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CLINICAL AND MOLECULAR STUDIES IN ANCA ASSOCIATED VASCULITIS

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Clinical and molecular studies in ANCA associated vasculitis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

ANCA associated vasculitis (AAV) is a heterogeneous group of diseases characterised by sterile pauci-immune systemic small vessel inflammation and closely associated with the presence of anti-neutrophil cytoplasmatic antibodies (ANCA). Although AAV can affect any organ, the kidney, skin, lungs and upper and lower airways are most commonly involved. In some patients there is granuloma formation and in some asthma and eosinophilia, and based on this patients can be further classified as microscopic polyangiitis (MPA) (no granuloma), granulomatosis with polyangiitis (GPA) or eosinophil granulomatosis with polyangiitis (EGPA). Induction treatment of AAV consists of a cytotoxic agent in combination with glucocorticoids and is usually effective, although relapse, infections and drug toxicity remain a problem. The aim of this thesis was to investigate the role of novel proinflammatory molecules in the pathogenesis of AAV and as markers of disease. We also wanted to investigate the outcome of new therapies.

In paper I we investigated the pro-inflammatory mediator high-mobility group box-1 protein (HMGB1) in patients with active AAV and in remission. Elevated levels of circulating HMGB1 were found in active disease compared to inactive disease. We found increased expression of HMGB1 in renal tissue of patients with active renal AAV compared to inactive AAV, indicating a role for HMGB1 in AAV.

In paper II we carried out a long time follow-up on patients treated with rituximab as a second line treatment in patients with refractory or relapsing disease. We found that a high proportion of patients achieved remission but there were several adverse events including two cases of hepatitis B reactivation. Patients with a negative conventional ANCA but positive capture ANCA seemed to be at risk of relapse after rituximab treatment.

In paper III macrophage inhibitory factor and thyroid hormones were investigated prospectively in 30 patients with active AAV. We found that levels of MIF were elevated in active disease compared to remission and that MIF levels correlated with disease activity. There were thyroid hormone alterations with lower triiodothyronine in active disease, similar to what has previously been described in critically ill patients. In addition there was an indication of an interaction between MIF and thyroxine similar to what has previously been described in sepsis.

In paper IV we investigated pentraxin – 3 (PTX3) and soluable Tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) in 40 patients with active AAV and found that PTX3 was elevated in active disease compared to remission and correlated with disease severity. sTWEAK levels in active disease did not significantly change compared to remission, but seemed to vary with disease phenotype. Patients with high sTWEAK and low PTX3 were less likely to achieve remission.

In conclusion several proinflammatory molecules may be implicated in the pathogenesis and exacerbation of AAV and are possible targets of therapy. There seems to be thyroid hormone alterations in active AAV and there may be an interaction between MIF and thyroxine in

AAV. PTX3 may be a useful marker of disease. Rituximab may be a useful treatment in refractory or relapsing disease but it is important to assess hepatitis status and adverse events are a concern.

LIST OF SCIENTIFIC PAPERS

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

I. Bruchfeld A, Wendt M, Bratt J, Qureshi AR, Chavan S, Tracey KJ, Palmblad K and Gunnarsson I. ”High-mobility group box-1 protein (HMGB1) is increased in antineutrophilic cytoplasmatic antibody (ANCA)-associated vasculitis with renal manifestations*” Mol Med. 2011 17: 29-35.*

II. Wendt M, Gunnarsson I, Bratt J and Bruchfeld A.”Rituximab in relapsing or refractory ANCA-associated vasculitis: a case series of 16 patients” *Scand J Rheumatol. 2012 41:116-9.*

III. Wendt M, Börjesson O, Avik A, Bratt J, Anderstam B, Qureshi AR, Miller EJ, Gunnarsson I and Bruchfeld A. ”Macrophage Migration Inhibitory Factor (MIF) and Thyroid Hormone Alterations in Antineutrophil Cytoplasmatic Antibody (ANCA)-Associated Vasculitis (AAV)” *Mol Med. 2013 20:109-14.*

IV. M Wendt, O Börjesson, A Avik, J Bratt, AR Qureshi, I Gunnarsson and A Bruchfeld. PTX3 and soluble TWEAK levels in active ANCA associated vasculitis (AAV) patients undergoing treatment – correlation to disease activity and relapse” *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AAV	ANCA Associated Vasculitis
ACR	American College of Rheumatology
ANCA	Anti-neutrophil cytoplasmic antibodies
AZA	Azathioprine
BVAS	Birmingham Vasculitis Activity Score
C	Complement component
CKD	Chronic kidney disease
CMV	Cytomegalovirus
CRP	C-reactive protein
CSS	Churg-Strauss syndrome
CYC	Cyclophosphamide
DAPI	4',6-diamidino-2-phenylindole
eGFR	Estimated glomerular filtration rate
EGPA	Eosinophilic granulomatosis with polyangiitis
ELISA	Enzyme-linked immunosorbent assay
EULAR	The European league against rheumatism
EUVAS	European vasculitis study group
Fn 14	Fibroblast growth factor-inducible molecule 14
GC	Glucocorticoids
GPA	Granulomatosis with polyangiitis (Wegener's granulomatosis)
HbcAg	Hepatitis B core antigen
HbeAg	Hepatitis B e antigen
HbsAg	Hepatitis B Surface antigen
HLA	Human Leukocyte antigen
HMGB1	High mobility group box 1
IG	Immunoglobulin
IgG	Immunoglobulin G
IL	Interleukin
i.v.	Intravenously
LPS	Lipopolysaccharide

MCP-1	Monocyte chemoattractant protein – 1
MDRD	Modification of diet in renal disease
MIF	Macrophage migration inhibitory factor
MMF	Mycophenolate Mofetil
MPA	Microscopic Polyangiitis
MPO	Myeloperoxidase
MTX	Methotrexate
NET	Neutrophil Extracellular Trap
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen-associated molecular patterns
PRR	Pattern recognition receptors
PR3	Protinase 3
PSGL-1	P-selectin glycoprotein ligand-1
PTX3	Pentraxin – 3
RA	Rheumatoid arthritis
RTX	Rituximab
SLE	Systemic lupus erythematosus
T3	Triiodothyronine
T4	Thyroxine
T-cells	Thymic-derived lymphocytes
TNF	Tumour necrosis factor
TSH	Thyroid-stimulating hormone
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
VDI	Vasculitis Damage Index

1 INTRODUCTION

1.1 OVERVIEW OF THE IMMUNE SYSTEM

The major function of the immune system is to protect the body from invading microorganisms.

1.1.1 Innate immunity

The innate immune system reacts to invading pathogens in a generic way and carries no long lasting memory (immunity) against a microorganism. The innate immune system recognises molecular patterns found in a broad group of microorganisms (pathogen-associated molecular patterns, PAMPSs) by pattern recognition receptors (PRR). The best-characterised PRRs are a group of transmembrane proteins called Toll-like receptors. They recognise extracellular PAMPs and trigger the synthesis and secretion of cytokines necessary for the immune response.

Cells involved in innate immunity include macrophages, neutrophils, dendritic cells, eosinophils and natural killer cells. Innate immunity also consists of biological and chemical barriers, inflammatory mediators such as cytokines and the complement system.

1.1.2 Inflammation

Inflammation is a complex biological response of vascular tissues to pathogens, injured cells or irritants amongst others. The classic signs are pain, heat, redness, swelling and loss of function. On a cellular and molecular level, cells in the innate immune system are activated when they recognise PAMPs, and releases inflammatory mediators such as the cytokines TNF, IL-1 and IL-10. Neutrophils are recruited to the injured area by the process of chemotaxis and the complement system is activated. Inflammatory mediators have short half-lives, hence the acute inflammatory response needs constant stimulation to be sustained.

1.1.3 Adaptive immunity

Adaptive immunity is characterised by an immunological memory, high specificity and a stronger reaction to subsequent exposures to the same antigen. Adaptive immune responses are dependent on antigen-presenting cells displaying antigens. Probably the most important antigen-presenting cells are the dendritic cells (DC), abundantly situated in tissue exposed to potential pathogens such as skin, lungs, intestine and kidneys. DC are an important link between innate and adaptive immunity. Basically adaptive immune reactions comprise humoral immunity, which is mediated by B-cells and antibodies, and cellular-mediated immunity, mainly mediated by T-cells.

1.1.4 Autoimmunity

A fundamental property of the immune system is to discriminate between self and non-self. Lymphocytes with auto reactivity are inactivated or eliminated thus maintaining immunological tolerance. Failure to do so will result in an immune response to the body's

own cells and tissues. A low level of autoimmunity can be found in healthy individuals and may in fact be beneficial but a specific and effective immune reaction against self-antigens will result in injury and autoimmune disease. The development of autoimmunity and autoimmune disease is generally seen as a combination of genetic, hormonal and environmental factors. Genes implicated in autoimmunity include genes related to immunoglobulin's, T-cell receptors and the human leukocyte antigen (HLA). Most autoimmune diseases predominantly affect women. Drugs, microorganisms and cigarette smoke are environmental factors linked to autoimmunity [1].

1.2 SYSTEMIC VASCULITIS

The systemic vasculitides are a heterogeneous group of diseases characterised by inflammation of the blood vessel wall leading to narrowing, obstruction or other destruction of the vessel with subsequent infarction and tissue damage. Both arteries and veins are affected. Broadly speaking, the underlying cause of disease can be divided into infectious and non-infectious. Infectious vasculitis is caused by an invading pathogen affecting the vessel wall, as for instance in the case of syphilitic aortitis, whereas non-infectious vasculitis is not caused by a direct invasion and proliferation in the vessel wall by a pathogen. The non-infectious vasculitides are further categorised primarily according to the size of the affected vessels [2].

