling the American College of Rheumatology (ACR) criteria [15], patients with SpA fulfilling the European Spondyloarthropathy Study Group criteria [16] and patients with knee osteoarthritis (OA) fulfilling the ACR criteria [17] were included in this study. All patients undergoing needle arthroscopy had active synovitis (RA and SpA) or joint effusions (OA) of the knee.
Serum samples were collected from 6 RA and 6 SpA patients.
The clinical characteristics of these patients are summarized in table 1. The study was conducted after approval by the local ethics committee. Written informed consent was obtained from all participating patients. Detailed information on the samples used in each of the experiments is given in table I.

Protein extraction

The cytosolic proteins (hereafter also referred to as the soluble proteins) were extracted from the synovial tissue samples using the ReadyPrep Sequential Extraction Kit from Biorad (Hercules, CA, USA) according to the manufacturer's instructions. A cocktail of phosphatase inhibitors containing cantharidin, bromotetramisole, microcystin LR, sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole (Sigma, Steinheim, Germany) was added to each sample. Endonucleases (Sigma, Steinheim, Germany), protease inhibitors for a broad range of serine, cysteine and metalloproteases, as well as calpain (Roche diagnostics, Mannheim, Germany) were also added to each sample. Before protein extraction, synovial biopsy samples were transferred to an Eppendorf tube and homogenized during 2 minutes

using a Turrax homogenizer (Ika-Werke, Stanfer, Germany) in buffer I of the ReadyPrep Sequential Extraction kit (Biorad, Hercules, CA, USA) containing 40mM Trisbase. Protein concentrations were measured using the Coomassie Protean Reagent assay from Pierce (Rockford, USA) according to the instructions provided by the manufacturer.

Table I: Clinical data on synovial biopsy samples and serum samples.

Diagnosis	Date of birth	Sex	NSAID's	DMARD's	Biologicals	SJC	CRP (mg/dl)	ESR (mm/hour)	Anti-CCP (U/ml)	Disease duration (years)
Synovium										
RA966	23/9/53	F	+	+[(a)]	+[(d)]	1	0.2	5	960	24
RA969	31/12/59	F	+	-	+[(d)]	5	ND	31	608	18
RA947	27/11/34	F	+	-	+[(e)]	1	3.2	65	1600	25
RA882	21/2/68	M	+	+[(a)]	+[(d)]	5	4.7	90	328	4
RA658	4/9/31	F	+	+[(a)]	+[(e)]	4	ND	ND	ND	12
RA130	28/1/51	M	+	-	-	11	16.4	71	1775	3
RA208	29/3/46	M	+	-	-	3	0.8	16	2	1
RA234	11/7/24	F	+	+	-	3	0.1	4	2	6
RA262	21/2/35	M	+	+	-	1	4.9	39	1600	15
RA482	22/4/64	F	+	-	-	0	3.2	46	828	0.3
RA571	18/9/37	F	+	+	-	2	1.6	38	101	6
SpA524	27/12/46	M	+	+[(b)]	-	1	9.9	21	2	10
SpA350	4/2/51	F	+	+[(b)]	-	3	5.2	69	1	0.17
SpA511	7/2/32	M	-	-	-	3	6	40	2	5
SpA713	28/7/52	F	+	-	-	3	0.1	7	1	4.5
SpA142	4/1/46	F	+	-	-	2	0.3	7	1	9
SpA673	26/6/81	F	+	+	-	2	0.5	6	1	3
SpA720	14/8/77	M	+	-	-	1	0.2	2	2	0.58
SpA958	19/1/88	F	+	-	-	4	1.9	18	1	0.17
SpA1085	17/2/83	M	+	-	-	1	11	103	ND	0.06
OA162	16/3/57	M	-	-	-	1	0.3	2	3	15
OA315	14/5/55	F	+	-	-	2	0.3	14	ND	15
OA377	21/1/32	F	+	-	-	1	0.4	33	2	0.17
OA768	14/6/33	F	+	-	-	2	1.9	37	2	7
OA19	19/9/43	M	+	-	-	0	0.2	3	1	35
OA58	8/9/14	M	-	-	-	1	ND	ND	1	20
OA7	2/10/30	F	-	-	-	1	0.5	13	2	4
OA1	26/9/41	F	-	-	-	1	1.4	25	3	6
Sera										
RA812[(*)]	29/9/34	F	+	+[(a)]	-	2	3.2	31	484	9
RA709[(*)]	2/12/51	M	+	+[(a)]	+[(e)]	8	0.1	1	687	13
RA597[(*)]	4/9/31	F	+	+[(a)]	+[(e)]	3	4.3	ND	1600	12
RA562[(*)]	17/3/52	F	+	+[(a)]	-	12	1.3	33	871	19
RA53328[(§)]	6/8/1959	F	_	-	+[(f)]	0	0.4	3	10	11
RA70105[(§)]	2/6/1966	F	+	+[(a)]	-	0	1	13	2	6
RA74579[(§)]	8/7/1944	M	+	+[(c)]	-	0	0.8	15	>1600	8
RA55625[(§)]	7/5/1935	M	+	+[(a)]	-	2	0.9	17	706	4
RA75378[(§)]	8/9/1947	F	+	+[(a)]	-	0	2	39	312	8
RA69841[(§)]	24/1/1938	M	+	+[(b)]	-	0	1.5	34	1600	12
SpA59200[(§)]	11/2/1967	M	-	-	+[(e)]	0	ND	ND	11	24

SpA67965[(§)]	28/5/1982	M	-	+[(b)]	-	0	0	1	19	8
SpA53861[(§)]	24/8/1949	M	+	-	-	3	1.1	20	11	5
SpA66508[(§)]	14/4/1955	M	+	-	-	1	0.1	2	1	4
SpA78973[(§)]	20/7/1970	F	+	-	-	0	0.2	15	2	13
SpA80598[(§)]	19/3/1987	M	+	-	-	1	0.4	10	2	3
SpA27[(*)]	11/10/55	M	+	+[(b)]	-	1	2.6	42	ND	22
SpA182[(*)]	20/2/37	M	+	-	+[(g)]	4	0.1	1	ND	3
SpA194[(*)]	27/3/58	F	+	-	-	1	0.4	7	ND	14
SpA111[(*)]	13/4/79	F	+	-	+[(g)]	0	0.6	22	ND	6

Except where mentioned otherwise: M = male; F = female; (+) = receiving; (-) = not receiving; (ND) = no data available; NSAID's = nonsteroidal antiinflammatory drugs; DMARD's = disease-modifying antirheumatic drugs where (a)= methotrexate and (b)= sulfasalazine, (c)= D-penicilamine,; Biologicals = biological modifiers where (d) = Adalimumab, (e)= Infliximab, (f)= Leflunomide ,(g)= etanercept. Patients RA709 and RA562 also received corticosteroids (prednisolon). Treatment characteristics were determined at the time of tissue sampling; RA = rheumatoid arthritis, OA = osteoarthritis, USPA = undifferentiated spondyloarthropathy, PsA = psoriatic arthritis, AS = ankylosis spondylitis, SLE = systemic lupus erythematosus; ND = no data available; SJC = swollen joint count; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; anti-CCP = antibody against cyclic citrullinated peptides determined by ELISA (Immunoscan RA, mark 2, Euro-diagnostica AB (Arnhem, The Netherlands) according to the manufacturer's instructions with a cut-off value of 25U/ml; the disease duration displayed was determined at the time of tissue sampling. Synovium samples of the same pathology were either pooled together or used separately for gel electrophoresis; serum samples indicated by [(*)] and [(§)] were used for 1-D or 2-D immunoblotting experiments as specifically indicated in the text.

Gel electrophoresis

1-dimensional gel electrophoresis

10µg of protein extract was diluted with sample buffer containing 0.5M TrisHCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and incubated at 95°C for 5 minutes. The samples were subsequently subjected to SDS-PAGE (10%, Laemmli) at 150V for 30min followed by 200V for 1hour. For visualization of citrullinated proteins 50µg of soluble protein extract was loaded on the SDS-PAGE. 1-D gels were further prepared for Western Blotting.

2-Dimensional gel electrophoresis

For 2-D analysis, 50µg of soluble synovial protein extract was precipitated with cold acetone overnight at –20°C. After centrifugation at 20000xg for 5 minutes, the pellet was air dried. 2-DE was performed as described before (IPG 3-10 (17cm) + 12% SDS-PAGE (Laemmli)) [18]. The gels were stained with Sypro Ruby (Molecular probes, Eugene, OR, USA) and analyzed by PD Quest software (Biorad, Hercules, CA, USA).

For 2-D immunoblotting experiments, 40µg soluble protein extract was prepared as described above and incorporated in a lineair IPG pH4-7 (11cm) strip (Biorad, Hercules, CA, USA) and rehydrated overnight. After in-gel rehydration, the strips were iso-electrically focused on the Protean IEF cell (Biorad, Hercules, CA, USA) at 18°C, using 100V for 30 min (linear ramping), 250V for 30 min (linear ramping), 500V for 1h (linear ramping), 1 kV for 1h (linear ramping), rapid ramping to 8kV in 2h and steady state at 8 kV for 25 kVh. After iso-electric

focussing, the IPG strips were equilibrated as described before [18], and placed on a Laemmli 10% (29:1) resolving polyacrylamide gel and run in sets of two at 150V for 30 minutes followed by 60 minutes at 200V.

Western Blotting

Protein transfer onto nitrocellulose membranes (Biorad, Hercules, CA, USA), was performed by tank blotting using the Trans-Blot Cell (Biorad, Hercules, CA, USA) at 50V for 3h. Prior to tank blotting, both the polyacrylamide gels and nitrocellulose membranes were incubated in 1x CAPS (pH=11) for at least 15 minutes.

Ponceau S visualization of the membranes was conducted to check the blotting efficiency, and to verify if the 2-DE was done successfully. Blocking was performed for 1 hour, using PBS/0.3% Tween-20 and followed by probing the membrane with patient sera (1/100 in PBS/0.3% Tween-20) overnight. Immune reactive spots were detected using HRP labelled goat anti-human IgG (Pierce, Rockford, IL, USA) (1/5000 in PBS/0.3%Tween-20) and visualized by enhanced chemiluminescent (ECL) using the Supersignal West Dura Extended Duration Substrate from Pierce (Rockford, IL, USA). Blots were stripped using the Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA) at 37°C for 30 min and reincubated with blocking buffer for 1h. Sufficient stripping was confirmed by reprobing the membrane with secondary antibody and subsequent detection by ECL.

Vimentin was visualized using mouse anti-human vimentin anti-body diluted 1/400 in PBS/0.3% Tween-20 (overnight incubation) (Clone V9, Sigma, St. Louis, MI, USA). The vimentin cluster was visualized by ECL following incubation with HRP labelled rabbit anti-mouse IgG (Pierce, Rockford, IL, USA) (1/1000 dilution in PBS/0.3% Tween-20) for 1h.

For the detection of citrullinated proteins, blots were first stripped as described above and subsequently chemically modified prior to immunostaining using the anti-modified citrulline (AMC) detection kit (Upstate, Charlottesville, VA, USA) as indicated by the manufacturer's instructions.

Protein patterns were scanned and digitized using the VersaDoc Imaging System (Biorad, Hercules, CA, USA) and band detection analysis was performed using Quantity One Analysis Software (Biorad, Hercules, CA, USA). Bands were represented as Gaussian models where the Gaussian model trace, representing the area under the profile curve calculated in pixel intensity x millimetres (INT x mm), was used to quantify the bands.

For all Western Blot experiments, negative controls were included in which the nitrocellulose membranes were incubated solely with the secondary antibody. In addition, to determine

disease specific autoantibody reactivity, blots were incubated with a pool of serum obtained from 4 healthy individuals.

Protein identification by mass spectrometry

Spots of interest were excised from the gel and digested with modified sequence grade trypsin (Porcine) (Promega, Madison WI, USA) and analyzed on a Q-TOF Ultima mass spectrometer (Waters, Milford, USA) using MALDI or ESI as previously described [18]. Processing and database searching of the data against the Swiss-prot database was performed by ProteinLynx Global Server v2.2.5 software (PLGS) (Waters, Milford, USA) or manually using MASCOT search engine (http://www.matrixscience.com).

In vitro caspase-3 cleavage of vimentin

Human recombinant vimentin (Progen Biotechnik GmbH, Heidelberg, Germany) was cleaved by human recombinant caspase-3 (Chemicon International Inc., Temecula, CA, USA) overnight at 37°C at 1U/1µg vimentin.

RESULTS

Processed vimentin is observed as a specific pattern on 2-D images of soluble inflammatory synovial tissue protein extracts

Analysis of the soluble synovial tissue proteome of inflammatory arthritides revealed a rather specific pattern on the acidic side of the 2-D gel image. This figure consisted of different forms of the protein vimentin and this was confirmed by mass spectrometry (table II - fig. 1). We focussed on two vimentin protein isoform spots, further referred to as VIM_1 and VIM_2 and indicated on figure 1. Detailed examination of the mass spectrometric data revealed a clear difference between the higher MW form VIM_1 and VIM_2, which was found at a lower MW. For the identification by ESI-Q-TOF of VIM_2, no peptides were retrieved within the first 100 amino acids of the protein sequence (fig. 2A) in contrast with the identification of VIM_1 (fig. 2B).

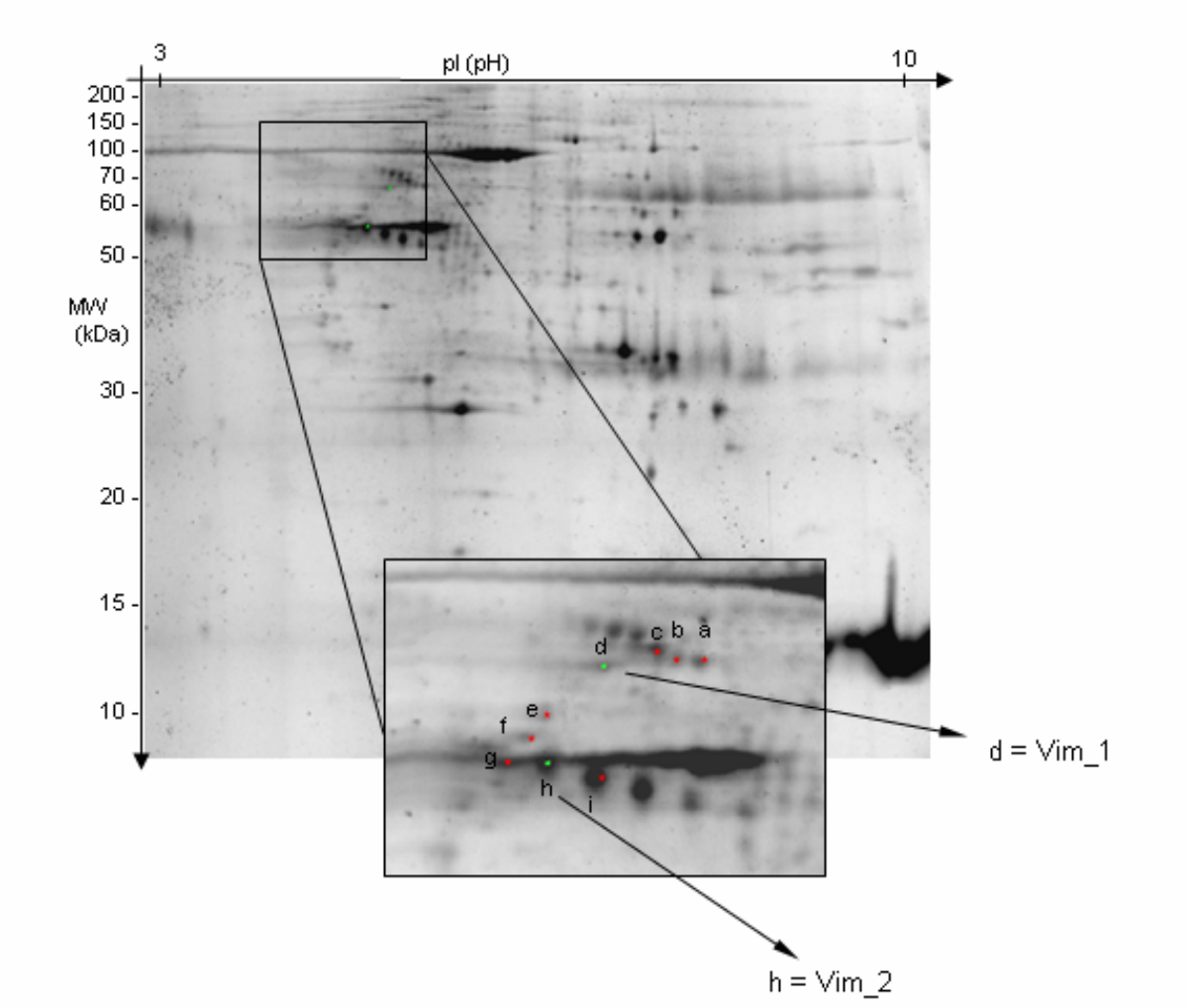


Figure 1: Raw 2-D image of the cytosolic proteome of synovial tissue obtained from a knee biopsy sample of a patient diagnosed with SpA.
The vimentin cluster is organized in a specific figure on the 2-D image of the synovial tissue soluble protein extract. Spots that were analyzed by mass spectrometry are indicated from (a) to (i). The green spots represent VIM_1 and VIM_2.

In addition, MALDI-Q-TOF identification of these isoforms confirmed this data. The peptide mass fingerprint (PMF) responsible for the identification of VIM_1 showed peptides derived from the first 100 amino acids with high ion count intensity (>50% of total ion count), contrary to the PMF for VIM_2, where these peptides had very low ion count intensity (<20% of total ion count) or were absent from the mass spectrum in multiple analyses (data not shown).

Table II: Identification of processed vimentin isoforms obtained soluble synovial tissue extracts analyzed by 2-D gel electrophoresis.

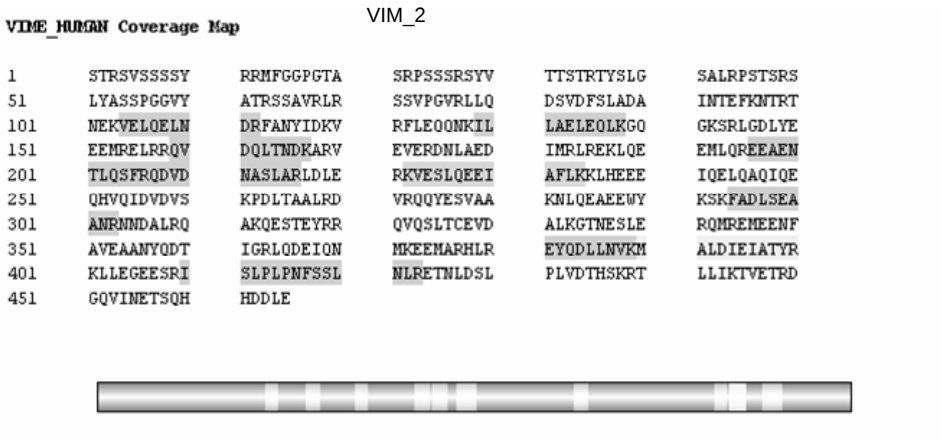
Spot (fig.1)	**Name**	**Peptide sequence matches by ESI-Q-tof**	**Sequence coverage (%) by Maldi-Q-tof**
a	VIME_human	(K)VELQELNDR(F) (R)EEAENTLQSFR(Q) (R)EYQDLLNVK(M) (R)ISLPLPNFSSLNLR(E)	21.1%
b	VIME_human	(K)ILLAELEQLK(G)	-
c	VIME_human	(K)VELQELNDR(F) (K)MALDIEIATYR(K)	14.6%
d	VIME_human	(K)FADLSEAANR(N) (K)VELQELNDR(F) (R)EYQDLLNVK(M) (K)ILLAELEQLK(G) (R)ISLPLPNFSSLNLR(E (R)SLYASSPGGVYATR(S) (R)EEAENTLQSFR(Q)	38.5%
e	VIME_human	(K)VELQELNDR(F) (K)ILLAELEQLK(G) (R)QVDQLTNDK(A) (R)EEAENTLQSFR(Q) (R)QDVDNASLAR(L) (R)ISLPLPNFSSLNLR(E) (K)FADLSEAANR(N) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (R)KVESLQEEIAFLK(K)	-
f	VIME_human	(K)VELQELNDR(F) (R)EEAENTLQSFR(Q) (K)ILLAELEQLK(G) (R)QDVDNASLAR(L) (R)ISLPLPNFSSLNLR(E) (K)FADLSEAANR(N) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (K)VESLQEEIAFLK(K)	-
g	VIME_human	(K)VELQELNDR(F) (R)EEAENTLQSFR(Q) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (R)ISLPLPNFSSLNLR(E)	16.3%
h	VIME_human	(K)VELQELNDR(F) (K)ILLAELEQLK(G) (R)QVDQLTNDK(A) (R)EEAENTLQSFR(Q) (R)QDVDNASLAR(L)	52.04%

		(R)ISLPLPNFSSLNLR(E) (K)FADLSEAANR(N) (R)EYQDLLNVK(M) (K)MALDIEIATYR(K) (R)KVESLQEEIAFLK(K)	
i	VIME_human	(K)MALDIEIATYR(K) (R)EYQDLLNVK(M) (R)DNLAEDIMR(L)	-

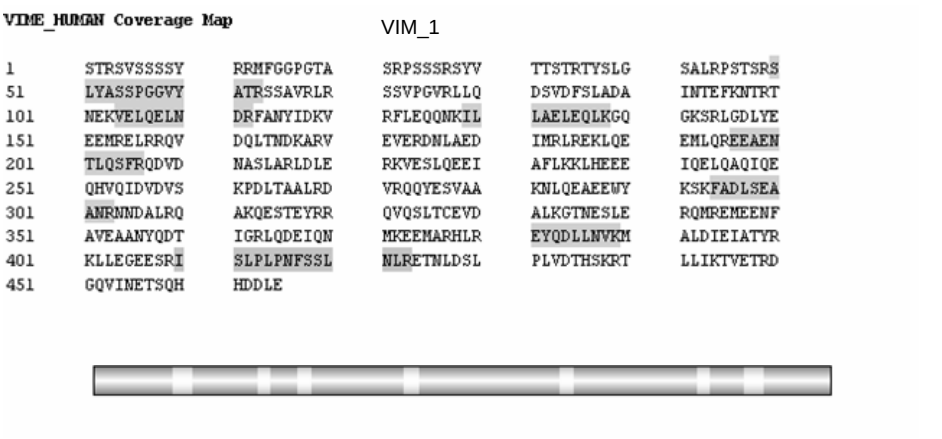
'Spot' resembles the letter indicated on figure 1; 'Name' indicates the protein name of vimentin described in the protein identification format of the Swiss-Prot database (http://www.expasy.org/); Peptides identified by ESI tandem mass spectrometry and protein coverage obtained by peptide mass fingerprinting are shown; protein spots that were not analyzed by maldi are indicated as '-'.

Vimentin has a defined structure consisting of a head, starting from the second amino acid up to amino acid 95, a body containing the coils 1A, 1B and coil 2 and a tail (amino acid 408-466) (fig. 2C) [19]. The mass spectrometric data clearly showed that the N-terminal head (comprising of the first 95 amino acids) of vimentin form VIM_2 was absent (fig.2A).

