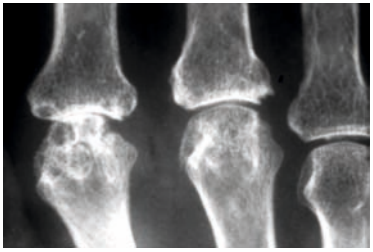
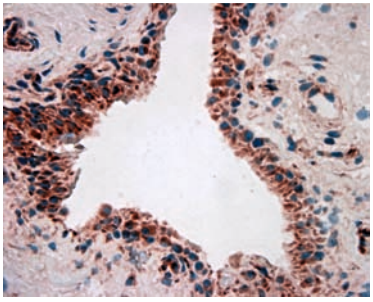
The role of the adipokines visfatin and leptin in the pathogenesis of rheumatoid arthritis

Florian Matthias Peter Meier





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Inauguraldissertation
zur Erlangung des Grades eines Doktors der Medizin
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vorgelegt von

Florian Matthias Peter Meier
aus Kronberg im Taunus

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Meiner Familie

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1 INTRODUCTION

RA is a chronic multisystem disease of unknown cause. There are a variety of systemic manifestations, but the characteristic feature of RA is a persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. The hallmark of the disease is a change in joint integrity, which is due the potential of the synovial inflammation to cause cartilage damage and bone erosions. Although the destructive potential may be severe, the course of RA can be quite variable. Some patients may experience only a mild oligoarticular illness of brief duration with minimal joint damage, whereas others will have a relentless progressive polyarthritis with marked functional impairment.[1]

1.1 Rheumatoid Arthritis

For a better description and integration of the necessity of cytokine research in RA, the major goal of this study, this introduction refers to the terms *"Mr Outside"* and *"Mr Inside"* which were coined by Professors Georg Schett, MD (University of Erlangen, Erlangen, Germany) and Gary S. Firestein, MD (University of California San Diego, La Jolla, CA, USA) in a review article about the pathogenesis of RA, published in 2010.[2] This viewpoint perfectly outlines two basic theories, which are currently used by the research community to explain the events taking place in the painful joints of patients suffering from that chronic inflammatory arthropathy.

The terms *"Mr Outside"* and *"Mr Inside"* refer to the two US college football stars Felix Blanchard and Glenn Davis, who have been nicknamed for their way of playing football. While *"Mr Inside"* was running directly towards the line of defense carrying the ball in his hands drawing all the attention, *"Mr Outside"* was circling around defense making it quite difficult for the other team to be prepared for both attacks. This strategy was successful in the 1940s. In RA pathophysiology, certain similarities to this strategy can be seen, thus leading to the *"Inside-out"* and the *"Outside-in"* hypotheses (Figure 1).

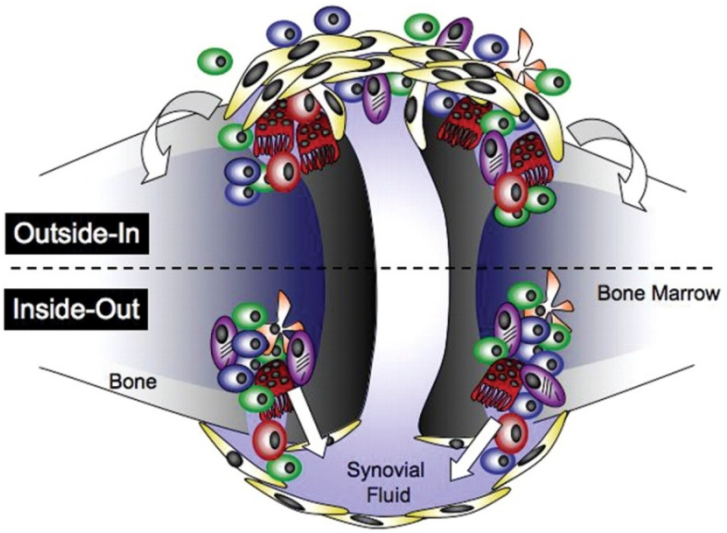


Figure 1: *"Outside-in"* and *"Inside-out"* hypotheses. Diagram illustrating the *"Outside-in"* (arthritis begins within the synovium) and *"Inside-out"* (arthritis begins in the bone and bone marrow) hypotheses of RA pathophysiology. Adapted from: Schett G. et al. Ann Rheum Dis 2010;69:787.[2]

"Inside-out" means that immune abnormalities are initiated within the juxta-articular bone marrow and then progress outwards to induce synovial inflammation. The *"Outside-in"* hypothesis, in contrast, postulates that the disease process starts in the synovial membrane and then spreads to surrounding structures such as cartilage, ligaments, tendons, bone and other supportive tissues. For both hypotheses, evidence is at hand and they should not be regarded as mutually exclusive but as synergistic when it comes to explain the full development of this joint disease.[2]

To further illustrate the pathogenesis of RA, it is important to describe the scenery in detail. A true joint is defined as a discontinuous bone connection made up by two or more bones, which are covered by hyaline articular cartilage at their communicating ends. The joint is surrounded by a joint capsule and, if needed, completed by special features such as ligaments, tendons, menisci and bursae (Figure 2).[3] Each joint capsule has an inner surface called the synovial membrane, which produces synovial fluid serving as a lubricant for smooth joint motion and a nutrient for the articular cartilage. The joint capsule inserts into the bone surface near the cartilage-bone junction. Of note, arthritic bone erosions usually begin in this area (Figure 4). The synovial membrane is subdivided into two layers which can be discriminated into the lining layer, a stratum comprising two to three rows of closely packed cells (synoviocytes types I and II) interspersed with little matrix, and the sublining, a stratum with less cells, more connective tissue matrix and blood vessels.

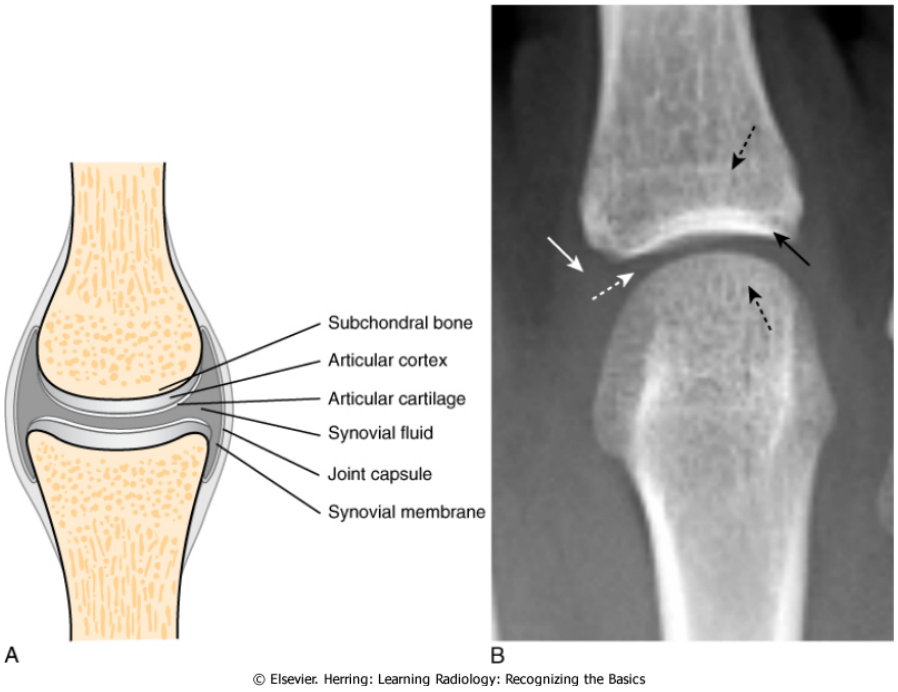


Figure 2: True joint. Diagram (**A**) and radiograph (**B**) of a true joint. A representation of a synovial joint shows the articular cortex, which corresponds to the thin, white line (closed black arrow) within the joint capsule (closed white arrow), which is usually capped by articular cartilage (dotted white arrow). The bone immediately beneath the articular cortex is called subchondral bone (dotted black arrows). Inside the joint capsule are the synovial membrane and synovial fluid. Adapted from: Herring W. Learning radiology: recognizing the basics - 1st. ed. Copyright © by Mosby, Inc. an affiliate of Elsevier, p.252.[4]

The *"Outside"* of the joint with respect to the mentioned theories is the synovial membrane, also called synovium. The physiological synovium does not possess epithelial structures like a delimiting membrane, cell-cell interfaces like tight junctions or a basal lamina. But apart from that most of the proteins which characterize an epithelium are present.[5] The dominant cellular constituents are the fibroblast-like synoviocytes type II, also referred to as synovial fibroblasts (SFs) and the macrophage-like synoviocytes type I. The functions of the synovium are to maintain viscosity of the synovial fluid, to filter molecules and to provide the inner area of the joint and the cartilage with oxygen and proteins. Nutrition of the cartilage is maintained by diffusion from the synovial fluid and synovial capillaries because healthy hyaline cartilage is devoid of blood vessels.[3] Joints are usually under mechanical stress and small lesions arise frequently. In that case, SFs contribute to the process of repair. In RA, however, the physiological balance between repair and destruction is severely disturbed thus leading to irreversible damage of cartilaginous and bony structures.[6,7] Beside SFs, synovial macrophages with a proportion of approximately 15% are present in the synovial membrane. Their major tasks are to phagocytise cell detritus, nonviable cells,

and bacteria and to function as antigen-presenting cells.[5] In RA, they actively perpetuate the inflammatory state of SFs by for example releasing microparticles and are further involved in the chronicity of the inflammatory processes by secreting proinflammatory molecules such as tumor-necrosis factor (TNF-) α.[8,9]

In terms of the hypotheses of Schett and Firestein, the bone, or rather the bone marrow, in contrast to the synovium represents the *"Inside"* of the joint. The bone itself contains three different cell types, namely osteoblasts, osteocytes, and osteoclasts. Osteoblasts are strongly active cells mainly producing collagen type I, the predominant extracellular matrix protein in the bone. In the phase of calcification, osteoblasts are walled into small lacunae and then termed osteocytes. Osteocytes, less productive than osteoblasts, maintain the extracellular matrix. Osteoclasts, which are motile, multinucleated, giant cells, act as opponents to anabolic osteoblasts by resorbing mineralized tissue.[10] In RA, osteoclast-driven bone resorption is initiated by of one of the key pathways of physiological osteoclast activation, the receptor activator of nuclear factor (NF)-κB ligand (RANKL)-RANK-interaction. RANKL is expressed by a variety of cell types involved in RA pathophysiology, including T cells and SFs.[11] The bone marrow itself is a central lymphoid organ and therefore highly capable of participating in processes of the immune system. *In vitro*, bone marrow cells show intense activity of tartrate-resistant acid phosphatase (TRAP), a standard marker of osteoclasts.[12]

Different imaging techniques are nowadays used to detect synovitis (*"Outside-in"*) or bone marrow edema (*"Inside-out"*) and other signs of early disease as far as possible. Besides being tools to support the diagnosis of RA, they can be used to monitor the effects of therapy, to detect silent progression (progressive joint destruction despite clinical response to therapy) and to draw conclusions about the steps of RA pathogenesis.

Ultrasound (US) is broadly used to identify joint synovitis, either in gray scale mode only or with additional power Doppler mode (Figure 3). The activated synovium, also referred to as pannus, can be seen as thickening of the joint capsule. In addition, hyperperfusion of the synovium, a result of neoangiogenesis and dilatation of local vessels displayed by power Doppler imaging, and early erosive bone changes are readily detectable by US supporting the *"Outside-in"* theory. The major disadvantage

of this method is that visualization of events beneath the cortical bone surface is not feasible and therefore signs of *"Inside-out"* processes cannot be detected.

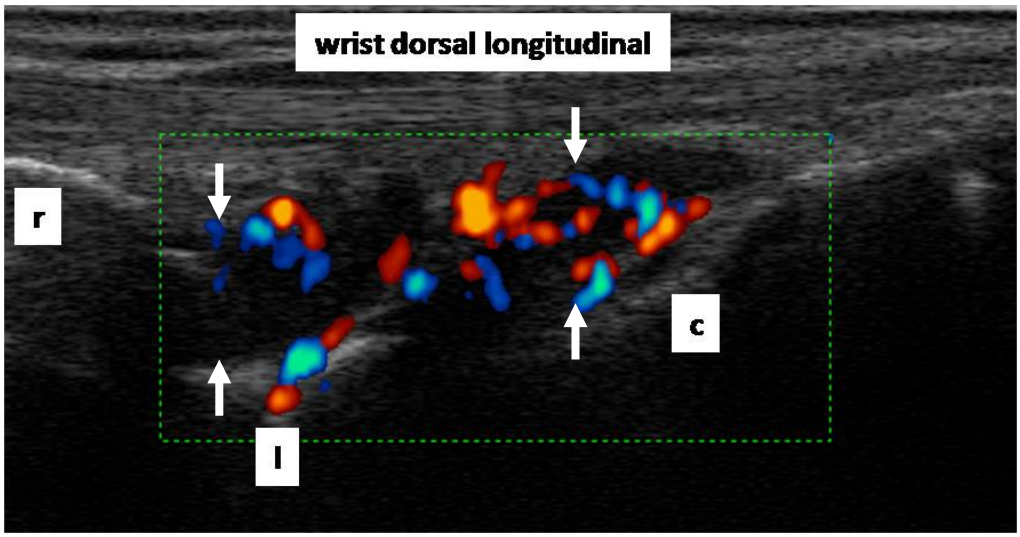


Figure 3: Image of an ultrasound examination. Grey-scale mode displays thickened synovium and use of the power-doppler mode (green rectangle) detects hyperperfusion of the inflamed synovium of the wrist joint. r = radius, l = lunate bone, c = capitate bone, arrows = thickened synovium. With kind permission of L.G. Meier, MD, Rheumapraxis Hofheim, Germany.

Conventional radiography (CR) is the most widely used imaging modality. Four stages of radiographic progression of RA are defined according to Steinbrocker et al.[13] Plain x-ray studies show signs of inflammation such as tissue swelling surrounding the affected joint, bone loss of three distinct forms (generalized osteoporosis distal from diarthrodial joints, periarticular osteopenia adjacent to inflamed joints, and erosions of marginal and subchondral bone at the bone-pannus interface[12]), and derangement of joint integrity at later stages of the disease (Figure 4). First signs of arthritis are periarticular osteopenia and, if present clinically, also tissue swelling. Only later in the course of the disease erosions are present, mainly close to where the synovial membrane inserts near the cartilage-bone junction. From this viewpoint, *"Inside-out"* might start before *"Outside-in"*. However, x-rays cannot reveal the active inflammatory processes in synovial tissue and therefore this method still cannot distinguish which one of the two theories represents the beginning of the disease.

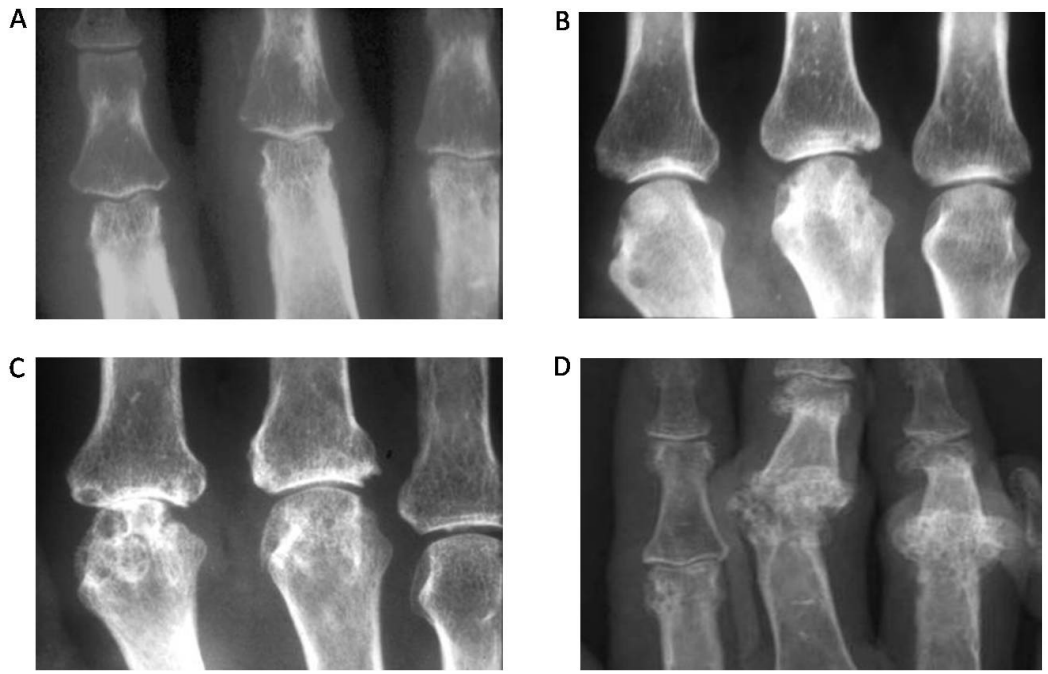


Figure 4: Four stages of radiographic progression of RA. Radiographs of the metacarpophalangeal and proximal interphalangeal joints displaying the radiographic stages of disease defined by Steinbrocker et al.[13] Soft tissue swelling and periarticular decalcification (**A**), small erosions and subchondral cysts (**B**), large erosions and subluxation (**C**), mutilation and destruction (**D**). With kind permission of the Department of Diagnostic Radiology, Kerckhoff-Klinik Bad Nauheim, Germany.

In recent years, a substantial amount of knowledge about disease initiation, duration and progression was gained by the implementation of magnetic resonance imaging (MRI) and its comparison to histological examinations. The most important finding of these studies was that so-called bone marrow edema in MRI is an independent predictor of progression to bone erosion.[14] Furthermore, histopathological analysis of these regions revealed lymphocyte infiltration replacing fatty bone marrow with a kind of inflammatory tissue consisting of mature B cells and activated T cells, both forming aggregates.[15–18] To explain the role of theses aggregates and to draw conclusions about their consequences, Schett and Firestein name pathophysiological implications, namely: (a) B cell growth and T cell survival, (b) forming of endothelial cells, and (c) expression of proinflammatory cytokines such as TNF-α, interleukin- (IL-) 6 and IL-8 maintaining and spreading inflammation.[2,19–22] MRI studies and subsequent analysis of bone marrow activities support the impact of the *"Inside-out"* theory by implementing facts about the way activated bone marrow might influence or initiate synovitis. Yet, it is still critical to discriminate whether osteitis or synovitis appears first taking into account that the respective MRI findings are usually seen simultaneously.

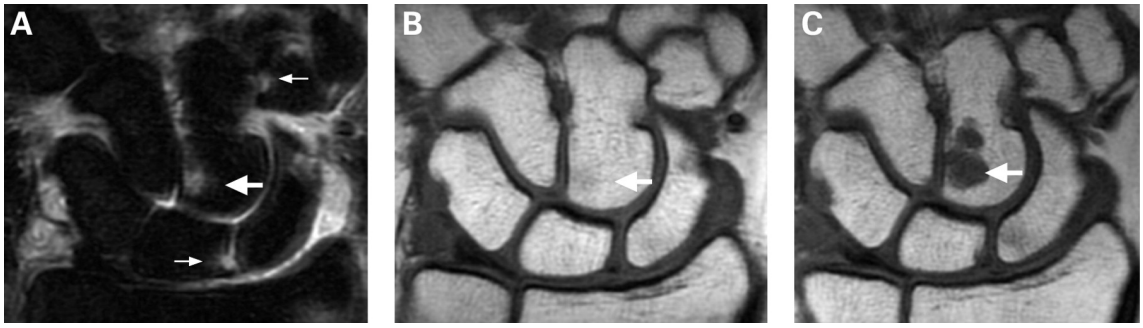


Figure 5: Magnetic resonance imaging of RA. MRI of the wrist, at baseline (**A**, **B**) and 1-year follow-up (**C**). **A**, Baseline coronal short-tau inversion recovery (STIR) image showing bone marrow edema in the capitate (thick arrow) and the lunate and trapezoid (thin arrows). (**B**), Baseline T1-weighted image without MRI erosion but with a hypointense signal corresponding to the bone marrow edema in (**A**) in the capitates (arrow). (**C**), T1-weighted image at 1-year follow-up, showing erosive progression in the capitate (arrow). Erosive progression was also seen in the trapezoid and the lunate, even though not optimally displayed in the presented slices. Adapted from: Haarvaldsholm EA et al. Ann Rheum Dis 2008;67:799.[14]

Even if the comparison between football strategies and hypotheses on RA pathophysiology might not be obvious at first sight, the underlying concepts are noteworthy for the development of scientific strategies in the investigation of RA pathogenesis. To discover specific cellular and molecular pathways, which are operative locally in RA synovium will be important for our understanding of this disease. In the past, investigation of the role of proinflammatory cytokines like TNF-α, IL-1 or IL-6 lead to the development of new therapeutic, so-called *"biologic"* agents, which have improved the outcome of our patients substantially.[23–26] Development of new and innovative therapies or improvement of therapeutic regimens needs extensive basic science effort to deal with the complexity of the immune processes which form the basis of this disease. As depicted above, imaging studies have revealed certain disease characteristics and their value is definitely high in terms of diagnostic and therapeutic decisions, but only in combination with histopathological studies their full significance could be unfolded. Hence, *in vitro* studies concentrating on central cell types acting in RA, such as RASFs, which were investigated in this study, need to be performed in order to identify new targets in the pathophysiological microcosmos of RA and to determine whether *"Inside-out"* or *"Outside-in"* has to be blocked to ameliorate disease activity.

1.2 Diagnosis

Indeed, synovitis is the hallmark of the disease, but diagnosis of RA requires more than detecting synovitis. Until 2010, the classification criteria of the American College of

Rheumatology (ACR) from 1987 were used by clinicians to classify RA (Table 1).[27] The impressive improvement of therapeutic strategies and development of new effective agents in the last years raised the question whether these criteria are still up-to-date.

Table 1: 1987 ACR criteria for the classification of acute arthritis of RA.

Criterion	*Definition*
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

For classification purposes, a patient shall be said to have RA if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable RA is *not* to be made.[27,28]

The first limitation of these criteria is a poor sensitivity and specificity for classifying patients with early inflammatory arthritis who in fact have RA and a second limitation is that they fail to identify very early arthritis in patients who in the course of the disease develop RA. Two of the seven criteria - erosive joint disease and extraarticular manifestations - describe late changes, which are in part prevented by modern treatment. Furthermore, the 1987 criteria do not include anti-citrullinated peptide antibodies (ACPAs) and acute-phase reactants, which are both used in clinical practice to assess and define the prognosis and the severity of disease. In the 1987 criteria, only rheumatoid factors (RF) are listed as laboratory parameters. However, their sensitivity and specificity compared to ACPAs are lower and the criteria fail to mention that the level of RFs have an impact on disease prognosis. Higher levels (thrice as much as the laboratory standard) have been shown to be associated with a more severe disease progression. ACPAs show a good sensitivity and very good specificity (79% and 97%) compared to RF (78% und 62%).[29] Acute-phase reactants reflect the inflammatory state regularly seen in patients with a disease flare. In a joint working group from the ACR and the European League Against Rheumatism (EULAR) new classification criteria

were devised in September 2010.[30] The new set of criteria consists of four criteria, which are evaluated in patients in whom presence of synovitis in at least one joint has been confirmed. An alternative diagnosis, which explains synovitis better than RA, e.g. reactive arthritis, should be excluded (Table 2).

Table 2: The 2010 ACR-EULAR classification criteria for RA.

Criterion	Score
A. Joint involvement	
1 large joint	***0***
2-10 large joints	***1***
1-3 small joints (with or without involvement of large joints)	***2***
4-10 small joints (with or without involvement of large joints)	***3***
>10 joints (at least 1 small joint)	***5***
B. Serology (at least 1 test result is needed for classification)	
Negative RF *and* negative ACPA	***0***
Low-positive RF *or* low-positive ACPA	***2***
High-positive RF *or* high-positive ACPA	***3***
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP *and* normal ESR	***0***
Abnormal CRP *or* abnormal ESR	***1***
D. Duration of symptoms	
<6 weeks	***0***
≥6 weeks	***1***

Score-based algorithm: add score of categories A–D. A score of ≥6/10 is needed to classify a patient as having definite RA.[30,31]

Of note, if a patient presents with negative RF and ACPA the sensitivity of the 2010 criteria decreases significantly leaving the treatment decision open to the physician.[32] Therefore, use of the 2010 criteria in patients having synovitis for more than 6 weeks does not necessarily answer the challenging question whether early treatment, as recommended for patients with a worse prognosis, is required or not.

The 2010 criteria account more for earlier stages of disease than the 1987 criteria and thus underline the importance of an early diagnosis and an early, aggressive treatment of RA. Nevertheless, the diagnosis of RA is still based on clinical signs and symptoms and the physician's experience since both sets of criteria have been established for classification but not diagnostic purposes.