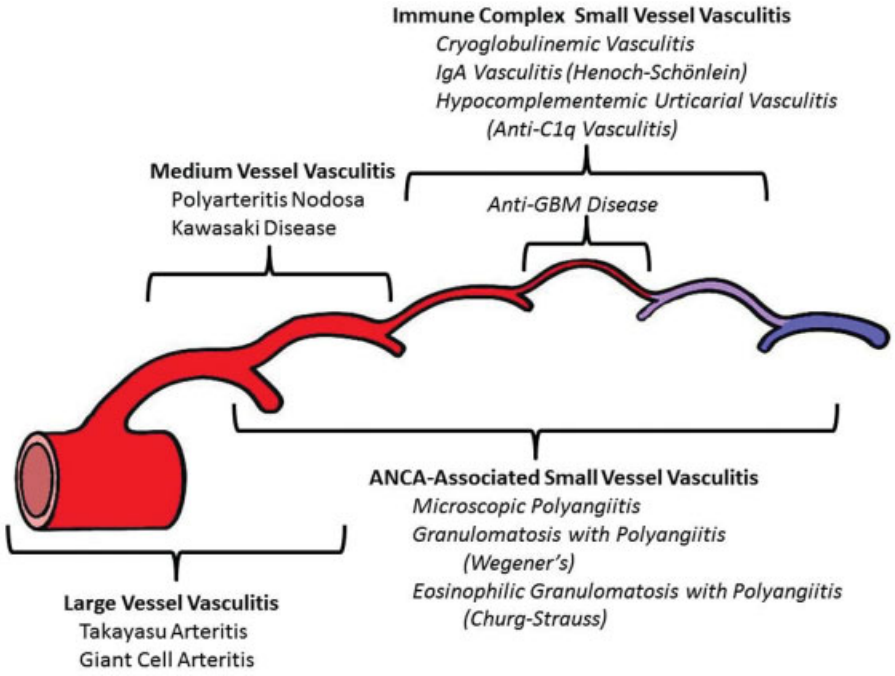


Figure 1. Distribution of vessel involvement by the different vasculidities. Categorisation is made according to the vessel predominantly affected (i.e. large vessel vasculitis, medium vessel vasculitis and small vessel vasculitis.) A single disease entity can affect many types of vessels. Reproduced from Jennette et al [2] with permission from the publisher.

1.3 ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA)

Anti-neutrophil cytoplasmic antibodies are a group of autoantibodies directed against antigens in the cytoplasm of neutrophils. Although found in a number of autoimmune conditions, ANCA is particularly associated with a group of systemic small vessel vasculitides, the so-called ANCA associated vasculitides (AAV). Typically ANCA is of IgG type and directed against proteinase 3 (PR3) or myeloperoxidase (MPO) however there are several other known ANCA antigens of unclear significance [3, 4].

1.3.1 ANCA testing

ANCA can be detected by immunofluorescence (IF), where two typical patterns can be seen: cytoplasmic ANCA (c-ANCA) and perinuclear ANCA (p-ANCA). The typical c-ANCA antigen is PR3 and the typical c-ANCA antigen is MPO, but there are several atypical ANCA antigens that can give rise to p- and c-ANCA patterns. Usually an Enzyme-Linked ImmunoSorbent Assay (ELISA) is carried out after a positive IF, testing for PR3 and MPO. Testing for ANCA with ELISA is traditionally done with a direct ELISA, where the antigen binds directly to the plastic in the wells. A variant of the solid phase ELISA is the bead-based multiplex assay in which capture antigens are bound to colour-coded beads in suspension that are then analysed using flow cytometry. With this method one can simultaneously detect multiple autoantibodies [5, 6]. Newer generations of ELISAs use a capture molecule, usually a monoclonal antibody, to bind the antigen. Theoretically, capture ANCA will further purify the antigen and preserve the three-dimensional structure leading to increased sensitivity and specificity [6, 7].

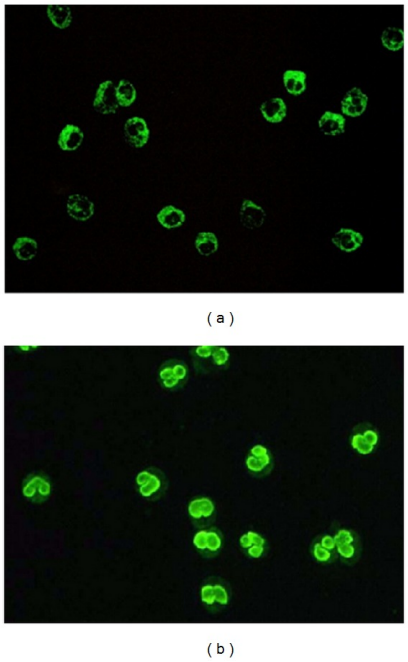


Figure 2. Appearance of cytoplasmic indirect immunofluorescence pattern (C-ANCA, Figure 1(a)) and perinuclear (P-ANCA, Figure 1(b)) on ethanol-fixed human neutrophil cells. Reproduced from Schulte-Pelkum et al [8].

1.3.2 The significance of ANCA

In the clinic, ANCA testing is primary used for diagnosing vasculitis. The presence of ANCA is not pathognomonic of AAV however and a negative ANCA test does not exclude AAV. The result of an ANCA test needs to be correlated with clinical signs and symptoms [9]. A reappearance of ANCA in a known AAV patient may indicate a relapse but several patients have a positive ANCA without any detectable disease activity [10, 11].

Although still controversial, most current theories attribute ANCA a causal role in the development of AAV [12]. It is believed however, that only some of the epitopes recognised by ANCA are pathogenic, thus explaining why there is no clear link between ANCA levels and disease activity [13-15].

1.3.3 The origin of ANCA

Low titres of non-pathogenic ANCA can be found in healthy people [16] but why some patients develop high titres and disease remains elusive. A genome-wide association study suggests a genetic contribution to the development of ANCA in ANCA associated vasculitis with different HLA specificities correlating to the development of MPO ANCA vasculitis and PR3 ANCA vasculitis. This association was stronger than with the clinical phenotype. In addition genes coding for α_1-antitrypsin and PR3 ANCA were associated with PR3 ANCA vasculitis [17].

Environmental factors are also probably important and microorganisms such as Staphylococcus aureus [18] and Ross River virus [19] has been implicated in the induction of ANCA associated disease. Furthermore, antithyroid drugs such as propylthiouracil can induce high ANCA titres and sometimes AAV [20] and exposure to silica [21] are related to the development of AAV.

1.4 ANCA ASSOCIATED VASCULITIS (AAV)

The ANCA associated vasculidities consists of three diseases: Microscopic polyangiitis (MPA), Granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis) and Eosinophilic Granulomatosis with Polyangiitis (EGPA, formerly known as Churg-Strauss syndrome). They have a similar clinical presentation, affecting small- to medium-sized vessels and are associated with the presence of ANCA. Immunopathologically, AAV has an absence of immunoglobulin dispositions in injured vessels (pauci-immune vasculitis), separating AAV from immune-complex-mediated small vessel vasculitis. AAV can involve virtually any organ but lungs, kidneys, skin and peripheral nerves are the most commonly affected organs. In addition, some variants of AAV (GPA and EGPA) have extravascular necrotising granulomatous inflammation (granuloma) most commonly affecting upper and lower airways [2].

1.4.1 Clinical presentation and disease progression

AAV is very heterogeneous in its clinical presentation but an archetypical patient is a man or a woman in their 5th to 7th decade of life [22] with upper and lower airway involvement and rapidly progressive renal failure. Other common presentations are limited granulomatous disease without signs of systemic angiitis, and organ limited vasculitis, most commonly renal limited vasculitis. AAV can also affect younger individuals and even children.

The natural history of AAV is grim and before causal treatment was available mean survival was only five months and one year mortality was 82% [23]. After the introduction of cyclophosphamide (CYC) in the 1970s outcome improved drastically with survival rates of 80% at follow up after eight years [24]. With modern treatment, the disease has a typical remitting-relapsing course although some patients enjoy lifelong remission. With conventional immunosuppression, 38% of ANCA patients included in four different European vasculitis study group (EUVAS) trials relapsed within five years and 25% died [25]. Some patients demonstrate residual disease activity after treatment and some patients are refractory to standard treatment altogether.

Risk factors for relapse in AAV patients include positive PR3 ANCA [26], upper and lower airway involvement and diagnosis of GPA[27]. Recent findings suggest that ANCA titres correlate more strongly with relapse in patients with renal vasculitis [28].

1.4.2 Epidemiology

In a study from southern Sweden, the incidence of AAV was about 21 new cases per million/year. About half of the cases were GPA and half MPA. EGPA was a very rare disease with an incidence of about one case per million/year. The male to female ratio was 1:1 and the median age 67.6 years at diagnosis. The one- and five-year survival rates were 87.8% and 71.6% for all patients with MPA carrying a worse prognosis due to higher age and lower glomerular filtration rate (GFR). Mortality was 2.77 times higher than in the general population [29]. Similar incidence of AAV has been reported from England whereas lower incidence has been reported from Germany [22, 30]. From reported data it seems as if the incidence of these diseases is increasing, this may be due to increased recognition of AAV following the introduction of ANCA testing. Furthermore, methodological differences such as whether AAV is defined by ACR criteria or Chapel hill consensus criteria or cases are captured by international classification of disease codes (ICD) may be of relevance when comparing different incidence rates [22].

Relatives of AAV patients have only a slightly increased risk of acquiring AAV [31]. Generally AAV is rarely seen in concurrence with other autoimmune diseases with the exception of thyroid disease [32].

1.4.3 Diagnosis

The diagnosis of AAV is based on clinical presentation, histopathology and ANCA testing. If there is pauci-immune small vessel vasculitis present without granuloma or asthma the patient is categorised as MPA, if there is granuloma but no asthma the patient is categorised aGPA and if there is granuloma, asthma and eosinophilia the patient is categorised as EGPA [2, 33] (figure 3). Patients can also be classified according to ANCA specificity (MPO or PR3 positive) [12]. MPA patients most commonly display MPO ANCA and GPA patients display PR3 ANCA although there is a substantial overlap. If ANCA is present in EGPA patients it is usually MPO ANCA [34].

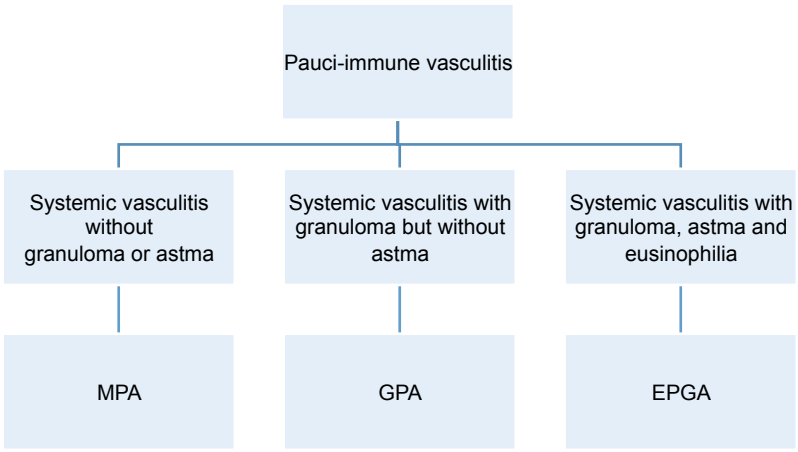


Figure 3. Categorisation algorithm of AAV.

The American College of Rheumatology (ACR) has issued classification criteria for GPA [35] and EGPA [36]. No ACR classification criteria exist for MPA.

