Resistin is a modulator of inflammation and autoimmunity

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Abstract

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Resistin is a protein with proinflammatory properties in man and a regulator of glucose metabolism in mice. Human resistin activates the nuclear factor kappa-B dependent cytokine cascade; however, its full role in inflammation in terms of regulation, expression, and cellular source is not entirely understood. Resistin may have a role in insulin growth factor signaling. The main aim of this thesis was to investigate the role of resistin in inflammation, with emphasis on rheumatic diseases and diseases of the liver and gastrointestinal tract; in addition, the neutrophil was evaluated as a possible source of resistin. Another aim was to investigate the effect of resistin on IGF signaling in a new mouse model of arthritis.

In paper I, resistin was shown to be associated with local inflammation in patients with primary Sjögren's syndrome. This conclusion was based on the finding of elevated resistin levels in saliva and in the salivary gland tissue of patients. Moreover, resistin levels were significantly higher in patients with high focal infiltration of leukocytes in glandular tissue than in those with no or low leukocyte infiltration. In paper II, resistin levels were found to be elevated in a wide variety of inflammatory and autoimmune conditions of the liver and gastrointestinal tract. Furthermore, they were significantly higher in patients who were seropositive for anti-nuclear antibodies than those who were seronegative. In paper III, a new cell source of resistin in inflammation was identified, namely the neutrophil. Subcellular fractionation of the neutrophil confirmed the presence of resistin in the azurophil granules and the specific granules. In a rheumatoid arthritis (RA) model in paper IV, resistin was shown to modulate IGF signaling. Levels of IGF-1 were significantly lower in RA patients, especially those with systemic inflammation, than in controls with non-inflammatory joint conditions, and they were inversely related to resistin levels. Resistin expression was abrogated in a transplantation mouse model of RA synovia. This led to downregulation of IGF-1R expression and intracellular Akt activity.

Taken together, these results indicate that resistin is an immunomodulatory molecule that is expressed locally at the site of inflammation. It is produced by neutrophils and possibly modulates IGF signaling. These findings suggest that resistin regulates both inflammation and could affect growth factor-related signaling in humans.

Key words: Resistin, inflammation, autoimmunity, rheumatoid arthritis, Sjögren's syndrome.
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List of publications

This thesis is based upon the following papers, referred to in the text by Roman numerals (I-IV)

I

Bostrom EA, Forsblad D'Elia, Dahlgren U, Simark-Mattsson C, Hasséus B, Carlsten H, Tarkowski A, Bokarewa M

Salivary resistin reflects local inflammation in Sjögren's Syndrome
J. Rheumatology, 2008 Oct;35(10):2005-11.

II

Bostrom EA, Ekstedt M, Kechagias S, Sjöwall C, Bokarewa M, Almer SH

Resistin is elevated in autoimmune disease of the gastrointestinal tract reflecting ANA positivity
Submitted

III

Bostrom EA, Tarkowski A, Bokarewa M

Resistin is stored in neutrophil granules being released upon challenge with inflammatory stimuli
Biochim Biophys Acta. 2009 Sep 18

IV

Bostrom EA, Andersson S, Gustafson B, Ekwall AK, Eisler, T, Dahlberg L, Smith U, Bokarewa M

Resistin and insulin/insulin-like growth factor signaling in rheumatoid arthritis
Manuscript

Table of contents

List of abbreviations

ADSF	Adipocyte-specific secretory factor
AIH	Autoimmune hepatitis
ANA	Anti-nuclear antibody
AMA	Anti-mitochondrial antibody
CD	Crohn's disease
CRP	C-reactive protein
DC	Dendritic cell
DHEA	Dehydroepiandrosterone
FIZZ	Found in inflammatory zone
fMLF	N-formylmethionyl-leucyl-phenylalanine
FOCI	Focal lymphocytic infiltration
FPR	Formylated peptide receptor
GPCR	G-protein coupled receptor
GSK	Glycogen synthase kinase
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin growth factor
IHC	Immunohistochemistry
IL	Interleukin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histability complex
MPO	Myeloperoxidase
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NFkB	Nuclear factor kappa B
OA	Osteoarthritis
PBMC	Peripheral blood mononuclear cells
PI3k	Phosphatidyl-inositol 3-kinase
PPAR	Peroxisome proliferator-activated receptor
PSC	Primary sclerosing cholangitis
PTEN	Phoshatase and tensin homolog
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SCID	Severe combined immunodeficient
siRNA	Small interference Ribonucleic acid
SMA	Smooth muscle antibody
SS	Sjögren's syndrome
TLR	Toll like receptor
TNF	Tumor necrosis factor
UC	Ulcerative cholitis

Introduction

General introduction

Inflammation is the body's response to infection or tissue injury and is also a hallmark of a variety of complex human diseases. The inflammatory response can be directed at the hosts own tissues, which is the case in autoimmune disorders (*auto* referring to *self*). An inflammatory response can also be triggered by environmental factors, bacteria, or viruses. Inflammatory diseases are complex. Examples of inflammatory conditions are rheumatoid arthritis (RA), Sjögren's syndrome (SS), inflammatory bowel disease, asthma, lupus erythematosus, and hepatitis. The inflammatory process is mediated by immunoactive molecules such as cytokines, and specialized cells. Advances in pharmacological treatment of inflammatory diseases have led to the targeting of cytokines, with promising results. It is, however, important to identify and widen our knowledge of other immunomodulating molecules to better direct future treatment. Resistin is one such molecule.

In this thesis, my aim has been to clarify the role of resistin in inflammation and autoimmunity. Relevant background information on inflammation, cell types, and important molecules is given in the following sections to put the work of the thesis in context.

Inflammation

The inflammatory response is usually local and is characterized by the classical signs of *rubor, calor, tumor, dolor,* and *functio laesa*. During inflammation, different types of leukocytes leave the circulation and enter the affected tissues. The signs of inflammation occur in response to increased vascular permeability and also accumulation of immune cells and immune modulating substances. Cells active in inflammation are leukocytes of the bloodstream and tissue-resident cells such as macrophages. The cells function in response to immune-modulating substances such as cytokines.

Cell types in inflammation

Monocytes

Monocytes are antigen-presenting cells that constitute 5–10% of the circulating leukocytes. They originate from a myeloid precursor in the bone marrow, from where they transmigrate into tissues[1]. Monocytes are classified into two subsets

according to the presence of CD14 and CD16. The two subsets are heterogeneous in size, morphology, granularity, and surface antigen expression[2]. The $CD14^{+}CD16^{-}$ cells account for 90% of monocytes; they produce higher levels of cytokines and are more active in bacterial clearance than the $CD14^{+}CD16^{+}$ cells, which account for 10% of circulating monocytes[3]. The $CD14^{+}CD16^{+}$ cells are instead potent in antigen presentation. Monocytes express high numbers of toll-like receptors, TLRs, which are important in mediating host-protective immunity to external danger signals in the course of bacterial and viral infection[4].

Macrophages

Proinflammatory or metabolic stimuli give rise to the recruitment of monocytes into tissues where they differentiate into macrophages or dentritic cells. Tissue macrophages are phagocytic cells important for tissue remodeling, recognition of pathogens, and discrimination of pathogens host tissues. Phagocytosis of pathogens and apoptotic cells and also antigen presentation are the main tasks of the macrophage.

Dendritic cells

Dendritic cells (DCs) are monocyte-derived antigen presenting cells in the tissue. An important feature of DCs is the presence of major histobility complex (MHC) class II. DCs that reside in tissues are immature. In the immature state, they are incapable of activating T cells. Cytokines are involved in the maturation of DCs. During maturation, expression of MHC is upregulated and DCs induce primary immune T cell responses to antigens. After activation of T cells by DCs, they interact with other antigen presenting cells.

Neutrophils

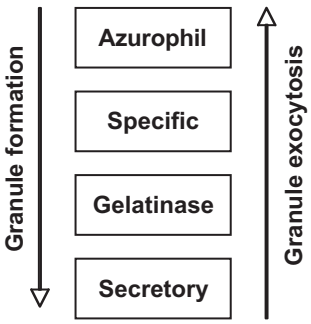
Neutrophils are phagocytic cells representing the major fraction of leukocytes in circulation (50–70%). These are short-lived cells that migrate into tissues when activated, which prolongs their lifespan[5]. When unactivated, neutrophils are cleared by macrophages. In contrast to macrophages, neutrophils are not antigen presenting cells.

Neutrophils are equipped with several receptors: the pathogen recognizing receptors (PPRs), complement receptors[6], TLR2 and TLR4[7, 8], and other receptors belonging to the G protein-coupled receptor family (GPCRs). The most studied receptor here is the formylated peptide receptor 1 (FPR1).

Formylated peptides are among the main activators of neutrophils. N-formyl-methionyl-leucyl-phenylalanine (fMLF) is one of the best known chemoattractants; it binds to FPRs leading to the release of proinflammatory cytokines, chemotaxis, phagocytosis and release of granular contents. [9-14]

Granules are membrane-sealed organelles containing degradative enzymes and antimicrobial agents. Traditionally, the granules have been classified as being peroxidase-positive (containing myeloperoxidase, MPO) or peroxidase-negative ones. However, granules are heterogeneous in content, function, and structure, and four subsets of granules are expressed in human neutrophils: the primary or azurophil granules, secondary or specific granules, gelatinase granules, and secretory vesicles[15]. During maturation, granules are formed in the order: azurophil, specific, gelatinase, and secretory granules. When activated, the granules are mobililized in the opposite order.

The granular proteins are responsible for bacterial killing and are involved in tissue damage during inflammation[15-18]. Azurophil granules are characterized by the presence of MPO, The specific granules have high content of lactoferrin and vitamin B12. The gelatinase granules are rich in gelatinase and lactoferrin whereas the secretory vesicles contain plasma proteins. The exact granule content of each subset is still not fully characterized.



T lymphocytes

T lymphocytes, or T cells, mediate cellular immune responses and regulate the functions of B lymphocytes, macrophages, and neutrophils through production of cytokines and cellular interactions. The main function of T lymphocytes is antigen recognition and presentation. Subsets of T cells are referred either to molecular markers or functional properties. Helper T cells (Th) are $CD4^+$ and recognize antigens in the context of MHC class II molecules. Th cells stimulate B cell differentiation into plasma cells or memory B cells through cytokine release and cell surface receptor-mediated interactions. Two classes of Th cells are defined by their cytokine secretion profile: Th1 and Th2. Cytotoxic T cells

are $CD8^+$ and recognize antigens in the context of MHC class I. Cytotoxic T cells are capable of lysing or inducing apoptosis in cells bearing MHC class I. Apart from this division there are memory T cells, regulatory T cells, and natural killer T cells. Regulatory T cells suppress inflammation in an antigen-specific manner. Therapies aimed at increasing the number of regulatory T cells have been proposed for treatment of rheumatoid arthritis (RA)[19] due to their induction of durable remission of inflammation.

B lymphocytes

The primary function of B cells is to produce antibodies. Plasma B cells, often called plasma cells, produce antibodies. Antibodies are composed of any of the five general isotypes of immunoglobulin and bind to antigens or pathogens, inactivating them or targeting them for elimination by phagocytic cells. B cells can also act as antigen presenting cells that activate autoreactive T cells. B cells respond to and secrete cytokines and chemokines.

NK cells

NK cells account for 10% of circulating lymphocytes. They are large granular lymphocytes that are capable of eliminating tumors in a non-MHC-dependent manner.

Fibroblasts

Fibroblasts are mesenchyme-derived cells. Fibroblasts are the most common cell type in connective tissue, their main function being to synthesize matrix constituents such as collagen. Fibroblasts can be activated by cytokines and are a source of several cytokines and growth factors.

