On the pathogenesis and clinical outcome of ANCA-associated vasculitis

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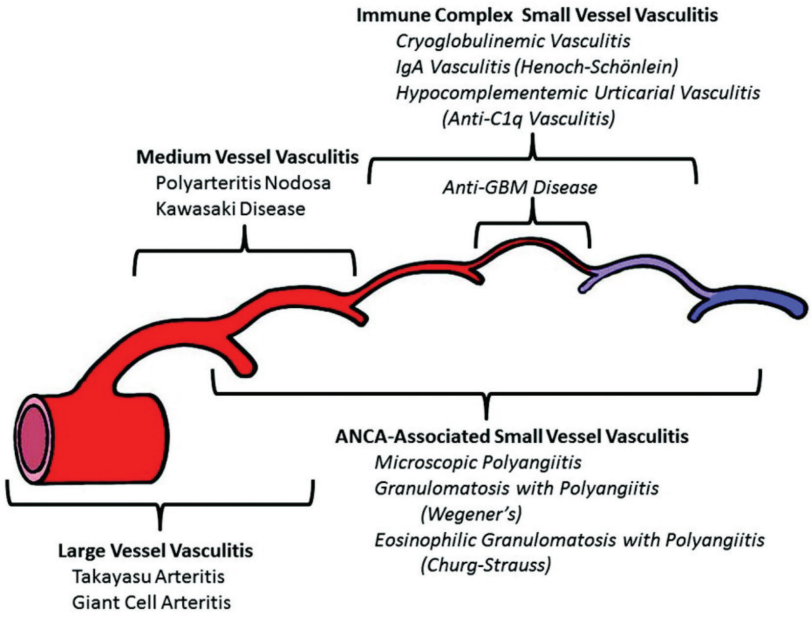
Chapter I

General introduction and outline of this thesis

Vasculitis means inflammation of blood vessels. The International Chapel Hill Consensus Conference Nomenclature of Vasculitides (CHCC) provides definitions for the different vasculitides and subcategorises them into three major categories: large-vessel vasculitis, medium-vessel vasculitis, and small-vessel vasculitis (**figure 1**).[1, 2]

Figure 1. The International Chapel Hill Consensus Conference Nomenclature of Vasculitides (CHCC)



The International Chapel Hill Consensus Conference Nomenclature of Vasculitides (CHCC) subcategorises the different vasculitides into three major categories: large-vessel vasculitis, medium-vessel vasculitis, and small-vessel vasculitis. The figure depicts (from left to right) aorta, large artery, medium artery, small artery/arteriole, capillary, venule, and vein. Anti-GBM = anti–glomerular basement membrane; ANCA = antineutrophil cytoplasmic antibody. Reproduced from Jennette et al.[2] with permission.

Large-vessel vasculitis predominantly affects the aorta and its major branches. Two important vasculitides within this category are Takayasu arteritis and giant cell arteritis. Medium-vessel vasculitis predominantly affects medium sized arteries. Kawasaki disease is an example of a medium-vessel vasculitis. Small-vessel vasculitis is further subcategorised into immune complex small-vessel vasculitis and antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis. Immune complex small-vessel vasculitis shows immunoglobulin (Ig) and complement deposits in the blood vessel walls. Two important vasculitides within this category are IgA vasculitis and anti–glomerular basement membrane

(anti-GBM) disease. ANCA-associated vasculitis is a necrotizing vasculitis with few or no immune deposits that is typically associated with ANCA-seropositivity by indirect immunofluorescence or enzyme-linked immunosorbent assay (ELISA).[3]

ANCA-associated vasculitis

This thesis focuses on different aspects of ANCA-associated vasculitis. ANCA first became widely recognized after a key publication in The Lancet in 1985 in which van der Woude *et al.* described circulating antibodies in patients with vasculitis that were in several ways similar to the granulocyte-specific antinuclear antibodies (GS-ANA) described in rheumatoid arthritis.[4] It should however be mentioned that Davies *et al.* had already described the presence of this class of antibodies in patients with pauci-immune glomerulonephritis in 1982.[5] Van der Woude *et al.* first named these antibodies ACPA (anticytoplasmic antibodies). This term was later replaced by ANCA, as the antibodies were found to be directed against neutrophil (and monocyte) constituents. The two most important ANCA antigens are proteinase 3 (PR3)[6-8] and myeloperoxidase (MPO)[9].

ANCA-associated vasculitis comprises the clinical diagnoses granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis[10-12]), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss Syndrome).[2]

GPA is a necrotizing granulomatous inflammation usually involving the upper and lower respiratory tract.[13] Approximately 90-95% of patients with active, generalized GPA are ANCA-positive; a small subset of GPA patients is ANCA-negative.[14-17] Thus, the absence of ANCA does not exclude the diagnosis of GPA. About 70% of the ANCA-positive GPA patients are PR3-ANCA positive; the remainder are MPO-ANCA positive.[18]

MPA is characterized by a non-granulomatous necrotizing systemic vasculitis. Nearly 90% of patients with MPA are ANCA-positive.[14-17] About 60% of the ANCA-positive MPA patients are MPO-ANCA positive; the remainder are PR3-ANCA positive.[9, 18] Because PR3-ANCA and MPO-ANCA may occur in both GPA and MPA, these conditions cannot be distinguished on the basis of ANCA-serotype.