1.4.4 Pathogenesis

The pathogenesis of AAV is largely unknown, especially the early stages of the disease. In current theories, it is suggested that neutrophils and monocytes in the circulation are primed by inflammatory stimuli and as a result display ANCA antigens on or near the cell surface. ANCA then interacts with these antigens leading to neutrophil activation and thereby initiating the vascular inflammation. In a similar fashion, granuloma formation is due to primed extravascular neutrophils interacting with interstitial ANCA, causing necrotising extravascular inflammation as the first stage in granuloma formation [12].

1.4.4.1 ANCA

There are several observations and findings that implicate ANCA in the pathogenesis of AAV. In the clinic, a strong association between ANCA and disease (MPA >90%, GPA >90 %, EGPA >40 %) can be observed, as well as a correlation between ANCA titre and disease

activity (albeit only partial correlation). Also, these diseases response well to B-cell depletion and plasmapheresis indicating that removal of autoantibodies resolves clinical disease [12].

In pathomechanistic studies of AAV, ANCA activate neutrophils and induce them to degranulate and produce oxygen radicals *in vitro* [37]. In rodent models of ANCA associated disease, anti MPO antibodies induce necrotising and crescentic glomerulonephritis either by direct injection, injecting splenocytes containing anti-MPO cells into immunodeficient mice or transplanting bone marrow that contains MPO positive myeloid cells into MPO knock-out mice [38, 39]. In some animals, systemic necrotising small vessel vasculitis and granuloma formation can be seen. These lesions closely resemble AAV in humans. Anti MPO IgG had this effect even in knock-out mice lacking functional T and B cells [40]. Although this strongly suggests the pathogenic role of ANCA, it should be pointed out that similar evidence does not exist for PR3 ANCA.

1.4.4.2 Complement

Although described as pauci-immune (no or minimal presence of immunoglobulins and/or complement), when closely examining renal biopsies from patients with active renal AAV, complement deposition is often found [41]. Furthermore, in patients with AAV plasma levels of the alternative complement pathway constituents: C3a, C5a and soluble C5b-C9 have been reported as being higher in active disease than in remission, whereas no difference could be seen for the classical pathway constituent C4d [42]. In mice models of AAV, blocking C5 either by knockout C5-/- or by a C5 inhibiting monoclonal antibody prevented the development of pauci immune glomerulonephritis that developed in control animals [43, 44]. This supports the view that complement, acting through the alternate pathway, mediates the necrotising vasculitis found in AAV. C5a is a strong neutrophil activator and recruits additional neutrophils through chemotaxis. These neutrophils exposed to C5a can activate the alternate pathway creating more C5a, thus creating a positive feedback loop [45].

1.4.4.3 Neutrophils and monocytes

A number of observations put the neutrophil at the centre of AAV pathogenesis. Obviously, neutrophils contain the antigen for ANCA and neutrophils are found in significant amounts in early glomerular and granulomatous lesions. The fact that AAV lesions are pauci-immune also suggests a direct cellular action pathogenesis [12]. There is strong evidence that neutrophils are recruited by chemotaxis to sites of vascular inflammation and that ANCA induces a respiratory burst and degranulation. In this setting neutrophils also undergo netting, a process in which chromatin fibres, so called neutrophil extracellular traps (NETs), are released. NETs display the MPO and PR3 antigens, suggesting that NET formation and deposition can trigger vasculitis and promote its exacerbation [46]. Apart from ANCA, many other events can induce and sustain neutrophil netting, for instance bacterial infections with Staphylococcus aureus [47] and the antithyroid drug propylthiouracil [48], both implicated in the pathogenesis of AAV.

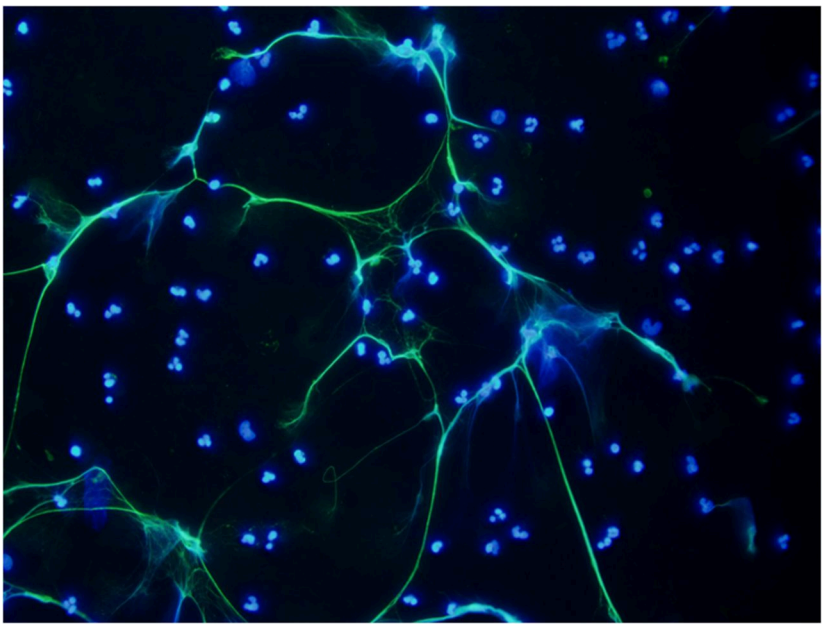


Figure 4. NETs induced *in vitro* by LPS in human neutrophils. NETs are visualised by the costaining of neutrophil elastase (green) and nuclear material (DAPI, blue). Reproduced from Kaplan et al. [49]. *Copyright 2012. The American Association of Immunologists, Inc*.

Monocytes also contain ANCA antigens and can be activated by ANCA [50]. ANCA activated monocytes release IL-8, a proinflammatory cytokine that attracts and activates neutrophils [51]. It also releases monocyte chemoattractant protein – 1 (MCP-1) that attracts monocytes and macrophages [52] and may be the reason why AAV lesions fairly quickly transform from a neutrophil-rich inflammation to an inflammation dominated by monocytes and macrophages. Monocytes are especially found in AAV lesions after the first day or two of inflammation and are systemically activated in active AAV [53].

1.4.4.4 T and B cells

Several observations implicate that cell mediated immune responses are important in the pathogenesis and exacerbation of AAV. Activation and expansion of thymic-derived lymphocytes (T cells) are reported in AAV [54]. This T-cell activation seems to persist during remission [55, 56]. Peripheral T cells have been shown to be activated by PR3 [57] . In GPA, T cells expressing IL-17 (a cytokine involved in activation of endothelial and epithelial cells and attraction of neutrophils) increase [58] and in an animal model of anti-MPO, necrotising glomerulonephritis IL-17 promoted the development of disease [59]. IL-17 producing cells have been shown to be major effector cells in autoimmunity in general [60].

T-regulatory cells (Tregs) have been reported dysfunctional in AAV and the frequency has been reported as increased and decreased by different investigators [61-63]. Although the picture is a little bit obscure, there seems to be an imbalance between Tregs and T-effector cells in AAV. The lack of function and/or numbers of Tregs may perpetuate ANCA formation.

The effectiveness of B-cell depletion in AAV as shown in randomised trials has highlighted B cells as an important target of therapy [64, 65]. The most obvious role of B cells in the pathogenesis of AAV is the production of ANCA. B cells can also interact with T cells and contribute to abnormal T-cell function [66].

B-regulatory cells (Bregs) is a population of B cells characterised by the production of IL 10 [67]. Bregs seem to have immunoregulatory properties and in animal models ameliorates immune response [68]. In patients with active AAV there is a lower percentage of Bregs, whereas the percentage of Bregs in patients in remission is normal. Normalisation of Bregs after targeted B-cell therapy with rituximab correlated with more effective remission [68, 69]. Thus a dysregulation between pathogenic and protective B-cell functions seems to be a feature of active AAV.

1.4.4.5 Cytokines

In order to activate neutrophils *in vitro* with ANCA, it is necessary to prime them with a proinflammatory stimuli, such as circulating tumour necrosis factor – α (TNF-α) [37], bacterial lipopolysaccharide (LPS) [70] or C5a [71]. When mice with anti-MPO IgG induced pauci-immune glomerulonephritis were injected with LPS as a proinflammatory stimulus it aggravated the glomerulonephritis causing increased amounts of glomerular crescent formation and glomerular necrosis. There was also an immediate, transient induction of TNF-α. Anti-TNF-α treatment attenuated, but did not prevent, the LPS-mediated aggravation of the glomerulonephritis [70]. This demonstrates the importance of proinflammatory cytokines, at least TNF-α, in the pathogenesis of AAV.

Other implicated cytokines include the monocyte-derived IL-8 that attracts and activates neutrophils [51] and the monocyte chemoattractant protein-1 (MCP-1) that attracts monocytes and macrophages [52].

Novel proinflammatory mediators have gained a lot of interest in the field of autoimmunity, although they have been little studied in AAV. These include high mobility group box 1 (HMGB1), macrophage migration inhibitory factor (MIF), pentraxin – 3 (PTX3) and soluble tumour necrosis factor-like weak inducer of apoptosis (sTWEAK).

HMGB1 is a nuclear protein known as a transcription and growth factor [72]. HMGB1 is actively secreted by innate immune cells such as macrophages and monocytes upon endotoxin stimulation, is passively released by injured and necrotic cells, and has been shown to stimulate necrosis-induced inflammation [73-75]. Extracellular HMGB1 can act as a chemoattractant for leucocytes and as a proinflammatory mediator stimulating release of TNF-α and other cytokines [76]. HMGB1 exists in several redox forms with different biological activities [77]. For instance, HMGB1 release after apoptosis is substantial but fails to stimulate TNF release, whereas HMGB1 release from necrotic cells is a potent stimulator of TNF production in responding macrophages, depending on different oxidation states of the cysteine residue at position 106 [77]. HMGB1 has previously been studied in

proinflammatory conditions such as systemic lupus erythomtousus (SLE), rheumatoid arthritis (RA), sepsis and chronic kidney disease [75, 78-81].

MIF is an upstream proinflammatory mediator stimulating the release of multiple cytokines including TNF-α and it promotes the recruitment of neutrophils into inflammatory sites in a chemokine-like fashion [82]. Three-dimensional X-ray crystallography has revealed that the MIF molecule contains a hydrophobic pocket [83]. This hydrophobic pocket is likely to play an important role in MIF activity, as compounds binding to this region decrease downstream MIF signalling [84]. Thyroxin (T4) has been proven to bind to this pocket thereby inhibiting MIF in a dose-dependent manner. This interaction is highly specific and the structurally similar triiodothyronine (T3) is only a weak inhibitor of MIF [85]. MIF has been implicated in the pathogenesis of sepsis, autoimmune diseases such as RA, SLE, cardiovascular disease and chronic kidney disease [86-90].