Cytokines in inflammation

Cytokines are low-molecular-weight immunomodulating proteins. They are important messenger molecules in shaping an immune response to foreign or self antigens. The nomenclature previously referred to their cellular origin by name; adipokines (adipocytic origin), monokines (monocytic origin), lymphokines (lymphocytic origin) etc. Nowadays, they are more commonly referred to as being either pro- or anti-inflammatory (promoters versus suppressors of inflammation). Several cytokines are interleukins (IL) or interferons (IFN).

There are several cytokines with diverse functions:

i) Those that promote or mediate proinflammatory responses (e.g. TNF, IL-1, IL-8)
ii) Those that promote positive feedback for their own synthesis (e.g. TNF, IL-1, IFN)
iii) Those that downregulate macrophage activity (e.g. TGF-β, IL-10)
iv) Those that mediate activation of T cells and NK cells (e.g. IL-1, IL-12).

Several cytokines are implicated in the pathogenesis of autoimmunity and inflammation, TNF-α is the best described. At present, new effective immunomodulating treatments that block cytokine(s) are being used in the treatment of inflammatory and autoimmune conditions such as RA and Crohn's disease. Many more new target molecules/cytokines are currently undergoing preclinical trials as new treatment alternatives for autoimmune and inflammatory diseases.

TNF-α is considered the classical superior cytokine on top of the cytokine cascade. It is one of the earliest cytokines to be released by mononuclear cells and has diverse functions, both systemically and locally. TNF-α induces fever, induces acute-phase reactants, is a recruiter of neutrophils and monocytes to sites of infection, activates endothelial cells, and stimulates further secretion of chemokines and cytokines (IL-6, IL-8, IL-1). Furthermore, TNF-α is a growth factor for T and B cells. It is also a target molecule for treatment of autoimmune disease. Resistin levels are significantly reduced following TNF-α blockade in RA and inflammatory bowel disease (IBD) [20, 21].

IL-6 is mainly produced by mononuclear phagocytes, T cells, endothelial cells and fibroblasts. It is involved in both local and systemic inflammation as an inducer of fever, fatigue, and acute-phase reactants. Moreover, IL-6 induces proliferation of T cells and the terminal differentiation of B cells. In RA joints, IL-6 causes angiogenesis through induction of endothelial growth factors, leading to involution of the inflamed (hypertrophic) synovium. IL-6 is a target molecule for treatment of autoimmune disease.

IL-1 is a proinflammatory cytokine that exists in two forms: IL-1α and IL-1β, which possess similar biological activity. Both IL-1α and IL-1β are produced by macrophages, monocytes, dendritic cells, B cells, and activated T cells. IL-1 and TNF-α display synergistic actions and stimulate the release of each other, thus amplifying the cytokine cascade.

IL-8 is a chemokine produced by macrophages and neutrophils. The main function of IL-8 is recruitment of neutrophils to the site of inflammation.

MMPs, matrix metalloproteases, are enzymes that are capable of degrading the extracellular matrix such as the cartilage in the joints. In the joint, MMPs are secreted by synovial fibroblasts in response to proinflammatory stimuli and they have been implicated in joint destruction of the cartilage and subchondral bone.

Adipokines

Adipokines are peptides that are expressed at high levels in adipose tissue. These peptides are secreted by adipocytes or by infiltrating macrophages, fibroblasts, or monocytes, which constitute one-third of the cells in adipose tissue. The adipokine resistin is the subject of this thesis and it will be discussed in detail.

NFκB signaling

Nuclear factor kappa B, NFκB, plays a major role in the expression of genes that are central to inflammation, where it is the main transcription factor regulating cytokine production. NFκB is downstream of signaling through TLRs, where it plays major role in the immune system[22]. It regulates the expression of cytokines and growth factors. NFκB is implicated in many disorders such IBD[23] where steroids can reduce NFκB activity[24].

NFκB was first described as a B-cell specific factor; however, it is now clear that NFκB is expressed in all cell types[25]. It is dysregulated in inflammatory and autoimmune conditions. In RA, NFkB is implicated in the pathophysiology of joint inflammation through increased levels of cytokines that activate it. Efficient treatments of RA have been shown to inhibit the effects of NFκB. The NFκB pathway is composed of several related transcription factors, including the p65 and p50 subunits.

Resistin

Resistin (named after its ability to *resist insulin*) was originally described in 2000–2001 by three different research groups[26-28] as an adipocyte-secreted peptide involved in insulin resistance, type II diabetes and obesity. Resistin is also known as FIZZ3 (found in inflammatory zone) or ADSF (adipocyte-specific secretory factor), and belongs to the group of adipokines due to the protein being found originally in mouse adipose tissue. The initial studies in mice gave promising evidence for the role of resistin in the regulation of carbohydrate metabolism. In the transition to the study of human resistin, these properties of the protein were not evident and led to controversy about the role of resistin. Results regarding the possible metabolic role of resistin in humans are still inconclusive.

Molecular structure of resistin

Resistin is a cysteine-rich peptide hormone of 12.5 kDa that belongs to the resistin-like family (RELM). The resistin gene, RETN, is conserved in chimpanzees, dogs, cows, mice, and rats. Resistin is 108 amino acids long in humans and 114 amino acids long in mice. In the two species, the genes are present on different chromosomes, as the human gene is located on chromosome 19 and the murine gene is on chromosome 8[29]. The two proteins show similarities with respect to conserved cysteine residues. The first cysteine in the mouse protein (Cys22) is conserved, but is at position 26 (Cys26) in humans[30]. Human resistin can form a dimer through Cys26[31] and in line with this, murine resistin forms a dimer through Cys22[32]. Murine resistin exists in two isoforms: the more abundant high-molecular-weight hexamer and the low-molecular-weight monomer form. The potency in impairing the action of insulin differs between the two forms. Human resistin is present in a mixture of oligomer and trimer, which can change into monomer and oligomer form[33 34]. The oligomer shows more potent proinflammatory effects[33] and the amino acid sequence between residues 43 and 65 is suggested to carry the proinflammatory properties[35]. At the amino acid level, the two proteins exhibit 59% homology; at the mRNA level the nucleotide sequence identity is 64.4% whereas at the genomic DNA level this is reduced to 46.7%[36]. This reduction in gene homology mainly reflects the differences within the introns, where the identity is as low as 28.7%.

The expression and localization of resistin

The expression patterns of human and murine resistin are different[37]. Murine resistin is almost exclusively expressed in white adipose tissue[26], but it is also found in the pituitary gland[38], the hypothalamus[38], and in the circulation. In humans, only low levels of resistin are expressed in adipose tissue. In contrast, resistin expression in man is high in bone marrow, in the spleen, in lung tissue, and in the placenta; it is only scantily expressed in fat tissues of lean subjects[39]. Resistin is upregulated during monocyte/macrophage differentiation and mononuclear cells serve as the main source of resistin in humans.

Peripheral blood mononuclear cells (PBMCs) respond to proinflammatory stimuli, such as cytokine stimuli by IL-1β, IL-6, and TNF-α, by upregulation of resistin mRNA and resistin protein synthesis[40]. Microbial antigens (lipopolysaccharides) also induce resistin synthesis[41]. In addition, acute phase proteins (e.g. C-reactive protein) induce resistin mRNA and protein synthesis of resistin[42]. Intriguingly, resistin itself has pro-inflammatory properties by upregulating the synthesis of IL-6, TNF-α, and IL-12 in mononuclear cells[43, 44] and it has also been shown to trigger an inflammatory state in vivo[43].

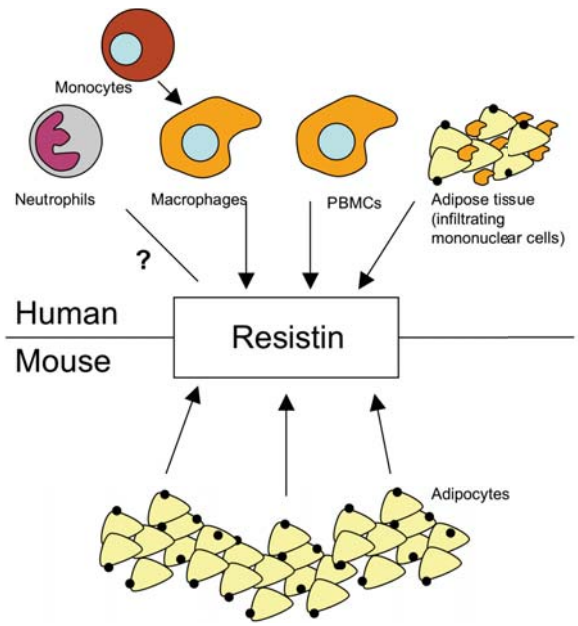


Figure 1. Patterns of expression of resistin in humans and mice. Human resistin is mainly expressed in mononuclear cells and murine resistin is almost exclusively expressed in adipocytes.

In humans, non-myeloid cell types have been tested for the presence of resistin. Pre-adipocytes and adipocytes are devoid of resistin mRNA expression[45], as are smooth-muscle cells[46], and in adipose tissue resistin is produced by infiltrating myeloid cells and not by adipocytes[47, 48]. Thus, human resistin has features similar to classical proinflammatory cytokines and has been shown to play a role in inflammation and immunity[49]. However, the regulation of such action is still not fully understood but it involves activation of the NFκB transcription pathway[43].

Receptor and signaling

Resistin was recently shown to mediate its proinflammatory effects via TLR-4[35]. Whether or not all the properties of resistin are mediated through activation of TLR-4 is as yet unknown. A search for additional receptor(s) mediating resistin activities is under way.

Resistin induces NFκB activity dose-dependently in PBMC nuclear extracts stimulated[35]. In addition, NFκB inhibitor has been shown to abrogate the proinflammatory effects of resistin[43] and blockade of IκBα, an inhibitor of NfkB, in monocytes leads to a reduction in the proinflammatory properties of resistin[42]. Induction of proinflammatory cytokines by resistin is mediated through complex formation by p65 and p50 subunits and translocation of these from the cytoplasm to the nucleus[44]. Moreover, resistin induces the phosphorylation of IκBα in hepatic stellate cells indicating NFκB activation[50].

PI3k/AKT signaling is an important mediator of cell proliferation in response to growth factors and other mediators. Resistin induces proliferation of smooth muscle cells through activation of PI3k/Akt where its effect is inhibited by PI3k inhibitor[51]. Resistin has also been shown to activate the mitogen-activated protein (MAP) kinase pathway[50 52]. There are three MAPK subfamilies, namely ERK, JNK, and p38 MAP. ERKs are activated by mitogens and growth factors, and JNK and p38 are activated by proinflammatory cytokines and cellular stress. Resistin phosphorylates ERK in hepatic stellate cells[50]. The MAPK subfamily p38 is related to proinflammatory cytokines and it has also been shown to be phosphorylated by resistin[52]. Taken together, this indicates that resistin has a role both in inflammation and growth.

Resistin has several features in common with proinflammatory cytokines. Like TNF-α and IL-1, resistin promotes inflammation through induction of a cytokine cascade of other cytokines[43]. This maintains an inflammatory state where more inflammatory cells are recruited and activated upon stimulation of

chemokines and cytokines. In contrast to TNF-α, however, resistin does not induce the suppression of adipose-specific markers suggesting that resistin's intracellular signaling pathway is distinct from that of TNF-α[45].

The proinflammatory effects of resistin are mediated via TLR-4[35]. Resistin-induced cytokine production by PBMCs is abolished by anti-TLR-4 antibodies[35]. Epithelial cells stably transfected with TLR-4 were found to respond to stimulation by resistin, which was not evident in untransfected cells or cells transfected with TLR-2[35]. The stably transfected TLR4-epithelial cells that were transfected with siRNA targeting TLR-4 lost their ability to respond to resistin stimulation[35]. SiRNA targeting myeloid differentiation factor 88 (Myd88), which is downstream of TLR-4, led to similar results. The part of resistin molecule from amino acid 43 to amino acid 64 has properties similar to that of the whole resistin lomecule, suggesting that this part is active in binding to TLR-4[35].