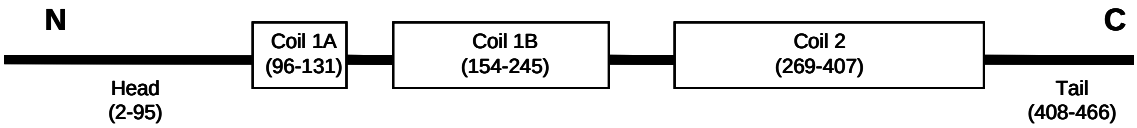
We reasoned that this may reflect protein processing. It is known that vimentin is a substrate for caspase cleavage [20]. Indeed, caspase-3 can cleave vimentin at amino acid 85, cleaving the head from the body of the protein resulting in a cleaved form of vimentin with theoretical MW=44.5kDa and pI= 4.75. This form would appear +/- 9kD lower than the native form which has a MW of 53.5kDa and would shift towards the acidic side on a 2-D gel as the native form has a pI of 5.06. When comparing these theoretical characteristics with the experimental data obtained from the 2-D gel (table III, fig. 1), in addition to the mass spectrometric data, we can assume that VIM_2 is a caspase-3 cleaved form of vimentin.



(A)



(B)



(C) (obtained from Fujita J. *et al.*)

Figure 2: Mass spectrometric identification of the high and low MW form of vimentin.
For the low MW form of vimentin (VIM_2), there were no peptides identified that were present in the first 100 amino acids of the protein sequence (A). Identification of the high MW form (VIM_1) indicates presence of peptides derived from the N-terminal head of the protein, represented by the first 100 amino acids of the protein (B). The data shown are obtained from ESI-Q-TOF MSMS analysis and are processed by ProteinLynxServer v2.2.5 (waters, Milford, USA). The protein work pad obtained from MALDI MS analysis showed the same results (not shown). Representation of the structure of vimentin describing its head, body and tail is shown (C).

Table III: Theoretical and experimental 2-D characteristics of vimentin isoforms

	Full length (Vim_1)	Cleaved (Vim_2)	$\Delta_{(VIM\_1 - VIM\_2)}$
pI_{th} (pH)	5.06	4.75	0.31
MW_{th} (Da)	53520.19	44534.56	8985.63
pI_{exp} (pH)	5.00	4.63	0.37
MW_{exp} (Da)	57300	49300	8000

'pI_{th}' and 'MW_{th}'= the theoretical isoelectric point and molecular weight calculated from the amino acid sequence of the full length protein as indicated in the Swissprot database; 'pI_{exp}' and 'MW_{exp}'= the experimental isoelectric point and molecular weight obtained from the position on the 2-D gel; '$\Delta_{(VIM\_1 - VIM\_2)}$' = difference in characteristics between VIM_1 and VIM_2.

In vitro caspase-3 cleavage of vimentin

In order to confirm these data, *in vitro* cleavage of vimentin with caspase-3 was analyzed by 1-DE and 2-DE. Vimentin cleavage by caspase-3 gives raise to a fragment at approximately 48kDa on SDS-PAGE [20]. Therefore, 1-DE analysis was performed in order to verify the caspase-3 cleavage. This indeed showed the appearance of a vimentin isoform at MW=48kDa (fig. 3B (P)).

The 2-D image showed several cleavage products. However, they did not show a dramatic shift towards the acidic pI of the IPG strip (data not shown). Nevertheless, the fragments were located at the correct MW, at approximately 48kDa. An explanation could be that the *in vivo* cleavage forms of vimentin bear an additional modification, making these products more acidic. Citrullination, a modification that changes an arginine to citrulline, is known to shift proteins towards a more acidic pI [21]. This possibility was explored by anti-modified citrulline (AMC) staining of the soluble synovial proteome.

Citrullinated vimentin in soluble protein extracts of inflammatory arthritides

Pooled synovial tissue extract of 4 RA patients (658 – 882 – 966 – 947) and 4 SpA patients (713 – 524 – 511 – 350) was analyzed by 2-DE. The proteins were transferred to nitrocellulose membranes and the vimentin cluster was first visualized. Protein citrullination was subsequently analyzed by AMC staining.

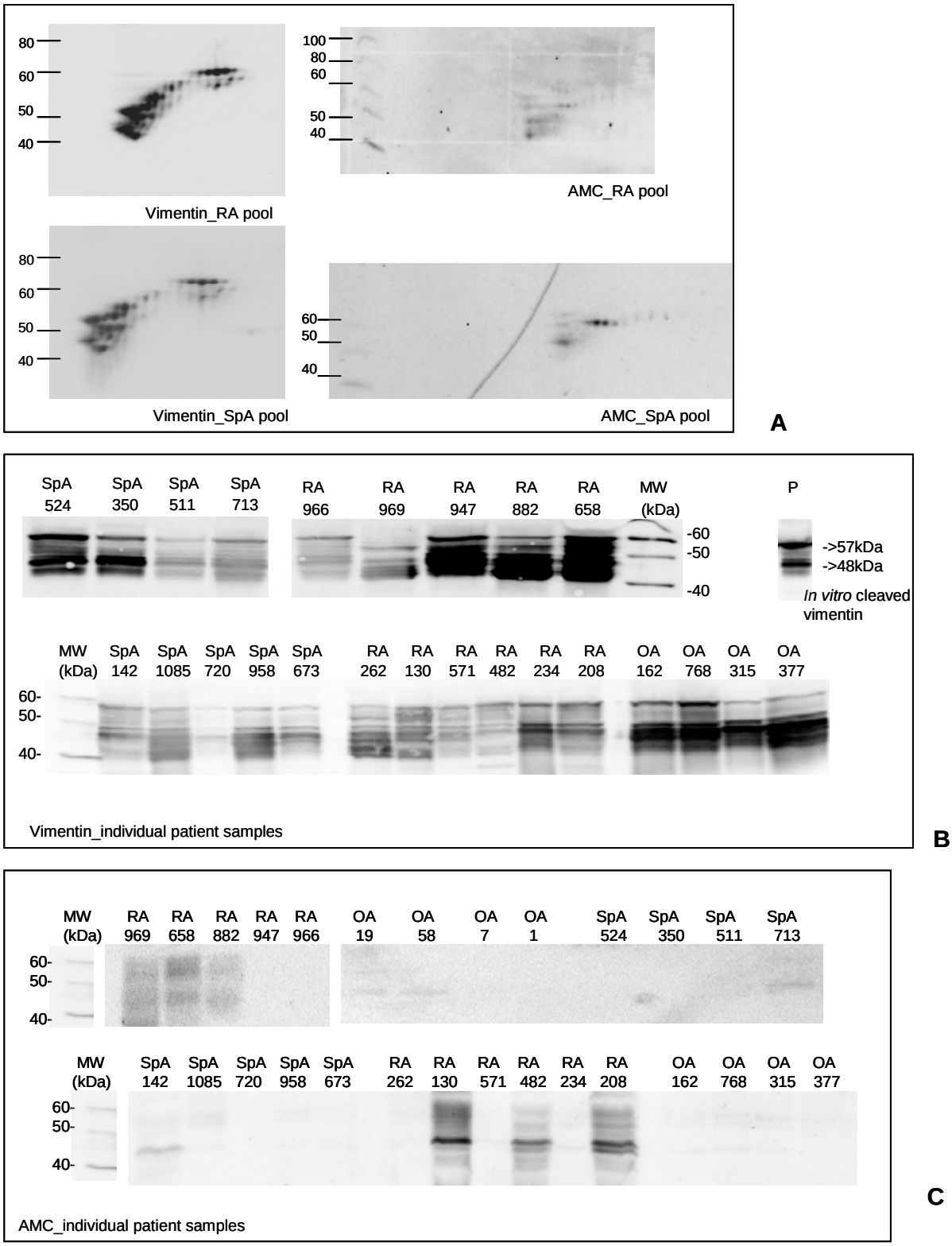


Figure 3: Detection of processed citrullinated vimentin in soluble synovial proteins in RA, SpA and OA

40µg of a pool of soluble synovial tissue protein extract was subjected to 2-DE (IPG 4-7, 10% SDS-PAGE) and transferred to nitrocellulose membrane. The vimentin cluster was first visualized in RA and SpA (panel A, left) after which the blot was stripped and citrullinated proteins were detected in RA and SpA using the AMC staining (panel A, right).

Individual patient protein extracts were analyzed by western blot on which vimentin (panel B) and citrullinated proteins were detected (panel C). *In vitro* capase-3 cleavage of vimentin was run as a positive control for the appearance of the 48kDa fragment (panel B, (P)).

The vimentin cluster was nicely visualized in both RA and SpA (fig. 3, panel A, left). We observed citrullination of vimentin fragments in both RA and SpA (fig. 3, panel A, right). However, the citrullinated vimentin fragments in RA appeared at a MW lower than 50kDa, whereas in SpA, no citrullinated vimentin fragments were seen below 50kDa.

This experiment was extended in order to verify patient variability. Therefore, cytosolic protein extracts of RA, SpA and OA patients were subjected separately to SDS-PAGE followed by Western Blotting. The vimentin fragments were visualized in all pathologies (fig. 3, panel B). Processed isoforms of vimentin were present in all pathologies. Strong AMC staining (Gaussion Model Trace of most intense band > 1000 INT x mm) was observed in 50% of the RA patients, weak detection in soluble protein extracts of SpA patients (Gaussion Model Trace of most intense band >300 INT x mm) and none in OA patients (Gaussion Model Trace of most intense band <100 INT x mm) (fig. 3, panel C).

Autoantibody reactivity of in vivo synovial citrullinated vimentin fragments

Citrullinated vimentin has been described as a potential autoantigen in RA; therefore autoantibody reactivity was examined by immunoblotting.

In a first experiment, we analyzed individual protein extracts on SDS-PAGE and incubated the blots with a pool of RA sera and SpA sera. A strong autoantibody reactivity against numerous cleavage forms of vimentin was observed in RA patients (fig. 4, panel A). The proteins that reacted with the serum were found at MW<50kDa. In SpA patients, the only immune reactive bands appear around 52kDa, which were artefacts since they were also found on blots incubated only with the secondary antibody (fig. 4, panel A (BL)).

Next, we analyzed the potential patient variability in autoantibody reactivity. Therefore, incubations with individual serum samples (table 1 '§') were analyzed on 2-D blots containing a pool of soluble synovial protein extracts of RA (969 – 882 – 966 – 947). In order to verify the autoantibody reactivity to the exact forms of cleaved vimentin, the blots were landmarked with narrow lines. After serum incubation, the nitrocellulose membranes were stripped and reprobed with anti-human vimentin in order to visualize the vimentin cluster. Using the landmarks, exact positioning of the autoimmune spots within the vimentin cluster was possible. These spots were indicated by '+' on the image. For easy interpretation of the data, the position of the vimentin cluster is indicated on each blot displaying the autoimmune reactivity of the patient's sera (fig. 4, panel B).

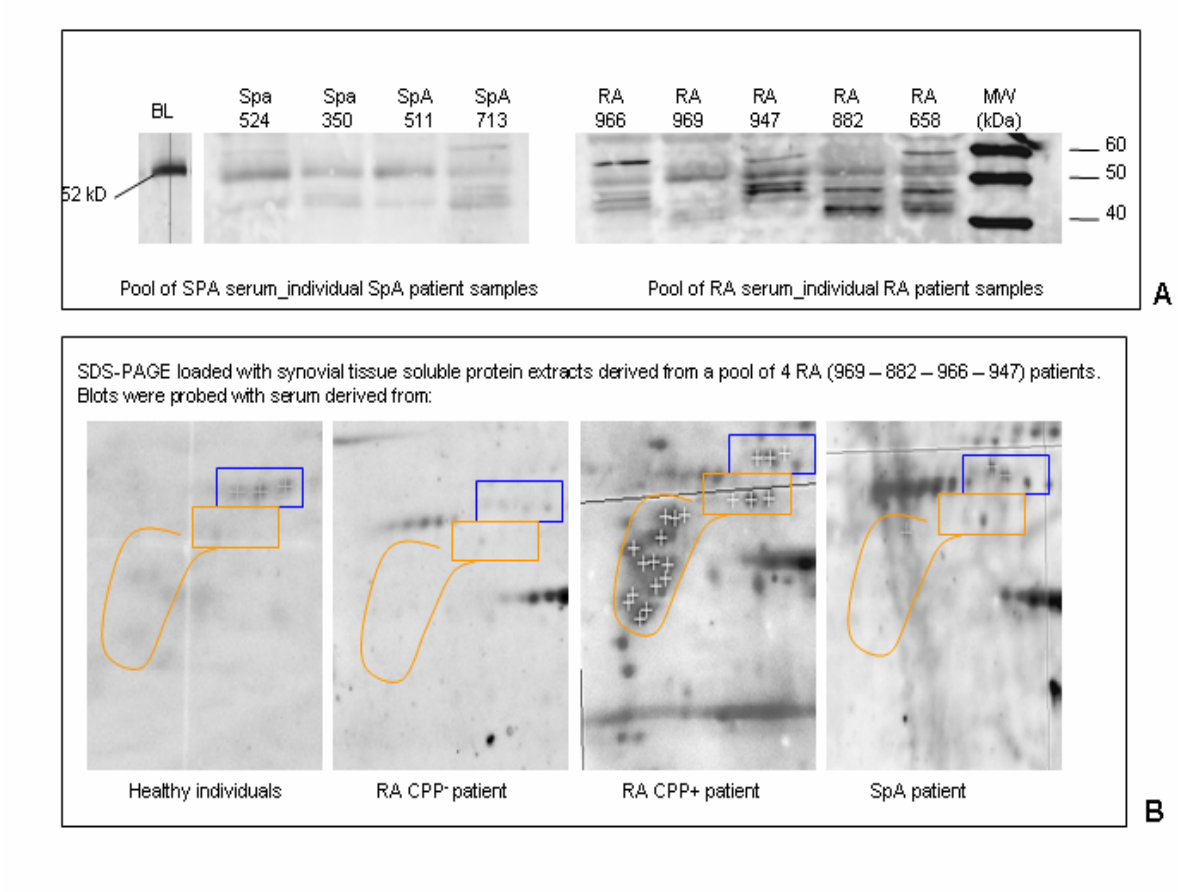


Figure 4: Presence of autoantibodies against vimentin in inflammatory arthritides.
(Panel A) Soluble synovial tissue extracts of individual patients diagnosed with RA or SpA were run on SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with a pool of RA and SpA sera, respectively. A strong positive reaction with cleaved synovial vimentin forms was observed in RA patients, in contrast with SpA sera, where immune reactivity was extremely weak. The immune reactive band at 52kD was IgG as indicated by the negative control strip (BL), only incubated with secondary antibody
(Panel B) Soluble synovial tissue extracts of a pool of RA patients was subjected to 2-D immunoblotting. In order to verify that the autoantibody reactivity was against vimentin, the blots were stripped and vimentin was detected. Immune reactive spots indicated by (+) correlated to vimentin isoforms subsequently detected on the same membranes. To facilitate the interpretation, the position of the vimentin cluster is indicated on the blots in colour. Reactivity of healthy sera is indicated in blue and should be regarded as non-disease specific. CCP(-) sera showed no reactivity for vimentin in contrast with CCP(+) RA sera in which strong positive detection of vimentin isoforms was observed. In SpA patients, autoantibody reactivity against cleaved products of vimentin was rather weak to absent. Figures are representative for sera of the same pathology as can be verified in the supplementary data of figure 4.

As a control for disease specific autoantibody reactivity, blots were incubated with a serum pool obtained from 4 healthy individuals, in which minor immune reactivity against the high MW forms of vimentin was observed (fig. 4, panel B). This reactivity was considered not disease specific and is indicated in blue on the blots.

When the 2-D blots were incubated with individual RA patient serum, we observed a different immune reactive pattern in serum derived from RA CPP^- patients in comparison to RA CCP^+ patients. In contrast to RA CCP^- patients (0/3 patients showed autoantibody reactivity against vimentin), there was a distinct reactivity of RA CCP^+ sera against processed forms of synovial

vimentin (3/3 showed positive autoantibody reactivity). When blots were probed with individual SpA serum, only the serum of 1 SpA patient was considered as positive autoantibody reactivity against vimentin fragments, since the blots probed with serum of other patients were negative or the signal was only slightly above the background noise level.

The data shown in figure 4 (panel B) are representative for each group. All 2-D immunoblotting results can be viewed as supplementary data for figure 4.

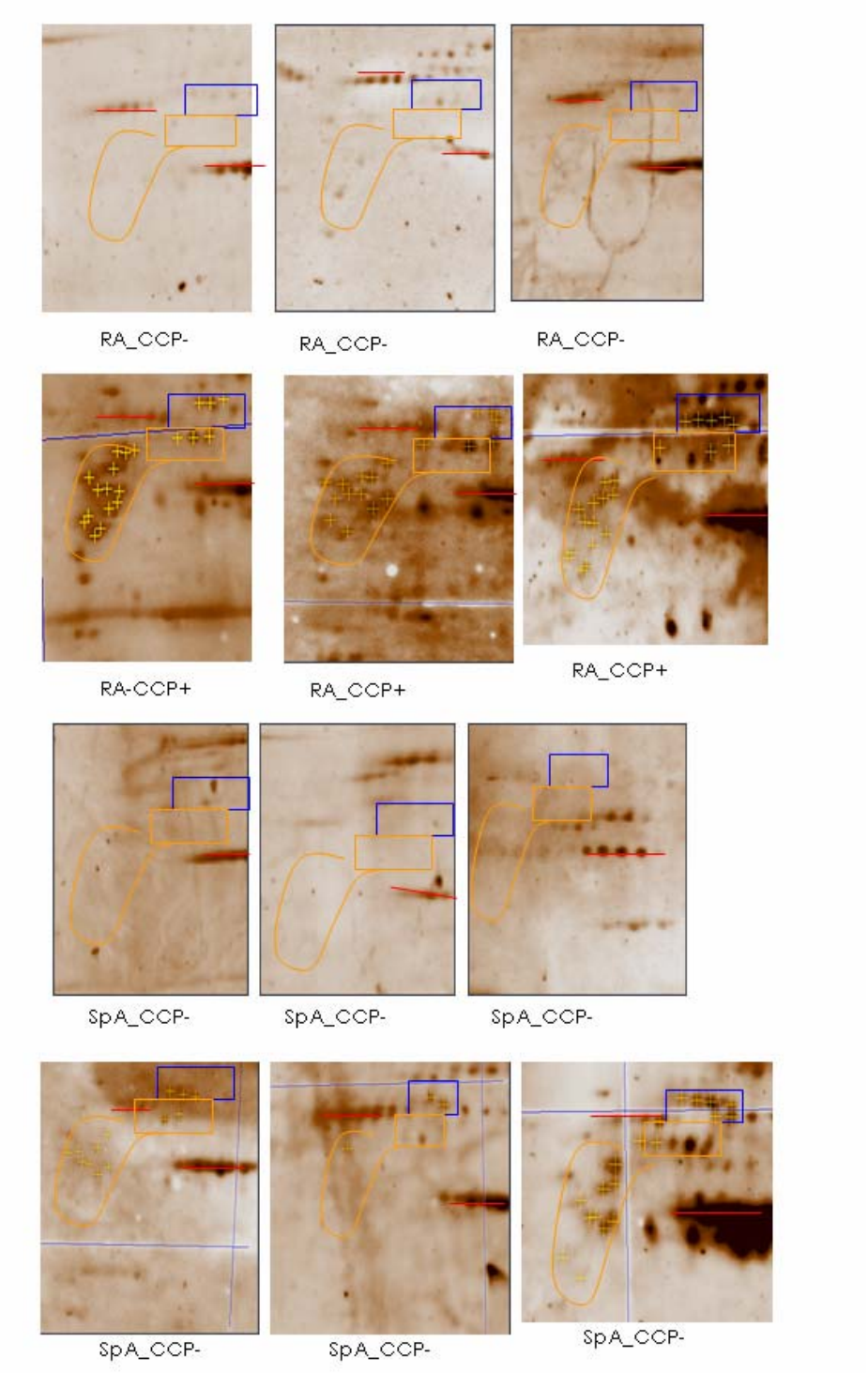


Figure 4: Supplementary data.

DISCUSSION

Processed forms of vimentin are citrullinated in RA and are accompanied with the development of autoantibodies in RA patients. Although it is known that citrullinated vimentin is an autoantigen in RA (previously known as the Sa-antigen) [5], the present report indicates a novel concept in the development of humoral autoimmunereactivity in RA since only processed fragments of soluble vimentin were found to be citrullinated.

Vimentin is known to be processed by caspases during the event of apoptosis [20]. Within 2h of apoptosis induction, when the majority of the cells are still viable, a 48kDa fragment of vimentin appears as the result of a caspase-3 like cleavage releasing the N-terminal head of the protein [20]. In our study, the low MW isoform of vimentin appeared at approximately 48kDa. In addition, careful interpretation of the mass spectrometric data revealed the absence of peptides which originated from the head of the protein (amino acids 1-85). We therefore concluded that this processed form of vimentin could be derived from caspase-3 cleavage.

When *in vitro* caspase-3 cleavage of vimentin was performed and subjected to 2-DE, we observed multiple cleavage fragments. However, we could not observe any fragments shifting towards the acidic pI. As this was in contrast with the *in vivo* situation, where fragments of vimentin were observed shifting towards the acidic side of the IPG strip, we investigated the presence of citrullinated residues in these fragments by AMC staining. Vimentin fragments appeared to be citrullinated in cytosolic synovial protein extracts of RA patients. In SpA, AMC staining was weak and no significant presence of citrullinated proteins was found in soluble protein extracts of synovial tissue obtained from OA patients.

Upon in vitro citrullination, vimentin is irreversibly disassembled resulting in an accumulation of soluble vimentin oligomers [22]. This could explain in part the lack of previous observations of citrullinated vimentin in synovial protein extracts as most extracts described in literature are ureum-DTT extracts as described by Masson-Bessière C. *et al*. [23]. These ureum-DTT extracts are enriched in deiminated fibrin, the most well known deiminated protein in synovial tissue [23].

It has been indicated that citrullination of proteins is not specific for RA, but inherent to chronic inflammation of the synovial tissue. Deiminated fibrin has been reported to be present in synovial protein extracts of other arthritides [24]. Our study showed the presence of citrullinated vimentin in soluble protein extracts obtained from the synovial tissue of RA patients, in contrary to SpA where the presence of citrullinated vimentin appeared to be very limited.

The reason why proteins are deiminated is not entirely known, although there are reports that indicate a possible role in apoptosis [25-27]. It has been shown that vimentin is selectively and rapidly citrullinated in macrophages undergoing apoptosis [27]. In addition, activated macrophages secrete fragments of vimentin [11]. Also neutrophils undergoing spontaneous apoptosis express fragments of the C-terminal tail of vimentin on their surface [28]. Whether these fragments are citrullinated has not been determined yet. It is possible that citrullination of vimentin is the only way towards a direct and irreversible disassembly of vimentin filaments resulting in cytoskeleton disruption leading to cell shrinkage in the process of apoptosis [20]. Therefore, citrullination of vimentin could be an early event in the processing of the protein. Disassembly of the intermediate filament by citrullination could be necessary for further processing of the protein by caspases or other proteases. However, the exact sequence of events is not known.

Not only the presence of processed citrullinated vimentin is shown in inflammatory arthritis, we also show the specific autoantibody reactivity in RA against these fragments. Indeed, RA CCP+ sera reacted strongly with low MW citrullinated vimentin isoforms, whereas in RA CCP- and SpA, this reactivity was absent or extremely weak. Antibodies against citrullinated proteins (ACPA) are highly specific for RA [29]. The presence of ACPA is significantly related to HLA-DR shared epitope (SE) [30]. This is explained by the fact that MHC with SE has a high affinity for negatively charged or uncharged polar amino acids while positively charged amino acids inhibit peptide binding [31]. Therefore, when arginine is deiminated, the positive charge is converted to a polar but uncharged residue increasing the affinity in the binding pocket. This was shown by Hill J. A. *et al*., where conversion of an arginine into citrulline in the N-terminal head of vimentin increased peptide affinity and resulted in CD4+ T cell activation in HLA-DR4-IE transgenic mice [32].