PTX3 is a CRP-like protein, but unlike CRP it is produced locally at sites of inflammation in response to TNF-α, IL-1 and LPS amongst others and it is not induced by IL6. Neutrophils store vast amounts of PTX3 in specific granules that are released upon respiratory burst and some of the released PTX3 stays associated with the parent cell by means of NETs [91]. PTX3s biological functions include C1q-mediated activation of the complement system through the classical pathway and the facilitation of pathogen recognition by phagocytes. It can also modulate immune response by restricting excess transmigration of neutrophils into tissue by interfering with the P-selectin/PSGL1 system [92] and inhibiting the amplification loop of the alternative complement pathway [93], thus dampening unwanted dissemination of inflammatory reactions.

sTWEAK is a ubiquitously expressed type II transmembrane glycoprotein of the TNF superfamily that circulates in the plasma in a soluble form (sTWEAK). Through binding to its receptor Fn14 it mediates its different biological functions including the exacerbation of the inflammatory response and tissue remodelling. Fn14 is highly inducible in the context of tissue injury and inflammation. The TWEAK/Fn14 pathway works through NF-κβ signalling which leads to target gene transcription [94]. sTWEAK is linked to cardiovascular disease, renal disease and autoimmune diseases amongst others and anti-TWEAK therapy has been tried experimentally in animal models of lupus nephritis [95, 96].

1.4.5 Scoring disease activity and damage

Since AAV is complex and heterogeneous in presentation, a structural evaluation is crucial when studying these diseases. There are two scoring instruments commonly used in research to asses disease activity and damage: the Birmingham Vasculitis Activity Score (BVAS) [97] and the Vasculitis Damage Index (VDI) [98]. Both have been adopted and used for clinical trials.

1.4.5.1 Birmingham Vasculitis Score (BVAS)

The Birmingham Vasculitis Score (BVAS) records signs and symptoms of active disease from nine different organ systems. The score is weighted so that more severe symptoms will score more points and new or worsening disease will score more points than persistent disease. Disease features are only scored when attributable to active vasculitis. There have been several versions of the BVAS since it was first introduced in 1994. The latest is version 3, which consists of 56 items with a maximum score of 63 [99].

1.4.5.2 Vasculitis Damage Index (VDI)

The Vasculitis Damage Index (VDI) records items of damage attributed to vasculitis or its treatment [98]. The damage must have occurred since the onset of vasculitis and been present for at least three months. Each item will score one point and over time the score can only remain unchanged or increase, even if an item of damage has been resolved.

1.5 TREATMENT OF AAV

Generally treatment of AAV consists of induction treatment in order to get the patient in remission and maintenance treatment in order to avoid relapse. When deciding on the appropriate induction treatment it is important to assess disease severity since different levels of disease severity respond to different treatment protocols [9]. Modern AAV treatment is evidence based and effective although drug toxicity and treatment failure remain a concern.

Category	Definition
Localised	Upper and/or lower respiratory tract disease without any other systemic involvement or constitutional symptoms
Early systemic	Any, without organ-threatening or life-threatening disease
Generalised	Renal or other organ-threatening disease, serum creatinine < 500 μmol/L (5.6 mg/dl)
Severe	Renal or other vital organ failure, serum creatinine > 500 μmol/L (5.6 mg/dl)
Refractory	Progressive disease unresponsive to glucocorticoids and cyclophosphamide

Table 1. EULAR disease categorisation of ANCA associated vasculitis [9].

1.5.1 Induction treatment

In principal induction treatment is given for 3–6 months and consists of a cytotoxic drug in combination with glucocorticoids. In severe disease plasmaphersis is considered [9].

1.5.1.1 Cyclophosphamide

Cyclophosphamide (CYC) was introduced in the 1970s as the first effective AAV treatment [100]. It can be given either orally (2 mg/kg/day) or intravenously (15 mg/kg) in pulsed therapy in combination with GC. Dose adjustments for age and renal function are generally recommended [101, 102]. In pulsed therapy a lower cumulative dose is given and there is less leukopenia and presumably less long-term complications [103]. The relapse rate seems to be higher with pulsed therapy however, especially in PR3 positive patients [104].

CYC metabolites are toxic and can cause haemorrhagic cystitis in the short term and malignancy in the long term [105]. For that reason 2-mercaptoethanesulfonate sodium (MESNA) is given. Antiemetics against infusion-related nausea and trimethoprim/sulphamethoxazole as prophylaxis against *Pneumocystis jiroveci* are also recommended [9]. Monitoring during the induction phase includes blood counts and leukopenia may necessitate dose changes or discontinuation of CYC [9, 106].

1.5.1.2 Rituximab

Rituximab (RTX) is a monoclonal mouse-human chimeric antibody directed against the CD 20 antigen on the B-cell surface, resulting in depletion of B cells in peripheral blood. In two randomised controlled trials, induction with RTX (375 mg/m^2 x IV) in combination with GC was equally effective to CYC in achieving remission [64, 65]. The rate of adverse events was similar in the two groups. A subgroup analysis suggested that RTX was more effective than CYC in relapsing disease [64].

Infusion reactions are common in RTX treatment [107] and patients are routinely given paracetamol, antihistamines and glucocorticoids before the infusion. Serious adverse events, such as hepatitis B reactivation [108, 109] and progressive multifocal leukoencephalopathy [110] have also been reported.

1.5.1.3 Methotrexate

In a randomised trial of early systemic AAV, methotrexate (MTX) (20-25 mg/week, oral or parenteral) was not inferior to CYC and in these circumstances MTX could be used as a less toxic alternative [111]. Some patients may respond more slowly to treatment [111] and some who develop more severe disease may have to be transferred to CYC or RTX [34]. Adverse events are, to some extent, dependent on renal function and include leukopenia, infections and hepatotoxicity [106].

1.5.1.4 Glucocorticoids

Despite being a mainstay of AAV therapy, no randomised trials have examined the role of glucocorticoids (GC) in AAV. Conventionally, 1 mg/kg/dag is given in most treatment protocols and then tapered out for a period of 6–18 months [111-113]. When a swift response is required pulses of methyl prednisolone (250 – 1000 mg) can be given i.v. [114]. It is important to consider osteoporosis prophylaxis [115] and monitor for diabetes [116] in all patients.

1.5.1.5 Plasmapheresis

Plasmapheresis is recommended in patients with severe disease and a creatinine > 500 µmol/L. This is based on the findings in the MEPEX trial where plasmapheresis (7 sessions within 14 days) proved superior to pulse treatment with i.v. methylprednisolone in terms of renal survival in the short term although the long-term benefits remain unclear [117, 118]. Although not studied in randomised controlled trials most centres will treat patients with lung haemorrhages with plasmapheresis based on published case series [119].

1.5.1.6 Second line therapies

Mycophenolate mofetil (MMF) has been tested in a randomised trial comparing it to cyclophosphamide as an induction agent in AAV. The remission rate and adverse events were similar although the study did not prove non-inferior to i.v. CYC (p= 0.06) [120] and there was a trend towards more relapses in the MMF-treated patients after 18 months [121].

In refractory or relapsing disease, RTX has been tested as rescue treatment in several case series with good results [108, 109, 122, 123] and as previously mentioned, RTX seemed to be superior to CYC in relapsing disease in the RAVE trial [64].

Other second line therapies include i.v. immunoglobulin [124], 15-deoxyspergualin [125] and anti-thymocyte globulin [126].

1.5.2 Maintenance treatment

Maintenance treatment is started when the patient has achieved remission. The purpose of maintenance treatment is to avoid relapse. The optimal length of maintenance treatment has not been determined but early cessation of therapy is associated with relapse and often 18–24 months of treatment is recommended [112, 127]. Long-term CYC therapy was previously used to maintain remission. Azathioprine (AZA) (2 mg/kg/day) has proven equally effective in preventing relapse at 18 months but without some of the drug toxicity concerns associated with CYC [112]. Other agents with proven efficacy in randomised trials are MTX (20-25 mg/kg/week) [128] and leflunomide (Leflunomide (20-30 mg/day) [129, 130]. These three agents are today the standard maintenance treatment together with a low dose of GC. MMF (2000 mg/day) was not as effective as AZA in maintaining remission in a study of 156 subjects over 42 months and must be considered as second line therapy [131].

Generally maintenance treatment has been studied after induction with CYC. Little is known about maintenance treatment after induction with RTX. Traditional maintenance agents such as AZA [132], watchful waiting [133] and pre-emptive treatment with renewed RTX treatment every six months [134] have all been used.

Chronic carriership of Staphylococcus aureus is associated with relapse in GPA patients [18] and continuous treatment with trimethoprim/sulphamethoxazole in addition to standard therapy may reduce the risk of relapse [135].

1.6 MONITORING AAV

Patient with AAV should periodically be evaluated using a structural clinical assessment since new organ involvement can develop at any time. In clinical practice, inflammatory markers, urine analyses, chest x-ray and renal function, are all helpful in assessing disease activity. A rise in ANCA titre may predict relapse but the monitoring of ANCA remains to be controversial [11].

For patients in remission on maintenance treatment the monitoring of their full blood count and liver functions should take place periodically to detect drug toxicity [106] and patients with GC therapy should have their blood sugar tested regularly in order to screen for diabetes [127].

Other long-term drug related complications of importance include osteoporosis [115], malignancy [50, 136] and gonad failure [137]. In RTX-treated patients transient neutropenia [138] and hypogammaglobulinemia [123] can be seen.

1.7 OBJECTIVES OF THE STUDIES

The overall aim of this study was to investigate the role of novel pro-inflammatory molecules and new therapies in AAV in order to optimise treatment, investigate the pathogenesis and find new biomarkers in these diseases.

The specific objectives were:

To study response to treatment in AAV by clinical assessment and routine and novel proinflammatory molecules **(paper I – IV)**.

To study long-term outcome in RTX-treated patients with relapsing or refractory disease in order to gain knowledge on long-term outcome, markers of prognosis and adverse events **(paper II).**

To study renal tissue expression and serum levels of novel pro-inflammatory molecules assumed to be involved in the pathogenesis of AAV in active disease and in remission **(papers I, III and IV)**.

