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# Prognosis in small vessel vasculitis

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Emma E. van Daalen

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## Colophon

**Title:** Prognosis in small vessel vasculitis
**Author:** Emma E. van Daalen

The studies described in this thesis were performed at the Department of Pathology (head: Prof. Dr. V.T.H.B.M. Smit) of the Leiden University Medical Center, the Netherlands.

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# Prognosis in small vessel vasculitis

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Door

**Emma Elisabeth van Daalen**

geboren te ’s-Hertogenbosch
in 1992

| | |
|---|---|
| Promotor: | Prof. dr. J.A. Bruijn |
| Co-promotor: | Dr. I.M. Bajema |
| Promotiecommissie: | Prof. dr. V.T.H.B.M. Smit |
| | Prof. dr. R. Goldschmeding (UMC Utrecht) |
| | Dr. A.E. Hak (AMC Amsterdam) |

Ich steh'im Dunkeln und ich mag das Licht

*(Nena, In meinem Leben, 2009)*

Voor mijn ouders, broer en zus

## Contents

# CHAPTER 1

## General introduction

## THE ROOTS OF MEDICAL TERMINOLOGY

Vasculitis descends from the Latin word *vasculum* and the Greek postfix *–itis*, meaning inflammation of vessels. Ancient Latin is regarded as the basis for medical terminology, providing an international language useful for universal understanding of medicine. The Romans adapted the ancient Greek language in their medical terminology. Therefore, about three-fourths of our medical terminology is actually of Greek origin.[1] During the last centuries, the identification of new conditions required expansion of medical terminology, resulting in the combination of Latin words and Greek endings. The Greeks were the founders of rational medicine. The Greek Hippocratics were the first to describe diseases based on observation, and the names given by them to many conditions are still used today, such as *nephritis* (inflammation of the kidney). The two medical terms abovementioned – vasculitis and nephritis – will be mentioned frequently, because they compromise the main focus of this thesis.

### Vasculitis

In medical terminology, the postfix *–itis* indicates inflammation of a tissue. *Vasculum* is the diminutive from *vas*, meaning vessel. Thus, the literal translation of vasculitis is inflammation of small vessels. However, vasculitis in medical practice does not refer to small vessels exclusively, but also takes into account large- to medium-sized blood vessels. This thesis focusses on the small vessel vasculitides, which can be further distinguished in either immune complex mediated or pauci-immune (from Latin *pauci*: few or little).[2] An example of immune complex small vessel vasculitis is anti-glomerular basement membrane (anti-GBM) disease. This disease is characterized by extensive immune complex deposition in the kidneys. In contrast, immune complexes are usually absent or low in number in tissues affected by antineutrophil cytoplasmic antibodies (ANCA-) associated vasculitis (AAV).[2]

### Nephritis

Nephritis is the medical term for inflammation of the kidneys. Two types can be distinguished: glomerulonephritis, i.e. inflammation of the glomeruli; and interstitial nephritis, i.e. inflammation of the spaces between the renal tubules. The glomerulus (diminutive of the Latin *glomus*: small ball) is a network of capillaries through which ultrafiltration takes place. A mature human kidney has approximately 617.000 glomeruli.[3] The glomeruli are susceptible targets in the small vessel vasculitides. AAV and anti-GBM disease often affect the kidneys, in which case the diseases can be referred to as ANCA-associated glomerulonephritis (AAGN) or anti-GBM glomerulonephritis, respectively.

After this linguistic introduction, a more extensive description of the focus of this thesis will follow. On the basis of a historical and contemporary case report, the advances in nomenclature, detection of the disease, understanding of the pathogenesis, treatment, outcome, and predicting this outcome will be introduced.

