ANCA-associated vasculitis : factors involved in disease induction, survival and long-term complications

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ANCA-associated vasculitis: Factors
involved in disease induction,
survival and long-term
complications

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ANCA-associated vasculitis: Factors involved in disease induction, survival and long-term complications

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
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Contents

Abbreviations	9
---------------	---

Chapter 1	11
------------------	-----------

Wegener's granulomatosis – an introduction.

Factors involved in disease induction

Chapter 2	21
------------------	-----------

Evaluation of antineutrophil cytoplasmic antibody seroconversion induced by minocycline, sulfasalazine, or penicillamine.

Chapter 3	35
------------------	-----------

Occurrence of antineutrophil cytoplasmic antibodies and associated vasculitis in patients with hyperthyroidism treated with antithyroid drugs: a long-term follow-up study.

Chapter 4	51
------------------	-----------

Evaluation of antibodies against human HSP60 in patients with MPO-ANCA associated glomerulonephritis: a cohort study.

Chapter 5	67
------------------	-----------

Immunoregulatory gene polymorphisms are associated with ANCA-related vasculitis.

Factors involved in patient, renal and disease-free survival

Chapter 6	83
------------------	-----------

Renal survival and prognostic factors in patients with PR3-ANCA associated vasculitis with renal involvement.

Chapter 7	101
------------------	------------

Differences in renal biopsies do not lead to difference in outcome between patients with PR3-ANCA and patients with MPO-ANCA.

Chapter 8	119
------------------	------------

A positive C-ANCA titer at switch to azathioprine therapy is associated with a disquieting relapse rate in PR3-ANCA-related vasculitis.

Atherosclerosis and ANCA-associated vasculitis

Chapter 9 **133**

Anti-oxLDL antibodies in MPO-ANCA positive vasculitis patients preferentially recognize hypochlorite modified low density lipoproteins.

Chapter 10 **149**

CD4⁺CD28^{null} T cells are related to previous cytomegalovirus infection but not to accelerated atherosclerosis in ANCA-associated vasculitis.

Chapter 11 **167**

Atherosclerosis and ANCA-associated vasculitis – a review.

Chapter 12 **187**

Summary and general discussion.

Samenvatting **199**

Dankwoord **205**

Voor Dave
en mijn ouders

Abbreviations

AAGN	ANCA-associated glomerulonephritis
AAV	ANCA-associated vasculitis
ACE	angiotensin converting enzyme
ANCA	antineutrophil cytoplasmic antibodies
APV	ANCA-positive vasculitis
bp	base pair
BVAS	Birmingham vasculitis activity score
C-ANCA	cytoplasmic ANCA
CCA	common carotid artery
CIMT	carotid intima media thickness
CMV	cytomegalovirus
CRP	C-reactive protein
CSS	Churg-Strauss syndrome
CSSRD	Cooperative Systematic Studies of the Rheumatic Diseases
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
Cu	copper
CYCAZAREM	Randomised trial of cyclophosphamide versus azathioprine during remission in ANCA positive Systemic Vasculitis
EDV	endothelium-dependent vasodilatation
ELISA	enzyme-linked immunosorbent assay
ENT	ear, nose, throat disease
ESR	erythrocyte sedimentation rate
ESRD	end-stage renal disease
EUVAS	European vasculitis study group
FACS	fluorescence activated cell sorting
GBM	glomerular basement membrane
GN	glomerulonephritis
HC	healthy controls
HD	hemodialysis
hHSP60	human heat shock protein 60
HLE	human leukocyte elastase
HSP70	heat shock protein 70
HWE	Hardy Weinberg equilibrium

IgG	immunoglobulin G
IIF	indirect immunofluorescence
IMT	intima media thickness
iNCGN	idiopathic necrotizing and crescentic glomerulonephritis
KIR2DS1/2	killer cell immunoglobulin-like receptor DS1 / 2
MAP	mean arterial pressure
MDA	malondialdehyde
mHSP65	mycobacterial heat shock protein 65
MIRA	Minocycline in Rheumatoid Arthritis
MPA	microscopic polyangiitis
MPO	myeloperoxidase
MTX	methotrexate
N/A	not applicable
oxLDL	oxidized low-density lipoprotein
P-ANCA	perinuclear ANCA
PD-1	programmed death-1 (also <i>PDCD1</i>)
PE	plasma exchange
PR3	proteinase 3
PTU	propylthiouracil
PWV	pulse-wave velocity
RA	rheumatoid arthritis
REMAIN	Randomized trial of prolonged remission-maintenance therapy in systemic vasculitis
RRT	renal replacement therapy
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphisms
TCR	T-cell receptor
TNF	tumor necrosis factor
TPO	thyroid peroxidase
UA	unstable angina
WG	Wegener's granulomatosis

Chapter 1

Wegener's granulomatosis – an introduction

Slot MC, Cohen Tervaert JW

Published in part in Autoimmunity 2004;37:313-315

Wegener's granulomatosis (WG) is a disease of presumed autoimmune origin characterized by necrotizing granulomatous inflammation of the upper and lower airways and necrotizing vasculitis that is especially likely to involve the kidneys¹. Untreated, WG results in death within weeks to months. Since the introduction of cyclophosphamide and prednisolone as standard treatment, survival has improved dramatically from less than 20% at 1 year reported in 1958² to at least 74% 5-years survival reported in the past ten years³⁻⁶.

WG and other small-vessel vasculitides, such as microscopic polyangiitis (MPA) and the Churg-Strauss syndrome are strongly associated with antineutrophil cytoplasmic antibodies (ANCA) which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)⁷⁻⁹. There is evidence that ANCA play a pathophysiologic role in the induction of these vasculitides. First, rises in ANCA level predict disease reactivation^{10,11}. Second, *in vitro* it has been demonstrated that ANCA induce neutrophil activation, i.e. degranulation and production of a respiratory burst¹². In addition, ANCA activate other cells *in vitro* such as monocytes and endothelial cells¹³. Finally, *in vivo*, experimental data also suggest an important role for ANCA in the pathophysiology of vasculitis^{14,15}.

The mechanism by which ANCA are induced are as yet unclear. One favored hypothesis is that infections may trigger an ANCA response¹⁶. Certain drugs have been linked to the induction of ANCA and the onset of ANCA-associated vasculitis (AAV)¹⁷. Recently, it has been described that an autoimmune response may be induced by the presence of a peptide that is antisense or complementary to the autoantigen, for instance, PR3¹⁸. This immune response may induce anti-idiotypic antibodies (autoantibodies) that cross-react with the autoantigen.

Autoantibodies as diagnostic markers

In patients with systemic vasculitis and glomerulonephritis, ANCA were first described in 1982¹⁹. The link between ANCA and active WG emerged in 1985²⁰. Two ANCA patterns may be seen with indirect immunofluorescence (IIF) on ethanol-fixed neutrophils: a cytoplasmic pattern (C-ANCA), for which the major antigen is PR3; and a perinuclear pattern (P-ANCA), for which the major antigen is MPO⁷⁻⁹. Several groups have investigated the sensitivity and specificity of ANCA for small-vessel vasculitides. PR3- and MPO-ANCA have been the

subject of a meta-analysis on their diagnostic performance in the spectrum of systemic necrotizing vasculitides²¹. This meta-analysis revealed that MPO-ANCA has a low sensitivity for vasculitis on its own, but since nearly all patients with vasculitis have either PR3- or MPO-ANCA, a testing system combining P-/MPO-ANCA and C-/PR3-ANCA yields a pooled sensitivity of 85.5% and a pooled specificity of 98.6%. However, the sensitivity and specificity of an ANCA subtype may vary with the disease condition studied. For Wegener's granulomatosis, the presence of C-ANCA at any time has a pooled sensitivity of 66%²², which increases to 91% during active disease with a specificity of 99%. On IIF, ANCA has been detected in various other rheumatic diseases²³. On enzyme-linked immunosorbent assay (ELISA), sera from patients with SLE or RA may display reactivity to MPO, in addition to reactivity to multiple other neutrophil contents. PR3-ANCA are rarely detected in patients with rheumatic autoimmune diseases other than the small-vessel vasculitides²³. Nevertheless, even in a large cohort of patients with connective tissue disease, the specificity of a scoring system that combines immunofluorescence and ELISA may be as high as 99.5%²⁴. Even though the presence of ANCA is highly specific for the spectrum of small-vessel vasculitides that includes WG and MPA, a positive ANCA result should always be interpreted with consideration of the clinical setting and presentation of individual patients suspected of vasculitis. In addition to the diagnostic performance of the ANCA assays, the presence of specific clinical features plays a major role in determining the diagnostic probability of vasculitis²¹.

Autoantibodies as predictive markers

Several large series that describe patients with WG find high relapse rates for patients with this disease ($\geq 50\%$ within 5 years)³⁻⁶. Relapse is more common in patients with WG as compared to patients with another ANCA-associated vasculitis subtype, MPA²⁵. Since WG is more frequently associated with antibodies against PR3 (70-80%) than to MPO (10-30%), while the opposite is true for MPA, the question is whether it is the ANCA specificity or the clinical syndrome that is related to the relapse risk²⁶. Higher relapse rates in patients with PR3-ANCA as compared to patients with MPO-ANCA have been described^{27,28}. Moreover, even within the subgroup of patients with WG, PR3-ANCA specificity confers a higher risk of relapse²⁶.

Next to the difference in relapse rates between PR3- and MPO-ANCA, differences in both patient and renal survival have been described for the ANCA subtypes. Patients with cytoplasmic ANCA²⁹ or PR3-ANCA³⁰ have previously been described to have a higher risk of patient death, but this has been disputed by others^{31,32}. Vizjak *et al* reported a higher rate of renal failure in MPO- when compared to PR3-AAV³³, in contrast to previous studies that reported no difference^{31,32,34}. Causes of progression to renal failure may differ between PR3- and MPO-ANCA. In patients with MPO-ANCA, a slowly progressive course with patients developing dialysis dependency during follow-up without signs of clinically active disease is often observed. In these patients, proteinuria is the most important risk factor for renal failure during follow-up³⁵. Whether the same is true for patients with PR3-ANCA will be studied in this thesis.

In addition to ANCA specificity, ANCA levels at diagnosis and during follow-up have been shown to be of predictive value for patient, renal and disease-free survival. A high PR3-ANCA level at diagnosis in capture ELISA is a risk factor for both poor patient and renal survival⁶, and a consistently elevated MPO-ANCA level is a risk factor for poor renal survival as well³⁵.

During induction treatment with cyclophosphamide and prednisolone, ANCA levels fall or become negative in 30 to >80% of the patients within the first few months²⁶. Persistent or reappearing C-ANCA during the first year, however, is significantly related to subsequent relapse in WG^{27,36,37}. Several groups have addressed the predictive value of positive, or increasing, ANCA titers during long-term follow-up. More than 80% of patients, who were ANCA positive at diagnosis and who experience a relapse, have a persistently or renewed positive ANCA titer at the time of relapse^{11,27,28,36-38}. The relationship between a rise in ANCA titer, and a subsequent relapse, has been more controversial. Some studies clearly show an association between rise in ANCA titer and renewed disease activity^{10,11,39}, but this has been disputed by others^{27,36,38}. Nonetheless, two studies show that when a rise in ANCA titer of fourfold or greater is found in a patient, pre-emptive treatment reduces the risk of relapses, and may lead to a reduced exposure to immunosuppressive drugs^{39,40}.

Aim of the thesis

The aim of this thesis is to investigate factors involved in disease induction and factors involved in patient, renal and disease-free survival. Furthermore, a possible long-term consequence of ANCA-associated vasculitis was studied: atherosclerosis.

Chapters 2 and 3 discuss the association of several drugs with the occurrence of ANCA and associated vasculitis. Does the use of rheumatoid drugs (minocycline, sulfasalazine, or penicillamine) or the use of antithyroid drugs (carbimazol, methimazol, propylthiouracil) lead to a higher prevalence of ANCA-associated vasculitis?

In *Chapter 4*, the relationship between genetic factors and the presence of ANCA-associated vasculitis is investigated. Several inhibitory molecules that control the immune response have been described. Are genetic mutations (polymorphisms) in two of these molecules, CTLA-4 and PD-1, related to the presence of ANCA-associated vasculitis?

One favored theory is that infections may trigger an ANCA response, through molecular mimicry between bacterial and human proteins. In *Chapter 5* we investigate the possible role of bacterial and human Heat Shock Proteins in the induction of ANCA.

After the diagnosis of ANCA-associated vasculitis, aggressive treatment is often necessary to save the life of and the renal function in the patient. In order to select this treatment only for patients who really need it, prognostic factors are sought that may give insight in the disease course of these patients. *Chapters 6 and 7* describe the search for such prognostic factors.

The need for aggressive treatment in ANCA-associated vasculitis may be questioned; for example, remission maintenance therapy has been shown to be less aggressive than remission induction therapy and equally capable of controlling disease activity. In *Chapter 8* is studied whether less aggressive therapy might lead to a reduced disease-free survival.

With the current standards of therapy, ANCA-associated vasculitis has become a chronic illness. Due to the disease, or the given therapy, patients develop long-term complications; atherosclerosis may be one of them. In *Chapter 9*, we investigated whether antibodies against oxidized LDL, a marker for the presence of atherosclerosis, may already be present at diagnosis of ANCA-associated vasculitis. In

Chapter 10 we studied the presence of atherosclerosis using intima-media thickness and pulse-wave velocity measurements, and accompanying immunological phenomena (i.e., the presence of T-cells related to the presence of atherosclerosis) in patients with ANCA-associated vasculitis.

Finally, *Chapter 11* reviews the current concepts regarding atherosclerosis, and the presence of atherosclerosis in ANCA-associated vasculitis.

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Chapter 2

Evaluation of antineutrophil cytoplasmic antibody seroconversion induced by minocycline, sulfasalazine, or penicillamine

Choi HK, Slot MC, Pan G, Weissbach CA, Niles JL, Merkel PA

Arthritis Rheum 2000;43:2488-92

Objective. Case reports have suggested that minocycline, sulfasalazine, and penicillamine are associated with antineutrophil cytoplasmic antibody (ANCA)–positive vasculitis. This study evaluated ANCA seroconversion due to these agents in serum samples prospectively collected in randomized, double-blind, controlled trials.

Methods. The sources of study sera were 3 clinical trials: 1) a 48-week trial of minocycline for early rheumatoid arthritis, with 64 patients receiving minocycline compared with 68 receiving placebo; 2) a 37-week trial of sulfasalazine for rheumatoid arthritis, with 51 receiving sulfasalazine compared with 38 receiving placebo; and 3) a 104-week trial of penicillamine for early systemic sclerosis, with 15 undergoing high-dose penicillamine treatment versus 12 receiving low-dose penicillamine. ANCA were measured in the baseline and study-end serum samples by indirect immunofluorescence (IIF) for perinuclear ANCA (P-ANCA) and cytoplasmic ANCA (C-ANCA) patterns, and by antigen-specific enzyme linked immunosorbent assay (ELISA) for antibodies to myeloperoxidase (MPO-ANCA) and proteinase 3 (PR3-ANCA). Laboratory personnel were blinded to the group identity of the samples. ANCA results were interpreted using an ANCA scoring system that combines the results of IIF and ELISA testing.

Results. No patient in any of the active study drug groups demonstrated ANCA seroconversion according to the final interpretation of the combined IIF and ELISA results. Twelve of the 248 patients (5%) were positive for MPO-ANCA with P-ANCA at baseline. No subject was positive for PR3-ANCA with C-ANCA. There were no findings suggestive of vasculitis in any of these patients.

Conclusion. From our study results, there was no suggestion of ANCA seroconversion induced by minocycline, sulfasalazine, or penicillamine. However, these findings do not rule out the possibility of rare, sporadic cases of either ANCA seroconversion or true drug induced vasculitis with these drugs.

The triggers that induce antineutrophil cytoplasmic antibody (ANCA)–positive vasculitis (APV) are largely unknown. However, there have been a number of cases reported of APV associated with prior use of certain medications. The 2 drugs that have been most often implicated in the induction of APV are hydralazine and propylthiouracil¹⁻⁵. In addition, there have been cases linked to other drugs, including minocycline, sulfasalazine, and penicillamine^{4,6-16}. Drug associated APV can result in the same life-threatening vasculitic features as observed in idiopathic cases². Given the clinical severity of APV, even low rates of drug-induced disease would have important medical implications.

Seroconversion from ANCA negativity to ANCA positivity may occur even in the absence of clinical vasculitis in patients who are exposed to certain drugs. This process would be similar to that seen with antinuclear antibody seroconversion and would suggest a parallel between the pathogenetic steps of drug-associated lupus and vasculitis. ANCA seroconversion by drugs in the absence of clinical vasculitis would, if a real phenomenon, be a cause of false-positive ANCA findings (i.e., nonvasculitis condition with drug-induced ANCA positivity). Nevertheless, ANCA seroconversion might provide an important means of identifying patients at risk for developing drug-induced APV. No published report has addressed this important issue in a systematic way.

To evaluate ANCA seroconversion in patients exposed to minocycline, sulfasalazine, or penicillamine, we performed blinded ANCA testing of prospectively collected, paired pre- and postexposure serum samples from patients who were enrolled in therapeutic clinical trials for rheumatic diseases.

Patients and methods

Clinical trials and study groups

Three double-blind, randomized trials provided prospectively collected sera for ANCA testing in our study: the Minocycline in Rheumatoid Arthritis (MIRA) trial¹⁷, the Cooperative Systematic Studies of the Rheumatic Diseases (CSSRD) comparative study of sulfasalazine, injectable gold, and placebo¹⁸, and a trial of penicillamine for early systemic sclerosis (scleroderma trial) (Table 1)¹⁹. In the MIRA trial, patients with early rheumatoid arthritis (RA) received 200 mg of minocycline or placebo daily for 48 weeks. We used sera from both

MIRA groups for this study. In the CSSRD trial, patients with RA received 2,000 mg of sulfasalazine, placebo, or gold sodium thiomalate daily for 37 weeks. Sera from the first 2 groups were used for this study. In the penicillamine trial, patients with early systemic sclerosis received penicillamine at 750–1,000 mg (high dose) or 62.5 mg (low dose) daily for 2 years. We used the sera from both of these groups. Only sera that were paired, i.e., both a baseline (0 weeks) and a trial-end serum sample were available, were tested for ANCA. Thus, 132 paired MIRA sera (from 64 patients receiving minocycline and 68 placebo), 89 paired CSSRD sera (from 51 patients receiving sulfasalazine and 38 placebo), and 27 paired scleroderma trial sera (from 15 patients receiving high-dose penicillamine and 12 low-dose penicillamine) were tested. The total number of study subjects was 248 and total number of paired sera was 496 (Table 1).

Control groups and blinding

To ensure the quality of ANCA testing in our laboratory, various groups of control sera were also tested: samples from 15 patients with vasculitis that tested positive for antimyeloperoxidase antibodies (MPO-ANCA) and perinuclear ANCA (P-ANCA), samples from 15 patients with vasculitis that tested positive for anti–proteinase 3 (PR3-ANCA) and

Table 1. Source of study subjects and summary of drug exposures*

<i>Source trial, treatment arm (dosage)</i>	<i>Duration of drug exposure, mean ± SD weeks (range)</i>	<i>Number of subjects (n = 248)†</i>
MIRA trial		
Minocycline (200 mg/day)	51 ± 7.1 (10 – 72)	64
Placebo	50 ± 6.7 (6 – 56)	68
CSSRD RA trial		
Sulfasalazine (2000 mg/day)	31 ± 11 (4 – 39)	51
Placebo	30 ± 12 (2 – 49)	38
Scleroderma trial		
High-dose penicillamine (750-1000 mg/day)	58 ± 4 (51 – 69)	15
Low-dose penicillamine (62.5 mg/day)	61 ± 13 (50 – 100)	12

* MIRA, Minocycline in Rheumatoid Arthritis; CSSRD, Cooperative Systematic Studies of the Rheumatic Diseases; RA, rheumatoid arthritis

† Total number of paired sera = 496

cytoplasmic ANCA (C-ANCA), and samples from 30 normal blood donors. All study sera were assigned new, randomized identification numbers. To keep laboratory personnel blinded to the study group identity, samples were realiquoted into identical tubes labeled only with study identification numbers. The group identification of the sera was kept masked until all of the ANCA assay results were collected for the final interpretation and the analysis was ready to begin.

ANCA assays by indirect immunofluorescence (IIF)

IIF tests were done with the use of ethanol-fixed neutrophils as described elsewhere²⁰. The results of staining were classified as having 1 of 4 patterns: C-ANCA, P-ANCA, atypical (neither cytoplasmic nor perinuclear), or negative. Given the subjective nature of scoring of the results of IIF for ANCA, we utilized a comprehensive interpretation system as described in our previous work²⁰. Briefly, the interpretation was decided by agreement of initial independent readings by 2 investigators (MCS and JLN) or by resolution of their discrepancy by taking the majority as final after obtaining a third investigator's (CAW) reading. IIF staining was repeatedly performed to resolve discrepant readings. IIF and enzyme-linked immunosorbent assay (ELISA) results were determined independently.

ANCA assay by ELISA

Each sample was tested by direct antigen-specific ELISAs that used highly purified antigens to detect anti-MPO and anti-PR3 antibodies, as well as a sandwich anti-PR3 ELISA. These ANCA assays have been extensively characterized in prior reports from our laboratory²⁰⁻²³.

Final interpretation by combining IIF and ELISA ANCA results

A final interpretation of ANCA testing was determined by combining the IIF and ELISA results. Samples were considered ANCA-positive when positive for both PR3-ANCA and C-ANCA or when positive for MPO-ANCA and P-ANCA or atypical ANCA. Therefore, samples with IIF positivity without respective ELISA positivity or those with ELISA positivity without respective IIF positivity were interpreted as negative in our final interpretation. This is consistent with our previously published methods²⁰.

Statistical analysis

Comparisons between groups were analyzed by Fisher’s exact test for categorical variables. All tests used a 2-tailed significance level of 0.05. We derived 95% confidence intervals (95% CI) using exact methods for proportions. Analysis was performed using the Stata software package (Stata Corporation, College Station, TX).

Results

ANCA seroconversion by final combined interpretation

No patient in any of the active study drug groups demonstrated ANCA seroconversion according to the final combined interpretation (table 2). Serum from 1 patient receiving placebo in the minocycline trial

Table 2. ANCA test results, final interpretation*

Study group, time point	n	PR3-ANCA	MPO-ANCA
MIRA RA trial			
Minocycline			
Start	64	0 (0)	3 (4.7)
End	64	0 (0)	2 (3.1)
Placebo			
Start	68	0 (0)	4 (4.4)
End	68	0 (0)	2 (2.9)
CSSRD RA trial			
Sulfasalazine			
Start	51	0 (0)	4 (7.8)
End	51	0 (0)	3 (5.9)
Placebo			
Start	38	0 (0)	1 (2.6)
End	38	0 (0)	0 (0)
Scleroderma trial			
High-dose penicillamine			
Start	15	0 (0)	1 (6.7)
End	15	0 (0)	1 (6.7)
Low-dose penicillamine			
Start	12	0 (0)	0 (0)
End	12	0 (0)	0 (0)
Control groups			
Normal blood donors	30	0 (0)	0 (0)
MPO-ANCA vasculitis	15	0 (0)	14 (93.3)
PR3-ANCA vasculitis	15	15 (100)	0 (0)

* Note that conversion from negative to positive or positive to negative could occur over the course of the study for each ANCA test. Values are the number (%) of antineutrophil cytoplasmic antibody (ANCA)-positive patients. MPO-ANCA, antimyeloperoxidase antibodies; PR3-ANCA, anti-proteinase 3 antibodies (see Table 1 for other definitions)

converted from ANCA negative to ANCA positive, with a very low titer of MPO-ANCA and an atypical IIF ANCA pattern. Twelve other patients were positive for ANCA at baseline (by final combined interpretation), but 5 of these were found to be MPO-ANCA negative at their end-study time point. All 13 MPO-ANCA-positive patients were positive for P-ANCA or showed an atypical ANCA IIF pattern, with 2 of the patient serum samples changing their IIF pattern from atypical to P-ANCA and 2 changing from P-ANCA to atypical. No study patient was positive for combined PR3-ANCA/C-ANCA at any time.

ANCA seroconversion by IIF alone

When the results of ANCA testing by immunofluorescence were analyzed separately from the ELISA data, seroconversions were found only in those samples with atypical ANCA (table 3). Nineteen of the 248 serum samples (8%) seroconverted for atypical ANCA (3 receiving sulfasalazine, 2 high-dose penicillamine, 7 minocycline, 1 placebo in the CSSRD trial, 6 placebo in the MIRA trial). No patient in any group showed a seroconversion for the P-ANCA or C-ANCA patterns by IIF alone.

ANCA seroconversion by ELISA alone

None of the patient sera seroconverted for MPO-ANCA in the absence of IIF positivity (table 4). One patient was PR3-ANCA negative at baseline, but positive for PR3-ANCA by sandwich ELISA alone at follow-up, representing a seroconversion by ELISA only. One patient was positive for PR3-ANCA by direct ELISA alone at baseline, but negative at follow-up. Three additional patients were PR3-ANCA positive by sandwich ELISA alone at baseline, but negative at follow-up.

Disease classification of ANCA-positive patients at baseline

RA trials. Among the patients in the 2 RA source trials, 6 patients with early RA in the minocycline study were positive for ANCA at baseline (3 in the minocycline group and 3 in the placebo group), while 5 patients with RA in the CSSRD trial were positive for ANCA at baseline (4 in the sulfasalazine group and 1 in the placebo group). Of these 11 RA patients with ANCA positivity at baseline, 7 had a borderline positive MPO-ANCA ELISA titer and were positive for perinuclear or atypical IIF at baseline. The remaining 4 had high MPO-ANCA titers with

Table 3. ANCA Indirect Immunofluorescence test results*

<i>Study group, time point</i>	<i>n</i>	<i>C-ANCA</i>	<i>P-ANCA</i>	<i>Atypical ANCA</i>
MIRA RA trial				
Minocycline				
Start	64	0 (0)	2 (3.1)	26 (40.6)
End	64	0 (0)	0 (0)	25 (39.1)
Placebo				
Start	68	0 (0)	1 (1.5)	35 (51.5)
End	68	0 (0)	1 (1.5)	29 (42.6)
CSSRD RA trial				
Sulfasalazine				
Start	51	0 (0)	2 (3.9)	23 (45.1)
End	51	0 (0)	3 (5.9)	19 (37.3)
Placebo				
Start	38	0 (0)	3 (7.9)	18 (47.4)
End	38	0 (0)	2 (5.3)	19 (50.0)
Scleroderma trial				
High-dose penicillamine				
Start	15	0 (0)	0 (0)	1 (6.7)
End	15	0 (0)	1 (6.7)	2 (13.3)
Low-dose penicillamine				
Start	12	0 (0)	0 (0)	1 (8.3)
End	12	0 (0)	0 (0)	1 (8.3)
Control groups				
Normal blood donors	30	0 (0)	0 (0)	2 (6.7)
MPO-ANCA vasculitis	15	0 (0)	11 (73.3)	3 (20.0)
PR3-ANCA vasculitis	15	14 (93.3)	0 (0)	1 (6.7)

** Note that conversion from negative to positive or positive to negative could occur over the course of the study for each ANCA test. Values are the number (%) of antineutrophil cytoplasmic antibody (ANCA)-positive patients. C-ANCA, cytoplasmic ANCA; P-ANCA, perinuclear ANCA; MPO-ANCA, antimyeloperoxidase antibodies; PR3-ANCA, anti-proteinase 3 antibodies (see Table 1 for other definitions)*

perinuclear or atypical IIF patterns at baseline. There were no clinical findings suggestive of vasculitis beyond the features of RA (meeting the study entry criteria of source trials) in any of these patients. This false-positive ANCA testing rate (for the diagnosis of vasculitis) among patients with RA (5.4%) was not significantly different from our previous results²⁰.

Scleroderma trial. One patient in the high-dose penicillamine group was ANCA positive at baseline. This was the only patient in the study with a high titer of ANCA. This patient had diffuse scleroderma with lung and gastrointestinal involvement. He tolerated 1,000 mg of penicillamine throughout the trial. The MPO-ANCA ELISA titer rose from 78 to 2,061 units (normal <2.8 ELISA units) after 14 months. No

findings suggestive of vasculitis were observed, even after 4 years of clinical follow-up. The finding that only 1 of 27 patients with scleroderma (3.7%) was positive for MPO-ANCA with P-ANCA represented a rate that was also not significantly different from our prior data²⁰.

Control groups. Of the 60 control sera (15 with PR3-ANCA/C-ANCA, 15 with MPO-ANCA/P-ANCA, and 30 normal blood donors), 59 sera were correctly classified. One sample from an MPO-ANCA/P-ANCA-positive patient was interpreted as ANCA negative by our combined scoring method (false negative), whereas the other 14 samples were positive for MPO-ANCA/P-ANCA or atypical ANCA. All PR3-ANCA/C-ANCA-positive samples were confirmed positive, and all blood donor samples were ANCA negative.

Discussion

The current study evaluated potential ANCA seroconversion by minocycline, sulfasalazine, and penicillamine in prospectively collected sera from 3 major clinical trials on rheumatic disorders. The results do not support ANCA seroconversion induced by these 3 drugs.

Our ability to demonstrate drug-induced ANCA seroconversion might have been affected by a number of factors: 1) the dosages of the study medications, 2) the duration of drug exposures, and 3) the potential frequency of ANCA seroconversion. The dosages of medications used in source trials were similar to those used in previously published cases that implicated these drugs as a cause of ANCA-positive vasculitis^{6,7,9,12,13,15}. The duration of drug exposure was close to that reported in the previously published cases associated with minocycline (12 months versus 12–36 months)⁶. However, the duration was shorter than in the reported cases associated with sulfasalazine (7 months versus 18–40 months)^{7,15} or penicillamine (14 months versus 17–70 months)^{9,12,13}.

The lower bounds of the frequency of potential seroconversion were limited in this study by the number of paired sera available. Thus, this study does not rule out ANCA seroconversion rates <1.6% for minocycline (95% CI 0–5.8%), <1.7% for sulfasalazine (95% CI 0–7.2%), or <3.7% for penicillamine (95% CI 0–13.7%). In order to further investigate the possibility of rare ANCA seroconversion and drug-associated APV, case–control studies may be more feasible than

Table 4. ANCA ELISA test results*

<i>Study group, time point</i>	<i>n</i>	<i>Direct PR3-ANCA</i>	<i>Sandwich PR3-ANCA</i>	<i>Direct MPO-ANCA</i>
MIRA RA trial				
Minocycline				
Start	64	0 (0)	0 (0)	3 (4.7)
End	64	0 (0)	0 (0)	2 (3.1)
Placebo				
Start	68	0 (0)	1 (1.5)	3 (4.4)
End	68	0 (0)	1 (1.5)	2 (2.9)
CSSRD RA trial				
Sulfasalazine				
Start	51	0 (0)	1 (2.0)	4 (7.8)
End	51	0 (0)	0 (0)	3 (5.9)
Placebo				
Start	38	0 (0)	1 (2.6)	1 (2.6)
End	38	0 (0)	0 (0)	0 (0)
Scleroderma trial				
High-dose penicillamine				
Start	15	1 (6.7)	0 (0)	1 (6.7)
End	15	0 (0)	0 (0)	1 (6.7)
Low-dose penicillamine				
Start	12	0 (0)	0 (0)	0 (0)
End	12	0 (0)	0 (0)	0 (0)
Control groups				
Normal blood donors	30	0 (0)	0 (0)	0 (0)
MPO-ANCA vasculitis	15	0 (0)	0 (0)	14 (93.3)
PR3-ANCA vasculitis	15	13 (86.7)	15 (100)	0 (0)

** Note that conversion from negative to positive or positive to negative could occur over the course of the study for each ANCA test. Values are the number (%) of antineutrophil cytoplasmic antibody (ANCA)-positive patients. ELISA, enzyme-linked immunosorbent assay; MPO-ANCA, antimyeloperoxidase antibodies; PR3-ANCA, anti-proteinase 3 antibodies (see Table 1 for other definitions)*

prospective cohort studies; these would require large sample sizes with long periods of follow-up to demonstrate potential positive results. There are several strengths in our study. By testing ANCA in pre- and postexposure serum samples, we were able to eliminate the potential false impression of ANCA seroconversion that might be presumed by administering the drugs to patients who were already positive for ANCA at baseline. Among the reported cases implicating minocycline, sulfasalazine, or penicillamine in inducing ANCA-associated syndromes^{6,7,9,12,13,15}, we are aware of only 1 case of APV associated with penicillamine in which a negative MPO-ANCA status was documented prior to drug initiation¹². Thus, it remains possible that the

remaining cases described in the literature^{6,7,9,12,13,15} could have had positive ANCA titers before the respective drug therapy was started, as demonstrated in our current study. In addition, the blinding of laboratory personnel to sample group assignment helped eliminate the potential bias involved in the subjective nature of the interpretation of immunofluorescence results²⁰. Another strength of our study is the extensive clinical and research experience of our ANCA testing laboratory^{20,21}.