To study the interaction between thyroid hormones and the immune system in active AAV and remission **(paper III).**

2 MATERIALS AND METHODS

2.1 PATIENTS

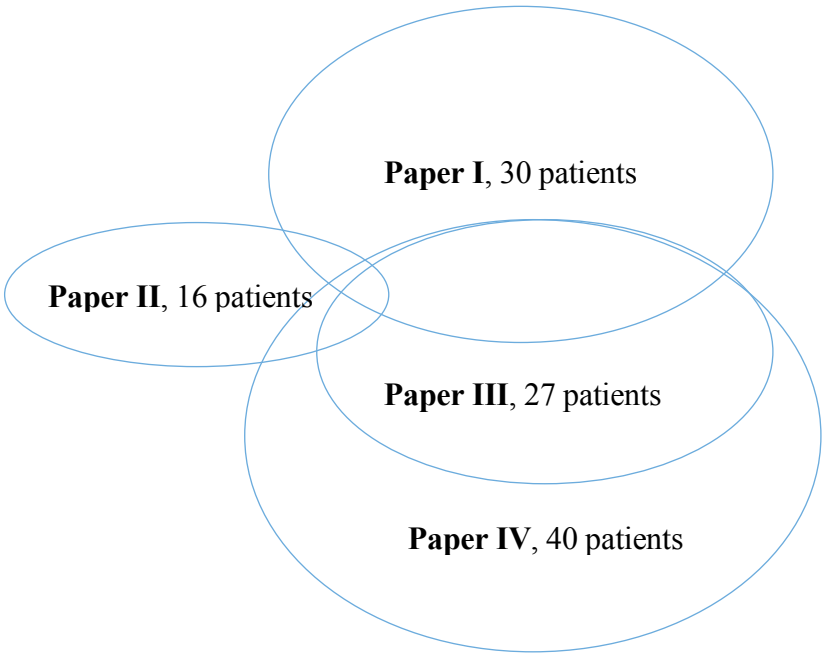
2.1.1 The VASKA cohort

Incident and prevalent AAV patients in the Stockholm area were included in the VASKA study in a joint project between the departments of rheumatology and nephrology at Karolinska University Hospital. During study visits, patients underwent clinical assessment, routine chemistry, immunological testing and microbial serology. Serum, plasma and urine research samples were spun and stored at -70° C for later analysis. Inclusion criteria were diagnosis of AAV, age of at least 18 years and a positive ANCA for MPO, PR3 or both by standard ELISA or capture ELISA methods at any time point. ANCA negative AAV patients or patients with anti-GBM antibodies were not included in the study. All patients gave informed consent.

Prevalent patients were assessed at one time-point and incident patients were assessed at 0, 3, 6, 24 and 60 months. The study is on-going and to date some 280 prevalent and 60 incident patients have been included in the study.

2.1.2 Study patients

In paper I, 30 patients with available renal biopsies were included. Most of these patients were prevalent patients with research data available from previous studies and some were incident patients within the VASKA study. In paper II, 15 prevalent patients with relapsing disease and one incident patient within the VASKA study who had been treated with RTX as a rescue treatment were followed up. In paper III, 27 incident patients were investigated regarding MIF and thyroid hormones and in paper IV, 40 incident patients were investigated regarding PTX3 and sTWEAK including the 27 patients in study III.



Paper	n	Age (Mean)	Men/ Women	Diagnosis GPA/MPA/EGPA	ANCA PR3/MPO/DP*
Paper I	30	59.0	14/16	17/12/1	16/12/1
Paper II	16	56.6	9/7	14/1/1	13/3/0
Paper III	27	57.5	15/12	17/9/1	15/12/0
Paper IV	40	58.0	20/20	23/15/1	22/16/1

*Double positive for both MPO and PR3

Table 2. Patient characteristics

2.1.3 Controls

Randomly selected healthy controls from a population-based cohort in the Stockholm area were recruited as a control group in papers I, III and IV. These patients were investigated as part of an on-going prospective study [139].

	Paper I	Paper III	Paper IV
n	48	53	20
Age (years)	62 ± 12	60 ± 12	63 ± 12
Gender (Male, %)	72	68	70
CRP (mg/L)	1.2 (0.2 – 32.0)	1.2 (0.2 – 32.0)	1.5 (0.8 – 8.1)
GFR (ml/min/1.73 m^2)	85 ± 20	85 ± 20	82 ± 14

Table 3. Controls' characteristics.

2.2 ASSESSMENT OF DISEASE ACTIVITY AND DAMAGE

Disease activity was assessed using the 2003 version of the BVAS at all study visits. The patients in **papers III** and **IV** had this data collected prospectively. Patients in **papers I** and **II** had the data collected prospectively when available, but in some instances the BVAS was estimated retrospectively. A patient with a BVAS of 0 was considered to be in remission and in **paper II** a patient with a reduction of the BVAS of at least 50% was considered to be in partial remission. Relapse was defined as an increase in disease activity, reflected in the BVAS, and requiring renewed induction treatment.

Disease and treatment-associated damage was assessed using the VDI.

2.3 TREATMENT

Most commonly, patients in the study were treated with pulsed i.v. CYC (15 mg/kg) in combination with GC according to EULAR guidelines or MTX (20-25 mg/week) in patients with localised or early systemic disease. Refractory or relapsing disease was generally treated with RTX 500 mg x II, 1000 mg x II or 375 mg/m^2 x IV in combination with GC. Patients with a creatinine > 500 μmol/L were given plasmapheresis. Some patients received MMF as induction treatment and some received oral CYC.

Patients in **paper II** were all given RTX as second line treatment and they had all previously received CYC at some time point.

Patients in remission were given maintenance treatment with MTX, AZA or MMF in combination with low dose GC.

2.4 LABORATORY MEASUREMENT

2.4.1 Routine laboratory parameters

Routine laboratory analyses were carried out using standard methods and these included CRP, albumin, creatinine, sedimentation rate (SR), urine dip slide, urine sediment and quantification of proteinuria by 24-hour urine-albumin excretion or creatinine/albumin ratio in morning urine. Estimated GFR (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula [140].

2.4.2 ANCA

ANCA was analysed at all study visits using standard ELISA methods (direct ELISA, Euro diagnostic) or multiplex (BIO-RAD, BioPlex TM 2200).). IF ANCA was not routinely

carried out. In selected cases, a capture PR3 ANCA was carried out at the discretion of the treating physician with the Wieslab Capture PR-3 ANCA kit (Eurodiagnostica AB, Malmoe, Sweden All patients in the studies had had a positive ANCA test at some time point.

2.4.3 Analysis of HMGB1

Serum levels of HMGB1 were analysed from cryopreserved serum by Western blot (Amersham Biosciences) and performed at The Feinstein Institute for Medical Research, New York, USA.

2.4.4 Analysis of MIF, PTX3 and sTWEAK

MIF, PTX3 and sTWEAK were analysed in serum using commercially available ELISA kits. In addition, PTX3 was measured in urine. ELISA kits for MIF were provided by Young In Frontier Co. Ltd. (Seoul, Korea), ELISA kits for PTX3 by R&D Systems Europe Ltd. (Abington, UK) and ELISA kits for sTWEAK by Bioscience (Hatfield, UK). All these analyses were carried out at the renal lab, KFC, Karolinska Institutet.

2.5 HISTOLOGICAL ASSESSMENT

Renal biopsies in **paper I** were performed by percutaneous ultrasonography-guided puncture. The renal tissue obtained was evaluated by light microscopy, immunofluorescence (IF) and electron microscopy according to standard techniques. Patients displaying pauci-immune focal necrotising glomerulonephritis or crescentic glomerulonephritis were considered as having active renal AAV and patients with the absence of these lesions were considered to be in renal remission.

Immunohistochemical stainings were performed using a monoclonal mouse IgG2b anti-HMGB1 antibody (Critical Therapeutics, Lexington, KY, USA) and Alexa Fluor© 488 conjugated goat anti-mouse IgG2b antibody (Molecular probes, Invitrogen, Eugene, OR, USA) for detection (figure 5a).

On paraffin embedded sections of renal tissue a biotin-labelled horse anti-mouse antibody (Vector laboratories Inc.) containing 2% normal horse serum was used for detection (figure 5b).

2.5 STATISTICS

Normally distributed variables were expressed as means ± SD (unless noted otherwise), and non-normally distributed variables were expressed as medians and ranges. Differences between the time points were examined using the Kruskal-Wallis analysis of variance (ANOVA), followed by a *post hoc* Dunn's test for non-parametric comparisons. A χ^2 test was

used for categorical variables. Correlations (ρ) were calculated by using the non-parametric Spearman rank test. A multivariate general linear model was used to assess the relationship between HMGB1 and various subgroups of vasculitis adjusting for eGFR. In paper II changes in CRP and BVAS were analysed by a Student's t-test. Statistical significance was set at a level of $p < 0.05$. All statistical analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA).

3 RESULTS AND DISCUSSION

3.1 PAPER I

High-mobility group box-1 protein (HMGB1) is increased in antineutrophilic cytoplasmic antibody (ANCA)-associated vasculitis with renal manifestations

High-mobility group box-1 protein (HMGB1) is a protein, or rather a group of proteins, that has gained a lot of attention in the field of autoimmunity. One of the reasons is that it seems to provide an answer to a key question in autoimmunity – how does a sterile inflammation arise? HMGB1 is a cytokine actively secreted in response to invading pathogens, but it is also released from dying and injured cells [76]. This means that products from cellular injury produced by the host (as in ischemia for instance) can give rise to an immune reaction indistinguishable from infection.

In this study 30 AAV patients with renal manifestations were included in the study at the time of renal biopsy with simultaneous collection of serum samples and evaluation of disease activity. In seven of the cases follow-up biopsies 6–9 months later were available with paired samples at these two time points.

A total of 38 biopsies showed active renal AAV in 25 cases and inactive in 13 cases. HMGB1 was significantly higher in active cases compared to inactive ones (120 +/- 48 ng/ml vs. 78 +/- 46 ng/ml, $p = 0.01$) and in the paired samples HMGB1 was significantly higher at baseline than after treatment and in remission 6-9 months later. HMGB1 staining appeared stronger with a more distinct extranuclear staining pattern in active disease than in remission. HMGB1 levels were much higher in AAV patients than in healthy controls (10.9 +/- 10.5 ng/ml), regardless of whether they were in remission or not. CRP and SR were significantly lower in inactive disease compared to active disease but did not significantly correlate with HMGB1. There was no statistically significant difference in HMGB1 levels between subgroups of AAV (GPA vs. MPA vs. EGPA) as has been proposed by other investigators [141], nor was there any difference between PR3 or MPO positivity.