In conclusion, we reported on processed vimentin isoforms in the cytosolic proteome of inflammatory arthritides. Our data suggest that these isoforms are probably the result of caspase cleavages. In addition, these cleaved forms of vimentin are citrullinated in soluble protein extracts of synovial tissue obtained form RA, in comparison to SpA patients, where there is little presence of citrullinated vimentin in soluble protein extracts. Furthermore, the presence of autoantibodies against these citrullinated fragments is highly specific for RA patients.

REFERENCES

[1.] Tarner, I. H., Harle, P., Muller-Ladner, U., Gay, R. E., *et al.*, *Best Pract Res Clin Rheumatol* 2005, 19, 19-35.

[2.] Baeten, D., Demetter, P., Cuvelier, C., Van Den Bosch, F., *et al.*, *Ann Rheum Dis* 2000, 59, 945-953.

[3.] van Boekel, M. A., Vossenaar, E. R., van den Hoogen, F. H. and van Venrooij, W. J., *Arthritis Res* 2002, 4, 87-93.

[4.] Despres, N., Boire, G., Lopez-Longo, F. J. and Menard, H. A., *J Rheumatol* 1994, 21, 1027-1033.

[5.] Vossenaar, E. R., Despres, N., Lapointe, E., van der Heijden, A., *et al.*, *Arthritis Res Ther* 2004, 6, R142-150.

[6.] Osung, O. A., Chandra, M. and Holborow, E. J., *Ann Rheum Dis* 1982, 41, 74-77.

[7.] Fuchs, E. and Weber, K., *Annu Rev Biochem* 1994, 63, 345-382.

[8.] Martys, J. L., Ho, C. L., Liem, R. K. and Gundersen, G. G., *Mol Biol Cell* 1999, 10, 1289-1295.

[9.] Eriksson, J. E., He, T., Trejo-Skalli, A. V., Harmala-Brasken, A. S., *et al.*, *J Cell Sci* 2004, 117, 919-932.

[10.] Eckes, B., Colucci-Guyon, E., Smola, H., Nodder, S., *et al.*, *J Cell Sci* 2000, 113 (Pt 13), 2455-2462.

[11.] Mor-Vaknin, N., Punturieri, A., Sitwala, K. and Markovitz, D. M., *Nat Cell Biol* 2003, 5, 59-63.

[12.] Moisan, E. and Girard, D., *J Leukoc Biol* 2006, 79, 489-498.

[13.] Vossenaar, E. R., Radstake, T. R., van der Heijden, A., van Mansum, M. A., *et al.*, *Ann Rheum Dis* 2004, 63, 373-381.

[14.] Baeten, D., Van den Bosch, F., Elewaut, D., Stuer, A., *et al.*, *Clin Rheumatol* 1999, 18, 434-441.

[15.] Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., *et al.*, *Arthritis Rheum* 1988, 31, 315-324.

[16.] Dougados, M., van der Linden, S., Juhlin, R., Huitfeldt, B., *et al.*, *Arthritis Rheum* 1991, 34, 1218-1227.

[17.] Altman, R., Asch, E., Bloch, D., Bole, G., *et al.*, *Arthritis Rheum* 1986, 29, 1039-1049.

[18.] Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I., *et al.*, *Proteomics* 2005, 5, 2247-2257.

[19.] Fujita, J., Bandoh, S., Yang, Y., Wu, F., *et al.*, *Microbiol Immunol* 2003, 47, 447-451.

[20.] Byun, Y., Chen, F., Chang, R., Trivedi, M., *et al.*, *Cell Death Differ* 2001, 8, 443-450.

[21.] van Venrooij, W. J. and Pruijn, G. J., *Arthritis Res* 2000, 2, 249-251.

[22.] Inagaki, M., Takahara, H., Nishi, Y., Sugawara, K., *et al.*, *J Biol Chem* 1989, 264, 18119-18127.

[23.] Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., *et al.*, *J Immunol* 2001, 166, 4177-4184.

[24.] Chapuy-Regaud, S., Sebbag, M., Baeten, D., Clavel, C., *et al.*, *J Immunol* 2005, 174, 5057-5064.

[25.] Chang, X., Yamada, R., Suzuki, A., Sawada, T., *et al.*, *Rheumatology (Oxford)* 2005, 44, 40-50.

[26.] Chang, X. and Han, J., *Mol Carcinog* 2005.

[27.] Asaga, H., Yamada, M. and Senshu, T., *Biochem Biophys Res Commun* 1998, 243, 641-646.

[28.] Moisan, E. and Girard, D., *J Leukoc Biol* 2005.

[29.] Zendman, A. J., van Venrooij, W. J. and Pruijn, G. J., *Rheumatology (Oxford)* 2006, 45, 20-25.

[30.] Cantaert, T., Coucke, P., De Rycke, L., Veys, E. M., *et al.*, *Ann Rheum Dis* 2005.

[31.] Hammer, J., Gallazzi, F., Bono, E., Karr, R. W., *et al.*, *J Exp Med* 1995, 181, 1847-1855.

[32.] Hill, J. A., Southwood, S., Sette, A., Jevnikar, A. M., *et al.*, *J Immunol* 2003, 171, 538-541.

Chapter 3

Hypoxia induced production of VEGF and Ceruloplasmin by synovial fibroblasts

Kelly Tilleman

Preliminary data

ABSTRACT

A hypoxic microenvironment is created in the joints affected by inflammatory arthritis. In response to the higher metabolic demand of the proliferating tissue, angiogenesis is triggered. The key regulator of angiogenesis is vascular endothelial growthfactor (VEGF) and in order to function properly, VEGF needs copper as cofactor. The majority of circulating copper is bound to Ceruloplasmin (Cp), a well known plasma protein secreted by the liver. Both VEGF and Cp are under the transcriptional control of hypoxia induced factor-1 (HIF-1) in hypoxic conditions.

In this study we investigated the hypoxia induced gene and protein expression of Cp and VEGF in RA and SpA fibroblast-like synoviocytes (FLS).

Hypoxia induced secretion of VEGF was observed in both RA and SpA FLS. Secreted levels of VEGF after 8h of hypoxia, were higher in SpA than in RA.

Our data showed that Cp can be locally produced by both RA and SpA FLS and there is a trend towards a higher hypoxia-induced gene expression of Cp in SpA compared to RA.

These finding suggest that the tissue response of FLS upon hypoxia is different in RA in comparison to SpA. It is conceivable that these differences account for the reported higher vascularity in SpA synovium.

INTRODUCTION

Synovitis, the chronic inflammation of the synovial tissue, is commonly considered as the initial change in the joints of patients with inflammatory arthritis. Synovitis is characterized by massive synovial proliferation and subintimal infiltration of inflammatory cells, which along with angiogenesis (the formation of new blood vessels) results in a very aggressive tissue called pannus [1, 2].

The oxygen supply to the synovium becomes impaired when the synovial lining starts to proliferate. The high metabolic state of the tissue and the fact that the cells become more distant from the closest blood vessel, compounds to the hypoxic state [3]. Early reports in the seventies, describe the arthritic joint as being hypoxic. Indeed, synovial fluid samples from patients with rheumatoid arthritis (RA) were hypoxic and acidotic, with low glucose and high lactate concentrations which indicated the presence of an anaerobic metabolism in the synovial tissue [4-6]. In these early reports the mean synovial fluid pO_2 in RA knee joints was reported to be as low as 27mm Hg compared to 43mm Hg in osteoarthritis (OA) and 63mm Hg in traumatic effusions in healthy control individuals. The group of Taylor P. recorded mean intra-articular pO_2 values of 13mm Hg in mice with collagen-induced arthritis and similar levels in patients with RA [7].

At such low oxygen levels, the hypoxia-inducible-factor-1 (HIF-1) starts to take control over the transcription rate of over 70 genes [8]. At oxygen concentrations below 5%, cellular levels of HIF-1α rise exponentially to a maximum of 0.5% O_2. In this situation HIF-1α is no longer targeted for degradation, and starts to accumulate. Subsequently, it translocates to the nucleus where it binds to HIF-1β and forms the HIF-1 complex [9] . This complex binds the hypoxia-regulating elements in the promoter region of several genes, hereby stimulating the cell to adapt to its new environmental conditions. The presence of HIF-1 has been indicated in the synovial lining of synovial tissue sections of RA and OA biopsy samples in comparison to healthy controls [10, 11].

In order to sustain its homeostasis in a hypoxic atmosphere, the cell starts to produce angiogenic factors. A key factor in this process is vascular endothelial growth factor (VEGF). VEGF, known to be regulated by HIF-1 [12], can be detected in synovial fluids of patients diagnosed with RA and SpA [13, 14] and the local production of VEGF by RA synoviocytes in hypoxic conditions has been reported [15].

Angiogenic factors, like VEGF, need cofactors in order to function properly [16]. Such a cofactor is copper [17]. In healthy individuals, 95% of total circulating copper is bound to ceruloplasmin (Cp), an abundant plasma alpha-2-glycoprotein which has been reported to be elevated in the serum of RA patients [18]. Although Cp is synthesized primarily in the liver, other cell types like monocytes, astrocytes and sertoli cells can express the protein [19]. Because of its ability to change its function in response to a change in substrate, localization or differential expression, Cp has been described as a moonlighting protein [20]. Cp is responsible for the transport of copper, plays a role in controlling iron homeostasis, and is a circulating anti-oxidant [21]. Its transcription is regulated by HIF-1 under hypoxia [22]. Reports on Cp in inflammatory arthritis are limited. However, tetrathiomolybdate (TM), a copper-lowering agent, which has been evaluated extensively in the treatment of Wilson's disease [23], can also attenuate angiogenesis and tumor growth in animal models [24]. A recent report by Omoto A. *et al.* showed that TM has a strong protective effect against the progression of adjuvant-induced arthritis in rats [25]. These data indicate a possible important role for Cp in arthritis.

It is known that the RA joint is hypoxic. Since RA and SpA are both characterized by synovitis, it is plausible that the environmental condition in the SpA joint is similar and therefore also hypoxic; however there are no studies that have explored this yet. Likewise, reports on the effect of hypoxia on FLS explants from SpA patients are lacking.
Neovascularization is a known consequence observed in tissues under hypoxic conditions. It has been reported that the inflamed synovial membrane in the SpA joint is more vasulated than in the RA joint [26]. Therefore, it could be possible that the tissue response to the hypoxic environment in the joint is different in SpA compared to RA.

In order to explore this we investigated the induction of protein expression of VEGF and Cp, two proteins involved in angiogenesis and transcriptionally activated under hypoxia via HIF-1α, in RA and SpA. Hypoxia induced expression of Cp appeared higher in SpA FLS than in RA FLS. These results also show that Cp can be locally expressed by FLS. In addition, we observed hypoxia activated secretion of VEGF by RA and SpA FLS. The VEGF secretion was shown to be higher in SpA FLS compared to RA FLS. Our results indicate a possible difference in tissue respons in RA and SpA upon hypoxia.

MATERIAL AND METHODS

Isolation and culture of synovial fibroblast

Synovial tissue was obtained from 6 patients with RA, 6 patients with SpA and 5 patients with OA at the time of needle arthroscopy of the knee. RA patients were diagnosed according to the criteria prescribed by the American College of Rheumatology (ACR) [27], patients with SpA fulfilled the European Spondyloarthropathy Study Group criteria [28] and patients with OA fulfilled the ACR criteria for the classification of OA [29]. All patients undergoing needle arthroscopy had active synovitis (RA and SpA) or joint effusion (OA) of the knee.

The study was conducted after approval by the local ethics committee. Written informed consent was obtained from all participating patients.

Tissue was immediately processed for synovial fibroblast culture according to Muller-Ladner U. *et al*. [30]. Briefly, Synovial tissue was digested with dispase (1.5mg/ml) in alpha Minimum Essential Medium (αMEM) (both from Gibco, Burlington, Ontario, Canada) twice 1 hour at 37°C. Cells were collected, centrifuged at 1000rpm for 15 minutes and resuspended in DMEM containing 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicilline/streptomycine (all obtained from Gibco, Burlington, Ontario, Canada) and cultured at 37°C and 7%CO_2 (this was regarded as passage 1). Cells were stored in liquid nitrogen upon passage 4 (P4).

For hypoxia experiments, fibroblasts (P4) were brought in suspension in 6-well polystyrene plates with coverslips at a concentration of 5 x 10^4cells/well for immunohistochemistry or without coverslips at a concentration of 3 x 10^5cells/well for real-time PCR. Before initiating hypoxic conditions, the cells were cultured overnight (16 hours) in serum-free medium to induce a quiescent state.

Hypoxia conditions

Cell preparations were exposed for 6h, 8h and 10h to hypoxic conditions in a Heraeus series 6000 incubator (Thermo Electron Corporation, Asheville, NC, USA), with 3%O_2, 5%CO_2 and 92% N_2. Fibroblast used as controls, were prepared in the same way, and were kept under normoxic conditions.

Fluorescence immunohistochemistry

Immunohistochemistry was used to determine the expression of HIF-1α in synovial tissue explants. Cells were fixed with 3.7% paraformaldehyde for 15 minutes and subsequently

quenched with 100mM glycine to reduce background fluorescence. Permeabilization was performed for the detection of HIF-1α using 0.25% Triton X-100 for 15 minutes. Cells were washed with PBS (Gibco, Burlington, Ontario, Canada) and blocked with blocking buffer containing 5% goat serum (Dako Cytomation, Glostrup, Denmark), 1% fish skin gelatine (45% from Sigma, Steinheim, Germany), 0.05% Tween-20 (Sigma, Steinheim, Germany) in PBS. After blocking, monoclonal mouse (IgG1) anti-human HIF-1α (BD Pharmingen, San Diego, CA, USA) (dilution 1/50 in PBS) or monoclonal mouse (IgG1) was applied for 2h at room temperature. Cells were washed with PBS and subsequently incubated with the Alexa Fluor-488 labeled goat anti-mouse IgG1 antibody from Molecular Probes (Eugene, OR, USA) (dilution 1/500). Nuclear staining was performed using DAPI (Sigma, Steinheim, Germany) at a concentration of 100ng/ml. IgG1 isotyope controls were included as negative controls.

VEGF protein expression determined by enzyme-linked immunosorbent assay (ELISA)

Hypoxia induced secretion of VEGF by synovial fibroblast was investigated by ELISA by R&D systems Inc. (Minneapolis, MN, USA). Culture supernatants of FLS derived from 6 RA, 6 SpA and 5 OA patients were collected after exposure to 8h of hypoxia (or normoxia). Each FLS line was derived from an individual patient and each sample was analyzed in triplicate. Data is displayed as the mean (pg/ml) for each group of patients and because of the small sample size, the standard error of the mean (SEM) was calculated to calculate the standard deviation of the sampling distribution.

Real-time PCR

RNA was isolated from 300.000 cells obtained from synovial fibroblast using samples of OA (n=5), RA (n=6) and SpA (n=6) patients using TRIzol (Life Technologies, Paisley, U.K.). The total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Before reverse transcription, digestion of DNA was performed using the RNase-Free DNase Set (QIAGEN). cDNA was synthesized using oligo(dT) priming the Superscript II kit (Life Technologies). cDNA was quantified using the Quant-iT Oligreen ssDNA Assay kit (Molecular Probes Inc., Eugene, OR, USA) and 5ng cDNA was used to quantify the gene expression.

Primers for the housekeeping enzyme HPRT were designed using the Primer Express software (Applied Biosystems, Foster City, CA). For HPRT, primers were TCA GGC AGT

ATA ATC CAA AGA TGG T (sense) and AGT CTG GCT TAT ATC CAA CAC TTC G (antisense) and CAA GCT TGC TGG TGA AAA GGA CCC C (probe). Assays on Demand (Applied Biosystems, Foster City, CA) were used for the amplification of Ceruloplasmin (Hs00236810) and VEGF (Hs00173626). Amplification and on-line detection of PCR products were carried out with optical 96-well plates (Applied Biosystems, Foster City, CA) in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). mRNA expression levels were calculated according to the delta delta Ct method.

RESULTS

Hypoxia induced expression of HIF-1α in synovial fibroblasts

The expression of HIF-1α was examined in FLS derived from biopsy samples obtained from 3 RA, 3 SpA and 3OA patients. Nuclear HIF-1α localisation could be detected after 6h of hypoxia (fig. 1). Strong perinuclear or nuclear expression of HIF-1α was observed in FLS obtained from RA patients (figures 1A and B). Also in FLS of SpA patients, patchy nuclear distribution was observed (fig. 1C). These expression patterns were observed in the majority of the FLS on each micropscopic slide. The perinuclear expression of HIf-1α, observed like a halo around the nucleus, is probably a high density of HIF-1α molecules queueing in order to enter the nucleus through its nuclear pores. We observed this phenomenon only in synovial fibroblasts from 2 RA patients.

HIF-1α expression in OA synovium explants was weak and cytoplasmic (fig. 1D).

No nuclear localization of HIF-1a was observed in normoxia. It is known that under aerobic conditions, HIF-1α is degraded by rapid ubiquination followed by proteasomal degradation [31]. This was in agreement with our HIF-1α stainings under normoxic conditions, where only a weak cytoplasmic distribution of HIF-1α could be observed in RA, SpA and OA FLS cultures.

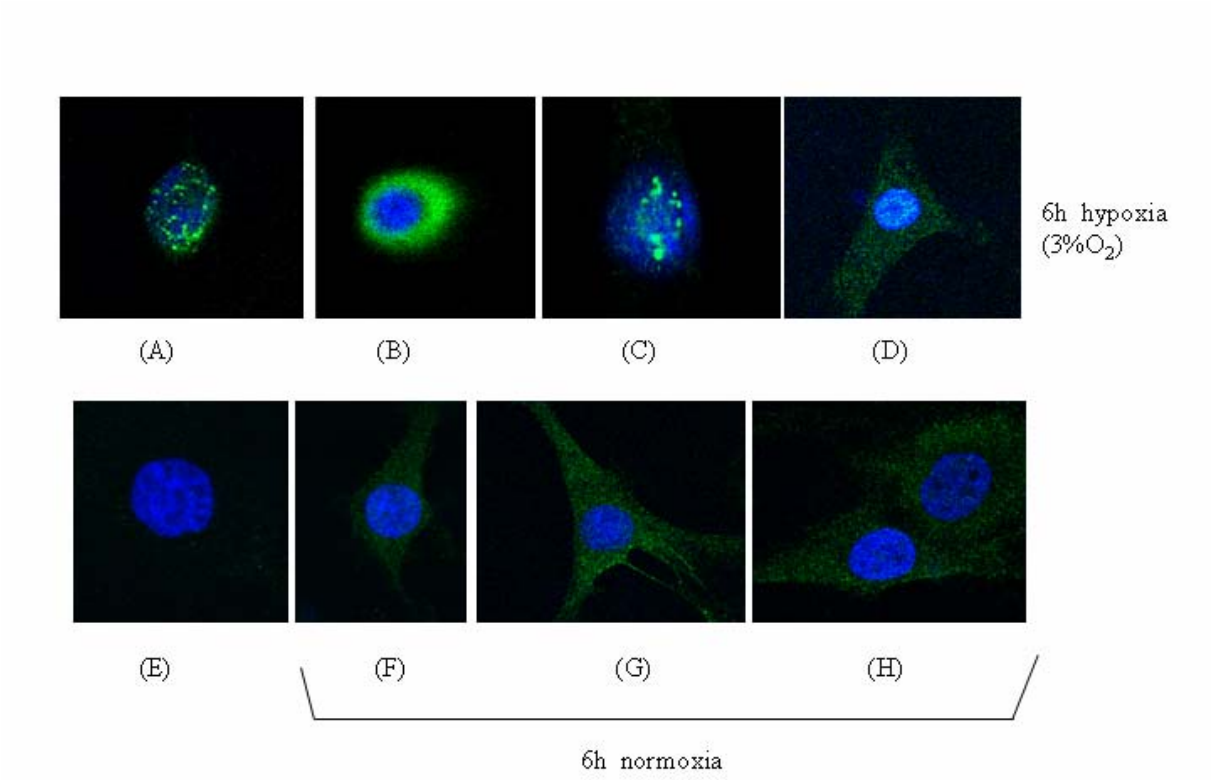


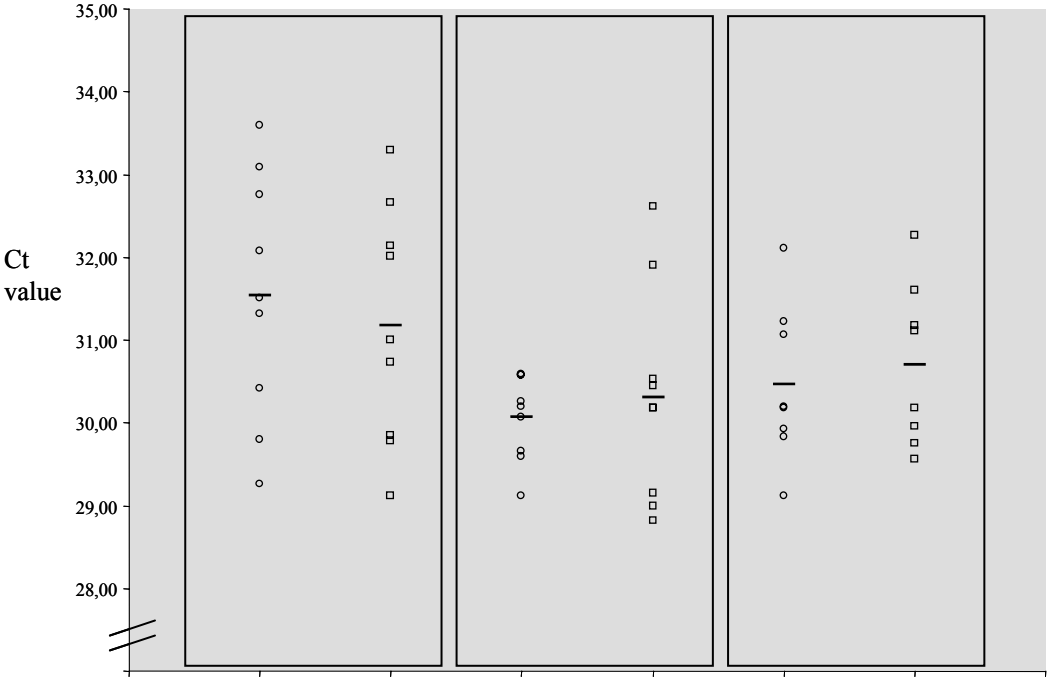
Figure 1: Merged images captured using confocal immunofluorescence microscopy (using sections of 0.8µm, magnification x 1000). FLS explants were incubated in a hypoxic environment (3% O_2) for 6h. FLS from 3 RA, 3 SpA and 3 OA were examined.
Strong nuclear (A) or perinuclear (B) staining of HIF-1α in FLS obtained from RA patients. Patchy nuclear expression in FLS derived from SpA. In OA synovium explants the expression of HIF-1α was very weak and its localization cytoplasmic (D). The cytoplasmic distribution of HIF-1α under normoxic conditions is displayed for FLS explants of RA (F), SpA (G) and OA (H). Isotype controls were included as negative controls (E).
HIF-1α was detected using alexa fluor-488 and is represented in green. The nucleus was visualized using DAPI stain represented in blue.

Hypoxia induced gene expression of VEGF and Cp in synovial fibroblasts

Synovial cells were harvested and RNA was extracted as described in the section 'Material and Methods'. The effect of hypoxia on VEGF and Cp mRNA expression was analyzed by real-time PCR. In order to analyze the stability of gene expression of household gene HPRT in hypoxic and normoxic conditions, FLS were incubated at 6h, 8h and 10h of normoxia and hypoxia. The results showed that the average gene expression of HPRT was relatively stable in both normoxic and hypoxic conditions and HPRT was further used to normalize gene expression (fig. 2).