EGPA is an eosinophil-rich and necrotizing granulomatous inflammation often involving the respiratory tract. In contrast to GPA and MPA, EGPA is associated with asthma and eosinophilia. Although variable from study to study, approximately 50% of patients with EGPA are ANCA-positive.[19, 20] About 70-90% of the ANCA-positive EGPA patients are MPO-ANCA positive.[20, 21]

The clinical diagnoses GPA and MPA are often grouped together on the grounds of striking similarities in clinical presentation and comparable histologic

findings.[18, 22] Nevertheless, there are marked differences between these disease entities, fuelling an ongoing debate as to whether GPA and MPA are different entities within the same disease spectrum or represent distinct auto-immune diseases.[23-25] Although there are overlaps, a clinical diagnosis of GPA usually involves PR3-ANCA positivity whereas a clinical diagnosis of MPA usually involves MPO-ANCA positivity. In this respect, data from a large genome-wide association study (GWAS) are interesting.[26] These data indicate that within the spectrum of ANCA-associated vasculitis distinct subtypes may be recognized. ANCA-serotype was demonstrated to be the main indicator of these subtypes.[26]

To date, the concept of a single disease spectrum has led to the inclusion of GPA and MPA patients in the same clinical trials and the development of similar treatment strategies for these patients.[14-17, 27, 28] Evidence that the different entities in ANCA-associated vasculitis actually represent distinct auto-immune diseases may in the future lead to the development of more specific therapeutic strategies. The existence of different subtypes within the spectrum of ANCA-associated vasculitis and their clinical implications are further investigated in **Chapter 2** and **Chapter 3** of this thesis.

Aetiology and pathogenesis of ANCA-associated vasculitis

The pathogenesis of ANCA-associated vasculitis has not been completely elucidated. However, over the past decades, significant progress has been made in the understanding of this complex disease. Environmental exposures, genetic factors, influences of the immune system, and the intensity and duration of the injury are all hypothesized to be involved in the pathogenesis of ANCA-associated vasculitis. Interestingly, the variability in aetiological and synergistic factors that may lead to the development of ANCA-associated vasculitis is hypothesized to contribute to the clinicopathologic differences between patients.[29]

Onset and exacerbation of ANCA-associated vasculitis occur more often during winter and spring. This points towards the existence of pathogenic, infectious or other environmental, factors that are typically present during these seasons.[30] Numerous studies demonstrated that pro-inflammatory factors, for example induced by infections, act synergistically in ANCA-associated vasculitis onset and exacerbation.[31, 32] In particular, *Staphylococcus aureus* infection was linked to ANCA-associated vasculitis onset.[33-35] Moreover, nasal carriage of *Staphylococcus aureus* was demonstrated to be an important risk factor for relapse in patients with GPA,[36] and treatment with trimethoprim-sulfamethoxazole was shown to aid in the induction of remission[37-46] and the prevention of relapses[37]. These treatment effects could be ascribed to the immunosuppressant and/or anti-staphylococcal properties of trimethoprim-sulfamethoxazole.

Evidence that genetic factors contribute to the pathogenesis of ANCA-associated vasculitis comes from familial association studies,[47-51] differences in the prevalence of ANCA-associated vasculitis between ethnic groups,[52] and numerous candidate gene associations studies[53]. Quite recently, two GWAS performed by the Vasculitis Clinical Research Consortium and the European Vasculitis Genetic Consortium also identified genetic associations in ANCA-associated vasculitis.[26, 54] Both GWAS found a strong association with a single nucleotide polymorphism (SNP) in human leukocyte antigen (*HLA*)-*DPB1*. The European Vasculitis Genetic Consortium GWAS also found a strong association between PR3-ANCA vasculitis and *PRTN3* (the gene encoding PR3) and *SERPINA1* (the gene encoding α1-antitrypsin; a major inhibitor of PR3), and between MPO-ANCA vasculitis and *HLA-DQ*. Moreover, this GWAS demonstrated genetic distinctions between GPA and MPA patients and between PR3-ANCA positive and MPO-ANCA positive patients. The numerous candidate gene association studies and two GWAS have revealed a great number of genetic variants that possibly contribute to the pathogenesis of ANCA-associated vasculitis. In **Chapter 2** of this thesis, we conducted a meta-analysis to investigate the genetic variants that are most likely associated with ANCA-associated vasculitis. We included raw data from the European Vasculitis Genetic Consortium GWAS to increase the validity of the meta-analysis.

Are ANCA pathogenic?