## HISTORICAL CASE OF WEGENER'S GRANULOMATOSIS

In 1933, a 39-year old man visited the Mount Sinai Hospital in New York with complaints of sinusitis and otitis media. He had a low-grade fever and had lost about 30 pounds over the past three months. On X-ray, infiltrations of both lung bases and a lesion in the left upper lobe were noticed. His urine contained an increased level of albumin and red cells and casts. The presumptive diagnosis was a tuberculosis infection. Seven months later he returned with symptoms of left middle ear and mastoid inflammation. A mastoidectomy was performed and the patient was discharged. Some months later he returned again, this time because of central nervous system symptoms, which were thought to be based on conversion hysteria. In the meantime, a saddle nose deformity had developed. The findings on X-ray and urine analysis had not changed. Two-and-a-half year later, he visited the hospital for difficulty in breathing. Two months later, he had an exacerbation of pansinusitis and otitis media, and a hemolytic streptococcal septicemia. Moreover, he developed an acute arthritis of his right elbow and shoulder. A sigmoid sinus thrombosis was suspected; therefore, ligation of the internal jugular vein was undertaken. The patient died two days after this operation, but the exact cause of his death was not reported. His autopsy revealed granulomatous inflammation of the larynx, lungs and bronchioles, and a focal necrotizing glomerulitis. In the literature of the time that this case report was published (around 1954), 18 autopsied cases similar to this case had been reported and were defined as Wegener's granulomatosis (WG). Therefore, Fahey *et al*. concluded that this patient had WG.[4]



## CONTEMPORARY CASE OF GRANULOMATOSIS WITH POLYANGIITIS

In 2016, a 45-year old male was admitted to Leiden University Medical Center with complaints of unexplained fatigue, fever, cough, and bloody rhinorrhea. Two months earlier, he had a sinusitis for which he received antibiotics. The complaints of the sinusitis improved in the meantime, but did not totally disappear. No significant somatic or psychiatric illnesses were noted in his medical history. The patient did report weight loss over the past few months. Laboratory examination revealed a serum creatinine level of 350 µmol/L, which progressively increased during the following days. He also had proteinuria and microscopic hematuria. A CT-scan showed bilateral non-cavitating nodules in the lungs. An indirect immunofluorescence assay for ANCA was positive with a C-ANCA pattern, and enzyme-linked immunosorbent assay demonstrated positivity for PR3-ANCA. The patient underwent a nasal and a renal biopsy, showing acute inflammation with necrosis in the nose, and a glomerulonephritis with a pauci-immune pattern and extensive crescent formation in the kidney. The combination of findings resulted in the establishment of the diagnosis granulomatosis with polyangiitis (GPA) with involvement of the kidneys, lungs, and ear-nose-throat-region. Since the pathologists scored the renal biopsy as crescentic class AAGN, the estimated risk for this patient to develop end-stage

renal disease (ESRD) at five years after diagnosis was 24%. In order to induce disease remission, treatment consisted of two infusions of cyclophosphamide, four infusions of rituximab, and three pulses of methylprednisolone, followed by tapering doses of oral prednisone. Disease remission and a stable renal function were achieved in three months.

## CASE DESCRIPTIONS

While reading the historical case from 1933 and the contemporary case from 2016, large differences can be noted although both patients have AAV. Research performed during the 20th and 21st century has extended our knowledge of AAV, for example knowledge concerning diagnostic procedures, pathogenesis, and treatment. This has improved patient outcomes and prognosis. The next sections of this thesis will elaborate on how these improvements have been established and will summarize our current knowledge of AAV. The following topics will be introduced: nomenclature, presentation, diagnostic procedures, epidemiology, pathogenesis, treatment, and outcome. Each paragraph will start with a short review of the historical and contemporary case regarding the topic. The last part of the general introduction of this thesis comprises the current knowledge of the prognosis of patients with AAV, as prognosis is the key subject of this thesis.

## IMPROVEMENTS: FROM THE EARLY BEGINNING TILL NOW

### Nomenclature

The two presented cases at the beginning of this introduction are patients with GPA. In the historical case description that originated from Fahey *et al.*,[4] this disease was designated WG. In 1939, Friedrich Wegener was the first to describe the full picture of the disease; a fulminant, life-threatening, necrotizing granulomatous inflammation of the upper and lower respiratory tracts, vasculitis, and glomerulonephritis (known as Wegener's triad).[5] In 2006, two investigators found evidence that Wegener was a follower of the Nazi regime.[6] Therefore, they suggested to abandon the eponym, which has led to a decreasing use of the term WG and increasing use of the term GPA.[7]