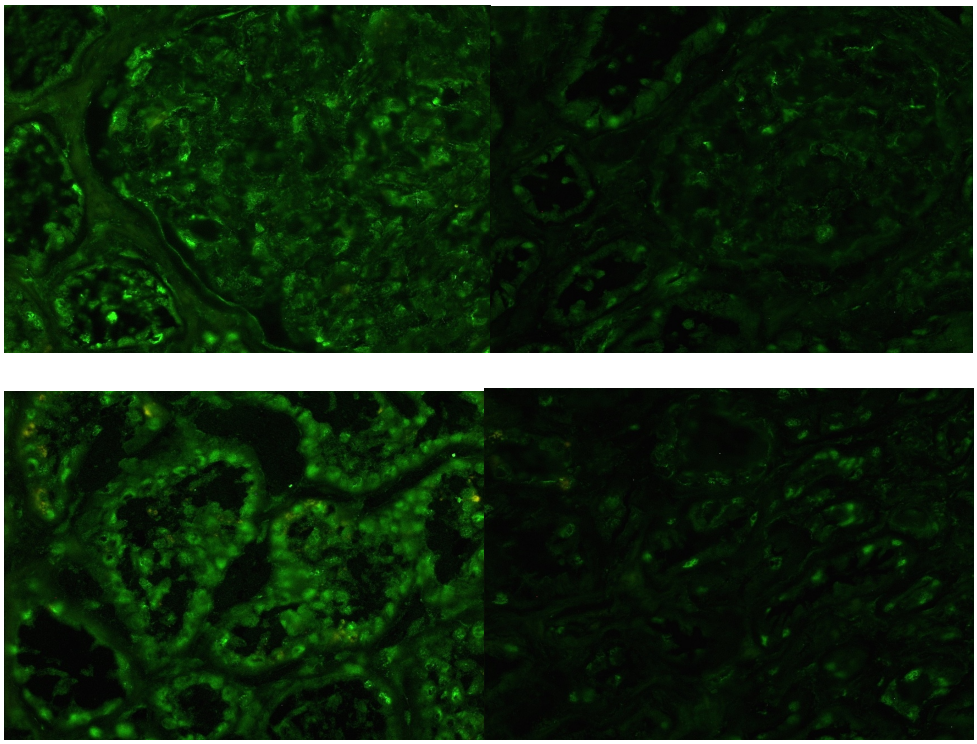


Figure 5a. Expression of extranuclear HMGB1 in necrotising renal AAV in paired kidney biopsies (active disease on the left and remission on the right).

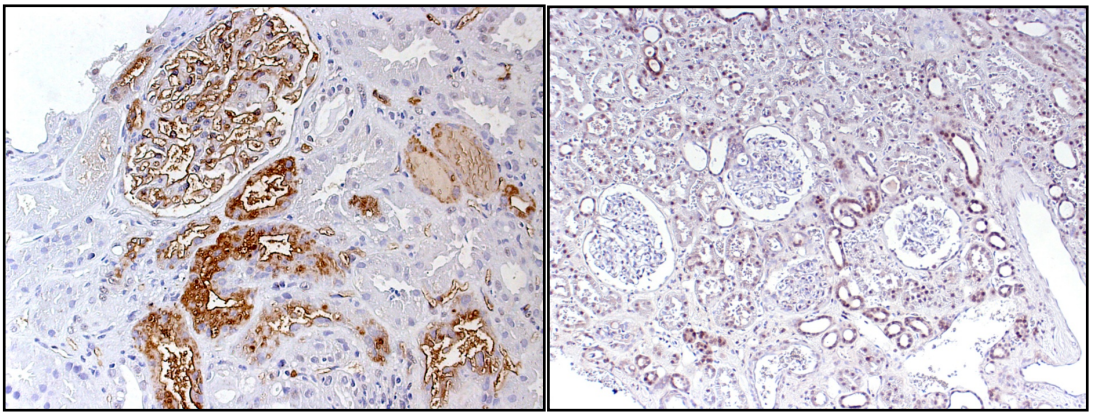


Figure 5b. Expression of extranuclear HMGB1 in necrotising renal AAV. Immunohistochemistry on paraffin embedded renal tissue. Active renal AAV on the left and healthy control on the right. Previously unpublished.

In conclusion, we found increased levels of HMGB1 in active renal AAV compared to inactive AAV and increased expression of HMGB1 in renal tissue in active disease clearly implicating HMGB1 in the pathogenesis of AAV. It is not clear whether increased serum levels of HMGB1 mainly derive from the kidney or other manifestations of the disease but the notion that necrotic tissue found in granulomatous disease (GPA) is the reason why excess amounts of HMGB1 are seen in AAV, as proposed by Wibisono *et al.* [141] is not supported by our data [142]. We did however find a higher level of HMGB1 in patients with pulmonary disease, but no correlation to granuloma, suggesting that the total burden of

disease is important. The discrepancy between our findings could be due to different methods in measuring HMGB1; we used a western blot method previously described [80] as opposed to an ELISA method used by Wibisono *et al*.

Levels of HMGB1 were much higher in AAV compared to healthy controls even when there was no clinically active disease, a finding that has been replicated in a cohort of SLE patients [78]. At the time, this result was puzzling but recent advances within the field of HMGB1 biology has shown that there are different forms of HMGB1 with different biological activity depending on the oxidative state of three cysteine residues within the molecule. Apoptotic cells oxidise the cysteine residue at position 106, creating a molecule without the proinflammatory properties of HMGB1 released by necrotic cells and secreted by inflammatory cells [77]. It is possible that the high levels of HMGB1 seen in inactive AAV derive from apoptotic cells but that needs to be confirmed in future studies.

3.2 PAPER II

Rituximab in relapsing or refractory ANCA-associated vasculitis: a case series of 16 patients

CYC is generally considered standard treatment of AAV but a small subset of patients is refractory or intolerant to this treatment. In these circumstances, RTX is often used as rescue therapy [122, 143, 144]. We wanted to study the outcome of these patients and identified 16 patients among our vasculitis patients who had received RTX on this indication and included them in the study.

We found that RTX was effective in this subset of patients that are otherwise difficult to manage. Twelve patients achieved complete remission and three patients achieved partial remission. During follow-up six patients relapsed. Patients positive for PR3 ANCA in the capture assay but not conventional ELISA seemed to be at risk of relapse. We could identify five such patients within our study. Four relapsed and the fifth received RTX every six months as pre-emptive therapy. Capture ANCA recognises different epitopes compared to conventional ANCA and it has previously been reported that capture ANCA is more effective in predicting relapse [145]. This could be consistent with the idea that different ANCA epitopes have different pathogenic effects [15, 146], but further conclusions from our study are limited by the sample size.

Infectious complications were a concern in the study and there were two cases of hepatitis B reactivation. One of the patients presented with elevated liver enzymes within two weeks of rituximab administration. Viral serology revealed anti-HBsAg positivity and HbeAg positivity. Virus load was quantified to 33.6 million copies of Hepatitis B virus per ml. blood. There was a hepatitis B serology 10 years prior to the rituximab treatment positive for anti-HbcAg and anti-HbsAg. The other patient received rituximab without any complication but on follow-up it was noted that the patient converted from anti-HbsAg and HbeAg negativity to positivity. The patient was anti-HbcAg positive at treatment start. Quantification showed > 110 million copies of hepatitis B virus per ml. blood. Both patients received antiviral

treatment with good control of viral load. Hepatitis B reactivation has mainly been reported in lymphoma treatment [147] but our data highlights the importance of assessing hepatitis B status and prescribing antiviral prophylaxis for patients at risk.

Other infectious complications included fatal sepsis, invasive fungus infection and CMV retinitis.

The efficacy and safety of RTX has been confirmed in randomised controlled trials, and in a subgroup analysis RTX appeared more effective in treating relapses [64, 65]. In our study RTX seems to be effective as rescue therapy but it also indicates that RTX treatment with other concurrent immunosuppression may be associated with a risk of serious infections.

3.3 PAPER III

Macrophage Migration Inhibitory Factor (MIF) and Thyroid Hormone Alterations in Antineutrophil Cytoplasmic Antibody (ANCA) Associated Vasculitis (AAV)

We investigated MIF in a prospective study of 27 incident patients before and after induction treatment (at 0, 3 and 6 months). There is a known interaction between thyroxine and MIF [85] and AAV is associated with thyroid disease and its treatment [32, 148]. We therefore assessed thyroid status as well along with CRP, creatinine, albumin and urine status. Disease activity was assessed using the BVAS 2003 at all time points and damage using the VDI. MIF and GC have reciprocal effects and it has been reported that MIF is inducible by GC [149]. Therefore exposure to GC was calculated and correlated with MIF levels.

We found that levels of MIF significantly decreased after induction treatment 8.6 pg/mL at baseline vs 5.7 pg/mL at 3 months and 6.2 pg/mL at 6 months ($p=0.001$) independently of CRP, creatinine, organ involvement or exposure to GC. Levels of MIF were much higher in AAV patients than controls regardless of whether they were clinically active or not.

T3 was depressed at baseline and increased during treatment similar to the thyroid alterations seen in critically ill patients, so-called ”Euthyroid sick syndrome” [150]. T3 correlated inversely with the BVAS, so these alterations were more pronounced in the most ill patients.

MIF did not correlate with GC, perhaps because GC dosing was unphysiologically high at the time of sampling. There was a strong correlation between the baseline MIF/T4 ratio and the MIF/T4 ratio at 6 months indicating an interaction between these molecules.

In conclusion, MIF is elevated in active AAV compared to remission and healthy controls indicating that MIF may be implicated in the pathogenesis and exacerbation of AAV. There seems to be an interaction between MIF and thyroxine as previously described in sepsis [85]. In animal models of sepsis, anti MIF treatment improves survival [85]. It is unknown whether anti MIF treatment would also be beneficial in AAV but this would be an excellent topic for future studies, since both animal models and anti MIF treatment exists [151]. The MIF – thyroxine interaction offers a possible causal link between AAV and thyroid disease.

3.4 PAPER IV

PTX3 and soluble TWEAK levels in active ANCA associated vasculitis (AAV) patients undergoing treatment – correlation to disease activity and relapse

In **paper IV** we examined the novel proinflammatory molecules PTX3 and sTWEAK in AAV. To do so we recruited 40 patients with active AAV (33 newly diagnosed, seven relapses) and measured levels of PTX3 and sTWEAK before and after induction treatment (at baseline and after six months) along with CRP, creatinine, urine samples and albumin. Disease activity was assessed at 0, 3 and 6 months. At three months, creatinine and urine samples were obtained for disease assessment purposes.

We found that PTX3 was elevated at baseline compared to follow-up (4.66 ng/ml vs.1.75 ng/ml $p < 0.001$) and that PTX3 correlated with disease activity but not with CRP or creatinine. The patients who were in remission at three months had a higher PTX3 level at baseline than the patients who failed to achieve remission: 5.3 ng/ml vs. 2.6 ng/ml ($p = 0.05$), at six months the difference was 4.8 ng/ml vs. 3.8 ng/ml (P=0.67). Urine levels of PTX3 were generally below detection threshold, even in patients with renal AAV.

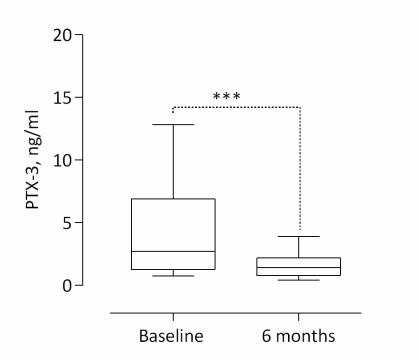


Figure 6. PTX3 levels at baseline and at follow-up.