In vitro and *in vivo* evidence support a pathogenic role for ANCA in the pathogenesis of ANCA-associated vasculitis.[55, 56] *In vitro* data include experiments demonstrating that ANCA IgG activates cytokine-primed neutrophils,[57-59] that ANCA-activated neutrophils induce endothelial cell injury,[60, 61] and that ANCA-activated neutrophils activate the alternative complement pathway[62].

The most convincing evidence that ANCA are pathogenic comes from animal models. Xiao *et al.* demonstrated that intravenous injection of purified murine anti-MPO IgG into wild type mice or immunodeficient Rag2-/- mice (these mice fail to generate mature T and B lymphocytes) induces proteinuria and haematuria in these mice. Moreover, the histopathologic lesions found in the kidneys of these mice were comparable to those in renal biopsies of patients with ANCA-associated glomerulonephritis.[56] Furthermore, Little *et al.* demonstrated that rats immunised with human MPO develop anti-human MPO-ANCA that binds these rats' neutrophils and induces pauci-immune crescentic glomerulonephritis and pulmonary haemorrhage with histologic evidence of lung vasculitis in these rats.[63] Thus far, it has not been possible to successfully reproduce these findings in a PR3-ANCA vasculitis animal model.[29, 64]

Clinical evidence for the pathogenicity of ANCA includes the occurrence of pulmonary-renal syndrome in a neonate shortly after birth from a mother with MPO-ANCA positive MPA, most likely caused by transplacental transfer of maternal MPO-ANCA.[65, 66] This clinical evidence is limited, however, in that it comprises only one case-report and until today no sequelae have been reported. Further clinical evidence for the pathogenicity of ANCA includes the induction of ANCA, particularly high titres of MPO-ANCA, by certain drugs (e.g. propylthiouracil and hydralazine[67]) and the subsequent onset of disease manifestations in humans. More clinical evidence is found in the beneficial effect of plasma exchange in the treatment of ANCA-associated vasculitis.[17]

One observation plaguing the contention that ANCA are pathogenic was the presence of ANCA in only 90-95% of ANCA-associated vasculitis patients.[14-17] However, the remaining 5-10% of patients that have long been assumed to be ANCA-negative based on the results of conventional clinical assays might be classified as ANCA-positive after all. Roth *et al.* demonstrated that, when using a highly sensitive epitope-excision method, ANCA-negative patients' purified IgG reacts with a specific MPO-epitope.[68] This MPO-ANCA epitope was blocked from reacting with ANCA IgG in serum because of competitive binding by ceruloplasmin, which is a natural inhibitor of MPO that is naturally present in serum.

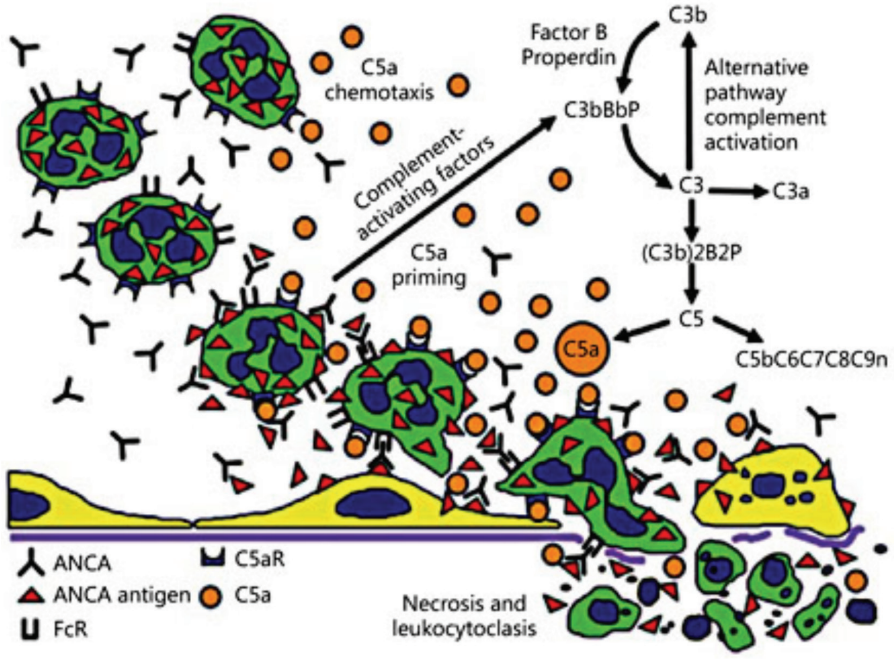
The pathogenicity of ANCA was also questioned since healthy individuals were also demonstrated to have ANCA.[69-72] Interestingly, Roth *et al.* demonstrated that compared with MPO-ANCA occurring in vasculitis, natural MPO-ANCA are present in lower titres, have lower avidity, have less subclass diversity, and are less capable of activating neutrophils *in vitro*.[68] Moreover, the epitope specificity of the repertoire of MPO-ANCA occurring in vasculitis patients was shown to differ from the epitope specificity of MPO-ANCA occurring in healthy individuals.[68] Thus, not all ANCA seem to be equal.[73]