Some important events in the field of vasculitis preceded the work of Wegener. Adolf Kussmaul and Rudolf Maier reported the first case of non-infectious vasculitis in 1866 and coined the term periarteritis nodosa (nowadays known as polyarteritis nodosa).[8,9] In 1931, Heinz Karl Ernst Klinger reported two cases which he called "borderline variants of periarteritis nodosa".[10] Eight years later, Wegener defined this variant as a distinct clinical and pathological entity.[5] Throughout the 20th century, other types of vasculitis were described, with a wide variety in suspected causes and symptoms. Distinction between the different vasculitides became urgent in order to define prognosis and treatment for each form of vasculitis. Therefore, in 1994, a first proposal for a standardized nomenclature system was made by a committee of international

internists, rheumatologists, nephrologists, immunologists, and pathologists in Chapel Hill, North Carolina (U.S.A.).[11] The proposed nomenclature of the Chapel Hill Consensus Conference (CHCC) became widely used and was updated last in 2012.[2] The definitions are not designed as sets of diagnostic criteria, but offer a guideline that can be used as a classification system in all types of studies. The CHCC nomenclature categorizes the primary, systemic vasculitides (i.e., vasculitides that are not related to an underlying disease and that involve multiple organ systems) according to the size of blood vessels involved and distinguishes large vessel vasculitis, medium vessel vasculitis, and small vessel vasculitis (Figure 1).



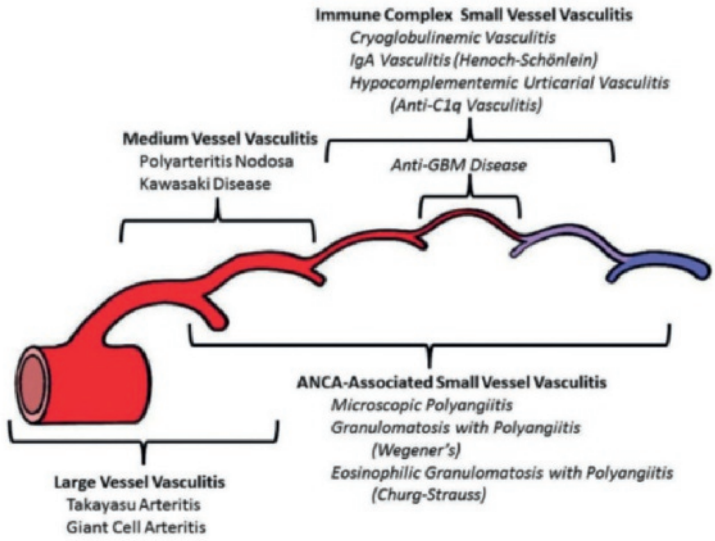


**Figure 1.** Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.

The major disease categories of AAV in addition to GPA are microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss syndrome). Another example of small vessel vasculitis as shown in Figure 1 is anti-GBM disease. This disease is also often referred to as Goodpasture's syndrome or Goodpasture's disease. However, most experts preserve the name Goodpasture's syndrome for the combination of glomerulonephritis and pulmonary hemorrhage, irrespective of the etiology, and the term Goodpasture's disease for these two clinical features in the presence of anti-GBM antibodies. Ernest Goodpasture was the first to report a patient with pulmonary-renal syndrome in 1919; however, it was unknown whether this patient had anti-GBM antibodies.[12] For the purpose of describing the

disease more precisely, it was suggested to replace the eponym by anti-GBM disease.[13] Nevertheless, Goodpasture's syndrome or disease are still frequently used names.

## Presentation

The first symptoms of the patients with AAV in the historical and the contemporary perspective were general, non-specific symptoms, such as fever, general malaise, and weight loss. In both cases, these symptoms were considered to result from an infection, and the patients were treated accordingly. However, other complaints emerged over time, originating from ear-nose-throat region and/or lower respiratory tract. In the historical case, the nose problems even progressed to the development of a saddle nose deformity due to collapse of the nasal septum. Another manifestation of the disease in both cases was the deterioration of renal function.