Our combined ANCA testing system that utilizes both ELISAs and IIF is highly specific^{20,21} and minimizes the potential false impression of ANCA seroconversion that could result from testing sera by IIF alone. Although we differentiate atypical from perinuclear patterns, IIF readings are subjective, and other laboratories may not read the slides the same way nor may they not have the samples read by at least 2 qualified personnel. The apparent rate of seroconversion among study subjects exposed to the test drugs would have been as high as 5% if determined by IIF alone, rather than the 0% found with the more comprehensive combined IIF and ELISA scoring method used.

In conclusion, our study results do not support ANCA seroconversion by minocycline, sulfasalazine, or penicillamine, which raises a possibility that previous reports of retrospective cases could have been premature in claiming the causality between these drugs and ANCA. However, these data do not rule out the possibility of rare, sporadic cases of either ANCA seroconversion or true drug-induced vasculitis with these drugs.

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Chapter 3

Occurrence of antineutrophil cytoplasmic antibodies and associated vasculitis in patients with hyperthyroidism treated with antithyroid drugs: a long-term follow-up study.

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Objective. To test whether antineutrophil cytoplasmic antibodies (ANCA) and ANCA-associated vasculitis (AAV) are not only induced during treatment with antithyroid drugs, but can also become manifest when medication has been ceased, possibly after years.

Methods. Patients who visited our hospital for the treatment of hyperthyroidism were included (n=209). Treatment consisted of antithyroid medications, radioactive iodide, thyroidectomy, or a combination of these treatment options. Patients were re-tested 3 to 6 years later to evaluate long-term effects of antithyroid drugs. Patients were tested for the presence of ANCA and, if positive, evaluated for the presence of AAV.

Results. Of 209 patients with hyperthyroidism, 12 patients (6%) were positive for myeloperoxidase- (MPO-), proteinase 3- (PR3-) and/or human leukocyte elastase- (HLE-) ANCA. Seventy-seven of 209 patients were re-tested; one patient, not treated with antithyroid drugs had developed MPO-ANCA. In 3 of 6 patients previously positive, ANCA could still be detected. The presence of ANCA was highly associated with (previous) treatment with antithyroid drugs (Odds ratio 11.8 (95% confidence interval 1.5 – 93.3). Of 13 patients with a positive ANCA result on ELISA, AAV with glomerulonephritis was diagnosed in 4 patients (31%).

Conclusion. The presence of ANCA with or without vasculitis is associated with previous treatment with antithyroid drugs, possibly after years.

Antineutrophil cytoplasmic antibodies (ANCA) with specificity for proteinase 3 (PR3) or myeloperoxidase (MPO) are strongly associated with small-vessel vasculitides such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA)¹⁻³. There is evidence that ANCA play a pathophysiologic role in the induction of these vasculitides. First, elevations in ANCA titers appear to correlate with risk of future disease flares, although they do not reliably predict the timing of such flares^{4,5}. Second, *in vitro* it has been demonstrated that ANCA induce neutrophil activation, i.e., degranulation and production of a respiratory burst⁶. In addition, ANCA activate other cells *in vitro* such as monocytes and endothelial cells⁷. Finally, *in vivo*, experimental data also suggest an important role for ANCA in the pathophysiology of vasculitis^{8,9}. Certain drugs have been linked to the induction of ANCA and the onset of ANCA-associated vasculitis (AAV)¹⁰. There are several case reports implicating hydralazine¹¹⁻¹⁴ and propylthiouracil (PTU)¹⁵⁻¹⁹ in the induction of AAV. Occasionally, other drugs are mentioned, including other antithyroid drugs such as carbimazole¹⁷ and methimazole^{20,21}. ANCA in drug-induced AAV are most frequently directed against MPO^{11-16,18-22}; however, cases in which patients were PR3-ANCA positive are also observed^{12,15,18,20,22}. ANCA in these patients may also be directed against human leukocyte elastase (HLE) and/or lactoferrin, and the presence of ANCA directed to two or more antigens may even be an indication of drug-induced disease^{15,23}. Clinically, drug-induced ANCA-associated vasculitis can be substantially milder than idiopathic vasculitis, and drug-induced AAV may resolve in many cases with discontinuation of the offending agent. However, severe disease courses may be observed and treatment with immunosuppressive therapy can be necessary.

To our knowledge, time span between medication use and induction of ANCA and AAV has not been studied. Furthermore, whether ANCA are induced by hyperthyroidism, or by antithyroid medication is still uncertain. We hypothesized that ANCA and AAV may not only be induced during treatment with antithyroid drugs, but can also become manifest when medication has been ceased, possibly after years. To test this hypothesis, we evaluated patients with hyperthyroidism, on various antithyroid treatment modalities, for the presence of ANCA. Additionally, we evaluated induction of ANCA after long-term follow-up by testing an additional sample > 3 years after previous testing for

ANCA.

Patients and methods

Patients

All consecutive patients diagnosed with hyperthyroidism (both new diagnosis and relapse) between January 1994 and July 1996 and treated in our hospital were included. Treatment consisted of antithyroid medications (carbimazole, methimazole, PTU), radioactive iodide, thyroidectomy, or a combination of these treatment options. Additionally, patients who were diagnosed with hyperthyroidism, visited the outpatient clinic of our hospital in 1999-2000 and had used antithyroid drugs (carbimazole, methimazole, and/or PTU), during the course of their disease for at least three months were included. Causes of hyperthyroidism were based on established clinical, laboratory, scintigraphy and ultrasonographic findings and are presented in table 1. Patients who were tested for the presence of ANCA between January 1994 and July 1996 were contacted for consent on re-testing in 1999-2000. Informed consent was obtained from each patient. When patients were found positive for ANCA (see below), they were invited for evaluation including physical and laboratory examination for signs of vasculitic disease according to our protocol²⁴.

Sera

Serum samples for ANCA testing were collected and stored at - 20 °C. Samples were coded and realiquoted in identical tubes. Laboratory personnel were blinded to patient identity and medication use.

Table 1. Causes of hyperthyroidism in groups A and B

	Group A N (%)	Group B N (%)
Graves and other autoimmune	87 (66)	58 (76)
Multinodular struma	25 (19)	14 (18)
Toxic adenoma	3 (2)	1 (1)
Unknown	17 (13)	3 (4)
Total	132	76

Testing for ANCA by indirect immunofluorescence (IIF)

Sera were tested for the presence of ANCA by IIF as described previously²⁵. Briefly, slides coated with human granulocytes were incubated with patient sera in two dilutions (1:20 and 1:40) or with positive controls (for P- and C-ANCA) or with a negative control (normal human serum). After addition of conjugate (FITC-labeled rabbit F(ab')₂ anti-human IgG, Dako, Denmark), slides were read independently by two experienced readers. Fluorescence patterns were scored as P-ANCA, C-ANCA, atypical, or negative. When readings were discordant, slides were read independently by a third reader without knowledge of prior fluorescence results. The IIF score was then decided by the majority.

Testing for ANCA by enzyme-linked immunosorbent assay (ELISA)

Each sample was tested by capture ELISA to detect PR3-, MPO- and HLE-ANCA as previously described¹.

Final combined interpretation

Results of IIF and ELISA were combined for final interpretation. Sera were considered positive for ANCA when capture ELISA was positive for MPO-, PR3- and/or HLE-ANCA. IIF staining showing C-, P- or atypical ANCA was helpful, but not necessary, for ANCA positivity. Sera were considered negative when IIF was positive without a positive ELISA result or when the results on both assays were negative.

Testing for thyroid microsomal antibodies

Titers of thyroid microsomal antibodies were determined by indirect haemagglutination using a commercial diagnostics kit according to the manufacturer's instructions (Murex Biotech, Dartford, UK). TPO has been determined as the thyroid microsomal antigen²⁶. Sera were considered positive when agglutination was present in at least a 1:100 dilution. All patients who were tested in 1999-2000 for the presence of ANCA were tested for anti-TPO antibodies.

Statistical analysis

Comparisons between groups were analyzed by Fisher's exact test for categorical variables. All tests used a 2-tailed significance level of 0.05. We derived 95% confidence intervals (95% CI) using exact methods

for proportions. Analysis was performed using the GraphPad Instat software package version 2.04a (GraphPad Software Inc., San Diego, CA).

Results

Cross-sectional evaluation of ANCA positivity in patients with hyperthyroidism

Between January 1994 and July 1996, 131 patients with hyperthyroidism were tested for the presence of ANCA. Additionally, 76 patients were tested between 1999 and 2000. Patient characteristics are presented in table 2.

On immunofluorescence, C-ANCA was present in 2 serum samples (1.0%). P-ANCA was present in 19 serum samples (9.1%). Atypical ANCA was present in 23 serum samples (11.1%). All other serum samples produced negative staining.

By ELISA, 12 patients of 132 (6.1%) reacted positive in one or more of the assays. Four patients (1.9%) were positive for MPO only and 4 patients were positive for HLE only. Additionally, 2 patients were positive for both PR3 and HLE (1.5%), 1 patient (0.8%) for both MPO and HLE, and 1 patient for MPO, PR3 and HLE. Ten patients who were positive by ELISA were also positive in IIF, with P-ANCA ($n = 6$) or atypical ($n = 4$) staining.

Table 2. Patient characteristics

<i>Group</i>	<i>Age*</i>	<i>Female (%)</i>	<i>Antithyroid drug use</i>
All patients N = 207	49.8 ± 15.1	165 (80)	107 (52)
Patients on antithyroid drugs N = 107	48.5 ± 15.2	84 (79)	
Patients without antithyroid drugs N = 100	51.2 ± 15.0	83 (83)	
Patients with follow-up sample N = 77	49.1 ± 14.7	65 (84)	56 (77)
Patients without follow-up sample N = 54	51.8 ± 15.3	44 (81)	43 (80)

* *Mean ± standard deviation*

Long-term follow-up of patients with hyperthyroidism

During follow-up, nine of 132 patients previously tested had died (of non-vasculitic causes). Forty-five patients refused to be re-tested. These patients did not differ from patients who were re-tested with respect to age, gender, and antithyroid drug use (table 2). Seventy-seven patients (6 of whom were previously positive for ANCA on ELISA) were re-tested in 2000. Median time between first and follow-up sample was 4.8 years (range, 3.3 to 6.0 years). We found 5 of 6 patients still positive for ANCA. One patient, who was previously positive for PR3- and HLE-ANCA, was now found negative. One of 71 patients previously negative for ANCA became positive for MPO-ANCA. The remaining 70 patients were persistently ANCA negative.

The presence of ANCA is associated with previous treatment with antithyroid drugs

Overall, 107 patients were or had been treated with one or more antithyroid drugs (27 with carbimazole, 69 with methimazole, and 38 with PTU); 162 patients were treated with radioactive iodide, and 1 patient had surgery. Eleven (10.3%) patients treated with antithyroid drugs were positive for MPO-, PR3 and/or HLE-ANCA, whereas 2 patients, not treated with antithyroid drugs (2%) were positive for ANCA. Treatment with antithyroid drugs was highly associated with the presence of ANCA (Odds ratio 11.8 (95% CI 1.5 – 93.3), $P = 0.005$). However, when patients only positive for HLE-ANCA were excluded, there was no significant association between antithyroid drug use and ANCA positivity ($P = 0.17$). ANCA positivity was not related to any individual antithyroid drug (data not shown). There was no relationship between time on/off antithyroid drugs and the presence of ANCA, nor the time interval between drug initiation and the onset of vasculitic symptoms. Fifty-three percent of patients who were ANCA negative had anti-TPO antibodies compared to 67% of patients who were ANCA positive ($P = 0.69$). Forty-one percent of patients who were ANCA negative had anti-colloid antibodies compared to 25% of patients who were ANCA positive ($P = 0.65$). Additionally, there was no association between cause of hyperthyroidism and the presence of ANCA ($P = 1.0$).

Clinical evaluation of ANCA-positive patients

In 13 patients who tested positive for ANCA, 4 patients (2 MPO-ANCA,

1 MPO- and HLE-ANCA, and 1 MPO-, PR3- and HLE-ANCA), all of whom had been or were treated with antithyroid drugs, had signs and symptoms of vasculitis, and were treated in our hospital (tables 3 and

Table 3. Laboratory results of ANCA-positive patients

<i>Gender (age)</i>	<i>Diagnosis</i>	<i>Drug use, duration (IV)</i>	<i>1st ANCA result</i>	<i>2nd ANCA result</i>
F (68)	Graves	MMI, 5 months (5 months)	IIF: P-ANCA ELISA: MPO	negative
F (49)	Graves	MMI, 8 months (7 years)	IIF: P-ANCA ELISA: MPO	negative
F (43)	Graves	PTU, 21 months (23 months)	IIF: P-ANCA ELISA: MPO/ PR3/ HLE	IIF: P-ANCA ELISA: MPO/ PR3/ HLE
F (51)	Graves	PTU, 4 years (4 years)	IIF: P-ANCA ELISA: MPO/ HLE	IIF: P-ANCA ELISA: MPO/ HLE
M (57)	Unknown	PTU, unknown	IIF: atypical ELISA: HLE	no FU
F (35)	Graves	None	IIF: atypical ELISA: PR3/ HLE	no FU
F (63)	Unknown	MMI, unknown	IIF: atypical ELISA: PR3/ HLE	negative
F (56)	Unknown	PTU, 6 years	IIF: atypical ELISA: HLE	IIF : P-ANCA ELISA : HLE
F (88)	MNS	None	IIF: atypical ELISA: negative	IIF: P-ANCA ELISA: MPO
F (31)	MNS	PTU, 6 months	IIF: P-ANCA ELISA: HLE	no FU
F (38)	Graves	MMI/ PTU, 2 years	IIF: negative ELISA: HLE	no FU
M (40)	Graves	CM/ MMI, 2 years	IIF: negative ELISA: MPO	no FU
F (26)	Graves	CM, unknown	IIF: P-ANCA ELISA: MPO	no FU

F, female; M, male; MNS, multinodular struma; IV, time interval between drug initiation and presence of vasculitic manifestations; MMI, methimazole; PTU, propylthiouracil; CM, carbimazole; ANCA, antineutrophil cytoplasmic antibodies; MPO, myeloperoxidase; PR3, proteinase 3; HLE, human leucocyte elastase; FU, follow-up

4). During follow-up, two patients with vasculitis continually tested positive for ANCA on IIF and ELISA, despite treatment with cyclophosphamide and steroids and discontinuation of their antithyroid

Table 4. Clinical features of ANCA-positive patients

<i>Gender (age)</i>	<i>Drug use, duration (IV)</i>	<i>ANCA result</i>	<i>Clinical</i>
F (68)	MMI, 5 months (5 months)	IIF: P-ANCA ELISA: MPO	Pleural effusion, renal functional deterioration but no active urine sediment → CY+ CS
F (49)	MMI, 8 months (7 years)	IIF: P-ANCA ELISA: MPO	NCGN in renal biopsy → CY+ CS
F (43)	PTU, 21 months (23 months)	IIF: P-ANCA ELISA: MPO/ PR3/ HLE	NCGN in renal biopsy, pulmonary infiltrates → CY+ CS
F (51)	PTU, 4 years (4 years)	IIF: P-ANCA ELISA: MPO/ HLE	NCGN in renal biopsy → CY+ CS
M (57)	PTU, unknown	IIF: atypical ELISA: HLE	No signs of vasculitis
F (35)	None	IIF: atypical ELISA: PR3/ HLE	No signs of vasculitis
F (63)	MMI, unknown	IIF: atypical ELISA: PR3/ HLE	No signs of vasculitis
F (56)	PTU, 6 years	IIF: atypical ELISA: HLE	No signs of vasculitis
F (88)	None	IIF: P-ANCA ELISA: MPO	Died of pneumonia and septic shock
F (31)	PTU, 6 months	IIF: P-ANCA ELISA: HLE	No signs of vasculitis
F (38)	MMI/ PTU, 2 years	IIF: negative ELISA: HLE	No signs of vasculitis
M (40)	CM/ MMI, 2 years	IIF: negative ELISA: MPO	No signs of vasculitis; glomerulosclerosis in biopsy
F (26)	CM, unknown	IIF: P-ANCA ELISA: MPO	No follow-up on clinical signs

F, female; M, male; MNS, multinodular struma; IV, time interval between drug initiation and presence of vasculitic manifestations; MMI, methimazole; PTU, propylthiouracil; CM, carbimazole; ANCA, antineutrophil cytoplasmic antibodies; CY, cyclophosphamide; CS, corticosteroids; NCGN, necrotizing and crescentic glomerulonephritis

medication. In the other 2 patients with vasculitis, ANCA titers normalized after treatment with cyclophosphamide and steroids and discontinuation of antithyroid drugs. In the remaining 9 patients, one patient with MPO-ANCA had impaired renal function and proteinuria (table). His renal biopsy showed changes, typical of hypertension in addition to focal segmental glomerulosclerosis of uncertain origin; necrotizing and crescentic glomerulonephritis was not observed. He had discontinued his antithyroid drugs prior to ANCA testing. Seven other patients who had tested positive for ANCA did not show signs of vasculitis. In the ninth patient positive for ANCA, follow-up on clinical signs was not possible. Two of these 8 ANCA-positive patients without signs of vasculitis were re-tested in the follow-up study. The first was again found positive for HLE-ANCA, without evidence of vasculitis, and without the present use of antithyroid drugs. The second patient (previously PR3- and HLE-ANCA positive) was now found negative, without being treated with any immunosuppressive drug such as cyclophosphamide and/or steroids.

Discussion

In the current study, we investigated the presence of ANCA in patients diagnosed with hyperthyroidism on various treatment modalities. In our cross-sectional evaluation, we found 12 patients (5.7%) positive for MPO-, PR3- and/or HLE-ANCA on first testing. Furthermore, during follow-up one patient became ANCA-positive. ANCA-positivity was significantly related to the use of antithyroid drugs. Clinical signs were present in two of six ANCA-positive patients who had used PTU, in two patients using other antithyroid drugs and in none of the patients that were not treated with antithyroid drugs.

The presence of ANCA, and of ANCA-associated vasculitis in patients using antithyroid drugs for treatment of hyperthyroidism have been recurrently reported^{15-23,27}. Reported prevalence of ANCA in patients treated with antithyroid drugs varies from 4% to 46%; prevalence of AAV is lower (0 to 1.4%^{22,27-29}). In our population ANCA and AAV were present in 11% and 4%, respectively, of patients treated with antithyroid drugs. Prevalence of primary systemic vasculitis (WG, MPA, Churg-Strauss syndrome, polyarteritis nodosa) has been estimated at 144.5/million (0.01%) in a population comparable to the general population in Groningen³⁰. Therefore, a prevalence of AAV of 4% in our

patients treated with antithyroid drugs clearly suggests a relationship between these drugs and the presence of AAV.

Although ANCA are present relatively often in patients treated with antithyroid drugs, the prevalence of AAV is much lower^{22,27-29}. In our study, 7 patients out of 11 treated with antithyroid drugs whom were positive for MPO-, PR3 or HLE-ANCA did not have, or develop, vasculitis (64%). This 'false positive' rate is much higher than could be expected from our earlier studies in which we found false positive results in less than 1% of patients with related disorders and/or healthy controls^{25,31}. Therefore, the use of PTU, carbimazole and methimazole seems an important cause for positive ANCA results not related to small-vessel vasculitis.

The presence of ANCA without vasculitis indicates that ANCA alone are not enough for the induction of vasculitis. Indeed, in patients with clinically quiescent AAV, high ANCA titers can persist without reactivation of vasculitis^{5,32-34}. It has been suggested that the characteristics of ANCA present in active disease may differ from those present in disease remission and in sera from patients without vasculitis, possibly in the mechanism of neutrophil activation^{7,35}.

The mechanism by which PTU, and other antithyroid drugs, may induce ANCA and AAV remains to be elucidated, although some clues do exist. A study by Jiang et al showed that propylthiouracil, among other drugs, was highly cytotoxic in the presence of activated neutrophils, at concentrations that are therapeutically relevant³⁶. Oxidation of PTU by activated neutrophils provides a way to generate reactive drug metabolites, and the finding of MPO-ANCA in patients with drug-induced lupus and vasculitis is consistent with an autoimmune reaction initiated by drug bioactivation mediated by neutrophil-derived MPO³⁷.

It is possible that AAV in the patients in our study was not induced by antithyroid drugs, but merely is associated with hyperthyroidism. Unfortunately, we did not have ANCA titers prior to the initiation of treatment in these patients. In a previous study on the induction of ANCA by minocycline, sulfasalazine and penicillamine, we showed that when ANCA were present in patients after treatment with these drugs, they had already been present prior to treatment start³⁸. The presence of MPO-ANCA may also be explained by the presence of anti-TPO antibodies which, given the partial homology between both proteins, may cross-react with MPO³⁹, although this has been disputed by

others⁴⁰. In our patients, the presence of MPO-ANCA was not related to anti-TPO antibodies. Moreover, cross-reactivity to TPO cannot explain the presence of HLE- and/or PR3-ANCA in our patients.

In conclusion, we showed that the presence of ANCA, and associated vasculitis, is not uncommon in patients treated with antithyroid drugs. The presence of ANCA and AAV extends beyond antithyroid treatment duration. This risk of using antithyroid drugs should be included in the decision to use these drugs for the treatment of hyperthyroidism.

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Chapter 4

Evaluation of antibodies against human HSP60 in patients with MPO-ANCA associated glomerulonephritis: a cohort study.

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Background. Human Heat Shock Protein 60 (hHSP60) has been implicated in autoimmunity through molecular mimicry, based on the high degree of homology with HSP65 of micro-organisms leading to autoimmune recognition of the human protein. Additionally, sequence homology between hHSP60 and myeloperoxidase (MPO) has been described. MPO is a major autoantigen in vasculitis associated with antineutrophil cytoplasmic antibodies (ANCA). We hypothesized that infections may trigger the ANCA response against MPO through hHSP60.

Methods. In 86 consecutive patients with ANCA-associated vasculitis (AAV), anti-hHSP60 and anti-mycobacterial HSP65 were measured by ELISA. Patients were compared with 69 healthy controls (HC). Continuous data between groups were compared using Wilcoxon signed rank test and Kruskal-Wallis test with Dunn's post-test when appropriate. Correlations between data were derived using Spearman correlation. Odds ratios and 95% confidence intervals were obtained using Fisher's exact test.

Results. At diagnosis, median anti-mHSP65 level was higher in AAV, and subsequently in MPO-ANCA, compared to HC. Anti-hHSP60 antibody levels in AAV were not higher compared to HC. However, in MPO-ANCA anti-hHSP60 levels were increased compared to PR3-ANCA and HC. We could not detect cross-reactivity between hHSP60 and MPO-ANCA. There was a correlation between anti-mHSP65 and anti-hHSP60 antibody levels but not between anti-hHSP60 and MPO-ANCA.

Conclusions. Antibodies against mHSP65 are higher in AAV compared to HC, and anti-hHSP60 antibodies are higher in patients with MPO-ANCA than in patients with PR3-ANCA and HC. Although this finding may be indicative for cross-reactivity between MPO-ANCA and hHSP60, additional assays did not support this hypothesis.

Small vessel vasculitides, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with antineutrophil cytoplasmic antibodies (ANCA), which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)¹⁻³. These diseases can occur in any organ system but the respiratory tract and the kidneys are most frequently involved. Untreated, WG results in death within weeks to months. Since the introduction of cyclophosphamide and prednisolone as standard treatment, survival has improved dramatically from less than 20% at 1 year reported in 1958⁴ to at least 60% 5-years survival reported in the past ten years⁵⁻⁸.

The mechanism by which ANCA are induced is as yet unclear. Certain drugs have been linked to the induction of ANCA and the onset of ANCA-associated vasculitis (AAV)⁹. Recently, it has been described that an autoimmune response may be induced by the presence of a peptide that is antisense or complementary to the autoantigen, for instance, PR3¹⁰. This immune response may induce anti-idiotypic antibodies (autoantibodies) that cross-react with the autoantigen. Another favored hypothesis is that infections may trigger an ANCA response¹¹. The proposed mechanisms by which infections break self tolerance can include bystander damage, unveiling of 'hidden' self epitopes, determinant molecular spreading and molecular mimicry¹¹.

Heat Shock Protein (HSP) 65 is an immunodominant antigen in micro-organisms. The human equivalent, hHSP60, has been implicated in autoimmunity through molecular mimicry, based on the high degree of homology with HSP65 of micro-organisms leading to autoimmune recognition of the human protein¹². In its turn, HSP60 shares sequence homology with MPO¹³. Thus, infections may trigger the ANCA response against MPO through hHSP60. To test this hypothesis, we determined the presence of antibodies against hHSP60 and mycobacterial HSP65 (mHSP65) in patients with MPO-ANCA and compared them to patients with PR3-ANCA and healthy controls (HC).

Methods

Patients

We retrospectively identified all patients in our renal biopsy registry^{14,15} diagnosed with crescentic glomerulonephritis between January 1977 and July 2003 without evidence of systemic lupus erythematosus, IgA nephropathy, Henoch Schönlein purpura, post-infectious

glomerulonephritis or cryoglobulinaemia. Serum samples, taken at the time of biopsy, were tested for the presence of ANCA, according to a multistep procedure that combines indirect immunofluorescence with direct and capture enzyme-linked immunosorbent assays (ELISAs)¹⁶. We thus identified 109 patients with AAV with biopsy-proven renal involvement: 46 patients with PR3-ANCA only (42%), 49 patients with MPO-ANCA only (45%), 4 patients with both PR3- and MPO-ANCA (4%) and 10 patients with MPO-ANCA with the presence of anti-glomerular basement membrane antibodies (9%). Only patients who were single positive for PR3- or MPO-ANCA, and had enough serum available for all tests, were included (n = 86). Additionally, 40 healthy controls (HC) (laboratory personnel) were tested to determine the cut-off value for the anti-hHSP60 test and, 69 HC were tested to determine the cut-off value for the anti-mHSP65 test. Demographic data on patients and controls are presented in table 1.

Sera

Serum samples, taken at the time of biopsy, were used to determine the presence of anti-hHSP60 and anti-mHSP65 in patients with AAV. These sera had been stored at -80°C, in some patients for over 20 years. However, when testing the antibody response, there was no difference in antibody level between patients included in 2003 and patients included in earlier years.

ELISA for the detection of antibodies against human HSP60 and mycobacterial HSP65

Recombinant hHSP60 (Stressgen, Victoria, Canada) was diluted to 2 µg/mL in coating buffer containing 0.1M NaHCO₃⁻ (pH 9.6), and 50 µL/well (i.e., 0.1 µg/well) was incubated in wells of microplates (Nunc

Table 1. Demographic data on patients and healthy controls

	AAV (N = 86)	MPO-ANCA (N = 42)	PR3-ANCA (N = 44)	HC (N = 69)
Age (years)*	62.2 ± 13.5	65.9 ± 10.9	59.7 ± 15.2	40.4 ± 9.8
Male gender (%)	69	64	73	59

AAV, ANCA-associated vasculitis; MPO-ANCA, antineutrophil cytoplasmic antibodies against myeloperoxidase; PR3-ANCA, ANCA against proteinase 3; HC, healthy controls

* Mean ± standard deviation

MaxiSorp™, Rochester, NY) overnight at 4°C. Control wells were incubated with coating buffer alone. Wells were then incubated overnight at 4°C with 100 µL/well coating buffer containing 1% grade V bovine serum albumin (BSA, Sigma, St. Louis, MO) to block non-specific binding. Plates were then washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). Plates were preincubated at room temperature with 200 µL/well of preincubation buffer, containing 0.05% Tween 20 and 1% BSA in PBS, for 1 hour. After washing 2 times with PBS-T, wells were incubated in triplicates with patient serum in a previously established optimal dilution of 1:50, 50 µL/well, in incubation buffer and kept overnight at 4°C. The next day, plates were washed 5 times with PBS-T and incubated with goat F(ab')₂ anti-human IgG (Fc)-horseradish peroxidase conjugate (Cappel-ICN Immunobiologicals, Costa Mesa, CA), diluted 1:2000 in incubation buffer, for 2 hours at room temperature. After washing 5 times with PBS-T, 100 µL of freshly made substrate (containing 0.5 mg/mL o-Phenylenediamine (Sigma) and 0.03% H₂O₂ in citrate/phosphate buffer, pH 4.9) was added to each well. After 10 minutes, the reaction was stopped with 50 µL 4N H₂SO₄ and absorbances were read at 490 nm. Results are expressed as anti-hHSP60 levels in arbitrary units (AU) /mL. Hereto, the mean OD of the triplicates was corrected for non-specific binding by subtraction of the mean OD in uncoated wells. Next, a standard curve was created by including in every assay a serial, 2-step dilution from 1:10 to 1:640 of a positive serum sample with anti-hHSP60 reactivity. The undiluted serum sample was arbitrarily assigned 100 AU/mL. Additionally, results are expressed as positive or negative. To establish the cut-off value for this test, the mean AU/mL and SD of the sera of 40 HC were determined, and the cut-off value was set at the mean + 2 SD.

For detection of antibodies against mHSP65, essentially the same procedure was followed. Recombinant mHSP65 (Stressgen) was diluted to 2 µg/mL and coated in a concentration of 0.1 µg/well. Serum was diluted 1:50 prior to incubation. To read results in AU, a standard curve was created as described for anti-hHSP60 antibodies. To establish the cut-off value for this test, the mean AU/mL and SD of the sera of 69 HC were determined, and the cut-off value was set at the mean + 2 SD.

Myeloperoxidase inhibition assays

To test cross-reactivity between MPO and hHSP60, serum from one patient being positive for both MPO-ANCA and anti-hHSP60 antibodies was incubated for 30 minutes at 37°C with MPO or hHSP60 in concentrations of 1, 3, 10 and 20 µg/mL. The serum was then tested in the anti-hHSP60 ELISA as described above, or in the MPO-ANCA ELISA using commercially available direct ELISA kits (Euro-diagnostica, Malmö, Sweden). Additionally, absorption tests were performed in which ELISA plates were coated with hHSP60 or MPO and incubated with serum. The next day, or the second day, anti-hHSP60 reactivity in this pre-absorbed serum was tested as described above for the anti-hHSP60 ELISA. Unfortunately, this was the only patient of whom enough serum was present to perform the described tests.