VEGF and Cp gene expression were calculated according to the delta delta Ct method where each delta delta Ct value represented the delta Ct value of the experimental gene observed under hypoxia compared to the delta Ct value in the normoxic environment. The data

displayed in figure 3 are represented as the ratio of gene expression in hypoxia in comparison to normoxia.



	RA	OA	SpA
NORM av	31.53	30.07	30.46
HYP av	31.18	30.31	30.70
NORM SD	1.49	0.51	0.95
HYP SD	1.43	1.29	0.98

Figure 2: HPRT gene expression in RA, OA and SpA fibroblast-like synovial cells under normoxic and hypoxic conditions. FLS obtained from 3 RA, 3 OA and 3 SpA were incubated at 6h, 8h or 10h of normoxia or hypoxia to verify the stability of the gene expression of HPRT.
Individual Ct values are represented by (°) in normoxic and by ( ) in hypoxic conditions; (-) represents the Ct average in the group. Average Ct values (NORM av; HYP av) and standard deviation of Ct values (NORM SD; HYP SD) are given in table format under the figure.

We observed no difference in VEGF gene expression in RA, OA or SpA FLS after hypoxic incubations (fig. 3, VEGF).

There was a trend in hypoxia induced gene expression of Cp in FLS cultures of SpA and OA patients (fig.3, Cp), although additional samples need to be analyzed, before these findings can reach statistical significance. These values were on average markedly higher after hypoxic incubation. In addition, Cp gene expression under hypoxic conditions was higher in SpA and OA than in RA.

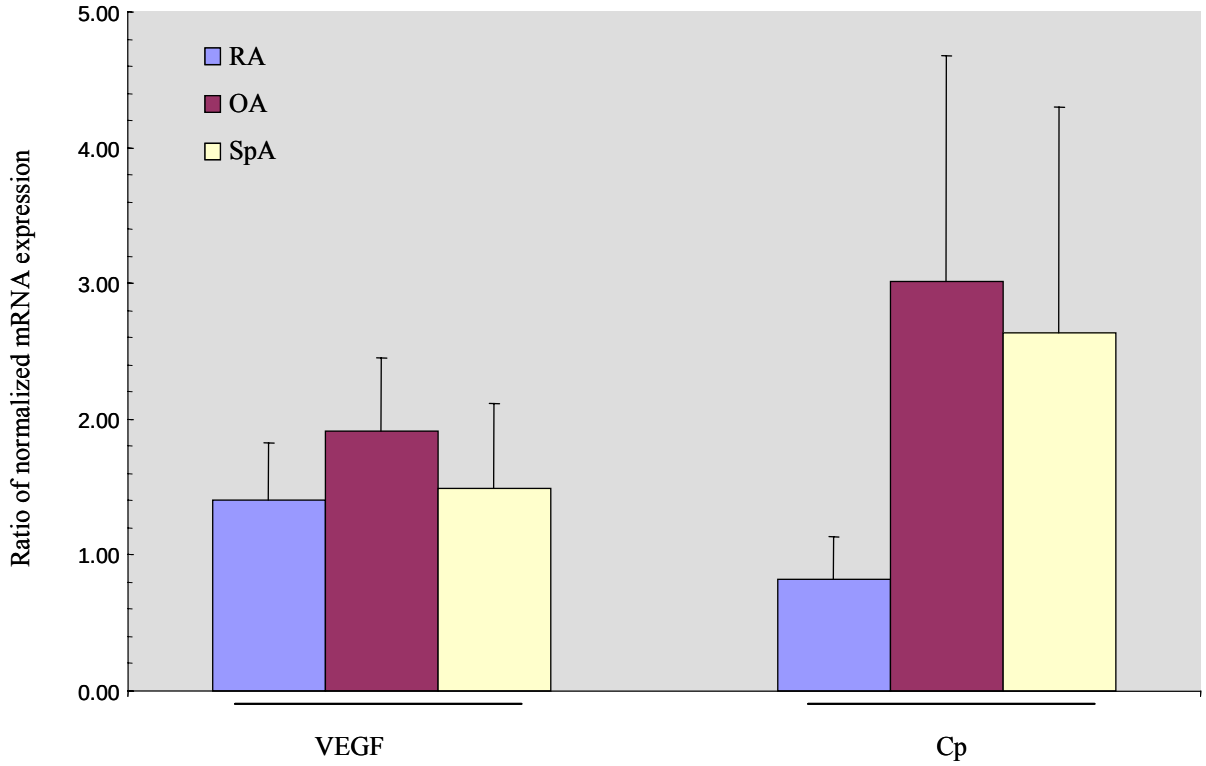


Figure 3: Gene expression of VEGF and Cp in FLS obtained from 6 RA, 6 OA and 6 SpA patients incubated in a hypoxic environment during 8h. Gene expression in hypoxic and normoxic conditions was normalized against HPRT. The mean of the individual ratios of gene expression in hypoxia compared to normoxia are displayed; error bars represent the SEM.

Hypoxia induced protein secretion of VEGF in synovial fibroblasts

The amount of secreted VEGF by synovial fibroblasts was detected using ELISA (fig. 4). VEGF production was increased in the supernatants of FLS cultures derived from synovial tissue of RA, SpA and OA patients after 8h hypoxia in comparison to normoxic conditions. The average secreted protein levels in RA, SpA and OA were 1.4, 1.8 and 1.7 times higher, respectively (fig. 4). These data also show that the level of VEGF produced by SpA FLS is higher than by RA FLS. There was a statistical difference between the amount of VEGF produced by the FLS in normoxic conditions between RA and SpA ($p<0.05$), showing that the basal secretion of VEGF is different in RA and SpA. Although no statistical significant difference between the secretion of VEGF in RA and SpA FLS incubated under hypoxic conditions in the currently analyzed was found, a similar trend was observed. Therefore, additional experiments to augment the sample size are underway.

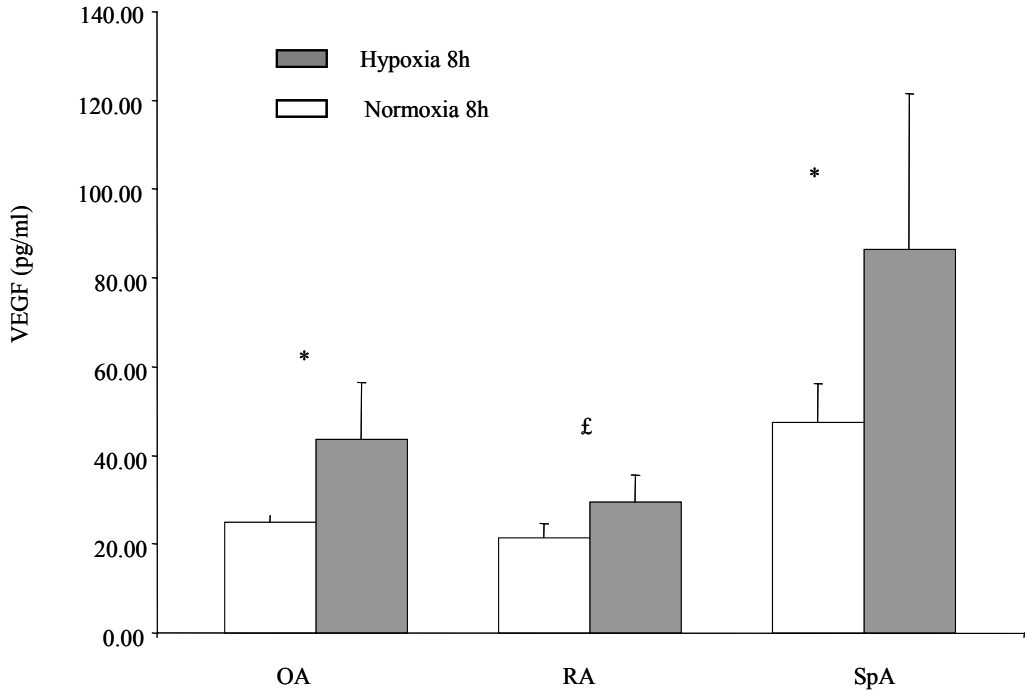


Figure 4: Hypoxia-induced production of VEGF by cultured FLS from 6 OA, 6 RA and 6 SpA patients. Synovial fibroblasts were cultured for 8h under hypoxic (3% O2) or normoxic conditions. VEGF values are detected by ELISA. *= p=0.1, £=p<0.05 using Student's T-test for hypoxic versus normoxia. Error bars represent the SEM.

DISCUSSION

Inflammatory arthritides, like RA and SpA, are accompanied by chronic synovitis. Persistent inflammation of the synovial tissue leads to hyperplasia of the synovial lining, resulting in a hypoxic environment in the joint. The molecular mechanisms in cellular adaptive response to hypoxia have been elicited and one principal regulator of this adaptive response is HIF-1α [9]. Nuclear staining of HIF-1α could be observed in RA and SpA FLS explants after 6h of hypoxia. These data are in agreement with Hitchon C. *et al.* which showed a strong nuclear staining of HIF-1α in synovial fibroblasts after 4-6h of hypoxia [32].

The synovial proliferation puts higher energy demands on the environment and this is often accommodated by the development of new blood vessels. A major cofactor in neovascularization is copper, of which the majority is bound to Cp.

Cp is also a major ferroxidase by catalyzing the oxidation from toxic ferrous iron (Fe^{2+}) to the safer ferric iron (Fe^{3+}) [33] in order to promote the incorporation of iron in to transferrin. Patients with aceruloplasminemia, a hereditary deficiency of ceruloplasmin, have severe intracellular iron accumulation in a number of organs [34]. Ferrous iron can generate highly

reactive superoxide and hydroxyl radicals, hereby promoting oxidative stress [35]. Iron deposits have been described in the synovium of patients with RA [36] and these deposits can perpetuate inflammation by aiding in the production of free oxygen radicals.

As Cp is implicated in angiogenesis and is regulated by HIF-1α in hypoxic conditions, the effect of hypoxia on the gene expression of Cp was investigated. Although we were unable to show a significant difference between gene expression in normoxia and hypoxia, there was on average a higher hypoxia induced gene expression of Cp in SpA and OA FLS than in RA FLS (fig. 3). However, these data need further confirmation in a large sample group.

Although the patient cohorts examined in this study were small and even though we observed patient variability, there is a tendency towards a difference in Cp gene expression between synovial tissue explants of RA and SpA cultured in hypoxic conditions. This difference in Cp gene expression between the inflammatory arthritides may, in part, explain the difference observed in synovial vascularity between RA and SpA [26]. Cp also plays a role in angiogenesis by transporting copper, a major cofactor for different angiogenic factors [16, 17]. Induction of Cp in synovial tissue of SpA could aid the formation of blood vessels, leading to the higher synovial vascularity observed in SpA [26]. In addition, Cp has been described as being differentially expressed in soluble protein extracts of synovial tissue in SpA in comparison to OA [37] (Chapter I). This difference could be explained by the active diffusion of Cp from the synovial fluid into the joint tissue. However, our real-time PCR data also show that local expression of Cp by synovial fibroblasts can play an important role in the inflammation of the synovial tissue by regulating iron oxidation and hereby protecting the cells form oxidative stress.

Another important key player in angiogenesis is VEGF. VEGF is also regulated by HIF-1 in hypoxic conditions [12]. Hypoxia induced mRNA expression of VEGF was investigated by real-time PCR. We could, however, not observe any up regulation of VEGF gene expression in RA or SpA FLS upon 8h of hypoxia. There was no difference in the geometric average mRNA expression of VEGF in normoxic conditions in comparison to hypoxic conditions. However, when protein production of VEGF was analyzed in the supernatants of the cultured fibroblasts, significant higher levels of VEGF could be detected after 8h of hypoxia. It is likely that the induction of VEGF transcription appears earlier as described by Hitchon C. *et al.* who observed markedly enhanced expression of VEGF mRNA after 6h of hypoxia [32]. Additionally, VEGF protein production and secretion was higher in SpA FLS than in RA FLS. This distinction could be the result of molecular differences between RA and SpA FLS

and their ability to react to certain stimuli. Our data suggests that SpA fibroblasts can produce and secrete higher levels of VEGF than RA fibroblasts when cultured in a hypoxic environment. This could indicate a difference in ability to produce new blood vessels and may contribute to the higher vascularity observed in SpA synovium [26].

As angiogenesis contributes to the chronic inflammation and proliferation of the joint tissue, especially in the formation and maintenance of the pannus tissue, the possibility of targeting the formation of new blood vessels by using anti-angiogenesis agents has gained interest over the past few years [38]. Especially in cancer, where angiogenesis is essential for the growth of most primary tumors and their subsequent metastasis, have VEGF receptor blockade (by monoclonal antibodies [39]) or VEGF Trap (a fully humanized soluble VEGF receptor fusion protein [40]), been successful in preventing tumor growth.
As described earlier, angiogenic factors are copper-containing or copper-binding proteins [16]. The majority of circulating copper is bound to Cp and serum Cp is a good surrogate marker of copper status, because the liver secretes the protein into the blood at a rate dependent on copper availability [24]. It has been shown that a decrease in copper levels attenuates angiogenesis and tumor growth in animal tumor models [24]. Tetrathiomolybdate (TM), which forms a stable tripartite complex with copper and protein has been successfully applied in the treatment of Wilson's disease [23]. Recently, it has been indicated that this copper-lowering agent could also be effective in arthritis as TM delayed the onset and suppressed the severity of clinical arthritis in adjuvant-induced arthritis in the rat [25]. Histological examination of the joint tissue of the treated animals clearly showed a significant reduction in synovial hyperplasia and inflammatory cell invasion in the joint tissue.

In conclusion, we show that Cp, a copper binding protein, is locally produced by RA and SpA synovial fibroblasts. We have indications that upon hypoxic incubation, the mRNA expression of Cp is higher in SpA in comparison to RA synoviocytes. However, additional experiments are required to confirm the observed trends in gene expression.
VEGF protein production and secretion is higher in SpA FLS, than in RA FLS cultured in 8h hypoxia.
As both Cp and VEGF play a role in the formation of new blood vessels, it could be that the differences in gene and protein expression observed in this study are related to the differences in synovial vascularity between RA and SpA [26].

Since angiogenesis is triggered by hypoxia, these results could also indicate that there could be a discrepancy in molecular response upon the hypoxic microenvironment in the SpA in comparison to the RA joint. Finally, these differences could be responsible for the histological and clinical differences observed between the two inflammatory arthritides.

REFERENCES

[1.] Feldmann, M., Brennan, F. M. and Maini, R. N., *Cell* 1996, 85, 307-310.

[2.] Koch, A. E., *Arthritis Rheum* 1998, 41, 951-962.

[3.] Stevens, C. R., Blake, D. R., Merry, P., Revell, P. A., *et al.*, *Arthritis Rheum* 1991, 34, 1508-1513.

[4.] Falchuk, K. H., Goetzl, E. J. and Kulka, J. P., *Am J Med* 1970, 49, 223-231.

[5.] Lund-Olesen, K., *Arthritis Rheum* 1970, 13, 769-776.

[6.] Treuhaft, P. S. and DJ, M. C., *Arthritis Rheum* 1971, 14, 475-484.

[7.] Etherington, P. J., Winlove, P., Taylor, P., Paleolog, E., *et al.*, *Clin Exp Rheumatol* 2002, 20, 799-805.

[8.] Semenza, G. L., *Nat Rev Cancer* 2003, 3, 721-732.

[9.] Brahimi-Horn, C., Mazure, N. and Pouyssegur, J., *Cell Signal* 2005, 17, 1-9.

[10.] Hollander, A. P., Corke, K. P., Freemont, A. J. and Lewis, C. E., *Arthritis Rheum* 2001, 44, 1540-1544.

[11.] Giatromanolaki, A., Sivridis, E., Maltezos, E., Athanassou, N., *et al.*, *Arthritis Res Ther* 2003, 5, R193-201.

[12.] Shweiki, D., Itin, A., Soffer, D. and Keshet, E., *Nature* 1992, 359, 843-845.

[13.] Drouart, M., Saas, P., Billot, M., Cedoz, J. P., *et al.*, *Clin Exp Immunol* 2003, 132, 158-162.

[14.] Paleolog, E. M. and Miotla, J. M., *Angiogenesis* 1998, 2, 295-307.

[15.] Jackson, J. R., Minton, J. A., Ho, M. L., Wei, N., *et al.*, *J Rheumatol* 1997, 24, 1253-1259.

[16.] Sen, C. K., Khanna, S., Venojarvi, M., Trikha, P., *et al.*, *Am J Physiol Heart Circ Physiol* 2002, 282, H1821-1827.

[17.] Raju, K. S., Alessandri, G., Ziche, M. and Gullino, P. M., *J Natl Cancer Inst* 1982, 69, 1183-1188.

[18.] Milanino, R., Frigo, A., Bambara, L. M., Marrella, M., *et al.*, *Clin Exp Rheumatol* 1993, 11, 271-281.

[19.] Fox, P. L., Mazumder, B., Ehrenwald, E. and Mukhopadhyay, C. K., *Free Radic Biol Med* 2000, 28, 1735-1744.

[20.] Bielli, P. and Calabrese, L., *Cell Mol Life Sci* 2002, 59, 1413-1427.

[21.] Shukla, N., Maher, J., Masters, J., Angelini, G. D., *et al.*, *Atherosclerosis* 2006, 187, 238-250.

[22.] Mukhopadhyay, C. K., Mazumder, B. and Fox, P. L., *J Biol Chem* 2000, 275, 21048-21054.

[23.] Brewer, G. J., Johnson, V., Dick, R. D., Kluin, K. J., *et al.*, *Arch Neurol* 1996, 53, 1017-1025.

[24.] Brewer, G. J. and Merajver, S. D., *Integr Cancer Ther* 2002, 1, 327-337.

[25.] Omoto, A., Kawahito, Y., Prudovsky, I., Tubouchi, Y., *et al.*, *Arthritis Res Ther* 2005, 7, R1174-1182.

[26.] Baeten, D., Demetter, P., Cuvelier, C., Van Den Bosch, F., *et al.*, *Ann Rheum Dis* 2000, 59, 945-953.

[27.] Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., *et al.*, *Arthritis Rheum* 1988, 31, 315-324.

[28.] Dougados, M., van der Linden, S., Juhlin, R., Huitfeldt, B., *et al.*, *Arthritis Rheum* 1991, 34, 1218-1227.

[29.] Altman, R., Asch, E., Bloch, D., Bole, G., *et al.*, *Arthritis Rheum* 1986, 29, 1039-1049.

[30.] Muller-Ladner, U., Kriegsmann, J., Franklin, B. N., Matsumoto, S., *et al.*, *Am J Pathol* 1996, 149, 1607-1615.

[31.] Maxwell, W. L., Donnelly, S., Sun, X., Fenton, T., *et al.*, *J Neurotrauma* 1999, 16, 1225-1234.

[32.] Hitchon, C., Wong, K., Ma, G., Reed, J., *et al.*, *Arthritis Rheum* 2002, 46, 2587-2597.

[33.] Osaki, S., *J Biol Chem* 1966, 241, 5053-5059.

[34.] Harris, Z. L., Takahashi, Y., Miyajima, H., Serizawa, M., *et al.*, *Proc Natl Acad Sci U S A* 1995, 92, 2539-2543.

[35.] de Silva, D. M., Askwith, C. C. and Kaplan, J., *Physiol Rev* 1996, 76, 31-47.

[36.] Blake, D. R., Gallagher, P. J., Potter, A. R., Bell, M. J.*, et al.*, *Arthritis Rheum* 1984, 27, 495-501.

[37.] Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I.*, et al.*, *Proteomics* 2005, 5, 2247-2257.

[38.] Pandya, N. M., Dhalla, N. S. and Santani, D. D., *Vascul Pharmacol* 2006, 44, 265-274.

[39.] Hurwitz, H., *Clin Colorectal Cancer* 2004, 4 Suppl 2, S62-68.

[40.] Konner, J. and Dupont, J., *Clin Colorectal Cancer* 2004, 4 Suppl 2, S81-85.

Chapter 4

In pursuit of B-cell synovial autoantigens in rheumatoid arthritis: confirmation of citrullinated fibrinogen, detection of vimentin and introducing carbonic anhydrase as a possible new synovial autoantigen

Kelly Tilleman[1]
Ann Union[2]
Tineke Cantaert[3]
Saskia Dekeyser[2]
Annick Daniels[2]
Dirk Elewaut[3]
Filip De Keyser[3]
Dieter Deforce[1]

[1] Laboratory for Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium
[2] Innogenetics NV, Ghent, Belgium , Ghent, Belgium
[3] Department of Rheumatology, University Hospital Ghent, Ghent, Belgium

Proteomics 2006, *in press*

ABSTRACT

We aimed to investigate potential synovial autoantigens in rheumatoid arthritis (RA) that could trigger the induction of B cell autoantibodies.

Total protein extract of synovial tissue obtained from 7 RA patients was pooled and separated by 1-DE and 2-DE. The corresponding blots were probed with sera from RA (n=30) and disease control samples (n=30). Protein spots showing a sensitivity of >15% were identified by mass spectrometry.

1-D immunoblots revealed one protein band with specificity in RA of 100%, a sensitivity of 43%, which was identified as fibrinogen β chain. The 2-D analysis revealed the subunits of fibrinogen, especially the β and γ chain as the most prominent synovial autoantigens. We also identified vimentin, the Sa-antigen and carbonic anhydrase I as a potentially new synovial autoantigen. The protein patterns of these immunoreactive spots were observed as trains. The spots showing the highest autoimmune reactivity occurred at the acidic side of these trains and were recognized by ACPA-positive RA sera. Anti-modified citrulline (AMC) staining of these patterns confirmed protein citrullination.

Therefore, post-translational modifications, such as citrullination due to alterations of PAD activity or generation of RA-specific epitopes, should be considered as trigger in tolerance break.

INTRODUCTION

The inflammatory, chronic joint disease rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane which can ultimately lead to the formation of an invasive tissue, the pannus. This pannus is capable of eroding adjacent cartilage and bone, primarily resulting in deformations of the joints of hands and feet. Synovial inflammation together with extra-articular manifestations, can lead to a severe decline in the quality of life. The presence of several autoantibodies with ranging sensitivities and specificities is one of the major arguments for an autoimmune nature of the disease. The first and most well known autoantibody is the rheumatoid factor. The family of anti-citrullinated protein/peptide antibodies (ACPA) recently described, are the most sensitive and specific markers for RA and occur before disease onset [1, 2]. These autoantibodies are directed against various proteins having one trait in common: all the proteins are modified post-translational. Indeed, some of their arginines have been converted to citrulline by the enzyme peptidylarginine deiminase (PAD) [3, 4].

The primary site of disease manifestations is the RA synovium. In RA, the synovium consists of a variety of cell types, including synovial macrophages, synovial fibroblasts and infiltrating B and T cell populations. The organisation of lymphoid aggregates in follicular structures and the presence of clonally related Ig heavy chain and kappa light chain sequences suggest that the synovial tissue may act as a secondary lymphoid organ where affinity maturation of the B cell response, based on antigen specificity *in situ* takes place [5,6]. This strongly suggests the presence of autoantigens in the synovium. The observation that ACPA are produced by local plasma cells in the pannus supports this hypothesis [7]. Therefore, the *in vivo* characterization of potential autoantigens present in the synovium can lead to a better understanding of the disease process.

Two-dimensional (2-D) gel analysis of synovial extracts gives an accurate image of the proteins expressed in the synovial membrane. Through comparison of protein patterns in different diseases, proteins related to a disease, or proteins implicated in the pathogenesis can be classified [8]. Potential autoantigens can be identified by probing the blots of these 2-D gels with sera from RA patients and controls [9]. This is in contrast with synovial protein microarrays in which only a limited protein repertory is offered to investigate specific antibody responses [10]. To this date, immunodetections are mainly performed on 1-D blots, with the obvious disadvantage of incomplete separation of a complex protein mixture such as synovial extracts [11]. A second disadvantage is that no protein charge isoforms (which are mostly due to posttranslational modification) can be observed although it is hypothesized that

breaking tolerance to self proteins could be induced by secondary modifications. One example is the oxidation and glycosylation of IgG, which may result in products targeted by rheumatoid factors [12, 13]. The usage of patient sera for immunodetection implies that only B cell reactivities are considered.