1.3 Synovial fibroblasts

RASFs are key players in the complex intercellular network of RA.[33] Several studies have led to the understanding that RASFs compared to healthy SFs differ in morphology and behaviour. Intracellular signaling cascades are activated, apoptotic responses delayed, the expression cluster of adhesion molecules is altered and enhanced production of matrix-degrading enzymes is observed. Thus, RASFs have adopted an aggressive phenotype which actively mediates joint erosion and cartilage destruction.[34–37] Their role as resident cells in the hyperplastic RA synovium is well explored, but recent studies indicate that RASFs are capable of leaving their natural environment thus spreading RA to other joints.[38]

Healthy synovial lining consists of 1-3 cell layers, but in RA this is increased to 10-15 cell layers, containing predominantly RASFs and macrophages.[34–37] Though non-immune cells by nature, fibroblasts of the hyperplastic synovium develop several immunological properties during the course of the disease such as expression of human leukocyte antigen (HLA-) DR molecules and synthesis of inflammatory cytokines.[35,36] The activation of RASFs is detectable early after disease onset. However, the mechanisms underlying this permanent activation are only partly understood. Studies in the severe combined immunodeficient (SCID) mouse model of RA showed that activation of RASFs is in some ways independent of the presence of inflammatory cells[35], so that up to now the process of RASF activation is thought to be based upon multiple factors: (a) attachment to articular cartilage matrix *via* adhesion molecules[34–36,39]; (b) stimulation by growth factors such as fibroblast growth factor (FGF), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and insulin-like growth factor 2 (IGF2)[35,40–44]; (c) indirect cell-cell interaction through microparticles released from other activated or apoptotic cells of the synovium[45–48]; (d) dysregulation of proto-oncogenes and tumor-suppressor genes such as p53, p21 and c-Myc[35,36]; (e) epigenetic alterations such as hyperacetylated and hypomethylated DNA as well as expression of distinct microRNAs (miRs) including miR-146a and -155[49–52]. Beside these factors, interactions of RASFs with the immune system play a central role in the perpetuation of the inflammation and chronic activation of the synovium. These interactions are represented by two distinct mechanisms: (a) inflammatory cytokines like TNF-α, IL-1, IL-6, and IL-17 enhance

and possibly stabilize the activated phenotype of RASFs[34–36]; (b) cell-cell interactions between RASFs and lymphocytes as well as endothelial cells are mediated by adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 or intercellular adhesion molecule (ICAM)-1.[35] Furthermore, interaction with exogenous and in part endogenous ligands with (Toll-like receptors (TLR), of which TLR-2, -3 and -4 are expressed on RASFs, leads to increased expression of proinflammatory and matrix-degrading factors such as VEGF, IL-6, IL-8, MMPs-1, -2 and -13 and RANKL.[53,54]

RASFs do not only maintain a long-term hyperplastic growth of synovium in RA by contributing to the influx and proliferation of proinflammatory cells as well as by altered apoptosis and proliferation rates, but also actively degrade and invade cartilage and mediate cartilage and bone erosion. RASFs in contrast to other synovial cells strongly produce matrix-degrading enzymes. MMP is an umbrella term for different proteinases with distinct functions including collagenases, stromelysins, gelatinases, and membrane-type (MT) MMPs, whereas cathepsins represent a separate type of matrix-component degrading enzymes.[55] RASFs are the main source for MMP-1, -2, -3, -9, -10, -13 and cathepsin K and L.[34–37] MT1-MMP (MMP14) and MT3-MMP (MMP-16) and to a lesser extent MT2-MMP (MMP-15) and MT-4 (MMP-17) are also expressed in RASFs cleaving matrix components and activating other MMPs. The balance between MMPs and their inhibitors, tissue inhibitors of metallo-proteinases (TIMPs) is disturbed in RA. The levels of TIMPs found in RA synovium are not sufficient to exert counteracting effects. By expressing RANKL, a stimulator of osteoclastogenesis and cathepsin K, RASFs also influence bone erosion.

Thickening of synovial lining as described above leads to a microenvironment, which lacks sufficient oxygen supply and is therefore called hypoxic. The local hypoxia, particularly at sites of cartilage or bone invasion, stimulates certain proangiogenic and chemotactic factors in RASFs, namely angiogenin, angiopoietin-1, FGF-2, inhibitor of DNA binding-2 (ID-2), hypoxia-inducible transcription factor (HIF)-1a and VEGF[56–62], which contribute to increased vessel formation (neoangiogenesis is detectable by US, cf. Figure 3) and activation of the endothelium leading to enhanced cell migration.

Cell migration is a physiological process to maintain tissue homeostasis and to deal with conditions such as wound healing, immune defense and matrix-remodelling.[63,64] Several cell types actively contribute to this process, including lymphocytes, macrophages,

fibroblasts and endothelial cells. Fibroblasts and macrophages are able to migrate locally within a tissue to sites of damage. Chemokines and adhesion molecules play an important role in this process and certain features of this process are well characterized such as the temporary change from a *"resting"* to an *"activated"* phenotype. Under pathophysiological conditions like in RA, chronic inflammation not only leads to an increased migration of inflammatory cells to the site of inflammation but also resident cells like SFs can acquire the potential for long distance migration comparable to tumor metastasis.[38] The potential of RASFs to migrate from one affected joint to another has been shown in the SCID mouse model of RA.[38] This might be one explanation for the clinical spreading of the disease when patients after brief periods of early mono- or oligoarthritis progress to develop severe and chronic polyarthritis.

In summary, RASFs require a variety of inflammatory and other stimuli to convert into an activated phenotype. Once activated and during chronic inflammation in RA, RASFs become the key cell type of the inflamed synovium by cross-talking with other inflammatory cells, attaching to and degrading cartilage and promoting bone erosion as well as contributing to synovial neoangiogenesis and therefore supporting increased cell invasion *via* activated endothelial cells and potentially spreading the disease from one joint to the another.

1.4 Adipokines

The so-called adipokines, also referred to as adipocytokines, are secretory products of preadipocytes and mature adipocytes, but their production is not restricted to this cell type. The fact that different mesenchymal cells, e.g. fibroblasts and adipocytes, derive from mesenchymal stem cells, explains why they are able to produce similar cytokines.[65] Adipokines have been reported to be involved in energy homeostasis, appetite/satiety, reproduction, and insulin sensitivity and influence neuroendocrine, endothelial, immunological, hematological, angiogenetic, and vascular functions in an endocrine, paracrine, and autocrine manner. Different adipokines are currently discussed as new targets for therapeutic intervention in different diseases with a background of atherosclerosis and inflammation. Mature adipocytes after several steps of differentiation are characterized by the production of highly specific late markers of differentiation such as adiponectin collagenous repeat-containing sequence of 26-kDa protein (CORS-26), resistin, leptin and visfatin.[65] While adiponectin has been

characterized as having anti-inflammatory properties in atherosclerosis, in RA it is regarded as a proinflammatory cytokine, as are resistin, leptin and visfatin. In contrast, CORS-26 appears to be anti-inflammatory. For this project, visfatin and leptin were of particular interest.

1.4.1 Visfatin

Visfatin was originally described in 1994 as a novel human pre-B cell colony-enhancing factor (PBEF) by Samal et al.[66] The novel gene coding for visfatin/PBEF was isolated from a human peripheral blood lymphocyte cDNA library. It is composed of 11 exons and 10 introns, spanning over 34.7 kb of genomic DNA. The predominant mRNA for visfatin was approximately 2.4 kb long and coded for a 52-kDa secreted protein.[66] The 3' untranslated region showed multiple TATT motifs (thymine, adenine) usually associated with cytokine and oncogene messages.[67] The analysis of the quaternary protein structure revealed that visfatin is a dimeric protein set up by two identical 499 amino acid chains.[68] This observation is confirmed by the characterization of the crystal structure of rat visfatin.[69] Kim et al. showed that rat visfatin forms a homodimer of two identical proteins of 491 amino acids in length. Further analysis of this protein revealed no evidence that it might form higher-order oligomers.[70] Each visfatin monomer contains 19 β-strands and 13 α-helices arranged into two structural domains. The first structural domain consists of a seven-stranded antiparallel β-sheet, two antiparallel β-strands, and an α-helix bundle. The second structural domain is a variant of the classical $(\beta/\alpha)_8$-barrel (Figure 6).

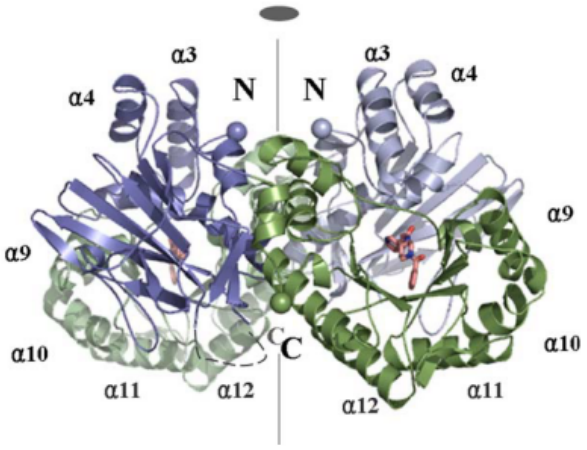


Figure 6: Ribbon diagram of visfatin showing the two monomers forming the visfatin homodimer in complex with the anti-cancer agent APO866. The two monomers are shown in slate and green (structural domain A) and in blue and pale green (structural domain B), respectively. The APO866 molecule is shown in red. Adapted from: Kim MK et al. J Mol Biol 2006;362(1):69.[71]

Up to now, visfatin has been found to act as a cytokine[66], a chemokine[72], a hormone[73], and an intracellular enzyme[74]. But the full spectrum of both, the intra- and extracellular effects of this molecule, remains an area of active investigation. Nonetheless, there is growing data available demonstrating the role of visfatin in physiological as well as pathophysiological conditions.

Samal et al., who first described visfatin as a cytokine-like molecule lacking a signal peptide, found that it had no activity on B cell precursors alone, but in the presence of stem cell factor and IL-7 it showed synergistic effects on pre-B cell colony formation. No effect was found with cells of myeloid or erythroid lineages. In 2002, Rongvaux et al. discovered that visfatin, whose expression was found to be up-regulated in activated lymphocytes, is in fact a cytosolic enzyme involved in the nicotinamide adenine dinucleotide (NAD^+) biosynthesis as nicotinamide phosphoribosyltransferase (NAMPT or NMPRTase).[75] NAMPT belongs to the dimeric class of type II phosphoribosyltransferases and is the rate-limiting enzyme of the NAD^+ salvage pathway recycling nicotinamide mononucleotide (NMN) from nicotinamide (NM, Figure 7).

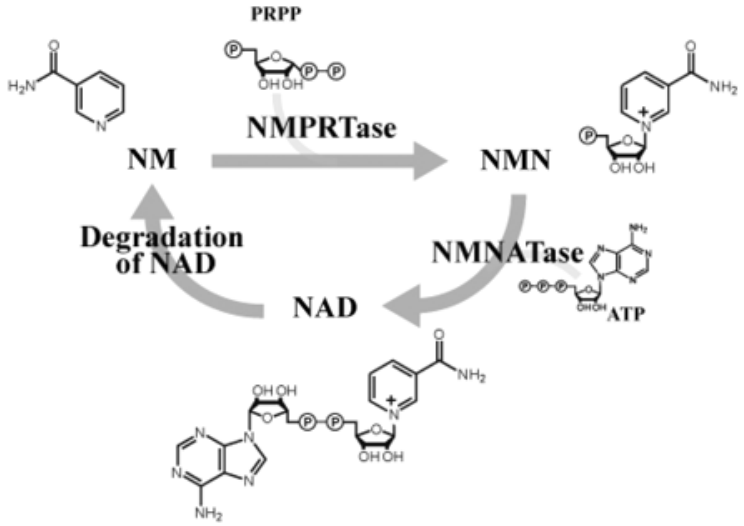


Figure 7: Cyclic metabolic pathway of NAD^+ salvage from NM. ATP is required for the synthesis of NAD^+ from NMN. PRPP = phosphoribosyl pyrophosphate. Adapted from: Takahashi R et al. J Biochem 2010;147(1):96.[68]

NAD^+ is an essential coenzyme which is also consumed by NAD^+-dependent sirtuin (Sir)-2 deacetylases.[76] Sir2 enzymes cleave NM from NAD^+ in the first step of their reaction, and NM is then used as a precursor for NAD^+ synthesis through the NAD^+ salvage pathway.[70] NAD^+ is synthesized *via* two major pathways, d*e novo* synthesis and the salvage pathway. Both pathways merge on the last metabolic step which is catalyzed by a NMN-ATPase.[69] This is of importance since several conditions require great

amounts of NAD^+ and are associated with an increase of NAMPT activity, namely: (a) sustained poly(ADP)-ribosylation activity; (b) mitogenic stimulation of human T cells, and (c) protein deacetylase activity during vascular smooth muscle cell maturation[77–80], all of which are to be found in activated synovium in RA.

Besides the finding that visfatin acts as an intracellular enzyme, its potential physiological function as a hormone is controversial. In 2005, Fukuhara et al. discovered that visfatin is abundantly expressed by visceral in contrast to subcutaneous fat in both humans and mice and therefore renamed it ***vis***ceral ***fa***t cytok***in***e (*"visfatin"*). Thus, the terms *"visfatin"*, *"NAMPT"* and *"PBEF"* refer to the same protein. They also showed that it exerts insulin-mimicking effects by binding to the insulin receptor at a site different from the binding site of insulin.[73] Several independent studies reported as well that visfatin has insulin-like effects.[72,81,82] However, investigators in academia and industry were unable to reproduce their results and by reporting errors of their work, the study was retracted from *Science* in 2007.[83]

Quite more evident are the findings, which describe visfatin as a cytokine involved in inflammatory conditions. Several studies found an increase of visfatin expression in a model of acute lung injury and clinical and experimental sepsis suggesting that visfatin might serve as a novel biomarker of inflammatory conditions.[84,85] In inflammatory bowel diseases such as Crohn's disease or ulcerative colitis, visfatin was detected in the epithelium of colonic biopsy specimens where its mRNA expression was increased.[86] Nemeth et al. and Ognjanovic et al. found elevated visfatin expression in acutely stressed human amniotic membrane, in pre-term membrane and in human amniotic epithelial cells by exogenous and endogenous inflammatory stimuli *in vitro*.[87,88] Moreover, Dahl et al. showed that visfatin was expressed by lipid-loaded macrophages which were located within unstable atherosclerotic plaques and suggested that visfatin potentially plays a role in plaque destabilization.[89] Visfatin was also expressed in cultured chondrocytes of patients with osteoarthritis and its expression was further enhanced by stimulation with IL-1β.[90] Moreover, visfatin was found to be upregulated during activation of immune cells such as monocytes, macrophages, dendritic cells, T and B cells, but its enhanced expression was not limited to these cells. Increased expression could also be detected in amniotic cells, chondrocytes, SFs and endothelial cells under the influence of inflammatory stimuli.[91] In patients with RA, visfatin was

found to be elevated in plasma, synovial fluid and synovial tissue and its expression correlated with the degree of inflammation, clinical disease activity and radiographic joint damage.[91]

In summary, visfatin is a very interesting molecule to be studied and its pathogenic role in several immune-mediated diseases needs to be further understood. It acts as a cytokine-like molecule in the extracellular microenvironment of activated and inflamed tissues and as an intracellular enzyme in the NAD^+ salvage pathway, which indeed is of great interest in cancer research. A specific inhibitor of enzyme activity, APO866, has been developed as an anti-cancer agent and has anti-tumoral, anti-metastatic and strong anti-angiogenic activities (Figure 6).[92] With regard to RA, several findings link visfatin to the imbalance of pro- *versus* anti-inflammatory cytokines and classify it as an adipokine with proinflammatory and immune-modulating properties. Hence, the definition of its role in RA needs further efforts. Some authors do already suggest that it is a potential therapeutic target for RA and that an inhibition of NAD^+ metabolism with APO866 may be efficacious in immune-mediated inflammatory disorders.[91,93–95]

1.4.2. Leptin

In 1994, Zhang and colleagues decoded the obese (ob) gene and found the obese protein which is nowadays called leptin.[96] It is a 16-kDa non-glycosylated peptide hormone, which is mainly produced by adipocytes. Structurally, the four-helix bundle of leptin shows homologies to the type I cytokine superfamily which led to the conclusion that leptin belongs to this family of molecules.[97] The discovery of leptin directed the traditional view of white adipose tissue as a storage tissue of triglycerides with low or no endocrine role towards an endocrine organ regulating energy homeostasis, metabolism and inflammatory processes.[65] Circulating leptin levels are directly correlated with the adipose tissue mass. Leptin acts as a satiety factor on the hypothalamus inducing a decrease in food intake and an increase in energy consumption. Long believed to be a potential therapeutic drug in obesity, the expected effect failed to appear. An explanation for this phenomenon might be that high levels of leptin in obese individuals lead to a reduced response, which is mainly due to an impaired transport of leptin across the blood brain barrier.

Leptin signals *via* specific leptin receptors (Ob-R), which are encoded by the diabetes (db) gene and belong like leptin itself to the class I cytokine receptor superfamily which also includes the receptors for IL-6, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M (OSM), granulocyte-colony stimulating factor (G-CSF) and the ubiquitously expressed gp130.[98–100] By alternative splicing of the db gene, six receptor isoforms have been discovered so far and they differ in terms of cytoplasmic domain length. There are one soluble form (Ob-Re), four short forms (Ob-Ra, -Rc, -Rd, -Rf) and one long-functional isoform (Ob-Rb), whose expression is almost ubiquitous.[101–103] The classical signaling pathway of leptin is activated *via* binding of leptin to its Ob-Rb-receptor. The cytoplasmic domain of the Ob-Rb receptor shares a Janus Kinase (JAK) like all other members of the cytokine I receptor superfamily. JAK-2 activation leads to phosphorylation of the cytoplasmic receptor residues then acting as binding sites for different transcription factors which include signal transducers and activators of transcription (STAT)-1, -3 and -5.[104–108] Of interest, leptin-induced STAT-3 activation leads to an upregulation of the suppressor of cytokine signaling (SOCS)-3 which in return inhibits leptin signaling, thus providing an inhibitory feedback loop.[109,110] Besides the classical signaling pathway, alternative pathways have been described to be involved in leptin signaling including activation of Ras, 5'-monophosphate kinase (AMPK), stearoyl-CoA desaturase 1 (SCD-1), protein kinase C (PKC), nitric oxide synthase (NOS), phosphoinositid (PI)-3 kinase, mitogen-activated protein kinase 1 (MEK-1) and p38 kinase.[111–118]

Food intake, hormones and different inflammatory mediators regulate leptin expression[101] and several inflammatory conditions such as type I diabetes, RA, inflammatory bowel disease, acute infection and sepsis are associated with elevated leptin levels.[119,120] However, the extent of leptin expression seems to differ between acute and chronic inflammatory states and study results are partially contradictory. Some studies, for instance, described that a chronic stimulation with proinflammatory markers causes a suppression of leptin expression.[121,122]

Immune responses require a well-balanced relation between energy intake and consumption.[123] Leptin has been described to link energy homeostasis and the immune system, for as a cytokine-like hormone with pleiotropic actions it is involved in glucose metabolism, $CD4^+$ T lymphocyte proliferation, cytokine secretion and hypothalamic-

pituitary-adrenal axis regulation.[101] These conclusions are based on several key studies which revealed the role of leptin in different pathophysiological conditions: (a) peripheral substitution of leptin to ob^-/ob^- mice reverses hyperglycemia and hyperinsulinaemia[124]; (b) leptin receptor deficient (db^-/db^-) mice suffer from thymus atrophy and leptin induces T cell activation in a way that T cell differentiation is shifted towards a T_{H1} response[125–127]; (c) the cytokine secretion pattern of monocytes/macrophages is modified by leptin *via* STAT-3-activated pathways[128]; (d) hypoglycaemia increases the release of corticotropin-releasing hormone (CRH) from hypothalamic neurons *in vitro*, but leptin is able to reverse this effect without altering the adrenocorticotropin (ACTH) secretion from pituitary cells.[129,130]

Leptin production by inflammatory cells is triggered by different inflammatory stimuli, which include TNF-α, IL-1β, IL-6 or lipopolysaccharide (LPS). Hence, it was suggested that leptin participates in the inflammatory processes through direct para- or autocrine actions.[131,132] In case of RA, leptin has been described as an interacting cytokine between the neuroendocrine and the immune systems, thus contributing to RA pathogenesis.[133] This statement is further emphasized by the findings of different studies: (a) ob^-/ob^- mice show leptin deficiency and are therefore resistant to antigen-induced arthritis (AIA) in comparison to wild-type mice[134]; (b) fasting of patients with RA leads to a decrease of leptin levels and is accompanied by a loss of $CD4^+$ lymphocyte activation and increased levels of the anti-inflammatory cytokine IL-4[135]; (c) circulating leptin levels are elevated in patients with RA.[136,137]

In summary, there is still increasing knowledge about the way leptin is involved in different inflammatory conditions such as RA and whether the link between energy homeostasis and immune responses is of clinical relevance in affected patients. Some authors even state that blocking leptin itself or leptin signaling using specific soluble receptors or monoclonal antibodies may be a promising target for future therapies.[97] Therefore, it is important to evaluate the pathophysiological implications of leptin in detail.

2 AIMS

There is growing evidence that adipose tissue can act as an inflammatory organ in human diseases by actively participating in and perpetuating inflammatory responses through the production of highly active cytokines now called adipokines.[65] Adipose tissue is ubiquitously distributed in the human body, but is also located adjacent to most joints. Of interest, the production of adipokines is not limited to adipocytes. Several different cell types in the joint tissue, including monocytes, SFs, and articular chondrocytes, can also express them upon stimulation. Furthermore, immune cells such as granulocytes, T and B lymphocytes, and macrophages are a source of adipokines during activation. Visfatin and leptin belong to the family of adipokines and several studies have already shown that both are linked to processes of metabolism and activity of the immune system. Because of these results visfatin and leptin are believed to be part of the inflammatory crosstalk of immune cells. RA is a chronic and painful arthropathy, which affects more and more joints during its course leading to severe disability. Autoimmunity is involved in the pathogenesis of this disease and immune cells as well as RASFs of the inflamed synovial tissue produce proinflammatory cytokines and matrix-degrading enzymes. Hence, it is of interest to investigate whether the local cytokine milieu also involves visfatin and leptin. Several studies suggest that both of them play an important role in RA and the influence of these adipokines on different cells of the synovial tissue has recently been demonstrated.[91,97] The aim of this study was to focus on the role of both cytokines in the activation of RASFs, which are key players in maintaining the immune response in the synovial tissue as well as in the degradation of articular cartilage and bone.[33]

To further clarify the role of the adipokines visfatin and leptin in the pathogenesis of RA the following questions were addressed by the experiments:

1. Are visfatin and leptin detectable in the joints of RA patients? For this purpose, synovial fluid and synovial tissue of RA patients were analyzed and the results were compared to samples from patients with osteoarthritis (OA).

2. If these adipokines are detectable in a significant amount in both synovial tissue and synovial fluid, do they have an influence on gene expression in SFs isolated from synovial tissues of patients with RA and OA?

3. If SFs react to the stimulatory effect of either visfatin or leptin, which pathways are involved in promoting the change in gene expression?

4. Does a visfatin- or leptin-induced change in gene expression lead to a modified local cytokine environment? If so, can autocrine stimulation promote a change in the behaviour of cells? Does a change in gene expression in some way alter the behaviour of SFs or lymphocytes *in vitro*?

3 MATERIALS

3.1 Materials and chemicals

Table 3: Applied chemicals and other materials.

Description	Source
5 x AMV-Buffer	Promega, Mannheim, Germany
6-, 12-, 24-, 48-well plates	Greiner, Frickenhausen, Germany
AEC substrate kit	Vector Laboratories, Burlingame, USA
Boyden chamber	Neuroprobe, Gaithersburg, USA
Bromophenol blue	Sigma-Aldrich, Taufkirchen, Germany
BSA (*bovine serum albumin*)	Roth, Karlsruhe, Germany
Cell culture flasks (25 cm²/75 cm²)	Corning, Wiesbaden, Germany
DMEM (*Dulbecco´s modified eagle medium*), 1 g/l glucose	PAN Biotech, Aidenbach, Germany
DMSO (*dimethyl sulfoxide*)	Sigma-Aldrich, Taufkirchen, Germany
Coverslips	Menzel Gläser, Braunschweig, Germany
Cryotubes (2ml)	Greiner, Frickenhausen, Germany
DTT (*dithiothreitol*)	Roche, Mannheim, Germany
EDTA (*ethylenediaminetetraacetic acid*)	Amresco, Karlsruhe, Germany
Eosin	Roth, Karlsruhe, Germany
Falcon tubes (15ml/50ml)	BD Biosciences, Heidelberg, Germany
FCS (*fetal calf serum*)	Sigma-Aldrich, Taufkirchen, Germany
Formaldehyde 37%	Merck, Darmstadt, Germany
Giemsa solution	Merck, Darmstadt, Germany
Hematoxylin	Roth, Karlsruhe, Germany
HEPES (*2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid*)	PAA Laboratories, Cölbe, Germany
Horseradish peroxidase-conjugated Streptavidin	Dianova, Hamburg, Germany
LightCylcer® capillaries (20µl)	Roche, Mannheim, Germany
May-Grünwald solution	Sigma-Aldrich, Taufkirchen, Germany
OCT TissueTek®	Sakura Finetek, Heppenheim, Germany
NaCl solution 0.9%, physiological	B. Braun, Melsungen, Germany
NF-κB activation inhibitor	Calbiochem, Darmstadt, Germany
nitrocellulose membrane	BioRad, München, Germany
p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580	Sigma-Aldrich, Taufkirchen, Germany
PBS (*phosphate buffered saline*)	PAA Laboratories, Cölbe, Germany

PCR nucleotide-mix	Roche, Mannheim, Germany
Penicillin/Streptomycin	PAA Laboratories, Cölbe, Germany
Polycarbonate PVPF-membrane, 8µm pore size	GE Osmonics, Minnetonka, USA
Protein kinase A (PKA) inhibitor 14-22 (cell-permeable myristoylated)	Calbiochem, Darmstadt, Germany
Protein kinase C (PKC) inhibitor 20-28 (cell-permeable myristoylated)	Calbiochem, Darmstadt, Germany
RNase-free dH_2O	Applied Biosystems, Darmstadt, Germany
RNasin (RNase inhibitor)	Promega, Mannheim, Germany
RT-PCR grade water	Applied Biosystems, Darmstadt, Germany
SuperFrost Plus slides	Menzel Gläser, Braunschweig, Germany
24-well Transwell® migration assay	Corning, Lowell, USA
Trypan blue	Sigma-Aldrich, Taufkirchen, Germany

Further chemicals, which are not listed, were either purchased from Roth, Karlsruhe, or Sigma-Aldrich, Taufkirchen.