Statistical analyses

All data are presented as median [range] unless stated otherwise. Continuous data between groups were compared using Wilcoxon signed rank test and Kruskal-Wallis test with Dunn's post-test when appropriate. Due to the perceived relevance of the results, we did not only perform Dunn's post-test for subgroup analysis, but we also compared subgroups using Wilcoxon signed rank test. Correlations between data were derived using Spearman correlation. Odds ratios (OR) and 95% confidence intervals (CI) were obtained using Fisher's exact test. Analyses were performed with GraphPad Prism version 3.00 and GraphPad InStat software package version 2.04a (both GraphPad Software Inc., San Diego, CA). A two-sided p-value <0.05 was considered to indicate statistical significance.

Results

Eighty-six consecutive patients with AAV (44 PR3- and 42 MPO-ANCA) were included in the study. Mean age was 62.2 ± standard deviation 13.5 (65.9 ± 10.9 in MPO-ANCA and 59.7 ± 15.2 in PR3-ANCA, $P = 0.07$). Fifty-nine patients (69%) were male (27 patients (64%) in MPO-ANCA and 32 patients (73%) in PR3-ANCA, $P = 0.49$).

Anti-mycobacterial HSP65 antibodies are elevated in MPO-ANCA compared to healthy controls

In patients with AAV, median anti-mHSP65 level was 42.5 AU/mL [0 –

Figure 1. Anti-mHSP65 antibody levels in healthy controls (HC) and in patients with ANCA-associated vasculitis (AAV). Anti-mycobacterial Heat Shock Protein 65 (anti-mHSP65) antibody levels are significantly higher in AAV compared to HC. Furthermore, anti-mHSP65 antibody levels in patients with PR3- and MPO-ANCA are shown. Anti-mHSP65 is comparable between patients with MPO-ANCA and PR3-ANCA. In patients with MPO-ANCA, anti-mHSP65 antibody level is significantly higher when compared to HC. Dotted line represents cut-off value; solid line represents median value.

Figure 2. Anti-hHSP60 antibody levels in healthy controls (HC) and in patients with ANCA-associated vasculitis (AAV). Anti-human Heat Shock Protein 60 (anti-hHSP60) antibody levels are not significantly higher in AAV compared to HC. Furthermore, anti-hHSP60 antibody levels are significantly higher in patients with MPO-ANCA compared to PR3-ANCA. In patients with MPO-ANCA, anti-hHSP60 antibody level is also significantly higher when compared to HC, whereas in PR3-ANCA, it is not. Dotted line represents cut-off value; solid line represents median value.

figure 1). In MPO-ANCA, antibody levels were significantly higher (44 AU [7 – 500]) compared to HC ($P = 0.006$, figure 1). In patients with PR3-ANCA, there was a trend to a higher antibody level (42.5 AU [0 – 500]) compared to HC ($P = 0.10$). With a cut-off value of 88.5 AU/mL, 13 of 69 HC (19%) were positive for anti-mHSP65 compared to 23 of 86 (27%) patients with AAV ($P = \text{NS}$). Eleven of 44 patients (25%) with PR3-ANCA were positive for anti-mHSP65 compared to 12 of 42 patients with MPO-ANCA (29%) ($P = \text{NS}$).

Anti-human HSP60 antibodies are elevated in MPO-ANCA compared to PR3-ANCA and healthy controls

In patients with AAV, median anti-hHSP60 level was 18 AU/mL [0 – 319], compared to 18.5 AU/mL [0 – 98] in healthy controls ($P = \text{NS}$, figure 2). In MPO-ANCA, antibody levels were significantly higher (32.5 AU [0 – 319]) compared to HC ($P = 0.02$) and PR3-ANCA (13 AU [0 – 79]) ($P < 0.0001$, figure 2). Patients with PR3-ANCA did not have higher antibody levels than HC. With a cut-off value of 80 AU/mL, 2 of 40 HC (5%) were positive for anti-hHSP60 compared to 12 of 86 (14%) patients with AAV ($P = \text{NS}$). One of 44 (2.3%) patients with PR3-ANCA was positive for anti-hHSP60 compared to 11 of 42 patients with MPO-ANCA (26%) ($P = 0.001$, OR 15.3, 95% CI 1.9 – 124).

Myeloperoxidase does not inhibit anti-hHSP60 antibody reactivity

To test whether anti-hHSP60 antibodies actually cross-react with MPO, we performed inhibition assays incubating serum from the patient with the highest anti-hHSP60 response with MPO or hHSP60 and testing for hHSP60 and MPO reactivity. Incubation with increasing concentrations of MPO inhibited the MPO-ANCA response in a dose-dependent manner (figure 3A). However, incubation of serum with hHSP60 did not inhibit the MPO-ANCA reactivity (figure 3A). Additionally, MPO was unable to inhibit the anti-hHSP60 antibody reactivity (figure 3B). However, incubation with hHSP60 did not inhibit the anti-hHSP60 response (figure 3B). To test for the ability of hHSP60 to inhibit anti-hHSP60 reactivity, absorption tests were performed. Results now showed inhibition of the anti-hHSP60 response by hHSP60 but not by MPO (figure 4).

Figure 3A. MPO-ANCA inhibition assay. Incubation with increasing concentrations of myeloperoxidase (MPO) (1 – 20 $\mu\text{g/mL}$) leads to a reduction in MPO-ANCA reactivity (■). In contrast, incubation with human Heat Shock Protein 60 (hHSP60) (1 – 20 $\mu\text{g/mL}$) does not decrease MPO-ANCA reactivity (●).

Figure 3B. Anti-human Heat Shock Protein 60 (hHSP60) inhibition assay. Incubation with increasing concentrations of myeloperoxidase (MPO) (1 – 20 $\mu\text{g/mL}$) does not lead to a reduction in anti-hHSP60 reactivity (■). However, incubation with hHSP60 (1 – 20 $\mu\text{g/mL}$) only slightly decreases anti-hHSP60 reactivity (●).

Figure 4. MPO and hHSP60 absorption assays. Incubation of serum in ELISA plates coated with human Heat Shock Protein 60 (hHSP60) for 1 or 2 days leads to a reduction in anti-hHSP60 reactivity (●). In contrast, incubation of serum in ELISA plates coated with myeloperoxidase (MPO) does not decrease anti-hHSP60 reactivity (■).

Follow-up anti-hHSP60 levels

To further exclude cross-reactivity between hHSP60 and MPO-ANCA, we tested whether anti-hHSP60 antibody levels were only present if there was presence of MPO-ANCA. In the patient with MPO-ANCA and the highest anti-hHSP60 level, follow-up serum samples were available to test for the presence of anti-hHSP60. As shown in figure 5, anti-hHSP60 levels dropped analogous to MPO-ANCA levels once treatment has commenced, but slightly increased with tapering of the treatment while MPO-ANCA levels remained undetectable.

Correlation between anti-mHSP65 and anti-hHSP60 but not between MPO-ANCA and anti-hHSP60 antibodies

Finally, we checked whether correlation exists between antibody levels against mHSP65, hHSP60, and MPO. There was a correlation between height of antibody levels against mHSP65 and hHSP60 (Spearman $r = 0.32$, $P = 0.003$), but no correlation existed between MPO-ANCA and anti-hHSP60 (Spearman $r = -0.064$, $P = 0.69$). Also, there was no correlation between anti-mHSP65 and MPO-ANCA (Spearman $r = 0.074$, $P = 0.65$).

Discussion

The mechanism by which ANCA are induced are as yet unclear. One

Figure 5. Follow-up of a patient positive for antibodies against human Heat Shock Protein 60 (anti-hHSP60). Anti-hHSP60 levels (■) drop as well as antibody levels against myeloperoxidase (MPO-ANCA) (◆) once treatment has commenced, but increase during follow-up, while MPO-ANCA levels remain undetectable.

favorable hypothesis is that infections may trigger an ANCA response¹¹. We hypothesized that infections may trigger the ANCA response against MPO through hHSP60. We found that anti-hHSP60 was almost exclusively found among patients with MPO-ANCA when compared to PR3-ANCA, supporting our hypothesis. Nevertheless, there was no correlation between MPO-ANCA and anti-hHSP60 antibody levels, and inhibition and absorption assays did not show cross-reactivity between hHSP60 and MPO-ANCA. Furthermore, when we determined the anti-mHSP65 response in AAV and HC, we found no difference between patients with MPO-ANCA and patients with PR3-ANCA, and only a weak correlation between anti-hHSP60 antibody levels and anti-mHSP65 levels. However, not every patient who was positive for anti-hHSP60 had antibodies against mHSP65, suggesting that anti-hHSP60 and anti-mHSP65 are not the same antibodies, and that an anti-hHSP60 response may not be triggered simultaneously with an anti-mHSP65 response.

The shared sequence homology described between hHSP60 and MPO is a theoretical one, based on amino acid sequence. This sequence similarity has a length of 17 amino acids and is present on the heavy

chain of myeloperoxidase¹³. The sequence is present in a region of myeloperoxidase that is recognized as an epitope by MPO-ANCA in some patients¹⁷. However, it is unknown whether anti-hHSP60 antibodies bind to the corresponding amino acid sequence of hHSP60. To test whether anti-hHSP60 antibodies actually cross-react with MPO, we performed inhibition assays incubating serum from the patient with the highest anti-hHSP60 response with MPO or hHSP60 and testing for hHSP60 and MPO reactivity. We could not detect a decrease in hHSP60 reactivity when serum was incubated with MPO, suggesting that although sequence homology exists, epitopes recognized by MPO-ANCA are different from those recognized by anti-hHSP60 antibodies. Obviously, this test cannot completely exclude that cross-reactivity exists in other patients, although the absence of correlation between MPO-ANCA and anti-hHSP60 also does not support such cross-reactivity.

Antibodies against hHSP60 are associated with the presence and severity of atherosclerosis¹⁸⁻²⁰, although this has been disputed by others²¹. Interestingly, we found elevated levels of anti-hHSP60 antibodies only in patients with MPO-ANCA. An explanation for this finding may be that diagnosis of MPO-AAV is often delayed, and this may lead to prolonged inflammation and eventually to elevated levels of anti-hHSP60 in MPO-ANCA patients when compared to PR3-ANCA patients. Our data might indicate that cardiovascular disease is more often present in patients with MPO-ANCA. However, follow-up data in our small study do not confirm this.

Interestingly, anti-hHSP60 reactivity could not be inhibited by our first inhibition assay. This may be due to the nature of the inhibition assay. In the first assay, hHSP60 was added to the serum of the anti-hHSP60 positive patient, whereas in the second assay, hHSP60 was coated to the ELISA plate. This second assay clearly demonstrated inhibition of the anti-hHSP60 response. An explanation for this phenomenon may be that anti-hHSP60 antibodies are unable to bind to fluid phase hHSP60, but can bind to solid phase hHSP60, as demonstrated by the second inhibition assay, and the positive antibody response demonstrated in the anti-hHSP60 ELISA.

In conclusion, we have shown that antibodies against hHSP60 are elevated in patients with MPO-ANCA in comparison with PR3-ANCA. These findings suggest a role for infections in the pathogenesis of MPO-

AAV through molecular mimicry between bacterial HSP65, human HSP60 and MPO. Additional absorption assays, however, did not support this hypothesis. Whether there is a relationship between anti-hHSP60 and cardiovascular disease in patients with MPO-ANCA remains to be studied.

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Chapter 5

*Immunoregulatory gene
polymorphisms are associated with
ANCA-related vasculitis*

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Submitted

Background. T cells have been implicated in the pathophysiology of ANCA-associated vasculitis (AAV), because increased T cell activation is present in patients with AAV. T cell activation is regulated by inhibitory molecules such as PD-1 and CTLA-4, whose expression in turn may be affected by gene polymorphisms. We investigated the relationship between polymorphisms in *PDCD1* and *CTLA4* and the presence of AAV.

Methods. Two single-nucleotide polymorphisms (SNPs) in *PDCD1* and five polymorphisms in *CTLA4* were investigated in 102 patients with AAV and 188 healthy controls (HC).

Results. The allelic distributions of the PD-1.3 and PD-1.5 SNPs did not differ between patients and HC. Additionally, the distributions of the *CTLA4* promoter polymorphisms

-1722T/C, -1661A/G, and -318 C/T, as well as the (AT)_n microsatellite in the 3' untranslated region of *CTLA4*, did not differ significantly between AAV patients and HC. However, the +49 G allele was significantly more often present in patients with AAV (70% in patients versus 55% in HC, $P = 0.01$). Furthermore, the co-occurrence of the PD-1.5 T allele with *CTLA4* +49 AA homozygosity was less often present in patients compared to HC (17% and 32%, respectively, $P = 0.007$).

Conclusions. AAV is associated with the presence of a G allele at the +49 position of *CTLA4* and this association is even stronger if also the PD-1.5 T allele is absent in the genome. These genetic polymorphisms may lead to hyperreactivity of T cells and thus may contribute to the pathogenesis of AAV.

Small vessel vasculitides, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with antineutrophil cytoplasmic antibodies (ANCA), which are either directed to proteinase 3 (PR3) or myeloperoxidase (MPO)¹⁻³. Evidence exists that ANCA play a pathophysiological role in the induction of these vasculitides. First, elevations in ANCA titers correlate with risk of future disease flares^{4,5}. Second, in vitro it has been demonstrated that ANCA induce neutrophil activation, i.e. degranulation and production of a respiratory burst⁶. In addition, ANCA activate other cells in vitro such as monocytes and endothelial cells⁷. Finally, in vivo experimental data also indicate an important role for ANCA in the pathophysiology of vasculitis^{8,9}.

Next to the role of ANCA, T cells have also been implicated in the pathophysiology of ANCA-associated vasculitis (AAV). Obviously, production of IgG ANCA by plasma cells is dependent on the presence of T helper cells¹⁰. Furthermore, PR3-specific and/or MPO-specific T cells are present in vasculitis patients, whether in active disease or in remission^{11,12}. Additionally, in active AAV, T cells are present in inflammatory infiltrates^{13,14}, suggesting direct involvement of T cells in the pathogenesis of AAV. Finally, patients with vasculitis often have a disturbed peripheral blood T cell compartment¹⁰. Several studies found increased serum markers of T cell activation^{15,16}, and percentages of activated CD4+ and CD8+ T cells in peripheral blood were higher in patients with WG than in healthy subjects, both during active disease and in remission^{17,18}. Why T cells remain activated in AAV, even in remission, is not yet elucidated.

The B7-1/B7-2 – CD28/CTLA-4 pathway is crucial in regulating T cell activation and tolerance. CD28 transmits a signal that synergizes with the T cell receptor (TCR) signal to promote T cell activation, whereas CTLA-4 engagement delivers a negative signal, inhibiting TCR- and CD28-mediated signal transduction. However, CTLA-4 is not the only molecule delivering an inhibitory signal; PD-1 is a second regulatory molecule in the activation of T cells. PD-1 deficient mice show autoimmune disease including arthritis and glomerulonephritis, disease manifestations also found in AAV. In *PDCD1*, the gene encoding PD-1, several single nucleotide polymorphisms (SNPs) have been described¹⁹ and two of these have been associated with autoimmune disease^{19,20}. In addition, certain polymorphisms in the CTLA-4 gene (*CTLA4*) have

been linked to a number of autoimmune diseases, including WG²¹⁻²³; these polymorphisms have been shown to reduce the expression or the inhibitory function of CTLA-4²⁴⁻²⁶. We hypothesized that these regulatory genes may be involved in the development of AAV. Thus, we compared the frequencies of the PD-1.3G/A and PD-1.5C/T SNPs in *PDCD1*, and the +49A/G, -318C/T, -1661A/G, -1722C/T SNPs and the (AT)_n polymorphism in *CTLA4* between patients with AAV and non-related healthy controls from the same region in the Netherlands.

Table 1. Patient characteristics*

	<i>AAV patients</i> <i>N = 102</i>	<i>Healthy controls</i> <i>N = 188</i>
Age (years)	59.0 ± 13.6	39.8 ± 15.8 [†]
Male gender	65 (64)	76 (40) [‡]
ANCA specificity		N/A
MPO-ANCA	34 (33)	
PR3-ANCA	49 (48)	
both	2 (2)	
none	17 (17)	
Disease subtype		N/A
Wegener's granulomatosis	50 (49)	
MPA	24 (24)	
Churg-Strauss syndrome	7 (7)	
Renal-limited vasculitis	21 (21)	
Disease extent[§]		N/A
Locoregional / limited	39 (38)	
Generalized	63 (62)	

AAV, ANCA-associated vasculitis; ANCA, Antineutrophil cytoplasmic antibodies; MPO, myeloperoxidase; PR3, proteinase 3; MPA, microscopic polyangiitis; N/A, not applicable.

*Data are given as mean ± standard deviation or N (%)

[†]In 11 healthy controls, age was unknown; *P* < 0.0001

[‡]In 10 healthy controls, gender was unknown; *P* = 0.0002

[§]Patients with renal-limited vasculitis were categorized as having generalized vasculitis.

Methods

Study population

One hundred and two patients with ANCA-associated vasculitis and 188 healthy controls (HC) were included. All patients fulfilled the definitions for WG, MPA, or the Churg-Strauss syndrome (CSS) as proposed by the Chapel Hill Consensus Conference²⁷. Additionally, diseased patients were categorized according to their disease extent: generalized when there was renal involvement, otherwise, patients were said to have limited disease. Patients with renal-limited vasculitis were categorized as having generalized vasculitis. Patients' spouses were included as healthy age-matched controls to exclude survival bias in our analysis. Furthermore, healthy volunteers from the southeast of the Netherlands were included. Characteristics of patients and controls are presented in table 1.

DNA extraction

EDTA- or heparin-anticoagulated venous blood samples were drawn and DNA was extracted using the Wizard[®] Genomic DNA purification kit (Promega, Leiden, the Netherlands). In some patients, blood samples were not available and DNA was extracted from renal biopsy material.

PDCD1 genotyping

A 381 bp fragment containing the PD-1.3G/A polymorphism (+7146G/A) was created using a forward primer 5'-GATCCACTGTGCCTTCCTTC-3' and reverse primer 5'-CAAGCTCTCCTGTCTCCAATG-3' (both Eurogentec, Liège, Belgium). The SNP was determined by digestion of the product with the restriction enzyme PstI (Fermentas Inc., Hanover, MD), resulting in two fragments of 314 and 67 bp if the G nucleotide is present and three fragments of 216, 98 and 67 bp when the A nucleotide is present.

The PD-1.5C/T polymorphism (+7785C/T) was determined in a 329 bp fragment created using a forward primer 5'-ACGGAGTATGCCACCATTGTC-3' and reverse primer 5'-CTGTGGGCATTGAGACATGAG-3' (both Eurogentec). Digestion of the product with the restriction enzyme PvuII (Fermentas) only results in two fragments of 261 and 68 bp when the T nucleotide is present. All digestion products were analyzed by electrophoresis in a 2% agarose gel.

CTLA-4 genotyping

The +49G/A polymorphism²⁸, the -318C/T polymorphism²⁹, and the -1661G/A and the -1722 C/T polymorphisms³⁰ were determined as described previously. All digestion products were analyzed by electrophoresis in a 2% agarose gel.

The (AT)_n polymorphism was determined using a 6-FAM labeled forward primer 5'-GCCAGTGATGCTAAAGGTTG-3'³¹, and reverse primer 5'-CCTCCACAAAACCTCCCTGGG-3' (both Sigma). These primers created a fragment of 171 – 215 bp depending on the length of the AT-repeat. The fragments were size-resolved on an ABI3100 capillary sequencer using the GeneScan Analysis v3.7 software package (Applied Biosystems). Alleles were manually called by a technician and successively checked by an expert. Using this method we found that amplification of the fragment containing the shortest (AT)_n repeat (mostly 171 bp) was highly favored over amplification of the longer fragment, possibly leading to an underrepresentation of heterozygosity for the shortest allele 171 and a longer allele. Therefore, we analyzed the Hardy Weinberg Equilibrium (HWE) dividing our control samples in 3 groups: homozygous for the shortest allele (171/171), heterozygous for the shortest allele (171/x), and carrying two longer alleles (x/x). Since HWE was present in our controls, we analyzed our patients and controls according to this method.

Statistical analyses

Genotype frequencies were compared between patients and controls using a Chi-square test for independence and Fisher's exact test when appropriate. Since AAV is a disease that manifests at an older age, we desired to exclude survival bias. Therefore, we also compared patients with age-matched healthy controls only. All analyses were performed with the GraphPad Instat software package version 2.04a (GraphPad Software, San Diego, CA). HWE was determined using the test provided by the Institute of Human Genetics, Technical University Munich, Germany (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). A two-tailed P-value ≤ 0.05 was considered to indicate statistical significance.

Results

PDCD1 polymorphisms

The genotype frequencies in patients and control subjects conformed to

the frequencies expected under conditions of HWE ($P > 0.05$). The distributions of the PD-1.3 and PD-1.5 SNPs did not differ between patients and controls (table 2). Subgroup analysis did not reveal any associations between disease extent, diagnosis or ANCA specificity and *PDCD1* genotype.

CTLA4 polymorphisms

The genotype frequencies in patients and control subjects conformed to the frequencies expected under conditions of HWE ($P > 0.05$). The distributions of the newly described promoter polymorphisms, -1722T/C and -1661A/G, did not differ significantly between AAV patients and healthy subjects (table 3). The distribution of the -318C/T SNP also did not differ between patients and controls (table 3). However, the +49 G allele was significantly overrepresented in patients with AAV (70% in patients versus 55% in HC, $P = 0.01$, table 4). The frequency distribution of the (AT)_n microsatellite in the 3' untranslated region of *CTLA4* did not differ between patients and controls. These results did not change when we used age-matched controls only (data not shown).

Subgroup analyses for CTLA4 polymorphisms

Fifty patients with WG were included in this study. Since an association

Table 2. *PDCD1* polymorphisms and ANCA-associated vasculitis

<i>PDCD1</i> polymorphism	Patients N (%)	Healthy controls N (%)	<i>P</i>	OR (95% CI)
PD-1.3: +7146				
GG	85 (83)	168 (82)		
GA	16 (16)	34 (17)		
AA	1 (1)	2 (1)		
A allele carrier	17 (17)	36 (18)	1.0	N/A
PD-1.5: +7785				
CC	45 (44)	78 (38)		
CT	41 (40)	102 (50)		
TT	16 (16)	24 (12)		
T allele carrier	57 (56)	126 (62)	0.32	N/A

PDCD1, programmed death 1; OR, Odds ratio; 95% CI, 95% confidence interval; N/A, not applicable.

Table 3. *CTLA4* polymorphisms and ANCA-associated vasculitis

<i>CTLA4</i> promoter polymorphism	Patients <i>N</i> (%)	Healthy controls <i>N</i> (%)	<i>P</i>	<i>OR</i> (95% <i>CI</i>)
-318				
CC	87 (84)	160 (85)		
CT	16 (15)	28 (15)		
TT	1 (1)	0 (0)		
T allele carrier	17 (16)	28 (15)	0.74	N/A
-1661				
AA	70 (68)	125 (68)		
AG	30 (29)	50 (27)		
GG	3 (3)	9 (5)		
G allele carrier	33 (32)	59 (32)	1.0	N/A
-1722				
TT	94 (90)	171 (91)		
CT	10 (10)	16 (9)		
CC	0 (0)	1 (1)		
C allele carrier	10 (10)	17 (10)	1.0	N/A

CTLA4, cytotoxic *T*-lymphocyte-associated antigen 4; *OR*, Odds ratio; 95% *CI*, 95% confidence interval; N/A, not applicable.

between WG and the −318C/T SNP has been previously described²², we performed an analysis including WG patients only. When analyzing these patients, we found no difference in this genotype between patients and controls ($P = 0.15$). Other *CTLA4* polymorphisms were also not associated with the presence of WG in this patient population. When patients were analyzed according to ANCA specificity, we found a difference in distribution of the G allele at the +49 position between patients with MPO-ANCA ($N = 34$) and HC (76% and 55%, respectively, $P = 0.02$) and patients with PR3-ANCA ($N = 49$) and HC (69% in patients with PR3-ANCA, $P = 0.009$) (table 5). There were no significant differences in distribution in the other polymorphisms studied.

In addition to diagnosis and ANCA specificity, we analyzed patients with generalized vasculitis (defined as vasculitis with renal involvement) and

patients with limited vasculitis (vasculitis without renal involvement). We found that patients with generalized vasculitis more often had a G allele at the +49 position than HC (76% and 55%, respectively, $P = 0.003$). Additionally, patients with limited vasculitis less often had the shortest allele in the (AT)_n-repeat and thus were more often homozygous for a longer allele compared to HC (49% and 28%, respectively, $P = 0.02$) and compared to patients with generalized vasculitis (25% homozygous for a longer allele, $P = 0.02$) (table 5).

The combination of CTLA4 and PDCD1 polymorphisms

When combining the *CTLA4* with the *PDCD1* polymorphisms, we found that in patients the co-occurrence of *CTLA4* +49AA and the PD-1.5 T allele was less often present in patients compared to HC (17% and 32%, respectively, $P = 0.007$). Additionally, the combination of the *CTLA4* 171 allele and PD-1.5 T allele tended to be less often present in patients (32%) compared to HC (44%, $P = 0.06$).

Discussion

The B7-1/B7-2 – CD28/CTLA-4 pathway is crucial in regulating T cell

Table 4. *CTLA4* polymorphisms and ANCA-associated vasculitis

<i>CTLA4</i> polymorphism	Patients N (%)	Healthy controls N (%)	<i>P</i>	OR (95% CI)
+ 49				
AA	31 (30)	83 (45)		
GA	51 (49)	73 (39)		
GG	22 (21)	29 (16)		
G allele carrier	73 (70)	102 (55)	0.01	1.9 (1.2 – 3.2)
(AT)_n repeat				
171/171	25 (24)	49 (28)		
171/x	44 (42)	77 (44)		
x/x	35 (34)	49 (28)		
x allele carrier	79 (76)	126 (72)	0.49	N/A

CTLA4, cytotoxic T-lymphocyte-associated antigen 4; OR, Odds ratio; 95% CI, 95% confidence interval; N/A, not applicable; x, allele fragment longer than 171 base pairs.

Table 5. *CTLA4* genotype frequencies in patient subgroups

Subgroup	Patients N (%)	Healthy controls N (%)	P	OR (95% CI)
ANCA specificity				
+ 49 G allele carrier				
MPO-ANCA (N = 34)	26 (74)	102 (55)	0.02	2.6 (1.1 – 6.2)
PR3-ANCA (N = 49)	34 (69)		0.009	2.4 (1.2 – 4.6)
Disease severity				
+ 49 G allele carrier				
generalized (N = 63)	48 (76)	102 (55)	0.003	2.7 (1.4 – 5.3)
limited (N = 39)	23 (59)		0.71	N/A
generalized vs. limited			0.10	N/A
<i>(AT)_n repeat x/x carrier</i>				
generalized (N = 63)	16 (25)	49 (28)	0.74	N/A
limited (N = 39)	19 (49)		0.02	2.4 (1.2 – 4.9)
generalized vs. limited			0.04	2.8 (1.2 – 6.5)

CTLA4, cytotoxic T-lymphocyte-associated antigen 4; ANCA, antineutrophil cytoplasmic antibodies; MPO, myeloperoxidase; PR3, proteinase 3; *(AT)_n repeat x/x*, homozygous for alleles longer than 171 base pairs; OR, Odds ratio; 95% CI, 95% confidence interval; N/A, not applicable.

activation and tolerance. CD28 transmits a signal that synergizes with the T cell receptor (TCR) signal to promote T cell activation, whereas CTLA-4 engagement delivers a negative signal, inhibiting TCR- and CD28-mediated signal transduction³². CTLA-4 deficiency in mice leads to lymphoproliferative disorder, autoimmune disease, and early mortality³³. Certain polymorphisms in *CTLA4* have been associated with reduced function of CTLA-4 as well. The presence of a G allele at the + 49 position leads to a significantly higher proliferation of T cells²⁶ and less expression of cell-surface CTLA-4²⁴ compared to the presence of an A allele. The –318C/T polymorphism, like the +49G/A, influences the availability of CTLA-4. When the C allele is present reduced cell-surface expression of CTLA-4 and reduced CTLA-4 mRNA is described compared to the presence of a T allele²⁴. Additionally, a dinucleotide repeat, when elongated possibly leading to more unstable mRNA³⁴, is present in the 3' untranslated region of *CTLA4*³⁵. Such an elongated (AT)_n repeat has been shown to lead to T cell hyper-reactivity²⁵. These

–318C/T, +49G/A, and (AT)_n polymorphisms in *CTLA4* have previously been associated with a variety of autoimmune diseases, like SLE^{36,37}, Graves' disease and Hashimoto's thyroiditis^{26,31,38} and multiple sclerosis³⁹. The –318C/T SNP and the (AT)_n microsatellite have been related to WG^{21–23}. In our study, the (AT)_n microsatellite was associated with the presence of limited AAV. Furthermore, the presence of a G allele at the +49 position was associated with AAV. In contrast, a Swedish study did not reveal an association between the +49G/A polymorphism and WG in 32 patients²². However, in our study not only WG patients were included, but also patients with MPA, CSS and renal-limited vasculitis as these diseases are also associated with the presence of ANCA. There were no other associations with polymorphisms in *CTLA4* and AAV in this patient population.

The promoter polymorphisms –1722T/C and –1661A/G have recently been described in patients with SLE. Patients with SLE more often were homozygous for the T allele at the -1722 position³⁰. We could not detect this association in AAV. Furthermore, we detected large differences between our control population and the Korean population as tested by Hudson et al³⁰. In their study, controls were more often homozygous for the C allele, in our population only one person was homozygous for this allele, and heterozygosity also was far less present. This clearly shows how different allele distributions can be between ethnically different populations and highlights the necessity of an ethnically matched control population. Our patients and controls all are from the southeast of the Netherlands and therefore, any disease association is less likely to be due to ethnic differences. This may also explain why we could not confirm earlier associations between polymorphisms in *CTLA4* and WG.

Recently it was discovered that not only CTLA-4 provides an inhibitory signal, but programmed death (PD)-1 also is involved in negative T cell regulation⁴⁰. Mice deficient for PD-1 develop a lupus-like disease including arthritis and glomerulonephritis⁴¹, disease manifestations also present in AAV. Polymorphisms in *PDCD1* have been associated with SLE^{19,42} and rheumatoid arthritis^{20,43}. We therefore hypothesized that next to polymorphisms in *CTLA4*, polymorphisms in *PDCD1* may be associated with the presence of AAV. We could not detect differences in genotype frequencies between patients with AAV and HC when analyzing *PDCD1* alone; however, in combination with *CTLA4*, we did

find an association with vasculitis. Both PD-1 and CTLA-4 regulate T cell responses once they are initiated, although PD-1 seems to be less potent than CTLA-4⁴⁴. This may explain why PD-1 alone is not associated with AAV. On the other hand, the (AT)_n microsatellite in CTLA-4 alone is not associated with AAV in this study, but in combination with PD-1 an association seems to be present. We therefore postulate that for autoimmune disease, a mutation in more than one inhibitory receptor predisposes to the development of disease, for instance, AAV.

In conclusion, we found that ANCA-associated vasculitis is associated with the presence of a G allele at the +49 position of *CTLA4*; other genetic associations that were previously described could not be confirmed in our population. Additionally, the absence of the PD-1.5 T allele is associated with AAV in combination with *CTLA4*. The combination of these genetic polymorphisms may lead to hyperreactivity of T cells and thus may contribute to the pathogenesis of AAV.

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Chapter 6

Renal survival and prognostic factors in patients with PR3-ANCA associated vasculitis with renal involvement

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Background. Severe renal disease is a feature of ANCA-associated small-vessel vasculitis. We evaluated patient and renal survival and prognostic factors in patients with PR3-ANCA associated vasculitis with renal involvement at diagnosis during long-term follow-up.

Methods. Eighty-five patients were diagnosed between 1982 and 1996 and followed until 2001 allowing ≥ 5 years of follow-up. All patients were treated with prednisolone and cyclophosphamide. Univariate and multivariate analyses with patient and renal survival as dependent variables were performed.