The aim of the present study was to list the spectrum of synovial autoantigens that induce an autoimmune humoral response. Therefore, 2-D immunoblotting with RA and control patients' sera to an RA synovial protein extract was performed, followed by identification by mass spectrometry.

PATIENTS AND METHODS

Patients

Synovial biopsy samples were obtained from 7 RA patients, fulfilling the American College of Rheumatology (ACR) classification criteria for RA [13], undergoing needle arthroscopy of the knee as described earlier [14]. The biopsy samples were immediately snap frozen in liquid nitrogen and stored in liquid nitrogen until further analysis. A total of 46 synovial biopsy samples were collected from 7 RA patients of which 6 patients showed active synovitis. Detailed information on clinical characteristics is shown in table 1.

Serum samples from 60 patients were collected comprising 30 patients diagnosed with RA fulfilling the ACR criteria [13], and 30 disease control patients. This control group consisted of 16 SpA patients fulfilling the European Spondyloarthropathy Study Group criteria [15], 10 OA patients fulfilling the ACR criteria [16], 2 patients diagnosed with gout, 1 patient with villonodulaire synovitis, and 1 patient diagnosed with sarcoidosis. The clinical characteristics of these patients are indicated in table 1. Within the SpA group, the precise clinical entities are indicated as well.

Table 1 also indicates which samples were used in the various experiments described in the manuscript.

The study was conducted after approval by the local ethics committee. Written informed consent was obtained from all participating patients.

Table 1: Clinical data of patients of whom synovial tissue or serum samples were included

Diagnosis	Age (years)	Sex	NSAID's	DMARD's	Biologicals	SJC	CRP (mg/dl)	ESR (mm/hour)	Anti-CCP (U/ml)	Disease duration
Synovium										
RA	57	F	+	-	-	8	10.5	72	4	0.2
RA	53	F	+	-	-	0	0.6	35	168	0.5
RA	65	F	+	+[(a)]	-	9	5.1	39	4	33
RA	55	F	+	+[(a)]	-	3	ND	29	556	2
RA	50	F	+	+[(a)]	-	9	7.5	77	1515	1.3
RA	57	F	+	+[(b)]	-	7	3.4	15	317	10
RA	50	F	+	+[(a)]	-	15	3.8	48	1515	1.5
Sera										
RA *	28	F	+	-	-	8	3.3	45	1600	1.5
RA *	72	M	+	-	-	7	9.3	45	2	0.1
RA * [f]	75	F	-	-	-	3	1	21	779	1
RA *	63	M	+	+[(b)]	-	4	6.5	55	2	12
RA *	52	F	+	-	-	2	0.7	6	140	0.2
RA *	55	F	+	-	-	8	10.5	72	4	0.2
RA *	60	M	+	+[(c)]	-	14	4	60	206	10
RA *	36	F	-	-	-	2	2.4	46	710	2
RA *	45	F	+	-	-	4	0.5	10	98	1.5
RA *	52	M	+	+[(a)]	-	11	16.4	71	1775	3
RA *	57	F	+	-	+[(e)]	13	12.2	55	146	9
RA * [f]	50	M	+	+[(a)]	+[(e)]	0	2.5	19	796	8
RA *	50	F	+	-	-	9	16.4	111	5	10
RA *	49	M	+	-	-	5	2.2	38	286	15
RA *	51	F	+	-	-	0	0.6	35	168	0.5
RA *	49	M	+	-	-	22	29	94	1600	2
RA *	63	F	+	+[(a)]	+[(e)]	0	1	26	497	7
RA * [f]	75	F	+	+[(b)]	-	3	0.8	26	265	30
RA *	57	M	+	+[(a)]	-	3	0.8	16	2	1
RA *	79	F	+	+[(c)]	-	3	0.1	4	2	6
RA	49	M	+	+[(a)]	-	3	1.6	44	153	10
RA	68	M	+	+[(a)(c)]	-	1	4.9	39	1600	15
RA	63	F	+	+[(a)]	-	9	5.1	39	4	33
RA	53	F	+	+[(a)]	-	3	111	29	556	2
RA	48	F	+	+[(a)]	-	9	7.5	77	1515	1.3

RA	55	F	+	+[(b)]	-	7	3.4	15	317	10
RA °	55	F	+	+[(b)]	-	0	9.2	45	1	1
RA °	49	F	+	+[(a)]	-	1	0.4	20	2933	6
RA °	50	F	+	+[(a)]	-	13	3	39	2362	22.5
RA °	73	M	+	+[(b)]	-	9	0.2	1	1	0.8
AS *	56	F	+	+[(b)]	-	1	3.5	60	2	16
USPA + SLE *	38	F	+	+[(b)]	-	1	1.2	27	4	1
USPA *	47	M	+	+[(b)]	-	1	2.6	42	1	22
USPA *	30	M	+	-	-	4	0.4	5	13	0.5
AS *	58	M	+	-	-	2	4	30	4	25
USPA *	47	M	+	+[(d)]	-	1	1.7	8	1	2
PsA *	29	M	+	-	-	2	0.7	9	9	5
AS *	57	F	+	+[(b)]	-	2	0.3	7	1	9
PsA *	33	M	+	-	-	1	6.8	39	4	10
USPA *	49	F	+	+[(b)]	-	6	0.9	13	2	4
PsA *	26	F	+	-	-	1	0	1	2	0.9
USPA *	26	F	+	+[(a)]	-	6	21.1	115	2	2
USPA *	58	M	+	-	-	1	0.1	3	2	12
PsA *	29	F	+	-	-	1	0.8	24	2	10
PsA *	34	M	+	+[(b)]	-	1	1.1	17	2	4
SPA *	29	M	+	-	-	2	14.3	70	5	0.1
OA and osteoporosis *	72	F	-	-	-	1	0.5	13	2	8
OA *	59	M	+	-	-	0	0.2	3	111	35
OA *	64	F	+	-	-	1	0.1	1	4	999
OA *	56	F	+	-	-	5	0.1	5	4	0.5
OA	58		+	-	-	1	0.2	29	4	7
OA	46	M	-	-	-	1	0.3	2	3	15
OA and chondrocalcinosis	72	F	+	-	-	4	0.3	5	6	1
OA	89	M	-	-	-	2	0.3	14	1	12
OA	60	F	+	-	-	0	0.7	11	3	20
Villonodular synovitis	32	M	+	-	-	1	0.6	4	1	2
Gout °	47	M	+	-	-	0	7	36	2	6
OA °	82	F	+	-	-	1	1.2	34	5	2
Gout °	36	M	-	-	-	2	4.	26	2	15
Sarcoidosis °	76	F	-	-	-	1	4.8	77	1	22

Except where mentioned otherwise: M = male; F = female; (+) = receiving; (-) = not receiving; NSAID's = nonsteroidal antiinflammatory drugs; DMARD's = disease-modifying antirheumatic drugs where (a)= methotrexate and (b)= sulfasalazine, (c)= leflunomide, (d)= Immuran; Biologicals = biological modifiers where (e)= Infliximab. Treatment characteristics were determined at the time of tissue sampling; RA = rheumatoid arthritis, OA = osteoarthritis, USPA = undifferentiated spondyloarthropathy, PsA = psoriatic arthritis, AS = ankylosing spondylitis, SLE = systemic lupus erythematosus; ND = no data available; SJC = swollen joint count;

CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; anti-CCP = antibody against cyclic citrullinated peptides determined by ELISA (Immunoscan RA, mark 2, Euro-diagnostica AB (Arnhem, The Netherlands) according to the manufacturer's instructions using a cut-off value of 25U/ml; the disease duration displayed was determined at the time of tissue sampling. Patients used for 2-D immunoblotting (pH 3-10 and pH 4-7) are indicated with '*'. Serum samples of patients used for 2-D immunoblotting with 4-12% Bis-Tris peptide gels are indicated with '°'. Serum samples used for 2-D immunoblotting of deiminated fibrinogen in vitro are indicated with 'f'

Protein extraction biopsy material

Prior to protein extraction, synovial biopsy samples were washed with DPBS (Gibco, Invitrogen Inc. Carlsbad, California, USA) to eliminate blood contamination. Protein extraction was performed using the PlusOne grinding kit (Amersham Biosciences, Uppsala, Sweden). Briefly, a maximum of 4 biopsy samples were transferred to an eppendorf tube and grinded for 4 minutes. 250µl extraction buffer (9.5M urea; 5% ampholine pH 3.5-10; 2% w/v CHAPS, 10mM DTT) containing a cocktail of protease inhibitors (Complete mini cocktail, Roche Applied Sciences, Indianapolis, USA) was added to the samples and incubated overnight at room temperature while gently shaking. Proteins were extracted by centrifugation for 10 minutes at 15000 x g at room temperature. Supernatant of 46 biopsy tissue samples was pooled and protein quantification was performed using PlusOne 2-D Quant kit from Amersham Biosciences (Uppsala, Sweden).

In vitro deimination of fibrinogen

Fibrinogen purified from human plasma (Plasminogen depleted fibrinogen, Calbiochem, Merck, Darmstadt, Germany) was dissolved at 1mg/ml in PBS pH 7.4 (37°). *In vitro* deimination was performed by incubation with rabbit skeletal PAD2 (Sigma-aldrich, St. Louis, MO, USA) (7 units/mg fibrinogen) overnight at 37° C in 100 mM Tris-HCl, pH 7.4, 10mM $CaCl_2$ with 5mM DTT. Reaction was stopped by adding EDTA to a final concentration of 50 mM.

Gel electrophoresis

1-Dimensional gel electrophoresis

For SDS-PAGE, 10 µg/cm of total protein extract was loaded on a precast gel containing one slot (8x8 cmxcm, 4-12% Bis-Tris zoomgel, 1.0mm; IPG well (7cm); Invitrogen Inc. Carlsbad, California, USA). The sample was run under reducing conditions in MOPS SDS running buffer according to the manufacturer's instructions.

2-Dimensional gel electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed according to Görg *et al*. [17] with minor adjustments. Briefly, 50µg of protein extract was dissolved in 100 µl rehydration buffer solution containing 9.5M urea, 2% v/w CHAPS, 10mM DTT and 5% Carrier Ampholyte solution (Amersham Biosciences, Uppsala, Sweden). The sample was centrifuged for 5 minutes at 13000rpm and subsequently incorporated in an immobilized pH gradient strip (IPG) of 7cm (Amersham biosciences, Uppsala, Sweden) containing a linear pH gradient of 3-10 or pH 4-7. The IPG strips were rehydrated overnight. After in-gel rehydration [18], the IPG strips were iso-electrically focused on the Multiphor II (Amersham Biosciences, Uppsala, Sweden) for a maximum of 7474Vh at 20°C using a three step program: 200V (10 min.-33Vh) – 200V (1h30 – 2775Vh) – 3500V (1h20 – 4666Vh). After iso-electric focusing, the strips were equilibrated in two steps by gently shaking for 15 minutes in a solution containing Tris-HCl buffer (50mM, pH 8.8), 6M urea, 30% v/w glycerol, 2% SDS containing 2% DTT. Subsequently, equilibration in the same buffer was performed for 15 minutes in the presence of 4.8% iodoacetamide. After equilibration, the IPG strips were placed on a 12.5% Laemmli, 4-12% Bis-Tris or 12% Bis-Tris peptide SDS-PAGE as specifically indicated further. The gels were run at 10°C applying 30V for 10 minutes followed by 150V until the bromophenolblue front reached the end of the gel (usually after 1h20).

Gels were further used for Western Blotting or were stained with Coomassie G-250 [19] or silver [20].

For the separation of fibrinogen, 10µg of the protein was dissolved in 180 µl rehydration buffer containing 7M ureum, 2M thioureum, 4% CHAPS, 20mM DTT and 0.2% Carrier Ampholyte Solution (Amersham Bioscience, Uppsala, Sweden) and incorporated in a linear IPG strip pH 3-10 or pH 4-7 (Biorad, Hercules, CA, USA) by overnight rehydration. The strips were iso-electrically focused on the Protean IEF cell (Biorad, Hercules, CA, USA) using the following program: 100V (30 min) – 250V (30 min) – 8000V (2h) – 8000V (25000Vh). The strips were equilibrated in two steps prior to SDS-PAGE by gently shaking in 50mM Tris-HCl buffer (pH 8.8) containing 6M urea, 20% glycerol, and 2% SDS. In the first step, 1.5% DTT was added followed by 4% iodoacetamide in the second step. The strips were subsequently placed on a 4-20% linear gradient Tris-HCl SDS-PAGE (Biorad, Hercules, CA, USA). The gels were run at 200V for 1h30 in the Criterion Cell (Biorad, Hercules, CA, USA) and stained with Sypro Ruby Protein Gel Stain (Biorad, Hercules, CA, USA).

Western blotting

Protein transfer to nitrocellulose Hybond C membranes (0.45µm) was performed by semi-dry Western blotting using the Multiphor II NovaBlot transfer unit (Amersham biosciences, Uppsala, Sweden) at 1,5mA/cm^2 for 1 hour. Transfer of fibrinogen and *in vitro* deiminated fibrinogen was done by tank blotting using the Trans-blot cell (Biorad, Hercules, CA, USA). In all Western blot experiments, Ponceau S visualization of the membranes was done first. This reversible low sensitive staining was used to check the blotting efficiency, and to verify the 2-D pattern. Prior to overnight incubation with human serum (1:100 v:v in PBS/0.3% Tween-20), blocking of the nitrocellulose membrane was done using PBS/0.3% Tween-20 for 1 hour. The membranes were subsequently incubated for 1 hour with goat anti-human IgG labeled with alkaline phosphatase (Sigma, Steinheim, Germany) (1:1000 v:v in PBS/0.3% Tween-20) and immunoreactivity was visualized using NBT/BCIP.

For the detection of citrullinated proteins, blots were chemically modified prior to immunostaining with a chemiluminescent substrate (Pierce, Rockford, IL, USA) as previously described [21] using the anti-modified citrulline (AMC) detection kit, (Upstate, Charlottesville, VA, USA).

To reveal the total protein pattern, the membranes were stained with AuroDye (Amersham Biosciences, Uppsala, Sweden) according to manufacturer's guidelines. This colloidal gold stain is highly sensitive and reveals all proteins transferred to the nitrocellulose membrane.

This protocol was used for all gel electrophoresis experiments, apart from little modifications specific for certain experiments described hereafter. For 1-D SDS-PAGE (4-12% Bis-Tris), nitrocellulose membranes were cut into small bands of 0.3cm and each membrane strip was probed with a different serum sample.

For Western blotting of 2-D peptide gels (12% Bis-Tris), PVDF membranes were used in stead of nitrocellulose membranes, because smaller molecules are better retained on PVDF membranes.

For each Western Blotting experiment, negative controls were included where the blot was only incubated with the specific secondary antibody.

The different visualizations of the blots were scanned and digitized with the ProXpress Imager from Perkin Elmer (Wellesly, MA, USA). At first, the Ponceau S-stained pattern was scanned, followed by the autoantigen detection and the AuroDye visualization. Digitized images were analyzed semi-manually using HT analyser Software (Perkin Elmer, Wellesly, MA, USA).

Pattern analysis

After Ponceau S visualization, marks were added onto the membranes, which served as landmarks for matching. The location of the major spots was highlighted by piercing the membrane. Matching of the immunoreactive spots to the total protein pattern visualized by Ponceau S and AuroDye was done semi-manually using the HT-Software. Finally, the total protein pattern revealed by AuroDye (Amersham biosciences, Uppsala, Sweden) was compared to the pattern in the 2-D gel pattern visualized by silver staining [20] and Coomassie G-250 [19], in order to correlate the immunodetected spots with the corresponding spots visualized in the gel.

Protein identification

Spots of interest were excised from semi-preparative 2-D gels, digested with modified sequence grade trypsin (Porcine) (Promega, Madison WI, USA) using the Montage In-gel Digest kit from Millipore (Billerica, MA, USA) and analyzed by nano-LC/MSMS on a Q-TOF I (Water, Milford, USA) equipped with an orthogonal Z-spray. Fragmentation spectra resulting from tandem mass spectrometry were searched against the mascot search engine (http://www.matrixscience.com) [22]. The mascot score is reported as $10*\log_{10}$ (P), where P is the absolute probability that the observed match is a random event.

RESULTS

Protein extraction of synovial biopsy tissue

Extraction of 46 pooled synovial tisuue biopsy samples obtained from the 7 RA patients yielded a total amount of 8.8mg protein at a concentration of 1.88mg/ml. Preliminary studies on different protein extraction protocols revealed that the protein yield from experiments with the PlusOne Grinding kit (Amersham biosciences, Uppsala, Sweden) was far superior to cutting the biopsy samples into little pieces or using a Wheaton Douncer. Also, overnight incubation of the biopsy samples in extraction buffer revealed higher protein yields compared to the recommended <2h extraction time.

Immunoblotting of total synovial extract

1-dimensional gel immunoblotting

A pre-screening was performed on 1-D blot strips with 30 RA and 30 control sera (table 1) to rapidly visualize immunoreactivity against synovial proteins. Probing the strips revealed two

major immunoreactive bands (fig.1). However, the 55kDa band was observed on all strips, including the negative control strip incubated only with the secondary antibody and represented the heavy chain of IgG. The second abundant immunoreactive band appeared just above the IgG heavy chain at 58.8kDa. This protein band was completely absent from immunostrips probed with non-RA sera (specificity 100%) and was detected in 13/30 immunostrips probed with RA sera (sensitivity 43%). The band was identified by mass spectrometry as the fibrinogen β chain (mascot score= 196).

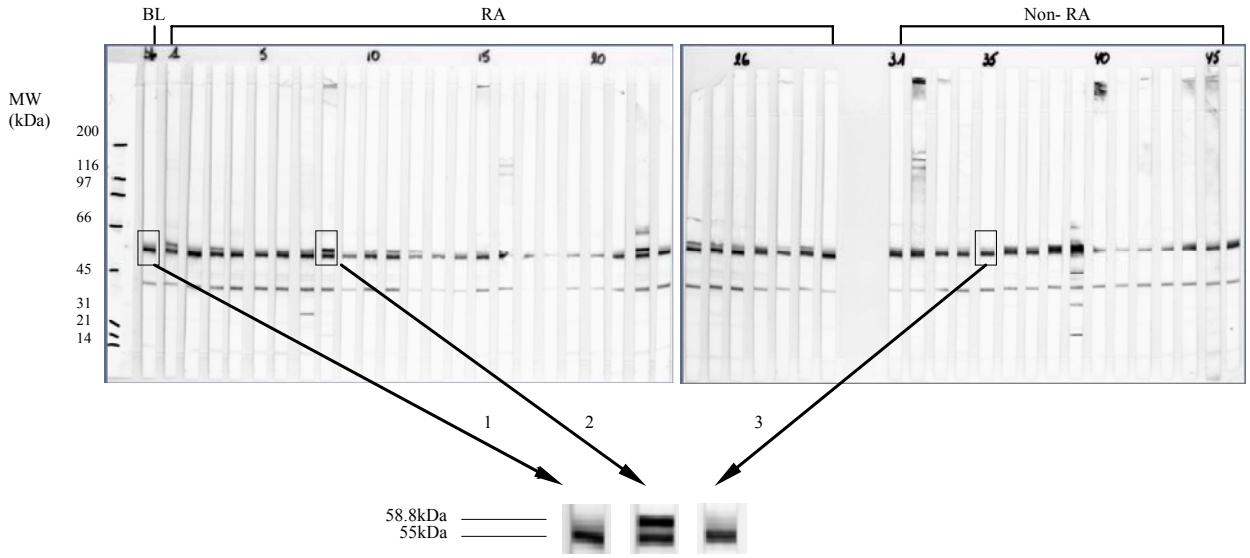


Figure 1: 1-D screening of immunoreactivity of RA sera (strip 1-30) against synovial tissue proteins in comparison with immunoreactivity of non-RA sera (strip 31-46) (4-12% Bis-Tris SDS-PAGE).
The 55kDa protein band is visual on all membrane strips including the negative control strip incubated only with the secondary anti-human IgG antibody (strip BL). This protein band corresponds to the IgG heavy chain.
A second abundant immunoreactive band is visible at 58.8 kDa band with 100% specificity and 43% sensitivity in RA patients. This band was identified as fibrinogen β chain.
The enlarged view shows the negative control **(1)**, a strip probed with RA serum **(2)** and non-RA serum **(3)**.

2-dimensional gel immunoblotting

A total amount of 50µg of synovial tissue proteins was subjected to 2-DE using IPG strips with pH 3-10 (fig. 2A) and pH 4-7 (fig. 2B). The gels were used for Western blotting, in which the nitrocellulose Hybond C membranes were probed with sera from 20 patients diagnosed with RA (see table 1, patients indicated with '*'). Sera from patients with other rheumatic diseases were used as control group (n=20). 2-D blots incubated with only the secondary antibody were completely negative.

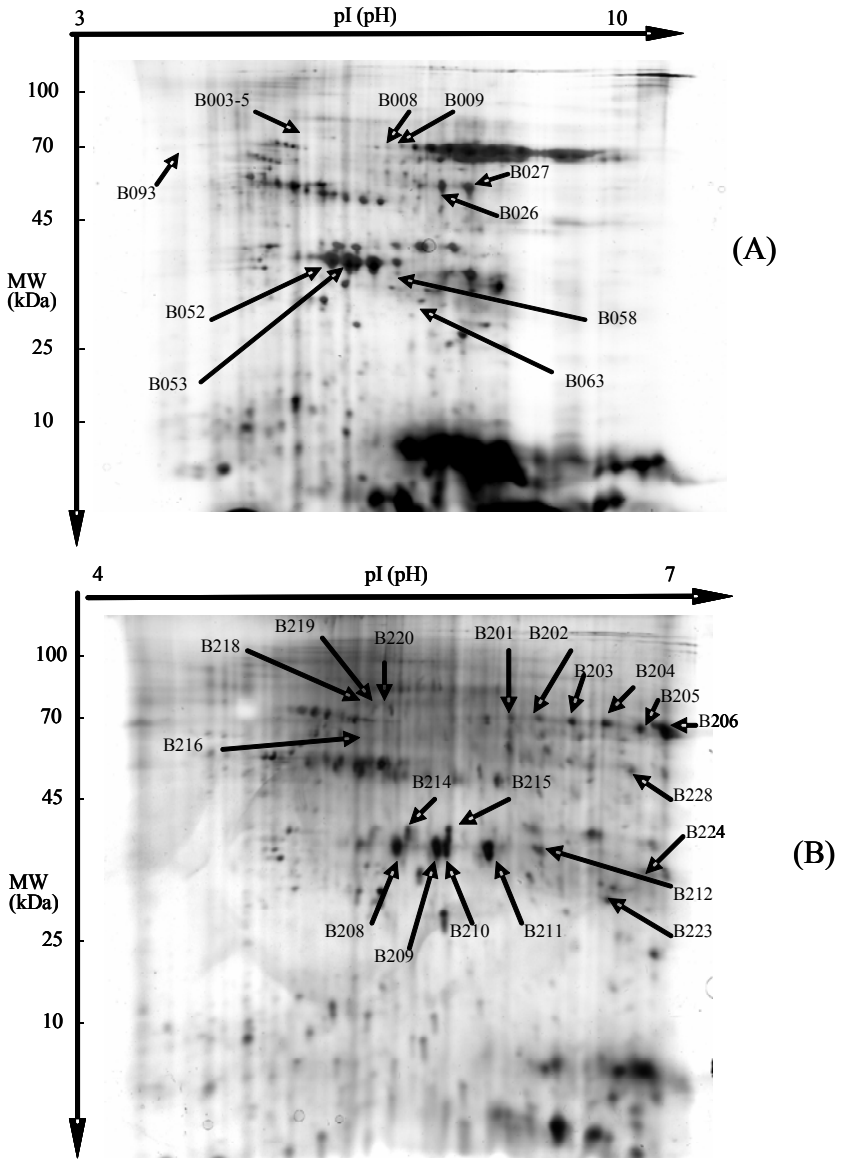


Figure 2: Total 2-D protein pattern of synovial tissue separated on 12.5% Laemmli resolving gel using IPG 3-10 (A) and IPG pH 4-7 (B) revealed by silver staining.
Immunoreactive spots with a sensitivity ≥15% were identified and are indicated on the gel. Some of the immunoreactive spots were not visible on the silver stained gel.