The majority of patients with AAV present with 'flu-like' symptoms, making it difficult for the physician to diagnose AAV. During progression of the disease, symptoms tend to become more specific and are confined to organ systems. The kidneys are vulnerable targets of the disease, and renal damage leads to hematuria and/or proteinuria. Ninety percent of patients with MPA, 80% of patients with GPA, and 45% of patients with EGPA have renal involvement (Table 1).[14] Ear, nose, and throat problems can present as hearing loss, otalgia, (bloody) rhinorrhea, sinusitis, nasal crusting, and recurrent otitis media. The respiratory tract can be affected, presenting as e.g. dyspnea, hemoptysis, and/or pleuritic pain. The pulmonary inflammation can be found by chest radiography, which shows nodules, infiltrates, or cavitations. Musculoskeletal symptoms are frequently reported manifestations, such as arthralgia or myalgia.[15,16] Other organ systems often affected by the disease are the skin, nervous system, and gastrointestinal tract (Table 1).

**Table 1.** Approximate frequency of organ-system manifestations in AAV[14]

| Organ system | MPA | GPA | EGPA |
|---|---|---|---|
| Cutaneous | 40 | 40 | 60 |
| Renal | 90 | 80 | 45 |
| Pulmonary | 50 | 90 | 70 |
| Ear, nose, and throat | 35 | 90 | 50 |
| Musculoskeletal | 60 | 60 | 50 |
| Neurologic | 30 | 50 | 70 |
| Gastrointestinal | 50 | 50 | 50 |

Numbers are percentages of patients with the type of AAV.

## Diagnosis: the role of ANCA

In the historical case, chest X-ray identified infiltrates, and urine analysis revealed proteinuria and hematuria. The diagnosis of WG was established at autopsy, creating an explanation for these radiographic and laboratorial findings. In the contemporary case, imaging was performed with a CT-scan of the lungs, showing non-cavitating nodules, and urine analysis also showed proteinuria and hematuria. The diagnosis was established earlier than in the historical case description, presumably as a result of the positive ANCA test.

The detection of AAV has improved dramatically since the discovery of ANCA. In 1982, Davies and colleagues were the first to describe serum antibodies that stained the cytoplasm of neutrophils by indirect immunofluorescence (IIF) in patients with glomerulonephritis.[17] However, this finding did not receive much attention until three years later when the hallmark paper of van der Woude *et al*. was published. This group of Dutch and Danish investigators showed that anticytoplasmic antibodies (ACPA) were specific for GPA and suggested that these autoantibodies could serve as a diagnostic and prognostic marker.[18] Once ACPA were known to be directed against neutrophils (and monocytes), the name ANCA became to be used synonymously. In 1988, Falk and Jennette showed that ANCA can also have a perinuclear staining pattern.[19] In the same period, studies using enzyme-linked immunosorbent assays (ELISA) demonstrated two major antigen specificities in ANCA: myeloperoxidase (MPO)[19] and proteinase 3 (PR3).[20] Subsequent studies found a close association between the presence of ANCA and GPA, MPA, and EGPA; approximately 90% of the patients with clinical and histopathological signs of AAV are positive for MPO- and/or PR3-ANCA.[14] From this discovery onwards, the diagnostic delay in AAV shortened substantially. This is illustrated by the finding that patients in the 1990s were diagnosed on average three months earlier than patients who presented in the 1970s or 1980s.[21]

The results obtained by IIF and ELISA are strongly associated. Perinuclear ANCA (P-ANCA) is in approximately 90% of patients directed against MPO, and cytoplasmic ANCA (C-ANCA) is in approximately 90% of patients directed against PR3. These specificities are related to type of AAV; most patients with GPA have PR3-ANCA (or C-ANCA), whereas most patients with MPA or EGPA have MPO-ANCA (or P-ANCA).[22] IIF is a more sensitive test, and ELISA is a more specific test.[15] Usually, it is recommended that both IIF and ELISA are performed and reported.[23] ANCA negativity does not rule out a diagnosis of AAV, since 10% of patients with typical GPA or MPA are negative for ANCA by using conventional assays. Moreover, other conditions are also associated with positivity for ANCA, such as inflammatory bowel syndrome, infections with hepatitis C virus or HIV, rheumatoid arthritis, and anti-GBM disease. Therefore, it is evident that a diagnosis of AAV cannot solely rely on ANCA test results.

## Diagnosis: the role of biopsies

The patient from the historical perspective did not undergo a biopsy. Examination of his tissue at autopsy revealed several important signs of the disease, such as granulomas in the lungs and necrotizing glomerulitis. In the contemporary case, a nasal and renal biopsy were performed. The nasal biopsy showed acute inflammation with necrosis, and the renal biopsy showed crescentic glomerulonephritis with a pauci-immune immunofluorescence pattern.