3.2 Media and solutions

Table 4: Applied media and solutions. If not mentioned otherwise, the substances were stored at room temperature.

Description	Source
Cell culture medium (CCM)	500 ml DMEM, 10% heat-inactivated FCS, 100 U/ml penicillin, 10 µg/ml streptomycin, 10 mM HEPES; storage at 4 °C[138–140]
Eosin solution	1% eosin, H_2O; filtered, stored in a dark place; addition of a few drops of 96% acetic acid before usage
Freezing medium	FCS, 10% DMSO; storage: 1 week at 4 °C
Hematoxylin solution	0.1% hematoxylin, 0.2 g/l sodium iodate, 50 g/l potassium aluminium sulfate; totally dissolved in H_2O; 50 g/l chloral hydrate, 1 g/l citric acid; filtered, stored in a dark place
NETN buffer	0.5% NP-40, 1mM EDTA, 20mM Tris-HCl at pH 8.0, 100mM NaCl and 10% glycerol

3.3 Enzymes, proteins and antibodies

Table 5: Applied enzymes, proteins and antibodies (for cell culture, reverse transcription (RT), immunohistochemistry, stimulation and inhibition as well as cell motility experiments).

Description	Source
Accutase	PAA Laboratories, Cölbe, Germany
Adiponectin (recombinant, human)	Biovendor, Heidelberg, Germany
AMV Reverse Transcriptase	Promega, Mannheim, Germany
Cyclophilin B	Abcam, Cambridge, UK
Dispase II	PAN Biotech, Aidenbach, Germany
Interleukin-1 (IL-1, recombinant, human)	R&D Systems, Wiesbaden, Germany
Histofine Simple Stain MAX PO (multi) anti-mouse, -rabbit antibodies	Nichirei Biosciences, Tokyo, Japan
Collagenase	Sigma-Aldrich, Taufkirchen, Germany
Leptin (recombinant, human)	Biovendor, Heidelberg, Germany
Mouse IgG isotype antibody	BD Biosciences, Heidelberg, Germany
Phosphorylated p38 antibody	Cell Signalling Technology, Beverly, MA, USA
Protease inhibitor cocktail	Roche Diagnostics, Mannheim, Germany
Proteinase K	Qiagen, Hilden, Germany
Rabbit IgG isotype antibody	Santa Cruz Biotechnology, Heidelberg, Germany
Total p38 antibody	Cell Signalling Technology, Beverly, MA, USA
Trypsin/EDTA	PAA Laboratories, Cölbe, Germany
Vimentin antibody (mouse anti-human antibody)	DAKO, Hamburg, Germany
Visfatin antibody (rabbit anti-human antibody)	Bethyl Laboratories, Montgomery, USA
Visfatin (recombinant, human)	Biovendor, Heidelberg, Germany

3.4 Kits for molecularbiological and immunological assays

Table 6: Applied kits.

Description	Source
BioArray HighYield RNA labeling kit	Enzo Diagnostics, Farmingdale, USA
ECL Plus Western Blotting Detection System	GE Healthcare, Little Chalfont, UK
Custom human antibody array	RayBiotech, Norcross, USA
Human Activin A Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human chemokine antibody array I kit	RayBiotech, Norcross, USA
Human ENA-78 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human GCP-2 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human genome U133 Plus 2.0 oligonucleotide probe array	Affymetrix, Santa Clara, USA

Human Gro-α Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human IGFBP-2 ELISA kit	R&D Systems, Wiesbaden, Germany
Human IGFBP-3 ELISA kit	R&D Systems, Wiesbaden, Germany
Human IL-6 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human IL-8 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human Leptin Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human MCP-1 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human MMP-3 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human OPG ELISA kit	R&D Systems, Wiesbaden, Germany
Human pro-MMP-1 Quantikine ELISA kit	R&D Systems, Wiesbaden, Germany
Human Visfatin EIA kit	Phoenix Europe GmbH, Karlsruhe, Germany
MycoSensor PCR assay kit	Stratagene, La Jolla, USA
QuantiTect SYBR Green PCR kit	Qiagen, Hilden, Germany
PicoPure RNA isolation kit	MDS Analytical Technologies, Sunnyvale, USA
RNeasy Mini Prep kit	Qiagen, Hilden, Germany
RNase-free DNase set	Qiagen, Hilden, Germany
SuperScript cDNA synthesis customer kit	Invitrogen, Basel, Switzerland
Taq PCR Master Mix kit	Qiagen, Hilden, Germany
Multi-Test Limulus Amebocyte Lysate (LAL)	Lonza, Cologne, Germany

3.5 Oligonucleotides (primer)

Oligonucleotides were designed by using the sequence of the corresponding gene or applying the program *"Primer 3"* (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Analysis for dimers or other secondary structures was checked by the *"OligoAnalyzer 3.0"* (http://eu.idtdna.com/Home/Home.aspx). The specificity of the appropriate primers was verified afterwards by *"BLAST search"* (http://www.ncbi.nlm.nih.gov/blast). Table 7 shows all pairs of oligonucleotides, optimal $MgCl_2$ concentrations as well as the annealing temperatures (T_a).

Table 7: Oligonucleotides. All oligonucleotides were purchased from Sigma-Aldrich, Taufkirchen, Germany and diluted with H_2O_{bidest} to gain a concentration of 100 pmol/µl.

Description	Sequence (5'→3')	$MgCl_2$ [mM]	T_a [°C]
CXCL1_for	CCAAGAACATCCAAAGTGTGAACG	4.5	62
CXCL1_rev	CAGTTGGATTTGTCACTGTTCAGC		
CXCL2_for	TGCAGGGAATTCACCTCAAGAAC	4.5	62
CXCL2_rev	TTGAGACAAGCTTTCTGCCCATTC		
CXCL3_for	GAAGTCATAGCCACACTCAAGAATGG	4.5	60
CXCL3_rev	TACTTCTCTCCTGTCAGTTGGTGC		
CXCL5_for	GCGTTGCGTTTGTTTACAGACCACG	4.5	60
CXCL5_rev	GCTACCACTTCCACCTTGGAGC		
CXCL6_for	GTGGTAGCCTCCCTGAAGAACG	4.5	62
CXCL6_rev	TCTTACTGGGTCCAGGGATCTCC		
CXCL11_for	TCAGAATTCCACTGCCCAAAGG	4.5	60
CXCL11_rev	TTGTAAACTCCGATGGTAACCAGC		
CCL2_for	CAATAGGAAGATCTCAGTGCAGAGG	4.5	60
CCL2_rev	GGAATCCTGAACCCACTTCTGC		
CCL13_for	CAAACTGGGCAAGGAGATCTGTGC	4.5	60
CCL13_rev	GAGTTCAAGTCTTCAGGGTGTGAGC		
18S_rRNA_for	CGGCTACCACATCCAAGGAA		
18S_rRNA_rev	GCTGGAATTACCGCGGCTGC		

Primers for 18S_rRNA were designed to be applicable for different concentrations of $MgCl_2$ and different T_a. Random primers p(DN)6 (Roche, Mannheim, Germany) were used for cDNA-synthesis.

3.6 Equipment and software

Table 8: Equipment.

Equipment	Source
BioPhotometer	Eppendorf, Hamburg, Germany
Centrifuge 5471C	Eppendorf, Hamburg, Germany
Cytospin II	Shandon Lipshaw Inc., Pennsylvania, USA
GeneAmp PCR System 9700	PE Applied Biosystems, Weiterstadt, Germany
Holten Lamin Air	Thermo Scientific, Waltham, USA
LC Carousel Centrifuge	Roche Diagnostics, Mannheim, Germany
Leica CM 3050S (Kryostat)	Leica Microsystems, Nussloch, Germany
Leica DC 200 (Camera)	Leica Microsystems, Wetzlar, Germany
Leica DM IRB (Microscop)	Leica Microsystems, Wetzlar, Germany

LightCycler 1.5	Roche Diagnostics, Mannheim, Germany
NanoDrop	Peqlab Biotechnologie, Erlangen, Germany
Rotamax 120	Heidolph Instruments, Schwabach, Germany
Sunrise ELISA Reader	Tecan, Crailsheim, Germany
VersaDoc imaging system	BioRad, Munich, Germany
Vortex Genie 2	Bender&Hobein AG, Zürich, Switzerland

Table 9: Software.

Software	**Source**
GraphPad Prism 5	GraphPad Software, La Jolla, CA, USA
IM 1000	Leica Microsystems, Wetzlar, Germany
LightCycler Software 3.5	Roche Diagnostics, Mannheim, Germany
Magellan 5	Tecan, Crailsheim, Germany
Microsoft Excel 2007	Microsoft Corporation, Redmond, USA
ND-1000 V3.5.2	Peqlab Biotechnologie, Erlangen, Germany

4 METHODS

4.1 Tissue specimens and cell culture

4.1.1 Tissue specimens

Synovial tissues were obtained from patients with RA and OA during routine synovectomy at the time of total knee joint replacement in the Department of Orthopedics of the University Hospital Gießen and Marburg in Gießen and in the Department of Orthopedics of the University Hospital Regensburg in Bad Abbach.

Synovial fluid samples were obtained during routine arthrocentesis from patients with articular effusions, mostly of the knee. Samples were centrifuged at 700 x g for 10 minutes and stored at -20° C until further evaluation.

All specimens were obtained with approval of the Ethics Committee of the Universities of Gießen and Regensburg. All patients gave signed informed consent. Patients with RA met the 1987 ACR classification criteria (cf. Table 1).[27]

4.1.2 Freezing of tissues

After joint surgery, the synovial tissue was immediately stored on ice to protect it from degeneration. The samples were processed immediately. Parts of the tissue samples were embedded in OCT Tissue Tek® medium and then carefully snap frozen in liquid nitrogen to protect proteins and RNA from enzymatic digestion. Until further use, the specimens were stored at -80° C.

4.1.3 Extraction of synovial fibroblasts

Parts of the synovial tissues were used for SF isolation. The tissues were cut manually into pieces of 0.5 mm^3. Enzymatic digestion with Dispase II at room temperature for 1 hour degraded the extracellular matrix. Subsequently, the solution was poured through a cell strainer and then centrifuged for 10 minutes at 300 x g. Cells were transferred into cell culture medium (CCM, Table 4). Depending on the amount, isolated RASFs and OASFs were cultured in 75 cm^2- or 25 cm^2-cell culture flasks and incubated at 37° C. Incubator conditions were set to a relative humidity of 95% and a CO_2 saturation of

10%. After 24 hours, the culture medium was exchanged to remove non-adherent cells and tissue residue. Further passaging was performed when the confluence of the cultured cells reached a level of 80-90%. Medium was exchanged every 48 hours. Cell cultures were routinely checked for mycoplasma contamination using the MycoSensor PCR Assay kit according to the manufacturer's protocol.

4.1.4 Passaging of synovial fibroblasts

Passaging of fibroblasts was performed under sterile conditions. Medium was removed, cells were washed with PBS, and 5 g/l trypsin was added. After incubation at 37°C for 3-5 minutes, the reaction was stopped by adding 5 ml of CCM. The suspension was centrifuged for 10 minutes at 300 x g. The pellet was resuspended and equally distributed into fresh cell culture flasks.

After 2-3 passages the cell culture is free of macrophages because they are not detached easily by using trypsin. Cultured SFs were exclusively used between passages 4-8 because Neumann et al. have demonstrated that SFs alter their gene expression cluster during higher passages.[141] This would have influenced the reproducibility of results.

4.1.5 Storage of synovial fibroblasts

SFs were cultured to a confluence of 90% in a cell culture flask (75 cm^2). Incubation with trypsin was followed by centrifugation with 300 x g for 10 minutes. The pellet was resuspended in 4 ml of iced freezing medium and distributed equally into cryovials. Those were placed on ice immediately and then stored overnight in a freezing container at -80° C. The next day, the tubes were transferred into liquid nitrogen for long-time storage.

4.1.6 Thawing of synovial fibroblasts

Tubes containing frozen SFs were removed from liquid nitrogen storage and directly placed on ice. Afterwards, the cells were thawed carefully and slowly. The suspension was transferred into 10 ml of warmed CCM and centrifuged at 300 x g for 10 minutes. The pellet was resuspended in CCM and transferred into cell culture flasks. The medium was changed after 24 hours in order to ensure that non-attached cells and

residues of DMSO were removed. Subsequently, cell culture was carried out as described above.

4.1.7 Cell count

Cell count was performed after staining the cells with trypan blue using a Neubauer® counting chamber.

4.2 Immunohistochemistry of synovial tissue

Immunohistochemistry was performed to detect visfatin at sites of inflammation. 5 µm sections of snap frozen synovial tissue from patients with RA and OA were prepared and fixed in cold acetone for 10 minutes. After rinsing the slides in PBS, nonspecific binding was blocked with 2% BSA followed by overnight incubation in a moist chamber at 4° C with rabbit anti-human visfatin antibodies. Next, the slides were washed in PBS and endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 100% methanol for at least 30 minutes. Sections were then incubated with Histofine Simple Stain MAX PO (multi) anti-mouse/-rabbit for 30 minutes. Color development with AEC-substrate at room temperature was stopped after microscopic examination. Counterstaining of the nuclei was performed using hematoxylin. Rabbit isotype-matched IgG sera served as isotype control and mouse anti-human vimentin antibodies as positive control. A staining without primary antibody was performed as negative control.

4.3 Enzyme-linked immunosorbent assay (ELISA)

The synovial fluid levels of visfatin and leptin of patients with RA and OA were measured using commercially available ELISA kits according to the manufacturer's protocol. Absorption was measured at 450 nm and data were analyzed using the Magellan 5 software.

4.4 Endotoxin control test for recombinant human visfatin

For stimulation assays of RASFs, recombinant human visfatin (subsequently referred to as visfatin) isolated from Escherichia coli was used in concentrations between 1 and 2500 ng/ml. The datasheet of visfatin states that Endotoxin levels are below 1.0 EU/µg

(1 EU = 0.092 ng) and the manufacturer recommends a quality control test with LAL to determine quantity of endotoxin. The LAL assay was performed according to the protocol.

4.5 Affymetrix GeneChip® expression analysis of stimulated fibroblasts

Affymetrix GeneChip® analysis was used as a sufficient and extensive screening method. RASFs were grown to a confluence of 90%, medium was removed, cells washed with PBS and then stimulated with either 500 ng/ml visfatin or 100 ng/ml recombinant human leptin (subsequently referred to as leptin) for 15 hours. RNA was extracted using the RNeasy™ MiniPrep Kit with additional DNase digestion. Extracted RNA of non-stimulated RASFs served as control (non-stimulated fibroblasts were incubated for 15 hours in CCM). Following total RNA extraction, RNA quantity was measured using the NanoDrop® system. RNA quality was analyzed by the Agilent 2100 Bioanalyzer considering a 2:1 ratio of the 28S:18S ribosomal peaks as an indicator of good quality. Target preparation and hybridization for the human genome U133 plus 2.0 GeneChip® were performed according to the manufacturer's protocol. Results of the two conditions design (stimulated *versus* non-stimulated RASFs) were analyzed using the GeneSpring microarray analysis software to obtain *"increase", "decrease"* or *"no change"* calls. Genes of interest, which were upregulated or downregulated at least 2-fold were used for further analysis if considered to be of relevance in the pathogenesis of RA.

4.6 Protein array of RASF supernatants

To verify whether the effects observed at the RNA level were translated into protein secretion, supernatants of RASF cultures were analyzed. For this purpose, commercially available human chemokine- and custom human cytokine-antibody-arrays were used.

RASFs were transferred into 25 cm² cell-culture flasks. When confluence reached 90%, RASFs were either stimulated or non-stimulated with 100 ng/ml visfatin or 10 ng/ml leptin. After 15 hours, supernatants were collected and stored immediately at -20° C. For incubation of each array, 500 µl of supernatant were used following the manufacturer's instructions. The two conditions design was evaluated by

chemiluminescence using the VersaDoc imaging system (BioRad, Munich, Germany) and spot intensities were quantified numerically with the Quantity One software (BioRad). Data processing included averaging of duplicate spot intensities, background correction and normalization. For each protein, an x-fold change value was calculated.

4.7 RASF and OASF stimulation assays

Prior to quantification of proteins in the supernatants of RASFs and OASFs, it was important to determine dose- and time-dependent effects during stimulation periods.

4.7.1 Dose-effect relationship

Cultured RASFs were grown to 90% confluence in 6-well plates. After rinsing the plates with PBS, cells were stimulated with increasing concentrations of visfatin (2.5, 5, 10, 50, 100, 250, 500, 2500, 5000, 10000 ng/ml) and leptin (1, 2, 5, 10, 20, 50, 100, 250 ng/ml) in a total volume of 1 ml per well for 15 hours. Non-stimulated RASFs and OASFs incubated with DMEM for 15 hours served as controls, whereas stimulation with adiponectin (25 µg/ml) served as positive control.[142] In addition, cell count was performed. Detection of a dose-dependent induction of cytokine expression (IL-6, IL-8, and MMP-3) was performed using commercially available ELISAs.

4.7.2 Time-dependent response

RASFs were cultured in 6-well plates to a confluence of 90% and stimulated with visfatin in a total concentration of 100 ng/ml. The volume per well was 1 ml. Stimulation was stopped at different time points from 4 hours to 48 hours (4, 6, 8, 10, 12, 15, 18, 20, 24, 28, 34, 43, 48 hours). To exclude effects of stimulation-related cell division, cell count was performed. ELISAs were performed to examine the effects of the time-response experiments (IL-6, IL-8, and MMP-3).

4.7.3 Quantification of regulated genes at the RNA and protein levels

In order to confirm and quantify the results of the Affymetrix GeneChip® at the RNA level and of the antibody array experiments at the protein level, RASFs and OASFs were grown in 25 cm² cell-culture flasks to a confluence of 90%. Stimulation with visfatin (100 ng/ml) was performed in a total volume of 2.5 ml. After 15 hours, stimulation was stopped and quantification of proteins, which had been induced or

reduced by visfatin, was performed using ELISAs following the manufacturer's protocol. For several chemokines (CXCL-1, -5, -6, -8, -11, CCL-2), cytokines (IL-1β, interleukin 1 receptor antagonist (IL-1Ra), IL-6, IL-8, TNF-α), matrix-degrading enzymes (pro-MMP-1 and MMP-3) and other molecules of interest (e.g. Activin A, osteoprotegerin (OPG), insulin-like growth factor binding protein (IGFBP)-2, -3 and TIMP-1, -2) data were analyzed and expressed as x-fold change (sample *versus* control).

4.8 Quantitative analysis of regulated genes

The data of the non-quantitative Affymetrix GeneChip® expression analysis revealed quite a broad regulation of different cytokines and chemokines. To prove the demonstrated effects of visfatin on RASFs, chemokines of the highest regulation were chosen and their induction was measured quantitatively by real time-PCR.

4.8.1 RNA isolation

Cultured RASFs and OASFs were either stimulated or non-stimulated with visfatin (100 ng/ml) for 15 hours. RNA was isolated using the RNeasy™ Mini Kit according to the manufacturer's instructions. To minimize DNA contamination, additional DNase digestion was performed applying the RNase-free DNase Set. Isolated RNA was stored at -80° C until further use.

4.8.2 Reverse transcription (RT)

To carry out RT of isolated RNA into cDNA, the following mix was prepared:

AMV buffer (5x)	8 µl	
PCR nucleotide mix	4 µl	(10 mM)
Random primer $p(dN)_6$	0.5 µl	(2 µg/µl)
RNase inhibitor	1.6 µl	(40 U/µl)
AMV reverse transcriptase	1.2 µl	(10 U/µl)
RNA	x µl	(100 - 300 ng)
H_2O_{bidest}	ad 40 µl	

The RT-PCR-program during the reaction was as follows:

25°C	10 minutes	interaction of primers and RNA
42°C	60 minutes	transcription of RNA into cDNA
99°C	5 minutes	inactivation of AMV reverse transcriptase
4°C	∞	

For further applications, cDNA was stored at -20° C.

4.8.3 Real-time PCR

Real-time PCR was performed using a LightCycler® with SYBR Green as detection system. The weak fluorescence of SYBR Green intensifies when binding to newly developed DNA double-strands. At the end of each elongation period total fluorescence in each capillary was measured. Fluorescence increased with each cycle and the increase was proportionate to the amount of newly synthesized DNA. In a parallel approach, a reference gene was measured to set the quantification into relation. In this case 18S RNA served as reference gene.

By using true melting curve analysis, the specificity of each primer was checked. SYBR Green attached to DNA double-strands independent of their sequence. Each product possessed a specific melting temperature at which SYBR Green was released and the fluorescence consequently decreased. The melting curve approach demonstrated the melting of double-stranded DNA in relation to the rising of the temperature (50° C → 90° C). If the melting curve analysis showed more than one peak, the reaction had yielded unspecific products and quantification was not possible. For exclusion of this error, optimization of real-time PCR conditions had to be done prior to the quantification.

4.8.4 Optimizing primers for real time-PCR

Each pair of primers had to be optimized as to T_a, $MgCl_2$-concentration and reference gene. This was performed initially using standard PCR.

Efficiency in the LightCycler® was tested using diluted probes of cDNA, e.g. 1:2, 1:4, 1:8. Dilution series determined standard curves. The LightCycler® software then calculated the ascending slope (logarithm to the base 10 of the concentration *versus* cycle threshold (ct-) value) and efficiency was calculated as follows:

$$E=10^{(-1/slope)}$$

According to the guidelines of the manufacturer, efficiencies of 2.00 ± 0.05 were considered acceptable for experiments meaning that optimized PCR conditions lead to a duplication of the product. Different $MgCl_2$-concentrations were investigated (2.5 mM, 3.5 mM and 4.5 mM). Each of the amplifications was performed as instructed by the manufacturer.

4.8.5 Real-time PCR approach

Each capillary was loaded with 20 µl real-time PCR reaction mix. The components were as follows:

SYBR Green Mix	10 µl	(1x)
forward primer	0.5 µl	(5 pmol)
reverse primer	0.5 µl	(5 pmol)
MgCl2	x µl	(2.5 mM - 4.5 mM)
cDNA template	2 µl	
H_2O_{bidest}	ad 20 µl	

After preparation, each sample was transferred into a capillary and passed on into the LightCycler carousel and the PCR was started. The program was as follows:

initiation:	95° C	15 minutes	initial denaturation
cycles (35-55x):	95° C	15 seconds	denaturation
	x° C	25 seconds	annealing
	72° C	30 seconds	elongation/measuring fluorescence
melting curve:	50-99° C	0.1° C/second	specifying
end:	40° C		cooling

4.9 Inhibition of signal transduction pathways

To determine if pivotal pathways of signal transduction in RASFs were involved in the observed effects, inhibition of signal transduction pathways with chemical inhibitors was performed.

Cultured RASFs were distributed equally in 48-well culture plates and grown overnight. Next, wells were rinsed with PBS and pre-incubation was carried out for 1.5 hours in a total volume of 250 µl with chemical inhibitors of signal transduction pathways.

Inhibitors were used as follows:

1) p38MAPK-inhibitor SB203580 (10, 20 µM)
2) cell-permeable myristoylated protein kinase C (PKC) inhibitor 20-28 (10, 40 µM)
3) cell-permeable myristoylated protein kinase A (PKA) inhibitor 14-22 (1, 2 µM)
4) cell-permeable NF-κB activation inhibitor (18-, 40 µM)

Subsequent to pre-incubation, supernatants were discarded and cells were stimulated with 100 ng/ml visfatin for 15 hours in the presence of the chemical inhibitors. Stimulation of RASFs with visfatin served as positive control, non-stimulated RASFs with or without inhibitors as negative control. Supernatants were obtained and stored at -20° C. Commercially available ELISAs for the detection of IL-6 and monocytes chemotactic protein (MCP)-1 were used to determine inhibition of visfatin-mediated effects. Inhibition of the p38MAPK pathway with the chemical inhibitor SB203580 in a total concentration of 20 µM was also performed over 6 hours, with a pre-incubation period of 1.5 hours. X-fold inductions were calculated for each approach, cell counts performed and data expressed as the ratio of visfatin-stimulated samples *versus* samples pre-treated and treated with chemical inhibitors of signal transduction pathways and visfatin.

4.10 Western blot analysis of p38 MAPK phosphorylation

Cell extracts were harvested in NETN buffer containing 1x complete protease inhibitor cocktail. Protein was separated on a SDS 10% (w/v) polyacrylamide gel and blotted onto nitrocellulose membranes. Proteins were detected by antibodies against phosphorylated and total p38. Detection was performed by horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence detection kit. All western blots were also probed for Cyclophilin B to ensure equal loading of samples.

4.11 Cell migration assay

Directed cellular migration was evaluated using a 48-well Boyden microchemotaxis chamber consisting of an upper and a lower compartment separated by a semipermeable

membrane. Cells migrate from the upper chamber towards chemo-attractive factors or media in the lower chamber. After an established time interval migration was stopped and the membrane was carefully removed from the chamber. Cells were fixed on the membrane, then stained and counted. The stronger the attraction of media was the more cells migrated through the membrane pores.

4.11.1 Preparation of conditioned media

RASFs were cultured in 25 cm² cell-culture flasks in DMEM containing no FCS to prepare conditioned media including chemo-attractive factors produced by RASFs. The total volume was 2.5 ml and the confluence of cells was 90%. The different approaches were as follows:

1. DMEM + 2% FCS (*"baseline"* medium)
2. DMEM + 100 ng/ml visfatin (stimulated medium)
3. DMEM + 20 µM SB203580 (stimulated medium)
4. DMEM + 100 ng/ml visfatin + 20 µM SB203580 (stimulated medium)

After 16 hours, the respective conditioned media were removed from the flasks, centrifuged to deplete detached cells and stored as aliquots at -20° C until further use.