Results. Of 85 patients included 17 (20%) died within 1 year after diagnosis. Of 25 patients (29%) dialysis dependent at diagnosis, 2 remained and 2 again became dialysis dependent < 1 year; 9 died early without renal recovery. Risk factors for death occurring within one year in univariate analysis (RR, 95% CI) were age > 65 years (6.5, 1.6-13.7) and dialysis dependency at diagnosis (3.6, 1.0-13). Twenty patients died beyond 1 year during long-term follow-up. Male gender (4.7, 1.6-10) and developing dialysis dependency during follow-up (4.1, 1.4-12) were associated with poor outcome.

Risk factor for renal failure within one year was dialysis dependency at diagnosis (29, 3.6-229). Of 64 patients dialysis independent 1 year after diagnosis, 12 patients became dialysis dependent during follow-up. A renal relapse was strongly associated with development of renal failure in long-term follow-up (17, 3.5 – 81).

Conclusions. Early death and failure to recover renal function in PR3-ANCA associated vasculitis is associated with age > 65 years and dialysis dependency at diagnosis. Long-term renal survival is determined by renal relapses during follow-up only. Slow, progressive renal failure without relapses is rarely observed in this group.

Small vessel vasculitides, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with antineutrophil cytoplasmic antibodies (ANCA), which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)¹⁻³. These diseases can occur in any organ system but the respiratory tract and the kidneys are most frequently involved. Untreated, WG results in death within weeks to months. Since the introduction of cyclophosphamide and prednisolone as standard treatment, survival has improved dramatically from less than 20% at 1 year reported in 1958⁴ to at least 60% 5-years survival reported in the past ten years⁵⁻⁸.

Although life-saving in many patients, treatment with cyclophosphamide and prednisolone is associated with severe morbidity and mortality, both at short-term and during long-term follow-up. Initiating aggressive immunosuppressive therapy in order to regain or conserve independent renal function in patients with ANCA-associated vasculitis with renal involvement is, therefore, dependent on the perceived prognosis of the patient. Previous studies have associated serum creatinine^{9,10} and kidney biopsy findings⁹⁻¹² at diagnosis, and the occurrence of renal relapses¹³ with reduced renal survival in patients with pauci-immune necrotizing glomerulonephritis. Recently, we and others observed differences between groups associated with the different antigenic specificity of ANCA (reviewed by Franssen et al¹⁴). Previously, we studied long-term outcome in patients with MPO-ANCA associated glomerulonephritis and found that proteinuria at diagnosis and during follow-up was the most important risk factor for the development of renal insufficiency¹⁵. So far, none of the studies have focused on long-term outcome of patients with PR3-ANCA associated vasculitis with renal involvement.

In the present study, we retrospectively analyzed patient and renal survival in patients with PR3-ANCA associated vasculitis with renal involvement that were diagnosed and followed in one single center. We investigated predictors of outcome at diagnosis and during follow-up, and we examined the role of non-renal and renal relapses on long-term survival and renal outcome in this patient population.

Methods

Patients

We retrospectively identified all consecutive patients diagnosed with,

treated and followed in our hospital for PR3-ANCA positive vasculitis with evidence of renal involvement (as determined by a biopsy showing necrotizing and/or crescentic glomerulonephritis¹ and/or by deterioration of renal function with glomerular erythrocyturia, erythrocyte casts and proteinuria) between January 1982 and January 1996. Patients were followed until January 2001. Surviving patients were thus followed for at least 5 years.

Treatment and follow-up

Patients were treated according to a standardized protocol¹⁵. The treatment consisted of oral cyclophosphamide (2 mg/kg) and prednisolone (1 mg/kg body weight) daily with a maximal oral prednisolone dose of 60 mg/day. Doses of cyclophosphamide were adjusted to maintain the leukocyte count above $4 \times 10^9/\text{L}$. Patients who presented with severe vasculitic manifestations such as alveolar hemorrhage, rapidly rising serum creatinine or gastrointestinal symptoms, were initially treated with intravenous methylprednisolone 1000 mg daily for 3 days. Plasma exchange was added to the treatment regimen in case of disease progression or persistence of active disease. The latter was defined as persistence of major extrarenal symptoms or the presence of active necrotizing renal lesions in a repeat renal biopsy after 2 to 4 weeks of immunosuppressive therapy. After 4 to 6 weeks, the daily prednisolone dose was tapered by 10 mg every 2 weeks until the dose reached 30 mg, and thereafter by 5 mg every 2 to 4 weeks. Once remission was achieved the daily dose of oral cyclophosphamide was tapered by 25 mg every 3 months. Patients who experienced a renal relapse, or a life-threatening non-renal relapse, were treated according to the same protocol. Minor non-renal relapses were treated with 1 mg/kg body weight cyclophosphamide daily and a maximal oral prednisolone dose of 40 mg/day.

Patients were hospitalized for at least 2 to 3 weeks or longer when indicated. After discharge, patients visited our outpatient clinic at least every 2 weeks during the first 3 months after diagnosis, every 4 weeks until 1 year after diagnosis, and every 6 weeks thereafter. At each visit, patients were evaluated by the same physician for signs and symptoms of vasculitic disease and/or adverse effects of treatment. In addition, blood pressure and weight were measured, serum creatinine,

erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, and ANCA titer¹⁶ were determined, microscopic urinalysis was performed, and proteinuria and creatinine were determined in a 24-hour urine collection.

Definitions

Remission was defined as a stabilization or improvement of creatinine clearance in combination with the absence of glomerular erythrocyturia and erythrocyte cell casts and symptoms or signs attributable to active extra-renal vasculitis with CRP <10 mg/l. A non-renal relapse was defined as new or recurrent findings indicative of vasculitic disease without signs of renal involvement¹⁷. A renal relapse was defined either histopathologically by the presence of new necrotizing lesions in a renal biopsy or clinically by recurrence of microscopic glomerular erythrocyturia, erythrocyte cell casts, proteinuria and a decrease in creatinine clearance in a patient with a previously stable renal function¹⁷. Microscopic glomerular erythrocyturia was defined as 10 or more red blood cells, with more than 50% dysmorphic cells, per high power field (magnification 400x) in the absence of a urinary tract infection or an indwelling catheter.

Statistical analyses

Data are presented as mean $\pm$ standard deviation unless stated otherwise. Continuous data between groups were compared using Student's t-test and the Wilcoxon signed rank test when appropriate. Survival curves for patient and renal survival were calculated using Kaplan-Meier estimates for survival distribution. End point for renal survival analysis was the start of renal replacement therapy or death due to renal failure. Differences between groups in survival were analyzed with log-rank test, and multivariate analysis with Cox proportional hazards analysis with survival time as dependent variable was performed. Renal survival rates (survival without end-stage renal disease (ESRD)) are calculated and analyzed with subjects dying with independent renal function censored.

Analyzing patient and renal survival, a large difference between both patient and renal survival ≤ 1 year and > 1 year after diagnosis became apparent. Survival differences between groups were, therefore, analyzed separately for the time period ≤ 1 year and > 1 year after

diagnosis (long-term follow-up), to identify risk factors for these specific time periods. Independent variables tested in the analysis of patient and renal survival ≤ 1 year were age, gender, pulmonary involvement (with or without need for mechanical ventilation), CRP level, serum creatinine level, dialysis dependency, and Birmingham Vasculitis Activity Score (BVAS)¹⁸ at diagnosis. Variables additionally tested for their association with long-term patient and renal survival (> 1 year) were the occurrence of non-renal and renal relapses, cumulative doses of medication, mean arterial pressure (MAP), creatinine clearances and proteinuria at 12 months and use of ACE inhibitors. In multivariate analysis the occurrence of non-renal and renal relapses were tested as time-dependent variables. Analyses were performed with SPSS version 10.0 (SPSS Inc., Chicago, IL). A two-sided p-value < 0.05 was considered to indicate statistical significance.

Results

Between April 1982 and January 1996, 85 patients (55 male, 30 female) were diagnosed with PR3-ANCA positive vasculitis with renal involvement in our hospital. In all patients, a diagnosis of small-vessel vasculitis was confirmed by biopsy. Renal involvement was confirmed in 64 by renal biopsy; in the remaining patients renal failure, erythrocyturia and erythrocyte casts, and proteinuria was present. Age

Table 1. Distribution of disease manifestations at diagnosis by organ system

<i>Organ system</i>	N	%
Renal	85	100
ENT ^a	72	85
Musculoskeletal	64	75
Pulmonary	47	55
Cutaneous	37	44
Neurologic	34	40
Ocular	29	34
Other ^b	19	22

^a ENT, ear nose throat disease

^b Including heart and gastrointestinal tract

Figure 1. Patient survival (◆) and survival without need for renal replacement therapy (■) in 85 patients diagnosed with PR3-ANCA associated vasculitis with renal involvement. Numbers indicate number at risk at 1, 5 and 10 years after diagnosis.

at diagnosis was 56 ± 17 years. Twenty-five patients (29%) were dialysis dependent at diagnosis. Forty-seven patients (55%) had pulmonary involvement, of which 9 patients (19%) needed mechanical ventilation. Organ manifestations are presented in table 1.

Overall

For the entire group of 85 patients, estimated patient survival at 5 and 10 years was 73 and 62%, respectively (figure 1). Causes of death are presented in table 2. Estimated median survival was significantly shorter in patients > 65 years at diagnosis (3.6 years) when compared to those < 51 years (17.8 years) and those 51 - 65 years of age (17.0 years) ($p < 0.0001$, figure 2). Estimated median survival for patients who were dialysis dependent at diagnosis was also significantly shorter than survival in patients who were not (9.4 vs. 17.0 years, respectively, $p = 0.013$, figure 3). Survival with independent renal function was 65% at 5 years and 51% at 10 years (figure 1).

Mortality ≤ 12 months

Of the 85 patients, 17 patients (20%) died within 1 year. Main causes

Table 2. Causes of death

	<i>N (%)</i>	<i>Within 1 year</i>	<i>Follow-up > 1 yr</i>
Active vasculitis	7 (19)	7	0
Infection ^a	12 (32)	6	6
Malignancy	3 (8)	0	3
Cardiovascular	13 (35)	4	9
Renal insufficiency	2 (5)	0	2
Total	37	17	20

^a 10 during immunosuppressive treatment

of death (table 2) were active vasculitis, usually within days after diagnosis (n=7), and infectious complications of therapy, between 3 and 6 months (n=6). Of 7 patients who died of uncontrolled vasculitis, 5 patients required dialysis at diagnosis; of 6 patients who died of infectious causes, 2 patients were dialysis dependent at diagnosis. Survival within the first year after diagnosis was age dependent: compared to patients < 51 years (n=26) and 51-65 years of age

Figure 2. Patient survival in 85 patients diagnosed with PR3-ANCA associated vasculitis with renal involvement according to age at diagnosis. (■) indicates patients aged < 51 at diagnosis, (●) indicates patients aged 51 – 65; (◆) indicates patients aged > 65 at diagnosis.

Figure 3. Patient survival in 85 patients diagnosed with PR3-ANCA associated vasculitis with renal involvement according to dialysis dependency at diagnosis. (■) indicates patients who were not dialysis dependent at diagnosis, (●) indicates patients who were.

($n=31$) at diagnosis, patients > 65 years ($n=28$) had a significantly higher risk for death ≤ 12 months with a relative risk (RR) of 6.5 (95% CI, 1.6 – 13.7) and 4.0 (1.4 – 10.5), respectively. Severe renal involvement was also associated with death ≤ 12 months (dialysis dependency at diagnosis: RR 2.9 (1.3 – 10.8); serum creatinine > 500 μmol at diagnosis: RR 2.2 (0.9 – 6.9)). In multivariate analysis, CRP level at diagnosis, age, and dialysis dependency at diagnosis were

Table 3. Predictors of patient mortality in multivariate analysis

Predictor	RR	95% CI	P value
Patient mortality ≤ 1 year ($n=85$)			
Age at diagnosis 1 yr increase	1.10	(1.04 - 1.16)	0.001
Dialysis dependency diagnosis	3.58	(1.01 - 12.7)	0.05
CRP level 1 mg/L increase	1.01	(1.00 - 1.02)	0.045
Patient mortality > 1 year ($n=68$)			
Age at diagnosis 1 yr increase	1.09	(1.03 - 1.15)	0.002
Male gender	5.75	(1.25 - 26.5)	0.025
Dialysis dependency follow-up	4.15	(1.43 - 12.0)	0.009

associated with mortality ≤ 12 months (table 3).

Independence of renal replacement therapy ≤ 12 months

Of 25 patients dialysis dependent at diagnosis, 2 did not regain renal function (8%) but survived at 1 year and 9 patients (36%) died within 1 year (median 1 month, range 4 days – 5 months) without regaining independent renal function. Of 14 patients (56%) who regained independent renal function, 2 where only temporarily dialysis independent but needed dialysis again within 1 year, and 1 died within 12 months. Only 11 of 25 patients dialysis dependent at diagnosis (48%) were alive at 12 months with functional renal status. In these 11 patients creatinine clearance at 12 months after diagnosis was 38 ± 14 ml/min. Only 1 of 60 patients dialysis independent at diagnosis (2%) developed renal failure requiring dialysis therapy within 1 year after diagnosis. With univariate and multivariate analysis dialysis dependency at diagnosis (RR 29, 95% CI 3.6 - 229) was the only variable associated with persistent or renewed dialysis dependency ≤ 12 months after diagnosis (table 4).

Long-term patient survival

Of 68 patients alive at 1 year, 27 patients (40%) did not relapse, 10 patients (15%) experienced only non-renal relapses, and 31 patients (46%) had at least 1 renal relapse, of which 10 also had one or more non-renal relapses. All relapses were accompanied by the presence of PR3-ANCA at the time of relapse. The median time to the first non-renal relapse was 3.3 years (range 1.6 – 10.5). The median time to the first renal relapse was 4.3 years (range 0.8 – 12.2). Twenty patients

Table 4. Predictors of renal failure in multivariate analysis

Predictor	RR	95% CI	P value
Renal failure ≤ 1 year (n=85)			
Age at diagnosis 1 yr increase			NS
Dialysis dependency	28.6	(3.58 - 229)	0.002
Renal failure > 1 year (n=64)			
Renal relapse	16.8	(3.51 – 80.8)	< 0.001
Creatinine clearance at 12 months 1 ml/min decrease	1.14	(1.04 - 1.22)	0.002

died > 1 year after diagnosis. In univariate analysis, age > 65 years at diagnosis was associated with mortality beyond 1 year of follow-up compared to age < 51 (RR 10.5, 3.6 - 51) and age 51-65 (RR 2.9, 1.3 - 11), as was male gender (RR 4.7, 1.6 - 10). In multivariate analysis, development of dialysis dependency was additionally associated with death, but occurrence of renal or non-renal relapses was not (table 3).

Long-term renal survival

Of 68 patients alive 1 year after diagnosis, 64 were dialysis independent with a creatinine clearance of 64 ± 31 ml/min (median 60 ml/min, range 18 – 142 ml/min) and proteinuria 0.8 ± 1.0 g/24h (median 0.3 g/24h, range 0 – 5 g/24h) at 1 year. Mean MAP was 101 ± 10 (median 100, range 83 – 127) at 1 year and did not change throughout the study period (data not shown). Forty-four patients (65%) were treated with antihypertensives, of which 24 with an ACE-inhibitor and/or angiotensin II antagonist. Twelve of 64 patients (19%) who were dialysis independent at 1 year progressed to ESRD during the study period; median time to ESRD in those 12 patients was 4.3 years after diagnosis (range, 2.0 – 10.4 years). Five patients who had to start RRT with the occurrence of a renal relapse (4 patients at the first renal relapse, 1 at the third) did not regain renal function during treatment of the relapse. Five patients developed ESRD after a median follow-up of 3.0 years after their first renal relapse. Only 2 patients progressed to ESRD without any signs of a renal relapse; these patients had been dialysis dependent at diagnosis. In multivariate analysis the occurrence of a renal relapse and creatinine clearance, but not proteinuria at 12 months were highly associated with development of dialysis dependency (table 4). In contrast, non-renal relapses were not associated with reduced renal survival ($p=0.98$). Dialysis dependency at diagnosis was also not associated with the development of ESRD in long-term follow-up.

Influence of non-renal and renal relapses on renal function

We further analyzed the influence of renal and non-renal relapses on renal function by comparing creatinine clearance and proteinuria before, at the moment of, and following a first relapse. In addition, the slope of the change in creatinine clearance over time was calculated until 3 months before the relapse and compared with the slope starting

Figure 4A. Creatinine clearances in 44 patients diagnosed with PR3-ANCA associated vasculitis before, during and after the first non-renal or renal relapse. (●) indicates patients with a renal relapse, (○) indicates patients with a non-renal relapse. Error bars represent SD.

3 months after a relapse. In 31 patients with a first renal relapse, creatinine clearance at the moment of relapse had decreased by 15 ± 13.0 ml/min compared to 12 months before relapse. At 12 months following the first renal relapse clearance was still 11 ± 11.0 ml/min lower than 12 months before the relapse (Figure 4A). Proteinuria increased from 0.9 ± 1.2 g/24h 12 months before, to 2.0 ± 1.6 g/24h at, and 1.1 ± 1.1 g/24h 12 months after relapse (Figure 4B). The slopes of the change in creatinine clearance, excluding the 4 patients who became dialysis dependent at their first renal relapse, tended to be more negative following as compared to prior to a renal relapse (-1.8 ± 4.5 ml/min/year before and -3.1 ± 5.2 ml/min/year after renal relapse; $p=0.06$). In contrast, a first non-renal relapse was not associated with renal function loss or increased proteinuria at the moment of or following the relapse (Figures 4A and B). The slopes of the change in creatinine clearance after the first non-renal relapse were not different before and after a non-renal relapse (-1.3 ± 2.0 ml/min/year before and -0.4 ± 1.5 ml/min/year after non-renal relapse).

Discussion

In the present study, we evaluated patient and renal survival in 85 patients with PR3-ANCA associated vasculitis with renal involvement during long-term follow-up. Age > 65 years and dialysis dependency at diagnosis were indicative of both poor patient and renal survival. In our

Figure 4B. Proteinuria in 44 patients diagnosed with PR3-ANCA associated vasculitis before, during and after the first non-renal or renal relapse. (●) indicates patients with a renal relapse, (○) indicates patients with a non-renal relapse. Error bars represent SD.

long-term follow-up analysis, renal function deteriorated during and after a renal relapse, and the occurrence of a renal relapse was the only predictor of an unfavorable long-term renal prognosis.

The patient survival in our study is comparable to previous studies considering patients with small-vessel vasculitides with renal involvement where it varied from 59 to 95% in various patient groups during various follow-up periods^{5,6,8,10,12,13,15,19}. The mortality rate in our series may be somewhat higher as our department is a tertiary referral center and patients with severe disease at diagnosis are referred to our hospital, while patients with less severe disease are often managed at their primary hospital. Mortality shortly after diagnosis is mostly due to uncontrolled vasculitis, while the other important cause is severe infectious complications of the treatment, usually occurring within 3 to 6 months after the initiation of treatment. These findings clearly highlight the dilemma the clinician treating these patients faces. Prognostic tools in this respect are therefore important to guide the intensity of treatment. Unfortunately, as reported by others^{5,7,10,13,19}, early mortality is strongly associated with older age and more advanced organ dysfunction caused by the vasculitic process. In our study dialysis dependency at diagnosis was associated with early mortality, but in contrast to some other studies^{9,13}, lung involvement (with or without the need for mechanical ventilation) was not.

As can be appreciated from the estimated survival curve, mortality in the first year after diagnosis is different compared to follow-up beyond

1 year. We therefore chose to make separate analyses for the time periods within and beyond 1 year after diagnosis. For long-term patient survival, we found no association of mortality with active vasculitis defined as the occurrence of a non-renal or renal relapse; no patients died from active vasculitis beyond 1 year. Long-term mortality was associated with advanced age (as reported previously^{7,8,10,20}), male gender and development of need for dialysis during follow-up.

Fourteen patients (16%) developed ESRD during follow-up (of whom 2 within one year), and 2 patients did not recover from dialysis. Dialysis dependency at diagnosis was highly associated with unfavorable renal outcome, as described previously^{12,19,20}. However, 11 of 25 patients dialysis dependent at diagnosis, who regained independent renal function, had a mean creatinine clearance of 38 ml/min at 1 year after diagnosis. We found that dialysis dependency at diagnosis, and not serum creatinine was associated with the development of ESRD which contrasts our study to others^{8-10,21}. Renal function at 1 year after diagnosis was associated with progression to ESRD for patients who experienced a renal relapse during follow-up.

In long-term follow-up, it does seem that relatively more patients who were dialysis dependent at diagnosis progress to end-stage renal disease (ESRD). Five patients of 11 who were dialysis dependent at diagnosis but had functional renal status at 1 year progressed to ESRD versus 5 patients of 53 who were dialysis independent at diagnosis and at 1 year. However, only 2 patients developed ESRD gradually, without the occurrence of a renal relapse. The other 3 patients progressed to ESRD after the occurrence of 1 or more renal relapses. The occurrence of a renal relapse was the strongest independent indicator of a worse renal outcome and carried a high risk for developing ESRD in our patient population. Dialysis dependency at diagnosis per se was not associated with progression to ESRD beyond 1 year. Long-term renal survival was not associated with blood pressure or proteinuria at 12 months after diagnosis, and renal survival was only related to creatinine clearance at 12 months in multivariate analysis in combination with renal relapse during follow-up. At 12 months after diagnosis renal function was rather well preserved with a median creatinine clearance of 60 ml/min. In addition, proteinuria was low (median 0.3 g/24h) and blood pressure well controlled (median MAP 100), characteristics suggesting a low risk for gradual loss of renal

function. As the occurrence of renal relapses of PR3-ANCA associated vasculitis was so strongly associated with renal failure, we analyzed the renal function loss associated with renal relapses and the possible influence on progressive renal functional decline. A renal relapse caused a mean long-term loss of creatinine clearance of 11 ml/min and an increase in proteinuria of 0.2 g/24h. In addition, renal function tended to deteriorate more rapidly after a renal relapse (as measured by the slopes of the change in creatinine clearance). It has been suggested before that the repetitive insults to the renal parenchyma would result in permanent damage¹³. Our observations clearly indicate the amount of damage that a renal relapse in PR3-ANCA associated vasculitis inflicts.

In our study, only 2 patients progressed to ESRD without signs of a renal relapse. In all other patients, we did not observe deterioration of renal function in long-term follow-up unless patients had a renal relapse, even if patients had been dialysis dependent at diagnosis. This is clearly in contrast to our findings in MPO-ANCA associated glomerulonephritis, where patients can have insidious renal disease ultimately leading to ESRD in about one third of the patients without signs of a renal relapse. In these patients, proteinuria is an important indicator of deteriorating renal function¹⁵, whereas in our PR3-ANCA vasculitic patients it is not. In the latter, close clinical follow-up for the occurrence of a relapse is needed^{22,23}.

In conclusion, for patients with PR3-ANCA associated vasculitis with renal involvement, there are no useful prognostic tools to guide the intensity of treatment. In long-term follow-up, our data demonstrate that renal relapses are highly associated with unfavorable renal outcome, and a renal relapse has a significant effect on renal function after this relapse. Since nearly half of the patients with PR3-ANCA experience relapses, physicians should be alert on the occurrence of a renal relapse and on possible consequences, and efforts to prevent relapses may be the way to preserve long-term renal function in this disease.

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Chapter 7

Differences in renal biopsies do not lead to difference in outcome between patients with PR3-ANCA and patients with MPO-ANCA

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Submitted

Background. Risk factors for the development of chronic renal insufficiency differ between MPO- and PR3-ANCA associated vasculitis (AAV) with renal involvement. We tested in a large group of patients with AAV with renal involvement whether differences already exist at diagnosis, and whether these differences explain distinctive outcomes in AAV.

Methods. All consecutive patients with biopsy-proven renal disease in AAV in the southeast of the Netherlands were included. Biopsies were scored for the presence of active and chronic lesions, and an activity index and chronicity index were calculated. Additionally, survival curves were estimated, and univariate and multivariate analysis with patient and renal survival as dependent variables was performed.

Results. Seventy-two patients (41 with MPO- and 31 with PR3-ANCA) were included. Overall patient and renal survival were 55% and 43% at 5 years, respectively. Risk factors for death were age >65 years, serum creatinine level and dialysis dependency at biopsy. The development of renal failure during follow-up was associated with serum creatinine, female gender, and renal biopsy findings. Evaluating patients with PR3- and MPO-ANCA, we found that more active lesions and a higher activity index were present in PR3-ANCA, whereas MPO-ANCA patients showed more chronic lesions, and a higher chronicity index. Despite the overt differences in renal biopsies, ANCA specificity was not a risk factor for patient death or the development of renal failure during long-term follow-up.

Conclusion. Renal lesions differ between patients with PR3- and MPO-ANCA, being more acute in PR3-ANCA, and more chronic in MPO-ANCA. These differences support the hypothesis that already at diagnosis more irreversible kidney damage exists in patients with MPO-ANCA compared to PR3-ANCA. The variation at diagnosis, however, does not result in differences in patient and renal outcome in PR3- and MPO-ANCA.

Small vessel vasculitides, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with antineutrophil cytoplasmic antibodies (ANCA), which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)¹⁻³. These diseases can occur in any organ system but the respiratory tract and the kidneys are most frequently involved. Untreated, WG results in death within weeks to months. Since the introduction of cyclophosphamide and prednisolone as standard treatment, survival has improved dramatically from less than 20% at 1 year reported in 1958⁴ to at least 60% 5-years survival reported in the past ten years⁵⁻⁸.

Although life-saving in many patients, treatment with cyclophosphamide and prednisolone is associated with severe morbidity and mortality, both at short-term and during long-term follow-up. Initiating aggressive immunosuppressive therapy in order to regain or conserve independent renal function in patients with ANCA-associated vasculitis with renal involvement is, therefore, dependent on the perceived prognosis of the patient. Previous studies have associated serum creatinine⁸⁻¹⁰ and kidney biopsy findings⁹⁻¹⁵ at diagnosis, and the occurrence of proteinuria^{15,16} and renal relapses^{17,18} with reduced renal survival in patients with pauci-immune necrotizing glomerulonephritis. Recently, we and others observed differences between groups associated with the different antigenic specificity of ANCA both with respect to clinical manifestations and follow-up (reviewed by Franssen et al¹⁹).

Previously, we studied long-term outcome in patients with ANCA-associated glomerulonephritis and found that clinical risk factors for the development of renal insufficiency differ between patients with MPO- and PR3-ANCA associated glomerulonephritis (AAGN). In PR3-AAGN, renal function is stable during remission, but declines with every renal relapse¹⁸. In MPO-AAGN, proteinuria at diagnosis and during follow-up was the most important risk factor for developing renal insufficiency¹⁶. It is unclear whether these different disease courses can be explained by biopsy findings at diagnosis.

We hypothesized that the differences in renal outcome in PR3- and MPO-AAV could be explained by differences in renal lesions. To test this, we retrospectively analyzed patient and renal survival in patients with biopsy-proven ANCA-associated glomerulonephritis in the southeast of the Netherlands. We investigated predictors of outcome in

renal biopsies and compared patients with PR3- and MPO-AAGN for the detection of differences at diagnosis and outcome.

Methods

Patients

We retrospectively identified all patients in our renal biopsy registry^{20,21} diagnosed with crescentic glomerulonephritis between January 1977 and July 2003 without evidence of systemic lupus erythematosus (SLE), IgA nephropathy, Henoch Schönlein purpura, post-infectious GN or cryoglobulinaemia. Serum samples, taken at the time of biopsy, were tested for the presence of ANCA. We thus identified 113 patients with AAGN: 47 patients (38%) with PR3-ANCA, 51 patients (46%) with MPO-ANCA, 5 patients (5%) with both PR3- and MPO-ANCA and 10 patients (10%) with MPO-ANCA with the presence of anti-glomerular basal membrane antibodies. Only patients whose biopsy was taken at the first disease episode and who were single positive for PR3- or MPO-ANCA were included in the current analysis ($N = 72$).

Treatment and follow-up

Patients were biopsied in 4 different hospitals in the southeast of the Netherlands. In most cases, follow-up was in the hospital where the biopsy was taken. Induction treatment consisted of prednisolone 1 mg/kg/day, usually combined with cyclophosphamide 2 mg/kg/day. In severe (renal) and / or life-threatening disease, methylprednisolone and / or plasma exchange were added. During follow-up, prednisolone was tapered and stopped; cyclophosphamide was tapered and stopped, or switched to azathioprine with or without ciclosporin A depending on the treating specialist.

Histological scoring

Renal biopsies were processed for light microscopy and immunofluorescence as described elsewhere²⁰. Biopsies had to contain five or more glomeruli per section to qualify for evaluation. To assess the activity and chronicity of the lesions serial 1 mm sections were stained with silver methenamin and counter stained with hematoxylin eosin. Numbers of normal glomeruli and numbers of glomeruli with global sclerosis were counted. Each glomerulus was separately assessed for the presence of necrosis and cellular and fibrous crescents

(the latter two had to cover at least 25 percent of the glomerular surface area). Percentages of affected glomeruli were calculated by dividing the number of glomeruli with lesions by the total number of glomeruli. Interstitial lesions, such as interstitial inflammation, interstitial fibrosis and tubular atrophy, were graded semiquantitatively on a scale of 0 to 3 (absent, mild, moderate, and severe, respectively). The presence of vasculitis of renal arteries and arterioles and (periglomerular) granulomatous inflammation was noted.

For calculation of activity scores, the presence of fibrinoid necrosis and cellular crescents was scored separately in a range of 0 to 3 (absent, less than 25% of glomeruli involved, 25 to 50% of glomeruli involved, and more than 50% of glomeruli involved, respectively). Additionally, the grade of interstitial inflammation (0 – 3, see above) was included in the activity score, resulting in a maximum score of 9. The chronicity score included the percentage of sclerosed glomeruli, the percentage of glomeruli with fibrous crescents and the grade of interstitial fibrosis and tubular atrophy, resulting in a maximum chronicity score of 12. Scoring methods are adapted from Franssen et al²². All biopsies were scored by one nephropathologist (PvBV) who was blinded to clinical diagnosis and ANCA specificity.

Statistical analyses

Data are presented as mean $\pm$ standard deviation unless stated otherwise. Continuous data between groups were compared using Student's t-test and the Wilcoxon signed rank test when appropriate. Survival curves for patient and renal survival were calculated using Kaplan-Meier estimates for survival distribution. End point for renal survival analysis was the start of renal replacement therapy or death due to renal failure. Differences between groups in survival were analyzed with log-rank test, and multivariate analysis with Cox proportional hazards analysis with survival time as dependent variable. Renal survival rates (survival without end-stage renal disease (ESRD)) are calculated and analyzed with subjects dying with independent renal function censored.

Analyzing patient and renal survival, a large difference between both patient and renal survival ≤ 1 year and > 1 year after diagnosis became apparent. Survival differences between groups were, therefore, analyzed separately for the time period ≤ 1 year and > 1 year after

diagnosis (long-term follow-up), to identify risk factors for these specific time periods. Variables analyzed included age, serum creatinine and dialysis dependency at the time of biopsy, gender, ANCA specificity, PR3-ANCA level in direct and capture ELISA (enzyme-linked immunosorbent assay), and renal biopsy findings. Analyses were performed with GraphPad Prism version 3.00 (GraphPad Software Inc., San Diego, CA) and SPSS version 10.0 (SPSS Inc., Chicago, IL). A two-sided P value < 0.05 was considered to indicate statistical significance.