In neutrophils, PTX3 is stored in specific granules that are released upon respiratory burst and can be found in the proximity of netting neutrophils [91], thus levels of PTX3 may reflect neutrophil reactivity in AAV. The correlation between disease activity and PTX3 levels may implicate PTX3 in the pathogenesis of AAV. PTX3 has proinflammatory properties but there is data that suggests that it actually may be protective by dampening the p-selectin function and thus recruitment and extravasation of leukocytes [92]. PTX3 also inhibits the amplification of the alternative complement pathway through interaction with factor H [152]. In light of this it is particularly interesting that high PTX3 in our study seemed to be associated with a more favourable prognosis suggesting that the role of PTX3 is of a more regulatory nature.

Our data thus suggests that PTX3 may be worth exploring as a marker of disease.

The other molecule we were interested in was sTWEAK. We found that sTWEAK did not significantly change throughout the study and that levels in AAV appeared similar to controls (566.6 pg/ml at baseline and 558.7 pg/ml at six months compared to 522.5 pg/ml in controls), however patients who failed to achieve remission at three and six months (BVAS > 0) had a significantly higher level of sTWEAK at baseline.

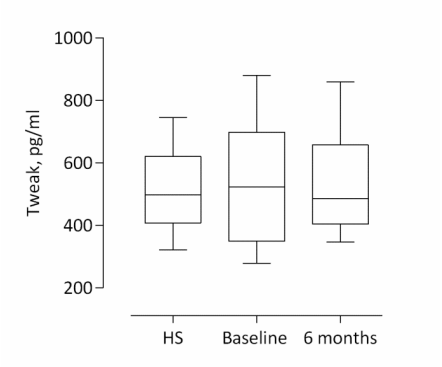


Figure 7. sTWEAK levels in healthy subjects, at baseline and at follow-up.

sTWEAK biology is firmly connected to its receptor, Fn14. A lot of work has been done on renal injury, and it has been shown that Fn14 in particular is upregulated in renal injury of various sorts [94]. We therefore separated out the patients with renal involvement and found a trend towards lower sTWEAK levels in these patients compared to patients without renal involvement ($p = 0.06$). In patients with renal vasculitis, sTWEAK levels increased after treatment and finally in patients without renal manifestations, levels fell after treatment although this did not reach significance.

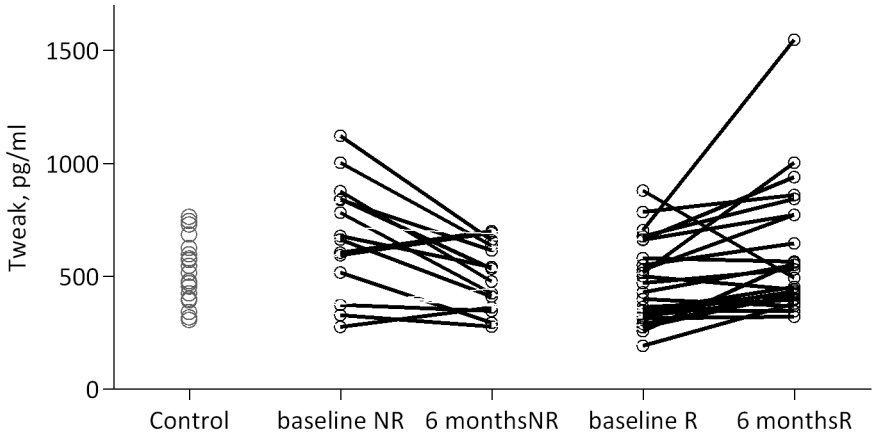


Figure 8. sTWEAK levels in controls, patients without renal vasculitis (NR) and patients with renal vasculitis (R).

Our data implies that sTWEAK has a tendency to vary with the phenotype of AAV and that sTWEAK may be involved in renal AAV, however that remains to be confirmed in a larger study.

4 GENERAL DISCUSSION

4.1 METHODOLOGICAL CONSIDERATIONS

4.1.1 Patient cohort

The ANCA associated vasculitides are rare and heterogeneous in presentation. The catchment area of Karolinska University Hospital is approximately 1.500.000 people. More or less all of the adult AAV patients are treated at the departments of rheumatology and nephrology and most were screened for inclusion in the VASKA study. Although a few patients declined participation and others were not enrolled as prevalent patients due to comorbidities, advanced age or social factors that would hinder follow-up, the cohort is highly representative of the spectrum of disease in our population. It is also a cohort of considerable size considering the rarity of these diseases. Few patients are lost to follow-up, partly on account of our general policy to never dismiss or refer these patients to general practitioners.

In the cohort there is a predominance of GPA, reflecting the fact that Scandinavia is a high endemic area of GPA [22]. Furthermore, we included relapses as incident patients, and relapses occur more frequently in GPA patients [26].

4.1.2 Treatment and response

With the exception of a few cases that were enrolled in international multicentre studies, all therapy decisions regarding the patients in this thesis were made by the treating clinician. Generally, treatment followed international guidelines. In systemic disease CYC was considered standard therapy and RTX was rarely used in novel patients. Dosage was generally according to EULAR guidelines [9], the main exception being RTX, where dosage varied considerably.

Obviously there are different treatment protocols for different phenotypes of the disease and since we tried to include all patients presenting with AAV willing to participate in the study, there was an intrinsic heterogeneity of treatment.

In refractory or relapsing disease, a topic explored in **paper II**, several immunosuppressive drugs were used concurrently and GC dosage varied considerably.

There were somewhat different response criteria in the studies. In **paper IV** patients were considered to be in remission if the BVAS was 0. In **paper II** patients were considered to be in complete remission if the BVAS was 0 and partial remission if there was a reduction of the BVAS of at least 50% (in concordance with definitions by previous investigators). Patients could still be on GC and considered to be in remission. Patients failing to achieve remission in **papers III** and **IV** were all on CYC as the original treatment and typically switched to RTX.

4.1.3 Strengths and limitations

This thesis is based on observational studies with the limitations that are associated with such studies. There is a clear possibility that the different treatment protocols used had an impact on the results, especially when measuring sTWEAK in renal and non-renal AAV. Exposure to GC also differed somewhat at baseline in **papers I, III** and **IV** although efforts have been made to secure samples at the earliest possible time point. A limited number of investigators who all had previous training and clinical experience of AAV carried out disease assessment. There were also some cases in **papers I** and **II** where the BVAS was estimated retrospectively, which could have influenced the quality of assessment.

In **Paper II** we investigated a highly selective subset of patients who were difficult to manage. Concurrent medication most certainly affected the result in terms of remission induction and adverse events. The number of patients was limited and the study was uncontrolled. Even though the study was descriptive in nature the cases were representative of how RTX is used in "real life" as a second line treatment, at least in our clinics.

In **Papers I, III** and **IV** proinflammatory molecules are measured before and after treatment and in some cases in healthy controls. An association between the molecule investigated and disease activity may suggest that the molecule is implied in the pathogenesis of the disease but it says little of the nature of that involvement regarding cause and effect. In this respect our results are hypothesis generating and should be followed by further studies.

A strength in **papers III** and **IV** is that the data is prospectively collected and that no patients within the cohort were excluded, thus reflecting a clinically relevant setting. Considering how rare these diseases are and that the thesis is based on a single centre cohort, it is a reasonable number of subjects. Patients in **paper II** reflect all consecutive AAV patients treated with RTX up to that time point at our clinics, giving it considerable descriptive power of RTX use in "real life" as second line therapy.

Our results from **paper I, II** and **IV** suggest that some of the molecules investigated may be used as biomarkers, however a promising biomarker candidate should be evaluated in subsequent studies.

As discussed elsewhere, HMGB1 was measured using a western blot in **paper I.** Recent findings have shown that the HMGB1 molecule exhibits different red-ox forms with different biological activity which this method does not discriminate between, making the results in **paper I** more difficult in interpret.

4.2 FINDINGS AND IMPLICATIONS

4.2.1 Persistently elevated levels of proinflammatory molecules after treatment

When comparing levels of the proinflammatory mediator MIF and HMGB1 to healthy controls we found persistently elevated levels even when disease was in remission. In fact the difference between patients in remission and healthy controls was more pronounced than the difference between active and inactive disease. In the case of HMGB1, newer findings have highlighted the importance of different isoforms of the molecule for the biological activity [77]. It is possible that we mainly see biologically inactive oxidised HMGB1 derived from apoptotic cells in inactive AAV. Although we did not find a correlation between MIF levels and glucocorticoid dosage there is a theoretical possibility of induction of corticosteroids [149] since patients were typically treated with 5-10 mg of prednisolone at follow-up. We did not measure PTX3 in healthy controls, but in a previously published paper PTX3 levels were similar to healthy controls in quiescent disease [153]. In this respect, PTX3 seems to more closely reflect disease activity than HMGB1 and MIF.

Another possible explanation of the elevated levels of HMGB1 and MIF is that we see many of the features of active disease still present in remission, but essential components necessary for the exacerbation of the disease, such as activated neutrophils, are lacking. This may predispose for relapse when discontinuing immunosuppression or when exposed to a disease-triggering event. It is also possible that this chronic inflammation enhances the progression of other vascular disorders such as cardiovascular disease [154-156] in concordance with what we see in other autoimmune systemic diseases such as SLE [157]. This is of particular interest in view of the higher incidence of stroke, heart infarction and accelerated atherosclerosis we see in AAV [155].

4.2.2 The role of rituximab as rescue treatment

RTX was originally licenced for treatment of B-cell lymphoma in 1997 [158]. It proved to be effective in autoimmune diseases as well and in 2006 it was licenced for RA [107]. In the lack of established second line therapies, off label prescription of RTX in AAV started to emerge as a form of rescue therapy. In published case series, RTX proved effective in inducing remission [109, 122, 143, 144, 159]. A significant proportion of the patients relapsed, often around the time point of B-cell return, but retreatment with RTX was generally effective. Based on these promising early experiences two randomised controlled trials were conducted comparing RTX with CYC [64, 65]. The AAV-patients included in these studies were newly diagnosed or relapsing and the studies showed that RTX was non-inferior to CYC as an induction agent. In relapsing patients it was in fact more effective than CYC [64]. These results have led to the common practice of using RTX as the treatment of choice for relapsing patients and in many ways RTX has revolutionised the management of difficult AAV cases [160]. Adverse events are however not less common with RTX and established maintenance treatment after RTX is lacking [64]. In **paper II** we report two cases of hepatitis B reactivations in RTX-treated patients among other serious adverse events. Early

on RTX was often used in combination with CYC [65]. The rationale for this was concern that the effect of rituximab was delayed in comparison to CYC. Our case series and others suggest that the combination of RTX and CYC may carry an increased risk of complications. This is not trivial since treatment is a major cause of morbidity and mortality associated with these diseases [161, 162], and during the first year after diagnosis, treatment-related deaths are by far more common than disease-related deaths [163].