Peroxisome proliferator-activated receptor γ, (PPARγ) is a ligand-activated nuclear receptor that regulates gene transcription and is the molecular target of anti-diabetic drugs (e.g. Thiazolidinedione, (TZD)). PPARγ negatively regulates resistin expression[36]. Treatment with the PPAR agonist Rosiglitazone decreased resistin mRNA expression as much as 80%. The decrease was also observed at the protein level[53]. Moreover, statins reduce resistin expression in diabetic patients and to downregulate its expression in PBMCs[42, 54]. Resistin expression is significantly reduced following TNF-α blockade in RA and IBD patients [20, 21].

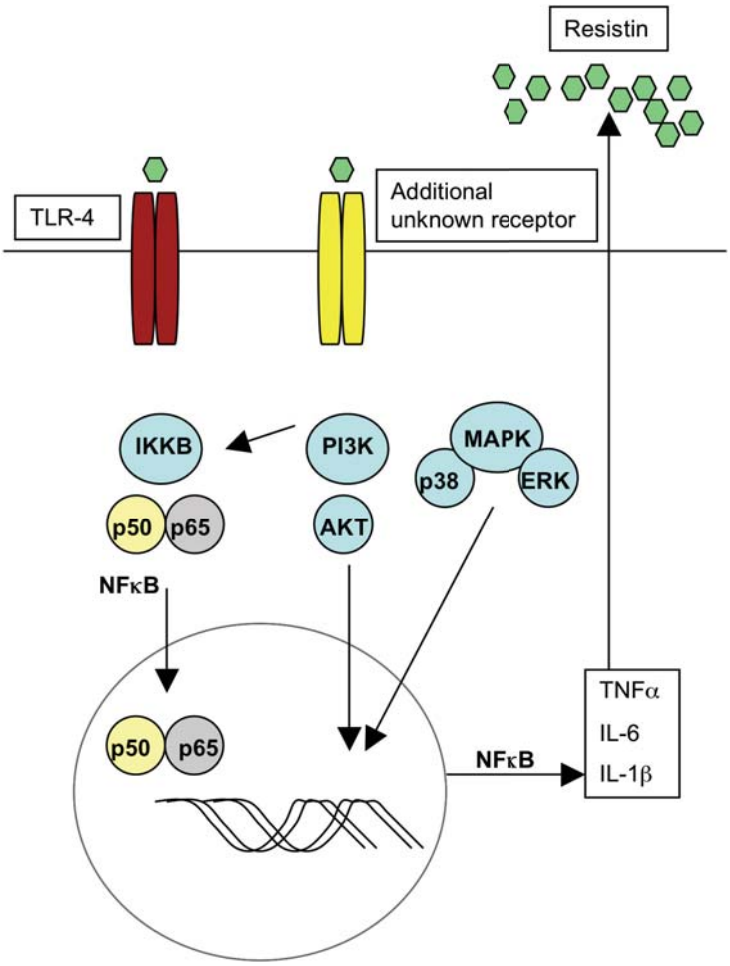


Figure 2. Resistin signaling in humans. The proinflammatory effects of resistin are mediated via TLR-4. Resistin activates the NFκB signaling pathway and leads to movement of the p65 subunit to the nucleus. No additional receptor(s) have been identified. Resistin has also been shown to activate PI3k/Akt signaling and MAPK signaling.

Resistin induces chemotaxis of $CD4^+$ T cells[55]. The chemotactic effects of resistin on T cells have been suggested to be mediated by a pertussis toxin-sensitive G-protein-coupled receptor. Downstream activation of Src kinase by resistin was observed, leading to activation of PI3k[55]. In inhibiting Src kinase, resistin-induced migration was also inhibited[55]. Apart from this, little is known about the interaction between resistin and T cells.

The NFκB pathway is used in mononuclear cells, but also in adipocytes. Activation of TLR-4 in adipocytes generates insulin-resistant adipocytes[56], and TLR-4 has been suggested as a link between inflammation and insulin resistance. Mice deficient in TLR-4 are protected from fatty acid-induced insulin resistance, suggesting that the innate immune system is involved in the regulation of insulin sensitivity[57].

Insulin and insulin growth factor signaling pathway

Insulin and IGFs are members of a family that include insulin, relaxin, and several other insulin-like peptides. IGF-1 and IGF-II have about 50% amino acid homology to insulin[58]. Circulating IGF-1 is mainly secreted by the liver and other tissues in a paracrine or autocrine manner. IGFs have been implicated in RA and other growth factor disorders, and may be at the borderline between inflammation and metabolism in RA[59]. In RA, IGF-1 stimulates synthesis of cartilage and bone extracellular matrix protein[60]. Resistin is upregulated in rodent models of diabetes, obesity and insulin resistance[26] and due to the simularities between insulin and IGF there is a possibility that resistin could affect growth.

The IGF-1 receptor is structurally related to the insulin receptor[61]. Like the insulin receptor[62], it undergoes ligand-induced phosphorylation, thus increasing the kinase activity, and further phosphorylates a number of substrate proteins resulting in growth or metabolic effects[63]. IGF-1 and insulin can bind to each other's receptors but with lower affinity and effect[64]. IGF-1 is active in mitogenesis and differentiation, inhibits apoptosis, and elicits insulin effects in most tissues of the body[65]. Most of the circulating IGF-1 is bound to binding proteins which modulate the levels of free IGFs[66].

Proinflammatory cytokines can induce a state of hormone resistance to insulin or IGFs. In hepatocytes, resistin induces insulin resistance through downregulation of insulin receptor substrate 2 (IRS-2) and upregulation of GSK-3b[67], and via induction of SOCS3 and supression of Akt phosphorylation[68]. In a humanized mouse model, macrophage-derived human

resistin was found to increase serine phosphorylation of IRS-1 in white adipose tissue, resulting in an insulin-resistant state[69]. In addition, insulin resistance of skeletal muscle was observed[69]. IGFs are expressed in RA synovium[59] and shown to contribute to the proliferation of synovial, and possibly in repair mechanisms of joint injury. High expression of IGFs is associated with low inflammation in the joints[70] and levels of IGF-1 are depressed in patients with RA[71]. The effect of resistin on IGF signaling is still unknown.

Resistin in inflammatory disease

Elevated levels of resistin in man are frequently found in association with inflammation and autoimmune disease. In this thesis, I concentrate on the role of resistin in rheumatic diseases and diseases of the liver and gastrointestinal tract.

Rheumatoid arthritis

Resistin has been implicated in RA by several research groups[43, 72, 73, 80]. RA is an progressive autoimmune disease characterized by a massive infiltration of leukocytes into synovial tissue, resulting in synoviocyte proliferation, cartilage injury, and bone erosion—possibly leading to further damage of the joints[74]. RA may affect movable joints, where the opposing bone surface is covered with cartilage, where there is a joint cavity containing synovial fluid, and where the joint cavity is lined by synovial membrane and a fibrous capsule. RA affects 0.5–1% of the population in the ration 3:1 women:men, and is associated with significant morbidity and increased mortality[75, 76]. Many cells participate in generating immunologically driven inflammation of the joints. T cells migrate into the synovium, where they recognize antigen presentation by monocytes, macrophages, and DCs. Normal synovium is almost an acellular structure covered by a thin lining of synoviocytes. In RA, the inflamed synovium is infiltrated by macrophages, T cells and B cells into the sublining layer. DCs are abundant in the synovial fluid of RA patients but not in other arthritic conditions. Synovial fluid is rich in several proinflammatory cytokines that may contribute to the maturation of DCs in situ.

Cell activity is mediated by functional cytokines and blockade of these has led to the development of effective new treatments for RA[77 78, 79]. Diagnosis of RA is based on several criteria, at least four of which have to be fulfilled for definitive diagnosis (Table 1).

Table 1. The revised ACR criteria (1987) for classification of RA

Criteria	Symptom
I	Morning stiffness in and around joints lasting at least 1 hour before maximal improvement
II	Arthritis of three or more joint areas
III	Arthritis of proximal interphalangeal, metacarpophalangeal, or wrist joints
IV	Symmetrical arthritis
V	Rheumatoid nodules
VI	Presence of rheumatoid factor
VII	Radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints

Resistin is present in the synovium in both RA and osteoarthritis (OA); however, its presence is more pronounced in the sublining layer of the RA synovia. Resistin is expressed in synovial fibroblasts, macrophages, B cells, and plasma cells however but not in T cells of the synovial tissue[73]. Resistin levels are elevated in the synovial fluid of RA patients compared to controls with non-inflammatory joint diseases[43, 73] and they are associated with elevated TNF-α, IL-6, disease activity, acute-phase reactants (CRP, IL-1Rα), and erythrocyte sedimentation rate[72, 80]. Expression of the resistin gene is upregulated in PBMCs [40]. Moreover, circulating resistin has shown correlations with levels of CRP, IL6 and TNF receptor 2[81]. In traumatic joint injury, resistin has a direct effect on cartilage matrix turnover[82]. Taken together, the data available support the hypothesis that resistin is a modulator of inflammation with potent regulatory functions. A possible metabolic role of resistin in RA has also been suggested[80]. This possible duality of possible roles of human resistin in inflammation and metabolism, studied in the context of rheumatism, could help to explain some of the previous controversies in the field.

Sjögren's syndrome

Sjögren's Syndrome (SS) is an autoimmune disorder that affects 0.5–1% of the world's population, predominantly women. The hallmarks of disease are dry eyes and dry mouth due to lymphocytic glandular exocrinopathy. One-third of patients develop extraglandular manifestations, including malignant lymphoma[83]. The disease is either present alone (primary) or in association with other connective tissue disease(s) (i.e. secondary). Autoantibodies directed at ribonucleoprotein molecules Ra/SSA and La/SSB are associated with a higher rate of extraglandular manifestation (cutanous, respiratory, renal, hepatic, vascular, and neurological)[84]. The majority of mononuclear cells that infiltrate tissues of exocrine glands in SS are $CD4^+$ T cells; however, B cells account for 20% of all cells in salivary tissue[85]. Furthermore, epithelial cell activation has been proposed in the pathological process, with increased expression of class II antigens[86], from its ability to produce cytokines[87]. A pathogenic role of cytokines and other signaling molecules that regulate the interaction between subsets of T cells and induce inflammation has been proposed[88]. The role of resistin in SS, if any, has not been explored.

Diagnosis of SS is based upon the criteria listed in Table 2. The diagnosis of primary SS is based on (i) the presence of any 4 of a total of 6 criteria (this is indicative as long as either the histopathology or the serology is positive) or (ii) the presence of any 3 of a total of 4 objective criteria (III–VI). Possibly associated diseases in combination with the presence of criterion I or II plus any of the criteria III–V is indicative for diagnosis of secondary SS. The histopathology is based on focal lymphocytic sialoadenitis where > 50 lymphocytes per 4 mm^2 gives a focus score of 1. This criterion is considered the single most important test of an oral component in SS.

Oral manifestations of disease are common, including increased risk of caries, fungal infections, dry mucosa, and depappilated tongue. Salivary electrolyte and protein content are changed in SS where Na^+,Cl^-, IgG, lysozyme, and MMP levels are elevated. Furthermore, the expression patterns of proinflammatory cytokines are altered in SS, e.g. TNFα, IL-6, and IL1 levels are elevated in saliva and in glandular tissue.