Immunoreactive spots with a sensitivity in RA of at least 15% were identified by mass spectrometry. The summary of these identifications is shown in table 2. If multiple proteins were identified in one spot, only the protein with the highest mascot score was listed. Identification of proteins such as keratin was not considered as the presence of this protein is most likely due to contamination during sample handling [23].

pH gradient 3-10

We first analyzed the synovial tissue proteome by 2-DE using IPG pH 3-10 (fig. 2A). A total of 101 spots showed immunoreactivity with RA sera. Protein spots with a sensitivity of at

least 15% were further analyzed (n=12). The specificity of these spots ranged from 65% to 100%. These spots were selected for identification by mass spectrometry (table 2). Most of immune reactive synovial proteins were identified as the subunits of fibrinogen (α, β and γ chain). Although spots B063 and B093 were almost invisible on the gel, sensitive mass spectrometric analysis revealed their identity being carbonic anhydrase I and vimentin, respectively.

In order to enlarge the resolution of the protein separation and since no reactivity was visible at the very basic site of the immunoblots; the analysis was repeated using IPG strips pH 4-7.

pH gradient 4-7

2-D immunoblots using IPG pH 4-7 revealed 73 spots showing autoimmune reactivity with RA sera. Spots that showed a sensitivity of at least 15 % (fig. 2B) were identified (n= 20) as fibrinogen β or γ chain. The specificity of these immunoreactive spots ranged from 70% to 100% (table 2).

Similarities between immunoblots IPG pH 3-10 and pH 4-7

By performing the immunoblot experiments with both the above mentioned pH gradients, all analyses were performed in duplex. Several immunoreactive spots could be matched between the gels of different pH gradients e.g. B008, B009 and B052, B053 on IPG 3-10 are identical to B202, B203 and B208, B209 on IPG 4-7, respectively. In addition, the identification of these spots confirmed these similarities. The separation resolution of the 2-DE with IPG 4-7 was improved dramatically. Although we could find some extra autoantigen spots, they did not represent new additional autoimmune proteins.

The 2-D protein patterns of immunoreactive spots were observed as trains consisting of adjacent spots of the same protein, but with a different isoelectric point (pI). Remarkably, immunoreactive spots with the highest specificity occurred at the acidic side of the spot train. For example, spot B201 and B202 (figure 2B), which were located on the acidic side of the train, showed a specificity of 100%. Spot B203, B204, B205 and B206 appearing at the basic side of the train, displayed a specificity in RA of 80%, 75%, 70% and 60%, respectively. Re-incubation with RA serum of membranes previously probed with a control serum confirmed these results and revealed additional spots on the acidic side of the spot train (fig. 3).

Table 2: Autoantigens present in synovial tissue visualized by 2-D immunoblotting on IPG pH 3-10 and pH 4-7 and identified by mass spectrometry

Spotnr.	Sens. (%)	Spec. (%)	Database protein name	Peptide score	Protein score	Expect	Peptide	Sequence coverage (%)	MW_{exp} (kDa)	pI_{exp} (pH)
pH 3-10										
B003-B004-B005	25-45-45	85-85-85	Fibrinogen alpha/alpha-E chain	82	163	0,000035	K.GLIDEVNQDFTNR.I	3%	73.3-74.5-74.5	5.6-5.7-5.8
				82		0,000034	R.GGSTSYGTGSETESPR.N			
B008-B009	35-50	100-85	Fibrinogen beta chain	35	602	0,66	R.SILENLR.S	28%	70.8-71.5	6.3-6.4
				17		41	K.YQISVNK.Y			
				68		0,00038	R.QDGSVDFGR.K			
				26		6,5	K.IRPFFPQQ.-			
				37		0,66	K.EDGGGWWYNR.C			
				57		0,0085	R.DNDGWLTSDPR.K			
				79		0,000054	K.QGFGNVATNTDGK.N			
				73		0,00024	K.LESDVSAQMEYCR.T			
				68		0,00093	R.TPCTVSCNIPVVSGK.E			
				80		0,000068	K.DNENVVNEYSSELEK.H			
				27		15	K.NYCGLPGEYWLGNDK.I			
				38		1,2	K.GGETSEMYLIQPDSSVK.L			
B026	50	75	Fibrinogen beta chain	14	60	200	K.HGTDDGVVWMNWK.G	8%	59.8	7.1
				31		4,6	K.DNENVVNEYSSELEK.H			
				15		220	K.NYCGLPGEYWLGNDK.I			
B027	45	65	Fibrinogen beta chain	27	487	6,4	K.IRPFFPQQ.-	26%	59.8	7.1
				43		0,17	K.EDGGGWWYNR.C			
				52		0,021	R.DNDGWLTSDPR.K			
				84		0,000017	K.QGFGNVATNTDGK.N			
				73		0,00025	K.LESDVSAQMEYCR.T			
				60		0,0062	R.TPCTVSCNIPVVSGK.E			
				23		30	R.MGPTELLIEMEDWK.G			
				67		0,0014	K.DNENVVNEYSSELEK.H			
				23		37	K.NYCGLPGEYWLGNDK.I			
				41		0,52	K.GGETSEMYLIQPDSSVK.L			
B052	20	90	Fibrinogen gamma chain	57	162	0,0057	R.DNCCILDER.F	7%	42.2	5.9
				48		0,06	K.QSGLYFIKPLK.A			

				56		0,012	R.YLQEIYNSNNQK.I			
B053	20	95	Fibrinogen gamma chain	39	393	0,31	R.LDGSVDFK.K	15%	42.2	6.0
				33		1,3	K.MLEEIMK.Y			
				39		0,33	K.RLDGSVDFK.K			
				60		0,0033	R.DNCCILDER.F			
				68		0,00053	K.DTVQIHDITGK.D			
				63		0,0021	K.QSGLYFIKPLK.A			
				32		3,2	K.YEASILTHDSSIR.Y			
				60		0,0057	R.YLQEIYNSNNQK.I			
B058	15	100	ND							
B063	30	95	Carbonic anhydrase I	40	210	0,26	K.VLDALQAIK.T	21%	34.8	6.7
				67		0,00066	K.ADGLAVIGVLMK.V			
				61		0,0055	K.LYPIANGNNQSPVDIK.T			
				43		0,36	K.HDTSLKPISVSYNPATAK.E			
B093	15	100	Vimentin	60	60	0,0031	K.FADLSEAANR.N	2%	69.6	4.3
pH 4-7										
B201	30	100	Fibrinogen beta chain	26	463	5,4	R.SILENLR.S	22%	65.6	5.8
				27		6,4	K.IRPFFPQQ.-			
				27		5,8	K.EDGGGWWYNR.C			
				55		0,011	R.DNDGWLTSDPR.K			
				91		0,000003	K.QGFGNVATNTDGK.N			
				59		0,0062	K.LESDVSAQMEYCR.T			
				69		0,00069	R.TPCTVSCNIPVVSGK.E			
				62		0,0037	K.DNENVVNEYSSELEK.H			
				50		0,071	K.GGETSEMYLIQPDSSVK.L			
B202	35	100	Fibrinogen beta chain	54	173	0,014	R.DNDGWLTSDPR.K	11%	65.3	5.8
				38		0,64	K.QGFGNVATNTDGK.N			
				59		0,0078	K.DNENVVNEYSSELEK.H			
				25		23	K.GGETSEMYLIQPDSSVK.L			
B203	45	80	Fibrinogen beta chain	26	353	6,5	K.IRPFFPQQ.-	21%	64.5	6.0
				42		0,21	K.EDGGGWWYNR.C			
				48		0,054	R.DNDGWLTSDPR.K			

				69		0,00051	K.QGFGNVATNTDGK.N			
				65		0,0019	R.TPCTVSCNIPVVSGK.E			
				65		0,0022	K.DNENVVNEYSSELEK.H			
				17		130	K.NYCGLPGEYWLGNDK.I			
				24		24	K.GGETSEMYLIQPDSSVK.L			
B204	35	75	Fibrinogen beta chain	66	66	0,0016	K.DNENVVNEYSSELEK.H	3%	63.4	6.1
B205	40	70	Fibrinogen beta chain	18	158	48	K.IRPFFPQQ.-	6%	63.5	6.3
				58		0,0063	R.DNDGWLTSDPR.K			
				82		0,000041	K.DNENVVNEYSSELEK.H			
B206	30	60	Fibrinogen beta chain	34	1031	1	R.SILENLR.S	47%	63.7	6.3
				27		6,3	K.IRPFFPQQ.-			
				55		0,012	K.EDGGGWWYNR.C			
				43		0,19	R.DNDGWLTSDPR.K			
				105		0,00000012	K.QGFGNVATNTDGK.N			
				105		0,00000017	K.AHYGGFTVQNEANK.Y			
				63		0,0026	K.HGTDDGVVWMNWK.G			
				80		0,00006	K.LESDVSAQMEYCR.T			
				97		0,0000011	R.TPCTVSCNIPVVSGK.E			
				34		2,1	R.YYWGGQYTWDMAK.H			
				34		2,7	R.MGPTELLIEMEDWK.G			
				74		0,00024	K.DNENVVNEYSSELEK.H			
				39		0,87	K.NYCGLPGEYWLGNDK.I			
				46		0,18	R.TMTIHNGMFFSTYDR.D			
				70		0,00073	K.GGETSEMYLIQPDSSVK.L			
				21		59	R.EEAPSLRPAPPPISGGGYR.A			
				37		1,6	R.KGGETSEMYLIQPDSSVK.L			
				78		0,00015	K.HQLYIDETVNSNIPTNLR.V			
B208	35	95	Fibrinogen gamma chain	41	323	0,19	R.LDGSVDFK.K	13%	34.3	5.6
				30		2,3	K.MLEEIMK.Y			
				31		2,1	K.RLDGSVDFK.K			
				44		0,12	R.DNCCILDER.F			
				62		0,0025	K.DTVQIHDITGK.D			
				49		0,064	R.YLQEIYNSNNQK.I			

				65		0,0015	K.VAQLEAQCQEPCK.D			
B209-B210	35-30	95-95	Fibrinogen gamma chain	39	421	0,32	R.LDGSVDFK.K	23%	34.1-34.0	5.7-5.6
				30		2,3	K.MLEEIMK.Y			
				41		0,24	K.RLDGSVDFK.K			
				46		0,083	R.DNCCILDER.F			
				71		0,0003	K.DTVQIHDITGK.D			
				50		0,037	K.QSGLYFIKPLK.A			
				34		2	K.YEASILTHDSSIR.Y			
				26		14	K.VAQLEAQCQEPCK.D			
				56		0,012	R.YLQEIYNSNNQK.I			
				29		16	K.AIQLTYNPDESSKPNMIDAATLK.S			
B211	30	95	Fibrinogen gamma chain	55	405	0,0067	R.LDGSVDFK.K	21%	34.1	5.7
				33		1,3	K.MLEEIMK.Y			
				44		0,11	K.RLDGSVDFK.K			
				44		0,12	R.DNCCILDER.F			
				74		0,00016	K.DTVQIHDITGK.D			
				62		0,0022	K.QSGLYFIKPLK.A			
				32		3,2	K.YEASILTHDSSIR.Y			
				55		0,018	R.YLQEIYNSNNQK.I			
				8		1700	K.AIQLTYNPDESSKPNMIDAATLK.S			
B212	25	90	Fibrinogen gamma chain	39	156	0,44	K.QSGLYFIKPLK.A	8%	33.9	5.9
				62		0,0029	K.YEASILTHDSSIR.Y			
				54		0,019	K.VAQLEAQCQEPCK.D			
B214	30	100	Fibrinogen gamma chain	45	240	0,11	R.VELEDWNGR.T	11%	36.0	5.4
				63		0,0016	R.TSTADYAMFK.V			
				42		0,2	R.DNCCILDER.F			
				51		0,026	K.DTVQIHDITGK.D			
				40		0,51	R.YLQEIYNSNNQK.I			
B215	30	90	Fibrinogen gamma chain	6	492	510	K.NWIQYK.E	24%	35.7	5.6
				35		0,76	K.MLEEIMK.Y			
				24		11	K.RLDGSVDFK.K			
				49		0,044	R.VELEDWNGR.T			
				59		0,0043	R.TSTADYAMFK.V			

				48		0,057	R.DNCCILDER.F			
				76		0,000089	K.DTVQIHDITGK.D			
				46		0,095	K.QSGLYFIKPLK.A			
				28		7,1	K.YEASILTHDSSIR.Y			
				62		0,0036	R.YLQEIYNSNNQK.I			
				61		0,0045	K.VAQLEAQCQEPCK.D			
B216	30	100	ND						62.4	5.2
B218-219-220	30-35-30	100-95-90	Fibrinogen gamma chain	48	47	0,053	R.TSTADYAMFK.V	2%	72.0-71.2-70.8	5.2-5.3-5.3
B223	20	90	Fibrinogen beta chain	47	47	0,13	K.DNENVVNEYSSELEK.H	3%	30.7	6.2
B224	30	95	Fibrinogen beta chain	40	146	0,37	R.DNDGWLTSDPR.K	7%	33.00	6.3
				84		0,000016	K.QGFGNVATNTDGK.N			
B228	25	80	Fibrinogen beta chain	53	53	0,036	K.DNENVVNEYSSELEK.H	3%	48.6	6.2

Sens.= sensitivity of autoimmunreactivity; Spec.= specificity of autoimmunoreactivity; the peptide score= the ion score for the individual peptide; the protein score= probability mowse score reported by matrix science where $p<0.05$ of the individual ions scores indicates identity or extensive homology; Expect= the expectation value for the peptide match (the lower this value, the more significant the result); Peptide= the sequence of the peptide; MW_{exp}= molecular weight of the protein spot obtained experimentally on the 2-D gel; pI_{exp}= isoelectric point of the protein spot measured experimentally from the 2-D gel.

Relation of immunoreactive spots with ACPA titers

In this table spots B008, B053, B052 and B009, were the only spots that were citrullinated, as described in the next section. These spots were only recognized by CCP positive sera. Fibrinogen α chain was also detected by 4 CCP negative sera. In addition, carbonic anhydrase I (B063) was recognized by 2 CCP negative RA sera. Both proteins did not show any citrullination on AMC staining as is indicated in the next section.

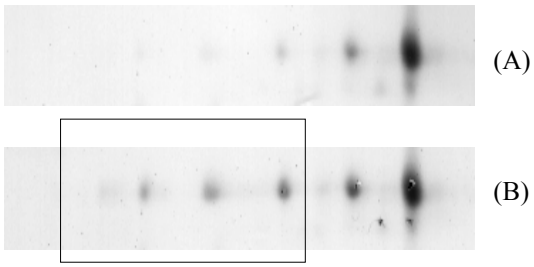


Figure 3: Re-incubation with RA serum (B) of membranes previously probed with control serum (A) (IPG 4-7; 4-12% Bis-Tris SDS-PAGE).
New immunoreactive spots appear at the acidic side of the spot train.

Table 3: Relation of immunoreactive spots with ACPA titers

Identification		FIBB	VIME	ND	CAH1	FIBG	FIBG	FIBB	FIBA	FIBA	FIBA
Spotnr.		**B008**	**B093**	**B058**	**B063**	**B053**	**B052**	**B009**	**B004**	**B005**	**B003**
Spec. (%)		100	100	100	95	95	90	85	85	85	85
Sens. (%)		35	15	15	30	20	20	50	45	45	25
Serum	**Anti-CCP**										
10	1175					x	x	x			
1	1600	x				x	x	x			
16	1600	x		x	x			x	x	x	x
3	779	x						x	x	x	x
12	796	x						x			
8	710	x						x	x	x	x
17	497				x						
14	286		x	x	x			x	x	x	x
18	265	x	x					x			
7	206	x			x	x	x	x	x	x	x
15	168		x								
11	146					x	x	x			
5	140										
9	98			x							
2	2								x	x	
4	2				x				x	x	
6	4										
13	5				x				x	x	
19	2								x	x	
20	2										

Autoimmunoreactivity against a certain spot is indicated by 'x'. The identification of the protein spots is given as the first part of the Swiss-prot entry name where FIBB= fibrinogen β chain; FIBG = fibrinogen γ chain; FIBA= fibrinogen α chain, VIME= vimentin; CAH1= carbonic anhydrase I and ND= no data. The spots that were citrullinated by AMC staining are underlined. The sensitivity (sens.) as well as the specificity (spec.) of the immunoreactivity is given in percentage. The amount of antibody against CCP determined by ELISA (Immunoscan RA, mark 2, Euro-diagnostica AB (Arnhem, The Netherlands) is presented by (anti-CCP).

Detection of citrullinated proteins

A possible explanation of the increasing specificity of the immunoreactive spots at the acidic side of the spot trains is that the antibodies in RA patients are directed against proteins with a specific post-translational modification that gradually lowers the pI of the protein. One such modification is deimination of arginine to citrulline. The replacement of 1 arginine with 1 alanine in β chain of fibrinogen theoretically lowers the pI with 0.32 units (http://us.expasy.org/tools/#proteome). This would result in a shift of 0.375cm on an IPG pH 4-7 strip of 8cm, which is exactly the distance between the individual spots in the spot trains observed on these 2-D gels (IPG 4-7, 12.5% Laemmli) (fig. 2B). Moreover, the relationship between the highly specific autoimmune spots with the ACPA titers (table 3) also supports this hypothesis.

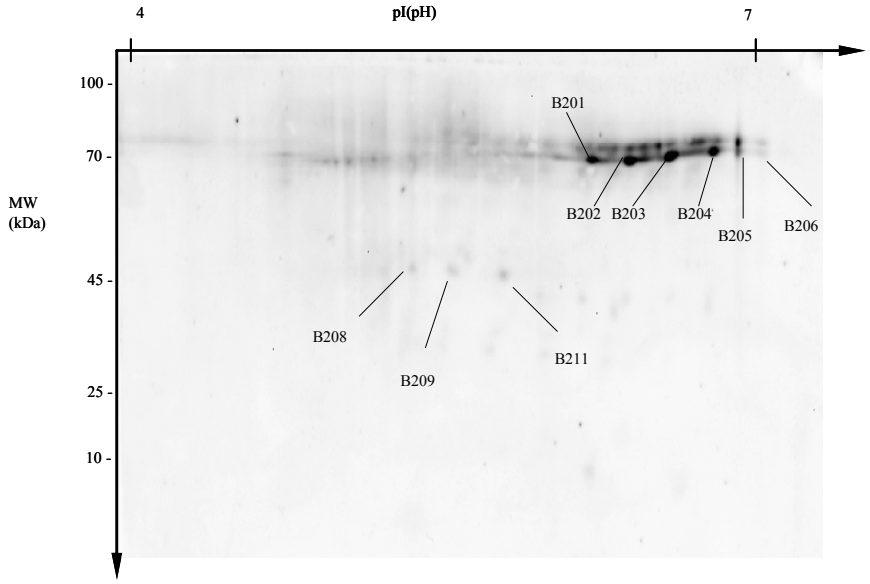


Figure 4: Anti-modified citrullline detection.
Synovial extract was separated on an IPG pH 4-7, 12.5 % Tris-HCl gel and blotted on a nitrocellulose membrane prior to chemical modification of the citrulline residues. Citrullinated protein spots B201-B206 were identified as fibrinogen β chain, spots B208-211 are identified as fibrinogen γ chain.

To explore this possibility further, 2-D blots of synovial extracts separated on IPG pH 4-7, 12.5% Laemmli resolving gels were probed with the anti-modified citrulline antibody (fig 4). This reactive pattern confirmed that citrullinated proteins are present in the human synovial membrane as demonstrated by synovial histology data and 1-D analysis performed by several groups [24], [25]. Fibrinogen β chain and fibrinogen γ chain were found to be citrullinated in

synovial protein extracts. These results were confirmed when AMC staining was performed on 2-D blots using IPG 3-10 (12.5%SDS-PAGE) (results not shown) where no other protein spots were citrullinated apart from fibrinogen β chain and fibrinogen γ chain.

In addition, all protein spots in a spot train seemed to be citrullinated, not only the spots appearing at the most acidic side. In order to address this phenomenon, we analyzed the behaviour of citrullination of fibrinogen on 2-DE.

Two-dimensional analysis of in vitro deiminated fibrinogen

Analysis of fibrinogen purified from plasma (Calbiochem, Merck, Darmstadt, Germany) on IPG pH 4-7 (4-20% lineair gradient gel) revealed the three subunits of fibrinogen. The individual spots of the α and the β chain were not well resolved (fig. 5A). They appeared compressed together at the end of the pH gradient. This is not surprising since the calculated pI of the non-deiminated chains of fibrinogen is 8.08 for the α chain, 7.15 for the β chain and 5.24 for the γ chain. Therefore, the non-deiminated fibrinogen was reanalyzed on IPG pH 3-10 (fig. 5B) and the different fibrinogen chains were observed clearly as different protein trains with well resolved individual spots.

In vitro citrullination of fibrinogen resulted not only in the appearance of more spots at the acidic side of the spot train, but also in a complete shift of the spot train towards a lower pI (fig. 5C). It was clear that the α and β chain had shifted to a lower pI, relative to the γ chain. Also, the most acidic spots in the α spot train shifted to a higher molecular weight, which is a known characteristic of citrullination [26]. In addition, citrullination was checked by AMC detection on the blotted membrane and revealed even more spots than on the corresponding 2-D gel (data not shown), visualized by Sypro Ruby total protein stain, because of the differences in sensitivity between the detection methods.

Immune reactivity against all three fibrinogen side chains was observed when *in vitro* citrullinated fibrinogen was probed with human RA serum (5D). Three RA sera (indicated in table 1 by '[f]') were used for immunoblotting against *in vitro* citrullinated fibrinogen and all 3 patients showed immune reactivity against the α, β and γ chains of *in vitro* deiminated fibrinogen. Moreover, the strongest immunoreactivity was observed against *in vitro* deiminated α chain.