In our case series and others there are patients treated and retreated with rituximab several times. Although there is a risk of hypogammaglobulinemia, (which is considered to be a relative contraindication for further treatments), clinical experience in AAV and other diseases such as RA supports multiple treatments with little risk to the patient [134]. This is significantly different when compared to CYC, where cumulative dose quickly becomes an issue.

4.2.3 Biomarkers

In the diagnosis of AAV, ANCA is very helpful and in a high proportion of patients ANCA is positive at the time of diagnosis, in particular in patients with systemic angiitis [164]. Subsequent monitoring of ANCA remains controversial, as titres do not always reflect disease activity [145, 165]. Previous investigators have found that capture ANCA is more sensitive without loss of specificity [7]. Albeit based on few cases, our study strengthens the notion that AAV patients with a negative conventional ANCA and positive capture ANCA may be at increased risk of relapse and should be monitored more closely.

In clinical practice CRP and sometimes sedimentation rate (SR) are followed. Although inflammation parameters are typically increased in active disease, these are crude markers of general inflammation and of little help in discriminating relapse from infection. CRP is also affected by corticosteroids and SR is commonly elevated in patients with impaired renal function and/or proteinuria [166]. Thrombocytosis is a common finding in AAV but unspecific and of little help in the clinical setting.

We have found three molecules that are all increased in active AAV compared to inactive AAV: HMGB1, MIF and PTX3. MIF correlated with disease activity at presentation and dropped after treatment reflecting less or no disease activity. A previous investigator has suggested that MIF may be a marker of disease worth exploring in AAV [167]. Any potential application in the clinic would be limited by the fact that we see persistently elevated levels of MIF in inactive disease compared to controls. GC, commonly used during the maintenance phase of treatment, also potentially affects MIF [149]. HMGB1 is also elevated in inactive AAV and there are several isoforms with different biological activity, limiting any conclusions regarding the molecules utility as a biomarker [77]. Recently it has been described that HMGB1 levels in urine are elevated in patients with active renal AAV compared to remission and healthy controls [168].

PTX3 has several interesting properties making it a candidate biomarker. It correlates well with disease activity independently of CRP and creatinine. It is not elevated in patients in remission. It is locally produced at sites of inflammation and it is unaffected by GC.

Urine samples are always investigated in AAV patients and haematuria is highly suggestive of glomerulonephritis. In a patient with renal AAV, haematuria and proteinuria is often persistent after induction treatment and may resolve several months after the patient has entered remission. We measured PTX3 in urine as well, but we could not consistently find elevated levels in patients with active renal disease. This may be due to tubular reabsorption.

sTWEAK did not correlate with disease activity and in that respect would make a poor biomarker. High levels of sTWEAK seemed to represent a phenotype of AAV with less favourable outcome in terms of remission. This is not as clear-cut as the difference between GPA and MPA as there was no clear difference in sTWEAK levels between these entities. Further conclusions are somewhat limited by the different treatment regimes.

4.2.4 Outcome and prognosis

Predicting outcome for the individual AAV patient is notoriously difficult. Airway granuloma, PR3 positivity and GPA diagnosis is associated with relapse but not necessarily with damage and mortality [25, 169, 170]. In fact, MPA seems to have a worse prognosis in this respect, most likely due to age and renal function [29, 171]. There are some observations in our studies that may relate to prognosis in terms of relapse and management of relapsing patients. Firstly, as discussed elsewhere, RTX is an excellent addition in the therapy arsenal in AAV, not least in relapsing patients who can be treated several times with good effect and with little toxicity. It is reasonable to assume that RTX treatment in this subset of patients results in lowered accumulative damage. Secondly, we observed that positive capture PR3 ANCA in the absence of a positive conventional ANCA was found in several relapsing, RTX-treated patients. And finally we saw that patients with high sTWEAK levels and low PTX3 levels at treatment start were less likely to achieve remission with standard treatment than other patients.

4.2.5 Future perspectives

Our studies suggest a potential role for a number of proinflammatory molecules in the pathogenesis and exacerbation of AAV and several of the findings merit further studies. In **paper I** we found elevated levels of HMGB1 in active disease compared to inactive and much higher levels of HMGB1 in patients in remission compared to healthy controls. Recent findings have shown that HMGB1 is actually a family of molecules with different redox states and biological functions [77]. This raises the question as to whether we see different redox states of HMGB1 in patients in remission and in active disease. Ideally, HMGB1 should be reanalysed with respect to the different redox forms in AAV patients.

In **paper III,** we see a clear indication of an interaction between MIF and thyroxine. This interaction has been demonstrated previously in sepsis and in animal models of sepsis

inhibition of MIF with biologically inert D-thyroxine (as opposed to the biologically active L-thyroxine) improved survival [85]. There are excellent and well-characterised animal models of MPO-mediated vasculitis [172]. A logical continuation of our research would be to feed MPO-vasculitis prone animals with D-thyroxine and compare them with untreated animals, with the hypothesis that MIF inhibition would diminish vasculitis activity.

The discrimination between remission and relapse is sometimes challenging and in addition there is a need to assess disease activity at any given time point in order to guide therapy. Today, there is a lack of reliable biomarkers in AAV presumably resulting in overuse and underuse of potentially harmful drugs resulting finally in increased morbidity and mortality. Of the molecules we investigated, PTX3 has shown the most promise as a marker of disease and should be evaluated in a larger study.

sTWEAK seems to be implicated in renal vasculitis, as in other kidney diseases. This could be further investigated by immunohistochemistry of TWEAK and the receptor, Fn 14, in renal biopsies from active disease and remission in analogy with our previous work on HMGB1.

As therapy becomes more effective in controlling disease, the research focus should shift somewhat to prevent long-term consequences such as diabetes, infections, cardiovascular disease, renal impairment and malignancies [161, 173-175]. One way is to optimise immunosuppressive therapy. Today, patients are to a large extent treated with “one size fits all” immunosuppressive therapy. This is not least the case with respect to GC. Tailoring treatment is a challenge for the future.

Most research today is carried out on active AAV but as our work and that of others highlights, there are immunological alterations in quiescent disease that may predispose patients for relapse and possible other diseases such as myocardial infarction and stroke. Better understanding of the immunological pathology in remission may help predict relapse as well as in finding targets for the prevention of cardiovascular disease in AAV patients.

5 CONCLUSIONS

AAV patients in remission still have persistent inflammation and signs of immune dysregulation compared to healthy subjects.

The proinflammatory molecules HMGB1, MIF and PTX3 are elevated in active AAV compared to remission, supporting the view that they may be involved in the pathogenesis and exacerbation of AAV. The increased tissue expression of HMGB1 in active ANCA associated renal vasculitis strongly supports a role of HMGB1 in the inflammatory process of pauci immune glomerulonephritis.

Thyroid hormone alterations are seen in AAV patients in concurrence with what is seen in critically ill patients and the known interaction between MIF and thyroxine may also be of clinical importance in AAV patients.

PTX3 is a potential biomarker in AAV and should be evaluated for that purpose in further studies. The sTWEAK/Fn14 system is implicated in renal vasculitis and should be further investigated, not least since TWEAK is a potential target of therapy.

RTX is an effective treatment in relapsing or refractory AAV but infectious complications are a concern. Concurrent immunosuppression may be a risk in these circumstances and it is important to assess hepatitis B status before treatment.

6 SVENSK SAMMANFATTNING

ANCA associerade vaskuliter (AAV) är en grupp ovanliga autoimmuna sjukdomar som drabbar kroppens minsta blodkärl och ibland också leder till inflammationshärdar (granulom), framför allt i övre och nedre luftvägar. Typiskt för AAV är förekomsten av ANCA, vilket är antikroppar som är riktade mot kroppsegna proteiner som normalt sett återfinns inuti vissa typer av vita blodkroppar (neutrofiler). Obehandlad leder sjukdomen till skador på inre organ och död inom något år, men med modern behandling kan nästan alla patienter bli fria från sjukdomsaktivitet. Oftast består behandlingen av ett cellgift i kombination med kortison vilket dämpar immunförsvaret och den autoimmuna reaktionen. Infektioner, läkemedelsbiverkningar och återfall i sjukdomen är problem som fortfarande är olösta och är föremål för mycket forskning. Eftersom symptomen av sjukdomen ofta kan vara svårtolkade för patient och doktor påbörjas behandling ibland för sent, då organskada redan utvecklats och AAV är en inte helt ovanlig orsak till att man behöver dialysbehandling.

Syftet med den här avhandlingen var att undersöka ett antal nykarakteriserade molekyler som man vet driver inflammation i andra, närbesläktade sjukdomar och se om dessa verkar vara inblandade i AAV också. Vi ville också undersöka effekten av nya behandlingar.

I den första artikeln undersökte vi molekylen HMGB1 och fann att nivåerna var högre hos patienter med aktiv sjukdom än hos patienter med behandlad sjukdom. När vi undersökte material från njurbiopsier fann vi att cellerna producerade mycket större mängder HMGB1 hos patienter som hade en aktiv vaskulit i njuren.

I den andra artikeln följde vi upp patienter med svårbehandlad sjukdom som inte hade haft fullgod effekt av cellgiftet cyklofosfamid och därför fått behandling riktad mot antikroppsproducerande vita blodkroppar (B-celler) med medicinen rituximab (Mabthera©). Vi fann att rituximab verkade vara effektivt hos dessa annars svårbehandlade patienter, men att det fanns en risk för allvarliga infektioner, speciellt då rituximab kombinerades med cellgifter.

I den tredje artikeln undersökte vi molekylen MIF och sköldkörtelhormoner vid AAV och fann att MIF nivåerna följde sjukdomsaktiviteten och att man kunde se förändringar i sköldkörtelhormoner som man normalt bara ser hos svårt sjuka patienter, till exempel patienter med blodförgiftning. Vi fann också tecken till att MIF och sköldkörtelhormoner interagerade med varandra, vilket är ett exempel på hur sköldkörtelhormoner kan påverka immunförsvaret.

Slutligen, i den fjärde artikeln undersökte vi molekylerna PTX3 och sTWEAK och fann att PTX3 korrelerade med sjukdomsaktivitet och att det finns tecken till att PTX3 kan vara skyddande vid AAV. sTWEAK föreföll vara inblandad i njurvaskulit och höga nivåer innan behandling verkade vara associerat med sämre behandlingssvar.

Sammanfattningsvis har vi funnit flera molekyler som verkar vara inblandade i uppkomst och utveckling av AAV. I framtiden kan dessa molekyler kanske underlätta diagnos av nyinsjuknade, följa sjukdomsförlopp eller skräddarsy behandling. De är också möjliga måltavlor för behandling med nya läkemedel. Rituximab förefaller vara en bra och effektiv behandling när standardbehandling inte är tillräcklig, men man bör vara uppmärksam på risken för allvarliga infektioner.

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