Table 2. The American-European revised classification criteria for Sjögren's Syndrome (2002)

	Classification criteria
I. Ocular symptoms	Daily, persistent, troublesome dry eyes for more than 3 months Recurrent sensation of sand or gravel in the eyes Use of tear substitutes more than 3 times a day
II. Oral symptoms	Daily feeling of dry mouth for more than 3 months Recurrently or persistently swollen salivary glands as an adult Frequent intake of liquids to aid in swallowing dry food
III. Ocular signs	Schirmer's I test, performed without anesthesia (<5 mm in 5 minutes) Rose Bengal score or other ocular dye score (>4 according to van Bijsterveld's scoring system)
IV. Histopathology	In minor salivary glands (obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score of > 1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm^2 of glandular tissue
V. Salivary gland involvement	Unstimulated whole salivary flow (< 1.5 ml in 15 min) Parotid sialography showing the presence of diffuse sialectasias (punctate, cavitary, or destructive pattern), without any evidence of obstruction in the major ducts Salivary scintigraphy showing delayed uptake, reduced concentration, and/or delayed excretion of tracer
VI. Autoantibodies	Antibodies to Ro(SSA) or La(SSB) antigens, or both

Diseases of the liver and gastrointestinal tract

The role of inflammatory cytokines, in particular TNF-α[89, 90] in liver disease, has been investigated. Resistin has also been implicated in non-alcoholic fatty liver disease (NALFD), non-alcoholic steatohepatitis (NASH), Chrohn's disease (CD), and ulcerative colitis (UC). Part of this thesis was to evaluate the role of resistin in autoimmune hepatitis (AIH), UC, CD, primary sclerosing cholangitis (PSC), and NAFLD. Of these, PSC and AIH are considered the major autoimmune liver diseases.

Resistin is expressed in hepatocytes and hepatic stellate cells[50]. In addition, hepatic stellate cells respond to resistin stimuli by increased expression of proinflammatory cytokines and NFkB activation[50]. In cirrhosis of the liver, there is a correlation between resistin and both TNF-α and free fatty acids[91]. Cirrhosis patients who have undergone transplantation have a reduced degree of insulin resistance that is not associated with a change in resistin levels[91]. Recognition of circulating antibodies to specific liver antigens is used in the diagnosis of liver and gut diseases; these antibodies are associated with more severe disease and thus suggest an unfavorable prognosis.

AIH is a progressive, chronic inflammation of the liver of unknown cause. Histologically, the appearance is similar to that of chronic hepatitis; however, they can be differentiated by the patient's response to immunosuppressive drugs. The suggested pathogenesis of AIH postulates that an environmental agent triggers a cascade of T cell-mediated events directed at liver antigens. This leads to an inflammatory and fibrotic process in the liver. Biochemical markers of AIH are aminotransferase elevations, elevated serum gamma globulins, and development of autoantibodies where the main serologic markers of type 1 AIH are anti-nuclear antibodies (ANA) and smooth muscle antibodies (SMA)[92]. Dysregulation of T cell-specific cytokines is present in AIH, where IL-2 and IL-4 levels are reduced whereas IL-1β, IL-6, TNF-α, IFN-γ levels are increased. The pathogenic role and possible mechanisms of autoantibodies in the pathogenesis of AIH are still unclear; however, they are important diagnostic tools. The role of adipokines in AIH was recently studied. Among these, there was found to be a correlation between resistin levels and elevated levels of aminotransferases, bilirubin, and TNF-α[93].

CD and UC are two chronic inflammatory conditions of the gastrointestinal tract, collectively referred to as inflammatory bowel disease (IBD). Cytokines have been implicated in the pathogenesis of the disease, and they have also been proposed in its diagnosis and as therapeutic targets in IBD[94, 95]. Resistin is

elevated in IBD[96]. Moreover, elevated levels correlate to CRP levels, disease activity, and white blood cell count[97].

PSC is a chronic liver disorder characterized by inflammation and fibrosis of the bile ducts, progressing to cirrhosis/end-stage liver disease. It is commonly associated with IBD. The etiology of PSC is not fully understood. No effective medication for halting the progression is presently known, and little is known about the pathogenesis. To our knowledge, no previous publications have dealt with the study of resistin in PSC.

Autoantibodies

Autoantibodies at high titers are indicative of autoimmune disease—in RA, SS and several pathological conditions of the liver and gastrointestinal tract. Autoantibodies are immunoglobulins directed against self antigens that attack self tissues and cause inflammation and tissue damage. The role of autoantibodies in autoimmune processes is unknown, but they are useful in the diagnosis. In RA, rheumatoid factor (RF) is the most relevant antibody. RF is present in 75–90% of RA patients. RA patients seropositive for RF tend to develop a more severe and progressive form of joint disease than seronegative patients[98, 99]. The most common autoantibodies in SS are those directed against the ribonucleoproteins Ro/SSA and La/SSB with high prevalence in patients[100, 101]. Several autoantibodies can be detected in patients with liver and gastrointestinal diseases. The most strongly associated ones are the ANAs, anti-mitochondrial antibodies (AMAs), and SMAs. ANAs are antibodies directed against the cell nucleus, and are present at the highest levels in AIH and PSC.

Resistin in metabolic conditions

Resistin expression is upregulated in rodent models of diabetes[102] and obesity[26], indicating its role in these diseases. Resistin inhibits insulin-mediated reduction of gluconeogenese. Moreover, glucose and fatty acid uptake in skeletal muscle is inhibited. Resistin knock-out mice have low blood glucose levels whereas high resistin disturbes glucose homeostasis. When treated with monoclonal anti-resistin antibodies, the condition in these animals becomes stable.

Several groups have failed to find any relationship between resistin and obesity, insulin resistance, or type 2 diabetes in man[34, 103-105]. Others have found a relationship between resistin and these conditions[106-112]. Thiazolidinedione treatment suppresses resistin production in patients with diabetes, and also suppresses resistin secretion from monocytes and macrophages in vitro[113, 114]. Chronic inflammation has been proposed to be important in obesity-related insulin resistance[115], and there is a correlation between resistin and inflammatory markers in patients with metabolic diseases[112, 114]. In a novel transgenic humanized mouse model human monocytes/macrophage-derived resistin was found to exacerbate insulin resistance in diet-induced obesity[69]. Moreover, inflammation of white adipose tisssue and free fatty acid release are increased by resistin[69]. Taken together, these results suggest that resistin does indeed have some metabolic effects. The balance between resistin functions in inflammation and in carbohydrate metabolism in man requires further study.

NAFLD is associated with metabolic syndrome. NAFLD ranges from steatosis (simple fatty infiltration), steatohepatitis (NASH), to cirrhosis (irreversible scarred liver) and end-stage liver disease, resulting in transplantation or morbidity. Elevation of liver enzymes is a hallmark of the disease. Resistin has been studied in NAFLD with contradictory results. Some authors have reported elevated resistin levels in patients where resistin was related to severity and not to insulin resistance[116], whereas others have failed to demonstrate any difference between NAFLD patients and healthy subjects[117, 118].

Resistin in pathological conditions

Resistin has been studied in several pathological conditions, as listed in Table 3.

Table 3. Summary of resistin in various pathological conditions

Pathology	**Resistin activity**	**Ref**
Rheumatoid arthritis	↑ proinflammatory cytokines Highly expressed in synovial fluid Induces arthritis-like inflammation in vivo Is decreased by anti-TNF therapy Suggested as therapeutic target	43, 73
Bone metabolism	↑ IL-6 ↓ RANKL/OPG Correlates to marker of bone metabolism but not to BMD	119, 120
SLE	Correlated to inflammation	121
Atherosclerosis	↑ proinflammatory cytokines Predictive factor for coronary atherosclerosis Correlates to markers of cardiac injury	81, 122-124
Insulin resistance, T2D, obesity	Humanized mouse model: exacerbates WAT inflammation and contributes to IR Unclear if elevated or unchanged levels Not correlated to metabolic parameters Resistin in adipose tissue of morbidly obese patients is higher than lean subjects	69, 125-127
Inflammatory bowel disease	↑ proinflammatory cytokines Highly expressed in both CD and UC Suggested as therapeutic target	94, 95
Chronic kidney disease	↑ proinflammatory cytokines Marker of disease severity associated with rate of glomerular filtration and inflammation status	128, 129
Asthma	Highly expressed Marker of disease severity	130
Non alcoholic liver disease	↑ IL-8, MCP-1 in hepatic stellate cells Unclear if elevated or unchanged levels	50, 116-118
Malignancies	Elevated in breast cancer, lymphoma Possibly involved in tumorigenesis by inducing MMPs, VEGFR and reducing TIMPs	131-133

Resistin and intervention

Endogenous cytokine inhibitors of several cytokines exist; thus, the approach of using cytokine inhibitors as therapeutic agents for inflammatory disease was launched and has been used as a therapy since 1992, with clinical success[77]. There is overlap in cytokine function; this gives difficulties in establishing which of them may be suitable targets. The strategy for cytokine blockade concerns how superior a cytokine is and how specific its action is. Resistin has immunoregulatory properties by inducing the NFκB-dependant cytokine cascade, which suggests that resistin also has superior characteristics such as those of TNF-α, IL-1, and IL-6. Moreover, resistin is negatively associated to several conditions. This in combination with its ability to mediate inflammation makes is an attractive therapeutic target.

There are several possibilities for resistin targeting therapies. As an extracellular molecule, it is available for treatment with monoclonal antibodies or soluble receptors that can bind to the secreted protein. Antibodies to its receptor are a second possibility. Extracellular approaches are somewhat less harmful than intracellular approaches regarding possible side effects. Intracellular targeting of downstream molecules by specific inhibitors is also possible. Intervention at the transcriptional level by the use of siRNA is another possibility. In combination with lentivirus-based techniques, this method could provide a durable suppression of resistin.

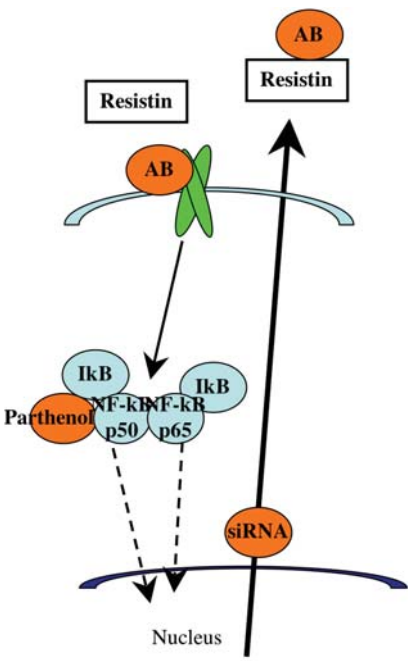


Figure 3. Some possibilities for intervention.

There are patients who do not respond to today's cytokine-targeting treatments. In RA patients, 20-40% only show a partial response or completely fail to respond to anti-TNF-α treatment[134]. These patients show low levels of TNF-α in synovial tissue biopsies suggesting that they might have a different cytokine profile or have a disease driven by other mechanisms[134].

Several pharmacological strategies have been suggested to modulate resistin. Monoclonal anti-TNF-α antibodies reduce resistin levels in RA and IBD patients[20, 21]. Moreover, anti-diabetic drugs lower resistin in overweight women, in patients with type II diabetes, and in macrophages in vitro. They have also been shown to reduce bone erosions in experimental arthritis[54].

It is tempting to picture resistin as a possible target molecule; however, there are still many questions that must be addressed. Some of them are addressed in the Results section of this thesis.

Aim

The aim of this thesis was to investigate the role of resistin in inflammatory conditions, with emphasis on rheumatic diseases and diseases of the liver and gastrointestinal tract.

In particular, it was important to determine:

- the extent to which resistin is present in patients with Sjögren's syndrome, autoimmune hepatitis, primary sclerosing cholangitis, ulcerative colitis, Crohn's disease, non-alcoholic fatty liver disease, and also the association between resistin and local inflammation, clinical characteristics of the disease, and metabolic parameters
- whether the neutrophil is a potential source of resistin
- the possible role of resistin in insulin-like growth factor signaling, by the use of an in vivo, orthotopic arthritic mouse model.