The procedure of performing biopsies has improved in the 20th century. In 1951, the Danish physicians Poul Iversen and Claus Brun were the first to describe the currently used method involving needle renal biopsy.[24] Before that moment, kidney tissue could only be obtained from open surgery. With the needle renal biopsy method, taking a biopsy became safer and thus, could be used more frequently in diagnostic processes. Although ANCA testing contributes in expediting the diagnostic process of AAV, histopathological examination remains the golden standard to establish a diagnosis of AAV. The tissue biopsies at the site of active disease are characterized by necrotizing inflammation. Biopsies from the skin, nose, and sinus have a low sensitivity. Open lung biopsy yields high sensitivity, but is a very invasive procedure. A renal biopsy is safer and has a similar sensitivity; therefore, it is often performed in AAGN.[25] Typical lesions found on renal biopsy are fibrinoid necrosis and crescents. GPA can be distinguished from other types of AAV by the presence of granulomatous inflammation, generally in the absence of asthma; EGPA is characterized by granulomatous inflammation as well, but in the presence of asthma and eosinophilia; in MPA, granulomatous inflammation is absent and asthma is infrequent.[14] However, granulomatous inflammation is often not detected in GPA and EGPA. Fortunately, the distinction between GPA and MPA is not relevant for therapeutic purposes, since patients are not treated differently. Next to the advances in the performance of biopsies, several histopathological techniques were developed during the 20th century, such as the immunofluorescence technique. The location of antibodies can be visualized on biopsy samples by using immunofluorescent staining techniques. The renal biopsy in AAV has a weak or absent staining pattern on immunofluorescence, hence the name pauci-immune glomerulonephritis.[26] In other small vessel vasculitides, immune complexes are often present, for instance the linear immunoglobulin G staining along the GBM in anti-GBM glomerulonephritis.

## Epidemiology

Both case descriptions are illustrative of the clinical course of the rare autoimmune disease GPA. The patient from the historical perspective lived in New York, and the patient from the contemporary perspective lived in the Netherlands. The patients had similar ages at disease presentation. The differences between the historical and the contemporary perspective regarding epidemiology is the knowledge gained about this topic; in the period of historical perspective, little was known about the epidemiology

of AAV, whereas in the contemporary perspective, knowledge of epidemiology has increased substantially.

AAV has a slight male predominance, and the incidence increases with age. Multi-ethnic studies have shown that patients with Caucasian ethnicity are more likely to develop AAV.[27] Before the discovery of ANCA in the 1980s, epidemiological studies were hampered by, among other things, lack of classification of vasculitides. There were very little data on incidence and prevalence of AAV. One study found an increased incidence of GPA and MPA in the 1980s; early in the 1980s, the incidence was 1.5 per million, which increased to 6.1 per million by the end of the 1980s.[28] This increment is most likely the result of increased awareness among physicians and routine ANCA testing.[27] In recent studies, the annual incidence rate of AAV in Europe and Northern America is reported to be 20 per million. A geographical difference is observed between different regions in Europe; the incidence of GPA is higher in the northern part of Europe, whereas MPA dominates in the southern regions.[29] Moreover, epidemiological studies from Asia report incidences similar to those in Europe. Interestingly, MPO positivity and MPA is much more common in Asia.[30] These geographical differences may reflect genetic variation between populations and/or may be the result of environmental differences. EGPA is more rare than GPA and MPA; reported incidences range between 1.3 and 6.8 per million per year.[31]



## Pathogenesis

In the article from which the historical case description was derived, Fahey *et al*. hypothesized that WG was a disease of hypersensitivity.[4] In the contemporary case description, the disease is still recognized as a hypersensitivity reaction, but the exact pathogenesis remains to be elucidated. Current research aims to further investigate the pathogenetic pathways in AAV, because up to now, these pathways are partly hypothetical.