Results

Of 113 renal biopsies, 72 biopsies were eligible for inclusion (31 PR3- and 41 MPO-ANCA). Fifty-three patients were male (74%). Age at biopsy was 61.9 ± 13.1 years. Serum creatinine level at biopsy was 445 ± 274 $\mu\text{mol/L}$; eight patients had serum creatinine levels below 120 $\mu\text{mol/L}$, 36 patients had creatinine levels between 120 and 500 $\mu\text{mol/L}$, and 28 patients had creatinine levels above 500 $\mu\text{mol/L}$. Seventeen patients (21%) were dialysis dependent at the time of renal biopsy; 15 of these had creatinine levels above 500 $\mu\text{mol/L}$. In four patients, it was unknown whether they required dialysis at biopsy (two patients had serum creatinine levels below 500 $\mu\text{mol/L}$, two patients above 500 $\mu\text{mol/L}$). Four patients were treated with corticosteroids alone, 57 patients were treated with cyclophosphamide and steroids; in two of these 57 patients plasma exchange was added. One patient was treated with methotrexate and steroids; one with mycophenolate and steroids; in seven patients treatment was withheld and in two patients, treatment is unknown.

Renal disease in PR3- and MPO-ANCA at diagnosis

In patients with PR3-ANCA, age at biopsy was 60.2 ± 12.4 versus 63.2 ± 13.7 in MPO-ANCA ($P = 0.07$). Mean serum creatinine with PR3-ANCA was 429 ± 273 $\mu\text{mol/L}$ compared to 456 ± 277 $\mu\text{mol/L}$ with MPO-ANCA ($P = \text{NS}$). Proteinuria was 1.9 ± 1.5 g/24h in patients with PR3-ANCA and 2.7 ± 2.6 g/24h in patients with MPO-ANCA ($P = \text{NS}$). Glomerular necrosis and cellular crescents were more often observed in PR3-ANCA positive biopsies (table 1, figures 1A and B), whereas in MPO-ANCA positive biopsies, fibrous crescents and glomerular sclerosis were more often present (table 1, figures 1C and D). Chronic interstitial injury, such as interstitial fibrosis and tubular atrophy, was also more

Table 1. Renal biopsy findings in PR3- and MPO-ANCA associated glomerulonephritis

	PR3-ANCA	MPO-ANCA	P value
% normal glomeruli	36.4 ± 28.5	29.7 ± 24.4	0.28
% glomerular necrosis	9.5 ± 11.7	3.8 ± 5.5	0.005
% cellular crescents	26.6 ± 23.0	18.4 ± 20.9	0.04
% fibrous crescents	15.2 ± 19.1	27.1 ± 24.6	0.02
% glomerular sclerosis	8.1 ± 13.8	13.9 ± 17.1	0.05
Interstitial inflammation ^a	1.4 ± 0.9	1.4 ± 0.9	0.68
Interstitial fibrosis ^a	0.7 ± 0.8	1.3 ± 0.7	0.0004
Tubular atrophy ^a	0.9 ± 0.9	1.6 ± 0.9	0.003
Activity score ^b	3.5 ± 1.7	2.9 ± 1.5	0.07
Chronicity score ^c	3.0 ± 2.7	5.1 ± 2.6	0.001

PR3, proteinase 3; MPO, myeloperoxidase; ANCA, antineutrophil cytoplasmic antibodies

^a Scored semiquantitatively on a scale of 0 to 3 (absent, mild, moderate, and severe, respectively)

^b Activity score consists of the grade of fibrinoid necrosis, cellular crescents, and interstitial inflammation

^c Chronicity score consists of the grade of fibrous crescents, glomerular sclerosis, interstitial fibrosis and tubular atrophy

common in MPO-ANCA. This resulted in a trend to a higher activity score in patients with PR3-ANCA and a higher chronicity score in MPO-ANCA. There were no differences in the frequency of granulomas in renal biopsies (8 in PR3- and 5 in MPO-ANCA) or vasculitis (7 and 3 for PR3- and MPO-ANCA, respectively).

Overall survival in ANCA-associated glomerulonephritis

In all 72 patients with eligible biopsies, follow-up data were available. Estimated survival at 5 years and 10 years was 54% and 34%, respectively (figure 2). Causes of death are presented in table 2. Estimated median survival was significantly shorter in patients >65 years at biopsy (3.1 years) when compared to those <50 years (undefined) and those between 50 and 65 years of age (7.0 years; $P = 0.05$). The estimated median survival in patients who were dialysis dependent at diagnosis was also significantly shorter than survival in

Figure 1. Renal biopsy findings in proteinase 3 (PR3)- and myeloperoxidase (MPO)-ANCA associated glomerulonephritis (AAGN). In all figures, 0 indicates no glomeruli involved; 1, less than 25% of glomeruli involved; 2, 25 to 50% of glomeruli involved; 3, 50% or more of glomeruli involved. A. Glomeruli with fibrinoid necrosis. Necrosis is more common in PR3-AAGN ($P = 0.005$). B. Glomeruli with cellular crescents. Cellular crescents are more common in PR3-AAGN ($P = 0.04$). C. Glomeruli with fibrous crescents. Fibrous crescents are more common in MPO-AAGN ($P = 0.02$). D. Sclerosed glomeruli. Glomeruli with global sclerosis are more common in MPO-AAGN ($P = 0.05$).

patients who were not on dialysis (0.46 vs. 8.7 years, respectively, $P = 0.002$). Survival with independent renal function was 47% at 5 years and 26% at 10 years (figure 2).

Mortality within one year

Of the 72 patients, 21 patients (29%) died within one year. In univariate analysis, severe renal involvement was associated with early death (dialysis dependency at the time of biopsy: relative risk (RR) 3.1, 95% confidence interval (CI) 1.4 – 12.6); serum creatinine >500 $\mu\text{mol/L}$: RR 4.8 (0.84 – 9.1) when compared to patients with serum creatinine < 120 $\mu\text{mol/L}$. In multivariate analysis, serum creatinine was associated with mortality within 12 months (Table 3).

Table 2. Causes of death

	<i>N (%)</i>	<i>Within one year</i>	<i>Beyond one year</i>
Vasculitis	1 (3)	1	0
Infection	12 (30)	8	4
Cardiovascular	7 (18)	4	3
Renal insufficiency	3 (8)	0	3
Malignancy	2 (5)	1	1
Other	4 (10)	2	2
Unknown	11 (28)	5	6
Total	40	21	19

Independence of renal replacement therapy within one year

Of the 17 patients who were dialysis dependent at diagnosis, five did not regain renal function (29%) but survived at one year and four patients (24%) died within one year without regaining independent renal function. Of five patients who regained independent renal function, three died within one year. In three patients, who died within

Figure 2. Patient survival (■) and patient survival without need for renal replacement therapy (◆) in 80 patients diagnosed with ANCA-associated glomerulonephritis. Numbers indicate number of patients at risk at 5 and 10 years after diagnosis.

Table 3. Predictors of patient mortality in multivariate analysis

<i>Predictor</i>	<i>RR</i>	<i>95% CI</i>	<i>P value</i>
Patient mortality ≤ 1 year (N = 72)			
Serum creatinine 1 μmol/L increase	1.002	(1.001 – 1.003)	0.001
Patient mortality > 1 year (N = 45)			
Age at biopsy 1 yr increase	1.05	(1.007 – 1.099)	0.006

one year, it was unknown whether they had regained independent renal function. Of 51 patients dialysis independent at biopsy, seven patients (14%) required dialysis within one year. In 4 patients, dialysis requirement at the time of biopsy was unknown. With univariate analysis, serum creatinine (*P* for trend 0.04) and the degree of interstitial fibrosis (absent or mild versus moderate or severe: RR 4.2, 1.1 to 34.9) were associated with the development of renal failure within one year. Additionally, the absence of glomeruli with necrosis (RR 5.3, 0.84 to 16.5) and the chronicity score (0 – 3 versus >3: RR 5.3, 0.84 to 16.6) tended to be associated with reduced renal survival. In multivariate analysis, only serum creatinine was associated with persistent or renewed dialysis dependency within one year after biopsy (Table 4).

Long-term patient survival

Of 46 patients with a follow-up of more than one year, 17 patients (37%) died more than one year after diagnosis. Estimated median survival was significantly shorter in patients >65 years at biopsy (8.3 years) when compared to those <50 years (undefined) and those between 50 and 65 years of age (18.6 years; *P* for trend = 0.05). Dialysis dependency at biopsy tended to be associated with reduced patient survival during long-term follow-up (RR 2.7, 0.86 to 20.3). In multivariate analysis, only age was associated with death (Table 3).

Table 4. Predictors of renal failure in multivariate analysis

<i>Predictor</i>	<i>RR</i>	<i>95% CI</i>	<i>P value</i>
Renal failure ≤ 1 year (N = 72)			
Serum creatinine 1 μmol/L increase	1.005	(1.001 – 1.009)	0.006
Renal failure > 1 year (N = 45)			
Not performed			

Long-term renal survival

Of 46 patients with a follow-up of more than one year, 36 patients (78%) were dialysis independent at one year. During long-term follow-up, seven patients (19%) progressed to ESRD; the median time to ESRD in these patients was 3.1 years (range, 1.3 to 7.8 years). In univariate analysis, female gender was the only factor associated with renal failure during follow-up (RR 6.6, 2.8 to 163); serum creatinine or dialysis dependency at biopsy, and renal biopsy findings, were not associated with reduced renal survival in long-term follow-up. Multivariate analysis was not performed due to the low amount of events in this group.

PR3- versus MPO-ANCA during follow-up

Eighteen of 34 patients (53%) with PR3-ANCA died versus 23 of 46 patients (50%) with MPO-ANCA ($P = \text{NS}$) (figure 3). ANCA specificity was not a risk factor for death within or beyond one year of follow-up. Comparing survival without need for renal replacement therapy, 8 of 34 patients (24%) with PR3-ANCA needed RRT during follow-up versus 17 of 46 patients (37%) with MPO-ANCA ($P = \text{NS}$). ANCA specificity was not a risk factor for developing need for RRT within or beyond one

Figure 3. Survival in ANCA-associated vasculitis according to ANCA specificity. Patient survival was comparable between patients with proteinase 3 (PR3)-antineutrophil cytoplasmic antibodies (ANCA) (◆) and patients with myeloperoxidase (MPO)-ANCA (■).

year of follow-up.

Discussion

In the present study, we evaluated patient and renal survival in patients with ANCA-associated glomerulonephritis during long-term follow-up and related these to histological findings in renal biopsies. In the patient population we studied, the prognosis of AAV was poor; both patient and renal survival were low in long-term follow-up. We found serum creatinine and dialysis dependency at the time of biopsy, the percentage of normal glomeruli, and the presence of moderate or severe interstitial inflammation were indicative of developing ESRD within one year. In patients who developed dialysis dependency later on, the percentage of normal glomeruli and the cumulative chronicity score were predictors for reduced renal survival. We found more acute lesions in patients with PR3-ANCA and more chronic lesions in patients with MPO-ANCA. However, there was no difference in renal outcome between patients with PR3-ANCA and patients with MPO-ANCA.

The patient survival in our study is lower than in previous studies considering patients with small-vessel vasculitis with renal involvement where it varied from 44 to 95% in various patient groups during various follow-up periods^{5,8,10,11,14,16-18,23-26} (table 5). This may be due to patients being treated in regional hospitals, instead of in a specialized center. The low amount of patients being treated with plasma exchange seems to strengthen this point. To analyze this hypothesis, we compared patient and renal survival in our previous study in Groningen¹⁸, a specialized center, to survival in the Limburg area. Overall patient survival was significantly lower in the Limburg area ($P = 0.04$), supporting our hypothesis. Survival without need for renal replacement therapy did not differ between Groningen and the Limburg area (data not shown). Additionally, although ANCA testing became locally available in 1994, patients biopsied before that year did not have a worse patient or renal survival than patients biopsied since 1994 (data not shown), ruling year of biopsy out as a cause of our high 10-year mortality rate.

Several studies have compared renal histology of PR3-ANCA with MPO-ANCA^{12,22,27-30}. Results have varied from comparable renal disease between PR3- and MPO-ANCA^{12,28,29} to more active lesions in PR3-ANCA and more chronic lesions in MPO-ANCA²² in addition to more diffuse

Table 5. Patient survival in ANCA-associated vasculitis

<i>Study</i>	<i>study period (years)</i>	<i>Patient survival at the end of study period</i>
Hedger (5)	1.8	59%
Westman (8)	10	52%
Wilkowski (10)	10	44%
Andrassy (11)	3	96%
Little (14)	5	63%
Franssen (16)	2	95%
Cohen (17)	2.7	87%
Slot (18)	10	62%
Aasarød (23)	5	74%
Booth (24)	5	76%
Lane (25)	5	66%
Rihova (26)	10	62%
Present study	10	36%

glomerulonephritis in MPO-ANCA³⁰. Furthermore, a mixture of both more chronic and more active lesions in patients with MPO-ANCA has also been described²⁷. Our study confirms the presence of more active lesions in PR3- and more chronic lesions in MPO-ANCA, resulting in a higher activity score in patients with PR3-ANCA and a higher chronicity score in patients with MPO-ANCA. Interestingly, the serum creatinine level was comparable between PR3- and MPO-ANCA, which has been described before^{17,22,24,30}. In spite of this, our results support the hypothesis that already at diagnosis more irreversible kidney damage exists in MPO-ANCA compared to PR3-ANCA.

During long-term follow-up, we could not detect a difference in patient or renal survival between patients with PR3- and MPO-ANCA. Patients with cytoplasmic ANCA⁹ or PR3-ANCA³¹ have previously been described to have a higher risk of patient death, but this has been disputed by others^{22,32,33}. A high PR3-ANCA level in capture ELISA is a risk factor for both poor patient and renal survival⁸, and a consistently elevated MPO-ANCA level is a risk factor for poor renal survival as well¹⁶. Vizjak *et al* reported a higher rate of renal failure in MPO- when compared to PR3-

ANCA³⁰, in contrast to previous studies that reported no difference^{22,32,33}, and one study even reported a higher rate of ESRD in patients with PR3-ANCA³¹. In the present study, 24% of patients with PR3-ANCA developed renal failure compared to 37% of patients with MPO-ANCA, a difference that was not statistically significant. Furthermore, we found no association between high PR3-ANCA level (both direct and capture ELISA) at renal biopsy and patient or renal survival.

To our knowledge, this is the first study investigating differences in renal biopsies between patients with PR3- and MPO-ANCA and determining the effect of these differences in long-term follow-up. We found that although differences existed in renal lesions, this did not lead to a difference in renal outcome in patients with AAV. This may, however, be due to the overall poor outcome in patients with AAV in our study making us unable to detect an existing difference. Another explanation may be the different risk factors for developing renal failure in PR3- and MPO-ANCA. In patients with PR3-ANCA, more acute lesions are found, which are expected to respond to treatment leading to a large improvement in renal function. However, a creatinine clearance above 60 mL/min is a risk factor for relapse³⁴, and renal relapse is an important risk factor for developing renal failure in patients with PR3-AAV¹⁸. In patients with MPO-ANCA, more chronic lesions are present, which are expected to result in glomerulosclerosis. Glomerulosclerosis may lead to persistent proteinuria, and proteinuria is the most important risk factor for developing renal failure in patients with MPO-AAV¹⁶.

In conclusion, patient and renal survival in ANCA-associated vasculitis is associated with both clinical variables and renal biopsy findings. There is, however, a large overlap in renal biopsy findings between patients who do and do not develop renal failure; therefore, renal biopsy alone should not guide the intensity of treatment. Furthermore, even though ANCA specificity results in different morphology in renal biopsy; outcome is comparable between PR3- and MPO-ANCA.

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Chapter 8

A positive C-ANCA titer at switch to azathioprine therapy is associated with a disquieting relapse rate in PR3-ANCA-related vasculitis

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Objective. To analyze disease-free survival in patients with ANCA-associated small-vessel vasculitis (AAV) treated with cyclophosphamide only or switched to azathioprine after 3 months of full remission with cyclophosphamide.

Methods. Analysis of disease-free survival in all consecutive patients diagnosed with ANCA-related small-vessel vasculitis between 1990 and 2000 at our center, who were followed at least 12 months, and treated with cyclophosphamide only (1990-1996) or switched to azathioprine after 3 months of remission on cyclophosphamide (1997-2000).

Results. Included were 128 patients of whom 44 (34%) switched to azathioprine. Fifty-three patients (41%) relapsed. Actuarial disease-free survival at 2 and 4 years was 76% and 65% in the cyclophosphamide group compared to 76% and 51% in the azathioprine group (RR 1.4, 95% CI 0.8-2.7; $p=0.20$). In patients with PR3-ANCA associated vasculitis switched to azathioprine ($n=33$) a positive C-ANCA titer at the moment of treatment switch ($n=13$) was significantly associated with the occurrence of relapse (RR 2.6, 95% CI 1.1 - 8.0; $p=0.04$). In patients with a negative ANCA titer at the time of switch to azathioprine, disease-free survival at 2 and 4 years was 80% and 62%, which was identical to patients treated with cyclophosphamide only. In patients who were ANCA positive at the time of treatment switch disease-free survival at 2 and 4 years was only 58% and 17%.

Conclusion. Switching cyclophosphamide to azathioprine after induction of remission in patients with PR3-ANCA associated vasculitis who are still ANCA positive at the time of treatment switch is associated with a high risk of developing relapses.

Small vessel vasculitides, such as Wegener's Granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with antineutrophil cytoplasmic antibodies (ANCA), which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)¹⁻³. ANCA-associated vasculitis (AAV) can occur in any organ system; the respiratory tract and the kidney are most frequently involved. Untreated, these diseases have a dismal prognosis. Since the introduction of cyclophosphamide and prednisolone as treatment regimen, survival has improved dramatically from less than 20% at 1 year⁴ to 60 – 90% at 10 years⁵⁻⁷. Although life-saving in many patients, treatment with cyclophosphamide is associated with severe morbidity and mortality, both at short-term and during long-term follow-up. Alternative maintenance regimens have been proposed to reduce cyclophosphamide exposure⁸. The European multicentre CYCAZAREM study showed that switching cyclophosphamide to azathioprine after induction of remission does not lead to more relapses within 18 months after diagnosis compared to more prolonged cyclophosphamide therapy (12 months) in patients with AAV⁹. Although the long-term safety has not been established, this switching is widely adopted in the treatment of patients with AAV. In our center, we adopted this switching regimen in 1996. We had the impression, however, that relapses occurred more frequently after ≥ 2 years of follow-up when patients with AAV were switched to azathioprine. To address this issue, we analyzed patients diagnosed with AAV, treated at our center with cyclophosphamide and switched to azathioprine after induction of remission, and compared long-term outcome with a historical control group that was treated with cyclophosphamide only.

Patients and Methods

Patients

All patients, diagnosed between 1990 and 2000 with AAV, followed at our center with ≥ 1 year follow-up, and treated with cyclophosphamide only (1990 – 1996) or switched to azathioprine after induction of remission (1997 – 2000) were included. All patients fulfilled the Chapel Hill definitions of WG, MPA, Churg-Strauss syndrome or renal limited vasculitis¹⁰, in addition to the presence of PR3- or MPO-ANCA. Patients were prospectively followed until December 2001.

Treatment

The treatment consisted of oral cyclophosphamide (2 mg/kg) and prednisolone (1 mg/kg body weight) daily with a maximal oral prednisolone dose of 60 mg/day¹¹. Doses of cyclophosphamide were adjusted to maintain the leukocyte count above $4 \times 10^9/L$. After 4 to 6 weeks, the daily prednisolone dose was tapered by 10 mg every 2 weeks until the dose reached 30 mg, and thereafter by 5 mg every 2 to 4 weeks. Once remission was achieved the daily dose of oral cyclophosphamide was tapered by 25 mg every 3 months (1990-1996), resulting in a treatment duration of approximately 18 months. From 1997 onwards, patients were switched to azathioprine (2 mg/kg body weight daily) after at least three months of therapy and induction of remission with cyclophosphamide. Azathioprine was then tapered by 25 mg every 3 months, resulting in a treatment duration of approximately 21 months. Actual duration of treatment was calculated until medication was stopped, or until patients were diagnosed with a relapse, whichever came first.

Detection of ANCA

ANCA were detected by indirect immunofluorescence (IIF) as described previously¹². ANCA titers were analyzed by serial dilutions of sera starting at 1:20. Sera were considered positive for ANCA when staining was present in at least a 1:40 dilution and a cytoplasmic pattern (C-ANCA) in patients diagnosed with PR3-ANCA associated vasculitis, or an atypical or perinuclear pattern (P-ANCA) in patients diagnosed with MPO-ANCA associated vasculitis. In all patients, the presence of PR3- or MPO-ANCA was confirmed by antigen-specific enzyme-linked immunosorbent assay (ELISA)². ANCA titers were recorded every 4 to 6 weeks.

Definitions

Remission was defined as the absence of clinical signs and symptoms of active vasculitis (Birmingham Vasculitis Activity Score (BVAS)¹³ = 0), and a normal C-reactive protein¹¹. A relapse was defined as clinical signs of vasculitic activity in combination with biopsy proven vasculitic disease activity, or the occurrence of nodular pulmonary lesions after exclusion of infectious or malignant diseases. Renal vasculitic disease was defined as biopsy proven necrotizing glomerulonephritis or a

combination of microscopic glomerular erythrocyturia, erythrocyte cell casts, proteinuria and a decrease in creatinine clearance¹⁴.

Statistical analyses

Data are presented as mean $\pm$ standard deviation unless stated otherwise. Continuous data between groups were compared using Student's t-test and the Wilcoxon signed rank test when appropriate. Survival curves were calculated using Kaplan-Meier estimates for survival distribution. Differences between groups in survival were analyzed with log-rank test with disease-free survival as dependent variable. End point for survival analysis was the occurrence of relapse. Patients without a relapse were censored at 31 December 2001, or death, whichever came first.

The primary planned analyses were the association of treatment with cyclophosphamide only or cyclophosphamide switched to azathioprine and relapse-free survival in the whole group of patients with AAV, and the PR3- and MPO-ANCA groups separately. Secondary analyses included the influence of ANCA positivity at 6 and 12 months, and at the moment of treatment switch from cyclophosphamide to azathioprine on relapse-free survival. Analyses were performed with GraphPad Prism version 3.00 (GraphPad Software Inc., San Diego, CA). A two-sided p-value < 0.05 was considered to indicate statistical significance.

Results

Between January 1990 and January 2000, 128 consecutive patients were eligible for entering the study. Median follow-up was 5.3 years (range 1.0 – 11.5 years). Ninety-three patients (73%) were positive for PR3-ANCA, whereas 35 patients (27%) were positive for MPO-ANCA. Forty-four patients (34%) were switched to azathioprine after a median follow-up of 0.48 years (range 0.23 – 1.43 years) after diagnosis. Ten patients, who switched to azathioprine, had also been included in the CYCAZAREM trial. Patients who switched to azathioprine were significantly younger than patients who remained on cyclophosphamide ($n = 84$) (table 1). As expected, duration of treatment was significantly longer in patients who were switched to azathioprine. Otherwise, the two groups did not differ with respect to clinical characteristics (table 1).

Table 1. Patient characteristics in treatment groups

Variable	Cyclophosphamide only (n = 84)	Treatment switch to azathioprine (n = 44)
PR3-ANCA specificity	60 (73%)	33 (75%)
Age at diagnosis*	57 ± 13	48 ± 18 [†]
Male gender	54 (64%)	26 (59%)
BVAS at diagnosis*	22 ± 8	24 ± 10
Pulmonary involvement	48 (57%)	23 (52%)
Renal involvement	62 (74%)	37 (84%)
ENT involvement	64 (76%)	34 (77%)
Duration of treatment (years)		
Cyclophosphamide / azathioprine*	1.38 ± 0.53	1.82 ± 0.54 [‡]
Prednisolone*	1.20 ± 0.46	1.20 ± 0.46

* Values are mean ± SD. PR3, proteinase 3; ANCA, antineutrophil cytoplasmic antibodies; BVAS, Birmingham vasculitis activity score; ENT, ear nose throat.
[†] p = 0.002 when compared to patients who were treated with cyclophosphamide only
[‡] p < 0.0001 when compared to patients treated with cyclophosphamide only

Fifty-three patients (41%) experienced at least one non-renal or renal relapse. Actuarial disease-free survival at 2 and 4 years was 76% and 65%, respectively, in the cyclophosphamide group compared to 76% and 51% in the group switched to azathioprine (relative risk (RR) for relapse 1.4, 95% CI 0.8 – 2.7; p = 0.20) (figure 1). In patients with PR3-ANCA (n = 93) and MPO-ANCA associated vasculitis (n = 35) no significant difference in disease-free survival between the treatment limbs was observed (figure 2). However, relapses were more frequently observed in patients with PR3-ANCA as compared to MPO-ANCA specificity (RR 3.2, 95% CI 1.4 – 4.4; p = 0.002).

A positive ANCA titer at 6 or 12 months after diagnosis was not significantly associated with the occurrence of a relapse (data not shown). However, when analyzed according to ANCA specificity, a positive C-ANCA titer in IIF at 12 months tended to be associated with the occurrence of a relapse with RR 1.7 (95% CI 0.9 – 3.0, p = 0.11) when compared to a negative C-ANCA titer at 12 months in patients with PR3-ANCA associated vasculitis.

Figure 1. Disease-free survival in 128 patients diagnosed with ANCA-associated vasculitis, according to treatment limb. There is no significant difference in disease-free survival between patients treated with cyclophosphamide only (■), and patients switched to azathioprine (▲).

Figure 2. Disease-free survival in 128 patients diagnosed with ANCA-associated vasculitis, according to ANCA specificity and treatment switch. There is no significant difference in disease-free survival in MPO-ANCA associated vasculitis between patients treated with cyclophosphamide only (■), or switched to azathioprine (▲). Similarly, there is no difference in PR3-ANCA associated vasculitis between patients treated with cyclophosphamide only (▼), or switched to azathioprine (◆). Disease-free survival in MPO- and PR3-ANCA associated vasculitis however is significantly different.

Table 2. Patient characteristics in PR3-ANCA associated vasculitis.

Variable	Cyclophosphamide only (n = 60)	Azathioprine ANCA negative at switch (n= 20)	Azathioprine ANCA positive at switch (n= 13)
Age at diagnosis* [†]	57 ± 11	47 ± 19	43 ± 16
Male gender	42 (70%)	13 (65%)	8 (62%)
BVAS at diagnosis*	23 ± 8	25 ± 10	27 ± 10
Pulmonary involvement	33 (55%)	11 (55%)	8 (62%)
Renal involvement	43 (72%)	16 (80%)	11 (85%)
ENT involvement	55 (92%)	16 (80%)	12 (92%)
Duration of treatment (years)			
Cyclophosphamide prior to switch*	N/A	0.49 ± 0.21	0.55 ± 0.32
Cyclophosphamide / azathioprine* [‡]	1.5 ± 0.8	1.9 ± 0.6	1.8 ± 0.5
Prednisolone*	1.2 ± 0.4	1.0 ± 0.3	1.3 ± 0.4

* Values are mean ± SD. PR3, proteinase 3; ANCA, antineutrophil cytoplasmic antibodies; BVAS, Birmingham Vasculitis Activity Score; ENT, ear nose throat; N/A, not applicable.

[†] Age was significantly higher in patients treated with cyclophosphamide only but not significantly different in patients who were ANCA positive versus patients who were ANCA negative.

[‡] p = 0.004 for the overall comparison

Thirty-three patients with PR3-ANCA associated vasculitis switched from cyclophosphamide to azathioprine following at least 3 months of complete remission. Nineteen patients of these 33 (58%) relapsed, of which 10 patients were still treated with azathioprine at the occurrence of a relapse. Thirteen patients (39%) of the 33 patients switched to azathioprine had a positive C-ANCA titer in IIF at the moment of treatment switch. In baseline clinical characteristics, these patients did not differ from patients who were C-ANCA negative at treatment switch (table 2). Ten of 13 patients, C-ANCA-positive at treatment switch, relapsed. Mean BVAS at relapse was 11 ± 3 (range 7 – 18); in 3 patients (30%), the relapse was renal. Eight patients (80%) were treated with cyclophosphamide and steroids again. A positive C-ANCA titer at treatment switch was significantly associated with the

occurrence of a relapse (RR 2.6, 95% CI 1.1 – 8.0; $p = 0.04$). In patients who were treated with cyclophosphamide only, there was no association between a positive C-ANCA titer at 6 months and the occurrence of a relapse (data not shown). Disease-free survival at 2 and 4 years after diagnosis was 58% and 17%, respectively, in patients who were positive for C-ANCA at treatment switch (figure 3). In contrast, patients who switched to azathioprine and were negative at the moment of treatment switch had a disease-free survival of 80% and 62% at 2 and 4 years, respectively, which was nearly identical to patients treated with cyclophosphamide only (figure 3).

Discussion

After the CYCAZAREM trial, switching to azathioprine after induction of remission is widely adopted as treatment regimen, since the CYCAZAREM trial showed that switching cyclophosphamide to azathioprine in AAV does not result in more relapses within 18 months after diagnosis compared to continued cyclophosphamide therapy⁹. Indeed, in our study, the relapse rate is similar between the two

Figure 3. Disease-free survival in 93 patients diagnosed with PR3-ANCA associated vasculitis, according to treatment limb. (▲) indicates patients positive for ANCA at treatment switch to azathioprine, (■) patients negative for ANCA at switch. (◆) indicates patients treated with cyclophosphamide only.

treatment limbs until 18 months (figure 3). Our results are comparable with the CYCAZAREM study: at 18 months, 10% of our patients switched to azathioprine had relapses versus 16% in the CYCAZAREM study; in patients treated with cyclophosphamide only relapse rate at 18 months was 10% and 17% in our study and the CYCAZAREM study, respectively.

Differences between our study and the CYCAZAREM trial do, however, exist. Our study is a non-randomized retrospective study comparing groups that have been treated according to two treatment regimens that have been consequently used in two sequential time periods. Despite this patients switched or not switched from cyclophosphamide to azathioprine were comparable with respect to clinical and disease characteristics. Our study has the advantage of longer follow-up after diagnosis and thus is able to show that the excess in relapse rate in patients treated with azathioprine does not occur until after 2 years. Several surveys on outcome in ANCA-associated vasculitis have been performed in which patients are usually treated with corticosteroids and cyclophosphamide (in some patients switched to azathioprine)^{11,15-17}. In these studies, reported mean disease-free survival varies from 9.8 to 27 months after remission (median 21.8 months). Follow-up in the CYCAZAREM study did not exceed 18 months and therefore may have been too short for detection of a difference in relapse rates. Second, in the CYCAZAREM trial, no distinction has been made between PR3- and MPO-ANCA related vasculitis. We have analyzed relapse rate in relation to ANCA specificity and we found that this is relevant, because in patients with PR3-ANCA associated vasculitis the relapse rate is significantly higher than patients with MPO-ANCA associated vasculitis. Third, continuation of cyclophosphamide therapy differed between our study and the cyclophosphamide limb of the CYCAZAREM trial. We treated patients with AAV with cyclophosphamide for 15 – 18 months, whereas in the CYCAZAREM trial patients were treated with cyclophosphamide for 12 months.

In our study, there was a significantly higher relapse rate in patients switching to azathioprine who had a positive C-ANCA titer at treatment switch. However, if patients were to remain on treatment with cyclophosphamide, serious drug toxicity may occur. Besides, 3 patients, positive for C-ANCA when treatment switch is considered, need to remain on cyclophosphamide to avoid one relapse. On the

other hand, our data show that patients, who are C-ANCA negative by IIF after 3 months of complete remission on cyclophosphamide, can safely be switched to azathioprine without an increased rate of relapses during long-term follow-up.