Presumed pathogenic sequence for acute vascular injury in ANCA-associated vasculitis

The putative pathogenic sequence inducing acute vascular inflammation in ANCA-associated vasculitis is depicted in **figure 2**.[29] Starting from the upper left, resting neutrophils have ANCA autoantigens (e.g. PR3 and MPO) sequestered in their cytoplasmic granules. Exposure to priming factors, e.g. cytokines induced by infection or pathogenic factors released by complement activation, leads to the exposure of ANCA-antigens on the neutrophil's surface and in the microenvironment. Circulating ANCA bind to these antigens and activate them by Fcγ receptor engagement and F(ab$'$)2 binding. ANCA-activated neutrophils penetrate vessel walls and release factors that initiate necrosis and

apoptosis of the neutrophils and the environment. Meanwhile, the alternative complement pathway is activated and generates C5a, a chemoattractant for neutrophils that also primes the arriving neutrophils for activation by ANCA, generating a positive feedback effect.

Figure 2. The putative pathogenic sequence inducing acute vascular inflammation in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis.



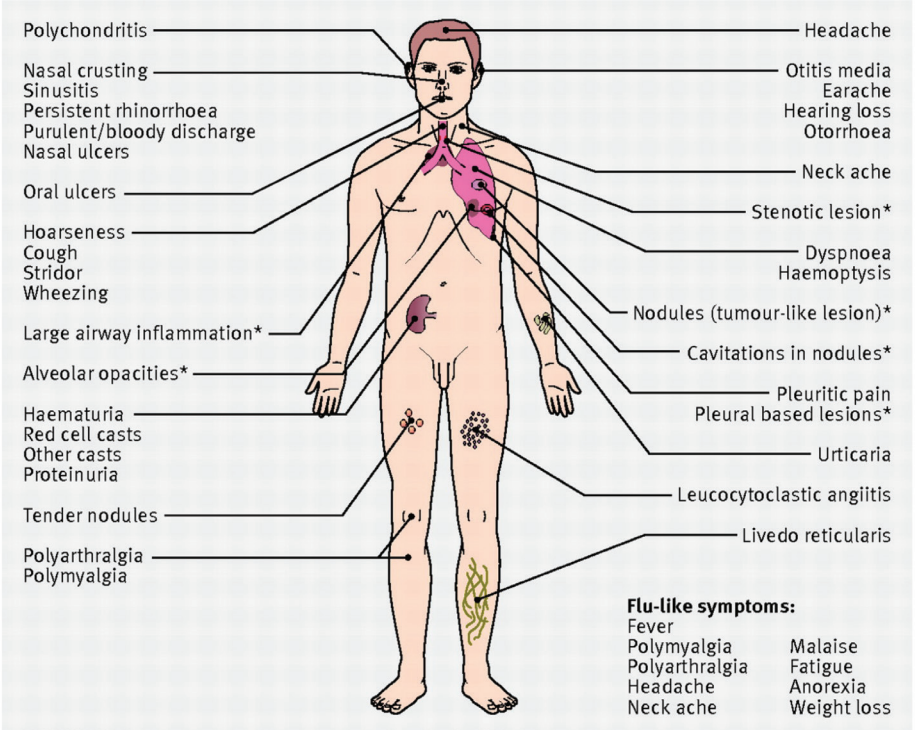
Starting from the upper left, resting neutrophils have ANCA-antigens (e.g. proteinase 3 (PR3) and myeloperoxidase (MPO)) in their cytoplasmic granules. Exposure to priming factors, e.g. cytokines induced by infection or pathogenic factors released by complement activation, leads to the exposure of the ANCA-antigens on the neutrophil's surface and in the microenvironment. Circulating ANCA bind to these antigens and activate the neutrophils. ANCA-activated neutrophils penetrate vessel walls and release factors that initiate necrosis and apoptosis of the neutrophils and the environment. Meanwhile, the alternative complement pathway is activated and generates C5a, a chemoattractant for neutrophils that also primes the arriving neutrophils for activation by ANCA, generating a positive feedback effect. Reproduced from Xiao et al.[29] with permission.

Disease manifestations

The peak age of ANCA-associated vasculitis onset is 65-74 years.[74] Men are more often affected than women, but when women are affected, they tend to have a younger age at disease onset than men.[75] Patients typically present with prodromal 'flu-like' symptoms that have been present for several weeks to months.[3] These symptoms include malaise, fever, headache, polyarthralgia, polymyalgia, and unintended weight loss.[13, 76] Presenting symptoms can be very similar to symptoms of non-vasculitic diseases such as infections, post-viral syndrome, and malignancies. It can therefore be challenging for clinicians to pinpoint the diagnosis early in the diagnostic process. A survey including 701 patients with ANCA-associated vasculitis demonstrated a lag of three to 12 months between disease onset and diagnosis, suggesting that diagnostic delay is a problem.[75] Correct diagnosis on the first visit to a physician was accomplished in only 7% of patients and 50% of patients had to visit at least four physicians before the correct diagnosis was made.