4.11.2 Migration of RASFs

For the chemotaxis assay, cultured RASFs were detached from the cell culture flask surface using ready-to-use accutase (less aggressive than trypsin) and counted. The required amounts of cells were obtained by centrifugation and re-suspended in *"baseline"* medium. 20000 cells in 50 µl *"baseline"* medium were placed into each upper chamber. The lower chambers contained 30 µl of either, *"baseline"* medium, CCM (positive control), DMEM without FCS (negative control), or each of the respective stimulated media of the approaches 2., 3., and 4. (cf. 4.11.1). The upper chambers were separated from the lower chambers by 8 µm pore-size filters. After a migration time of 7 hours, non-migrated cells on the filter facing the upper chamber were scraped off; filters were fixed in methanol, and subsequently stained with hematoxylin, May-Grünwald and Giemsa. Of each well, 6 areas were photographed, the amount of cells was counted and data were expressed as migration index (MI; ratio of sample to baseline).[143]

4.11.3 Migration of lymphocytes

Migration of lymphocytes was measured using a 24-well Transwell® migration assay. A total of 10^6 cells was added onto the filter (pore-size 5 µm). Into the lower well the prepared conditioned medium and stimulated media (as described above) were placed. In the control experiment, visfatin was diluted in CCM. Cells were allowed to migrate for 4 hours at 37° C. Cells in the lower well were enumerated with a Neubauer® counting chamber.

4.12 Cell motility assay

To differentiate between induced directed chemotaxis and increased undirected cell motility, a scrape motility assay was performed. Prior to the experiments, squares were scratched into the bottom side of 6-well cell culture plates as orientation guides. RASFs were added to the plates in equal amounts and grown to 100% confluence in CCM. The cells were preincubated with increasing concentrations of visfatin (5, 25, 50, 100, 250 ng/ml) for 1.5 hours. Beginning from the center of the crosses of each well, cells were removed by scraping using a 100 µl pipet tip. The wells were washed with PBS to remove free-floating detached cells. Preconditioned medium was re-added and the cells were incubated for 8 hours. To visualize cell motility within the scrape line, photos of the defined, cell-free scraped areas were taken every 90 minutes between 8 to 17 hours after scraping.[144] Cells moving in the scraped gap were counted and data was expressed as percentages of sample compared to baseline.

4.13 Statistics

Statistical analysis was performed using Students *t* test, Wilcoxon-Mann-Whitney *U* test and non-parametric Spearman's correlation. Arithmetic means and standard errors of the mean (SEM) were calculated for inter- and intra-assay replicates. If mentioned otherwise, standard deviations (SD) were assessed. Results were regarded as significant for $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). Statistical evaluation was performed using GraphPad Prism 5.

5 RESULTS

5.1 Detection of adipokines in synovial fluid and tissue

To investigate whether the adipokines visfatin and leptin exert pro- or anti-inflammatory effects on SFs, which are key players in the destructive inflammatory process of RA, synovial fluids and synovial tissues were analyzed for their presence. The identified levels in RA were compared to those in OA.

5.1.1 Visfatin levels in synovial fluid

The mean level (± SEM) of visfatin in synovial fluid of RA patients was 76.26 ± 7.22 ng/ml (n = 24), which was lower than in OA synovial fluid (88.92 ± 6.71 ng/ml, n = 38; Figure 8A), but this difference was not significant (p = 0.128). In RA, visfatin levels correlated positively with CRP levels (n = 24, ρ = 0.329, p = 0.117; Figure 8B) but not with erythrocyte sedimentation rate (ESR; n = 24, ρ = 0.236, p = 0.266). A significant negative correlation was found between visfatin levels and age of the patients (n = 24, ρ = -0.456, p = 0.026; Figure 8C), whereas the body-mass index (BMI) did not correlate with visfatin levels (n = 15, ρ = -0.111, p = 0.2; Figure 8D). In OA, visfatin levels were not correlated with BMI either (n = 29, ρ = 0.049, p = 0.8). Patient details are listed in Table 10.

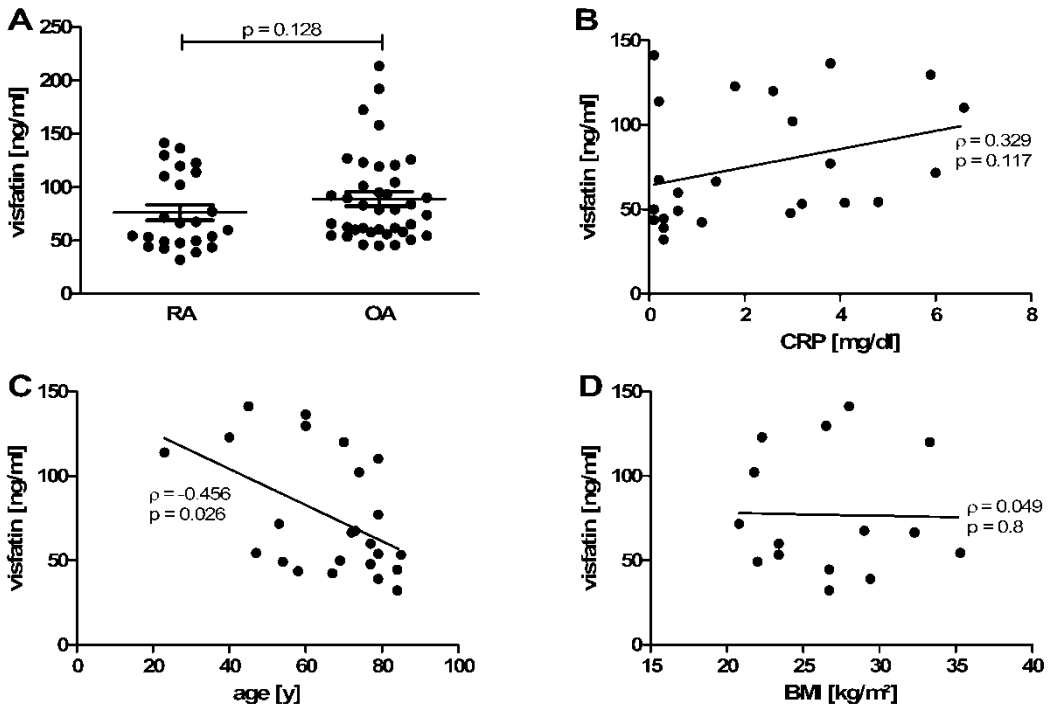


Figure 8: Expression levels of visfatin and correlations in RA and OA. (**A**), Expression of visfatin levels (ng/ml) in RA and OA synovial fluid. Data is presented as mean value ± SEM. (**B**), Correlation of visfatin concentration in RA synovial fluid to CRP levels (mg/dl), (**C**), to the age of patient in years, and (**D**), to the BMI (kg/m²).

5.1.2 Leptin levels in synovial fluid

The mean level (± SEM) of leptin in synovial fluids of patients with RA was 10.56 ± 1.94 ng/ml (n = 23). In OA, the mean synovial fluid concentration of leptin was significantly higher (15.52 ± 1.68 ng/ml, n = 38, p = 0.037; Figure 9A). In RA, leptin levels in synovial fluid were strongly correlated with BMI (n = 14, ρ = 0.801, p = 0.0006) but not with markers of inflammation such as CRP or ESR. By contrast, the levels of leptin in OA synovial fluid were not correlated with BMI. For patient details see Table 10.

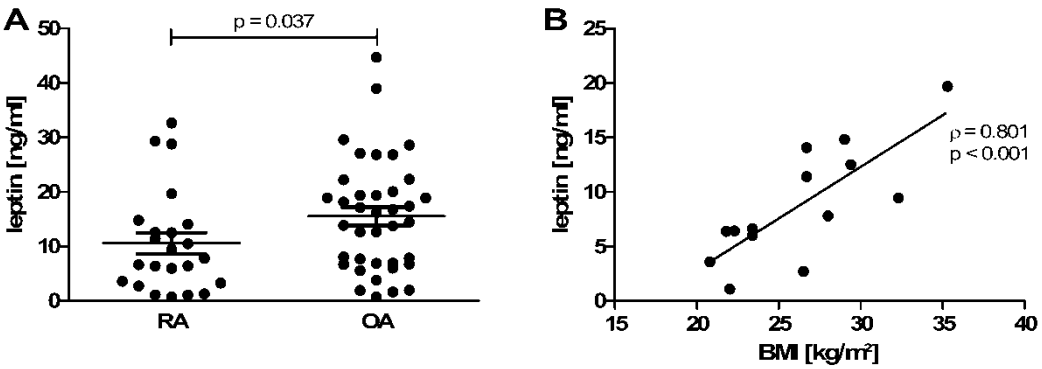


Figure 9: Expression levels of leptin and correlations in RA and OA. (**A**), Expression of leptin levels (ng/ml) in RA and OA synovial fluid. Data is presented as mean value ± SEM. (**B**), Correlation of leptin levels in RA synovial fluid with BMI (kg/m²).

Table 10: Patient characteristics.

diagnosis	age (years ± SD)	female (%)
RA (n = 24)	66.17±15.99	83.3%
OA (n = 38)	70.71±9.1	65.8%

Details of patients who presented with articular effusions and underwent routine arthrocentesis. Synovial fluid samples were examined for the presence and concentrations of the adipokines visfatin and leptin, respectively.

5.1.3 Detection of visfatin at sites of tissue inflammation

Immunohistochemistry using a monoclonal anti-human visfatin antibody was performed in order to detect visfatin protein in synovial tissues of patients with RA (n = 3) and OA (n = 2). All inflammatory tissues expressed visfatin, but RA synovium in comparison to OA synovium showed a stronger expression. In addition, expression of visfatin in RA synovium was not limited to perivascular areas as in OA synovium, but was also abundant in the lining layer and in lymphoid aggregates. Expression in the sublining and in the interstitium was overall lower (Figure 10).

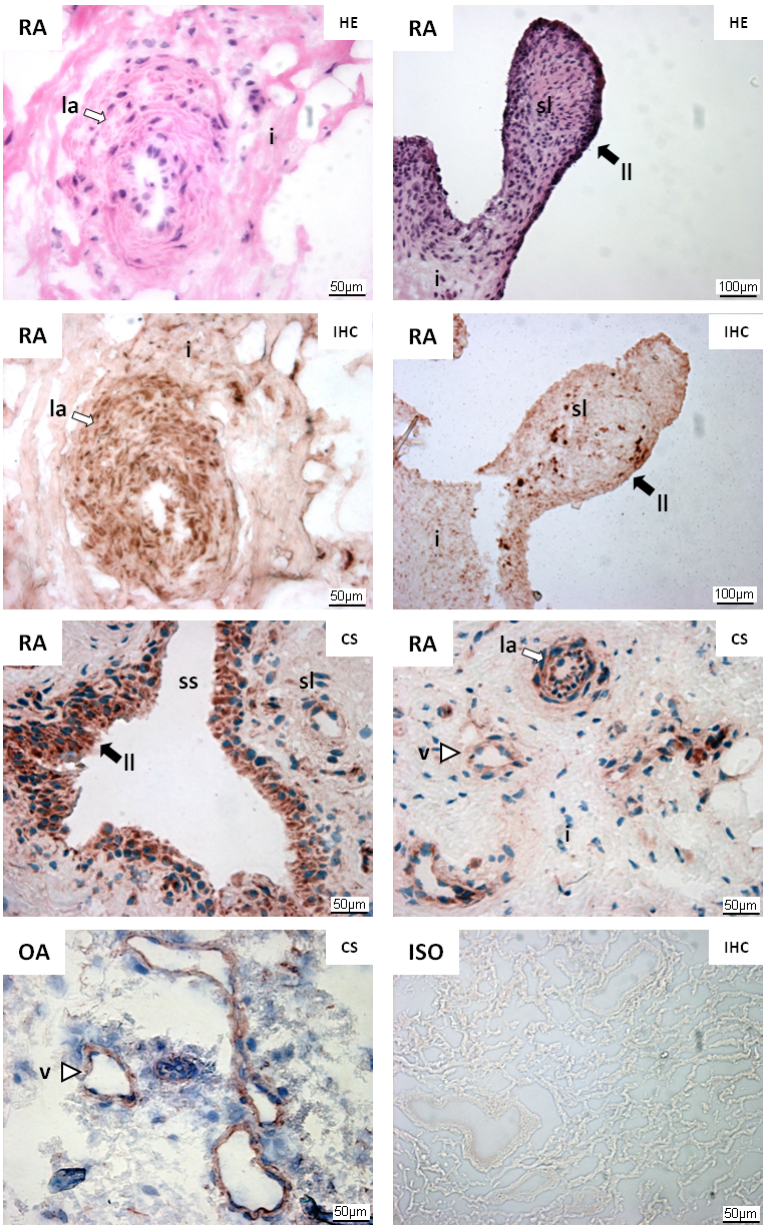


Figure 10: Immunohistochemical proof of visfatin protein expression in RA and OA synovial tissue. *First row*, HE-staining of RA synovium. *Second row*, immunohistochemical detection of visfatin in RA synovium. *Third row*, immunohistochemical detection of visfatin in RA synovium counterstained with hematoxylin. *Fourth row*, immunohistochemical detection of visfatin in OA synovium counterstained with hematoxylin (left) and isotype control (right). CS = counterstaining, i = interstitium, IHC = immunohistochemistry, la = lymphoid aggregate, ll = lining layer, ss = synovial space, sl = sublining, v = vessel.

5.2 Influence of visfatin on synovial fibroblasts

5.2.1 Endotoxin concentration in recombinant human visfatin

The source of recombinant proteins is usually bacteria, in case of visfatin, the protein was synthesized in Escherichia coli. Hence, endotoxin contamination of the protein needed to be clarified. As recommended by the manufacturer, endotoxin levels in the

stock solution of recombinant human visfatin were measured to exclude possible effects of endotoxin on the following experiments (n = 3). The endotoxin concentration of the stock solution was 2.44 ng/ml, which was reduced to 0.488 pg/ml if visfatin was used in a concentration of 100 ng/ml. In a previous study, it could be demonstrated that endotoxin concentrations of less than 0.1 ng/ml do not significantly change the expression of proinflammatory cytokines like for example IL-8 in RASFs.[145] In this study, the level of endotoxin in the relevant, used protein concentrations was approximately 0.5 pg/ml and therefore negligible.

5.2.2 Expression analysis of genes regulated by visfatin in RASFs

The first step in the analysis of visfatin-mediated effects in RASFs was to screen which genes are regulated by stimulation with visfatin. Therefore, gene chip expression analysis was performed (n = 1) applying the human genome U133 Plus 2.0 GeneChip (analysis of over 47,000 transcripts).

The expression of several genes was strongly altered by visfatin (Table 11). Most prominently, the group of chemokines was upregulated (CXCL-1, -2, -3, -5, -6, -8, -9, -10, -12, -16 and CCL-2, -5, -7, -8, -13, -20) and there was no recognizable difference between the CC- and the CXC-families. Furthermore, pro- and antiinflammatory cytokines were induced, namely IL-1β, -6, -7, -8, -15, -32, -33, IL-1Ra as well as adiponectin and visfatin itself. In addition, more groups of genes that play essential roles in the pathogenesis of RA were upregulated including proteinases (MMP-1, -3, -10, -12 and -19), adhesion molecules (VCAM-1, ICAM-1, and -2), growth factors (FGF-13, -18, ECGF-1, CSF-1, IGF-1 and VEGF-C), receptors (TLR-3, -5 and IL17RB) and intracellular signaling molecules like STAT-1, -2 and SOCS. Few genes were partly downregulated (FGF-9, IL1RI and TLR-6). The inductions of the CXCL11- and LEPR-genes were excluded from the gene chip analysis due to the high variability between the multiple spots.

These results indicate that visfatin is involved in chemotactic processes and activation of the respective intracellular pathways, which are necessary for the synthesis of proinflammatory and matrix-degrading proteins. Therefore, these aspects were investigated in more detail.

Table 11: Visfatin regulated genes in RASF. The selection focuses on genes, which are of putative relevance in RA.

Changes in gene expression of RASFs after stimulation with visfatin: Selection of genes regulated by visfatin		
Gene Name	**Gene Symbol**	**Fold Change**
Chemokines		
chemokine (C-C motif) ligand 20	CCL20	1024.00
chemokine (C-C motif) ligand 5	CCL5	473.71
chemokine (C-X-C motif) ligand 10	CXCL10	445.72
chemokine (C-X-C motif) / interleukin 8	CXCL8/IL8	164.03
chemokine (C-X-C motif) ligand 1	CXCL1	90.51
chemokine (C-X-C motif) ligand 3	CXCL3	51.98
chemokine (C-C motif) ligand 8	CCL8	42.22
chemokine (C-X-C motif) ligand 5	CXCL5	22.63
chemokine (C-X-C motif) ligand 6	CXCL6	18.38
chemokine (C-X-C motif) ligand 2	CXCL2	17.96
chemokine (C-C motif) ligand 13	CCL13	13.93
chemokine (C-C motif) ligand 2	CCL2	13.93
chemokine (C-C motif) ligand 7	CCL7	12.13
chemokine (C-X-C motif) ligand 9	CXCL9	4.59
chemokine (C-X-C motif) ligand 16	CXCL16	2.64
chemokine (C-X-C motif) ligand 12	CXCL12	2.46
Cytokines		
interleukin 1 receptor antagonist	IL1Ra	34.30
interleukin 6	IL6	10.56
interleukin 32	IL32	8.00
interleukin 33	IL33	6.96
adiponectin	ADIPOQ	4.29
interleukin 15	IL15	4.01
interleukin 1 beta	IL1B	3.61
interleukin 7	IL7	3.48
leukemia inhibitory factor	LIF	3.25
visfatin	PBEF	2.44
fibroblast growth factor 13	FGF13	3.25
fibroblast growth factor 18	FGF18	2.14
fibroblast growth factor 9	FGF9	-2.14
Growth Factors		
endothelial cell growth factor 1	ECGF1	9.85
colony stimulating factor 1	CSF1	2.65
insulin-like growth factor 1	IGF1	2.47
vascular endothelial growth factor C	VEGFC	2.14
Receptors		
interleukin 15 receptor alpha	IL15RA	11.31
toll-like receptor 3	TLR3	8.57

activin A receptor type IIB	ACVR2B	6.06
endothelin receptor type B	EDNRB	5.66
fibroblast growth factor receptor 3	FGFR3	4.92
interleukin 7 receptor	IL7R	3.49
interleukin 9 receptor	IL9R	2.30
interleukin 17 receptor B	IL17RB	2.30
toll-like receptor 5	TLR5	2.14
fibroblast growth factor receptor 1	FGFR1	2.14
chemokine (C-C motif) receptor-like 1	CCRL1	-2.00
insulin-like growth factor 1 receptor	IGF1R	-2.46
toll-like receptor 6	TLR6	-3.03
interleukin 1 receptor type I	IL1R1	-4.92
Proteinases & Peptidases		
matrix metallopeptidase 3	MMP3	59.71
matrix metallopeptidase 12	MMP12	34.30
matrix metallopeptidase 1	MMP1	16.00
matrix metallopeptidase 10	MMP10	4.92
matrix metallopeptidase 19	MMP19	2.14
Adhesion & Cytoskeleton		
vascular cell adhesion molecule 1	VCAM1	16.00
intercellular adhesion molecule 1	ICAM1	8.44
intercellular adhesion molecule 2	ICAM2	2.30
Signaling molecules		
signal transducer and activator of transcription 1	STAT1	4.59
signal transducer and activator of transcription 2	STAT2	3.69
suppressor of cytokine signaling 1	SOCS1	3.25

5.2.3 Dose-dependency of visfatin effects on RASFs

With regard to different concentrations of visfatin in synovial fluid of RA patients, stimulation experiments with different concentrations of visfatin were performed *in vitro* (2.5, 5, 10, 50, 100, 250, 500, 2500, 5000, 10000 ng/ml). Expression of IL-6 and IL-8 was regulated by visfatin stimulation in the gene chip expression analysis. Therefore, both cytokines were measured in supernatants of stimulated fibroblasts and dose-dependent effects were observed (Figure 11). For example, the basal concentration of IL-6 was 23.3 pg/10³ cells and was increased to 477.96 pg/10³ cells when stimulating with 100 ng/ml visfatin. Similarly, IL-8 levels increased from 0.72 pg/10³ cells to 199.49 pg/10³ cells using the same concentration of visfatin. For all subsequent experiments, visfatin was used at 100 ng/ml because the corresponding point for that concentration on the dose-effect-curve was located in the linearly increasing section and exerted a strong effect on the production of pro-inflammatory cytokines. Further

increase of the visfatin concentration did not result in additional cytokine production and, accordingly, the dose-effect curves reached a plateau (Figure 11).

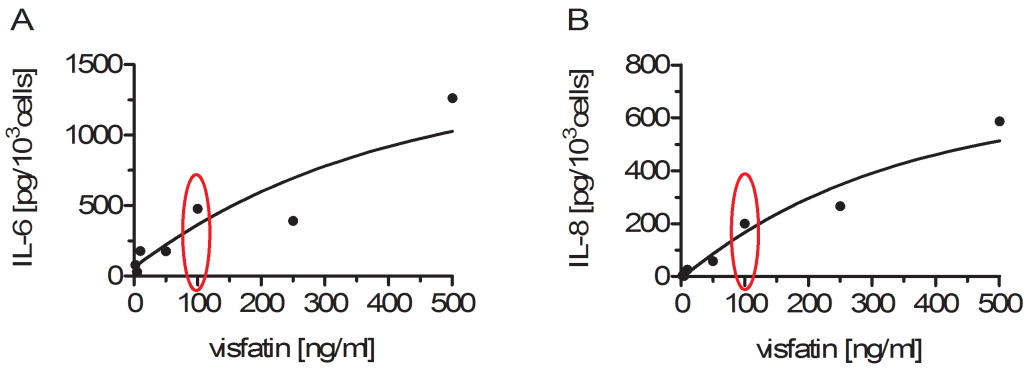


Figure 11: Dose-dependency of visfatin effects on RASFs. Dose-dependent relationships for different stimulation concentrations of visfatin are plotted against the concentrations of (**A**), IL-6 and (**B**), IL-8 in supernatants of stimulated RASFs. The presented range is between 2.5 to 500 ng/ml. The linearly increasing sections of the curves are marked in red. For subsequent experiments 100 ng/ml visfatin as stimulation concentration was established.

5.2.4 Time-dependency of visfatin effects on RASFs

Increasing the time intervals of stimulation assays may result in an increase or decrease of cytokine production. In previous experiments, the time period during which visfatin could influence protein production in RASFs was 15 hours.[93] To confirm the sufficiency of this interval, time-related responses were investigated. RASFs were stimulated with 100 ng/ml visfatin from 4 hours to 48 hours. Corresponding relationships are depicted in Figure 12. For example, the basal concentration of IL-6 was increased from 14.9 pg/10³ cells to 106.04 pg/10³ cells (Figure 12A) and of IL-8 from 3.97 pg/10³ cells to 67.42 pg/10³ cells (Figure 12B). Extension of the time period to 48 hours resulted in decrease of cytokine production. Thus, 15 hours were established as incubation period, also to avoid possible secondary regulatory mechanisms after longer incubation periods.

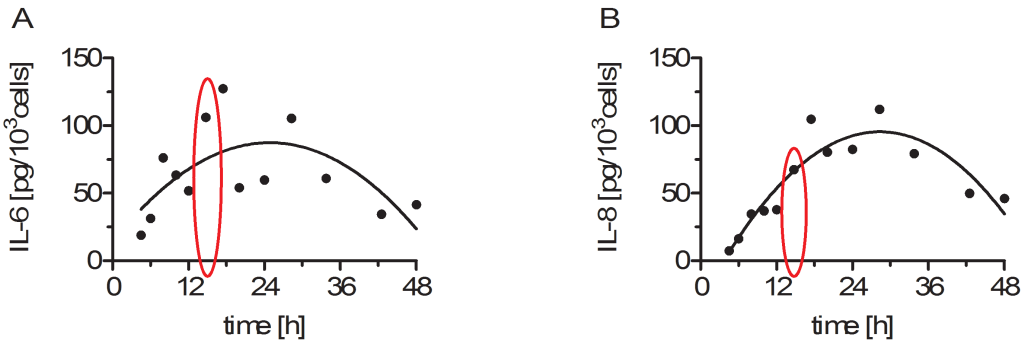


Figure 12: Time dependency of visfatin effects on RASFs. Time-dependent responses of cytokine production by RASFs are demonstrated. RASFs were incubated with a total concentration of 100 ng/ml visfatin. Increasing the stimulation intervals led to a decrease in cytokine production of (**A**), IL-6 and (**B**), IL-8, presumably due to secondary regulatory mechanisms or resistance to stimulation. The established stimulation time is marked in red.

5.2.5 Confirmation of visfatin-regulated genes by real time-PCR

After establishing standard conditions for the RASF stimulation experiments with visfatin (dose-dependency and time-dependency), confirmation of the Affymetrix results could be performed. With regard to the strong induction of chemokines, a focus was set on confirming most of them by a quantitative method. Real time-PCR was selected to quantify the fold-inductions of the following chemokines: CXC-family members CXCL-1, -2, -3, -5, -6 and CC-family members CCL-2 and -13 (n = 7-9, Table 12). The results verified the impressive up-regulation of genes coding for chemokines as seen in the Affymetrix gene chip analysis. The well-known high biological variability between cell populations of different patients was also evident in this experiment, thus reflecting the heterogeneity of human RA (see chapter 5.2.8). Of note, different regulatory factors for each chemokine were documented for the non-quantitative Affymetrix array and the quantitative real time-PCR due to the different concentrations of visfatin used in each experiment.

Table 12: Change in the gene expression profile of RASFs after stimulation with visfatin: Selection of genes regulated by visfatin.