As most relapses occur during or shortly after tapering of azathioprine, prolonged therapy with azathioprine may also be an option. In the present study, mean duration of treatment with azathioprine was within two years. In REMAIN, a second-wave trial by the European Vasculitis Study Group (EUVAS), long-term (4 years) low dose immunosuppressive therapy will be compared to the standard of two years of treatment¹⁸. Although this study is mainly designed to study patients in whom a second episode of renal vasculitis would seriously threaten their renal survival, results might be extrapolated to all patients with ANCA-associated vasculitis.

Other treatment regimens for maintenance therapy have been proposed, for instance, switching of cyclophosphamide to methotrexate (MTX). In an open-labeled study by Langford et al.¹⁹ in 31 patients with Wegener's granulomatosis, this led to a disease-free survival rate of 84% at a median follow-up of 16 months (maximum disease-free survival was 49 months). On the other hand, a study by Reinhold-Keller et al. showed that patients who were switched to MTX had a high rate of renal relapse²⁰. However, these studies have not considered the influence of ANCA titers at treatment switch on the occurrence of a relapse.

A fourth option in the treatment of patients with PR3-ANCA associated vasculitis may be intensification of induction therapy with the attempt to deplete circulating ANCA, for instance, with plasma exchange (PE)²¹, or anti-CD20 therapy²². PE has been shown to increase survival in patients with renal failure at diagnosis²¹. To our knowledge, benefit of PE on relapse rate has not been reported. Anti-CD20 therapy as induction therapy for a relapse led to the disappearance of C-ANCA and complete remission in a patient with WG²². Another option to intensify induction therapy may be anti-TNF targeted therapy; a randomized, placebo-controlled trial of etanercept plus standard therapy in WG has recently started²³.

In conclusion, our data challenge the safety of switching cyclophosphamide to azathioprine in patients with PR3-ANCA associated vasculitis who are still positive for ANCA. Whether

alternative treatment regimens can prevent relapses in this subgroup of patients is unclear. Based on our findings, we postulate that C-ANCA response during remission induction and early follow-up may provide a powerful tool for risk-adjusted treatment regimens.

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Chapter 9

*Anti-oxLDL antibodies in MPO-ANCA
positive vasculitis patients
preferentially recognize hypochlorite-
modified low density lipoproteins*

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Submitted

Aim of the study. Many patients surviving vasculitis are prone to accelerated atherosclerosis and often have enhanced levels of antibodies to oxidized low-density lipoprotein (oxLDL). To measure anti-oxLDL antibodies, oxidation of LDL is achieved with copper (Cu) or malondialdehyde (MDA). Since *in vivo*, LDL may be oxidized with myeloperoxidase (MPO) or its product hypochlorite, we measured anti-hypochlorite LDL antibodies in patients with vasculitis, hemodialysis patients, and healthy controls.

Methods. A newly developed ELISA-method was used to detect antibodies to oxLDL as modified by hypochlorite. Results are compared with data obtained by standard LDL oxidation using MDA-LDL or Cu-LDL as substrate. Results were compared between antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients (n=93), hemodialysis (HD) patients (n=59) and healthy controls (HC; n=43). Furthermore, patients with MPO-ANCA associated vasculitis (n=47) were compared with patients with PR3-ANCA associated vasculitis (n=46). Optimal cut-off points were determined by ROC-curve analysis.

Results. Anti-oxLDL antibodies are enhanced in AAV patients (MDA-LDL and hypochlorite-LDL) and in HD patients (hypochlorite-LDL), when compared to HC. Furthermore, patients with MPO-ANCA associated vasculitis had higher levels of antibodies to hypochlorite-LDL than patients with PR3-ANCA associated vasculitis.

Conclusion. Our newly developed assay, in which hypochlorite-LDL is used as substrate, seems a more sensitive assay than traditional assays to measure oxLDL antibodies. Furthermore, our results suggest that enhanced MPO-mediated LDL oxidation occurs in patients with MPO-ANCA reflecting the idea that circulating MPO-ANCA prevents inactivation of circulating MPO activity by its natural inhibitor ceruloplasmin.

Small vessel vasculitis, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA) are strongly associated with anti-neutrophil cytoplasmic antibodies (ANCA) which are either directed to proteinase 3 (PR3) or to myeloperoxidase (MPO)¹⁻³. ANCA associated vasculitis (AAV) patients have an increased risk to develop accelerated atherosclerosis^{4,5} and to experience subsequent cardiovascular events^{6,7}. Atherosclerosis is generally considered an inflammatory disease⁸, and chronic inflammation is a promoting factor for progression of atherosclerosis toward cardiovascular events⁹.

Antibodies to oxidized low density lipoproteins (oxLDL) are useful markers for atherosclerosis¹⁰, and levels of these antibodies are increased in AAV at diagnosis¹¹. Detection of antibodies to oxLDL can be accomplished by oxidation of LDL *in vitro* by exposing LDL to malondialdehyde (MDA), a reactive aldehyde generated during lipid oxidation and resulting in adduct formation on apoB100¹⁰. Alternatively, LDL can be oxidized *in vitro* by copper^{10,12}. An interesting *in vivo* pathway for LDL oxidation is catalyzed by MPO, a heme enzyme present in neutrophilic granulocytes. MPO catalyses the generation of several reactive oxidants, leading to products which have been found in atherosclerotic lesions^{13,14}. It is the only known enzyme capable of producing the highly toxic hypochlorite *in vivo* in the presence of chloride and hydrogen peroxide (H₂O₂).

MPO is not only intracellularly active. MPO is also released into the extracellular space after activation of granulocytes. Here, enzyme activity is inhibited by ceruloplasmin, a copper binding protein which attaches to the active site of MPO and hence regulates MPO enzyme activity in the circulation¹⁵. MPO-ANCA, isolated from AAV patients, is capable of partially reversing this MPO-inactivation¹⁶.

We hypothesize that patients who are prone to accelerated atherosclerosis have antibodies to hypochlorite-modified LDL. Furthermore, we hypothesize that in MPO-AAV patients, but not in PR3-AAV patients, increased circulating MPO activity may result in enhanced LDL modification by hypochlorite and subsequent generation of antibodies specific for hypochlorite-modified LDL epitopes.

To test these hypotheses, we analyzed anti-oxLDL antibody levels in 93 consecutive AAV patients with pauci-immune crescentic glomerulonephritis, 59 hemodialysis patients and 43 healthy controls. Forty-seven of the AAV patients had MPO-ANCA and 46 had PR3-ANCA.

Anti-oxLDL antibody levels were measured using hypochlorite-modified LDL as well as MDA- and copper-modified LDL.

Methods

Patients

Patients included in this study had biopsy-proven pauci-immune crescentic glomerulonephritis without evidence of systemic lupus erythematosus (SLE), IgA nephropathy, Henoch Schönlein purpura, post-infectious glomerulonephritis (GN), cryoglobulinaemia, or anti-glomerular basement membrane (GBM) nephritis¹⁷. Only patients whose biopsy showed active lesions (either at the first disease episode or at relapse) were included. Serum samples, taken at the time of biopsy, were tested for the presence of ANCA. Ninety-three patients with ANCA associated glomerulonephritis (AAGN) were included in this study, 46 of which had PR3-ANCA and 47 had MPO-ANCA; patients with both PR3- and MPO-ANCA and those with both MPO-ANCA and anti-GBM antibodies were excluded. As disease controls we used sera from all patients who are routinely dialyzed at the hemodialysis (HD) unit in the university hospital Maastricht (n=59). Patients with renal failure due to AAV were excluded from this latter group. Additionally, 43 healthy controls (HC; laboratory personnel) were tested. This study was performed in accordance with the 1997 Declaration of Helsinki of the World Medical Association.

LDL isolation and preparation of oxidized LDL

LDL was isolated from plasma of a healthy subject by ultracentrifugation in a KBr discontinuous gradient according to Redgrave et al¹⁸. KBr and EDTA were removed by rapid filtration through disposable desalting columns (Econo-Pac 10 DG, Bio-Rad, Hercules, USA). The LDL protein content was determined according to Lowry et al¹⁹. Native LDL was either freshly used for preparation of oxLDL or stored at 4°C in PBS under N₂, with 1 mg/ml EDTA as a preservative.

MDA-LDL was prepared as described by Palinski et al¹⁰; in brief, 0.5 M MDA (Merck, Darmstadt, FRG) was added to LDL in a ratio of 100 µl per mg LDL and incubated for 3 hours at 37°C. After removal of unbound MDA by exclusion chromatography, MDA-LDL was stored at 4°C in PBS/EDTA under N₂ after sterile filtration.

Copper oxidation (Cu-LDL) was performed by incubating a 0.2 mg/ml

solution of LDL in PBS with 20 μM CuCl_2 for 20 hours at 37°C; reaction was stopped by adding 100 μM EDTA.

Hypochlorite modification (hypochlorite-LDL) was done by coating 100 μl of native LDL, diluted to 100 $\mu\text{g}/\text{ml}$ in PBS, in microtiter plates (Nunc MaxiSorp™, Nalge Nunc, Rochester, NY) overnight at 4°C and, after washing three times with PBS, incubating half of each plate for two hours at 4°C in a solution of 36 μM sodium hypochlorite (Sigma, St. Louis, MO) in PBS; the other half of the plate was incubated in PBS. Incubation was followed by a threefold wash with PBS to stop the reaction, and plates were stored in PBS at 4°C for maximal 2 hours before starting the ELISA. Optimal concentration of hypochlorite was determined using a serial dilution and subsequent testing of antibody reactivity (data not shown).

ELISA for the detection of antibodies against MDA-LDL, Cu-LDL and hypochlorite-LDL

Native LDL and MDA-LDL or Cu-LDL were diluted to 100 $\mu\text{g}/\text{ml}$ in PBS, and 100 $\mu\text{l}/\text{well}$ was incubated in microtiter plates (Nunc) overnight at 4°C; for detection of antibodies to hypochlorite-LDL, native LDL was coated before hypochlorite modification as described above. Wells were then washed 5 times with a buffer containing 0.01 M Tris, 0.15 M NaCl and 0.05 % Tween 20 (pH 8.0) followed by incubation in triplicate with patient serum in a 1:100 dilution, 100 $\mu\text{l}/\text{well}$, in buffer containing 0.1 M Tris, 0.3 M NaCl and 0.05 % Tween 20 (pH 8.0) and kept overnight at 4°C; a positive control was used on each plate to test for intra assay variation. The next day, plates were washed 5 times with washing buffer and incubated with alkaline phosphatase conjugated goat F(ab')₂ anti-human IgG (γ)-specific conjugate (American Qualex, San Clemente, CA), diluted 1:3000 in incubation buffer, for 1 hour at 37°C. After washing 5 times with washing buffer, 100 μl of freshly made substrate containing 1 mg/ml of nitrophenyl phosphate (Sigma) in diethanolamine buffer at pH 9.8 was added to each well. After 30 minutes on a shaking platform at room temperature, plates were read at 405 nm. Results are expressed as mean anti-oxLDL levels in optical density (OD) from triplicate determinations and were calculated by subtracting binding to native LDL from binding to oxLDL. All results were obtained with native and oxLDL preparations that were stored for less than two weeks.

Statistical analyses

All data are presented as median [range] unless stated otherwise. Kruskal-Wallis test was used for comparing values of healthy controls and ANCA patients, and Dunn's post-test was used for testing differences between MPO-ANCA and PR3-ANCA groups. Receiver operator characteristic (ROC) curves were used to calculate appropriate cut-off values. Analyses were performed with SPSS version 11.0.1 (SPSS Inc., Chicago, IL). A two-sided p -value ≤ 0.05 was considered to indicate statistical significance.

Results

Anti-oxLDL antibody detection in patients with AAV, hemodialysis patients, and healthy controls.

We first confirmed previous findings that antibody levels to oxLDL are increased in AAV patients by using MDA-oxidized LDL as antigen¹¹. Anti-MDA-LDL antibody levels are higher in AAV patients than in healthy controls (0.250 vs. 0.131; $P < 0.001$) and/or HD patients (0.250 vs. 0.164; $P = 0.001$; Figure 1A). Antibody levels in HD patients were not significantly higher than in HC.

Next, we evaluated a second commonly used LDL oxidation procedure, i.e. copper oxidation. This did not reveal a significant difference in anti-Cu-LDL antibody levels between AAV patients (0.035), HD patients (0.021) or HC (0.032; Figure 1B).

Figure 1A. Anti-MDA-LDL reactivity is significantly higher in patients with ANCA-associated vasculitis (AAV) compared to healthy controls (HC) and chronic hemodialysis patients (HD). HD patients are comparable to HC. Lines represent median values.

Figure 1B. Anti-Cu-LDL reactivity is comparable between HC, patients with AAV, and HD patients. Lines represent median values.

Figure 1C. Anti-hypochlorite-LDL reactivity is significantly higher in patients with AAV compared to HD patients and HC. Reactivity is also slightly higher in HD patients compared to HC. Lines represent median values.

Third, we evaluated a new method for oxidation of LDL by using hypochlorite. The difference in anti-hypochlorite-LDL antibody levels between AAV patients (0.598) and healthy controls (0.389) was statistically highly significant ($P < 0.001$; Figure 1C). As for MDA-LDL, antibody levels against hypochlorite-LDL were significantly higher in AAV patients compared to HD patients (0.598, and 0.468, respectively, $P < 0.001$). Interestingly, detection of antibody levels against hypochlorite-LDL also enabled discrimination between HD patients and HC (0.468 and 0.389, respectively, $P = 0.03$).

Figure 2. ROC curves of ANCA patients vs. healthy controls. Assay sensitivity in comparing AAV patients to healthy controls is higher using hypochlorite-modified LDL or MDA-modified LDL than using copper-modified LDL.

Finally, we determined the discriminating performance of the respective assays for AAV patients, HD patients, and HC using ROC curve analysis (Figure 2). The ROC curves of hypochlorite- and MDA-LDL were almost identical and revealed, in contrast to the ROC curve of Cu-LDL, the ability to discriminate between the different study populations. For comparison we analyzed the number of positive samples (sensitivity) from AAV patients and HD patients at a cut-off linked to an arbitrarily chosen specificity of 88.4% (5 HC positive for anti-oxLDL; Table 1). With this given specificity, sensitivity is highest using hypochlorite-LDL (53 of 93 AAV patients positive (57.0%; OR 10.1, 95% CI 3.6 – 27.9, $P<0.001$). Additionally, 21 of 59 HD patients were positive (35.6%; OR

Table 1. Sensitivity of the three anti oxLDL assays at a given specificity

antigen	cut-off OD value	% specificity	% sensitivity
ANCA vs. HC			
MDA-LDL	0.242	88.4	54.8
Cu-LDL	0.108	88.4	18.3
Hypochlorite-LDL	0.559	88.4	57.0

4.2, 95% CI 1.4 – 12.3, $P=0.006$). With MDA-LDL, 51 AAV patients were positive (54.8%; OR 9.2, 95% CI 3.3 – 25.5, $P<0.001$) and 9 HD patients were positive (15.3%; NS). Finally, while using Cu-LDL we found only 17 AAV patients (18.3%) and 9 HD patients (15.3%) positive (Table 1).

AAV patients with MPO -ANCA have higher antibody levels to hypochlorite modified LDL than patients with PR3-ANCA

Antibody levels to the different oxLDL antigens were compared between patients with MPO-ANCA and PR3-ANCA. Anti-MDA-LDL antibody levels were not different in MPO-AAV patients (0.250) in

Figure 3A. Anti-MDA-LDL reactivity is comparable between patients with MPO-ANCA and patients with PR3-ANCA. Lines represent median values.

Figure 3B. Anti-Cu-LDL reactivity in patients with MPO-ANCA is significantly higher compared to patients with PR3-ANCA. Lines represent median values.

Figure 3C. Anti-hypochlorite-LDL reactivity is significantly higher in patients with MPO-ANCA compared to patients with PR3-ANCA. Lines represent median values.

comparison with PR3-AAV patients (0.256; Figure 3A). Using cut-off values as presented in Table 1, twenty-five of 46 PR3-AAV patients (54%) were positive compared to 26 of 47 MPO-AAV patients (55%; NS). Using Cu-LDL as an antigen, a significant difference was found between MPO-AAV patients (0.052) and PR3-AAV patients (0.025; $P=0.03$; Figure 3B). Thirteen of 47 MPO-AAV patients (28%) were positive compared to 4 of 46 PR3-AAV patients (8.7%, OR 4.0, 95% CI 1.2 – 13.5, $P=0.03$). The use of hypochlorite-treated LDL resulted in significantly increased antibody levels in MPO-AAV patients (0.697) versus PR3-AAV patients (0.507; $P<0.001$; Figure 3C). MPO-AAV patients more often had anti-hypochlorite-LDL antibodies (34 of 47, 72%) compared to PR3-AAV patients (19 of 46, 41%; OR 3.7, 95% CI 1.6 – 8.8, $P=0.003$).

In conclusion, modification of LDL with hypochlorite performs best when differentiating between both groups of AAV patients.

Discussion

Our results show that antibody levels to hypochlorite modified LDL are significantly increased when comparing AAV patients and HD patients to healthy controls and that these antibodies are more increased in patients with MPO-AAV than in patients with PR3-AAV. When comparing our new method for oxidation of LDL with previously established methods, we found that hypochlorite-LDL and MDA-LDL are equally sensitive for detection of anti-oxLDL antibodies in AAV patients,

while hypochlorite-LDL, but not MDA-LDL, enabled detection of anti-oxLDL antibodies in HD patients. Cu-LDL lacked sensitivity in both patient populations. The increase in anti-oxLDL antibodies, as detected by hypochlorite-LDL, in MPO-AAV patients in comparison to PR3-AAV patients was also observed by using Cu-LDL. However, the results obtained with Cu-LDL were statistically less significant.

These results are in accordance with our hypothesis that the MPO-catalyzed pathway of LDL oxidation is increased in patients with accelerated atherosclerosis. Several pathways for MPO-catalyzed modification of LDL have been described indicating that both the protein and lipid part are affected, which processes seem to interact. Oxidation of the apoB100 part of LDL activates lipid peroxidation^{20,21} while the latter can lead to protein alterations^{20,22}. In the presence of NO, MPO generates reactive nitrogen species initiating lipid peroxidation^{23,24}, and in the presence of chloride and H₂O₂, MPO catalyses chlorination of unsaturated fatty acids and phospholipids^{25,26} and conversion of LDL-cholesterol into chlorinated sterols¹³. However, protein modification seems to play a more pronounced role. Podrez²⁴ reported tyrosine nitration, while tyrosine chlorination was described by Hazen¹³ in turn causing formation of dityrosyl in the apoB100 part of LDL²⁷⁻²⁹. Cysteine, methionine, lysine and tryptophan are also targets of MPO-catalyzed modification³⁰⁻³². Another effect of tyrosyl radical formation is the promotion of peroxidation of the lipid part of LDL²¹.

MPO-derived epitopes on LDL are at least in part different from those generated by copper- and MDA-treatment; this is reflected in the above study where we showed that the use of hypochlorite-treated LDL to detect autoantibodies results in a more sensitive assay to differentiate between different groups of patients. In atherosclerosis patients, anti-oxLDL antibody levels are commonly tested using copper- or MDA-treated LDL as the antigen of choice. Indeed, in HD patients, antibodies against both antigens are increased^{33,34} and these antibodies are thought to mirror the presence of atherosclerosis³⁴, a well-known complication in these patients^{35,36}. In our study, anti-hypochlorite-LDL levels were increased in HD patients, whereas anti-MDA-LDL and anti-Cu-LDL levels were not, indicating that our hypochlorite-LDL method may be a more sensitive test for atherosclerosis. Further studies are needed to see whether the use of hypochlorite-treated LDL is a useful tool for this purpose.

Previously, we demonstrated enhanced levels of antibodies to MDA-modified LDL in vasculitis patients compared to healthy controls¹¹. In the present study, we confirm these findings in a much larger cohort of patients. Furthermore, we found that patients with MPO-ANCA have higher levels of anti-oxLDL antibodies than patients with PR3-ANCA, when using hypochlorite-modified LDL as an antigen. The presence of MPO-ANCA seems to influence the formation of antibodies to hypochlorite-modified LDL. These anti-MPO antibodies are thought to reverse inactivation of MPO by ceruloplasmin resulting in persistent MPO activity in the circulation which might result in oxidation of LDL in blood vessels^{16,37}. In HD patients, MPO activity increases during dialysis treatment as we have shown previously³⁸; this may have similar consequences as the presence of MPO-ANCA.

In conclusion, the use of hypochlorite modified LDL has advantage over other modifications of LDL when detecting antibodies to oxLDL. Overall, our newly developed assay proved to be a more sensitive assay to measure these antibodies than the traditional assays in which LDL is modified by MDA or copper. By using our new assay, we found enhanced levels of autoantibodies to oxLDL in MPO-AAV patients when compared to PR3-AAV patients, suggesting that MPO-ANCA may interfere with MPO inhibition by ceruloplasmin. Our study also suggests that measuring antibodies to hypochlorite-modified LDL may be a useful addition for testing anti-oxLDL antibodies in patients who are suspected of accelerated atherosclerosis.

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Chapter 10

CD4⁺ CD28^{null} T cells are related to previous cytomegalovirus infection but not to accelerated atherosclerosis in ANCA-associated vasculitis

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Manuscript in preparation

Objective. Previous studies have suggested accelerated atherosclerosis in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV). In this study, we analyzed the number of circulating $CD4^+CD28^{\text{null}}$ T cells in relation to the presence of atherosclerotic damage in patients with AAV. Furthermore, we determined whether antibodies against cytomegalovirus (CMV) and human Heat Shock Protein 60 (hHSP60) were related to the presence of $CD4^+CD28^{\text{null}}$ T cells in AAV.

Methods. Forty patients with inactive AAV were included in this study. Patients' spouses were recruited as healthy controls (HC, $N=38$). Intima-media thickness (IMT) and pulse wave velocity (PWV) were measured. Additionally, $CD4^+CD28^{\text{null}}$ T cells, anti-CMV and anti-hHSP60 levels were determined.

Results. $CD4^+CD28^{\text{null}}$ T cells are present in patients with AAV in a higher percentage (median 3.1, range 0.01 – 85) than in HC (0.28, 0 – 36, $P<0.0001$). There was a strong correlation between a previous CMV infection and the presence and percentage of $CD4^+CD28^{\text{null}}$ T cells (Spearman $r = 0.76$, $P < 0.0001$). No significant difference in IMT between patients and controls was detected (mean $0.77 \pm$ standard deviation 0.15 and 0.73 ± 0.11 , respectively, $P=0.20$). PWV standardized for MAP, however, was increased in AAV patients (9.80 ± 2.50 , compared to 8.72 ± 1.68 in HC, $P=0.04$). We could not detect a direct relationship between $CD4^+CD28^{\text{null}}$ T cells and/or a previous CMV infection and higher IMT or PWV values. There was no relation between anti-hHSP60 and $CD4^+CD28^{\text{null}}$ T cells.

Conclusions. There is evidence of atherosclerotic damage in patients with AAV. Plaque size, however, did not seem to differ. $CD4^+CD28^{\text{null}}$ T cells are relatively increased in AAV and related to previous CMV infection.

Small vessel vasculitides, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with ANCA, which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)¹⁻³. These diseases can occur in any organ system but the respiratory tract and the kidneys are most frequently involved. Untreated, WG results in death within weeks to months. Since the introduction of cyclophosphamide and prednisolone as standard treatment, survival has improved dramatically from less than 20% at 1 year reported in 1958⁴ to at least 60% 5-years survival reported in the past ten years⁵⁻⁸.

With prolonged survival, patients may experience long-term sequelae as a result of their vasculitis or its treatment^{6,7,9}. Several studies have shown that in long-term follow-up, cardiovascular disease is a major cause of mortality in patients with AAV^{5,8,10-16}. Furthermore, the annual incidence of myocardial infarction in patients with AAV is much higher than in the background population¹⁷.

Atherosclerosis is now widely regarded as an inflammatory disease¹⁸, and chronic inflammation seems to be important in the progression of atherosclerosis¹⁹. Several studies demonstrated that signs of atherosclerosis are apparent in patients with ANCA-associated vasculitis (AAV). Endothelial dysfunction, the earliest stage of atherosclerosis¹⁸, is present during active disease in patients with primary systemic vasculitis²⁰. Furthermore, antibodies against oxidized low-density lipoprotein (oxLDL), a marker for progression of atherosclerosis²¹, are also present in active vasculitis²². Antibodies against human Heat Shock Protein 60 (hHSP60) are another marker elevated in both atherosclerosis and vasculitis²³⁻²⁵.

In patients with unstable angina and myocardial infarction, the expansion of an unusual subset of CD4⁺ T cells has been described^{26,27}. Interestingly, these cells have been found in WG as well²⁸. The lack of the CD28 molecule on these cells suggests repeated antigenic exposure, with cytomegalovirus (CMV) and hHSP60 as candidate antigens^{29,30}. CD4⁺CD28^{null} T cells have acquired a cytotoxic phenotype, with expression of perforin and granzyme B, and have been shown to be cytotoxic to endothelial cells. These T cells may have a role in accelerating the atherosclerotic process in patients with WG. In this study, we analyzed the number of circulating CD4⁺CD28^{null} T cells in relation to the presence of atherosclerotic damage in patients with

AAV. Furthermore, we determined whether antibodies against CMV and hHSP60 were related to the presence of CD4⁺CD28^{null} T cells in AAV.

Methods

Subjects

Patients with AAV, who visited the outpatient clinic of our hospital, were included in this study. All patients fulfilled the disease definitions for WG, MPA, and Churg Strauss syndrome as proposed by the Chapel Hill Consensus Conference³¹. Only patients with inactive disease, as evaluated by the Birmingham Vasculitis Activity score (BVAS)³², were included. Patients' spouses were recruited as control subjects. The local medical ethics committee approved of the study. Written informed consent was obtained from each subject.

The traditional risk factors for atherosclerosis assessed were the presence or absence of a family history of premature cardiovascular disease (cardiovascular event before the age of 60 years), smoking status (pack years), the presence of hypertension (as defined by a blood pressure of at least 140 mmHg systolic or 90 mmHg diastolic, or the use of antihypertensive medications, at the study visit), the presence of diabetes mellitus, and cholesterol and triglyceride levels. All subjects were interviewed using a standardized data-collection form. Previous and current medication use was assessed through patient interviews and chart review.

Laboratory analyses

Anti-CD3, anti-CD4 and anti-perforin monoclonal antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ). Anti-CD28 monoclonal antibodies were purchased from Beckman Coulter (Fullerton, CA). The simultaneous presence of CD3, CD4, CD28 and perforin was evaluated on peripheral blood mononuclear cells using 4-color flow cytometry (FACSCalibur, BD) and analyzed using CellQuest software (BD). Additionally, we tested the presence of CD25, HLA-Dr, and killer cell immunoglobulin-like receptors (KIR2DS1 and KIR2DS2) (BD).

Antibodies against hHSP60 were determined as described previously³³. In short, ELISA plates were coated with recombinant hHSP60 (Stressgen, Victoria, Canada) overnight at 4°C. For blocking of nonspecific binding, wells were incubated the next day with coating

buffer and again left overnight at 4°C. The third day, plates were preincubated with preincubation buffer for one hour, washed, incubated with patient serum in 1:50 dilution, and kept overnight at 4°C. The fourth day, plates were incubated with goat F(ab')₂ anti-human IgG (Fc)-horseradish peroxidase conjugate (Cappel-ICN Immunobiologicals, Costa Mesa, CA), for 2 hours at room temperature. Then 100 µL of freshly made substrate (containing o-Phenylenediamine (Sigma)) was added to each well. After 10 minutes, the reaction was stopped with H₂SO₄ and absorbances were read at 490 nm. Results are expressed as anti-hHSP60 levels in arbitrary units (AU) /mL. The cut-off value for this test was previously established at 80 AU/mL.

Anti-CMV antibodies were determined using the axSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL). Results are expressed as anti-CMV levels in AU/mL. The cut-off value for this test is 15 AU/mL.

Measurement of the intima-media thickness

The carotid intima-media thickness (IMT) was determined in the common carotid artery (CCA) segment 10 mm to 20 mm proximal to the tip of the flow divider. B-mode images of the CIMT were acquired for the predefined 10 mm segment of the right and left CCA at defined interrogation angles using Meijers Arc. Standard images were obtained from 4 angles at each side. The mean IMT of the far walls was determined from a single enddiastolic B-mode image at each interrogation angle for both the right and left CCA with an automated edge-tracking method (M'Ath version 2.0.1.0, METRIS, Paris, France). Additionally, the largest IMT value in the predefined segment was noted for both the left and the right CCA.

Measurement of the pulse wave velocity

Pulse pressure waveforms were recorded from the radial and femoral artery using the Complior® SP (Colson, France). Pulse wave velocity was then calculated between the carotid and femoral artery, and the carotid and radial artery. The average of ten measurements was used. As PWV is related to blood pressure, and blood pressure was significantly higher in the HC, we did not only compare PWV between patients and controls, but also PWV corrected for the mean arterial pressure (MAP), standardized at a MAP of 94 (PWV@94), as this was

the mean MAP in AAV patients. Antihypertensive medication was continued in all subjects.

Statistical analyses

All data are presented as mean $\pm$ standard deviation unless stated otherwise. Continuous data between groups were compared using Student's *t* test and Wilcoxon signed rank test, ANOVA and Kruskal-Wallis test with Dunn's post-test when appropriate. Due to the perceived relevance of the results, we did not only perform Dunn's post-test for subgroup analysis, but we also compared subgroups using Wilcoxon signed rank test. Correlations between data were derived using Spearman correlation. Odds ratios (OR) and 95% confidence intervals (CI) were obtained using Fisher's exact test. Analyses were performed with GraphPad Prism version 4.00 and GraphPad InStat software package version 2.04a (both GraphPad Software Inc., San Diego, CA). A two-sided *p*-value <0.05 was considered to indicate statistical significance.

Results

Patient characteristics

Forty patients with AAV and 38 healthy controls were included. Mean age was 56.4 ± 10.5 in patients and 54.4 ± 9.7 in HC. Fifty-five percent of the patients were male compared to 45% of HC. The traditional risk factors for atherosclerosis are presented in table 1. These risk factors for atherosclerosis differed between patients and controls as patients had a higher body mass index, more often had hypertension (as defined by a blood pressure of at least 140 mmHg systolic or 90 mmHg diastolic, or the use of antihypertensive medications, at the study visit), higher triglyceride levels, and a lower blood pressure. Additionally, patients had significantly higher CRP levels than HC (8.0 and 2.2, respectively, $P = 0.0002$) even though only patients in clinical remission had been selected.

Median disease duration was 2.6 years (range, 0.6 to 16.3 years, table 2). Twenty-one patients had had one or more relapses. All but one patient had used prednisolone; 34 patients had used cyclophosphamide and 36 patients had used azathioprine.

Table 1. Clinical characteristics and risk factors in patients and healthy controls*

	<i>Patients (N = 40)</i>	<i>Controls (N = 38)</i>	<i>P-value</i>
Age, years	56.4 ± 10.5	54.4 ± 9.7	0.38
Male	22 (55)	17 (45)	0.50
Risk factors			
Smoking			
<i>ever</i>	24 (60)	18 (47)	0.36
<i>pack years</i>	13.5 ± 18.1	12.0 ± 18.2	0.53
BMI	27.8 ± 5.1	25.7 ± 3.6	0.04
DM	5 (13)	1 (2.6)	0.20
Current hypertension	23 (58)	8 (21)	0.001
<i>systolic</i>	130 ± 18	138 ± 18	0.04
<i>diastolic</i>	76 ± 10	82 ± 10	0.01
<i>MAP</i>	94 ± 11	101 ± 11	0.01
Total cholesterol	5.85 ± 0.97	5.86 ± 0.99	0.96
HDL	1.49 ± 0.33	1.55 ± 0.40	0.48
LDL	3.61 ± 1.01	3.57 ± 0.91	0.85
Triglycerides	2.09 ± 1.23	1.63 ± 0.87	0.06
Family history	10 (25)	14 (37)	0.22
CRP level	8.0 ± 10.4	2.2 ± 2.5	0.0002

* Values are given as mean ± standard deviation or N (%)

BMI, Body Mass Index; DM, diabetes mellitus; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein

Higher percentage of circulating CD4⁺CD28^{null} T cells in ANCA-associated vasculitis

In patients with AAV, the percentage of circulating CD4⁺CD28^{null} T cells was significantly higher (median 3.1, range 0.01 – 85) than in HC (median 0.28, range 0 – 36, table 3). Interestingly, the absolute number of CD4⁺CD28^{null} T cells did not differ between patients and controls (median 9.8, range 0.02 – 377 per mL in patients and 1.5 (0 – 321) in controls). CD8⁺CD28^{null} T cells did not differ between patients and controls, both as percentage and as absolute cell count (table 3).