Methodology

The purpose of this section is to provide a brief overview of the materials and methods used in the work of this thesis. In more detail this is described in the papers and manuscripts of the thesis.

Patients

Study I

Blood and saliva was collected from 37 patients with primary SS (pSS) fulfilling the American-European diagnostic criteria[135] and 32 age- and sex matched healthy controls. Resistin content in the samples was related to the intensity of sialoadenitis in biopsies of minor salivary gland tissues where resistin expression also was visualized. Intensity of sialoadenitis was graded by the infiltration of lymphocytes per $4mm^2$ where above 50 lymphocytes in $4mm^2$ corresponded to focus score 1. Stimulated saliva was collected during 5 minutes during stimulation by chewing paraffin. Patients were also enrolled in a double blind placebo controlled cross-over study with dehydroepiandrosterone (DHEA) substitution.

Study II

Blood samples of 46 patients diagnosed with AIH, 104 with IBD (53 UC and 51 CD), 27 with PSC, 50 with NAFLD and 40 healthy controls were collected and evaluated for resistin content in relation to autoantibodies.

Study III

Peripheral blood neutrophils were isolated from buffy coats to evaluate the neutrophil as a possible source of resistin. Surface resistin was evaluated by flow cytometry in cells of synovial samples of 6 patients; 4 RA according to criteria of the American College of Rheumatology[136] and 2 spondyloartritis, and in blood of 9 healthy donors. Intracellular resistin was evaluated after subcellular fractionation followed by ELISA, SDS-PAGE and mass spectrometry.

Study IV

The possible effect of resistin on IGF signaling was evaluated. Levels of resistin and IGF-1 were measured in blood and synovial fluid of 60 RA patients and in blood and synovial fluid of 39 patients with non-inflammatory joint diseases as controls. Synovial tissue was obtained from 3 RA and 3 OA

patients during joint replacement surgery and subjected to histological evaluation and in vivo transplantation onto severe immunodeficient (SCID) mice.

Animals (IV)

The differences between human and murine resistin limits the use of established arthritis mouse models or knock-out models. Therefore, in study IV, we used the orthotopic model of synovial tissue transplantation as described by Rendt et al[137] and by Sack et al[138]. We engrafted fresh human synovia, obtained during knee replacement surgery, subcutaneously onto the back of SCID mice (lacking B and T cells). Following 2 weeks of implantation, the synovia was subjected to anti-resistin therapy using siRNA targeting resistin versus non-targeting. Synovia obtained from one patient were transplanted to at least two mice subjected to resistin targeting RNA and control RNA (mock). The mice were kept in a pathogen free environment at the animal facility of the Department of Rheumatology and Inflammation Research, University of Gothenburg, Sweden and the requisitions of National Board for Laboratory Animals were followed.

Resistin measurements (I, II, III, IV)

Concentrations of resistin in blood and saliva (I), in blood (II), in supernatants, blood and synovial fluid (IV), and in neutrophil fractions (III) were determined by a sandwich ELISA. In study II where resistin was measured in neutrophil granule fractions the ELISA was adjusted due to the fact that MPO and alkaline phosphatase (ALP), enzymes enriched in neutrophil fractions, often are used in the final step of an ELISA and possibly could influense the results. To diminish such possible samples were diluted in Na- azide (MPO inhibitor) and resistin complex formation was visualized by two independent development systems - employing Streptavidin-HRP and Streptavidin-ALP conjugates. The obtained absorbance values were compared with serial dilution of recombinant human resistin.

Cell culture (I, III, IV)

PBMC were prepared from healthy donors by a density gradient. Human promyelocytic leukemia cells, HL60, obtained from the American Type Culture Collection were incubated with DHEA in order to assess a possible change in resistin synthesis (I). Fibroblast cell line MRC-5 was used in studies of resistin and IGF-signaling (IV). To study neutrophils as a possible source of resistin, peripheral blood neutrophils, isolated from buffy coats, were subjected to subcellular fractionation. Subcellular fractionation of neutrophils was

performed by incubation with the serine protease inhibitor diisopropyl fluorophosphate, pelleted, resuspended in disruption buffer and disrupted by nitrogen cavitation. The post-nuclear supernatant was collected after centrifugation and was applied on top of a Percoll gradient.

To separate azurophil granules, specific granules and secretory vesicles/plasma membrane, the postnuclear supernatant was layered on top of a two-layer Percoll gradient. After centrifugation, the three distinct fractions containing subcellular organelles appeared. To generate better separation of specific granules from gelatinase granules, a three-layer gradient was used. The distribution of subcellular organules in the fractions was analyzed by marker analysis (the plasma membrane/secretory vesicles marker alkaline phosphatase, the specific granule marker vitamin B12-binding protein and azurophilic granule marker myeloperoxidase).

Histological evaluations (I, III, IV)

Minor salivary gland tissues (I) and human synovial tissues (III, IV) were prepared and cut in 4 μm sections. Salivary gland tissues and synovial tissues were incubated with rabbit anti-human resistin abs and washed with PBS. Salivary gland tissues were incubated with secondary alkaline phosphatase-coupled anti-mouse antibodies and NBT/X-phosphate as substrate and synovial tissues were incubated with biotinylated secondary goat anti-rabbit IgG antibodies followed by Vecstrastain Elit ABC kit as substrate. Sections were counterstained with hematoxylin.

Flow cytometry (II)

Leukocytes were prepared from blood and synovial fluid by lysis of erythrocytes in sedimented cell pellets with NH_4Cl followed by washing with PBS containing 1% FCS and 0.5mM EDTA. The leukocytes were incubated with biotin-labeled anti-resistin antibodies or mouse IgG2B isotype control antibodies. Anti-CD16, anti-CD3 and anti-CD14 were used to separate the leukocyte populations.

SDS-PAGE and Western blot (III)

The presence of resistin in neutrophil fractions was evaluated by Western blot. Samples were loaded on 15% SDS-RAGE gel and transferred to nitrocellulose membranes by electroblotting. After blocking, incubation with monoclonal anti-resistin antibodies and HRP-conjugated anti-mouse IgG resistin was visualized by chemiluminesence.

Masspectrometry (III)

Following SDS-PAGE of fractions representing the azurophil, the specific and the gelatinase granules, pieces of gel were cut out, precipitated with anti-resistin antibodies and submitted to mass spectromic analysis. In short, proteins were tryptic digested in-gel, extracted peptides analysed by nano-LC ESI mass spectrometry. Data were searched against Swissprot protein database. The observed peptide mass and fragmented spectra were matched against theoretical peptides and combined into protein hits.

Real-time PCR (IV)

Total RNA was extracted from MRC-5 cells and transplanted tissues, and concentration was assessed spectrophotometrically. Gene expression was measured with TaqMan real-time PCR. The RT-PCR reaction was performed using 100nM probe, 200nM of forward and reverse primers and 10ng total RNA in a final volume of 20μl.

Ethical considerations (I-IV)

The studies I, III, and IV were approved by the Ethical Comittee of Gothenburg University, Sweden and study II by the Ethical Committee at the University Hospital of Linköping, Sweden. When including patient samples, written informed consent was obtained.

Statistics (I-IV)

Throughout the thesis non-parametric statistical methods, Mann Whitney U-statistics, are used in comparisons between groups. When having small groups to compare this is the most reliable test. Correlation between different variables in patients was performed by Spearman rank correlation test. All values are expressed as mean± standard error of the mean (SEM). $P<0.05$ is considered statistically significant.

Results

Salivary levels of resistin reflect the intensity of local inflammation in salivary glands of SS patients

The targeting of self tissues in autoimmune diseases can either be systemic or local. Resistin has proinflammatory properties through activation of the NFκB-dependent cascade and mononuclear cells have been identified as a local source of resistin[43]. The first objective of this thesis was to study the role of resistin at the site of inflammation, namely in the saliva and salivary gland tissue of patients with primary SS (pSS).

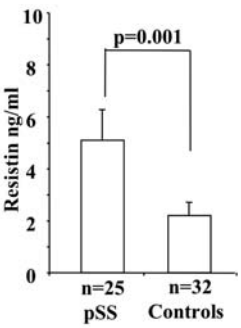


Figure 4. Resistin levels in saliva of pSS patients and controls.

Circulating and salivary resistin was measured in order to study the role of resistin in local inflammation. As shown in Figure 4, salivary resistin was 2.5 times higher in pSS patients than in controls. No difference in resistin levels was observed in serum samples, indicating that resistin has more of a local impact than a systemic one.

Possible explanations for accumulation of resistin in saliva were considered by evaluating the morphology of salivary gland biopsies as seen in Figure 5. Resistin was present in glandular tissue of pSS patients but it was only scantly expressed in controls (control tissue shown in manuscript I). Lymphocytic foci and infiltrating mononuclear cells were found to express resistin. Moreover, epithelial cells expressed resistin (scant expression in epithelium was also observed in controls); they possibly serve as an additional source of resistin in the absence of inflammation. In accordance with this, we observed high levels of resistin expression at the local site of inflammation in synovial tissue of RA patients in paper III.

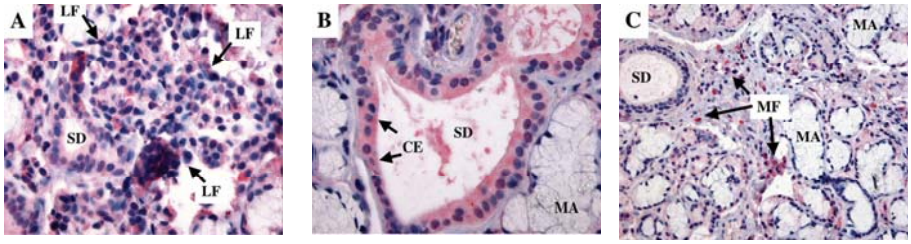


Figure 5.

Resistin expression in minor salivary glands of patients with pSS. Resistin is stained red. **A.** Resistin is expressed in mononuclear cells within the lymphocytic focus. **B**. Resistin is expressed in the epithelium within striated ducts. **C**. Monunuclear cells infiltrating stromal tissue are stained for resistin. The histology is previously published in paper I[139].

In order to evaluate a possible relationship between resistin and grade of inflammation in salivary glands, resistin was analyzed in relation to morphological findings in biopsies of salivary gland tissues. Patients with high-grade inflammation (> 1 foci/4 mm^2) were found to have significantly higher resistin levels than those with low-grade inflammation (< 1 foci /4 mm^2) ($p = 0.023$), see Figure 5.

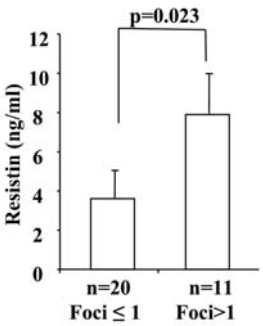


Figure 6. Resistin levels in pSS patients according to the presence of high or low focal inflammation (foci).

One of the hallmark symptoms of SS is inability to produce saliva. To study the possible effects of DHEA on resistin levels, pSS patients were enrolled in a double-blind placebo-controlled crossover study of DHEA over a total period of 9 months. The levels of resistin were measured before treatment, during the washout period, and after placebo. Resistin levels were unchanged following DHEA treatment. One positive clinical outcome was a slight increase in stimulated saliva volume. In vitro studies performed in parallel with the clinical study supported the observations in patients. In vitro treatment of myelocytic cells with DHEA induced resistin levels slightly. Taken together, our findings did not support the hypothesis of a potential effect of DHEA in modulation of resistin in patients with pSS.