Gene Name	Gene Symbol	Fold Change AFFX	Fold Change Real-Time	Fold Change ELISA
Chemokines				
chemokine (C-C motif) ligand 20 (CCL20)	MIP-3α	1024.00		28.97±11.5
chemokine (C-C motif) ligand 5 (CCL5)	RANTES	473.71		30.56±13.79
chemokine (C-X-C motif) ligand 8 (CXCL8)	IL-8	164.03		51.29±13.79*
chemokine (C-X-C motif) ligand 1 (CXCL-1)	GRO-α	90.51	17.21±4.69**	18.28±8.05
chemokine (C-X-C motif) ligand 3 (CXCL3)	GRO-γ	51.98	14.77±2.82**	
chemokine (C-X-C motif) ligand 5 (CXCL5)	ENA-78	22.63	14.32±2.79**	6.39±1.22*
chemokine (C-X-C motif) ligand 6 (CXCL6)	GCP-2	18.38	114.82±35.6*	12.88±3.26*
chemokine (C-X-C motif) ligand 2 (CXCL2)	GRO-β	17.96	10.95±2.72**	
chemokine (C-C motif) ligand 13 (CCL13)	MCP-4	13.93	6.96±1.49**	
chemokine (C-C motif) ligand 2 (CCL2)	MCP-1	13.93	4.83±1.37*	3.29±0.95
Cytokines				
interleukin 6	IL6	10.56		6.07±0.94*
Proteinases & Peptidases				
matrix metallopeptidase 3	MMP3	59.71		3.63±0.77*
matrix metallopeptidase 1	MMP1	16.00		1.19±0.14

Data are presented as mean value ± SEM; * = <0.05; ** = <0.01

5.2.6 Expression analysis of proteins regulated by visfatin in RASFs

The expression of a broad variety of different cytokines, chemokines and MMPs was regulated in RASFs in response to stimulation with visfatin. Since the observed effects pertained to the RNA level, further investigations were performed to prove that they were actually translated to the protein level. For this purpose, protein arrays were carried out.

Up-regulation of protein expression in the chemokine array was observed for IL-8, CXCL-10 and CCL-2 (10.13-, 3.41- and 4.23-fold; n = 2). In the custom-cytokine array, inductions of IL-8 and IL-6 (6.8- and 5.6-fold; n = 2) could be seen. Neither in the chemokine array nor in the custom-cytokine array, any down-regulations of greater than 2-fold were observed. High inter-assay variations were seen due to either strong differences between cell populations of distinct patients (see chapter 5.2.8) or uncertain results of the protein array itself. As the reliability of the test was not sufficient, ELISAs, specific for the individual target proteins, had to be performed.

5.2.7 Confirmation of visfatin regulated proteins by ELISA

Since the results of the protein array were not sufficiently reliable, ELISA was chosen to identify and to quantify changes in the protein secretion of RASFs. Induction of pro-inflammatory factors, matrix-degrading molecules and most prominently chemokinetic and chemoattractive cytokines could be validated. In detail, confirmation of up-regulation could be observed for IL-6 and MMP-3 as well as for CXCL-1, -5, -6, -8, and CCL-2, -5, -20 (n = 4, Table 12, Figure 13). Significant regulations were seen for IL-6, -8, MMP-3, and CXCL-5, and -6 (p-value <0.05). The illustrated changes in protein expression of CXCL-1, CCL-2, -5, and -20 were not statistically significant, but there was consistent up-regulation in all samples examined. Significance could not be reached due to high variability of each sample (see chapter 5.2.8). No regulatory effect was found for pro-MMP-1, activin A, OPG, IGFBP-2 and -3 and TIMP-1 and -2. The levels of TNF-α and IL-1β were below the detection limit and thus no further evaluation was performed. The results confirm in part that visfatin stimulated changes in gene expression of RASFs leads to increased protein production of mostly chemokines, but also pro-inflammatory cytokines in comparison to unstimulated RASFs.

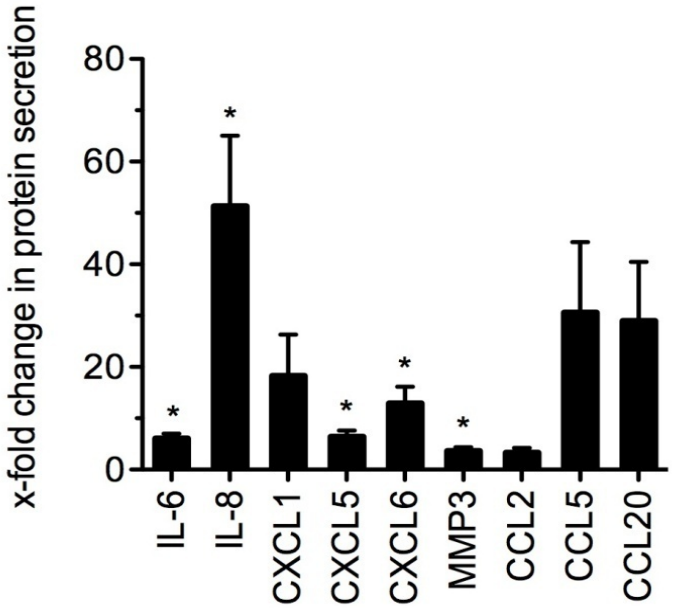


Figure 13: Regulation of protein expression in RASFs by visfatin. The bar diagram demonstrates the fold-change induction for each named protein after stimulation of RASF with 100 ng/ml visfatin. Data is presented as mean ± SEM. Asterisk (*) indicates significant results (p<0.05).

5.2.8 Interindividual differences of RASFs and OASFs regarding gene and protein expression levels

Throughout the experiments it could be observed that high interindividual differences of expression levels exist between fibroblasts obtained from different patients with RA. These differences were measurable on the protein as well as on the RNA levels (Figure 14). In the evaluations performed, x-fold changes in stimulated *versus* unstimulated fibroblasts were calculated and overall regulation was expressed as mean value (± SEM). This explains why the error bars representing the SEM may be relatively pronounced. Of note, however, the single values are all pointing into the same (either positive or negative) direction as can be seen in Figure 14. OASFs obtained from different patients behave similarly (Figure 14A).

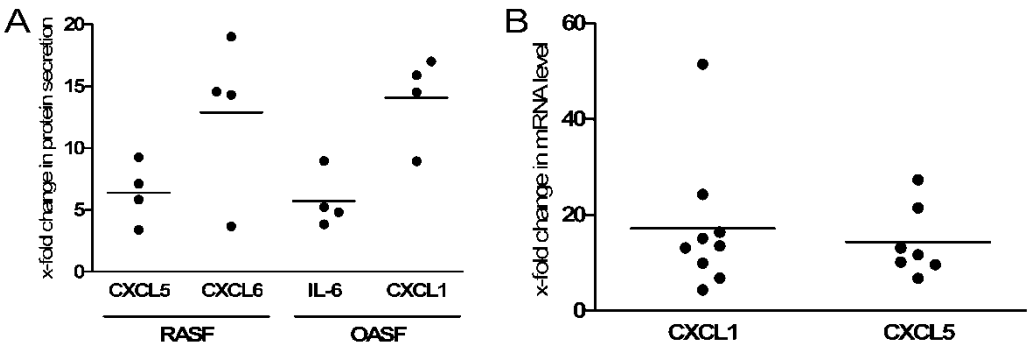


Figure 14: Interindividual differences of RASFs and OASFs regarding gene and protein expression. The grouped column scatters display the differences of RASFs and OASFs obtained from different RA and OA patients with regard to (**A**), protein secretion of CXCL5 and CXCL6 from RASFs and IL-6 and CXCL1 from OASFs (n = 4), and (**B**), mRNA level of CXCL1 and CXCL5 from RASFs (n = 7-9). Each cell population is represented by a dot. Horizontal lines demonstrate the mean values.

5.2.9 Effects of specific signal transduction inhibitors on visfatin-mediated IL-6 and CCL-2 induction

Several signal transduction pathways (p38 MAPK[86,146], PKA[147], PKC[148,149] and NF-κB[93]) are known to be connected with pro-inflammatory cytokine (IL-6) and chemokine (CCL-2) production in RASFs. Therefore, inhibition of these pathways by specific chemical inhibitors was performed to identify the signaling pathways, which are activated in RASFs in response to visfatin stimulation.

Incubation with the p38 MAPK inhibitor at the final concentration of 20 µM for 15 hours resulted in pronounced reduction of the visfatin-mediated production of IL-6 and CCL-2 by RASFs. The expression of IL-6 was reduced by 66% from 439.3 ± 103.09 pg/10³ cells to 150.4 ± 50.31 pg/10³ cells (n = 3) in comparison to visfatin-stimulated RASFs without p38 MAPK inhibitor (Figure 15A). The expression of CCL-2 was reduced by 28% from 463.4 ± 54.46 pg/10³ cells to 341.0 ± 86.15 pg/10³ cells (n = 3; Figure 15B).

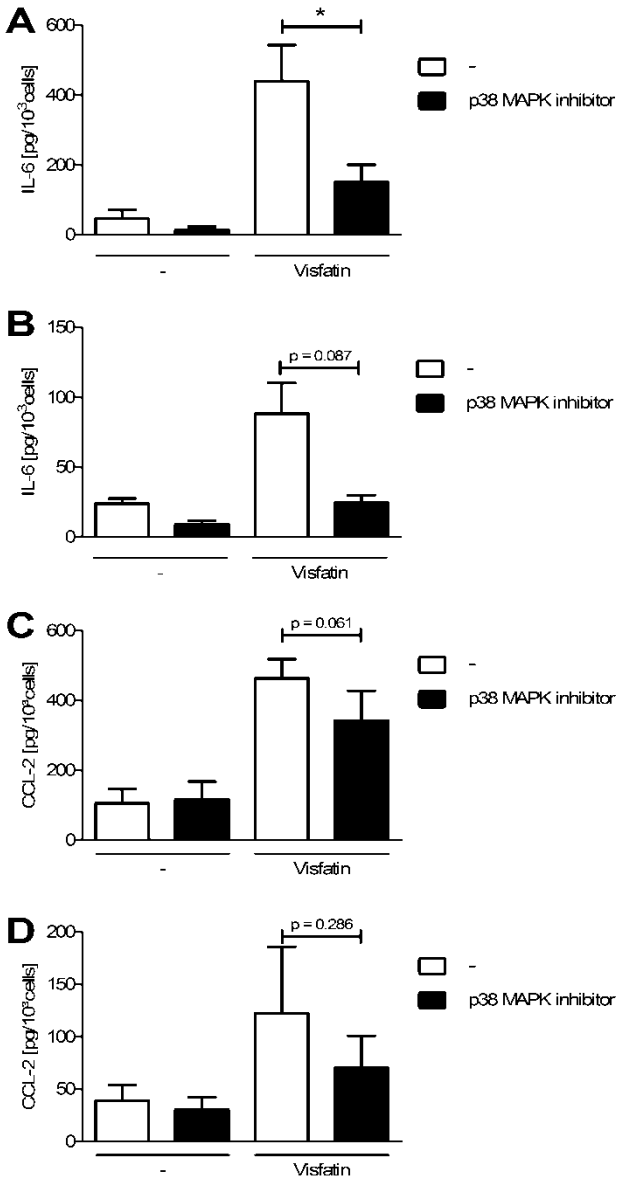
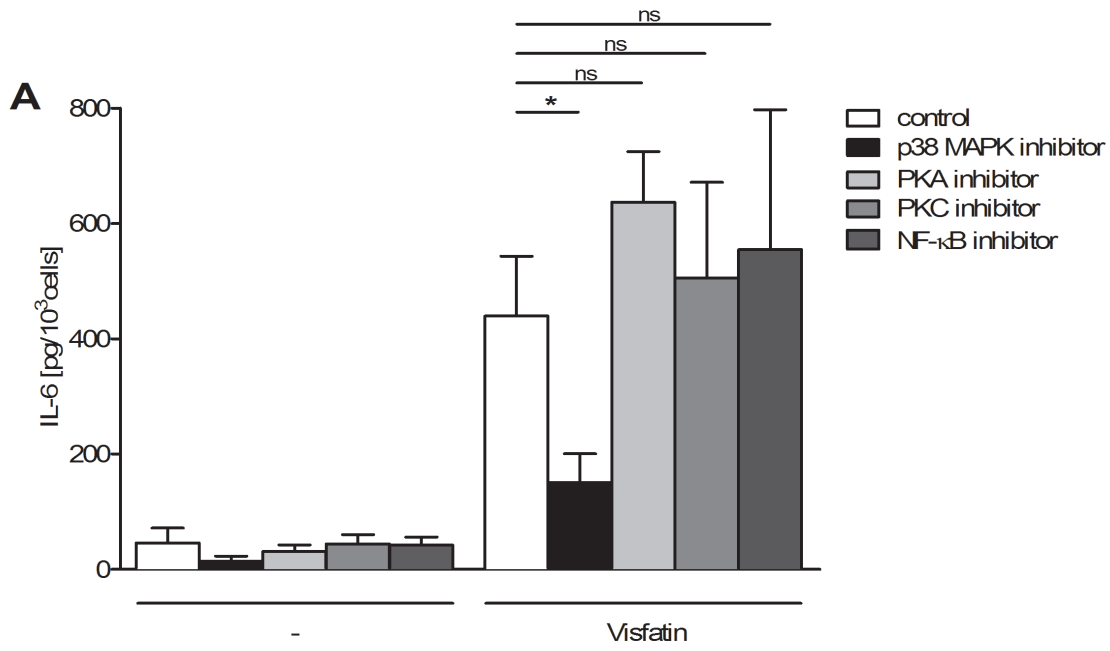


Figure 15: Effects of the inhibition of the intracellular signaling pathway p38 MAPK on visfatin-induced expression of IL-6 and CCL-2 by RASFs. The bar diagrams show the levels of the respective cytokine. Black bars indicate the presence of the chemical inhibitor. Data presented as means ± SEM. (**A+C**), cytokine production after 15 hours of stimulation. (**B+D**), cytokine production after 6 hours of stimulation and 1½ hours of pre-incubation with the p38 MAPK inhibitor.

Evaluation of the same setting was performed with a pre-incubation period of the p38 MAPK pathway inhibitor of one and a half hours followed by a co-incubation period of visfatin and the inhibitor for six hours. This was done to exclude time-dependent effects of p38 MAPK salvage due to the chemical inhibition. In keeping with the previous experiment, the expression of IL-6 was reduced by 72% from 88.2 ± 38.4 pg/10³ cells to 24.5 ± 9.2 pg/10³ cells (n = 3) in comparison to visfatin-stimulated RASFs without p38 MAPK inhibitor (Figure 15C). The levels of CCL-2 were reduced by 57% from 122.5 ± 110 pg/10³ cells to 70.3 ± 52.6 pg/10³ cells (n = 3; Figure 15D), but high individual levels were observed in the samples.

In contrast, the other inhibitors (PKC inhibitor 20-28, PKA inhibitor 14-22, NK-κB activation inhibitor) did not induce any relevant change in IL-6 or CCL-2 production (Figure 16).



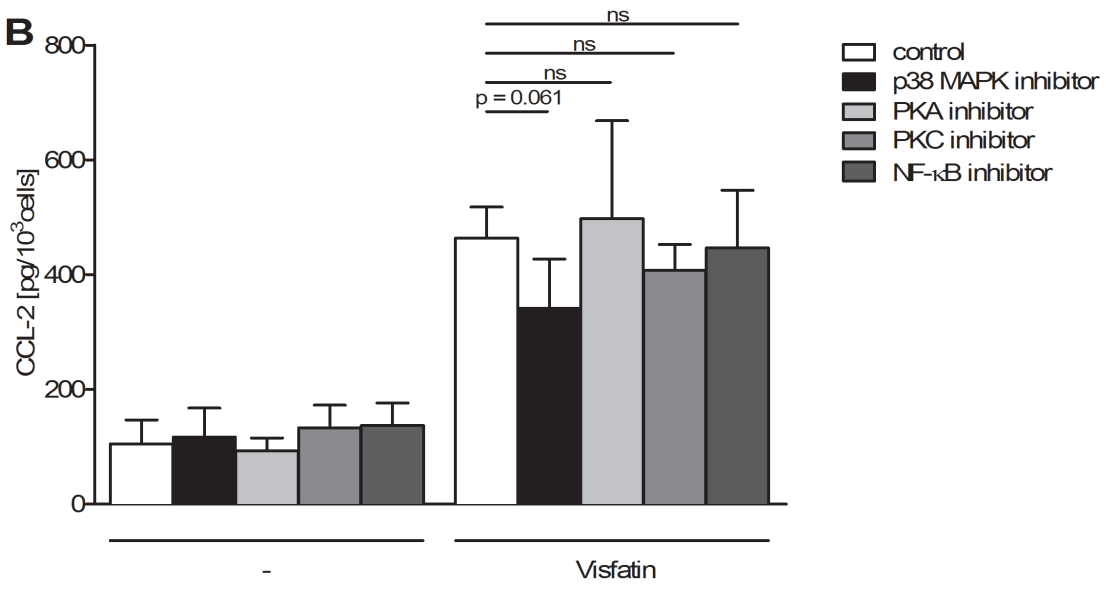


Figure 16: Effects of the inhibition of the intracellular signaling pathways of p38 MAPK, PKA, PKC and NF-κB on visfatin-induced expression of IL-6 and CCL-2 by RASFs. The bar diagrams show the levels of the respective cytokine. Legend indicates which bar represents the presence of which chemical inhibitor. Data presented as means ± SEM. (**A**), IL-6 production after 15 hours of stimulation. (**B**), CCL-2 production after 15 hours of stimulation. A pre-incubation of 1½ hours was performed with each of the chemical inhibitors. Asterisk (*) indicates significant results ($p<0.05$). ns, not significant.

5.2.10 Phosphorylation of p38 MAPK induced by visfatin

Inhibition of the p38 MAPK signaling pathway by using a chemical inhibitor of p38 MAPK led to a reduction of the visfatin-induced production of IL-6 and CCL-2 by RASFs. In order to verify whether visfatin signals *via* this pathway or whether the observed effects were due to secondary stimulatory effects after longer incubation periods, Western blot analysis of p38 MAPK phosphorylation was performed.

After exposure of RASF to visfatin for 5 minutes, an elevation of the basal phosphorylation of p38 MAPK could be observed (Figure 17). This indicated an activation of the p38 MAPK pathway in RASFs by visfatin stimulation.

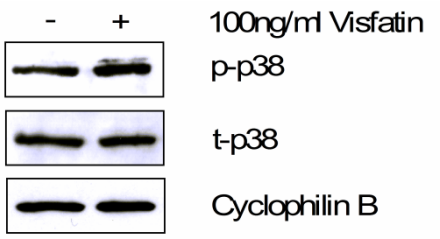


Figure 17: Analysis of visfatin-mediated p38 MAPK pathway activation by Western blotting. The signals demonstrate chemiluminescence of Cyclophilin B, total p38 MAPK (t-p38) and phosphorylated p38 MAPK (p-p38) prior to (-) and 5 minutes after (+) visfatin stimulation (100 ng/ml).

5.2.11 Effects of visfatin on cell migration of RASFs and lymphocytes

Recently, it was shown that RASFs have the potential to leave the synovium and migrate to other joints.[38] Therefore, it is of interest whether visfatin may contribute to this process. A chemotaxis assay using a Boyden microchemotaxis chamber was performed to evaluate whether stimulation of RASFs with visfatin creates a cytokine environment that increases directed cell motility of fibroblasts and lymphocytes. Besides using different cell populations (n = 2), experimental replicates were performed as well (n = 3).

As shown in 5.2.6, stimulation of RASFs with visfatin leads to increased levels of chemokines, e.g. CXCL-1, -5, -6 or CCL-2, -5 and -20. In 5.2.9, inhibition of the p38 MAPK signaling pathway in the presence of visfatin resulted in decreased levels of chemokines. Migration towards media containing visfatin-induced factors could be detected for fibroblasts and lymphocytes, and migratory activity could be reduced back to baseline levels by blocking the signal transduction of p38 MAPK. As shown in figure 18A, the migration index (MI) for RASFs was increased by visfatin-induced factors to 2.73 ± 0.45 and reduced by inhibition of the p38 MAPK pathway to 1.08 ± 0.59 (60% reduction). The p38 MAPK inhibitor itself reduced the migratory capacity of RASFs to some extent (MI: 0.39 ± 0.31).

When lymphocyte migration was analyzed, a similar increase in migration of and a reduction with the p38 MAPK inhibitor was observed in accordance with the effects of visfatin-induced factors on RASFs (Figure 18B). In order to exclude direct effects of visfatin on cellular migration, the cells were incubated with 100 ng/ml visfatin.

However, as shown in figure 18C, visfatin-induced lymphocyte migration can be neglected.

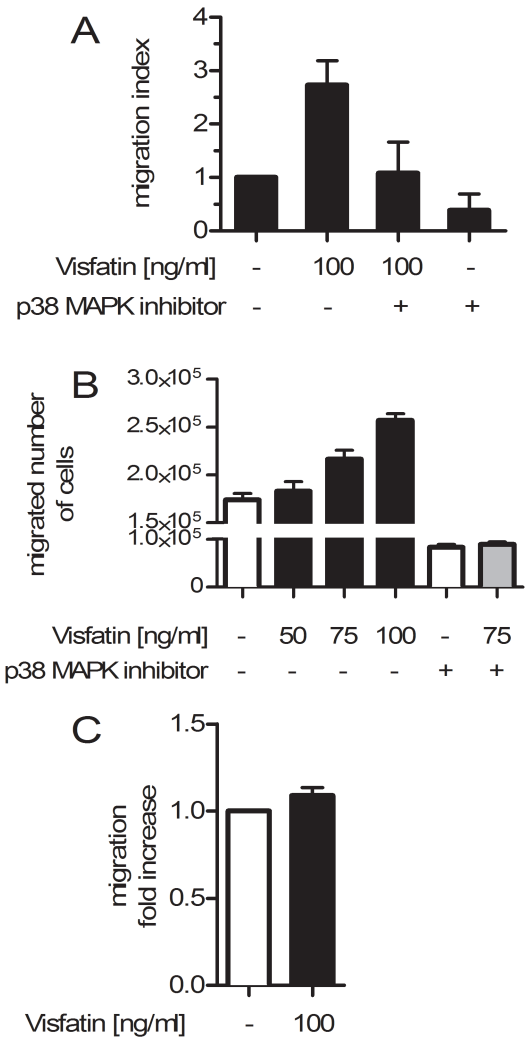


Figure 18: Effects of visfatin on cell migration of RASFs and lymphocytes. (**A**), The bar diagram demonstrates the migration index for RASFs. The marking of the x-axis indicates whether RASFs were migrating towards baseline media (visfatin (-), p38 MAPK (-)) or stimulated media as indicated by the labeling of the x-axis (see 4.11.1). (**B**), The bar diagram shows the number of migrated lymphocytes towards baseline and stimulated media as indicated by the labeling of the x-axis. (**C**), The bar diagram shows the migration index for lymphocytes to baseline and to media containing 100 ng/ml of visfatin. Data presented as mean ± SEM.

5.2.12 Evaluation of changes in RASF cell motility induced by visfatin

A scrape assay was performed to evaluate whether visfatin exerts effects on the undirected cell motility of SFs. RASFs were incubated with increasing concentrations of visfatin (5, 25, 50, 100, 250 ng/ml) and the filling of the scraped gap at defined areas

was observed over time (n = 3). In order to minimize confounding by cell proliferation, serum free-media was used. Figure 19 gives an example for increased cell motility of RASFs in the presence of visfatin compared to the negative control.

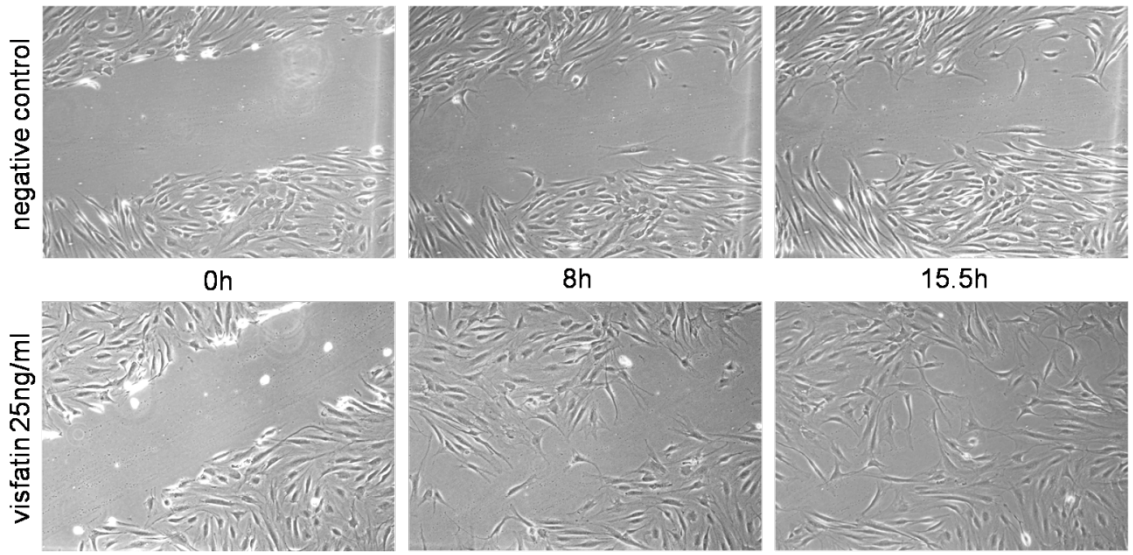


Figure 19: Increased RASF motility after visfatin stimulation. The pictures demonstrate the scraped gap in a confluent RASF cell culture at the beginning of the assay, after 8h and after 15.5h in the absence (negative control) or presence of visfatin (25ng/ml). It is clearly visible that the gap is filled over time by motile RASFs and that visfatin can enhance this undirected fibroblast motility.