Figure 3 depicts the various manifestations of ANCA-associated vasculitis. Although disease symptoms in GPA and MPA overlap, the incidences of these symptoms can differ significantly between the two conditions. For instance, ear, nose, and throat (ENT) symptoms occur in 90% of GPA patients and in only 35% of MPA patients.[13, 76] Large observational studies demonstrated that the airways and lung parenchyma are commonly affected, as are the kidneys, although renal involvement can be asymptomatic until renal failure occurs.[76-78] Approximately half of patients develop skin manifestations.[76-78] Clearly, ANCA-associated vasculitis can become manifest in virtually all organs. Therefore, thorough physical examination is important to determine the full extent of the disease.[3]

Figure 3. The various manifestations of antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis.



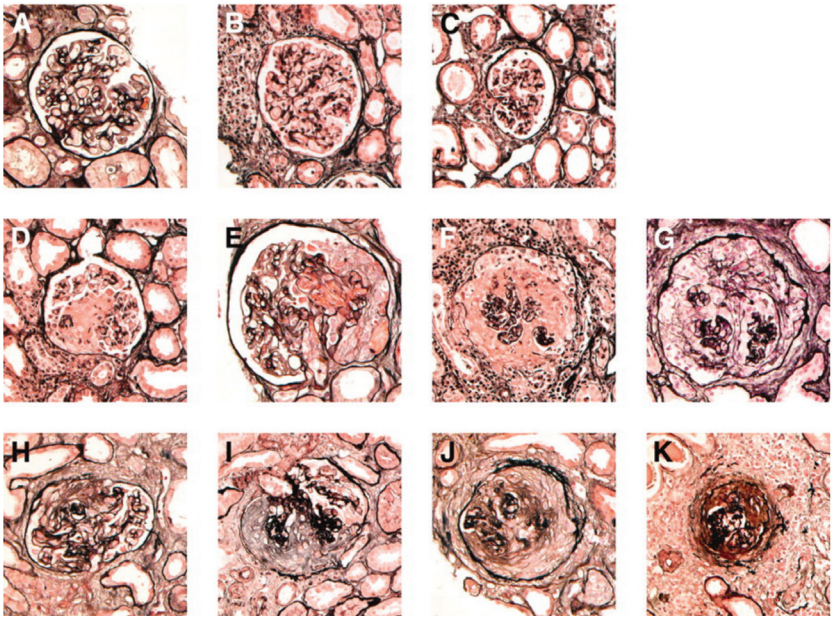
*These lesions can be seen on chest radiography and computed tomography. Reproduced from Berden et al.[3] with permission.

Chapters 3 and 4 of this thesis focus on renal involvement in ANCA-associated vasculitis. Renal involvement is common and is important with respect to patient morbidity and mortality.[22] The kidneys become involved in approximately 80% of GPA patients and 90% of MPA patients.[13] A key study published in 1958 demonstrated that the main cause of death in untreated ANCA-associated vasculitis patients is uraemia due to rapidly progressive renal failure.[79]

Rapidly progressive renal failure with an active sediment (i.e. red cell casts and/or proteinuria) in patients who are seropositive for ANCA is suggestive of ANCA-associated glomerulonephritis. The morphologic changes in the renal biopsy are the gold standard for establishing the diagnosis of ANCA-associated glomerulonephritis.[80, 81] In these biopsies, light microscopy shows necrotizing and crescentic glomerulonephritis.[82] Immunofluorescence microscopy shows little or no immunoglobulin or complement staining (the so-called pauci-immune staining pattern). By electron microscopy, subendothelial edema, microthrombosis, and degranulation of neutrophils are present, but immune deposits are absent.[83]