In summary, based on measurements of resistin, grading of focal lymphocytic infiltration, and staining of salivary gland tissue, we conclude that resistin is expressed in glandular tissue and in the saliva of pSS patients. Patients with high focal inflammation had highest resistin levels.

Resistin levels reflect systemic inflammation and are associated with the presence of anti-nuclear antibodies

In paper I, no change in circulating resistin was observed between pSS patients and controls. In RA and SS, the affected tissues—mainly the exocrine glands and joints—are rather small areas. In other diseases such as those of the liver and the gut, however, the affected area is larger. Thus, a possible effect of resistin in these diseases could possibly be observed as elevated circulating resistin levels. Indeed, previous studies have suggested that circulating resistin may have a role in IBD and NAFLD, where elevated circulating resistin levels are observed[94, 97, 116].

In paper II, we broadened the spectrum of inflammatory diseases and our objectives were (i) to determine the presence of resistin in AIH, PSC, UC, CD, and NAFLD, and (ii) to determine the possible relationship between resistin and both disease characteristics and autoantibodies.

Resistin levels are illustrated in Figure 7. They were significantly elevated in AIH, PSC, and CD as compared to NAFLD. The highest levels of resistin were detected in PSC patients who had levels that were almost three times above the levels of NAFLD patients. We analyzed resistin levels between those patients with hepatobiliary inflammation (AIH and PSC) and those with IBD (UC and CD). Patients with hepatobiliary inflammation had significantly higher resistin (15.6 ± 1.8 ng/ml) than IBD patients (12.4 ± 1.6 ng/ml) (p = 0.013).

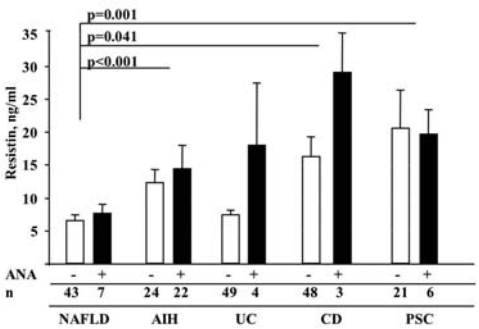


Figure 7. Resistin levels in diseases of the liver and gastrointestinal tract according to the presence of anti-nuclear antibodies. Figure as shown in manuscipt II.

As resistin is a modulator of inflammation, we evaluated possible associations with clinical inflammation data. Resistin levels were correlated to those of inflammatory parameters (IL-6, IL-8, and IgM), supporting its role as an immunomodulating molecule. Since resistin has been suggested to reflect disease severity, we investigated an association between resistin and disease activity. However, no difference in resistin levels was observed between patients with active disease and those in clinical remission. No correlation was found between resistin levels and the levels of liver enzymes.

We evaluated the relation between resistin and the presence of autoantibodies. Figure 6 shows resistin levels according to the presence (black bars) or absence (white bars) of ANA. Overall, resistin levels were significantly higher in ANA-positive patients than in ANA-negative patients (16.9 ± 2.7 ng/ml versus 12.8 ± 1.4 ng/ml; p=0.025, Figure 8). The best distinction was seen in IBD patients. Furthermore, the ANA-positive patients had higher total levels of IgG and IgM and higher systemic inflammation than ANA-negative patients.

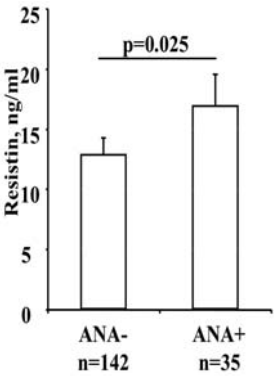


Figure 8. Resistin levels in ANA-positive patients and in ANA-negative patients.

We observed higher resistin levels in patients treated pharmacologically than in untreated patients. ANA-positive patients were more often treated with immunomodulators, namely thiopurins and corticosteroids, than the ANA-negative ones. The patient groups showing high resistin levels (AIH, PSC, and CD) were more often treated with corticosteroids (AIH 65%, PSC 26%, CD 33%) than UC patients (18%) and NAFLD patients (0%). Moreover, thiopurine-treated patients had higher resistin levels than patients who did not receive thiopurins, suggesting that patients with a severe disease (thus treated) have higher resistin levels. An alternative explanation might be that the effects of treatment stimulate resistin production.

The view on the role of resistin in metabolic disease is controversial. Our objective was therefore to evaluate a possible relationship between resistin and metabolic parameters in NAFLD patients. Our findings failed to demonstrate elevated resistin levels in NAFLD. There was no correlation with any metabolic parameters in NAFLD. Moreover, no relation to morphological stage

of fibrosis in liver biopsies of NAFLD patients was observed. This is in accordance with the results of other studies, where resistin levels were not found to be elevated in NAFLD[117, 118]. This contrasts with the results of one previous study in which elevated resistin was observed. Taken together, our findings do not support the hypothesis that resistin is a marker of fibrosis or metabolic disturbances in NAFLD patients.

Neutrophils contain resistin

Mononuclear cells have been considered to be a main source of resistin. The most predominant leukocyte in the circulation is, however, the neutrophil. Moreover, neutrophils are actively recruited to the site of inflammation such as to the synovial fluid of RA patients. Local inflammatory compartments are also characterized by high levels of resistin.

In paper III, we hypothesized (i) that resistin is stored in neutrophil granules, and (ii) that resistin is released after inflammatory stimuli.

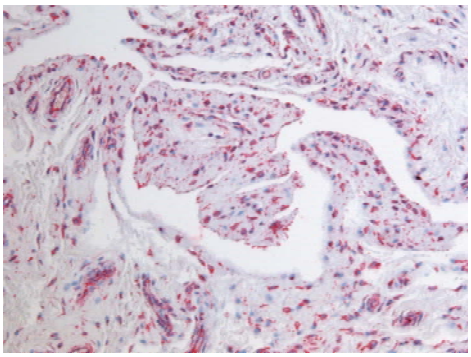


Figure 9.
Resistin expression in RA synovial tissue.

Resistin is enriched in synovial fluid of RA patients[43]. Thus, we investigated the presence of resistin in synovial tissue. Histological staining of synovia showed the presence of resistin in leukocytes of the lining layer (Figure 9). To continue the investigation on the cellular origin of resistin, human leukocytes were subjected to flow cytometry in order to visualize resistin on the surface. This was performed to demonstrate whether the possible presence of resistin is of intracellular or surface origin. The expression of resistin in synovial fluid leukocytes was higher than the expression on the leukocytes circulating in peripheral blood. Surface resistin was detected on 95–98% of the $CD16^+$ synovial neutrophils. Monocytes, marked by $CD14^+$, and T cells, marked by $CD3^+$, were also expressing surface resistin, but to lower extents. We also

evaluated the impact of proinflammatory stimuli in altering surface resistin. Stimulus with fMLF resulted in an increase in surface resistin, which was also observed with TNF-α stimuli.

To study the source of intracellular-derived resistin, neutrophils were activated by fMLF, one of the most potent neutrophil activators. Following activation, resistin content of the supernatant was measured and lactoferrin—an enzyme of the specific granules—was measured in parallel. fMLF induced resistin secretion in a dose-dependent manner (Figure 10). The increase in resistin was almost 300% compared to the unstimulated cells. The induction was similar to the observed release in lactoferrin, indicating the presence of resistin in the same granules as lactoferrin, namely in the specific granules (Figure 10). Taken together, these findings indicate that resistin is released from the intracellular compartments of the neutrophil.

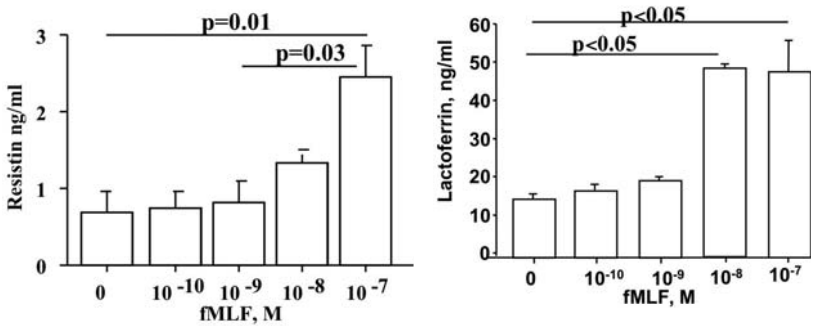


Figure 10. Release of resistin and lactoferrin from human neutrophils following fMLF stimulus.

To study the origin of resistin at the subcellular level, neutrophils were cavitated and divided into fractions representing the subsets of granules. They were fractionated on a two-layer ficoll gradient that separates the content of neutrophils into azurophil granules (the α-fraction), specific/gelatinase granules (the β-fraction), and plasma membrane/secretory vesicles (the γ-fraction). By immunoblot with anti-resistin antibodies, we found that resistin was present in fractions 7–9, corresponding to the β-fraction (the specific and gelatinase granules). The study was continued with a 3-layer fractionation, permitting better distinction between the specific and the gelatinase granules. The immunoblot of 3-layer fractions demonstrated resistin present in fractions 10–18, corresponding to the specific granules. Simultaneous immunoblot to detect gelatinase indicated its presence in fractions 14–18. Thus, resistin was localized to the β-fraction, i.e. the specific granules of the neutrophil.

Resistin was quantified in the subcellular fractions by ELISA. To avoid any possible influence of MPO and ALP, enzymes rich in azurophil and specific granules, the final step in the assay was modified. Resistin was identified in the fractions corresponding to the azurophil and the specific granules, but not in the secretory vesicles. In order to determine whether resistin was present in the specific granules alone, or also in the azurophil granules, we performed immunoprecipitation of resistin in fractions 3, 8, and 20 followed by mass spectrometry. This identified resistin in fractions corresponding to the azurophil granules and the specific granules.

Taken together, our results suggest that resistin is present in neutrophils in the azurophil granules and in the specific granules. This study supports the role of resistin in inflammation where neutrophil-derived resistin is possibly vital for maintenance of an inflammatory state.

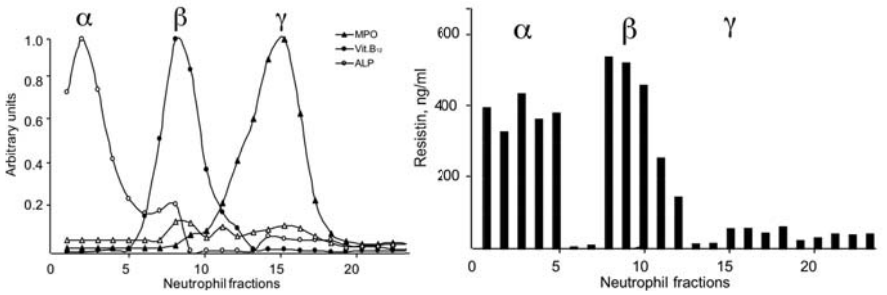


Figure 11. Subcellular fractioning of human neutrophils into α, β and γ fractions. Black bars represent measured levels of resistin in each fraction measured by an ELISA. Resistin is present in fractions of the α and β granules (azurophil and specific).

Inflammatory resistin modulates IGF signaling

IGFs are growth factors implicated in synovial growth in RA by their contribution to proliferation and growth. Moreover, IGFs exhibit metabolic effects. We examined the role of resistin in IGF-1 signaling. The specific objectives were (i) to compare the presence of IGF-1R in synovial tissue and the presence of IGF-1 and IGFBP3 in synovial fluid and blood of RA patients and controls, (ii) to study downstream effects of IGF-1R following resistin stimulation of the human fibroblast cell line MRC-5, and (iii) to study the expression of IGF-1 and Akt following resistin abrogation in a new mouse model of arthritis.