Increased cell motility was observed for concentrations of visfatin of 5, 25 and 50 ng/ml. Increase of cell motility was strongest at the visfatin concentration of 25 ng/ml. Data was expressed as the ratio of cell motility of the sample compared to baseline (see Methods 4.11). Significant, enhanced cell motility was found for visfatin at concentrations of 25 ng/ml after 8 hours (13.28±2.69%; p=0.039), 11 hours (14.1±2.99%; p=0.042), and 15.5 hours (18.4±3.56%; p=0.036), and of 50 ng/ml after 8 hours (11.67±1.42%; p=0.014; Figure 20). Decreased or unchanged fibroblast motility at earlier time points of the assay was observed for the applied visfatin concentrations of 100 and 250 ng/ml. Of note, cell motility declined at the latest time points of the assay due to complete filling of the gap. Interestingly, no significant increase of cell motility was found for visfatin at a concentration of 100 ng/ml after 15 hours, which were the conditions used for the chemotaxis assay.

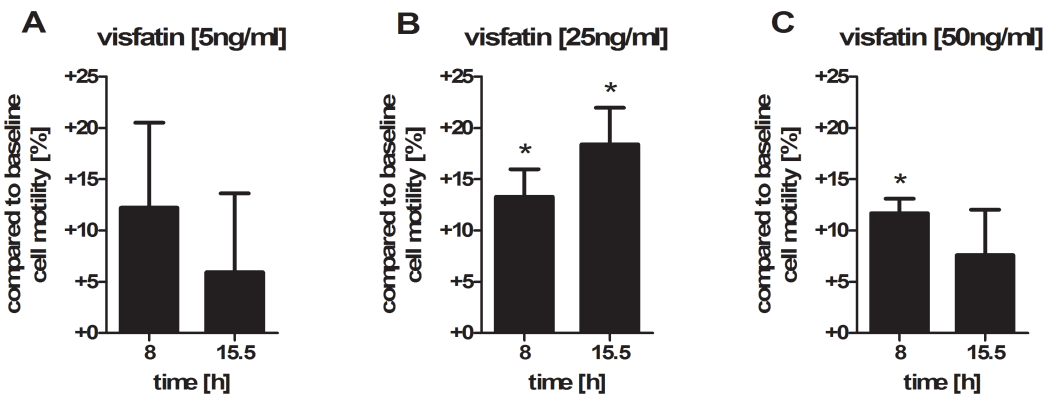


Figure 20: Evaluation of changes in RASF cell motility by visfatin. Observed changes are expressed as the ratio of cell motility of the sample compared to baseline. Visfatin concentrations: (**A**), 5ng/ml. (**B**), 25 ng/ml. (**C**), 50 ng/ml. Data presented as mean ± SEM. Asterisks (*) denote significant changes compared to baseline ($p<0.05$).

5.3 Influence of leptin on synovial fibroblasts

5.3.1 Expression analysis of genes regulated by leptin in RASFs

Analogous to the expression analysis of genes regulated by visfatin in RASFs, potential regulatory effects of leptin on gene transcription in RASFs were evaluated.

Expression of several genes was altered by leptin (Table 13). Amongst them, distinct chemokines such as CCL-7 and CXCL-9 were upregulated, whereas others including CXCL-5 and -10 were down-regulated. Anti-inflammatory cytokines such as IL-11 or IL-1Ra were also regulated differentially with IL-11 being increased and IL-1Ra being reduced. Furthermore, matrix-degrading enzymes and signaling molecules (MMP-10, -12 and STAT-2) were increased to some extent while the growth factor FGF-1 was decreased by leptin stimulation. Induction of CXCL11 was excluded from the gene chip analysis due to the high variability between multiple spots. In comparison to the gene expression analysis of RASFs stimulated by visfatin, the observed regulatory effects were not consistent, less pronounced, and affected a smaller number of genes.

Table 13: Leptin-regulated genes in RASFs. The selection was made with focuses on genes, which are known to be involved in RA pathophysiology.

Change in gene expression of RASFs after stimulation with leptin: Selection of genes regulated by leptin		
Gene Name	**Gene Symbol**	**Fold Change**
Chemokines		
chemokine (C-C motif) ligand 7	CCL-7	3.252
chemokine (C-X-C motif) ligand 9	CXCL9	2.616
chemokine (C-X-C motif) ligand 5	CXCL5	0.393
chemokine (C-X-C motif) ligand 10	CXCL10	0.331
Cytokines		
interleukin 11	IL11	3.929
interleukin 1 receptor antagonist	IL1Ra	0.257
Growth Factors		
fibroblast growth factor 1	FGF1	0.49
Proteinases & Peptidases		
matrix metallopeptidase 10	MMP10	3.343
matrix metallopeptidase 12	MMP12	2.44
Signaling molecules		
signal transducer and activator of transcription 2	STAT2	2.375

5.3.2 Confirmation of leptin-regulated proteins by ELISA

In the gene chip expression analysis, a few genes of interest were either slightly increased or decreased by leptin stimulation but the overall change in the gene expression pattern was of low significance. To further evaluate possible inductions of cytokines with key functions in the pathophysiology of RA, changes in protein expression were measured by ELISA.

IL-1β, -6 and -8 as well as TNF-α and MMP-3 could not be evaluated, because signals were below detection levels (n = 4). Thus, pivotal cytokines known to be involved in pro-inflammatory mechanisms of synovial pathology were not regulated in RASFs by leptin stimulation.

5.4 Differences between RASFs and OASFs regarding protein expression levels

Recent studies have shown that the induction of protein expression mediated by adiponectin is stronger in RASFs than in OASFs.[145] Hence, it was of interest to

elucidate potential differences in visfatin-induced protein expression by RASFs in comparison to OASFs.

The results of this comparison were, however, variable. Some factors such as CXCL-1 showed stronger expression in RASFs than in OASFs, whereas other such as MMP-3 were more prominent in the supernatants of OASFs compared to RASFs after visfatin stimulation. Overall, effects of visfatin on either RASFs or OASFs were comparable and the respective changes in protein secretion were not significantly different between RASFs and OASFs for any of the parameters (IL-6, -8, CXCL-1, -5, -6, CCL-2, MMP-1, -3, activin A, OPG, IGFBP-2, -3, TIMP-1 and-2; n = 4; Figure 21).

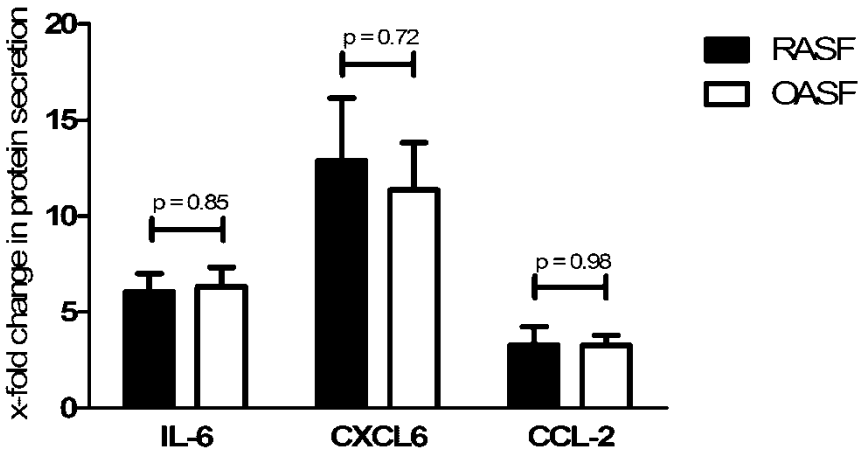


Figure 21: Comparison of protein expression levels of RASFs and OASFs in response to visfatin. IL-6, CXCL-6 and CCL-2 are shown as representative examples. The bars represent the mean ± SD. No significant differences between RASFs and OASFs could be observed.

6 DISCUSSION

In this work, the potential of visfatin and leptin to act as effector molecules in RA was investigated. The significant changes in gene expression of RASFs mediated by visfatin, especially the induction of a variety of chemokines and moreover proinflammatory and matrix-degrading factors, could be confirmed on the RNA and protein levels. This supports the hypothesis that visfatin contributes to the inflammatory molecular environment in RA and to the increased fibroblast and leukocyte motility within RA synovial tissue. In comparison to visfatin, the changes in gene expression of RASFs induced by leptin were less prominent supporting the idea of a low or absent proinflammatory role of leptin in RA synovial pathophysiology with respect to RASFs.

Synovial fluid is usually obtained from patients with RA and OA presenting with joint effusions. Levels of proinflammatory cytokines in RA synovial fluid can therefore be elevated compared to healthy controls, and usually also when compared to OA. In this study, visfatin was detectable in a significant amount not only in RA, but also in OA synovial fluid (Figure 8). Taking into account that effusions in OA generally resemble an activated or inflammatory status of the joint, it is reasonable that visfatin is present as a marker of inflammation. This is also supported by the fact that stimulation of OASFs by visfatin leads to an increase of proinflammatory cytokine levels comparable to those of stimulated RASFs (Figure 21). Similarly, it has been shown that OASF react to the adipokine adiponectin as well, although to a lesser extent than RASFs.[142,145] Thus, SFs from joints affected by either OA or by RA seem to react to proinflammatory stimuli in a similar way. Other studies, in contrast, report higher visfatin levels in RA compared to OA.[93] This might be caused by different study populations. In this regard, it is of interest that long-term disease activity may affect the degree of visfatin production (Figure 8). In contrast to leptin, visfatin production is independent of adipose tissue, but correlates to markers of inflammation or disease activity scores such as CRP, ESR, or DAS28 which is in accordance with results of published studies.[93,150]

Inflammation of the synovium is a hallmark of RA and activated RASFs play a pivotal role in local pathophysiological mechanisms. While the normal synovial lining is made up of 1-3 cell layers, this architecture is severely altered in RA. Predominantly RASFs and macrophages increase the lining thickness to 10-15 layers.[55] The structural change

of the synovial membrane could therefore be the primary step in disease onset. In the inflamed synovium of RA patients, accumulation of visfatin was most intense in the *"lining layer"* as well as at the sites of cartilage invasion[93] as shown by immunohistochemistry (Figure 10). Hence, it was of interest whether visfatin exerts effects on cell motility or influences cell migration within the synovium either as a chemoattractant or indirectly by creating an environment of high chemokine levels. If so, visfatin would contribute to the conspicuous accumulation of RASFs in the synovium and at the cartilage invasion zone. Besides, hypoxia or proinflammatory molecules, both highly effective stimuli for visfatin induction, contribute further to the strong visfatin expression.[151,152] A combined effect appears likely.

In healthy joints, SFs contribute to tissue repair and support wound healing. Therefore, they inherit a motile phenotype which is increased in RA as demonstrated by their potential to leave the synovium.[38] Although they have been regarded to act only as resident cells, recent research has demonstrated that RASFs have the potential to leave their compartment and migrate to other joints.[38] In this study, visfatin showed the potential to contribute to this phenotype of RASFs as the cell motility assay revealed increased cellular motility in the presence of visfatin (Figure 20). In terms of RA synovial pathophysiology, visfatin could further trigger or at least contribute to the tumor-like growth of the synovial tissue over the underlying cartilage. RA has been referred to as a *"locally invasive tumor"*, an observation based on several aggressive characteristics of RA synovium reminiscent of neoplastic tissue.[11] These include defective contact inhibition of RASFs and their overexpression of embryonic genes. In addition, similar to neoplastic disorders, neo-angiogenesis providing the blood supply for the ever growing hyperplastic synovial tissue can also be found.[153–155] Mutations in key genes are also discussed, for instance p53 tumor suppressor gene alteration or microsatellite instability.[156–159] Moreover, the potential of RASFs to drive the progression of disease from oligo- to polyarticular manifestation is reminiscent of a metastatic distribution.[38]

The *in vitro* results of this study show that visfatin promotes RASF motility and induces pro-inflammatory and pro-destructive molecules. Whether visfatin is thus a major factor for inducing the development of RASFs to their aggressive phenotype *in vivo* or

whether it is a co-factor, which further aggravates that development is speculative at the moment.

TLRs, receptors activated *via* pattern recognition, can bind microbial components but also endogenous molecules resulting in the production of cytokines, chemokines and MMPs. TLR-3 for example is overexpressed in RA synovial tissue and release of RNA by necrotic synovial fluid cells serves as an endogenous ligand to that receptor which is also expressed by RASFs.[53,160] Poly(I-C), structurally similar to double-stranded RNA, can mimic this effect, acting as an immunostimulant. TLR-3 activation by poly(I-C) leads to an upregulation of visfatin in RASFs.[93] The results of the Affymetrix array further underlined the role of this specific receptor in visfatin pathophysiology. An upregulation of TLR-3 by visfatin stimulation of RASFs could be observed (Table 11) suggesting a positive feedback loop. Furthermore, abundant levels of IL-6 can mediate STAT-3 activation *via* trans-signaling in RASFs, leading to increased visfatin synthesis. Taken together, these findings establish a role for visfatin as a new marker of inflammation in RA.[93]

The lining layer contains high numbers of activated RASFs characterized by a high basal production of IL-6 and MMPs advancing chronic inflammatory responses and contributing to T-cell and B-cell activation and progressive cartilage destruction.[161] Visfatin enhances these effects by increasing the production of IL-6 and MMP-3 and thus intensifies the aggressive phenotype of RASFs (Table 12). This is of interest as IL-6 induces visfatin as well, suggesting a positive feedback loop between these two molecules. Further *in vitro* studies will have to prove whether inhibition of IL-6 trans-signaling by therapeutic antibodies such as tocilizumab, a so-called *"biologic response modifier"* approved for RA therapy, can interrupt this positive feedback regulation.[162] This would then be another explanation for the therapeutic efficacy of tocilizumab.

With regard to MMP induction, previous studies have revealed visfatin-mediated induction of both MMP-1 and MMP-3 mRNA levels.[93] The results of the Affymetrix array in this study also suggested an increased production of MMP-10, -12, and -19 (Table 11), whereas the upregulation of MMP-1 could not be confirmed at the protein level suggesting that increased expression of mRNA is not translated into significant protein synthesis, possibly due to posttranscriptional or posttranslational processes.

Nevertheless, the data of this study strongly indicate that visfatin promotes cartilage destruction *via* MMP induction in RASFs (Table 12).

Looking more closely at visfatin-induced alterations of the gene expression profile of RASFs, a strong up-regulation of a broad variety of chemokines of both, the CXC- and the CC-cluster was observed suggesting that visfatin mediates chemoattraction in RA synovium to a significant extent (Table 11). The results of the Affymetrix array, highlighting this broad induction, were confirmed on the RNA and on protein levels (Table 12). Chemoattraction plays a key role in RA synovial pathophysiology for the initiation, aggravation and perpetuation of local inflammation. Attracting immune cells to the synovium is a central mechanism of disease development and chemokines of the CXC, CC, and fractalkine families seem to be of great importance in this respect.[163] Of particular interest, chemokines do not only act as chemoattractants but are also involved in synovial neovascularization thus supporting inflammation in several ways.

Accelerated angiogenesis leads to an increase in the total endothelial surface, thus contributing to the intense influx of inflammatory cells. This cell ingress further drives the inflammatory process in RA synovium and activated interstitial vessels play a major role in the extravasation of leukocytes. Of note, increased expression of visfatin was observed around interstitial vessels as well as in lymphoid aggregates (Figure 10). This finding further supports the idea that on the one hand visfatin production is driven locally by synovial cells, particularly RASFs, lymphocytes and endothelial cells, and on the other hand these cells are activated by increased levels of visfatin expression in an autocrine and paracrine fashion (Figure 10).[152,164,165] In addition, a marked induction of factors with strong pro-angiogenic effects, namely IL-8, CXCL-1 and -5, VEGF, and ECGF, could be observed.[163,166,167] It is therefore likely that visfatin also promotes RA synovial angiogenesis to a certain extent. This notion is in keeping with other studies which have demonstrated activation of ERK-1/-2 pathways and an increase of FGF-2 in human endothelial cells in response to visfatin.[163,168–170] Moreover, visfatin increases the expression of adhesion molecules like VCAM-1, ICAM-1 and -2 by RASFs thus facilitating not only attachment of RASFs to cartilage but also RASF migration.[33]

Effective chemoattraction requires enhanced cellular motility. Our results support the idea that visfatin operates as a cell motility increasing molecule for RASFs *in vitro* similar to what has been shown for $CD14^+$ monocytes and $CD19^+$ B cells under

different pathophysiological conditions.[86] The visfatin-induced cytokine environment exerts chemoattractive properties promoting directed fibroblast motility towards the site of visfatin expression (Figure 18). Furthermore, the visfatin-induced cytokine environment contributes to lymphocyte recruitment (Figure 18). This supports the idea that visfatin facilitates the accumulation of RASF at sites of cartilage invasion[33,171] and the aggregation of lymphocytes in inflammatory infiltrates within the synovium resulting in increased destruction and remodeling of articular cartilage (Figure 10).

Due to the fact that IL-6 and MMP-1 production is closely linked to PKC and p38MAPK activation[148,146,149,172–174] and that initiation of common signaling pathways such as PKA and NF-κB result in inflammatory responses of RASFs[148,147], investigation of those pathways was performed to clarify whether the observed effects were mediated by them. Although it has been shown that visfatin activates the NF-κB pathway in RASFs[93], the only detectable, significant effects in this study were seen for inhibition of the p38MAPK pathway (Figure 15, 16). The involvement of this pathway in visfatin signaling could be confirmed by detection of p38 MAPK phosphorylation directly after stimulation (Figure 17). The same pathway was also described to be required for the activation of monocytes by visfatin, supporting a strong connection between visfatin and p38 MAPK signaling.[86] Of interest, inhibition of p38MAPK in animal models of arthritis leads to improvement of the disease severity, thus reflecting its central role in arthritis.[175,176]

There is an ongoing discussion about the way by which visfatin exerts its biologic effects on cells. It has been described that visfatin can bind to the insulin receptor mimicking insulin effects but does not share the same binding site with insulin.[73] This is supported by the finding that inhibition of insulin receptor activity abrogates the effects of visfatin in chondrocytes.[177] Previous studies further revealed that visfatin also functions as an intra- and extracellular enzyme called NAMPT, a key enzyme in the salvage pathway of NAD^+ and therefore an important molecule in regulating energy homeostasis and apoptosis of cells.[178–181] Recently, promising results have been reported for the inhibition of this enzymatic function using the specific inhibitor APO866 in the animal model of collagen-induced arthritis (CIA) in two independent studies.[94,95] NAMPT inhibition with APO866 ameliorated established CIA and reduced the production of proinflammatory cytokines in human PBMCs treated with LPS. The

effects were closely linked to intracellular NAD levels and were reversed by NMN supplementation suggesting that the enzymatic activity of NAMPT may cause the inflammatory cell reactions. The authors compared the effect of APO866 to TNF-α blockade by etanercept and demonstrated similar efficacy.[94] In an independent study, it could be demonstrated that the same selective NAMPT inhibition led to a suppression of leukocyte infiltration and cartilage degradation in CIA.[95] Interestingly, these authors could show the same effects of visfatin by inhibiting its function *in vivo* as the work of the present study does *in vitro*. Blocking of the enzymatic activity of visfatin by APO866 resulted in suppression of leukocyte infiltration into the inflamed synovium of arthritic joints and of cartilage destruction mediated by MMP-3 and -13.[95] However, it remains unclear, whether the effects of visfatin/PBEF are only due to its enzymatic activity or if the effects are also dependent on binding to the insulin receptor or to an as yet unidentified receptor. Involvement of the insulin receptor is indirectly proven, because inhibition of its activity led to amelioration of visfatin-mediated effects and possible insulin receptor-dependent downstream activation of ERK-1/-2 has been demonstrated in response to visfatin.[86,169,177] In contrast, the observed activation of the p38 MAPK pathway remains to be explained, because neither insulin receptor signaling nor the NAD^+ salvage pathway include phosphorylation of p38 MAPK (Figure 17), thus the possibility of an yet unidentified receptor is reasonable.[94,95]

Of interest, APO866 and inhibitors of p38MAPK are the subject of phase II clinical studies. APO866 is currently tested in melanoma, B-cell chronic lymphocytic leukemia, and cutaneous T-cell lymphoma (http://www.clinicaltrials.gov; NCT00432107, NCT00435084, NCT00431912). Different inhibitors of p38MAPK have already been tested in phase II clinical trials of active RA reporting disappointing results.[182–184] The p38MAPK inhibitors, namely pamapimod, SCIO-469, and VX-702, though well-tolerated, either failed to show greater efficacy than placebo or were less effective than methotrexate. Compared to the results from pre-clinical studies, there is a discrepancy in terms of efficacy, an observation, which is reported relatively frequently since translation from bench to bedside is not successful in any case.

Leptin, on the contrary, was detectable in the synovial fluid of RA patients, but to a higher extent in OA patients and correlated with the BMI as shown before (Figure 9).[185–188] Even though some authors suggest a proinflammatory role of this molecule in

RA[97,101], the conclusion is controversial as some studies show that increased levels in RA patients compared to healthy controls are associated with reduced levels of radiographic damage.[188] On the other hand, several studies have shown a role of leptin in the pathogenesis of RA. In comparison to plasma levels of leptin in RA patients, the levels in synovial fluid of the same patients have been found to be significantly lower, suggesting an *in situ* consumption of this molecule.[137] However, leptin consumption in inflamed joints is not explained any further. In this study, leptin was found to induce significantly less changes in the gene expression pattern of RASFs in comparison to visfatin. Pivotal proinflammatory cytokines such as IL-1, -6, or TNF-α or matrix degrading enzymes such as MMP-3 were not regulated by leptin (Table 13). In contrast, it has been demonstrated that leptin induces, in synergy with IFN-γ or IL-1, NOS type II activation in chondrocytes, thus leading to increased levels of NO.[118] NO is known to act as a proinflammatory mediator in joint cartilage, where it generates chondrocyte phenotype loss, apoptosis and activation of metalloproteinases.[117] These results are further underlined by the observation that leptin increases the production of IL-8 in human chondrocytes.[189] The same has also been demonstrated for human SFs.[190] However, this result could not be confirmed by the current work. Results concerning leptin and its potential role in the pathogenesis of RA are still controversial and more efforts are needed to clarify whether or not leptin is involved in the cytokine cross-talk of activated immune cells in the inflamed RA synovium and if so, which cells are the corresponding effector cells. In contrast to chondrocytes, this study has shown that RASFs do not significantly react to stimulation by leptin.

The current study focussed on the evaluation of possible effects of visfatin and leptin on RASFs, key cells in local pathophysiology of RA synovial tissue. RASFs were isolated from synovial tissue of RA patients who underwent knee joint replacement surgery. Thus, each synovial sample came from a different patient with an individual disease. While all patients fulfilled the 1987 ACR criteria, their individual disease duration, history of therapies and antibody status (rheumatoid factor or anti-citrullinated peptide antibodies) varied and could not be matched because human specimens are rare, valuable, and unique. Because of the difficulty in obtaining human specimens from a homogeneous population of patients, a possible bias secondary to heterogeneity of the cell populations cannot be excluded. In this study, however, individual rather than experimental replications were preferred. Therefore, x-folds were calculated to interpret

the data. Because of the high inter-individual variability in expression levels of measured cytokines, statistical significance was not achieved in every case, whereas the regulation of the respective cytokine was always significant (Figure 14).

To summarize the present work, visfatin in contrast to leptin appears to be a novel potent effector molecule in the pathophysiology of RA. Visfatin is detectable at key sites of RA synovium as well as in synovial fluid. RASFs strongly react to stimulation by visfatin, thus supporting a microenvironment of increased proinflammatory, matrix-degrading, and neoangiogenic cytokines that further aggravate synovial inflammation. Furthermore, visfatin exerts chemoattraction, which contributes to both the ingress of inflammatory cells into the synovium and the accumulation of RASFs at sites of cartilage invasion. This is supported by the observation that visfatin triggers cell motility. Therefore, visfatin according to recent research is a new proinflammatory marker in RA. As the *in vivo* and *in vitro* studies with the specific inhibitor of the enzymatic activity of visfatin, APO866, show promising results, and taking into account that Kim et al. state[69], upon analyzing the crystal structure of visfatin in complex with APO866, that inhibition can be further optimized for therapeutic usage, the results of our experiments support also the idea that visfatin can be regarded as potential future therapeutic target for RA.

7 SUMMARY

Rheumatoid arthritis (RA) is a chronic, inflammatory joint disease, which is mainly characterized by a painful affection of multiple joints. The synovial tissue lining the joint cavity is clearly thickened in inflamed joints and activated synovial fibroblasts (SFs) align in the *"lining layer"* closely to the cartilage-bone junction. SFs can adhere to, invade into and degrade the cartilage by producing different matrix-metalloproteinases (MMPs). Visfatin and leptin belong to the group of adipokines, molecules released from adipose tissue. Visfatin has been demonstrated to function as an extracellular cytokine, but also as an intracellular enzyme regulating energy metabolism on the cellular level. Leptin on the other hand is known as a hormone regulating food intake at the level of the central nervous system. Recent studies have shown that both proteins are actively participating in the inflammatory immune response affecting arthritic joints in RA. The aim of this work was to detect both leptin and visfatin in the joints of RA patients and to investigate their influence on joint inflammation. Thus, synovial fluid and synovial tissue of RA patients were analyzed. Responses of SFs to the stimulatory effect of visfatin and leptin as well as effects of possible alterations in the cytokine microenvironment on fibroblasts and lymphocytes were studied. Leptin and visfatin are present in the synovial fluid of RA patients, whereas only visfatin was positively correlated to parameters of inflammation such as C-reactive protein (CRP). Visfatin was detectable in the synovial tissue around interstitial vessels, lymphoid aggregates and in the *"lining layer"* by immunohistochemistry. A clear change in the gene expression pattern of RASFs was induced by visfatin showing an upregulation of proinflammatory cytokines and MMPs. The most distinct change was seen for the subgroup of chemokines. Stimulation of RASFs with visfatin led to an alteration of the cytokine environment, which was chemoattractive not only for RASFs in an autocrine fashion but also for lymphocytes. Visfatin-conditioned medium increased directed fibroblast migration towards a gradient as well as undirected cell migration and therefore overall cell motility. These and other previously published results lead to the hypothesis that visfatin acts as a proinflammatory cytokine in arthritic joints by enhancing the joint-destructive potential of RASFs and by promoting their accumulation in the *"lining layer"*. In contrast, leptin did not significantly influence the gene expression profile of RASFs, neither on the

RNA nor the protein levels. Therefore, leptin appears not to be a main trigger of RASF activation. Of note, results from animal models of RA showed that inhibition of the enzymatic activity of visfatin led to an amelioration of the disease. Taken together, the results of this study suggest that visfatin is an important proinflammatory cytokine, which could be a novel therapeutic target in RA.