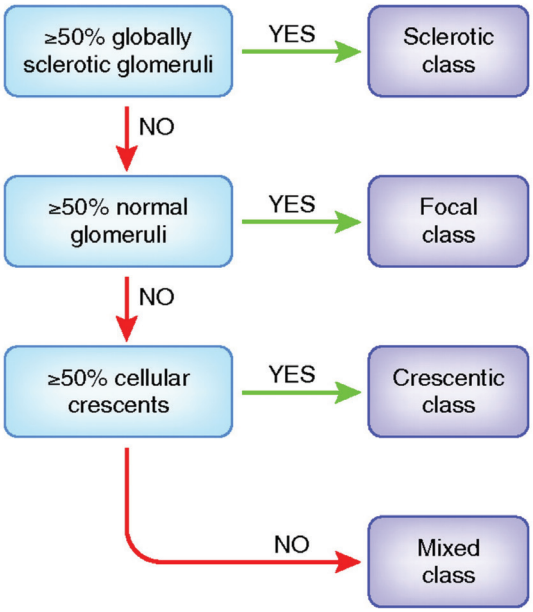
Several studies have demonstrated strong associations between histopathological parameters in the renal biopsy and renal outcome. The most consistent findings were associations between the percentage of normal glomeruli and favorable renal outcome and between the percentage of sclerotic glomeruli and poor renal outcome.[84-87] Moreover, the presence of active lesions such as cellular crescents was found to be positively associated with renal recovery under immunosuppressive treatment.[84, 85] In 2010, the histopathological classification of ANCA-associated glomerulonephritis was devised within the collaboration of the European Vasculitis Society (EUVAS) with the aim of further adding to the prognostication of patients with ANCA-associated glomerulonephritis.[88] This classification system is built around glomerular pathology and distinguishes four classes: focal class biopsies contain ≥50% normal glomeruli; crescentic class biopsies contain ≥50% glomeruli with cellular crescents; sclerotic class biopsies contain ≥50% sclerotic glomeruli; and biopsies without predominant lesions are assigned to the mixed class. Examples of glomerular lesions typically for each of the four classes are depicted in **figure 4**. The classification flowchart is depicted in **figure 5**.

Figure 4. Examples of glomerular lesions that can be observed in the four classes of the histopathological classification of antineutrophil cytoplasmic antibodies (ANCA)-associated glomerulonephritis.



A-C show examples of normal glomeruli; D-G show examples of cellular crescents; H-J show examples of fibrous crescents; and K shows an example of global glomerulosclerosis. Reproduced from Berden et al.[88] with permission.

Figure 5. The classification flowchart for the histopathological classification of ANCA-associated glomerulonephritis.



Reproduced from Berden et al.[88] with permission.

The study by Berden *et al.* wherein the classification system was proposed incorporated a validation study including 100 patients.[88] Multiple regression analysis showed that baseline renal function and the histopathological classification were the only independent predictors of renal outcome. The histopathological classification of ANCA-associated glomerulonephritis has been validated in various cohorts.[89-109] The outcomes of these validation studies, points of consideration, and future perspectives are discussed in **Chapter 4** of this thesis.

Treatment of ANCA-associated vasculitis

Early diagnosis of ANCA-associated vasculitis is of major importance as prompt instigation of treatment is essential to prevent progressive organ damage, particularly end-stage renal disease, and death.[79] In the last 20 years, a significant number of large, international trials were performed with the aim of improving the treatment modalities in ANCA-associated vasculitis.[14-17, 27, 28, 110-112]

Treatment of ANCA-associated vasculitis traditionally consists of two phases.[113] Phase I consists of aggressive remission-inducing immunosuppression classically consisting of cyclophosphamide and corticosteroids for the duration of three to six months. Phase II consists of remission maintenance treatment with,

amongst others, azathioprine or methotrexate while corticosteroids are tapered and if possible stopped.[114] The choice of treatment depends on disease severity and organ manifestations. Recently, rituximab was introduced for both induction and remission maintenance treatment in ANCA-associated vasculitis.[27, 28, 115]

Induction treatment

Induction treatment of ANCA-associated vasculitis consists of cyclophosphamide treatment until complete clinical remission is achieved, which is often after three to six months. The CYCLOPS trial demonstrated that, while achieving similar remission rates, intravenous pulsed cyclophosphamide treatment results in a lower cumulative cyclophosphamide dose than oral cyclophosphamide treatment.[15] However, long-term analysis of this cohort demonstrated a higher relapse risk in the intravenous group than in the oral group.[116] The increased frequency of relapses in the intravenous group was not associated with increased mortality or renal damage.

Two randomised controlled trials, RITUXVAS[27] and RAVE[28], investigated rituximab – a chimeric monoclonal antibody directed against pre-B cells and mature B lymphocytes – in the treatment of ANCA-associated vasculitis. RITUXVAS included only newly diagnosed patients, whereas in RAVE both new and relapsing patients were included. In both trials, rituximab was non-inferior to cyclophosphamide for remission induction. Moreover, both trials demonstrated no significant difference between the number of adverse events in the rituximab group and the cyclophosphamide group. However, concerns were raised about a possible higher malignancy rate in patients treated with rituximab.[117, 118] **Chapter 5** of this thesis reports the first 10-year follow-up study investigating malignancy risk in patients with ANCA-associated vasculitis treated with cyclophosphamide according to current treatment regimens. **Chapter 6** reports the first study to compare the long-term malignancy risks between rituximab-based treatment and cyclophosphamide-based treatment in ANCA-associated vasculitis.