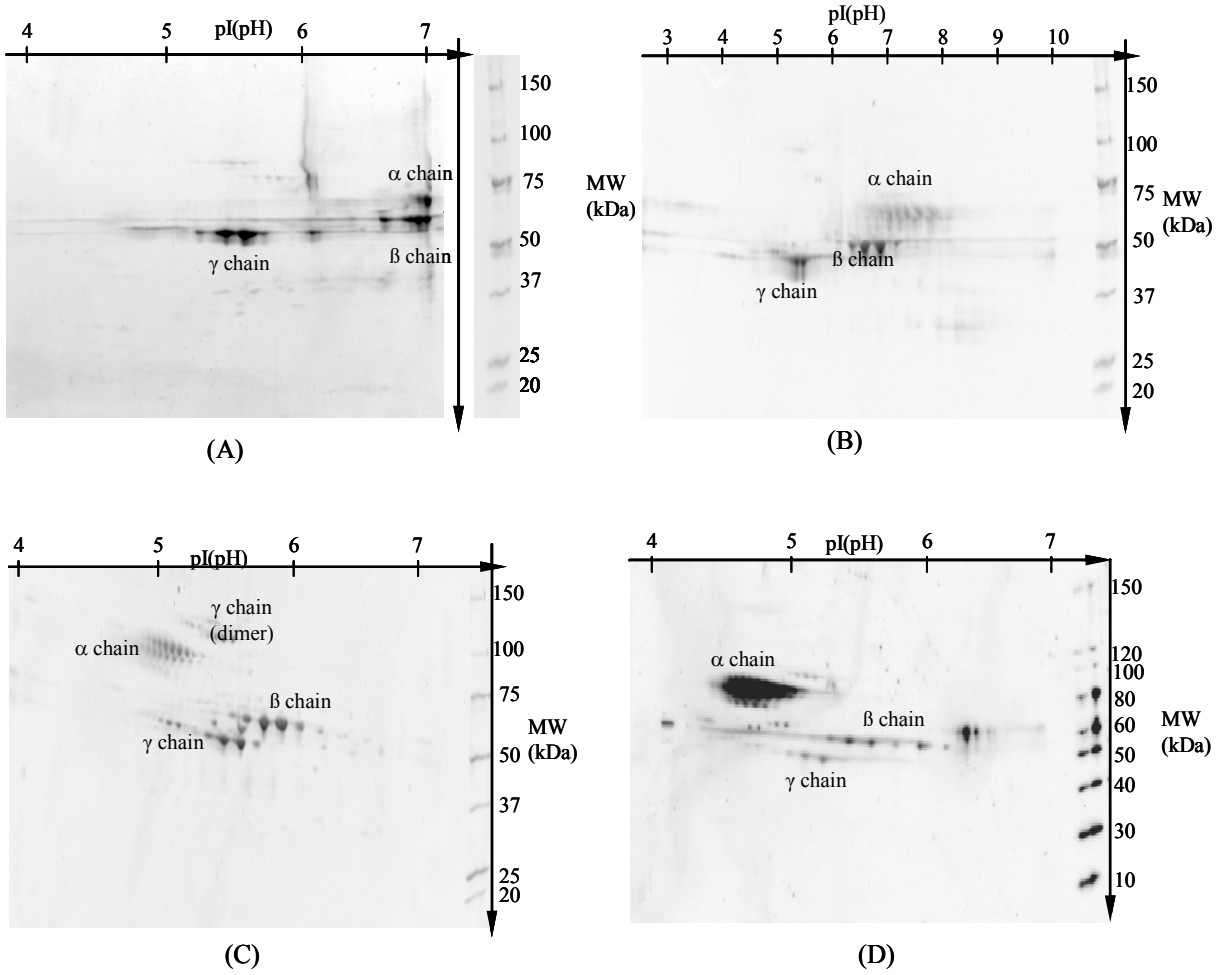


Figure 5: *in vitro* citrullination of fibrinogen.
2-DE of non-deiminated fibrinogen revealed distinct spot trains comprising of the three side chains of the protein (IPG pH 4-7 (A), IPG pH 3-10 (B); 4-20% linear gradient Tris-HCl gel; Sypro Ruby staining). Deimination of fibrinogen overnight with PAD2 revealed a shift towards the lower pI (IPG pH 4-7; 4-20% linear gradient Tris-HCl gel, Sypro Ruby staining) (C) and immune reactivity against in vitro deiminated fibrinogen was observed (IPG pH 4-7; 4-20% linear gradient Tris-HCl gel, serum detection) (D).

Immunoblotting of total synovial extract on peptide gels

The protein spots showing immunoreactivity with RA sera were located in the high MW region (>40kDa) of the gels. In order to investigate also potential immunoreaction in the low MW range, 4-12% Bis-Tris peptide gels were run. Four RA and 4 disease control serum samples (table 1, patients indicated with '°') were used for immunoblotting. Despite the presence of numerous protein spots in the range of 2-30kDa as revealed by AuroDye staining, no immunoreactivity was detectable in the low MW region (data not shown).

DISCUSSION

RA is an autoimmune disease which is characterized by the production of autoantibodies. Several autoantibody systems have been described like anti-RA33 (hnRNP-A2) antibodies or anti-collagen type II antibodies, but some of these autoantibodies are also present in a variety of other diseases [28, 29]. Other antibody systems, like antibodies against BiP (heavy chain binding protein) have been reported to be RA specific [30]. However, ACPA are the most specific and sensitive autoantibodies detected in 60%-70% of the RA patients [31]. As the synovium is the primary site of manifestation of the disease, we aimed to investigate potential autoantigens present in this tissue that could trigger the induction of autoantibodies. To achieve a representative image of the total proteome of the inflamed RA synovium, biological material of 7 RA patients was pooled. The 1-D screening with 30 RA sera and 30 control sera was performed to enable a more specific choice of second dimension gel in the 2-DE approach. The 100% specific autoreactive protein band revealed in the 1-D analysis contained the fibrinogen β chain.

In the current 2-D immunoblotting, most of the autoreactive spots with a high specificity were identified as the components of fibrinogen. Other identified proteins were carbonic anhydrase isoform I and vimentin. Also, additional autoreactive proteins were present in the synovium, but they were not retained for further identification analysis because of their too low specificity for RA.

Citrullinated vimentin has been reported as a potential autoantigen in RA as it has been identified as the Sa antigen which is highly specific for RA [32]. Although autoimmunity against carbonic anhydrase I has not been reported in RA to date, antibodies to carbonic anhydrase have been considered pathogenic factors in the development of autoimmune pancreatitis [33] and are present in sera from patients with Sjögren's syndrome [34]. Early reports also describe autoimmunoreactivity against carbonic anhydrase I in systemic lupus erythematosis [35, 36]. This zinc enzyme assists rapid inter-conversion of carbon dioxide and water into carbonic acid, protons and bicarbonate ions and therefore plays an important role in tissue rehydration and secretory activities.

The presence of antibodies against fibrinogen have been described in RA [11]. This 340 kDa protein, is encoded on chromosome 4 and synthesized by hepatocytes. It is composed of two identical subunits, each containing three polypeptide chains, the α chain with fibrinopeptide A, the β chain with fibrinopeptide B and the γ chain which are linked by disulfide bonds. Thrombin cleaves fibrinopeptides A and B from fibrinogen, resulting in the formation of

strands of insoluble fibrin monomers and polymers. These polymers can be cross-linked by transglutaminase to form a relatively stable and insoluble meshwork. Interstitial amorphous fibrin deposits are frequently seen in the synovial tissue as a consequence of the inflammatory process [37].

Taking a closer look at the 2-D immune protein pattern, we observed that immunoreactive spots occurred in spot trains comprised of the same protein. It was also noticed that the immunoreactive spots with the highest specificity arose at the acidic site of these spot trains. Re-incubation with RA serum of membranes probed with disease control serum, revealed the appearance of additional spots at the acidic side of the spot train. Furthermore, these most specific spots are only recognized by ACPA positive RA sera. Combination of the protein spots that showed a specificity of 100% in RA augmented the sensitivity in RA up to 50%.

Although, reactivity against fibrinogen α was also observed in CCP negative RA sera, combining of antigen substrates which leads to a higher sensitivity in RA could have implications in the design for new diagnostic tests for inflammatory arthritis. Fibrinogen β and γ, were only recognized by ACPA positive sera and these protein spots were found to be citrullinated in the synovium.

Autoimmune reactivity of ACPA positive sera against the most acidic proteins in the autoimmunoreactive protein trains, suggests the presence of citrulline in these proteins, as citrullination is a posttranslational modification that lowers the pI of proteins. This was also illustrated by the *in vitro* deimination of purified human fibrinogen. The spots containing the α and β fibrinogen shifted to a lower pI and a higher apparent MW relative to the γ chain. AMC staining on the synovial extracts confirmed the presence of citrullinated proteins in the synovial membrane. Autoreactive spots with high sensitivity and specificity that contained citrulline were identified as the β and γ chain of fibrinogen. There were some citrullinated protein spots visible which were not identified because their sensitivity was too low.

So far, fibrinogen is the only protein that is shown to contain citrulline residues *in vivo* in the synovial membrane. The deiminated forms of the α and β chain of fibrinogen in interstitial amorphous deposits are autoantigenic targets for ACPA [11]. In the present study, deiminated forms of the β and γ chain of fibrinogen were identified, but we could not detect any citrullinated forms of vimentin or carbonic anhydrase I. Moreover, there was some weak reaction in the MW and pI region of fibrinogen α chain on the nitrocellulose membrane on which AMC staining was performed, however, there was only a clear positive signal for fibrinogen β and a minor signal was observed for the γ chain. This could possibly be due to

the low amount of citrullinated α fibrinogen in the synovial protein extract used by our group, as the only autoimmune spot that was identified as fibrinogen α (B003) was not visible on the 2-D gel visualized by a sensitive silver stain [21]. We started our experiments with a total protein extract of synovial tissue without prior sequential extraction steps in comparison with previous reports which used an enriched protein extract in fibrin [11]. This could in part explain the differences between our results and the results of the group of Serre G. *et al* [11] which report on fibrinogen α and β, being the most prominent synovial antigens.

On the contrary, if we incubated *in vitro* deiminated fibrinogen with sera, the most prominent autoantigen was the α chain of fibrinogen, which is in agreement with what has been reported in the literature. These findings illustrate the potential discrepancy that may arise between autoimmune studies based on *in vitro* deiminated potential autoantigens and autoimmune experiments based on *in vivo* autoantigens present in inflamed tissue.

It is now known, that deiminated fibrinogen is not RA specific, and that it is a phenomenon nonspecifically associated with synovitis. It is not necessarily associated with the production of ACPA [25]. However, the RA specific immunoreactivity of the most acidic spots in a protein spot train, emphasizes the importance of considering posttranslational modifications as a trigger in tolerance break. It is possible that these RA-specific epitopes are generated by citrullination of arginine in a different amino acid context than usual due to altered PAD enzyme reactivity which is dependent on the amino acids flanking the arginine [27], [38].

A known genetic factor that may influence the induction of ACPA is the presence of the HLA-DR shared epitope (SE) [39]. SE is significantly associated with ACPA levels as these were increased in SE positive versus SE negative RA patients [40].

In summary, the present study describes a comprehensive inventory of a large array of synovial tissue B cell autoantigens by 2-D immunoblotting. The most abundantly and specifically recognized proteins were the β and γ chains of fibrinogen. By using 2-D immunoblotting, we were able to observe the *in vivo* synovial autoantigens as spot trains of which the most acidic protein spots were more specifically recognized by RA autoantibodies. Therefore, post-translational modifications, like citrullination, should be considered as crucial intermediates for the generation of autoantibodies. Indeed, both β and γ fibrinogen chain were found to be citrullinated. In addition, we also identified vimentin, which has already been reported as being the Sa antigen [32] and carbonic anhydrase I, which could be a potential new synovial autoantigen in RA.

ACKNOWLEDGMENTS

We thank Anneleen Devos and Liesbet Amerijckx for their skilful technical assistance.

GRANT SUPPORTERS

This work was supported by the Fund for Scientific Research Flanders project number G.0180.05 and the Research council of Ghent University (Bijzonder Onderzoeksfonds).

REFERENCES

[1.] Peene, I., De Rycke, L., Baeten, D., Hoffman, I., *et al.*, *Int J Immunopathol Pharmacol* 2004, 17, 107-116.

[2.] Vincent, C., Nogueira, L., Clavel, C., Sebbag, M., *et al.*, *Autoimmunity* 2005, 38, 17-24.

[3.] Girbal-Neuhauser, E., Durieux, J. J., Arnaud, M., Dalbon, P., *et al.*, *J Immunol* 1999, 162, 585-594.

[4.] Schellekens, G. A., de Jong, B. A., van den Hoogen, F. H., van de Putte, L. B., *et al.*, *J Clin Invest* 1998, 101, 273-281.

[5.] Kim, H. J., Krenn, V., Steinhauser, G. and Berek, C., *J Immunol* 1999, 162, 3053-3062.

[6.] Gause, A., Gundlach, K., Carbon, G., Daus, H., *et al.*, *Rheumatol Int* 1997, 17, 145-150.

[7.] Masson-Bessiere, C., Sebbag, M., Durieux, J. J., Nogueira, L., *et al.*, *Clin Exp Immunol* 2000, 119, 544-552.

[8.] Tilleman, K., Van Beneden, K., Dhondt, A., Hoffman, I., *et al.*, *Proteomics* 2005, 5, 2247-2257.

[9.] Hueber, W., Kidd, B. A., Tomooka, B. H., Lee, B. J., *et al.*, *Arthritis Rheum* 2005, 52, 2645-2655.

[10.] Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., *et al.*, *J Immunol* 2001, 166, 4177-4184.

[11.] Tai, A. W. and Newkirk, M. M., *Clin Exp Immunol* 2000, 120, 188-193.

[12.] Uesugi, M., Yoshida, K. and Jasin, H. E., *J Immunol* 2000, 165, 6532-6537.

[13.] Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., *et al.*, *Arthritis Rheum* 1988, 31, 315-324.

[14.] Baeten, D., Van den Bosch, F., Elewaut, D., Stuer, A., *et al.*, *Clin Rheumatol* 1999, 18, 434-441.

[15.] Dougados, M., van der Linden, S., Juhlin, R., Huitfeldt, B., *et al.*, *Arthritis Rheum* 1991, 34, 1218-1227.

[16.] Altman, R., Asch, E., Bloch, D., Bole, G., *et al.*, *Arthritis Rheum* 1986, 29, 1039-1049.

[17.] Gorg, A., Obermaier, C., Boguth, G., Harder, A., *et al.*, *Electrophoresis* 2000, 21, 1037-1053.

[18.] Rabilloud, T., Valette, C. and Lawrence, J. J., *Electrophoresis* 1994, 15, 1552-1558.

[19.] Anderson, L., Hofmann, J. P., Anderson, E., Walker, B., *et al.*, 1989, 288-297.

[20.] O'Connell, K. L. and Stults, J. T., *Electrophoresis* 1997, 18, 349-359.

[21.] Senshu, T., Sato, T., Inoue, T., Akiyama, K., *et al.*, *Anal Biochem* 1992, 203, 94-100.

[22.] Raymackers, J., Daniels, A., De Brabandere, V., Missiaen, C., *et al.*, *Electrophoresis* 2000, 21, 2266-2283.

[23.] Stone, K. L., DeAngelis, R., LoPresti, M., Jones, J., *et al.*, *Electrophoresis* 1998, 19, 1046-1052.

[24.] Chapuy-Regaud, S., Sebbag, M., Baeten, D., Clavel, C., *et al.*, *J Immunol* 2005, 174, 5057-5064.

[25.] Chang, X., Yamada, R., Suzuki, A., Sawada, T., *et al.*, *Rheumatology (Oxford)* 2005, 44, 40-50.

[26.] Tarcsa, E., Marekov, L. N., Mei, G., Melino, G., *et al.*, *J Biol Chem* 1996, 271, 30709-30716.

[27.] van Boekel, M. A., Vossenaar, E. R., van den Hoogen, F. H. and van Venrooij, W. J., *Arthritis Res* 2002, 4, 87-93.

[28.] Corrigall, V. M. and Panayi, G. S., *Crit Rev Immunol* 2002, 22, 281-293.

[29.] Blass, S., Union, A., Raymackers, J., Schumann, F., *et al.*, *Arthritis Rheum* 2001, 44, 761-771.

[30.] De Rycke, L., Peene, I., Hoffman, I. E., Kruithof, E., *et al.*, *Ann Rheum Dis* 2004, 63, 1587-1593.

[31.] Vossenaar, E. R., Despres, N., Lapointe, E., van der Heijden, A., *et al.*, *Arthritis Res Ther* 2004, 6, R142-150.

[32.] Bartolome, M. J., de las Heras, G. and Lopez-Hoyos, M., *ScientificWorldJournal* 2002, 2, 1560-1568.

[33.] Kino-Ohsaki, J., Nishimori, I., Morita, M., Okazaki, K., *et al.*, *Gastroenterology* 1996, 110, 1579-1586.

[34.] Inagaki, Y., Jinno-Yoshida, Y., Hamasaki, Y. and Ueki, H., *J Dermatol Sci* 1991, 2, 147-154.

[35.] Itoh, Y. and Reichlin, M., *Arthritis Rheum* 1992, 35, 73-82.

[36.] Weinberg, J. B., Pippen, A. M. and Greenberg, C. S., *Arthritis Rheum* 1991, 34, 996-1005.

[37.] Kubota, K., Yoneyama-Takazawa, T. and Ichikawa, K., *Rapid Commun Mass Spectrom* 2005, 19, 683-688.

[38.] Hill, J. A., Southwood, S., Sette, A., Jevnikar, A. M., *et al.*, *J Immunol* 2003, 171, 538-541.

[39.] Cantaert, T., Coucke, P., De Rycke, L., Veys, E. M., *et al.*, *Ann Rheum Dis* 2005.

Summary and final discussion

Proteomics: a close encounter with rheumatology

Kelly Tilleman

Rheumatoid arthritis (RA) and spondyloarthropathy (SpA) are two frequent forms of inflammatory arthritis with an important morbidity and socio-economic impact on our society. Although similar joints can be affected in both diseases, their clinical presentation is different. Nevertheless, both pathologies are characterized by synovitis, the chronic inflammation of the synovial tissue. The synovial tissue lines the joint cavity and is responsible for the homeostasis in the joint. This delicate serous membrane is subject to acute inflammatory reactions upon stress and injury or to chronic inflammation upon unknown triggers which is the case in RA or SpA synovitis. In both RA and SpA, the synovial membrane of the affected joint undergoes several profound changes in synovial architecture. Infiltration of inflammatory lymphocytes initiates inflammatory processes, resulting in thickening of the synovial lining layer. This hyperplasia, due to infiltration of macrophages and monocytes, along with the local proliferation of synoviocytes, results in a hypoxic microenvironment associated with neovascularisation. Despite intensive investigations into the nature of chronic synovitis, the molecular differences between RA and SpA synovial inflammation are poorly understood.

This project was intended to examine synovitis from a different point of view. The majority of reports on inflammatory arthritis has a histological or immunological character and has indeed resolved important issues concerning the chronic inflammation of the synovial tissue. The use of a molecular biochemical approach could shed a different light upon synovial inflammation and may reveal unknown differences between RA and SpA synovitis. Additionally, they could explain reported variation in synovial histology and could be fundamental to the distinctive clinical outcome observed between the inflammatory arthritides.

We initiated our study by a differential screening of the synovial protein expression of cytosolic proteins derived from knee biopsy samples of patients diagnosed with RA, SpA and OA (**chapter 1**). The method of choice was a classic proteome analysis, which gave us the possibility to study protein expression profiles of several hundred proteins at the same time. The results of this study showed that the synovial cytosolic proteome of SpA as opposed to RA patients consisted of a unique set of proteins with a defined expression pattern for each form of chronic arthritis. Proteins unique to either RA or SpA inflammation in the synovial tissue were identified. Proteins which had been implicated in inflammatory arthritis were detected.

Our attention was drawn to a rather explicit feature on our 2-D gels. Analyzing the cytosolic proteome revealed a specific cluster of spots consisting of vimentin isoforms. This pattern

was further analyzed in **chapter 2** and appeared to be the result of protein processing. Vimentin is known to be processed by caspases [1] and our mass spectrometry data confirmed this. Imitating this processing *in vitro* showed the lack of post-translational modifications that would shift the cleaved fragments of the protein towards the acidic side of the 2-D gel.
Since citrullination, a modification that changes an arginine to citrulline, is known to shift proteins towards the acidic pI [2], and because citrullinated vimentin is a known autoantigen in RA, the possible presence of this modification was examined. Indeed, fragments of processed vimentin were citrullinated in cytosolic protein extracts of RA, contrary to SpA, where there is little occurrence of citrullinated vimentin. This is in contrast with the occurrence of citrullinated fibrinogen, another well known citrullinated synovial protein, which is observed both in RA and SpA synovial tissue [3].
Citrullination is a protein modification that has been implicated in many pathological processes [4] and it occurs in extreme conditions, since the enzyme responsible for the conversion is only activated in environments where calcium concentrations are high [5]. Therefore, on could attribute these finding to apoptosis as deimination of proteins has been associated with apoptosis of certain cell types [6, 7], although the exact sequence of events is unknown.
The question remains however, as to why citrullinated vimentin is present in RA synovitis and not (or limited) in SpA synovitis. It is tempting to speculate that this is related to a difference in apoptosis or a better clearing of citrullinated residues in SpA in comparison to RA. The residence of citrullinated vimentin in RA synovium probably contributes to the development of autoantibodies and the reported high specificity of the ACPA family of antibodies in RA [8, 9]. Indeed, in chapter 2 we additionally show evidence that the presence of autoantibodies against processed citrullinated isoforms of vimentin was highly disease specific in RA. These findings indicated a novel concept in the development of autoantibodies in RA by which humoral autoimmunity is targeted against caspase cleaved and citrullinated fragments of an intermediate filament in synovial tissue, in a highly disease specific manner.

Not only apoptosis, but also hypoxia can increase the amount of citrullination in tissue [10]. This was previously reported in astrocytes exposed to varying durations of hypoxia (2%O_2) and showed upregulation of PAD and citrullinated glial fibrillary acidic protein (GFAP) [10].
It is known that the affected joints of RA patients are hypoxic [11-13]. Due to the hyperplasia of the synovial lining, the distance between the cells and the nearest blood vessels increases, depriving the cells of oxygen, hereby creating a hypoxic microenvironment [14]. When

oxygen levels drop below 5%, HIF-1 start to take control over the transcription of over 70 genes [15].

In our intial proteomics study we indentified proteins, related to SpA inflammation, to be regulated by HIF-1. Alpha-enolase, aldolase A, triose phosphate isomerase and ceruloplasmin, were found to be up regulated in SpA versus OA (chapter 1). All of them have been described to be regulated by HIF-1 in hypoxic conditions [16, 17]. As the SpA joint is also subject to synovitis, we can assume the environment to be hypoxic. However, no present data have examined this.

On the other hand, it is known that the SpA synovial tissue is highly vasculated and neovascularisation is indeed a reaction upon hypoxia. There is a difference in synovial vasculature between RA and SpA, where the amount of vascularity is reported to be significantly higher in SpA compared to RA [18]. We hypothesized that the tissue response to hypoxia was distinct in SpA versus RA. To address this, we investigated the ability of RA and SpA synovial fibroblasts (FLS) to produce VEGF and Cp, two proteins involved in angiogenesis, in hypoxic conditions (**chapter 3**). VEGF has been described as the key factor in angiogenesis [19, 20] and Cp binds and transports the majority of the circulating copper, a major co-factor for angiogenic factors [21]. Both elevated levels of VEGF, copper and Cp have been reported in serum and synovial fluid of patients diagnosed with inflammatory arthritis [22-25].

Results obtained from these experiments showed that elevated gene expression of Cp and higher protein production of VEGF in SpA FLS in comparison to RA FLS both cultured in hypoxia. Although additional confirming experiments are underway, these data suggest that the observed differences in synovial vascularity between RA and SpA [18, 26] could be at least partly attributed to this distinct response to hypoxia.

In the final chapter, **chapter 4**, we describe a study searching for new synovial autoantigens. RA is an autoimmune disease characterized by the production of autoantibodies. Since the primary site of inflammation is the synovium and because of the characteristics of a proteome analyses, this allowed us to list the spectrum of synovial autoantigens that induce an autoimmune humoral response. By screening the presence of autoantibodies in 30 RA and 30 non-RA serum samples against synovial tissue proteins, we confirmed that the most autoantibody reactivity in the synovial tissue was directed to subunits of fibrinogen (especially fibrinogen β and γ), confirming the data of Serre's group [27].

However, we also identified vimentin, better known as the Sa-antigen and carbonic anhydrase I as potential new synovial autoantigens. The novelty in this report related to the use of a total protein extract in order to find new synovial autoantigens and the use of 2-D blots. Moreover, because of the characteristics of 2-DE, we were able to show that these autoimmune targets consisted of trains of adjacent spots, containing the same protein. Remarkably, the protein spots present at the more acidic side of the train, showed the highest specificity for RA. A modification, like citrullination could be crucial in the tolerance break and the development of autoantibodies and we could indicate these spots, representing fibrinogen β and γ, were citrullinated.