Histological staining showed the presence of IGF-1R in both RA and OA synovia. Levels of IGF-1 in synovial fluid were significantly lower in RA patients than in OA patients. The decrease in IGF-1 was most pronounced in RA patients with systemic inflammation, defined by acute phase reactants. Moreover, IGFBP3 levels were higher in the blood of RA patients than in that of OA patients, and were inversely related to IGF-1 levels. The relative excess of IGFBP-3 may partly explain the low IGF-1 levels observed in the synovial fluid of RA patients. Resistin levels were inversely related to the levels of IGF-1 in synovial fluid, suggesting a possible role of resistin in IGF signaling. Taken together, these results show that IGF expression in RA synovia is reduced.

In vitro incubation of human MRC-5 cells with resistin significantly reduced the levels of IGF-1. The IGF-1 production by MRC-5 cells was restored by resistin-specific monoclonal antibodies. In contrast, no decrease in IGF-1 levels was observed following incubation with TNFα. Short-term incubation of MRC-5 cells with resistin induced the phosphorylation of IGF-1R and the phosphorylation of Akt. The phosphorylation was suppressed by resistin and insulin incubation combined. This suggests that resistin has a regulatory role in IGF signaling.

Possible effects of resistin downstream of the IGF-1R was evaluated by gene expression analysis. Resistin significantly induced mRNA expression of IRS-1 both 1 h and 12 h after stimulation. Combined stimulation of MRC-5 cells with resistin and insulin increased IRS-1 transcription further. Thus, insufficient mobilization of IRS-1 was not a reason for the suppressed Akt phosphorylation observed with the combination of resistin and insulin.

Resistin significantly induced mRNA expression of the Akt inhibitors PTEN, GSK-3b, and PTPN, effects that were also observed by insulin. Transcription of inhibitors was potentiated severalfolds by the combination of resistin and insulin. This may be a reason for the limited phosphorylation of Akt in the presence of both stimuli.

Resistin stimulation of MRC-5 fibroblasts induced the production of IL-6, IL-8, and MMP-3. Survivin synthesis was downregulated by resistin. Similar downregulation was observed with insulin stimulation. The downstream effect of insulin and IGF-1 receptor stimulation is associated with production and activation of iNOS. Stimulation of MRC-5 cells with resistin and insulin resulted in a significant activation of iNOS in the cell cultures, as long as 24 h and 48 h after stimulation. Stimulation of MRC-5 cells with insulin resulted in no improvement in iNOS activity. Moreover, simultaneous stimulation of MRC-5 cells with insulin and resistin significantly reduced the effect of resistin on iNOS activity, indicating the possible presence of competing mechanisms (Figure 12).

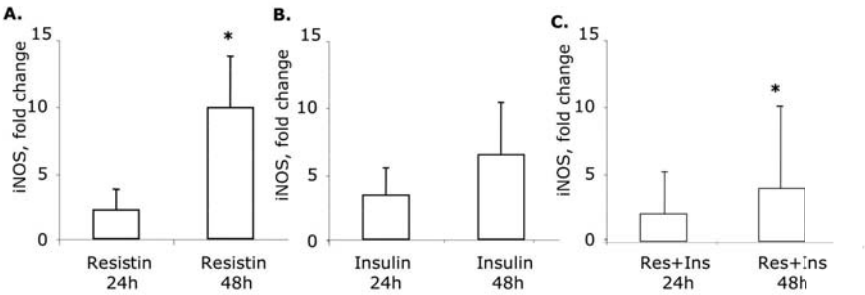


Figure 12. Levels of iNOS in supernatants of MRC-5 fibroblasts following resistin, insulin and resistin+insulin incubation at 24h and 48 h.

Resistin is highly expressed in RA synovial fluid and IGF-1 expression is reduced. Moreover, the levels are inversely related to each other. Thus, we evaluated the possible effect of resistin on the IGF-1R pathway following resistin suppression in vivo. Human synovia were transplanted into SCID mice and subjected to resistin abrogation by the siRNA technique. As demonstrated in Figure 13, synovia injected with siRNA targeting resistin showed a decrease in resistin expression whereas resistin was highly expressed in synovia that were subjected to control RNA sequence. Morphologically, resistin-poor synovia were characterized by a reduced cellularity and accumulation of fat deposits. No fat deposits were found in mock-treated synovia. Resistin-abrogation was associated with reduced phosphorylation of Akt and a decrease in the expression of IGF-1R. The tissues that were subjected to resistin

abrogation were evaluated for gene expression of the Akt inhibitors PTPN, PTEN, and GSK-3b. Downregulation of resistin resulted in a decrease in PTPN, PTEN and GSK-3b, suggesting that resistin may have a role in IGF signaling. This is in accordance with in vitro studies where resistin was found to induce the expression of Akt inhibitors.

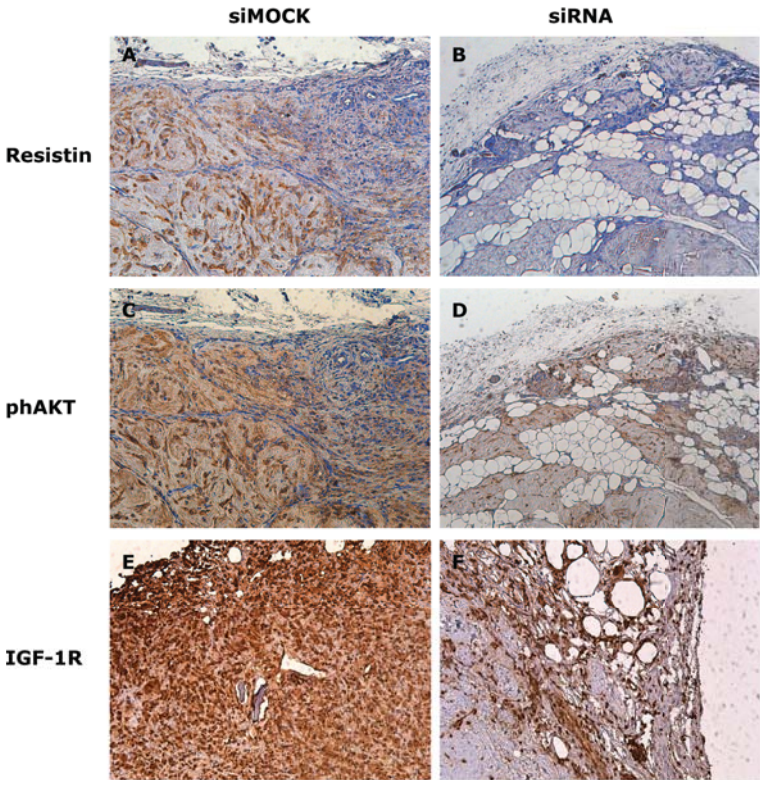


Figure 13. Human synovia were used in an orthotopic mouse model of arthritis and subjected to siRNA targeting resistin or mock RNA (control).

Discussion

In this thesis, the role of resistin in inflammation and autoimmunity was studied, with emphasis on rheumatic diseases and diseases of the liver and gut. The impact of resistin on IGF signaling was also assessed. These are the main conclusions drawn from the work of this thesis:

- Resistin is a modulator of inflammation and autoimmunity, is abundantly expressed at the local site of inflammation, and is correlated to disease characteristics in patients with pSS and in diseases of the liver and gastrointestinal tract

- Resistin is present in human neutrophils, which serve as an important source of resistin at the site of inflammation. Resistin is released in response to inflammatory stimuli from intracellular granules.

- Resistin has dual properties in man, acting as a modulator of inflammation and as a regulator of insulin growth factor (IGF) signaling.

In paper I, it was shown that resistin is expressed in glandular tissue and in the saliva of pSS patients; the levels in patients with high focal inflammation are the most pronounced. This is based on measurements of resistin, morphological grading of focal leukocyte infiltration, and staining of salivary gland tissue.

One reason for the accumulation of resistin in saliva could stem from the reduced saliva volume, thus resulting in more dense saliva with a high protein content. Uunstimulated saliva is considered unspecific[140] and increased levels of electrolytes have been reported in saliva of SS patients[140, 141]. Arguing against this is the specific staining of resistin in SS glandular tissue in our study. Moreover, the fact that there was no correlation between total protein content and resistin content indicates differently. Taken together, the data strongly suggest that there are elevated salivary resistin levels in SS patients.

Resistin was found to be elevated locally at the site of inflammation whereas the circulating levels of resistin remained unchanged. In addition to this, resistin was expressed in salivary gland tissue of SS patients but not in that of healthy controls. It is possible that resistin may be produced at a remote location and accumulate in glandular tissue and saliva or that resistin is produced locally. However, in favor of local rather than a systemic or remote production, is the observed accumulation of immune cells in glandular tissue.

Immunohistochemical staining of resistin showed its presence in infiltrating mononuclear cells, leukocytes, and epithelial cells. Previous studies have shown the presence of other proinflammatory cytokines, and staining for TNF-α and IL-6 has been reported with a similar pattern of expression in mononuclear cells and epithelial cells[87]. A possible role of resistin in glandular tissue and saliva could be to interact with TNF-α and IL-6 to maintain the inflammatory state. Based on measurements of resistin in saliva and blood, and staining of glandular tissue, the data suggest that there is mainly local production of resistin in SS.

In paper II, we determined resistin levels and their relation to clinical parameters in patients with autoimmune hepatitis, inflammatory bowel disease (ulcerative colitis, Crohn's disease), primary sclerosing cholangitis, non-alcoholic fatty liver disease, and healthy controls. We found elevated resistin levels in AIH, PSC, and IBD; they were significantly elevated in ANA-positive patients compared to ANA-negative patients.

The increase in resistin expression in IBD is in accordance with previous studies[94, 142, 143]. Little is known about resistin in AIH and PSC. One previous study measured adipokines in type 1 AIH and showed a positive correlation between resistin levels and levels of both TNF-α and bilirubin[93]. The authors did not observe any difference in resistin levels between AIH patients and controls. We observed elevated resistin levels in AIH patients relative to controls. The discrepancy between our results and the results of Durazzo et al[93] on resistin levels is possibly due to the different patient populations, where a heterogeneous patient group can explain the lack of findings. Effects of pharmacological treatment and severity of disease of the patients included in the studies are other possible reasons.

Resistin was shown to be elevated in ANA-positive patients compared to ANA-negative patients. Recognition of circulating antibodies to specific liver antigens is used in the diagnosis of liver and gut diseases, and that is associated with a more severe disease. Thus, the presence of such antibodies is unfavorable for the prognosis. Little is previously known of resistin an T cells. Resistin in shown to act as a chemokine for $CD4^+$ lymphocytes[55] and we show elevated resistin levels at the site of inflammation in saliva of pSS, being considered a T cell driven disease. This together with the elevated resistin levels in ANA-positive patients suggests a role of resistin on lymphocytes.

The AIH group had the highest percentage of ANA–positive patients. In this group alone, however, the difference in resistin levels between AIH-positive and AIH-negative patients failed to reach statistical significance. AIH-patients were also treated with thiopurins and corticosteroids significantly more often than AIH-negative patients.

Patients with high resistin levels were more often treated with immunomodulators such as thiopurins and corticosteroids. Whether resistin expression is affected by the treatment or whether patients with more severe disease are have high resistin and heavier treatment is unknown. The latter statement is supported by previous reports of reduction of resistin expression by immunomodulators. This reasoning could explain the lack of statistical significance in comparison of resistin between ANA-positive and ANA-negative patients in the AIH group alone. Many of these patients received immunomodulators. It is possible that the treatment reduces the levels of resistin, however, it is likely that patients who receive treatment have a more severe form of the disease, which is thus associated with elevated resistin levels; however, it is not possible t to make any firm conclusions regarding the importance of treatment or pathogenesis for the levels of resistin measured.