The NORAM trial investigated methotrexate induction treatment in patients with ANCA-associated vasculitis with early systemic disease without significant renal involvement.[14] Methotrexate was demonstrated to have similar efficacy to cyclophosphamide for remission induction, but was associated with a higher relapse rate. Long-term follow-up of these patients demonstrated that methotrexate treatment is associated with less effective disease control and prolonged use of steroids.[119]

Evidence from a retrospective study[120] and a prospective pilot trial[121] indicates that mycophenolate mofetil is effective in the induction treatment of ANCA-associated vasculitis. An advantage of mycophenolate mofetil over cyclophosphamide includes a more favourable safety profile in terms of toxicity. The MYCYC trial compares mycophenolate mofetil to cyclophosphamide

for induction treatment in ANCA-associated vasculitis (ClinicalTrials.gov NCT00414128). The MYCYC trial results are expected in 2018.

Maintenance treatment

Maintenance treatment aims at the prevention of relapses. The landmark EUVAS trial CYCAZAREM demonstrated that cyclophosphamide exposure in patients with ANCA-associated vasculitis can be safely reduced by the substitution of cyclophosphamide by azathioprine shortly after remission achievement.[16] Relapse risk and severe adverse event occurrence were similar between the cyclophosphamide group and the azathioprine group. Currently, azathioprine, alongside with methotrexate, is the first choice for maintenance treatment in ANCA-associated vasculitis.[122]

Methotrexate can be used as an alternative to azathioprine for remission maintenance in patients with ANCA-associated vasculitis in whom renal function is not severely impaired. The WEGENT trial demonstrated similar remission maintenance and adverse events rates between methotrexate and azathioprine maintenance treatment.[114]

Rituximab was recently introduced for remission maintenance treatment in ANCA-associated vasculitis patients who relapsed on other maintenance therapies or who are at high risk of relapse. The MAINRITSAN trial demonstrated a superiority of rituximab over azathioprine as maintenance treatment after cyclophosphamide induction treatment.[115] Adverse events rates were similar in the rituximab group and the azathioprine group. The ongoing RITAZERAM trial compares rituximab to azathioprine as maintenance treatment in relapsing patients who achieved remission following rituximab induction treatment (ClinicalTrials.gov NCT01697267).

The IMPROVE trial demonstrated that mycophenolate mofetil is less effective than azathioprine in remission maintenance after remission induction with cyclophosphamide.[111] Therefore, mycophenolate mofetil maintenance treatment is only considered in ANCA-associated vasculitis patients in whom azathioprine and methotrexate are contraindicated.

Prognosis of patients with ANCA-associated vasculitis

As stated previously, the natural history of untreated ANCA-associated vasculitis is that of a rapidly progressive, usually fatal disease.[79] The introduction of immunosuppressive therapy in the 1960s has dramatically reduced the 1-year mortality rate of patients with ANCA-associated vasculitis from 82% to 10%.[32, 79, 123] Nevertheless, patients continue to have an increased mortality risk compared to the general population.[123]

A large, prospective 5-year follow-up study that included 535 patients demonstrated that ANCA-associated vasculitis patients have a 2.6 times increased mortality risk compared to the general population.[123] Infection (48%) and active vasculitis (19%) were the main causes of death during the first year after diagnosis. This finding emphasizes the importance of finding the right balance between disease control and immunosuppressive treatment. After the first year of diagnosis, patients continued to have a 1.3 times increased mortality risk.[123] Cardiovascular disease (26%), malignancy (22%), and infection (20%) accounted for the majority of these deaths.

About 14% of patients will experience at least one major cardiovascular event within 5 years after ANCA-associated vasculitis diagnosis.[124] Patients with GPA are reported to have a 3.6 times increased myocardial infarction risk compared to the general population.[125] In addition, when matched for renal function and other traditional risk factors, cardiovascular risk is still doubled in patients with ANCA-associated vasculitis.[126] PR3-ANCA positivity, older age, and diastolic hypertension were shown to be independent determinants of poor cardiovascular outcome.[124] Factors contributing to the increased cardiovascular risk in vasculitis include the chronic inflammatory state, endothelial dysfunction, renal dysfunction, and the use of corticosteroids, which accelerates the development of hypertension, dyslipidaemia, and diabetes.[127, 128]

Malignancies were demonstrated to be the second leading cause of death after the first year of ANCA-associated vasculitis diagnosis.[123] A number of studies, using data from retrospective monocentre cohorts,[76, 129-131] prospective multicentre clinical trials,[132, 133] and nationwide registry linkage,[134] demonstrated that patients with ANCA-associated vasculitis have an increased malignancy risk compared to the general population.[135] In particular, the risks of leukaemia, lymphoma, bladder cancer, and non-melanoma skin cancer (NMSC) were increased. Except for one 7-year follow-up study published in 1992,[76] average follow-up in these studies was at most five years[129-132]. Moreover, the observation period in most studies was 1960-1990. Thus, the patients included in these studies were treated with treatment regimens that are outdated and, consequently, the findings of these studies do not represent current malignancy risks.[76, 129, 131, 134] As explained previously, treatment regimens in ANCA-associated vasculitis have changed significantly in recent years based on efforts to reduce cumulative cyclophosphamide exposure.[14-17] Moreover, rituximab has emerged as a promising substitute for cyclophosphamide in the treatment of ANCA-associated vasculitis.[27, 28, 136, 137] **Chapter 5** of this thesis reports the first 10-year follow-up study that investigates malignancy risk in ANCA-associated vasculitis patients treated with cyclophosphamide according to current treatment regimens. **Chapter 6** reports the first study to compare the long-term malignancy risks between rituximab-based treatment and cyclophosphamide-based treatment in ANCA-associated vasculitis.