The hallmark in the clarification of the pathogenesis was the discovery of ANCA, which is generally considered to be the pathogenic agent in AAV.[32,33] The hypotheses on the etiology of ANCA production have not been fully proven; however, it is widely assumed that the production of ANCA is caused by interplay between both genetic and environmental factors.[7] Several genetic variants are associated with AAV, of which variants in *HLA-DPB1*0401* and the Z allele for alpha-1 antitrypsin are most strongly associated with AAV. Interestingly, subdivision according to ANCA serotype (PR3- and MPO-ANCA) has a stronger genetic basis than subdivision according to clinical diagnosis (GPA and MPA).[34] In addition to genetic predisposition, several studies found associations between the production of ANCA and specific environmental factors. Among these, silica exposure, antithyroid and antihypertensive drugs, and several microbial agents, in particular Staphylococcus Aureus, have been suggested to play a role.[35] In addition to the presence of ANCA, it is generally accepted that a 'second hit' is required in the pathogenetic pathway of AAV. This second hit comprises the priming of neutrophils with

e.g., TNF-α or complement component 5a (C5a), resulting in expression of MPO or PR3 at the cell surface. Circulating ANCA bind to these autoantigens, leading to neutrophil activation. Activated neutrophils release damaging factors and activate the complement system, causing inflammation of the vessel wall.[7] Less is known about the pathogenesis of EGPA, but it is considered to be quite different from the pathogenesis of MPA and GPA. Only 30-40% of the patients with EGPA are ANCA positive. Therefore, in addition to clinical differences, it remains a matter of debate whether EGPA should be clearly distinguished from MPA and GPA.[25]

### Treatment

The patient from 1933 was not adequately treated for AAV, but one of the other cases described by Fahey *et al*. was the first reported patient with WG who was treated with cytotoxic chemotherapy in the form of nitrogen mustard.[4] Of the seven cases described by Fahey *et al.*, this patient was the only case who had long-term survival. Before the publication of Fahey *et al*. in 1954, other treatments such as antibiotics, chelating agents (EDTA) and local radiotherapy were reported. The patient in the contemporary description was treated with corticosteroids, low dose cyclophosphamide, and rituximab, i.e. a monoclonal antibody against B-cells. A number of immunosuppressive agents, such as rituximab, were proven effective in AAV in the period between the historical and contemporary case. The use of different therapies was based on case reports in the early beginnings, but these have expanded to large, international, randomized clinical trials nowadays. Based on results from these clinical trials, international guidelines have been established.

Around the same time Fahey *et al*. introduced treatment with alkylating agents, the benefit of corticosteroids in the treatment of AAV also became apparent, followed a couple of years later by promising results with azathioprine.[36,37] The evidence for alkylating agents became firm with the publication of Fauci *et al*. in 1973.[38] They treated 15 patients with oral cyclophosphamide or azathioprine; most of these patients also received corticosteroids. In three patients with very severe disease, cyclophosphamide was given intravenously for approximately one week, followed by oral administration. Thirteen of the 15 patients achieved complete remission. After the publication of this observation, cyclophosphamide became widely used in patients with AAV, leading to a dramatic improvement in outcome. However, it was already known that the benefit of treatment with cyclophosphamide comes at the cost of toxicity including infertility, infections, and malignancies. Therefore, from the start of the use of cyclophosphamide in AAV, there is an ongoing search for the optimal dose and duration of this therapy. Fauci *et al*. recommended to use 1 to 2 mg/kg/day of oral cyclophosphamide, which should be gradually tapered and stopped one year after all signs of disease activity have disappeared.[38] Currently, the initial daily dose of oral cyclophosphamide has not changed; however, the duration of treatment with cyclophosphamide has shortened

substantially. This is largely the result of the landmark paper of Jayne *et al*. in 2003, reporting results from the CYCAZAREM trial conducted within the collaboration of the European Vasculitis Society (EUVAS).[39] All included patients received induction therapy (i.e. induction of remission with initial immunosuppressive therapy) consisting of at least three months of oral cyclophosphamide and prednisolone. After achieving remission within a maximum of six months, half of the patients received maintenance therapy (i.e. therapy for a variable period to prevent relapse) with azathioprine and the other half continued cyclophosphamide therapy for maintenance. Both groups continued to receive prednisolone. The relapse rate in the azathioprine and continued cyclophosphamide group was similar, as was the percentage of patients experiencing a severe adverse event. Jayne *et al.* concluded from these results that cyclophosphamide duration could be safely reduced by switching to azathioprine for maintenance therapy. Another key clinical trial also led by the EUVAS was CYCLOPS, which compared daily oral cyclophosphamide to intravenous cyclophosphamide every two to three weeks.[40] No difference in remission rate and time to remission was observed; however, the advantage of intravenous administration is that patients receive a lower cumulative cyclophosphamide dose. Of note, patients in the CYCAZAREM and CYCLOPS trials all had generalized disease. These patients might respond differently to treatment than patients with less severe disease. Therefore, disease severity should be taken into account when defining the optimal treatment strategy for an individual patient.