8 SUMMARY (GERMAN VERSION)

Die rheumatoide Arthritis (RA) ist eine primär chronisch entzündliche Gelenkerkrankung, die hauptsächlich durch eine schmerzhafte Beteiligung mehrerer Gelenke charakterisiert ist. In einem entzündeten Gelenk zeigt sich die Synovialis deutlich verdickt und es finden sich aktivierte synoviale Fibroblasten (SF), die sich in einem sogenannten *„lining layer“* am Knorpel-Knochenübergang sammeln. Sie adhärieren aktiv am Gelenkknorpel, wandern in ihn ein und zerstören ihn, unter anderem durch die Produktion verschiedener Matrixmetalloproteinasen (MMP). Visfatin und Leptin sind Zytokine aus dem Fettgewebe und werden dementsprechend als Adipozytokine bezeichnet. Visfatin zeigt neben der Funktion eines extrazellulären Zytokins auch intrazelluläre enzymatische Aktivität im Energiehaushalt der Zellen. Leptin ist bekannt als Hormon, das zentral das Hungergefühl hemmt. Für beide konnte kürzlich gezeigt werden, dass sie aktiv an der entzündlichen Immunantwort in rheumatischen Gelenken beteiligt sind. Ziel dieser Arbeit war es herauszufinden, ob sowohl Visfatin als auch Leptin im Gelenk von RA Patienten vorkommen und ob sie einen Einfluss auf die Entzündung im Gelenk nehmen. Hierzu wurden die Synovialflüssigkeit und die Synovialis von RA Patienten analysiert, die Antwort synovialer Fibroblasten auf die Stimulation mit Visfatin und Leptin untersucht und festgestellt, ob sich durch diese Einflussnahme das Zytokinmilieu verändert und welcher Einfluss dabei auf die SF und Lymphozyten entsteht. Es konnte gezeigt werden, dass beide Visfatin und Leptin in der Synovialflüssigkeit von RA Patienten vorliegen, wobei sich nur für Visfatin eine Korrelation zu Entzündungsparametern, wie dem C-reaktiven Peptid, zeigte. Im Synovialgewebe konnte nur Visfatin und hier nur im *„lining layer“*, in Endothelien und Lymphaggregaten gefunden werden. Visfatin hatte einen deutlichen Einfluss auf die Genexpression von RASF, indem proinflammatorische Zytokine und MMP induziert wurden. Am deutlichsten jedoch wurden Chemokine reguliert. Die Stimulation von RASF mit Visfatin führte zu einem veränderten Zytokinmilieu, das chemoattraktiv für RASF selbst, aber auch für Lymphozyten war. Es konnte gezeigt werden, dass das durch Visfatin konditionierte Medium sowohl die gerichtete Migration von Fibroblasten als auch die ungerichtete Migration und somit sie generelle Beweglichkeit der SF verstärkt. Diese Beobachtungen führen neben anderen bereits bekannten zu der Annahme, dass Visfatin als proinflammatorisches Zytokin im

entzündeten Gelenk fungiert, indem es die gelenkzerstörende Antwort der RASF verstärkt und zu deren vermehrten Anordnung im *„lining layer“* beiträgt. Leptin zeigte im Vergleich zu Visfatin keine wesentliche Beeinflussung der RASF, weder auf die Genexpression noch auf die Proteinproduktion proinflammatorischer Zytokine. In Tiermodellen der RA konnte bereits gezeigt werden, dass die Inhibition der enzymatischen Aktivität des Visfatins zu einer deutlichen Besserung des Verlaufs führt. Die Ergebnisse dieser Studie untermauern, dass Visfatin ein neues entzündungsförderndes Zytokin und die Blockade der enzymatischen Aktivität ein mögliches, neuartiges Therapiekonzept in der RA ist.

9 LIST OF ABBREVIATIONS

RA - rheumatoid arthritis
SFs - synovial fibroblasts
MMP - matrix metallo-proteinase
RASFs - rheumatoid arthritis synovial fibroblasts
TLR - toll-like receptor
MD - medical doctor
TNF - tumor-necrosis factor
RANKL - receptor activator of the nuclear factor κB ligand
NF-κB - nuclear factor κB
TRAP - tartrate-resistant acid phosphatase
US - ultrasound
CR - conventional radiography
MRI - magnetic resonance imaging
IL - interleukin
STIR - short-tau inversion recovery
ACR - American College of Rheumatology
ACPAs - anti-citrullinated peptide antibodies
RF - rheumatoid factor
EULAR - European League Against Rheumatism
HLA - human leukocyte antigen
SCID - severe combined immunodeficiency
FGF - fibroblast growth factor
TGF - transforming growth factor
VEGF - vascular endothelial growth factor
PDGF - platelet-derived growth factor
IGF - insulin-like growth factor
miR - microRNA
VCAM-1 - vascular cell adhesion molecule
ICAM-1 - intercellular adhesion molecule
TIMP - tissue inhibitor of metallo-proteinases
ID - inhibitor of DNA binding
HIF - hypoxia inducible transcription factor
CORS26 - collagenous repeat-containing sequence of 26-kDa protein
PBEF - pre-B cell colony-enhancing factor
NAD - nicotinamide adenine dinucleotide
NAMPT - nicotinamide phosphoribosyltransferase
NMN - nicotinamide mononucleotide
NM - nicotinamide
Sir - sirtuin
QA - quinolinic acid
NA - nicotinic acid
PRPP - phosphoribosyl pyrophosphate
NaMN - nicotinate mononucleotide
QaPRTase - quinolinate phosphoribosyltransferase
NaPRTase - nicotinate phosphoribosyltransferase
NMNATase - NMN-ATPase
ob - obese
db - diabetes
LIF - leukemia inhibitory factor
CNTF - ciliary neurotrophic factor
OSM - oncostatin M
G-CSF - granulocyte-colony stimulating factor
gp - glycoprotein
JAK - janus kinase
STAT - signal transducers and activators of transcription
SOCS - suppressor of cytokine signaling
AMPK - 5'-monophosphate kinase
SCD - stearoyl-CoA desaturase
PKC - protein kinase C
NOS - nitric oxide synthase
PI - phosphoinositid
MEK - mitogen-activated protein kinase
CD - cluster of differentiation
CRH - corticotropin-releasing hormone
ACTH - adrenocorticotropin hormone
LPS - lipopolysaccharide
AIA - antigen-induced arthritis
OA - osteoarthritis
RT - reverse transcription
T_a - annealing temperature
CCM - cell culture medium
PBS - phosphate-buffered saline
BSA - bovine serum albumin
IL-Ra - interleukin receptor antagonist
OPG - osteoprotegerin
IGFBP - insulin-like growth factor binding protein
PCR - polymerase chain reaction
LAL - limulus amebocyte lysate
MAPK - mitogen-activated protein kinase
MCP - monocyte chemotactic protein
FCS - fetal calf serum
DMEM - Dulbecco's modified eagle medium
ECGF - endothelial cell growth factor
MI - migration index

10 LIST OF FIGURES AND TABLES

10.1 Figures

10.2 Tables

11 REFERENCES

1. Kasper DL, Braunwald E, Fauci AS, et al. Rheumatoid Arthritis. In: *Harrison's Principles of Internal Medicine*. 16th Ed. McGraw-Hill Companies, Inc. 2005:1968.

2. Schett G, Firestein GS. Mr Outside and Mr Inside: classic and alternative views on the pathogenesis of rheumatoid arthritis. *Ann Rheum Dis*. 2010;69(5):787–789.

3. Platzer W. Allgemeine Skelettlehre. In: *Taschenatlas der Anatomie*.Vol 1. 8th Ed. Georg Thieme Verlag; 2003:20–29.

4. Herring W. Recognizing Joint Disease: An Approach to Arthritis. In: *Learning Radiology: Recognizing the Basics*. 1st Ed. Mosby Elsevier; 2007:250–262.

5. Harris E. *Kelly's Textbook of Rheumatology*. 7th Ed. W.B. Saunders Company; 2004.

6. Gay S, Gay RE, Koopman WJ. Molecular and cellular mechanisms of joint destruction in rheumatoid arthritis: two cellular mechanisms explain joint destruction? *Ann Rheum Dis*. 1993;52(Suppl 1):S39.

7. Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? *Arthritis Rheum*. 1996;39(11):1781–1790.

8. Distler JH., Jüngel A, Huber LC, et al. The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles. *PNAS*. 2005;102(8):2892.

9. Burmester GR, Stuhlmüller B, Keyszer G, Kinne RW. Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum*. 1997;40(1):5–18.

10. Junqueira LC., Carneiro J. Knochen. In: *Histologie*. 5th Ed. Springer Verlag; 2002:91–105.

11. Firestein GS, others. Evolving concepts of rheumatoid arthritis. *Nature*. 2003;423(6937):356–361.

12. Gravallese EM, Harada Y, Wang JT, et al. Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis. *Am J Pathol*. 1998;152(4):943.

13. Steinbrocker O, Traeger CH, Batterman RC. Therapeutic criteria in rheumatoid arthritis. *JAMA*. 1949;140(8):659.

14. Haavardsholm EA, Bøyesen P, Ostergaard M, Schildvold A, Kvien TK. Magnetic resonance imaging findings in 84 patients with early rheumatoid arthritis: bone marrow oedema predicts erosive progression. *Ann Rheum Dis*. 2008;67(6):794.

15. Jimenez-Boj E, Nöbauer-Huhmann I, Hanslik-Schnabel B, et al. Bone erosions and bone marrow edema as defined by magnetic resonance imaging reflect true bone marrow inflammation in rheumatoid arthritis. *Arthritis Rheum*. 2007;56(4):1118–1124.

16. Jimenez-Boj E, Redlich K, Türk B, et al. Interaction between synovial inflammatory tissue and bone marrow in rheumatoid arthritis. *J Immunol*. 2005;175(4):2579.

17. Dalbeth N, Smith T, Gray S, et al. Cellular characterisation of magnetic resonance imaging bone oedema in rheumatoid arthritis; implications for pathogenesis of erosive disease. *Ann Rheum Dis*. 2009;68(2):279.

18. Bugatti S, Caporali R, Manzo A, et al. Involvement of subchondral bone marrow in rheumatoid arthritis: lymphoid neogenesis and in situ relationship to subchondral bone marrow osteoclast recruitment. *Arthritis Rheum*. 2005;52(11):3448–3459.

19. Kaisho T, Oritani K, Ishikawa J, et al. Human bone marrow stromal cell lines from myeloma and rheumatoid arthritis that can support murine pre-B cell growth. *J Immunol*. 1992;149(12):4088.

20. Filer A, Parsonage G, Smith E, et al. Differential survival of leukocyte subsets mediated by synovial, bone marrow, and skin fibroblasts: Site-specific versus activation-dependent survival of T cells and neutrophils. *Arthritis Rheum*. 2006;54(7):2096–2108.

21. Hirohata S, Yanagida T, Nampei A, et al. Enhanced generation of endothelial cells from CD34+ cells of the bone marrow in rheumatoid arthritis: possible role in synovial neovascularization. *Arthritis Rheum*. 2004;50(12):3888–3896.

22. Jongen-Lavrencic M, Peeters HRM, Wognum A, et al. Elevated levels of inflammatory cytokines in bone marrow of patients with rheumatoid arthritis and anemia of chronic disease. *J Rheumatol*. 1997;24(8):1504–1509.

23. Moreland LW, Baumgartner SW, Schiff MH, et al. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)–Fc fusion protein. *NEJM*. 1997;337(3):141–147.

24. Nishimoto N, Yoshizaki K, Miyasaka N, et al. Treatment of rheumatoid arthritis with humanized anti–interleukin-6 receptor antibody: A multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum*. 2004;50(6):1761–1769.

25. Elliott MJ, Maini R, Feldmann M, et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor [alpha](cA2) versus placebo in rheumatoid arthritis. *Lancet*. 1994;344(8930):1105–1110.

26. Drevlow BE, Lovis R, Haag MA, et al. Recombinant human interleukin-1 receptor type I in the treatment of patients with active rheumatoid arthritis. *Arthritis Rheum*. 1996;39(2):257–265.

27. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*. 1988;31(3):315–324.

28. 1987 Criteria for the Classification of Acute Arthritis of Rheumatoid Arthritis. Available at: http://www.rheumatology.org/practice/clinical/classification/ra/ra.asp.

29. Vasishta A. Diagnosing early-onset rheumatoid arthritis: the role of anti-CCP antibodies. *Am Clin Lab*. 2002;21(7):34–36.

30. Aletaha D, Neogi T, Silman AJ, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum*. 2010;62(9):2569–2581.

31. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis. Available at: http://www.rheumatology.org/practice/clinical/classification/ra/ra_2010.asp.

32. Kaneko Y, Kuwana M, Kameda H, Takeuchi T. Sensitivity and specificity of 2010 rheumatoid arthritis classification criteria. *Rheumatology*. 2011;50(7):1268–1274.

33. Huber LC, Distler O, Tarner I, et al. Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology*. 2006;45(6):669–675.

34. Noss EH, Brenner MB. The role and therapeutic implications of fibroblast-like synoviocytes in inflammation and cartilage erosion in rheumatoid arthritis. *Immunol Rev*. 2008;223(1):252–270.

35. Müller-Ladner U, Ospelt C, Gay S, Distler O, Pap T. Cells of the synovium in rheumatoid arthritis. Synovial fibroblasts. *Arthritis Res Ther.* 2007;9(6):223.

36. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol Rev*. 2010;233(1):233–255.

37. Perlman H, Pope RM. The synovial lining micromass system: toward rheumatoid arthritis in a dish? *Arthritis Rheum.* 2010;62(3):643–646.

38. Lefèvre S, Knedla A, Tennie C, et al. Synovial fibroblasts spread rheumatoid arthritis to unaffected joints. *Nat Med*. 2009;15(12):1414–1420.

39. Chang SK, Gu Z, Brenner MB. Fibroblast-like synoviocytes in inflammatory arthritis pathology: the emerging role of cadherin-11. *Immunol Rev.* 2010;233(1):256–266.

40. Malemud CJ. Growth hormone, VEGF and FGF: involvement in rheumatoid arthritis. *Clinica chimica acta*. 2007;375(1-2):10–19.

41. Rico MC, Rough JJ, Del Carpio-Cano FE, Kunapuli SP, DeLa Cadena RA. The axis of thrombospondin-1, transforming growth factor beta and connective tissue growth factor: an emerging therapeutic target in rheumatoid arthritis. *Curr Vasc Pharmacol*. 2010;8(3):338–343.

42. Rosengren S, Corr M, Boyle DL. Platelet-derived growth factor and transforming growth factor beta synergistically potentiate inflammatory mediator synthesis by fibroblast-like synoviocytes. *Arthritis Res Ther.* 2010;12(2):R65.

43. Terabe F, Kitano M, Kawai M, et al. Imatinib mesylate inhibited rat adjuvant arthritis and PDGF-dependent growth of synovial fibroblast via interference with the Akt signaling pathway. *Mod Rheumatol*. 2009;19(5):522–529.

44. Martin-Trujillo A, van Rietschoten JGI, Timmer TCG, et al. Loss of imprinting of IGF2 characterises high IGF2 mRNA-expressing type of fibroblast-like synoviocytes in rheumatoid arthritis. *Ann Rheum Dis.* 2010;69(6):1239–1242.

45. Ardoin SP, Pisetsky DS. The role of cell death in the pathogenesis of autoimmune disease: HMGB1 and microparticles as intercellular mediators of inflammation. *Mod Rheumatol*. 2008;18(4):319–326.

46. Distler JHW, Huber LC, Gay S, Distler O, Pisetsky DS. Microparticles as mediators of cellular cross-talk in inflammatory disease. *Autoimmunity*. 2006;39(8):683–690.

47. Boilard E, Nigrovic PA, Larabee K, et al. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science*. 2010;327(5965):580–583.

48. Messer L, Alsaleh G, Freyssinet J-M, et al. Microparticle-induced release of B-lymphocyte regulators by rheumatoid synoviocytes. *Arthritis Res Ther.* 2009;11(2):R40.

49. Jüngel A, Ospelt C, Gay S. What can we learn from epigenetics in the year 2009? *Curr Opin Rheumatol*. 2010;22(3):284.

50. Huber LC, Brock M, Hemmatazad H, et al. Histone deacetylase/acetylase activity in total synovial tissue derived from rheumatoid arthritis and osteoarthritis patients. *Arthritis Rheum*. 2007;56(4):1087–1093.

51. Karouzakis E, Gay RE, Michel BA, Gay S, Neidhart M. DNA hypomethylation in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum*. 2009;60(12):3613–3622.

52. Sánchez-Pernaute O, Ospelt C, Neidhart M, Gay S. Epigenetic clues to rheumatoid arthritis. *J Autoimmunity*. 2008;30(1-2):12–20.

53. Brentano F, Kyburz D, Gay S. Toll-like receptors and rheumatoid arthritis. *Methods Mol Biol.* 2009;517:329–343.

54. Maciejewska Rodrigues H, Jüngel A, Gay RE, Gay S. Innate immunity, epigenetics and autoimmunity in rheumatoid arthritis. *Mol Immunol*. 2009;47(1):12–18.

55. Neumann E, Lefèvre S, Zimmermann B, Gay S, Müller-Ladner U. Rheumatoid arthritis progression mediated by activated synovial fibroblasts. *Trends Mol Med*. 2010;16(10):458–468.

56. Lioté F, Champy R, Moenner M, Boval-Boizard B, Badet J. Elevated angiogenin levels in synovial fluid from patients with inflammatory arthritis and secretion of angiogenin by cultured synovial fibroblasts. *Clin Exp Immunol.* 2003;132(1):163–168.

57. Gravallese EM, Pettit AR, Lee R, et al. Angiopoietin-1 is expressed in the synovium of patients with rheumatoid arthritis and is induced by tumour necrosis factor alpha. *Ann Rheum Dis.* 2003;62(2):100–107.

58. Presta M, Andrés G, Leali D, Dell'Era P, Ronca R. Inflammatory cells and chemokines sustain FGF2-induced angiogenesis. *Eur Cytokine Netw.* 2009;20(2):39–50.

59. del Rey MJ, Izquierdo E, Caja S, et al. Human inflammatory synovial fibroblasts induce enhanced myeloid cell recruitment and angiogenesis through a hypoxia-inducible transcription factor 1alpha/vascular endothelial growth factor-mediated pathway in immunodeficient mice. *Arthritis Rheum.* 2009;60(10):2926–2934.

60. Kurowska-Stolarska M, Distler JHW, Jüngel A, et al. Inhibitor of DNA binding/differentiation 2 induced by hypoxia promotes synovial fibroblast-dependent osteoclastogenesis. *Arthritis Rheum.* 2009;60(12):3663–3675.

61. Akhavani MA, Madden L, Buysschaert I, et al. Hypoxia upregulates angiogenesis and synovial cell migration in rheumatoid arthritis. *Arthritis Res Ther.* 2009;11(3):R64.

62. Westra J, Molema G, Kallenberg CGM. Hypoxia-inducible factor-1 as regulator of angiogenesis in rheumatoid arthritis - therapeutic implications. *Curr Med Chem.* 2010;17(3):254–263.

63. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen*. 2008;16(5):585–601.

64. van Beurden HE, Snoek PAM, Von den Hoff JW, Torensma R, Kuijpers-Jagtman A-M. Fibroblast subpopulations in intra-oral wound healing. *Wound Repair Regen*. 2003;11(1):55–63.

65. Schäffler A, Müller-Ladner U, Schölmerich J, Büchler C. Role of adipose tissue as an inflammatory organ in human diseases. *Endocr Rev*. 2006;27(5):449–467.

66. Samal B, Sun Y, Stearns G, et al. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol*. 1994;14(2):1431.

67. Shaw G, Kamen R. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell*. 1986;46(5):659–667.

68. Takahashi R, Nakamura S, Nakazawa T, et al. Structure and reaction mechanism of human nicotinamide phosphoribosyltransferase. *J Biochem.* 2010;147(1):95–107.

69. Kim MK, Lee JH, Kim H, et al. Crystal structure of visfatin/pre-B cell colony-enhancing factor/nicotinamide phosphoribosyltransferase, free and in complex with the anti-cancer agent FK-866. *J Mol Biol*. 2006;362(1):66–77.

70. Wang T, Zhang X, Bheda P, et al. Structure of Nampt/PBEF/visfatin, a mammalian NAD+ biosynthetic enzyme. *Nat Struct Mol Biol*. 2006;13(7):661–662.

71. Zhang F, Basinski MB, Beals JM, et al. Crystal structure of the obese protein Ieptin-E100. *Nature.* 1997;387:206-209.

72. Moschen AR, Kaser A, Enrich B, et al. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *J Immunol.* 2007;178(3):1748–1758.

73. Fukuhara A, Matsuda M, Nishizawa M, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science*. 2005;307(5708):426–430.

74. Rongvaux A, Shea RJ, Mulks MH, et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol*. 2002;32(11):3225–3234.

75. Rongvaux A, Shea RJ, Mulks MH, et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol.* 2002;32(11):3225–3234.

76. Sauve AA, Wolberger C, Schramm VL, Boeke JD. The biochemistry of sirtuins. *Annu Rev Biochem.* 2006;75:435–465.

77. Bredt D, Snyder S. Nitric oxide: a physiologic messenger molecule. *Annu Rev Biochem*. 1994;63(1):175–195.

78. Berger SJ, Manory I, Sudar DC, Berger NA. Induction of the pyridine nucleotide synthesis pathway in mitogen-stimulated human T-lymphocytes* 1. *Exp Cell Res*. 1987;169(1):149–157.

79. Zhang J, Dawson VL, Dawson TM, Snyder SH. Nitric oxide activation of poly (ADP-ribose) synthetase in neurotoxicity. *Science*. 1994;263(5147):687.

80. van der Veer E, Nong Z, O'Neil C, et al. Pre-B-cell colony-enhancing factor regulates NAD+-dependent protein deacetylase activity and promotes vascular smooth muscle cell maturation. *Circ Res.* 2005;97(1):25–34.

81. Dahl TB, Yndestad A, Skjelland M, et al. Increased Expression of Visfatin in Macrophages of Human Unstable Carotid and Coronary Atherosclerosis. *Circulation*. 2007;115(8):972–980.

82. Xie H, Tang SY, Luo XH, et al. Insulin-like effects of visfatin on human osteoblasts. *Calcif Tissue Int*. 2007;80(3):201–210.

83. Fukuhara A, Matsuda M, Nishizawa M, et al. Retraction. *Science*. 2007;318(5850):565.

84. Jia SH, Li Y, Parodo J, et al. Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. *J Clin Invest*. 2004;113(9):1318–1327.

85. Ye SQ, Simon BA, Maloney JP, et al. Pre-B-cell colony enhancing factor as a potential novel biomarker in acute lung injury. *Am J Respir Critical Care Med*. 2005:171(4):361-70.

86. Moschen AR, Kaser A, Enrich B, et al. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. *J Immunol*. 2007;178(3):1748–1758.

87. Nemeth E, Tashima LS, Yu Z, Bryant-Greenwood GD. Fetal membrane distention: I. Differentially expressed genes regulated by acute distention in amniotic epithelial (WISH) cells. *Am J Obstet Gynecol.* 2000;182(1 Pt 1):50–59.

88. Ognjanovic S, Bao S, Yamamoto SY, et al. Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes. *J Mol Endocrinol*. 2001;26(2):107–117.

89. Dahl TB, Yndestad A, Skjelland M, et al. Increased expression of visfatin in macrophages of human unstable carotid and coronary atherosclerosis: possible role in inflammation and plaque destabilization. *Circulation*. 2007;115(8):972–980.

90. Gosset M, Berenbaum F, Salvat C, et al. Crucial role of visfatin/pre-B cell colony-enhancing factor in matrix degradation and prostaglandin E2 synthesis in chondrocytes: possible influence on osteoarthritis. *Arthritis Rheum.* 2008;58(5):1399–1409.

91. Bao JP, Chen WP, Wu LD. Visfatin: a potential therapeutic target for rheumatoid arthritis. *J Int Med Res*. 2009;37(6):1655–1661.

92. Drevs J, Löser R, Rattel B, Esser N. Antiangiogenic potency of FK866/K22.175, a new inhibitor of intracellular NAD biosynthesis, in murine renal cell carcinoma. *Anticancer Res.* 2003;23(6C):4853–4858.

93. Brentano F, Schorr O, Ospelt C, et al. Pre-B cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities. *Arthritis Rheum*. 2007;56(9):2829–2839.

94. Busso N, Karababa M, Nobile M, et al. Pharmacological inhibition of nicotinamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD. *PLoS ONE*. 2008;3(5):e2267.

95. Evans L, Williams AS, Hayes AJ, Jones SA, Nowell M. Suppression of leukocyte infiltration and cartilage degradation by selective inhibition of pre-B cell colony-enhancing factor/visfatin/nicotinamide phosphoribosyltransferase: Apo866-mediated therapy in human fibroblasts and murine collagen-induced arthritis. *Arthritis Rheum.* 2011;63(7):1866–1877.

96. Zhang Y, Proenca R, Maffei M, et al. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;372(6505):425–432.

97. Otero M, Lago R, Gomez R, et al. Towards a pro-inflammatory and immunomodulatory emerging role of leptin. *Rheumatology*. 2006;45(8):944–950.