In paper II, elevated resistin levels were observed in the circulation. One possibility would be that resistin is produced at a remote location, e.g. by circulating cells or in other organs. Another possibility would be local production of resistin. Possible local sources of resistin might be hepatocytes, hepatic stellate cells[50] and infiltrating mononuclear cells.

In paper III, resistin was identified at the site of inflammation in neutrophil granules, which represent a new and important source of resistin. Proinflammatory stimuli induce the release of resistin and boost the extracellular resistin concentration at the site of inflammation.

Mononuclear cells were previously thought to be the main source of resistin in humans[43]. In this work, a new cellular source of resistin was found, namely the neutrophil. Neutrophils have the same same progenitor cell as mononuclear cells and they have similar properties in phagocytosis and killing of bacteria. These similarities support the possibility of the cells sharing the function of producing at the site of inflammation. This is supported by previous studies on resistin in mononuclear cells which may synthesize resistin after stimulation[43]. We demonstrated that resistin was present in the azurophil granules and the

specific granules. The presence of resistin in granules, shown in paper III, is supported by a parallel study by Johansson et al[144]. They also reported the presence of resistin in neutrophil granules, namely in the azurophil granules in septic shock[144]. In accordance with our study, they also found that resistin was abundantly expressed on the surface of neutrophils.

Whether or not neutrophil-derived resistin represents a physiologically significant source of resistin is not fully known. In favor of its importance is the fact that neutrophils constitute two-thirds of all circulating leukocytes. Moreover, the impact of cytokine release from neutrophils has been demonstrated by previous studies on TNF-α, where neutrophils account for a larger source than monocytes/macrophages[145]. Since the latter cells were previously considered to be the major source of resistin, one can suggest that neutrophils do indeed count as a substantial source in acute inflammation. In support of the importance of neutrophil-derived resistin are the findings of Johansson et al., where there was found to be a positive correlation between resistin levels and neutrophil count. We suggest that neutrophil-derived resistin is a significant proportion of the total resistin present at the site of inflammation.

The role of resistin in inflammation is supported by its release by neutrophils. A possible model of the situation at the site of inflammation is presented in Figure 14. In the case of inflammation in a rheumatic joint, resistin is highly expressed in the synovial fluid. The neutrophil-derived resistin potentiates the release of NfκB-dependent cytokines and chemokines from mononuclear cells. This leads to further neutrophil recruitment but also provides positive feedback to mononuclear cells to produce more proinflammatory cytokines (including resistin). What we have is a state of chronic inflammation, but with an innate component because of the presence of neutrophils.

This argues for resistin having a role in inflammation where neutrophil-derived resistin contributes to the pathology of disease.

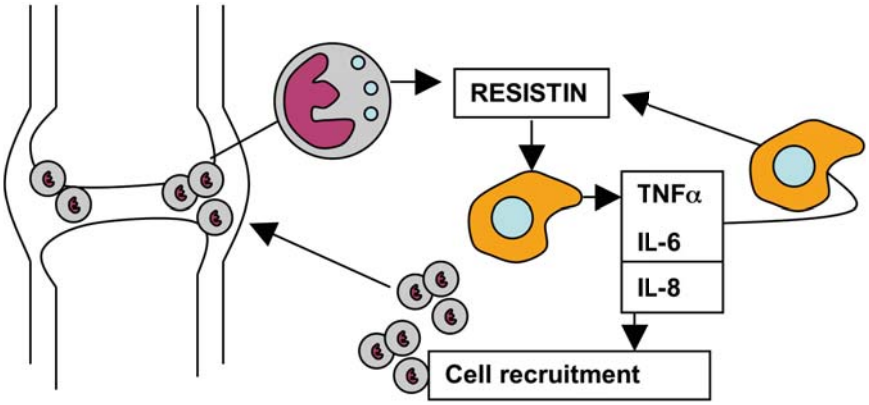


Figure 14. Model of neutrophil-derived resistin in inflammation of a rheumatic joint. Resistin activates proinflammatory cytokine release, including further release of resistin and chemokines, from mononuclear cells. This leads to cell recruitment of more neutrophils and a positive-feedback loop, resulting in an inflammatory state.

In paper IV, resistin was shown to modulate IGF-1 signaling in a model of RA. This conclusion was based on in vitro data on the effect of resistin on IGF signaling, gene expression analysis, and histological evaluations. A new humanized mouse model of arthritis was used.

Reduced levels of IGF-1 were observed in synovial fluid of RA patients. This is in accordance with a previous study that showed decreased IGF-1[71] whereas another study failed to find differences in IGF-1 levels between RA patients and controls was observed[146]. In juvenile chronic arthritis, low IGF-1 levels in serum have also been reported[147].

There are several possible reasons for low IGF-1 levels. IGFBP3 can form a complex with IGFs, thus regulating the available IGFs. In favor of this is inverse relationship observed between IGF-1 and IGFBP3 levels. In addition, we observed higher IGFBP3 in RA than in OA. Low IGF-1 could, on the other hand be an effect in response to high resistin levels. This is supported by the fact that RA patients have high resistin levels in synovial fluid[43, 72, 73]; also, in vitro data from our study have shown a decrease in IGF-1 in fibroblasts incubated with resistin. The IGF-1 production by fibroblasts was restored by the use of specific anti-resistin antibodies. Moreover, in accordance with the observed decrease in IGF-1 caused by resistin, work on other proinflammatory cytokines, namely TNF-α and IL-1β has shown that injection of these cytokines into healthy animals can cause a 30-40% reduction in circulating and tissue IGF-1[148, 149]. Moreover, IL-1 has been shown to inhibit the action of IGF-

1[150]. Taken together, our data suggest that reduced IGF-1 levels in RA are partly due to a regulatory role of resistin in IGF signaling.

The IGF-1 receptor is critical for malignant transformation and possibly for pannus formation in RA, but is less involved in normal cell growth[151]. Targeting of IGF-1 receptor may modulate synovial hyperplasia. We abrogated resistin expression in vivo in a transplantation model of arthritis and evaluated gene expression and morphology of the transplanted tissues. Resistin abrogation led to a decrease in the expression of both IGF-1R and phosphorylated Akt. IGFs are shown to be modulated by TNF and IFN where they decrease IGF-1R expression[152] in contrast to our findings of decreased IGF-1R when abrogating resistin. Thus, a possibility of different mechanisms in affecting IGFs is probable.

Gene expression analysis of the transplanted synovial tissues showed a decrease in Akt inhibitors when resistin was abrogated. This was in accordance with the in vitro studies suggesting that resistin induced the expression of Akt inhibitors. Moreover, in vitro, resistin was shown to induce phosphorylation of IGF-1R and Akt. From this, we propose that resistin has a regulatory role in IGF signaling and IGF-1R expression in RA. It may also have a role in synovial growth, and it may be possible to improve hyperplasia in RA by inhibiting the expression and activity of resistin.

Autoimmune diseases, with the exception of RA are rare however, considered as a group of diseases they affect 5% of the population in western countries[153, 154]. They lead to morbidity and mortality and are costly for the society. The targeting of the single cytokines TNF-α, IL-1, and IL-6 is proven effective[77] however not in all patients[134]. Non-responding patients are proposed to have a different cytokine profile, possibly a resistin profile. Resistin is capable to mediate inflammation and could thus be a possible target molecule in non-responding patients identified to have resistin as the driving force of inflammation. Possible future anti-resistin targeting therapies will however need good in vivo systems for pre-clinical trials.

The differences between human and mouse resistin has limited the use of knock-out mouse models, classical disease mouse models, and humanized models of resistin. Recently a humanized mouse model with macrophage-derived resistin was introduced[69]. We applied the orthotopic transplantation model of arthritis. Models have their limitations and are far from the real situation in humans. The orthotopic model has the limitations to study local rather than systemical inflammation. However, in studies of RA local inflammation is the case so the model is still useful. Some research groups have continued to study intracellular regulation of murine resistin rather than studying human resistin. It is tempting to hope that some of this data will become applicable also in humans since there are many questions that are still unanswered.

Future possibilities for in vivo studies of resistin are to study the protein in animals with greater homology to the human counterpart than rodent resistin has. Possible such animals are chimpanzee, dog or cow where the sequence homology is 74%, 74%, and 72% respectively[155] as compared to mouse and rat where the sequence homology is 58% versus 54%. Resistin reduces vasodilation in Bradykinin treated dogs[156]. Gene expression of resistin in lactating cows is increased and possibly involved in insulin resistance. The cDNA sequence homology in cows is 83% to the human protein[157] (compared to mouse where this is 47%). If the information of resistin from humanized mouse models is promising these animal could represent the possible next step for preclinical studies.

Populärvetenskaplig sammanfattning

Immunförsvaret har till uppgift att skydda organismen mot bakterier och virus genom att framkalla så kallad inflammation. Vid vissa sjukdomar blir inflammationen alltför kraftig och skadar de egna vävnaderna. Detta kan leda till inflammatoriska och autoimmuna sjukdomar. Exempel så sådana sjukdomar är ledgångsreumatism, tarm och leversjukdomar.

Inflammationen förmedlas till stor del av små proteiner. Avhandlingen presenterar betydelsen av ett sådant protein kallat *resistin* vid inflammatoriska och autoimmuna sjukdomar.

Resistin är i möss kopplat till övervikt, diabetes och insulinresistens där proteinet reglerar glukosmetabolism. Hos människan har resistin istället förmågan att framkalla inflammation genom att starta en kaskad av andra inflammationsproteiner, så kallade cytokiner. Avhandlingen berör resistins roll vid reumatisk sjukdom, samt sjukdom i tarm och lever. Den tar också upp om den vanligaste vita blodkroppen, neutrofilen, producerar resistin.

Vid lokal inflammation i saliv och spottkörtel hos patienter med Sjögrens syndrom, en sjukdom som drabbar körtlar i kroppen, är resistin förhöjt. Vidare har de patienter med hög inflammationsgrad i spottkörtlarna högre nivåer av resistin i saliven än de med låg inflammationsgrad. Då resistin i blodet inte är högre talar dessa resultat för en lokal verkan av resistin snarare än en systemisk.

Mätningar av resistin i lever- och tarmsjukdomar visar på höga resistinnivåer i blodet vid autoimmun hepatit, primär skleroserande kolangit, Crohns sjukdom och ulcerös kolit. Patienter med anti-nukleära antikroppar, dvs. antikroppar riktade mot cellkärnan i kroppsegna vävnader, har signifikant högre nivåer av resistin är de som saknar dessa.

Tidigare studier har visat att resistin främst produceras av en typ av vita blodkroppar som heter monocyter. En av studierna här visar att neutrofiler, de vanligaste vita blodkropparna, utsöndrar resistin och att resistin finns inuti neutrofilen i organeller som kallas granule. Detta är en tidigare okänd källa till resistin vid inflammation.

Resistin har metabola egenskaper i möss men hos människan är det ännu osäkert huruvida resistin har dessa egenskaper. Tillväxtfaktorer, därbland insulin growth factor 1 (IGF-1), är proteiner med metabola egenskaper som bl.a. stimulerar celltillväxt. En av studierna visar att resistin sänker nivåerna av IGF-1. Vidare är IGF-1 sänkt i ledvätska hos patienter med ledgångsreumatism medan nivåerna av resistin hos dessa patienter är förhöjda. I en ny djurmodell där mänsklig ledvävnad transplanteras till immundefekta möss, sänks mängden IGF-1 receptor då resistin tas bort. Detta visar på att det är möjligt att resistin kan reglera IGF-1 vid ledgångsreumatism.

Sammanfattningsvis visar avhandlingen att resistin är ett protein som deltar i inflammation och är förhöjt vid autoimmuna och inflammatoriska sjukdomar. Resistin utsöndras av neutrofiler som är den vanligaste vita blodkroppen. Resistin påverkar inflammation och möjligen celltillväxt hos människan.

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