Table 2. Disease characteristics in ANCA-associated vasculitis*

Characteristic	AAV (N = 40)
Disease duration, years	2.6 (0.6 – 16.3)
ANCA specificity	
PR3	23 (58)
MPO	10 (25)
both	1 (3)
none	6 (15)
Disease subtype	
Wegener’s granulomatosis	25 (63)
MPA	7 (18)
Churg-Strauss syndrome	4 (10)
Renal-limited vasculitis	4 (10)
Renal involvement	18 (45)
Cumulative medication	
Prednisolone (g)	7.6 (0 – 80.2)
Cyclophosphamide (g)	18.0 (0 – 302)
Azathioprine (g)	34.2 (0 – 303)
Number of relapses	1 (0 – 5)
Number of patients with relapses	21 (53)

* Values are given as median (range) or N (%)

AAV, ANCA-associated vasculitis; ANCA, Antineutrophil cytoplasmic antibodies; PR3, proteinase 3; MPO, myeloperoxidase; MPA, microscopic polyangiitis

Additional FACS analysis showed that CD4⁺CD28^{null} T cells were CD25 negative, perforin positive, and to some extent positive for HLA-Dr and KIR2DS1 and KIR2DS2 (figure 1).

CD4⁺CD28^{null} T cells in AAV are related to anti-CMV but not to anti-hHSP60 antibodies

As antigenic specificity of CD4⁺CD28^{null} T cells has been described to be for hHSP60 and CMV, we determined the relationship between antibodies against these antigens and the presence of CD4⁺CD28^{null} T cells. Prevalence of antibodies against hHSP60 was comparable between patients and controls (table 3). The prevalence of CMV

infection was comparable between patients and controls as well (table 3). We could not detect a correlation between antibodies against hHSP60 and the presence or percentage of CD4⁺CD28^{null} T cells. However, there was a strong correlation between a previous CMV infection and the presence and percentage of CD4⁺CD28^{null} T cells (Spearman $r = 0.76$, $P < 0.0001$). Interestingly, AAV patients with a previous CMV infection had a significantly higher percentage of CD4⁺CD28^{null} T cells than HC with a previous CMV infection ($P = 0.006$, figure 2).

Carotid intima-media thickness is not increased in patients with ANCA-associated vasculitis

Both the left and the right common carotid artery were evaluated for the presence of early atherosclerotic damage. Results of the IMT measurements are presented in table 4. Patients with AAV did not have a significantly higher IMT compared to HC (0.77 ± 0.15 and 0.73 ± 0.11 in the right CCA, respectively, $P = 0.20$). As the IMT was not always homogenous in patients with AAV, the maximum IMT value was additionally determined; these also did not differ between patients and

Table 3. Laboratory evaluation

Test	AAV patients (N = 40)	HC (N = 38)	P
Flow cytometry			
% CD4 ⁺ CD28 ^{null} T cells	3.1 (0.01 – 85)	0.3 (0 – 36)	0.003
CD4 ⁺ CD28 ^{null} cells/ μ L	9.8 (0.02 – 377)	1.5 (0 – 321)	0.68
% CD8 ⁺ CD28 ^{null} T cells	28 (0.91 – 92)	21 (4.5 – 71)	0.19
CD8 ⁺ CD28 ^{null} cells/ μ L	36 (1.1 – 539)	63 (14 – 547)	0.08
ELISA			
Anti-hHSP60	19 (0 – 256)	18 (0 – 491)	0.98
Positive (%)	6 (15)	5 (13)	1.0
Anti-CMV	176 (0 – 250)	5 (0 – 250)	0.20
Positive (%)	24 (60)	18 (47)	0.36

AAV, Antineutrophil cytoplasmic antibody-associated vasculitis; HC, healthy controls; ELISA, enzyme-linked immunosorbent assay; anti-hHSP60, antibodies against human Heat Shock Protein 60; anti-CMV, antibodies against cytomegalovirus

Figure 1. FACS analysis of $CD4^{+}CD28^{null}$ T cells. $CD4^{+}CD28^{null}$ T cells lack expression of CD25 (upper left). On the other hand, they are positive for perforin (lower left). Subsets of $CD4^{+}CD28^{null}$ T cells express HLA-Dr (upper right) and CD158b (a marker for killer cell immunoglobulin-like receptors, lower right).

controls (table 4).

Pulse-wave velocity is increased in patients with ANCA-associated vasculitis

To determine vascular elasticity, pulse-wave velocity was measured over the carotid-femoral and carotid-radial arterial segments. Since PWV is related to the blood pressure, and blood pressure was significantly higher in our HC, we standardized the PWV in both groups

Figure 2. CD4⁺CD28^{null} T cells in healthy controls and vasculitis patients. CD4⁺CD28^{null} T cells are relatively more often present in patients with ANCA-associated vasculitis (AAV) who have had a cytomegalovirus (CMV) infection (ACMV+). However, they are also present in healthy controls with a previous CMV infection (HCMV+). In AAV patients and controls without a previous CMV infection, CD4⁺CD28^{null} T cells are scarcely detected (ACMV- and HCMV-, respectively).

at a MAP of 94 and compared both PWV and PWV@94. Femoral PWV was comparable between AAV patients and controls, as was radial PWV (table 4). However, when PWV values were corrected for MAP, femoral

Table 4. Vascular tests in patients and healthy controls*

	Patients	Controls	P-value
IMT			
Right, mean	0.77 ± 0.15	0.73 ± 0.11	0.20
Right, max	1.027 ± 0.20	0.98 ± 0.13	0.22
Left, mean	0.73 ± 0.12	0.72 ± 0.11	0.59
Left, max	0.96 ± 0.18	0.96 ± 0.14	0.99
PWV			
Femoral	9.73 ± 2.76	9.27 ± 2.18	0.44
Femoral@94	9.80 ± 2.50	8.72 ± 1.68	0.04
Radial	8.86 ± 1.40	8.47 ± 1.91	0.32
Radial@94	8.88 ± 1.42	8.07 ± 1.89	0.02

*All values are mean ± standard deviation

IMT, intima media thickness; PWV, pulse-wave velocity; Femoral@94, Femoral PWV standardized at a MAP of 94; Radial@94, Radial PWV standardized at a MAP of 94.

PWV was higher in AAV patients (9.80 ± 2.50 , compared to 8.72 ± 1.68 in HC, $P = 0.04$). Additionally, radial PWV@94 was higher in patients with AAV (8.88 ± 1.42) than in HC (8.07 ± 1.89 , $P = 0.01$).

CD4⁺CD28^{null} cells and previous CMV infection not associated with increase in IMT or PWV

Previously, an increase in IMT was described in patients with rheumatoid arthritis who had an increased percentage of CD4⁺CD28^{null} T cells³³. However, we could not detect a difference in IMT between subjects with >5% CD4⁺CD28^{null} T cells and subjects with <5%. Furthermore, PWV measurements also did not differ between patients with a high or low percentage of CD4⁺CD28^{null} T cells. Additionally, we could not detect a difference between patients and controls with or without a previous CMV infection.

Discussion

In the present study, we determined the presence of early atherosclerotic damage, comparing carotid IMT and femoral and radial PWV between AAV patients and healthy age- and sex-matched volunteers. No difference in IMT between patients and controls was detected. PWV standardized for MAP, however, was increased in AAV patients, suggesting acceleration of atherosclerosis compared to healthy controls. Furthermore, CD4⁺CD28^{null} T cells were found to be present in patients with AAV in a higher percentage compared to controls and were found to be related to a previous CMV infection. No direct relationship, however, between CD4⁺CD28^{null} T cells and IMT and/or PWV was found.

Patients with AAV who survive the acute phase of their vasculitis often die of cardiovascular disease in long-term follow-up^{5,8,10-16}. In addition, patients with AAV have a higher incidence of myocardial infarction than their background population¹⁷. During active vasculitis endothelial dysfunction can be demonstrated²⁰. Furthermore, Booth et al recently described a higher PWV in patients with AAV during active disease but not during remission³⁴. In our study, we found higher PWV in patients with quiescent disease corrected for MAP. The number of patients with AAV in remission ($N = 16$), as studied by Booth et al³⁴, was relatively small compared to our study group, and as PWV was slightly higher in their patients in remission group compared to their HC, this difference

may not have reached statistical significance due to the sample size.

Recently, de Leeuw et al studied IMT in patients with WG ($N = 29$) and compared findings to matched controls³⁵. With B-mode evaluation, no differences were found in mean IMT levels between patients and controls. When evaluating maximum IMT levels, they did detect higher IMT values in WG patients. In the present study, however, we did not find differences in mean or maximum IMT levels in our larger cohort of AAV patients when compared to healthy controls. The finding that IMT and/or PWV between patients and their spouses did show only minor differences is surprising. Risk factors for cardiovascular disease between the two groups differed largely and patients with AAV did have prolonged periods of inflammation, which are supposed to accelerate the atherosclerotic process. We hypothesize that cytostatic therapy with cyclophosphamide and/or azathioprine may have protected our patients with AAV since these drugs may influence the development of atherosclerotic lesions^{20,34} as has been found in patients with SLE³⁶. On the other hand, increase in body weight, hypertension and diabetes mellitus are well-known side-effects of the use of corticosteroids and also risk factors for developing atherosclerosis. In fact, a recent study by Wei and colleagues showed that taking glucocorticoids by prescription is associated with an increase of cardiovascular events³⁷.

The increased incidence of myocardial infarction in AAV without an increase in IMT and only marginal differences in PWV raises the possibility that it is not plaque size but plaque stability that may be different between AAV patients and healthy subjects. Liuzzo and colleagues reported the presence of CD4⁺CD28^{null} T cells in patients with unstable angina or myocardial infarction. CD4⁺CD28^{null} T cells are resistant to apoptosis and the percentage of these cells remains remarkably stable during follow-up³⁸. Furthermore, these cells are cytotoxic for endothelial cells³⁹ and produce large amounts of interferon γ ⁴⁰, both factors that are relevant for plaque instability. Interestingly, these cells had only invaded the “culprit plaques”, i.e., plaques that have ruptured and seemed to have caused myocardial infarction. Patients with AAV have an increased percentage of circulating CD4⁺CD28^{null} T cells. So, we postulate that although plaque size is comparable between patients and controls, the increase in CD4⁺CD28^{null} T cells in AAV patients may result in an increased propensity for myocardial infarction. It may therefore be interesting to

look at the presence of rupture-prone plaques in patients and controls⁴¹.

The reason why CD4⁺CD28^{null} T cells are relatively increased in AAV is not yet clear. Previous studies suggested that hHSP60 and/or CMV may be candidate antigens that are recognized by these T cells^{29,30}. In our study, we could not detect an association between CD4⁺CD28^{null} T cells and the presence of anti-hHSP60 antibodies. A previous CMV infection, however, was highly associated with the presence of CD4⁺CD28^{null} T cells. The percentage of CD4⁺CD28^{null} T cells was even higher in AAV patients with a previous CMV infection compared to the percentage of these cells in healthy controls with a previous CMV infection. This may be due to (subclinical) CMV reactivation in AAV patients with a previous CMV infection, caused by the immunosuppressive therapy that is needed to control active vasculitis. The lack of the CD28 molecule on CD4⁺CD28^{null} T cells suggests repeated antigenic exposure³⁰, supporting this hypothesis. Interestingly, CMV infection has been associated with the presence of atherosclerosis as well⁴². In mice, CMV infection increases atherosclerosis⁴³. Perhaps CMV is causing plaque instability in AAV, acting both directly and through the emergence of CD4⁺CD28^{null} T cells. We are currently performing a long-term follow-up study in these patients to evaluate whether patients with increased CD4⁺CD28^{null} T cells indeed have more cardiovascular events than patients without these cells.

In conclusion, we found only minor evidence of accelerated atherosclerosis in patients with ANCA-associated vasculitis when studying IMT and PWV. CD4⁺CD28^{null} T cells, however, are relatively increased in AAV and found to be related to previous CMV infection. The occurrence of these cells may be relevant for plaque instability and responsible for the observed increased incidence of myocardial infarction in these patients.

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Chapter 11

Atherosclerosis and ANCA-associated vasculitis – a review

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Manuscript in preparation

Atherosclerosis and vasculitis associated with antineutrophil cytoplasmic antibodies (ANCA) are both considered to be inflammatory diseases of the blood vessels. Whereas in atherosclerosis, primarily the large- and medium-sized vessels are involved¹, ANCA-associated vasculitis (AAV) is a disease of the smaller blood vessels², including arterioles, capillaries and venules.

Atherosclerosis is the most common vascular disease of the western world. Atherosclerotic lesions may be present throughout a person's lifetime; indeed, the earliest lesion in atherosclerosis, the so-called fatty streak, may often be found in children. The progression of the fatty streak to an advanced, complicated lesion mainly occurs later in life; eventually leading to complications of atherosclerosis including myocardial and cerebral infarction.

The spectrum of AAV includes Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) and idiopathic necrotizing and crescentic glomerulonephritis (iNCGN). These vasculitides are rare diseases; their prevalence has been estimated at 144.5/million (0.01%)³. AAV, like atherosclerosis, typically becomes manifest later in life, with a median age at diagnosis between 50 and 65³⁻⁵. Although vasculitis can lead to injury to the vascular wall and ischemic damage to organs supplied by these vessels, cerebral or myocardial infarction due to active vasculitis is only rarely observed.

While it seems that AAV and atherosclerosis are different disease processes evolving in different types of blood vessels, several recent discoveries have led to believe that patients with AAV do have an increased risk of developing atherosclerotic complications, and that development of atherosclerosis may be accelerated in patients with AAV. This review will focus on the role of immunity in atherosclerosis and whether there are immunological mechanisms in AAV that may lead to accelerated atherosclerosis in patients with AAV.

ANCA-associated vasculitis

Small vessel vasculitides, such as Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), are strongly associated with ANCA, which are either directed to myeloperoxidase (MPO) or proteinase 3 (PR3)^{2,6,7}. These diseases can occur in any organ system but the respiratory tract and the kidneys are most frequently involved. Untreated, WG results in death within weeks to months. Since the

introduction of cyclophosphamide and prednisolone as standard treatment, survival has improved dramatically from less than 20% at 1 year reported in 1958⁸ to at least 60% 5-years survival reported in the past ten years⁹⁻¹².

With prolonged survival, patients may experience long-term sequelae as a result of their vasculitis or its treatment^{10,11,13}. Several studies have shown that in long-term follow-up, cardiovascular disease is a major cause of mortality in patients with AAV^{9,12,14-20}. Furthermore, the annual incidence of myocardial infarction in patients with AAV is markedly increased compared to the background population²¹.

Atherosclerosis

Generally, atherosclerosis is believed to be a response to injury, with endothelial dysfunction as the first step in the disease process²². Endothelial dysfunction increases both the adhesiveness of the endothelium and its permeability. Increased permeability of the endothelium may lead to trapping of lipoproteins, including low-density lipoprotein (LDL). LDL can be oxidized upon entrapment in the vessel wall and subsequently taken up by macrophages. The accumulation of oxidized LDL in macrophages leads to the formation of foam cells. The presence of foam cells is characteristic for the second step in atherosclerosis, the fatty streak²². As fatty streaks progress to intermediate and advanced lesions, they form a fibrous cap to separate the lesion from the lumen of the blood vessel. Inside, a necrotic core may be formed, consisting of leukocytes, lipid and debris. Ultimately, the fibrous cap becomes thin which results in rupture of the cap or ulceration of the plaque. This is the final stage of atherosclerosis resulting in complications like myocardial or cerebral infarction.

Inflammation seems to be an important part of the development and progression of atherosclerosis²². Although the nature of the inflammatory response in the atherosclerotic lesion is not yet clear, immunological mechanisms seem to play an essential role. The immune response in atherosclerosis may be the result of antigenic stimulation. Several antigens have been implicated in the pathogenesis of atherosclerosis; in particular human cytomegalovirus (CMV), *Chlamydia pneumoniae*, oxidized LDL and human Heat Shock Protein 60 (hHSP60). We will next discuss evidence that has arisen from immunological studies involving these antigens in the pathogenesis of

atherosclerosis.

T lymphocytes

T lymphocytes are present in all stages of development of the atherosclerotic plaque²². Indeed, the earliest lesions of atherosclerosis consist only of monocyte-derived macrophages and T cells²³. De Boer and colleagues showed that these T cells express costimulatory molecules indicative of recent local contact with a specific antigen²⁴. Several groups have tried to determine the antigenic specificity of T cells in the atheromatous lesion. Stemme et al showed that a subset of these T cells respond to oxidized LDL²⁵. Additionally, Mosorin and colleagues identified *Chlamydia pneumoniae* as a specific microbial antigen recognized by in vivo activated plaque T cells²⁶.

A role for T cells in the development may thus be suspected; evidence for this suspicion comes from animal models in which atherosclerosis-prone mice (apolipoprotein E-deficient or LDL-receptor-deficient mice) are crossed into mice with severe combined immunodeficiency, generating hypercholesterolemic mice lacking both T and B cells. When these mice are analyzed for the presence of atherosclerosis, it was shown that under certain circumstances, the effect of immune deficiency results in a decrease in the extent of lesions formed²⁷⁻²⁹. Additionally, when CD4⁺ T cells were transferred into these mice, they developed atherosclerosis similar to that of immunocompetent controls³⁰.

Of particular interest is a subgroup of CD4⁺ T cells, i.e., CD4⁺CD28^{null} T cells. These T cells are clonally expanded³¹, autoreactive³¹, and have acquired a cytotoxic phenotype³². Originally described in severe rheumatoid arthritis (RA) and rheumatoid vasculitis³³, this unusual subset of T cells has also been reported in patients with unstable angina (UA)³⁴. Liuzzo et al showed that unstable plaques are invaded by clonally expanded CD4⁺CD28^{null} T cells but stable plaques are not³⁵. These cells may contribute directly to plaque instability by stimulating tissue-infiltrating macrophages through production of interferon- γ . Additionally, CD4⁺CD28^{null} T cells may mediate plaque instability through lysis of local endothelial cells³⁶. Two recent studies have gained some insight in the triggers of activation and expansion of CD4⁺CD28^{null} T cells. Zal et al reported that hHSP60 is an antigen recognized by CD4⁺CD28^{null} T cells of patients with an acute coronary

syndrome³⁷. In renal transplant patients, however, CD4⁺CD28^{null} T cells are to a large extent CMV-specific³⁸. Interestingly, CMV is one of the antigens that has also been repeatedly associated with atherosclerosis³⁹⁻⁴². Nevertheless, since the patients in this investigation were renal transplant recipients and not studied for the presence of atherosclerosis³⁸, no correlation between these cells and atherosclerotic complications could be made.

Auto-antibodies

Although B lymphocytes are not found in atherosclerotic plaques^{24,43}, or only in small percentages⁴⁴, they do seem to be involved in the development of atherosclerosis. In two studies, the protective effect of B cells has been demonstrated^{45,46}. In both studies, atherosclerosis-prone mice were made deficient in B cells, resulting in increased atheromatous plaque formation in these mice. These findings were suggested to be related to a deficiency in antibody production. Indeed, the disease-aggravating effect was associated with a significant reduction of antibody titers against malondialdehyde (MDA)-modified LDL⁴⁶. In humans, the presence of antibodies against oxidized LDL or MDA-LDL has repeatedly been associated with the presence and severity of atherosclerosis^{47,48}, although the role of these antibodies is still largely unknown. Recent data suggest that immunoglobulin M (IgM) against oxidized LDL may be protective against atherosclerosis, as high anti-oxidized LDL antibody levels were negatively related to intima-media thickness, a marker for the presence of atherosclerosis⁴⁹. A protective role for antibodies is also suggested by immunization studies with oxidized LDL. Immunization with oxidized LDL has been found to inhibit the progression of atherosclerosis in several animal models⁵⁰⁻⁵³. The protective effect of these antibodies may result from the ability to block the binding and degradation of oxidized LDL by macrophages, although this has only been demonstrated in vitro⁵⁴.

Antibodies against other antigens have also been determined in patients with atherosclerosis, to demonstrate involvement of these antigens and / or the antibodies against them in the development of atherosclerosis. Much research has been devoted to the heat shock proteins (HSP), either from bacterial or human origin. Xu and colleagues reported an association between antibodies against mycobacterial HSP65 and the presence of carotid atherosclerosis⁵⁵.

They found that sustained existence of these antibodies in patients with severe atherosclerosis was predictive for mortality in these patients⁵⁶. These anti-HSP65 antibodies were found to cross-react with its human homologue, hHSP60⁵⁷. HSP60 is present on the surface of stressed endothelial cells, including cells in atherosclerotic lesions⁵⁸. Schett et al reported that reaction of anti-HSP65 antibodies with hHSP60 on stressed endothelial cells may result in endothelial cytotoxicity⁵⁹. These findings suggest an important role for antibodies against hHSP60 in the pathogenesis of atherosclerosis. The association between antibodies against hHSP60 and coronary atherosclerosis has been demonstrated by some^{60,61}, but has been disputed by others⁶².

Interestingly, increased serum levels of HSP70 are associated with low risk of coronary artery disease (present on coronary angiography), possibly through its multiple protective effects on a cell's response to stress⁶³. Furthermore, patients with high serum levels of HSP70 had a lesser increase in IMT over a period of 4 years⁶⁴. In both studies, antibodies against HSP70 were not related to the presence or development of atherosclerosis^{63,64}.

Atherosclerosis in ANCA-associated vasculitis

Cardiovascular risk factors

Prevalence of cardiovascular risk factors has been studied in patients with AAV²¹. Diabetes and hypertension were much more common in patients than in the background population; however, the incidence of smoking was lower in patients with AAV. Obesity and hyperlipidemia did not differ between patients and background population.

Indirect measurements of atherosclerosis

Several indirect markers for atherosclerosis have been studied in patients with AAV. Raza et al assessed endothelial function in patients with active AAV by measuring flow-mediated, endothelium-dependent brachial artery vasodilatation (EDV)⁶⁵. They found that EDV was significantly impaired in patients with active vasculitis compared to healthy controls. Interestingly, after remission induction, EDV was comparable between patients and healthy controls⁶⁵.

A second indicator for atherosclerosis, intima-media thickness of the carotid artery (figure 1), has also been described in WG^{66,67}. In both studies, it was found that patients had an increased IMT in comparison

Figure 1. Intima-media thickness (IMT, arrow) in a vasculitis patient.

to age- and sex-matched healthy controls. De Leeuw and colleagues hypothesized that IMT increase may be very focal in WG, and therefore studied both mean and maximum IMT value in the carotid artery. They found a difference in maximum IMT, but not mean IMT, between patients and controls⁶⁷. In contrast, Zaenker et al found a difference in mean IMT between patients and controls; furthermore, atheromatous plaques were seen more frequently in WG⁶⁶. Traditional cardiovascular risk factors were comparable in the groups studied^{66,67}.

Recently, Booth et al described a third marker in patients with AAV, pulse wave velocity (PWV) and its augmentation index (AIx)⁶⁸. They found that AAV is associated with increased arterial stiffness, and that stiffness correlates with the degree of active inflammation. In this study, patients in remission also were comparable to healthy controls. These data support the hypothesis that during active disease, AAV patients experience acceleration of the atherosclerotic process in comparison with controls matched for age, sex and cardiovascular risk factors. However, when inflammation is controlled in these patients, endothelial function and arterial stiffness return to values comparable to healthy subjects. Of course, damage that has occurred in the blood vessels may still be present possibly resulting in higher IMT values in patients with AAV. Furthermore, with every disease reactivation, a state of endothelial dysfunction and arterial stiffness may again be reached resulting in a faster increase in IMT compared to healthy age-matched persons.

T cells

Patients with vasculitis often have a disturbed peripheral blood T cell compartment⁶⁹. Several studies found increased serum markers of T cell activation^{70,71}, and percentages of activated CD4⁺ and CD8⁺ T cells in peripheral blood were higher in patients with WG than in healthy subjects, both during active disease and in remission^{72,73}. Circulating T cells have been further characterized by Moosig and colleagues⁷⁴. They found a lack of expression of CD28 on T cells in patients with WG, but the significance of this finding is not yet clear. We recently discovered a strong association between previous CMV infection and circulating CD4⁺CD28^{null} T cells in patients with AAV⁷⁵ (figure 2). Whether these CD4⁺CD28^{null} T cells, like those in myocardial infarction, are causing plaque instability in AAV is yet unknown.

Auto-antibodies

Antibodies, associated with atherosclerosis have also been studied in AAV. We have previously described the presence of anti-MDA-LDL in patients with active vasculitis, decreasing during immunosuppressive treatment⁷⁶. Recently, we also observed higher levels of antibodies against hypochlorite-modified LDL in patients with AAV⁷⁷. These antibodies may arise due to the disease process, especially in patients

Figure 2. CD4⁺CD28^{null} T cells in healthy controls and vasculitis patients. CD4⁺CD28^{null} T cells are relatively more often present in patients with ANCA-associated vasculitis (AAV) who have had a cytomegalovirus (CMV) infection (ACMV+). However, they are also present in healthy controls with a previous CMV infection (HCMV+). In AAV patients and controls without a previous CMV infection, CD4⁺CD28^{null} T cells are scarcely detected (ACMV- and HCMV-, respectively).

with MPO-ANCA. In MPO-AAV, the presence of MPO-ANCA may hinder the binding of MPO to its natural inhibitor, ceruloplasmin⁷⁸. Current evidence suggests that MPO has an important role in the oxidative conversion of LDL⁷⁹. Patients with MPO-ANCA may have prolonged oxidation of LDL resulting in higher levels of antibodies against hypochlorite-modified LDL. However, this oxidative process may be a result of the disease process per se, leading to a different mode of oxidation of LDL compared to the oxidation of LDL in atherosclerosis. Thus, patients with MPO-AAV may have antibodies against oxLDL regardless of the presence or absence of atherosclerosis.

Recently, we described the presence of another autoantibody associated with atherosclerosis in patients with AAV⁸⁰. Anti-hHSP60 levels were elevated in patients with MPO-ANCA compared to patients with PR3-ANCA and healthy controls. Whether this is due to chronic inflammation in patients with MPO-ANCA, or cross-reactivity between myeloperoxidase and anti-hHSP60, is yet unknown.

Accelerated atherosclerosis in SLE and rheumatoid arthritis

In recent years, several studies have described the presence of accelerated atherosclerosis in patients with rheumatoid arthritis or systemic lupus erythematosus (SLE). In patients with SLE, the incidence of myocardial infarction seems to be at least 5 times as high as in the general population⁸¹. Svenungsson et al compared carotid IMT in SLE patients with a history of cardiovascular events with SLE patients without such a history. They found a significant increase in the former patient group⁸². On the other hand, IMT values were comparable between the latter group without cardiovascular events, and the healthy control group studied. Asanuma and colleagues studied coronary-artery atherosclerosis using electron-beam computed tomography and found that the prevalence of coronary-artery atherosclerosis is increased in patients with SLE, and the age of onset is reduced. Furthermore, a third study showed that patients with SLE have lower endothelium dependent vasodilatation than sex and age-matched controls, even in patients without traditional cardiovascular risk factors⁸³. The presence of CD4⁺CD28^{null} T cells has been demonstrated in patients with SLE⁸⁴.

Over the past several decades, patients with RA were found to have an increased mortality^{85,86}, mostly due to cardiovascular disease⁸⁷. In

patients with RA, PWV⁸⁸ and carotid IMT^{89,90} were found to be elevated, and EDV was found to be decreased^{89,91} indicating accelerated atherosclerosis in these patients. Furthermore, circulating CD4⁺CD28^{null} T cells are increased in patients with RA³¹ and have been shown to be associated with increased IMT values in these patients⁸⁹.

Conclusions and recommendations for future studies

Recent evidence indicates that several markers of atherosclerosis are more pronounced in patients with ANCA-associated vasculitis (AAV) compared to healthy controls. Endothelial dysfunction, the earliest stage of atherosclerosis, is present during active disease in patients with primary systemic vasculitis. Furthermore, antibodies against oxidized LDL and human HSP60, markers for progression of atherosclerosis, are also elevated during active vasculitis. Subtle increases of the intima-media thickness may be present in patients with AAV, possibly as a result of periods of active disease.

We believe that these findings call for a search for treatment of accelerated atherosclerosis in patients with AAV. Angiotensin converting enzyme (ACE) inhibitors, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) and aspirin are widely used agents in patients with coronary artery disease. ACE inhibitors traditionally elicit their effects by inhibiting conversion of angiotensin I to angiotensin II⁹². Angiotensin II may directly enhance synthesis and release of proinflammatory cytokines, for instance, interleukin-6⁹³, and ACE inhibition can blunt this⁹⁴. Because interleukin-6 mediates B cell maturation, complement activation, cytokine release, and release of C-reactive protein⁹⁴, ACE inhibition may act immunomodulatory and anti-inflammatory. In addition, ACE inhibitors decrease the breakdown of bradykinin. Bradykinin causes vasodilatation, enhanced endogenous fibrinolysis, and improved endothelial function⁹⁵. Thus, ACE inhibition may improve endothelial function in patients with AAV in addition to immunomodulatory and anti-inflammatory effects.

Statins are the most powerful class of lipid-lowering drugs available. Interestingly, additional actions of statins unrelated to cholesterol reduction have been identified, including anti-inflammatory and immunomodulatory properties. For instance, statins act as direct inhibitors of induction of major histocompatibility complex (MHC) class II expression and thus suppress T lymphocyte activation. Furthermore,

statins prevent downregulation of nitric oxide synthase induced by oxidized lipoproteins⁹⁶, improving endothelial function and reducing oxidative stress, two factors important for the development of atherosclerosis and present in AAV.

The use of acetylsalicylic acid, or aspirin, results in decreased production of prostaglandins and thromboxane A₂, likely accounting for the antiaggregatory and anti-inflammatory effects of aspirin. Aspirin may help to decrease the progression of atherosclerosis by protecting LDL from oxidative modification and also by improving endothelial function in atherosclerotic vessels⁹⁷.

Treatment of existing risk factors for atherosclerosis seems logical, but additional treatment may be necessary. Raza and colleagues suggested that suppression of inflammation may be the most important factor in patients with AAV, as endothelial function restores to values found in normal subjects after immunosuppressive treatment⁶⁵. Additionally, immunosuppressive treatment results in lowering of anti-oxLDL antibody levels⁷⁶. Indeed, in systemic lupus erythematosus, patients with atherosclerotic lesions in the carotid artery were less likely to have been treated with cyclophosphamide or prednisone⁹⁸. However, increase in body weight, hypertension and diabetes mellitus are well-known side-effects of the use of corticosteroids and also risk factors for developing atherosclerosis. Cyclophosphamide use can have serious side effects, both in short- and long-term follow-up. Other medications, for instance biologicals may be an alternative treatment option. Booth et al reported that treatment with infliximab in patients with AAV may result in improved endothelium-dependent vasomotor responses⁹⁹. Additionally, therapy with infliximab may decrease the CD4⁺CD28^{null} T cell compartment in AAV, as was described in patients with rheumatoid arthritis¹⁰⁰.