Following the European League Against Rheumatism (EULAR) recommendations, patients with localized or early systemic AAV disease may be withheld from treatment with cyclophosphamide; a less toxic regimen consisting of methotrexate and glucocorticoids can suffice in these patients.[41] In patients with rapidly progressive severe renal disease, plasma exchange may be added to conventional therapy.[42] For patients who receive maximal doses of cyclophosphamide, but do not achieve remission or still relapse , alternatives have been sought. Of these, intravenous administrations of immunoglobulins, mycophenolate mofetil, 15-deoxyspergualin, anti-thymocyte globulin, infliximab, and rituximab were approved alternatives in the EULAR recommendations of 2009.[41] After these recommendations, results from two clinical trials on rituximab were published: RAVE and RITUXVAS. The RAVE trial showed a similar remission rate in patients treated with either a cyclophosphamide- or rituximab-based induction regimen. Another interesting finding was superiority of rituximab in patients with relapsing disease.[43] The RITUXVAS trial included patients with generalized disease only and concluded that the sustained remission rate and number of adverse events were similar in patients treated with intravenous cyclophosphamide and patients treated with rituximab.[44] Both trials only included patients with either GPA or MPA. The effectivity of rituximab in EGPA has been studied in a retrospective analysis, showing a remission rate of 49% after 12 months.[45] Given these study results on rituximab, the updated EULAR guidelines from 2016 now recommend treatment with glucocorticoids in combination with either

cyclophosphamide or rituximab as first line therapy in patients with generalized AAV (Figure 2).[46] Other amendments to the 2009 recommendations are the second line option of mycophenolate mofetil in patients with non-organ threatening disease and extended possibilities of maintenance therapy with other options than azathioprine, namely rituximab, methotrexate or mycophenolate mofetil. These amendments were a result of increased evidence provided by clinical trials and retrospective analyses.

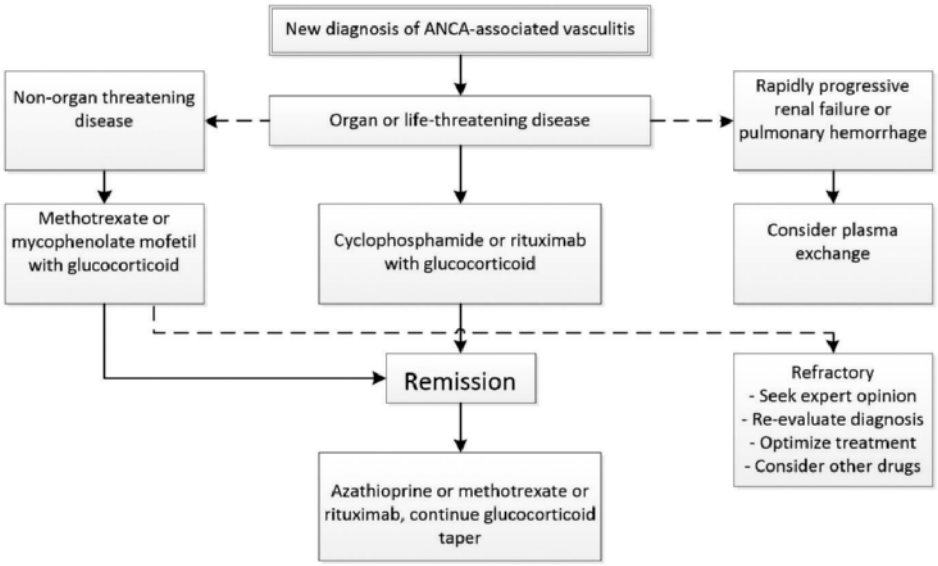


**Figure 2.** Algorithm to describe the management of patients with a new diagnosis of AAV.[46]

## Outcome

The difference in patient outcome between the historical and contemporary case is striking. The patient in 1933 died most likely as a consequence of untreated disease, whereas the patient in 2016 was treated successfully and achieved remission of the disease. Although this remission is a favorable status, the patient is still at increased risk of death. Moreover, the patient is at risk to develop ESRD, because he has renal involvement. This risk is estimated to be 24% at five years of follow-up given the finding that the renal biopsy contained 50% or more cellular crescents.[47] The development of ESRD has an enormous life-impact as it requires renal replacement therapy, i.e. dialysis or renal transplantation.