98. Tartaglia LA, Dembski M, Weng X, et al. Identification and expression cloning of a leptin receptor, OB-R. *Cell*. 1995;83(7):1263–1271.

99. White DW, Tartaglia LA. Leptin and OB-R: body weight regulation by a cytokine receptor. *Cytokine Growth Factor Rev*. 1996;7(4):303–309.

100. Münzberg H, Björnholm M, Bates S, Myers M. Leptin receptor action and mechanisms of leptin resistance. *Cellular Mol Life Sciences*. 2005;62(6):642–652.

101. Otero M, Lago RI, others. Leptin, from fat to inflammation: old questions and new insights. *FEBS letters*. 2005;579(2):295–301.

102. Lee GH, Proenca R, Montez J, et al. Abnormal splicing of the leptin receptor in diabetic mice. *Nature.* 1996;379(6566):632-5.

103. Fei H, Okano HJ, Li C, et al. Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *PNAS.* 1997;94(13):7001.

104. Good DJ. How Tight Are Your Genes? Transcriptional and Posttranscriptional Regulation of the Leptin Receptor, NPY, and POMC Genes. *Hormones and behavior*. 2000;37(4):284–298.

105. Frühbeck G. Intracellular signalling pathways activated by leptin. *Biochem J*. 2006;393(Pt 1):7.

106. Sanchez-Margalet V, Martin-Romero C. Human leptin signaling in human peripheral blood mononuclear cells: activation of the JAK-STAT pathway. *Cellular Immunol*. 2001;211(1):30–36.

107. Ghilardi N, Ziegler S, Wiestner A, et al. Defective STAT signaling by the leptin receptor in diabetic mice. *PNAS*. 1996;93(13):6231.

108. Banks AS, Davis SM, Bates SH, Myers MG. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem*. 2000;275(19):14563.

109. Bjørbaek C, El-Haschimi K, Frantz JD, Flier JS. The role of SOCS-3 in leptin signaling and leptin resistance. *J Biol Chem.* 1999;274(42):30059–30065.

110. Bjørbaek C, Elmquist JK, Frantz JD, Shoelson SE, Flier JS. Identification of SOCS-3 as a potential mediator of central leptin resistance. *Mol Cell*. 1998;1(4):619–625.

111. Gualillo O, Eiras S, White D, Dieguez C, Casanueva F. Leptin promotes the tyrosine phosphorylation of SHC proteins and SHC association with GRB2. *Mol Cellular Endocrinol*. 2002;190(1-2):83–89.

112. Zhang F, Chen Y, Heiman M, DiMarchi R. Leptin: structure, function and biology. *Vitamins & Hormones*. 2005;71:345–372.

113. Zarkesh-Esfahani H, Pockley G, Metcalfe RA, et al. High-dose leptin activates human leukocytes via receptor expression on monocytes. *J Immunol*. 2001;167(8):4593.

114. Mancuso P, Canetti C, Gottschalk A, Tithof PK, Peters-Golden M. Leptin augments alveolar macrophage leukotriene synthesis by increasing phospholipase

activity and enhancing group IVC iPLA 2 (cPLA2gamma) protein expression. *Am J Physiol-Lung Cellular Mol Physiol*. 2004;287(3):L497.

115. Dixit VD, Mielenz M, Taub DD, Parvizi N. Leptin induces growth hormone secretion from peripheral blood mononuclear cells via a protein kinase C-and nitric oxide-dependent mechanism. *Endocrinology*. 2003;144(12):5595.

116. Raso GM, Pacilio M, Esposito E, et al. Leptin potentiates IFN-gamma-induced expression of nitric oxide synthase and cyclo-oxygenase-2 in murine macrophage J774A. 1. *Brit J Pharmacol*. 2002;137(6):799–804.

117. Goldring MB, Berenbaum F. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. *Clin Orthop Rel Res*. 2004;427:S37.

118. Otero M, Lago R, Lago F, Reino J, Gualillo O. Signalling pathway involved in nitric oxide synthase type II activation in chondrocytes: synergistic effect of leptin with interleukin-1. *Arthritis Res Ther*. 2005;7(3):R581–91.

119. Sarraf P, Frederich RC, Turner EM, et al. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. *J Exp Med*. 1997;185(1):171.

120. Gualillo O, Eiras S, Lago F, Diéguez C, Casanueva FF. Elevated serum leptin concentrations induced by experimental acute inflammation. *Life Sciences*. 2000;67(20):2433–2441.

121. Popa C, Netea MG, Radstake TRDS, et al. Markers of inflammation are negatively correlated with serum leptin in rheumatoid arthritis. *Ann Rheum Dis*. 2005;64(8):1195.

122. Zhang HH, Kumar S, Barnett AH, Eggo MC. Tumour necrosis factor-[alpha] exerts dual effects on human adipose leptin synthesis and release. *Mol Cellular Endocrinol*. 2000;159(1-2):79–88.

123. Buttgereit F, Burmester GR, Brand MD. Bioenergetics of immune functions: fundamental and therapeutic aspects. *Immunol Today*. 2000;21(4):194–199.

124. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ. Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature*. 1997;389(6649):374–377.

125. Matarese G, Moschos S, Mantzoros CS. Leptin in immunology. *J Immunol.* 2005;174(6):3137–3142.

126. Farooqi IS, Matarese G, Lord GM, et al. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J Clin Invest*. 2002;110(8):1093–1104.

127. Lord GM, Matarese G, Howard JK, et al. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature*. 1998;394(6696):897–900.

128. Williams L, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol*. 2004;172(1):567.

129. Heiman ML, Ahima RS, Craft LS, et al. Leptin inhibition of the hypothalamic-pituitary-adrenal axis in response to stress. *Endocrinology*. 1997;138(9):3859.

130. Wabitsch M, Jensen PB, Blum W, et al. Insulin and cortisol promote leptin production in cultured human fat cells. *Diabetes*. 1996;45(10):1435.

131. Faggioni R, Feingold KR, Grunfeld C. Leptin regulation of the immune response and the immunodeficiency of malnutrition. *FASEB J.* 2001;15(14):2565–2571.

132. Sanna V, Di Giacomo A, La Cava A, et al. Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses. *J Clin Invest*. 2003;111(2):241–250.

133. Imrich R. The role of neuroendocrine system in the pathogenesis of rheumatic diseases (minireview). *Endocr Regul*. 2002;36(2):95–106.

134. Busso N, So A, Chobaz-Péclat V, et al. Leptin signaling deficiency impairs humoral and cellular immune responses and attenuates experimental arthritis. *J Immunol*. 2002;168(2):875.

135. Fraser D, Thoen J, Reseland J, Førre O, Kjeldsen-Kragh J. Decreased CD4+ lymphocyte activation and increased interleukin-4 production in peripheral blood of rheumatoid arthritis patients after acute starvation. *Clin Rheumatol*. 1999;18(5):394–401.

136. Otero M, Lago R, Gomez R, et al. Changes in plasma levels of fat-derived hormones adiponectin, leptin, resistin and visfatin in patients with rheumatoid arthritis. *Ann Rheum Dis*. 2006;65(9):1198.

137. Bokarewa M, Bokarew D, Hultgren O, Tarkowski A. Leptin consumption in the inflamed joints of patients with rheumatoid arthritis. *Ann Rheum Dis*. 2003;62(10):952.

138. Müller-Ladner U, Evans CH, Franklin BN, et al. Gene transfer of cytokine inhibitors into human synovial fibroblasts in the SCID mouse model. *Arthritis Rheum*. 1999;42(3):490–497.

139. Neumann E, Kullmann F, Judex M, et al. Identification of differentially expressed genes in rheumatoid arthritis by a combination of complementary DNA array and RNA arbitrarily primed-polymerase chain reaction. *Arthritis Rheum*. 2002;46(1):52–63.

140. Neumann E, Judex M, Kullmann F, et al. Inhibition of cartilage destruction by double gene transfer of IL-1Ra and IL-10 involves the activin pathway. *Gene Ther*. 2002;9(22):1508–1519.

141. Neumann E, Riepl B, Knedla A, et al. Cell culture and passaging alters gene expression pattern and proliferation rate in rheumatoid arthritis synovial fibroblasts. *Arthritis Res Ther.* 2010;12(3):R83.

142. Ehling A, Schäffler A, Herfarth H, et al. The potential of adiponectin in driving arthritis. *J Immunol*. 2006;176(7):4468–4478.

143. Brühl H, Mack M, Niedermeier M, et al. Functional expression of the chemokine receptor CCR7 on fibroblast-like synoviocytes. *Rheumatology*. 2008;47(12):1771–1774.

144. Schedin PJ, Eckel-Mahan KL, McDaniel SM, et al. ESX induces transformation and functional epithelial to mesenchymal transition in MCF-12A mammary epithelial cells. *Oncogene*. 2004;23(9):1766–1779.

145. Frommer KW, Zimmermann B, Meier FMP, et al. Adiponectin-mediated changes in effector cells involved in the pathophysiology of rheumatoid arthritis. *Arthritis Rheum*. 2010;62(10):2886–2899.

146. Kunisch E, Gandesiri M, Fuhrmann R, et al. Predominant activation of MAP kinases and pro-destructive/pro-inflammatory features by TNF alpha in early-passage synovial fibroblasts via TNF receptor-1: failure of p38 inhibition to suppress matrix metalloproteinase-1 in rheumatoid arthritis. *Ann Rheum Dis*. 2007;66(8):1043–1051.

147. DiBattista JA, Martel-Pelletier J, Morin N, Jolicoeur FC, Pelletier JP. Transcriptional regulation of plasminogen activator inhibitor-1 expression in human synovial fibroblasts by prostaglandin E2: mediation by protein kinase A and role of interleukin-1. *Mol Cell Endocrinol*. 1994;103(1-2):139–148.

148. Onodera S, Nishihira J, Koyama Y, et al. Macrophage migration inhibitory factor up-regulates the expression of interleukin-8 messenger RNA in synovial fibroblasts of rheumatoid arthritis patients: common transcriptional regulatory mechanism between interleukin-8 and interleukin-1beta. *Arthritis Rheum*. 2004;50(5):1437–1447.

149. Chiu Y-C, Fong Y-C, Lai C-H, et al. Thrombin-induced IL-6 production in human synovial fibroblasts is mediated by PAR1, phospholipase C, protein kinase C alpha, c-Src, NF-kappa B and p300 pathway. *Mol Immunol*. 2008;45(6):1587–1599.

150. Alkady E, Ahmed H, Tag L, Abdou M. Adiponectin, Resistin und Visfatin in Serum und Gelenkflüssigkeit bei Patienten mit rheumatoider Arthritis. *Z Rheumatol*. 2011:1–6.

151. Bae S-K, Kim S-R, Kim JG, et al. Hypoxic induction of human visfatin gene is directly mediated by hypoxia-inducible factor-1. *FEBS Lett*. 2006;580(17):4105–4113.

152. Nowell MA, Richards PJ, Fielding CA, et al. Regulation of pre-B cell colony-enhancing factor by STAT-3-dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis. *Arthritis Rheum*. 2006;54(7):2084–2095.

153. Imamura F, Aono H, Hasunuma T, et al. Monoclonal expansion of synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* 1998;41(11):1979–1986.

154. Pap T, Aupperle KR, Gay S, Firestein GS, Gay RE. Invasiveness of synovial fibroblasts is regulated by p53 in the SCID mouse in vivo model of cartilage invasion. *Arthritis Rheum.* 2001;44(3):676–681.

155. Walsh DA, Wade M, Mapp PI, Blake DR. Focally regulated endothelial proliferation and cell death in human synovium. *Am J Pathol.* 1998;152(3):691–702.

156. Firestein GS, Echeverri F, Yeo M, Zvaifler NJ, Green DR. Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium. *PNAS.* 1997;94(20):10895–10900.

157. Rème T, Travaglio A, Gueydon E, et al. Mutations of the p53 tumour suppressor gene in erosive rheumatoid synovial tissue. *Clin Exp Immunol.* 1998;111(2):353–358.

158. Inazuka M, Tahira T, Horiuchi T, et al. Analysis of p53 tumour suppressor gene somatic mutations in rheumatoid arthritis synovium. *Rheumatology*. 2000;39(3):262–266.

159. Lee S-H, Chang DK, Goel A, et al. Microsatellite instability and suppressed DNA repair enzyme expression in rheumatoid arthritis. *J Immunol.* 2003;170(4):2214–2220.

160. Brentano F, Schorr O, Gay RE, Gay S, Kyburz D. RNA released from necrotic synovial fluid cells activates rheumatoid arthritis synovial fibroblasts via Toll-like receptor 3. *Arthritis Rheum*. 2005;52(9):2656–2665.

161. Ospelt C, Neidhart M, Gay RE, Gay S. Synovial activation in rheumatoid arthritis. *Front Biosci*. 2004;9:2323–2334.

162. Smolen JS, Beaulieu A, Rubbert-Roth A, et al. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. *Lancet*. 2008;371(9617):987–997.

163. Szekanecz Z, Pakozdi A, Szentpetery A, Besenyei T, Koch AE. Chemokines and angiogenesis in rheumatoid arthritis. *Front Biosci (Elite Ed)*. 2009;1:44–51.

164. Samal B, Sun Y, Stearns G, et al. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol*. 1994;14(2):1431–1437.

165. Storka A, Vojtassakova E, Mueller M, et al. Angiotensin inhibition stimulates PPARgamma and the release of visfatin. *Eur J Clin Invest*. 2008;38(11):820–826.

166. Rofstad EK, Halsør EF. Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts. *Cancer Res*. 2000;60(17):4932–4938.

167. Bodolay E, Koch AE, Kim J, Szegedi G, Szekanecz Z. Angiogenesis and chemokines in rheumatoid arthritis and other systemic inflammatory rheumatic diseases. *J Cell Mol Med*. 2002;6(3):357–376.

168. del Rey MJ, Izquierdo E, Caja S, et al. Human inflammatory synovial fibroblasts induce enhanced myeloid cell recruitment and angiogenesis through a hypoxia-inducible transcription factor 1alpha/vascular endothelial growth factor-mediated pathway in immunodeficient mice. *Arthritis Rheum*. 2009;60(10):2926–2934.

169. Kim S-R, Bae S-K, Choi K-S, et al. Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2. *Biochem Biophys Res Commun*. 2007;357(1):150–156.

170. Bae Y-H, Bae M-K, Kim S-R, et al. Upregulation of fibroblast growth factor-2 by visfatin that promotes endothelial angiogenesis. *Biochem Biophys Res Commun*. 2009;379(2):206–211.

171. Iwamoto T, Okamoto H, Toyama Y, Momohara S. Molecular aspects of rheumatoid arthritis: chemokines in the joints of patients. *FEBS J*. 2008;275(18):4448–4455.

172. Suzuki M, Tetsuka T, Yoshida S, et al. The role of p38 mitogen-activated protein kinase in IL-6 and IL-8 production from the TNF-alpha- or IL-1beta-stimulated rheumatoid synovial fibroblasts. *FEBS Lett*. 2000;465(1):23–27.

173. Miyazawa K, Mori A, Miyata H, et al. Regulation of interleukin-1beta-induced interleukin-6 gene expression in human fibroblast-like synoviocytes by p38 mitogen-activated protein kinase. *J Biol Chem*. 1998;273(38):24832–24838.

174. Reunanen N, Li S-P, Ahonen M, et al. Activation of p38 alpha MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. *J Biol Chem*. 2002;277(35):32360–32368.

175. Badger AM, Griswold DE, Kapadia R, et al. Disease-modifying activity of SB 242235, a selective inhibitor of p38 mitogen-activated protein kinase, in rat adjuvant-induced arthritis. *Arthritis Rheum*. 2000;43(1):175–183.

176. Nishikawa M, Myoui A, Tomita T, et al. Prevention of the onset and progression of collagen-induced arthritis in rats by the potent p38 mitogen-activated protein kinase inhibitor FR167653. *Arthritis Rheum*. 2003;48(9):2670–2681.

177. Jacques C, Holzenberger M, Mladenovic Z, et al. Pro-inflammatory actions of visfatin/Nampt involve regulation of insulin signaling pathway and Nampt enzymatic activity. *J Biol Chem*. 2012;287(18):15100-8.

178. Rongvaux A, Shea RJ, Mulks MH, et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol*. 2002;32(11):3225–3234.

179. van der Veer E, Nong Z, O'Neil C, et al. Pre-B-cell colony-enhancing factor regulates NAD+-dependent protein deacetylase activity and promotes vascular smooth muscle cell maturation. *Circ Res*. 2005;97(1):25–34.

180. van der Veer E, Ho C, O'Neil C, et al. Extension of human cell lifespan by nicotinamide phosphoribosyltransferase. *J Biol Chem*. 2007;282(15):10841–10845.

181. Revollo JR, Grimm AA, Imai S. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J Biol Chem*. 2004;279(49):50754–50763.

182. Genovese MC, Cohen SB, Wofsy D, et al. A 24-week, randomized, double-blind, placebo-controlled, parallel group study of the efficacy of oral SCIO-469, a p38 mitogen-activated protein kinase inhibitor, in patients with active rheumatoid arthritis. *J Rheumatol.* 2011;38(5):846–854.

183. Damjanov N, Kauffman RS, Spencer-Green GT. Efficacy, pharmacodynamics, and safety of VX-702, a novel p38 MAPK inhibitor, in rheumatoid arthritis: results of two randomized, double-blind, placebo-controlled clinical studies. *Arthritis Rheum.* 2009;60(5):1232–1241.

184. Cohen SB, Cheng T-T, Chindalore V, et al. Evaluation of the efficacy and safety of pamapimod, a p38 MAP kinase inhibitor, in a double-blind, methotrexate-controlled study of patients with active rheumatoid arthritis. *Arthritis Rheum.* 2009;60(2):335–344.

185. Palmer G, Gabay C. A role for leptin in rheumatic diseases? *Ann Rheum Dis*. 2003;62(10):913.

186. Anders HJ, Rihl M, Heufelder A, Loch O, Schattenkirchner M. Leptin serum levels are not correlated with disease activity in patients with rheumatoid arthritis. *Metabolism*. 1999;48(6):745–748.

187. Hizmetli S, Kisa M, Gokalp N, Bakici MZ. Are plasma and synovial fluid leptin levels correlated with disease activity in rheumatoid arthritis? *Rheumatol Int*. 2007;27(4):335–338.

188. Rho YH, Solus J, Sokka T, et al. Adipocytokines are associated with radiographic joint damage in rheumatoid arthritis. *Arthritis Rheum*. 2009;60(7):1906–1914.

189. Gómez R, Scotece M, Conde J, et al. Adiponectin and leptin increase IL-8 production in human chondrocytes. *Ann Rheum Dis.* 2011;70(11):2052–2054.

190. Tong K-M, Shieh D-C, Chen C-P, et al. Leptin induces IL-8 expression via leptin receptor, IRS-1, PI3K, Akt cascade and promotion of NF-kappaB/p300 binding in human synovial fibroblasts. *Cell Signal.* 2008;20(8):1478–1488.

12 LIST OF PUBLICATIONS

1. **Meier FMP**, Frommer KW, Peters MA, Brentano F, Lefèvre S, Schröder D, Kyburz D, Steinmeyer J, Rehart S, Gay S, Müller-Ladner U, Neumann E. Visfatin/Pre-B Cell Colony-Enhancing Factor (PBEF): a Proinflammatory and Cell Motility-Changing Factor in Rheumatoid Arthritis. J Biol Chem 2012;287(34):28378-85.
2. **Meier FMP**, Frommer KW, Dinser R, Walker UA, Czirjak L, Denton C, Allanore Y, Distler O, Riemekasten G, Valentini G, Müller-Ladner U, EUSTAR co-authors. Update on the Profile of the EUSTAR cohort - an analysis of the EULAR Scleroderma Trials and Research group database. Ann Rheum Dis 2012;71(8):1355-60.
3. **Meier FM**, Geyer M, Tiede H, Rieth A, Ghofrani HA, Müller-Ladner U, Dinser R, Hermann W. Nailfold videocapillaroscopy in pulmonary hypertension: a valuable diagnostic tool? Rheumatology 2012;51(Suppl 2):ii77-8.
4. Strohbeck C, **Meier FM**, Riemekasten G, Himsel A, Herrgott I, Blank N, Bretzel RG, Distler J, Seidel M, Müller-Ladner U. Clinical Features of Gastrointestinal Involvement in Patients with Systemic Sclerosis. Arthritis Rheum 2011;63(10):S569.
5. Strohbeck C, **Meier FM**, Riemekasten G, Himsel A, Herrgott I, Blank N, Bretzel RG, Distler J, Seidel M, Müller-Ladner U. Gastrointestinale Beteiligung bei systemischer Sklerose (SSc) im Vergleich zu anderen rheumatischen Erkrankungen. Z Rheumatol 2011;70(Suppl 1):39.
6. **Meier FM**, Broll M, Bachmann G, Lange U, Müller-Ladner U, Tarner IH. Investigation of Intensified Rituximab Therapy for RF- and ACPA-Positive Rheumatoid Arthritis with Low-field MRI and Power-Doppler Ultrasound – A Pilot Project. Ann Rheum Dis 2011;70(Suppl3):721.
7. Tony HP, Burmester G, Schulze-Koops H, Grunke M, Henes J, Kotter I, Haas J, Unger L, Lovric S, Haubitz M, Fischer-Betz R, Chehab G, Rubbert-Roth A, Specker C, Weinerth J, Holle J, Muller-Ladner U, Koenig R, Fiehn C, Burgwinkel P, Budde K, Soerensen H, Meurer M, Aringer M, Kieseier B, Erfurt-Berge C, Sticherling M, Veelken R, Ziemann U, Strutz F, von Wussow P, **Meier FM**, Hunzelmann N, Schmidt E, Bergner R, Schwarting A, Eming R, Hertl M, Stadler R, Schwarz-Eywill M, Wassenberg S, Fleck M, Metzler C, Zettl U, Westphal J, Heitmann S, Herzog AL, Wiendl H, Jakob W, Schmidt E, Freivogel K, Dorner T, Investigators G. Safety and clinical outcomes of rituximab therapy in patients with different autoimmune diseases: experience from a national registry (GRAID). Arthritis Res Ther 2011;13(3):R75.
8. Frommer KW, Zimmermann B, **Meier FM**, Schröder D, Heil M, Schäffler A, Büchler C, Steinmeyer J, Brentano F, Gay S, Müller-Ladner U, Neumann E. Adiponectin-mediated changes in effector cells involved in the pathophysiology of rheumatoid arthritis. Arthritis Rheum 2010;62(10):2886-99.
9. Specker C, Laubenthal E, Unger L, Tony HP, Fleck M, Fiehn C, Schulze-Koops H, Grünke M, Kötter I, Haas J, Haubitz M, Lovric S, Rubbert-Roth A, Aringer M, Müller-Ladner U, **Meier F**, Sörensen H, Freivogel K, Burmester GR, Dörner T**.** Efficacy of Treatment with Rituximab in 26 Patients with Refractory Dermato-Polymyositis: Data of the German Registry of Autoimmune Diseases (GRAID). Ann Rheum Dis 2010;69(Suppl3):423.

10. Roll P, Ostermeier E, Tony HP, Schulze-Koops H, Specker C, Kötter I, Fischer-Betz K, Rubbert-Roth A, Müller-Ladner U, Fiehn C, Sörensen H, Aringer M, Fleck M, Wassenberg S, **Meier F**, Grünke M, Schwarting A, Schwarz-Eywill M, Burmester GR, Dörner T. Efficacy and Safety of Rituximab Treatment in ANCA-Associated Vasculitis: Results of the German National Registry. Ann Rheum Dis 2010;69(Suppl3):233.
11. **Meier FM**, Frommer K, Brentano F, Lefèvre S, Knedla A, Schroeder D, Büchler C, Schäffler A, Steinmeyer J, Stürz H, Gay S, Müller-Ladner U, Neumann E. Visfatin (PBEF) – a Dominant Mediator of Chemotaxis in Rheumatoid Arthritis. Arthritis Rheum 2008;58(9):S910-1.
12. Frommer KW, **Meier F**, Schäffler A, Büchler C, Steinmeyer J, Brentano F, Gay S, Müller-Ladner U, Neumann E. Adiponectin-Mediated Changes in Gene Expression of Rheumatoid Synovial Fibroblasts. Arthritis Rheum 2008;58(9):S522.
13. **Meier FM**, Frommer K, Brentano F, Lefèvre S, Knedla A, Schröder D, Schäffler A, Büchler C, Steinmeyer J, Stürz H, Gay S, Müller-Ladner U, Neumann E. Inflammatory Response of Synovial-Like Fibroblasts to Stimulation with Visfatin/PBEF. Ann Rheum Dis 2008;67(Suppl II):452.
14. Frommer KW, **Meier F**, Schäffler A, Büchler C, Steinmeyer J, Brentano F, Gay S, Müller-Ladner U, Neumann E. Adiponectin-mediated Effects on Gene Expression of Rheumatoid Synovial Fibroblasts. Ann Rheum Dis 2008;67(Suppl II):153.
15. **Meier FM**, Knedla A, Lefèvre S, Frommer K, Schäffler A, Büchler C, Steinmeyer J, Stürz H, Gay S, Müller-Ladner U, Neumann E. The Adipokine Visfatin and its Influence on the Inflammatory State in Rheumatoid Arthritis. Arthritis Rheum 2007;56(9):S105.
16. **Meier FM,** Knedla A, Lefèvre S, Schäffler A, Büchler, C, Stürz H, Müller-Ladner U, Neumann E. Das Adipokin Visfatin und dessen Einfluss auf die Entzündung in der rheumatoiden Arthritis. Z Rheumatol 2007;66(Suppl I):24.
17. Neumann E, Knedla A, **Meier F**, Tarner IH, Büchler C, Schäffler A, Müller-Ladner U. Adipocytokines as driving forces in rheumatoid arthritis, Z Rheumatol 2007;66(2):139-41.

13 DECLARATION

Erklärung zur Dissertation

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