In conclusion, there is increasing evidence that immunological mechanisms in atherosclerosis are also present in patients with AAV. These mechanisms could be responsible for the observed acceleration of atherosclerosis in AAV. Clinicians should be alerted to the increased risk of cardiovascular disease in patients with AAV and reduce the presence of inflammation and traditional risk factors for atherosclerosis in these patients as much as possible.

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Chapter 12

Summary and general discussion

Vasculitis associated with antineutrophil cytoplasmic antibodies (ANCA), including Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS), predominantly affects small- and medium-sized blood vessels. These ANCA-related syndromes form a distinct subset with overlapping features within the spectrum of primary vasculitic diseases; the respiratory tract and kidney are organ systems frequently involved.

The ANCA-associated vasculitides can be distinguished in two ways. First, a distinction based on diagnosis; subcategories then include WG, MPA, CSS, and idiopathic necrotizing and crescentic glomerulonephritis (iNCGN). A drawback of this method is that a disease pattern first being diagnosed as MPA can evolve into WG several years after diagnosis. A second drawback is that a diagnosis of WG can almost never be excluded, as granulomas must be present in a biopsy for this diagnosis to be made, and they may not be present in a particular biopsy, even though the patient has WG.

A second distinction is based on ANCA specificities. Proteinase 3 (PR3) and myeloperoxidase (MPO) are the major autoantigens in ANCA-associated vasculitis (AAV), although other ANCA specificities occasionally are seen. However, patients with a biopsy-proven "ANCA-related" vasculitic syndrome may very well be ANCA negative, especially when the vasculitis is limited or locoregional (for instance, patients with vasculitis of the upper respiratory tract with granulomatous inflammation in a nasal biopsy). Especially patients with CSS are often ANCA-negative (up to 70% of the cases).

As an introduction to the diseases studied in the subsequent chapters, *Chapter 1* gives a general overview of the ANCA-associated vasculitides and the current role of ANCA in the diagnosis and prognosis of patients with this disease.

Several drugs have been associated with the onset of AAV, including antirheumatic and antithyroid drugs. In *Chapter 2* we evaluate the potential of the antirheumatic drugs minocycline, sulfasalazine and penicillamine to induce ANCA. We tested 248 patients who had participated in clinical trials, providing us with pre- and post-exposure blood samples. From our study results, there was no suggestion of ANCA seroconversion due to these drugs.

Chapter 3 describes a related study in which we investigated the presence of ANCA after the use of antithyroid drugs. We hypothesized that ANCA and AAV may not only be induced during treatment with antithyroid drugs, but can also become manifest when medication has been ceased, possibly after years. To test this hypothesis, we evaluated patients with hyperthyroidism, on various antithyroid treatment modalities, for the presence of ANCA. We found that of 209 patients with hyperthyroidism, 12 patients (6%) were positive for myeloperoxidase- (MPO-), proteinase 3- (PR3-) and/or human leukocyte elastase- (HLE-) ANCA, and that the presence of ANCA was highly associated with (previous) treatment with antithyroid drugs.

Human Heat Shock Protein 60 (hHSP60) has been implicated in autoimmunity through molecular mimicry, based on the high degree of homology with HSP65 of micro-organisms leading to autoimmune recognition of the human protein. Additionally, sequence homology between hHSP60 and myeloperoxidase (MPO) has been described. MPO is a major autoantigen in vasculitis associated with antineutrophil cytoplasmic antibodies (ANCA). In *Chapter 4*, we hypothesized that infections may trigger the ANCA response against MPO through hHSP60.

In 86 consecutive patients with ANCA-associated vasculitis (AAV), anti-hHSP60 and anti-mycobacterial HSP65 were measured by ELISA. Patients were compared with 69 healthy controls (HC). We found that antibodies against mHSP65 are higher in AAV compared to HC, and anti-hHSP60 antibodies are higher in patients with MPO-ANCA than in patients with PR3-ANCA and HC. Although this finding may be indicative for cross-reactivity between MPO-ANCA and hHSP60, additional assays did not support our hypothesis.

In *Chapter 5* we investigated whether genetic factors, regulating T cell activation, are involved in the onset of AAV. T cell activation is regulated by inhibitory molecules such as PD-1 and CTLA-4, whose expression in turn may be affected by gene polymorphisms. We investigated the relationship between polymorphisms in *PDCD1* and *CTLA4* and the presence of AAV, and found that AAV is associated with the presence of a G allele at the +49 position of *CTLA4*; this association is even stronger if also the PD-1.5 T allele is absent in the genome.

This led us to hypothesize that for autoimmune disease, a mutation in more than one inhibitory receptor predisposes to the development of disease, for instance, AAV. Furthermore, disease subsets are associated with different genetic polymorphisms in *CTLA4*.

Chapter 6 presents a long-term follow-up study in patients with PR3-ANCA associated vasculitis with renal involvement. Eighty-five patients that were diagnosed between 1982 and 1996 were followed for at least 5 years, to investigate prognostic factors for patient and renal survival. We discovered that early death and failure to recover renal function in PR3-ANCA associated vasculitis is associated with age above 65 years and dialysis dependency at diagnosis. Furthermore, long-term renal survival is determined by renal relapses during follow-up only. Slow, progressive renal failure without relapses, not infrequently found in patients with MPO-ANCA associated vasculitis, is rarely observed in patients with PR3-ANCA associated vasculitis with glomerulonephritis.

In patients with PR3-ANCA, renal function is stable during remission, but declines with every renal relapse. In patients with MPO-ANCA a slowly progressive course with patients developing dialysis dependency during follow-up without signs of clinically active disease is often observed. In *Chapter 7* we studied the clinical and histological differences at diagnosis between patients with PR3-ANCA and patients with MPO-ANCA, and we investigated whether these differences explain the distinctive outcomes in AAV. Eighty patients (46 with MPO- and 34 with PR3-ANCA) were included. Renal lesions differed between patients with PR3- and MPO-ANCA, being more acute in PR3-ANCA, and more chronic in MPO-ANCA. However, despite the overt differences in renal biopsies, ANCA specificity was not a risk factor for patient death or the development of renal failure during long-term follow-up. Risk factors for death were female gender, age 65 years or higher at diagnosis and serum creatinine level at biopsy. The development of renal failure during follow-up was associated with female gender, a lower percentage of normal glomeruli, and the chronicity score in renal biopsy.

Next to patient and renal survival, we investigated factors that are associated with disease-free survival. In *Chapter 8*, we analyzed

disease-free survival in patients with AAV treated with cyclophosphamide only or switched to azathioprine after 3 months of full remission with cyclophosphamide. Included were 128 patients of whom 44 (34%) switched to azathioprine. In patients with PR3-ANCA associated vasculitis switched to azathioprine (n=33) a positive C-ANCA titer at the moment of treatment switch (n=13) was significantly associated with the occurrence of relapse. In patients with a negative ANCA titer at the time of switch to azathioprine (n=20), disease-free survival at 2 and 4 years was 80% and 62%, which was identical to patients treated with cyclophosphamide only. In patients who were ANCA positive at the time of treatment switch disease-free survival at 2 and 4 years was only 58% and 17%. Thus, switching cyclophosphamide to azathioprine after induction of remission in patients with PR3-ANCA associated vasculitis who are still ANCA positive at the time of treatment switch is associated with a high risk of developing relapses.

A serological marker for the extent and progression of atherosclerosis, anti-oxidized low-density lipoprotein (oxLDL), is studied in AAV in *Chapter 9*. Three methods for detection of anti-oxLDL are compared between patients with MPO-ANCA and patients with PR3-ANCA. We measured antibody levels to LDL after treatment with hypochlorite, MDA or copper in sera of ANCA-positive vasculitis patients, and compared them to antibody levels in sera of hemodialysis patients and healthy controls. We found that anti-oxLDL antibodies are enhanced in AAV patients (MDA-LDL and hypochlorite-LDL) and in dialysis patients (hypochlorite-LDL), when compared to controls. Furthermore, patients with MPO-ANCA associated vasculitis had higher levels of antibodies to hypochlorite-LDL than patients with PR3-ANCA associated vasculitis. We concluded that our newly developed assay to measure antibodies to ox-LDL, in which hypochlorite-LDL is used as substrate, seems a more sensitive assay than traditional assays to measure these antibodies. Furthermore, our results suggest that enhanced MPO-mediated LDL oxidation in patients with MPO-ANCA reflects the idea that circulating MPO-ANCA prevents inactivation of circulating MPO activity by ceruloplasmin in these patients.

In *Chapter 10*, we analyzed the number of circulating CD4⁺CD28⁻ T

cells in relation to the presence of atherosclerotic damage in patients with AAV. Furthermore, we determined whether antibodies against cytomegalovirus (CMV) and human Heat Shock Protein 60 (hHSP60) were related to the presence of $CD4^+CD28^{\text{null}}$ T cells in AAV. Forty patients with inactive AAV were included in this study. Patients' spouses were recruited as healthy controls (HC, $n=38$). Intima-media thickness (IMT) and pulse wave velocity (PWV) were measured. Our results showed that there is evidence of atherosclerotic damage in patients with AAV, as measured by PWV, although carotid plaque size did not seem to differ. $CD4^+CD28^{\text{null}}$ T cells are relatively increased in AAV and related to previous CMV infection. However, there was no correlation between $CD4^+CD28^{\text{null}}$ T cells and increased IMT or PWV.

Finally, in *Chapter 11*, we summarize the current perceptions on involvement of the immune system in atherosclerosis, with particular attention to possible immunological causes of accelerated atherosclerosis in AAV.

General conclusions

The work described in this thesis is focused on ANCA-associated vasculitis. Its subjects are factors involved in disease onset (*Chapters 2, 3, 4, 5*), factors involved in patient and renal survival (*Chapters 6, 7*) and disease-free survival (*Chapter 8*), and the presence and development of atherosclerosis at diagnosis and during follow-up of AAV (*Chapters 9, 10*). Although we are still a long way from curing this disease, some issues have become clear.

First of all, if the factors involved in disease onset are known, therapies or strategies may evolve that might be able to prevent vasculitis, or cure this disease at an early stage. We studied several drugs that have been associated with the induction of ANCA, and possibly of AAV. A relationship between the use of antithyroid drugs and the presence of AAV was established (*Chapter 3*); on the other hand, a connection between certain antirheumatic drugs and vasculitis is still questionable (*Chapter 2*).

Although medication use has sometimes been associated with vasculitis, infections (and especially infection with *Staphylococcus aureus*) have often been linked to vasculitis. Antibodies against bacterial and human Heat Shock Proteins (HSPs) are elevated in AAV,

especially in MPO-AAV (*Chapter 4*). Nevertheless, we could not establish a correlation between the presence of antibodies against HSPs and the presence of ANCA.

Next to the possible induction of ANCA by infection or medication use, we studied a possible cause for the involvement of T cells in the onset of AAV. We found that certain gene polymorphisms that lead to a reduced expression or function of the T cell inhibitory molecule CTLA-4, in combination with a gene polymorphism in a second inhibitory molecule (PD-1), are associated with the presence of AAV (*Chapter 5*). This may indicate that reduced inhibition of T cells influences the occurrence of AAV.

Second, we investigated whether we could identify factors that are involved in patient, renal and disease-free survival, possibly leading to a more tailored approach of patients with AAV. We observed that age and reduced renal function are primarily associated with abridged short-term patient and renal survival (*Chapter 6, 7*), whereas long-term renal survival is strongly dependent on the occurrence of renal relapses (*Chapter 6*). In our long-term survival analysis, we found that atherosclerosis and malignancy are the most common causes of death in patients with AAV (*Chapter 6*). Among other reasons, the high chance of malignancy with the use of the standard therapy against AAV (cyclophosphamide and prednisolone), has led investigators to propose a less aggressive treatment for remission maintenance, giving patients azathioprine instead of cyclophosphamide. The use of azathioprine, however, may not be as successful as cyclophosphamide in preventing the occurrence of relapses of disease activity during follow-up. In patients that remain positive for ANCA, the use of azathioprine may lead to a strongly reduced disease-free survival (*Chapter 8*).

Third, we investigated the presence of atherosclerosis as a consequence of ANCA-associated vasculitis, at diagnosis and during follow-up. One serological marker of atherosclerosis, antibodies against oxidized LDL, was found to be already present at diagnosis of AAV, particularly in patients with MPO-ANCA (*Chapter 9*). During long-term follow-up, we also found evidence for the presence of accelerated atherosclerosis in patients with AAV (*Chapter 10*). Furthermore, immunological abnormalities were found in patients with AAV that are also common in patients with atherosclerosis (*Chapter 10*).

Future perspectives

Pathophysiology of ANCA-associated vasculitis

In the last years, more insight has been gained regarding the pathophysiological role of ANCA in ANCA-associated vasculitis, leading some investigators to propose the term “ANCA vasculitis” instead¹. These investigators have demonstrated in a mouse model that ANCA alone are sufficient for disease induction¹. The MPO knockout mice they used were immunized with murine MPO, leading to the development of MPO-ANCA in these mice. Injecting these antibodies in mice with MPO led to the development of vasculitis and glomerulonephritis. Of course, the question still remains how a person can suddenly develop an autoimmune response against MPO or, for that matter, PR3. Many theories have been developed, with molecular mimicry between bacterial and human proteins as a favored one; one that has also been studied by us (*Chapter 4*). Recently, a second theory has gained much attention after proving its pathogenicity in a mouse model. Pendergraft and colleagues described an autoimmune response induced by the presence of a peptide that is antisense or complementary to PR3². After immunizing mice with antisense PR3, they found an autoimmune reaction to blood vessels in these mice. The mice had not only developed antibodies against antisense PR3, but also to the sense peptide PR3. Furthermore, antibodies in sera from patients with Wegener’s granulomatosis reacted not only with PR3, but also with antisense human PR3. An explanation for this remarkable finding is that when the sense and antisense molecules of a peptide are complementary to each other, an immune response against the antisense peptide may induce anti-idiotypic antibodies (autoantibodies) that cross-react with the autoantigen. The antisense immune response may, in turn, be a result of molecular mimicry with bacterial proteins – the antisense PR3 protein, for instance, shares sequence homology with a protein of *Staphylococcus aureus*², a pathogen that has been repeatedly linked to WG.

The role of T cells in the pathogenesis of AAV is still much less clear. Obviously, T cells are necessary for the production of IgG ANCA³, and T cells are present in inflammatory lesions in AAV^{4,5}. On the other hand, in the mouse model previously described, infusion of only T cells does not lead to inflammation in mice, in contrast to infusion of only MPO-ANCA. Nevertheless, infusion of both T cells and MPO-ANCA leads to

much more inflammation in the mouse model than infusion of MPO-ANCA alone¹.

Since the T cell response is necessary for the production of ANCA, it is interesting to investigate why a T cell would react with autoantigens. We have investigated one hypothesis for hyperreactivity of T cells; impairment of counterregulation due to polymorphisms in genes coding for the immunoregulatory molecules CTLA-4 and PD-1 (*Chapter 5*). Of course, these molecules are not the only ones involved in the regulation of the immune response. Besides, since these gene polymorphisms are also found in many healthy persons, and various other autoimmune diseases, it seems that more than a gene polymorphism is necessary for the development of AAV. It could be interesting to look whether a combination of factors that are thought to be at least partially responsible for the induction of AAV coincide in patients with AAV. For instance, are patients with immunoregulatory polymorphisms more susceptible to drug-induced vasculitis?

Treatment and follow-up of ANCA-associated vasculitis

Many studies have described potential prognostic factors for patient and renal survival (summarized in *Chapters 6 and 7*). Unfortunately, most of these prognostic factors can not readily be influenced by therapy. An exception to this is renal failure at diagnosis, defined as serum creatinine above 500 $\mu\text{mol/L}$ or dialysis dependency. Renal failure at diagnosis is highly associated with early death and failure to recover renal function (*Chapters 6 and 7*). A recent study has shown that plasma exchange may be beneficial to these patients, drastically improving their survival without need for renal replacement therapy within one year⁶.

Induction therapy with cyclophosphamide and prednisolone is still the golden standard for generalized ANCA-associated vasculitis. Long-term use of cyclophosphamide, however, leads to serious morbidity, even mortality, mostly due to malignancies several years after diagnosis⁷. To decrease the chance on developing these long-term side effects, other drugs have been tested for remission maintenance and remission induction. Azathioprine at first seemed equally able to suppress disease activity⁸; however, during long term observation, we showed that azathioprine use does lead to more relapses of disease activity, especially when patients were C-ANCA positive at the time of treatment

switch from cyclophosphamide to azathioprine (*Chapter 8*). Two trials are currently on their way; one testing the effect of longer use of azathioprine in patients that remain C-ANCA positive; the other testing mycophenolate mofetil (MMF) as treatment for remission maintenance. On the other hand, perhaps induction should be made more aggressive and aiming at an absent ANCA titer when treatment switch is considered. Adding anti-CD20 or anti-TNF α to induction treatment can be interesting in this perspective.

Atherosclerosis and ANCA-associated vasculitis

Since the introduction of cyclophosphamide and prednisolone as standard treatment, AAV has evolved from an almost uniformly fatal disease to a chronic disorder. Long-term follow-up, however, has shown that late consequences of the disease and / or its treatment can result in serious morbidity. Cardiovascular events occur in a substantial percentage of patients with AAV⁹, leading us to investigate the presence of accelerated atherosclerosis in these patients. Although we did not find marked differences between patients and healthy, age-matched controls, subtle distinctions were there. It would be very interesting to know how these differences progress.

Of course, the methods we used to detect atherosclerosis in AAV patients may not have been sensitive enough for us to detect large differences between patients and controls. With a recent method described by Kooi et al¹⁰ it would be possible to specifically look at rupture-prone atherosclerotic plaques, which may more adequately assess the increased risk of cardiovascular events for patients with AAV than intima-media thickness measurements or pulse-wave velocity. Assessment of myocardial perfusion could also be a way to detect atherosclerotic lesions¹¹.

Since the presence of CD4⁺CD28^{null} T cells was clearly related to myocardial infarction in the studies by Liuzzo et al^{12,13}, one point for follow-up could be the occurrence of cardiovascular events, though the number of patients we studied is small. Animal studies could perhaps give us more insight in the role of CD4⁺CD28^{null} T cells in atherosclerosis. Furthermore, the natural course of atherosclerosis in vasculitis could be studied in the mouse model for AAV.

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Samenvatting

Vasculitis geassocieerd met antineutrofiele cytoplasmatische antistoffen (ANCA), zoals Wegener's granulomatose, microscopische polyangiitis (MPA), en het syndroom van Churg-Strauss (CSS), komt met name voor in de kleine en middelgrote bloedvaten. Deze ANCA-gerelateerde aandoeningen vormen een speciale categorie met overlappende symptomen binnen het spectrum van de vasculitiden van de kleine vaten; de luchtwegen en de nieren zijn daarbij vaak aangedaan.

Er kan op 2 manieren onderscheid gemaakt worden binnen de ANCA-geassocieerde vasculitiden. De eerste manier is gebaseerd op de verschillende diagnoses; subcategorieën zijn dan WG, MPA, CSS en idiopathische necrotiserende en crescentische glomerulonefritis (iNCGN). Een nadeel van deze methode is dat een syndroom dat eerst gediagnosticeerd wordt als MPA, jaren na de diagnose kan evolueren naar een WG. Een tweede nadeel is dat de diagnose WG bijna nooit geëxcludeerd kan worden, aangezien de aanwezigheid van granulomen in minimaal een biopt voorwaarde is voor deze diagnose. Deze kunnen echter bij het maken van het biopt gemist worden, waardoor de diagnose WG niet gesteld kan worden zelfs als deze wel de onderliggende oorzaak is.

Een tweede onderscheid tussen de aandoeningen is gebaseerd op ANCA specificiteiten. Proteinase 3 (PR3) en myeloperoxidase (MPO) zijn veruit de belangrijkste autoantigenen in ANCA-geassocieerde vasculitis (AAV), hoewel sporadisch andere specificiteiten gevonden worden. Echter, patiënten met een biopt bewezen "ANCA-gerelateerd" syndroom kunnen ANCA negatief zijn, vooral wanneer de ziekte gelimiteerd of locoregionaal is (bijvoorbeeld patiënten met vasculitis van de bovenste luchtwegen en granulomateuze ontsteking in een neusbiopt). Met name patiënten met CSS zijn vaak ANCA negatief (tot 70% van de patiënten).

Hoofdstuk 1 geeft als introductie tot het ziektebeeld een overzicht van de ANCA-geassocieerde vasculitiden en de huidige rol van ANCA in de diagnose en prognose van patiënten met deze ziekte.

Verscheidene medicamenten zijn geassocieerd met het ontstaan van AAV, onder andere anti-reumatische medicijnen en thyreostatica. In *Hoofdstuk 2* onderzoeken we of de anti-reumatische medicijnen minocycline, sulfasalazine en penicillamine ANCA kunnen induceren. We testten hiervoor 248 patiënten die hadden geparticipeerd in clinical

trials, waardoor wij serum van voor en na de blootstelling tot onze beschikking hadden. Onze resultaten lieten geen aanwijzingen zien voor het ontstaan van ANCA door het gebruik van deze medicijnen.

Hoofdstuk 3 beschrijft een gerelateerde studie waarin wij de aanwezigheid van ANCA na het gebruik van thyreostatica onderzochten. We hypothetiseerden dat ANCA niet alleen kan ontstaan tijdens het gebruik van thyreostatica, maar ook jaren later, wanneer de thyreostatica gestopt zijn. Om deze hypothese te testen onderzochten wij patiënten met hyperthyreoïdie, met verschillende vormen van behandeling, op de aanwezigheid van ANCA. Van de 209 patiënten met hyperthyreoïdie hadden 12 patiënten (6%) MPO-, PR3- of human leukocyte elastase (HLE)-ANCA; de aanwezigheid van ANCA was sterk geassocieerd met thyreostatische behandeling.

Humaan heat shock protein 60 (hHSP60) wordt een rol toegedicht bij het ontstaan van autoimmunitet door molecular mimicry, veroorzaakt door een sterke overeenkomst met HSP65 dat gevonden wordt bij micro-organismen. Daarnaast is overeenkomst tussen hHSP60 en MPO beschreven. MPO is een van de belangrijkste autoantigenen in ANCA-geassocieerde vasculitis. In *Hoofdstuk 4* hypothetiseerden we dat infecties het ontstaan van ANCA gericht tegen MPO kunnen induceren via kruisreactiviteit met hHSP60. In 86 patiënten met AAV werden antistoffen tegen hHSP60 en mycobacterieel HSP65 gemeten met behulp van ELISAs. Deze patiënten werden vergeleken met 69 gezonde controles (HC). We zagen dat antistoffen tegen mHSP65 hoger waren in AAV patiënten in vergelijking met HC, en anti-hHSP60 antistoffen waren hoger in patiënten met MPO-ANCA dan in patiënten met PR3-ANCA en HC. Dit zou kunnen wijzen op kruisreactiviteit, maar in aanvullende onderzoeken bleef bewijs van kruisreactiviteit uit.

In *Hoofdstuk 5* onderzochten we of genetische factoren, van invloed op de T-cel regulatie, betrokken zijn bij het ontstaan van AAV. T-cel activatie wordt gereguleerd door inhibitoire moleculen zoals PD-1 en CTLA-4. De expressie van deze moleculen wordt beïnvloedt door genetische polymorfismen. We onderzochten de relatie tussen polymorfismen in *PDCD1* en *CTLA4* en de aanwezigheid van AAV, en we zagen dat AAV geassocieerd is met de aanwezigheid van het G allel op

de positie +49 van *CTLA4*; deze relatie wordt sterker als ook het PD-1.5 T allel niet aanwezig is in het genoom. Dit resultaat leidde naar onze huidige hypothese; voor het ontstaan autoimmuunziekten zoals AAV is waarschijnlijk meer dan één mutatie nodig in inhibitoire receptoren. Daarnaast zijn verschillende subgroepen binnen AAV geassocieerd met verschillende *CTLA4* polymorfismen.

Hoofdstuk 6 presenteert een lange termijn follow-up studie van patiënten met PR3-ANCA geassocieerde vasculitis met nierbetrokkenheid. Vijfentachtig patiënten, gediagnosticeerd tussen 1982 en 1996 werden minimaal 5 jaar gevolgd om te onderzoeken welke factoren van invloed zijn op overleving van patiënten en nierfunctie. We zagen dat snel overlijden en blijvend verlies van nierfunctie in PR3-ANCA vasculitis geassocieerd is met leeftijd boven 65 jaar en dialyse-afhankelijkheid ten tijde van diagnose. Verder vonden wij dat alleen het optreden van renale recidieven voorspellend is voor overleving van de nierfunctie op lange termijn. Langzame achteruitgang van de nierfunctie zonder activiteit van de vasculitis, zoals regelmatig gezien wordt bij MPO-ANCA vasculitis, komt zelden voor bij PR3-ANCA vasculitis.

In patiënten met PR3-ANCA is de nierfunctie stabiel tijdens remissie, maar gaat achteruit met elk renaal recidief. In patiënten met MPO-ANCA wordt juist vaak gezien dat de nierfunctie versneld achteruit gaat, zonder dat er activiteit van de ziekte te herkennen is. In *Hoofdstuk 7* bestudeerden we welke klinische en histologische verschillen aanwezig zijn tussen patiënten met PR3-ANCA en patiënten met MPO-ANCA, en we onderzochten of deze verschillen de verschillen in outcome kunnen verklaren. Tachtig patiënten (46 met MPO-ANCA en 34 met PR3-ANCA) werden geïnccludeerd in de studie. De afwijkingen in het nierbiopt verschilden tussen patiënten met PR3- en MPO-ANCA; in PR3-ANCA zijn de laesies meer acuut, terwijl in MPO-ANCA meer chronische schade wordt gezien. Desondanks was ANCA specificiteit geen risicofactor voor overlijden van de patiënt of nierfalen tijdens follow-up. De risicofactoren voor overlijden die wij wel zagen waren vrouwelijk geslacht, leeftijd boven 65 jaar, en serum creatinine ten tijde van het biopt. Het ontstaan van nierfalen was geassocieerd met het vrouwelijk geslacht, een lager percentage van normale glomeruli,

en de chroniciteitsscore van het nierbiopt.

Naast patiënten- en renale overleving hebben we ook factoren onderzocht die van invloed kunnen zijn op ziektevrije overleving. In *Hoofdstuk 8* analyseerden we ziektevrije overleving in patiënten met AAV die ofwel met cyclofosfamide werden behandeld, danwel na 3 maanden volledige remissie van cyclofosfamide naar azathioprine overschakelden. Er werden 128 patiënten geïnccludeerd; 44 hiervan (34%) gingen over op azathioprine. In patiënten met PR3-ANCA (n=33), die een positieve ANCA titer hadden op het moment van switch (n=13), was de kans op een recidief significant hoger. In patiënten met een negatieve ANCA titer ten tijde van switch (n=20) was de ziektevrije overleving op 2 en 4 jaar respectievelijk 80% en 62%; dit was gelijk aan de ziektevrije overleving van patiënten die cyclofosfamide bleven gebruiken. In patiënten die ANCA positief waren, was de ziektevrije overleving op 2 en 4 jaar slechts 58% en 17%. Wij concludeerden dan ook dat de overstap van cyclofosfamide naar azathioprine bij patiënten met PR3-ANCA geassocieerde vasculitis, die ANCA positief zijn ten tijde van de overstap, het risico op recidieven sterk verhoogd is.

Een serologische marker voor de uitgebreidheid en progressie van atherosclerose, antilichamen tegen geoxideerd low-density lipoproteïne (oxLDL), werd bestudeerd in AAV in *Hoofdstuk 9*. Drie manieren om anti-oxLDL te detecteren werden vergeleken tussen patiënten met MPO-ANCA en patiënten met PR3-ANCA. We bepaalden de hoeveelheid antilichamen tegen LDL, nadat dit behandeld werd met hypochloriet, MDA of koper, in ANCA-positieve patiënten, en vergeleken deze met de hoeveelheid antilichamen in sera van hemodialysepatiënten en gezonde controles. We zagen dat de hoeveelheid anti-oxLDL antilichamen waren toegenomen in AAV patiënten (MDA-LDL en hypochloriet-LDL) en in dialysepatiënten (hypochloriet-LDL) in vergelijking met gezonde controles. Daarnaast zagen we dat patiënten met MPO-ANCA meer antilichamen tegen hypochloriet-LDL hadden dan patiënten met PR3-ANCA. We concludeerden dat onze nieuw ontwikkelde test voor het meten van anti-oxLDL, met hypochloriet-LDL als substraat, gevoeliger is voor het detecteren van deze antilichamen dan traditionele testen. Daarnaast lijkt het zo te zijn dat oxidatie van LDL met behulp van MPO

in patiënten met MPO-ANCA voorkomt en veroorzaakt wordt door de circulerende MPO-ANCA antilichamen, die door hun binding met MPO de binding van MPO aan zijn natuurlijke remmer, ceruloplasmine, voorkomen.

In *Hoofdstuk 10* analyseerden we de hoeveelheid circulerende CD4⁺CD28⁻ T cellen in relatie tot de aanwezigheid van atherosclerotische schade in patiënten met AAV. Daarnaast bepaalden we of antilichamen tegen cytomegalovirus (CMV) en humaan heat shock protein 60 (hHSP60) geassocieerd waren met de aanwezigheid van CD4⁺CD28^{null} T cellen in AAV. Veertig patiënten in remissie werden in de studie geïnccludeerd. De echtgenoten van de patiënten werden als gezonde controles (HC) gevraagd (n=38). De intima-media dikte (IMT) en pulse-wave velocity (PWV) werden bepaald. We zagen dat er bewijs voor atherosclerotische schade is in patiënten met AAV, zoals gemeten met de PWV; aan de andere kant werd er geen verschil in IMT gevonden. CD4⁺CD28^{null} T cellen zijn relatief verhoogd in AAV en geassocieerd met vroegere CMV infectie. We zagen geen relatie tussen CD4⁺CD28^{null} T cellen en verhoogde IMT of PWV.

Tot slot worden in *Hoofdstuk 11* de huidige opvattingen van de betrokkenheid van het immuunsysteem in atherosclerose samengevat, met speciale aandacht voor mogelijke immunologische oorzaken van versnelde atherosclerose in AAV.

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Curriculum vitae

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Marjan Carolien Slot werd geboren te Enschede op 5 juni 1977. Na het behalen van haar atheneumdiploma aan het Stedelijk Lyceum Zuid aldaar in 1995 startte zij haar studie geneeskunde aan de Rijksuniversiteit Groningen. Tijdens het tweede jaar van deze studie is zij begonnen aan de eerste stappen naar het huidige proefschrift onder begeleiding van Dr. J.W. Cohen Tervaert en Dr. C.A. Stegeman. Haar wetenschappelijke stage verrichtte zij in het Massachusetts General Hospital in Boston onder begeleiding van Dr. H. Choi, Prof. Dr. P.A. Merkel en Dr. J. Niles. In 2001 behaalde zij haar artsexamen in Groningen en ze startte in 2002 als assistent geneeskunde in opleiding tot klinisch onderzoeker bij het departement interne geneeskunde, afdeling klinische en experimentele immunologie van het academisch ziekenhuis Maastricht waar Dr. Cohen Tervaert recent als hoogleraar benoemd was. De resultaten van de studies in Boston, Groningen en Maastricht zijn gebundeld in dit proefschrift.

Momenteel werkt Marjan als stafarts bij Stichting Eurotransplant International alwaar zij verantwoordelijk is voor de orgaanallocatie, het verbeteren van de allocatiealgoritmen en het verrichten van wetenschappelijk onderzoek.