Another novel aspect of the current study relates to the use of synovial tissue extracts from RA patients to search for new autoantigens in RA. In contrast to previous studies searching for citrullinated autoantigens where *in vitro* deiminated protein extracts were used. Therefore, the currently identified autoantigens represent the *in situ* situation in inflamed synovial tissue and may therefore lead to a better understanding of the autoimmune character of RA.

Altogether, by using proteomics, we encountered different aspects of synovitis, pointing towards distinct molecular differences between RA and SpA synovitis. Expression patterns of proteins, modification of proteins and secretion of proteins appeared specific for each form of synovitis. We showed that proteomics is a fascinating tool to tackle the biology of synovitis and underline the role for 2-DE, the ‘old work horse’ of the classic proteomics approach, which allows protein modifications to be elucidated.

REFERENCES

[1.] Byun, Y., Chen, F., Chang, R., Trivedi, M., *et al.*, *Cell Death Differ* 2001, 8, 443-450.

[2.] van Venrooij, W. J. and Pruijn, G. J., *Arthritis Res* 2000, 2, 249-251.

[3.] Chapuy-Regaud, S., Sebbag, M., Baeten, D., Clavel, C., *et al.*, *J Immunol* 2005, 174, 5057-5064.

[4.] Gyorgy, B., Toth, E., Tarcsa, E., Falus, A., *et al.*, *Int J Biochem Cell Biol* 2006, 38, 1662-1677.

[5.] Vossenaar, E. R., Zendman, A. J., van Venrooij, W. J. and Pruijn, G. J., *Bioessays* 2003, 25, 1106-1118.

[6.] Chang, X., Yamada, R., Suzuki, A., Kochi, Y., *et al.*, *Rheumatology (Oxford)* 2005, 44, 1374-1382.

[7.] Asaga, H., Yamada, M. and Senshu, T., *Biochem Biophys Res Commun* 1998, 243, 641-646.

[8.] El-Gabalawy, H. S. and Wilkins, J. A., *Arthritis Res Ther* 2004, 6, 86-89.

[9.] Zendman, A. J., van Venrooij, W. J. and Pruijn, G. J., *Rheumatology (Oxford)* 2006, 45, 20-25.

[10.] Sambandam, T., Belousova, M., Accaviti-Loper, M. A., Blanquicett, C., *et al.*, *Biochem Biophys Res Commun* 2004, 325, 1324-1329.

[11.] Falchuk, K. H., Goetzl, E. J. and Kulka, J. P., *Am J Med* 1970, 49, 223-231.

[12.] Lund-Olesen, K., *Arthritis Rheum* 1970, 13, 769-776.

[13.] Etherington, P. J., Winlove, P., Taylor, P., Paleolog, E., *et al.*, *Clin Exp Rheumatol* 2002, 20, 799-805.

[14.] Stevens, C. R., Blake, D. R., Merry, P., Revell, P. A., *et al.*, *Arthritis Rheum* 1991, 34, 1508-1513.

[15.] Semenza, G. L., *Nat Rev Cancer* 2003, 3, 721-732.

[16.] Gaber, T., Dziurla, R., Tripmacher, R., Burmester, G. R., *et al.*, *Ann Rheum Dis* 2005, 64, 971-980.

[17.] Distler, J. H., Wenger, R. H., Gassmann, M., Kurowska, M., *et al.*, *Arthritis Rheum* 2004, 50, 10-23.

[18.] Baeten, D., Demetter, P., Cuvelier, C., Van Den Bosch, F., *et al.*, *Ann Rheum Dis* 2000, 59, 945-953.

[19.] Ferrara, N. and Davis-Smyth, T., *Endocr Rev* 1997, 18, 4-25.

[20.] Ferrara, N. and Keyt, B., *Exs* 1997, 79, 209-232.

[21.] Sen, C. K., Khanna, S., Venojarvi, M., Trikha, P., *et al.*, *Am J Physiol Heart Circ Physiol* 2002, 282, H1821-1827.

[22.] Paleolog, E. M. and Miotla, J. M., *Angiogenesis* 1998, 2, 295-307.

[23.] Drouart, M., Saas, P., Billot, M., Cedoz, J. P., *et al.*, *Clin Exp Immunol* 2003, 132, 158-162.

[24.] Milanino, R., Frigo, A., Bambara, L. M., Marrella, M., *et al.*, *Clin Exp Rheumatol* 1993, 11, 271-281.

[25.] Yazar, M., Sarban, S., Kocyigit, A. and Isikan, U. E., *Biol Trace Elem Res* 2005, 106, 123-132.

[26.] Reece, R. J., Canete, J. D., Parsons, W. J., Emery, P., *et al.*, *Arthritis Rheum* 1999, 42, 1481-1484.

[27.] Masson-Bessiere, C., Sebbag, M., Girbal-Neuhauser, E., Nogueira, L., *et al.*, *J Immunol* 2001, 166, 4177-4184.

Samenvatting

Een ontmoeting tussen proteomics en reumatologie

Kelly Tilleman

Rheumatoide artritis (RA) and spondyloartropathie (SpA) zijn twee frequent voorkomende vormen van inflammatoire artritis met een belangrijke morbiditeit en socio-economische impact op de samenleving. Alhoewel dezelfde gewrichten aangetast kunnen worden in beide aandoeningen, zijn de klinische verschijnselen verschillend. RA wordt als een aggressieve en destructieve gewrichtsaandoening beschouwd, integenstelling tot SpA dat veel minder erosief blijkt en waar bovendien pogingen tot herstel waargenomen worden. Desondanks worden zowel RA als SpA gekenmerkt door synovitis, de chronische ontsteking van het synoviaal membraan.

Het synoviaal membraan bekleedt het gewrichtskapsel en is verantwoordelijk voor de homeostase van het gewricht. De ontsteking van dit delicate vlies kan accuut zijn door belasting en/of blessure, of er kan een chronische onstekingsreactie ontstaan waarvan, zoals in het geval van RA en SpA synovitis, de oorzaak ongekend is. Bij zowel RA als SpA ondergaat de synoviale architectuur van het aangetaste gewricht een aantal ingrijpende veranderingen. De inflammatoire initiatie gebeurt door de infiltratie van inflammatore cellen. Deze leiden tot een verdikking van het synoviaal weefsel, ten dele door de inflammatoire infiltratie, maar ook door de locale proliferatie van de synoviocyten. Deze hyperplasie resulteert in een hypoxische micro-omgeving geassocieerd met neovascularisatie. Ondanks diepgaand onderzoek naar de chronische natuur van synovitis, blijft de kennis omtrent het moleculair verschil tussen RA en SpA synoviale inflammatie gebrekkig.

Dit project had de intentie om synovitis vanuit een ander perspectief te bekijken. Het overgrote deel van studies naar inflammatoire artritis hebben een histologisch of immunologisch karakter. Alhoewel deze analyses heel wat aspecten betreffende chronische ontsteking van het synoviaal membraan hebben opgehelderd, kan een meer moleculair, biochemische benadering een ander licht werpen op mogelijke verschillen tussen de RA en SpA synovitis aantonen. Daarnaast, kunnen dergelijke studies de beschreven verschillen in synoviale histologie verklaren en mogelijks een bijdrage leveren aan de zoektocht naar de oorzaak van de uiteenlopende klinische verschijnselen tussen de twee vormen van inflammatoire artritis.

Dit project startte met een differentiële screening van de expressie van de synoviaal cytosolische eiwittten afkomstig van knie biopsie stalen verkregen bij RA, SpA en OA patiënten (**hoofdstuk 1**). De verkozen methode was een klassieke proteome analyse en dit gaf ons de mogelijkheid om het expressie patroon van honderden eiwitten terzelfdertijd te

bestuderen. De resultaten van deze studie toonden aan dat het synoviaal cytosolisch proteome van SpA in vergelijking met RA uit eiwitten bestond met een uniek expressie profiel voor elke vorm van artritis. Eiwitten uniek voor RA of SpA synovitis werden vervolgens geïdentificeerd en proteïnen gerelateerd aan inflammatoire artritis werden aangetroffen.

Gedurende deze analyse werd onze aandacht gevestigd op een groep van eiwit spots die in een uitgesproken vorm gegroepeerd lagen op de twee-dimensionele (2-D) gels. Deze groep van spots werd geïdentificeerd als het eiwit vimentine. Het verdere onderzoek naar de afkomst van deze cluster van spots werd beschreven is **hoofdstuk 2** en bleek het resultaat te zijn van eiwit processing.

Het is gekend dat vimentine verknipt kan worden door caspasen en de massaspectrometrie data afkomstig van de verschillende eiwiten uit de groep bestigde dit. Wanneer we echter dit proces *in vitro* wensten na te bootsen, vertoonden de fragmenten van vimentine een ander patroon. De fragmenten waren niet opgeschoven naar de zure kant van de 2-D gel zoals op het in vivo beeld van het synoviaal cytosolisch proteome.

Een afwezigheid van een specifieke eiwit modificatie lag aan de basis van dit verschil. Citrullinatie, een modificatie waarbij een arginine omgezet wordt tot citrulline, veroorzaakt een shift op een 2-D beeld naar de zure kant en bovendien is gecitrullineerd vimentine een gekend autoantigen in RA. Om deze redenen werd de mogelijke aanwezigheid van deze modificatie onderzocht. Inderdaad, fragmenten van verknipt vimentine waren gecitrullineerd in cytosolische eiwit extracten van RA, in tegenstelling tot SpA, waar deze gemodificeerde fragmenten bijna volledig afwezig bleken te zijn. Deze resultaten zijn in strijd met het voorkomen van gecitrullineerd fibrinogeen, een ander gekend gecitrullineerd synoviaal eiwit, dat zowel in RA als SpA aangetoond is.

Citrullinatie is een eiwit modificatie dat verwikkeld is in verschillende pathologieën en het komt voor in extreme omstandigheden aangezien het enzyme dat verantwoordelijk is voor de omzetting enkel geactiveerd wordt in hoge calcium concentraties. Men denkt dan onmiddellijk aan apoptose en inderdaad, er zijn rapporten die citrullinatie associëren met apoptose van bepaalde celtypes, echter de precieze volgorde van stappen in het proces is niet gekend.

De vraag waarom gecitrullineerd vimentine zo prominent aanwezig is in het RA synovium en niet (of beperkt) in het SpA synovium blijft onbeantwoord. Een mogelijks verschil in apoptose of een betere opruiming van gecitrullineerde vimentine residues zou misschien aan de basis ervan kunnen liggen. Het verblijf van gecitrullineerd vimentine in het RA synovium

is ongetwijfeld geassocieerd met de ontwikkeling en de hoge specificiteit van antilichamen tegen gecitrullineerde proteïnen (ACPA) in RA.
Inderdaad, bijkomend in hoofdstuk 2 toonden we aan dat de aanwezigheid van autoantilichamen tegen gecitrullineerde fragmenten van vimentine specifiek was voor RA. Deze bevindingen wezen op een nieuw concept in de ontwikkeling van autoantilichamen in RA waarbij humorale autoimmuniteit, gericht op caspase geknipt and gecitrullineerde fragmenten van een intermediair filament in het synoviaal membraan, ziekte-specifiek is.

Niet enkel apoptose, maar ook hypoxie is geassocieerd met citrullinatie. Dit werd voorheen gerapporteerd in astrocyten die geïncubeerd werden in hypoxische omstandigheden (2% O_2) van verschillende duur waarbij er een verhoging van peptidyl arginine deiminase (PAD) en gecirullineerd gliaal fibrillair zuur eiwit (GFAP) aangetoond werd.
Het is gekend dat de getroffen gewrichten van RA patiënten hypoxisch zijn. Wegens de hyperplasie van het synoviaal weefsel wordt de afstand tussen de cellen en het nabijgelegen bloedvat groter. Hierdoor verkleint de zuurstoftoevoer naar de cellen waardoor een hypoxisch microklimaat gecreëerd wordt. Als het zuurstofgehalte onder de 5% valt, neem hypoxie geïnduceerde factor- 1 (HIF-1) de controle over de transcriptie van meer dan 70 genen.
In onze initële proteome analyse werden eiwitten geïdentificeerd die in hypoxie onder invloed staan van HIF-1. De transcriptie van alfa-enolase, aldolase A, triose fosfaat isomerase en ceruloplasmine (Cp); eiwitten die verhoogde expressie vertoonden in SpA in vergelijking met OA (hoofdstuk 1), wordt geïnduceerd door HIF-1.
Aangezien het SpA gewricht gekenmerkt wordt door synovitis, kunnen we aannemen dat ook hier de synoviale omgeving hypoxisch is. Er zijn echter geen studies die dit onderzocht hebben. Toch is het beschreven dat het SpA synoviaal membraan sterk gevasculeerd is en de vorming van nieuwe bloedvaten is een gekend effect van hypoxie. Bovendien blijkt er een verschil in synoviale vasculatuur te zijn tussen RA en SpA waar men bij SpA een verhoogde hoeveelheid aan bloedvaten waarneemt.
Een mogelijkse hypothese was dat de weefsel respons op hypoxie verschillend is tussen RA en SpA. Hiervoor werd de mogelijkheid van RA en SpA synoviale fibroblasten (FLS) onderzocht om vasculair endotheliale groeifactor (VEGF) en Cp, twee eiwitten die een rol spelen in angiogenese, te produceren in hypoxische omstandigheden (**hoofdstuk 3**).
VEGF is beschreven als dé sleutelfactor in angiogenese en Cp bindt en vervoert koper; de belangrijkste cofactor voor factoren die instaan voor de vorming van nieuwe bloedvaten.

Zowel VEGF, koper als Cp zijn verhoogd in serum en synoviaal vocht van patiënten met inflammatoire artritis.
De resultaten toonden een verhoogde genexpressie van Cp en een hogere produktie van VEGF aan in SpA FLS in vergelijking met RA FLS, beiden gegroeid in hypoxische omstandigheden. Alhoewel er extra confirmerende experimenten op de agenda staan, suggeren deze data dat het beschreven verschil in synoviale vascullariteit tussen RA en SpA gedeeltelijk zou kunnen verklaard worden hierdoor.

Finaal, in het laatste hoofdstuk (**hoofdstuk 4**) beschrijven we een zoektocht naar nieuwe synoviale autoantigenen specifiek in RA. RA is een autoimmuun ziekte gekarakteriseerd door de produktie van autoantilichamen. We hadden de mogelijkheid om de synoviale autoantigen te catalogeren door gebruik te maken van een grootschalige techniek zoals proteomics.
Voor deze experimenten werden 30 RA en 30 non-RA serum stalen geïncubeerd ten opzichte van een synoviaal eiwit extract. De autoantilichamen aanwezig in het serum van RA patiënten waren gericht tegen dat de subunits van synoviaal fibrinogeen (vooral de β en γ subunit). De data confirmeerden de resultaten van de groep van Prof. Serre. Additioneel identificeerden we ook synoviaal vimentine, gekend as het Sa-antigen, en carbonic anhydrase I als potentiële nieuwe synoviale autoantigenen.
Door het gebruik van 2-D gel electroforese (2-DE) en door de specifieke karakteristieken van deze techniek konden we aantonen dat deze autoimmune eiwitten uit treinen bestonden van aaneensluitende spots afkomstig van het zelfde eiwit. Merkwaardig genoeg, vertoonden spots die zich aan de zure kant van deze spot trein bevonden, de hoogste specificiteit voor RA. Een modificatie, zoals citrullinatie kan dus cruciaal zijn in de ontwikkeling van autoimmuniteit en we toonden ook aan dat zowel fibrinogeen β als γ gecitrullineerd waren.
Er is een verschil tussen de beschreven studie in hoofdstuk 4 en wat reeds verschenen is in de literatuur omtrent synoviale autoantigenen. Door te vertrekken van een eiwit extract afkomstig van synoviaal weefsel afkomstig van RA patiënten benaderden de bekomen resultaten de *in situ* situatie in het inflammatoir gewricht, in tegenstelling tot het beschreven gebruik van *in vitro* gecitrullineerde cel extracten.

Gedurende onze proteomics trip, zijn we met verschillende aspecten van synovitis in contact gekomen. Allen wezen ze in de richting van een moleculair verschil tussen RA en SpA synovitis. Expressie patronen van eiwitten, modificaties van eiwitten en de secretie ervan

bleken specifiek te zijn voor elke vorm van inflammatoire artritis. We toonden aan dat proteomics een fascinerend instrument is om de biologie van synovitis uit te spitten en onderlijnen dat er nog steeds nood is aan 2-DE, het ‘oude werkpaard’ van de klassieke proteomics, die als enige de post-translationele modificatie zo knap in beeld kan brengen.

Bedankt....

Coming together is a beginning
Keeping together is a progress
Working together is success
--Henry Ford

Alhoewel mijn naam op de voorpagina van deze thesis staat, kon dit werk niet verwezenlijkt worden door mij alleen en het is dan ook, traditiegewijs, in deze sectie dat anderen in de spotlight staan:

Mijn promotoren Prof. Deforce en Prof. Elewaut:
Dieter en Dirk, oftwel kortweg D&D genoemd door de docstudenten, jullie bijdrage aan dit werk is enorm geweest. Ik wil jullie bedanken voor de input aan financiële resources en wetenschappelijk visie. De discussie liepen soms hoog op, mede omdat ik ook altijd mijn zegje wou doen en zelden gemakkelijk overtuigbaar was. Toch denk ik, dat we mooi werk geleverd hebben en ik kijk er naar uit om ook na dit doctoraat met jullie verder te werken.

Prof. De Keyser, ook u bent onmisbaar geweest in de creatie van resultaten beschreven in deze thesis. Uw expertise in het klinische maar ook uw interesse naar het moleculaire hebben een grote indruk op mij gemaakt.

De groep van Ann Union by Innogenetics zou ik willen bedanken voor vlotte samenwerking en het mooie werk omtrent de synoviale antigenen.
Ook Prof. De Sutter voor het gebruik van de hypoxie-incubator en Sylvie voor de leuke sfeer in het keldertje van de P3.

Wetenschap kan enkel z'n vruchten afwerken wanneer men voldoende relativerend, ontspannend en ludiek de dingen kan benaderen.
En op wie kon ik hiervoor beter rekenen dan mijn mede docstudentjes. Steek 6 jonge gasten in een lokaal van 4 op 10 en dat komt wel allemaal vanzelf: Ann-Sophie, Ben, Ivan, Stijnie, Alientje, Martin en nu ook Mado en Katleen, merci voor de koffie'tjes, de koekskes, de Duvels en vooral de sfeer, het begrip en jawel ook soms de stilte waardoor je alleen maar 'tokkel tokkel' hoorde van 60 vingers op een toetsenbord. Daarnaast was er Filip die een bureau had van 4 op 10 voor hem alleen (ja, we waren jaloers, maar daar komt weldra verandering in ☺). Bedankt voor alle pc-stuff en de psychologische groepssessies (ya know.....) over de middag.
De mensen van de routine, of 'de DNA'ers', nog zo'n bonte boel en zeker op feestjes en trouwfeesten. De foto's, niet voor publicatie vatbaar, bewijzen dat. Wie moet er trouwens nog bevallen?!!! ☺
Speciaal een thanks voor David, Nadine en Astrid die de administratie van ons labo in goede banen leiden. Merci voor de hulp bij crashende kopiemachines, verloren gaande faxen, te laat komende bestellingen, ingewikkelde SAP dossiers en zoveel meer! Ook merciekes aan Sabrina om ons labo'ke proper te houden en de leuke babbels over de bouwperikelen. Ik kom zeker eens langs!
Een speciale vermelding geniet Sofie Vande Casteele, ookwel Sofie van de massa's: wat zouden we zonder jou doen. Het gepruts aan de nanoLC met die mini mini schroevendraaiertjes en die naaldjes en pfff, ik was soms content dat ik geen stalen moest runnen als je in verlof was. Heel heel heel erg bedankt voor je inzet, expertise en enthousiasme waarmee je het mass spec werk deed en elke dag doet. Hopelijk lukt hetgeen we willen uittesten op dat nieuw machien! Spannend....
En dan zijn er nog de aanhangsels, ik noem ze graag de 'aliens'; docstudenten van andere labo's die bij ons wat praktisch werk komen doen: Céline, Heleen, Kristien, Kinjip (eigenlijk bijna een volwaardige FBT'er), Matthias, Phebe....Ook de reumaboys en girls (die de eerste jaren bij ons kampeerden tot het MRB af was): Pierre, Katrien, Tineke (merci voor het blotwerk en de AMC's), Peggy, Ann, Sara, Ken, ...allen bedankt voor de wetenschappelijke

en klinische info (vooral thx hier aan Bert en Ilse), maar vooral ook voor de grappige momenten, de Duvels (alweer), de feestjes (om 17h ☺), de BBQ, de etentjes..... .
Ook mijn vele thesisstudentjes: Erin, Liesbeth, JP, Laurence, Loreke, An-Sofie, Nele en Barbara, merci voor het werk en de leute.

Ook de andere vrienden (ja, jullie daar, bende ongeregelden ☺): ik kan al een doctoraatsthesis op zich schrijven over alle docfeestjes, MC's, Werchters, trouwfeestjes, tobbe-dansen, Sjakosj-stuiken, Salamandervoetballen, Abou-tequila-slammen, tequila-disco party's (when Floor n° 10?, for all time sake!), Gentsche Fiesten, Pukkelpopjes, tuinfeestjes, oudejaartjes-nieuwejaartjes, etc. die ik met jullie mee gemaakt heb. Jullie (en jullie kleintjes) waren dan ook de ideale uitlaatklep na een weekje labo-zwoegen. Ook mijn sportpartnerkes, ik noem ze graag mijn volleybabes....hihi...Luv you much!
Een speciale vermelding voor mijnen 'manager' Wim: thx voor het over en weer gemail zo'n 4,5 jaar geleden, het luisterende oortje en de 'Dobbie-analyse' van de vele situaties. Thx!
En dan mijn drie musketiers: Saar en Cathy, mijn supergirls, die me zeer nauw aan het hart liggen samen met Steven, mijn rots in de branding.

Tom, merci voor je nuchtere kijk op de dingen en de steun. Je weet wat je voor mij betekent!
Ook mijn kleine Sofie: je bent een toffe meid en je was de ideale verstrooiing na een frustrerende werkdag.

En dan een speciaal stukje om mijn ouders te bedanken. Ik ben tenslotte officieel "student" af. Ma en pa, merci! Pa, ik weet pa dat je me dikwijls gevraagd hebt wat ik daar eigenlijk zat te doen, ganse dagen in dat labo. Ehwel, ik weet niet of je na het bekijken van dit boekske daar veel wijzer uit zult geraken. Mijn job is eigenlijk geen 'job'. Het is een interesse, een drang (een wat groot woord, maar kom...) naar kennis, denk ik. De frustratie en ontgoocheling kan soms zeer groot zijn. En je moet content zijn met weinig en het feit dat alles ultra traag vooruit gaat. Maar dan lukt het experiment toch en zijn de data goed en dat zorgt ervoor dat je toch blijft doorgaan. Ik weet niet of je het nu zult begrijpen. De resultaten van mijn werk zijn niet te meten in bakstenen, muren, vloeren of huizen. Ik kan niet zeggen, kijk daar eens: daar heb ik met mijn handen op zitten zwoegen. Je naam op een publicatie in een vooraanstaand tijdschrift is de top in mijnen job.
Ik wil jullie vooral bedanken om mij te leren met m'n voeten op de grond te staan en om vooruit, en niet naast mij, te kijken. Merci!

It is amazing what can be
accomplished when nobody
cares about who gets
the credit.
--Robert Yates