Several factors have been hypothesized to contribute to the increased malignancy risk in patients with ANCA-associated vasculitis. Firstly, immunosuppressive treatment may decrease the immune system's ability to recognize and eradicate malignant cells. The importance of a well-functioning immune system in the prevention of malignancies is well-demonstrated by the increased malignancy risk observed in HIV-positive patients.[138] Moreover, immunosuppressive therapy itself may have direct mutagenic properties, as, for example, demonstrated in cyclophosphamide-induced bladder cancer.[139-143] Furthermore, long-standing immune activation per se may be oncogenic; for example, long-standing immune activation is hypothesized to contribute to the increased lymphoma risk seen in a number of chronic autoimmune rheumatic conditions.[144]

The European Vasculitis Society and the European Vasculitis Genetics Consortium

Research in ANCA-associated vasculitis is hindered by the low incidence of this complex disease. Therefore, in 1994 the EUVAS was founded with the objective of uniting vasculitis researchers and clinicians and promoting the study of vasculitis. The apparent need for clinical trials was demonstrated by the wide heterogeneity in treatment, poor patient outcomes, and high levels of treatment-related toxicity.[122] Thus far, the EUVAS has conducted a significant number of clinical trials with the aim of optimizing the treatment of ANCA-associated vasculitis.[14-17, 27, 110, 111, 145] Long-term follow-up studies of the first four EUVAS trials (CYCAZAREM,[16] NORAM,[14] MEPEX,[17] and CYCLOPS[15]) have been published and longer follow-up is pending. The aim of these studies was to investigate several long-term outcomes in ANCA-associated vasculitis, including long-term patient survival,[123] disease relapse,[146] cardiovascular events,[124] malignancies,[132] disease-related damage,[147-149] and severe adverse events[150]. To harmonize the clinical trials, scoring systems were created for both presenting clinical manifestations[151-153] and renal histology[154]. Other studies conducted by the EUVAS include ANCA assay standardization studies[18, 155, 156] and renal histopathological studies[84, 85, 88, 157-162]. The study presented in **Chapter 3** of this thesis was performed within the collaboration of the EUVAS. The European Vasculitis Genetics Consortium has grown out of EUVAS activity and focuses on the genetics of ANCA-associated vasculitis. In 2010, the European Vasculitis Genetic Consortium published the first GWAS in ANCA-associated vasculitis.[26] The study presented in **Chapter 2** of this thesis was performed within the collaboration of the European Vasculitis Genetic Consortium.

Outline of this thesis

The role of genetic variants in ANCA-associated vasculitis is explored in **Chapter 2** of this thesis. The numerous candidate gene association studies and two GWAS have revealed a great number of genetic variants that could contribute to the pathogenesis of ANCA-associated vasculitis. In **Chapter 2** we report the results of a meta-analysis investigating the genetic variants that are most likely associated with ANCA-associated vasculitis. We included raw data from the European Vasculitis Genetic Consortium GWAS to increase the validity of the meta-analysis.[26] Moreover, in the light of the ongoing debate as to whether the different subtypes within ANCA-associated vasculitis represent different entities within the same disease spectrum or are distinct auto-immune diseases, we investigated whether these subtypes have distinct genetic backgrounds. In **Chapter 3** we investigated whether ENT involvement represents a separate phenotype in ANCA-associated vasculitis by exploring the relationship between ENT manifestations and renal outcome.

As discussed previously, renal disease is a common and severe manifestation of ANCA-associated vasculitis that can lead to end-stage renal disease and death. **Chapter 4** reviews the outcomes of studies validating the histopathological classification of ANCA-associated glomerulonephritis and discusses points of consideration and future perspectives.

The prognosis of patients with ANCA-associated vasculitis has improved dramatically in recent years, shifting attention towards the long-term complications these patients experience. In **Chapter 5** the malignancy risk in patients with ANCA-associated vasculitis treated with current immunosuppressive regimens was investigated and its relationship with cyclophosphamide treatment was explored. Recently, rituximab was introduced for both induction and remission maintenance treatment in ANCA-associated vasculitis with the promise of, amongst others, further reducing malignancy risk. **Chapter 6** reports the first study to date to compare the long-term malignancy risks between rituximab-based treatment and cyclophosphamide-based treatment in ANCA-associated vasculitis.

The findings of this thesis will be summarized and placed in a more general perspective in **Chapter 7**.

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