As illustrated in the historical case description, untreated GPA is fatal; the mortality is 80% at one year follow-up.[48] This mortality rate is similar for patients with MPA, whereas EGPA is considered to have a milder clinical course, because renal involvement is less common (Table 1). Since the introduction of treatment with cyclophosphamide and corticosteroids, the high mortality rate has decreased tremendously; nowadays, the

1-year mortality rate in GPA and MPA is approximately 12%.[48] In patients diagnosed with EGPA, the 1-year mortality rate is reported to be 4%.[49] Thus, AAV has been converted from being almost always fatal to a chronic illness with a slightly higher mortality risk compared to the general population.[21,48] As a consequence, research on AAV started to investigate long-term outcomes with a focus on disease control and side-effects of therapy. Predictors of patient outcomes have been widely studied, and knowledge of these predictors will be extended from the work presented in this thesis. The next section will summarize the current knowledge on long-term outcome in patients with AAV.



## PROGNOSIS – THE CURRENT STATE

### Patient survival

The prognosis of patients with AAV consists of several outcome parameters. Obviously, patient survival is the most important parameter. As previously discussed, patient survival has increased dramatically since the introduction of cyclophosphamide therapy in AAV. Nevertheless, the disease is associated with an excess risk of mortality compared to the general population.[48] The main causes of death differ between phases of the disease. During the first year after diagnosis, most deaths are caused by infections or active vasculitis. After this period, infections remain major contributors to mortality together with cardiovascular disease and malignancies.[48,49] In a study from 1996, the risk of death was associated with pulmonary hemorrhage, C-ANCA positivity, and treatment with corticosteroids in the absence of cyclophosphamide.[50] A more recent study showed that advancing age, estimated glomerular filtration rate (eGFR) <15 ml/min, and higher Birmingham Vasculitis Activity Score (BVAS [i.e. a clinical tool to evaluate disease severity]) are predictors of death.[48] Patients with eGFR <15 ml/min are categorized as having ESRD. These patients have a three-fold increased risk of death compared to patients with no or mild renal involvement. Therefore, renal involvement in AAV is extremely important with respect to morbidity and mortality.

### Renal survival

Renal survival is another outcome parameter in the prognosis of patients with AAV. It is usually defined as the time between the date of diagnosis and the occurrence of ESRD, requiring dialysis and/or transplantation. Fortunately, the risk of developing ESRD at 5-year follow-up has significantly decreased from 61% in 1985 to 30% in 2009.[51] Most recent data showed that ESRD still occurs in approximately 20-25% of patients with AAV within a few years after diagnosis.[52] ESRD causes serious health problems and reduces quality of life; in patients with kidney disease who develop ESRD, quality of life is significantly poorer compared to patients with other chronic diseases.[53] It is important to identify patients who are prone to develop ESRD, as they might need more aggressive therapy to prevent this event. Therefore, a substantial number of studies

investigated prognostic markers for the development of ESRD. One of the most consistent findings is the correlation between renal function at presentation and renal survival. Even after adjustments for age, race, ANCA specificity, and pulmonary involvement, renal function in terms of serum creatinine or eGFR remains the strongest predictor for the occurrence of ESRD.[50] Some studies also found proteinuria to be a marker of kidney disease progression.[54-57] Most patients with AAGN present with proteinuria, but the amount is quite variable, ranging from slightly elevated protein loss to nephrotic range proteinuria. Since little is known about proteinuria in AAGN, the symptom was investigated in one of the studies presented in this thesis (chapter 5). In addition to eGFR and proteinuria as predictors of ESRD, a Swedish study reported a 2.6-fold increased risk of ESRD for MPO-ANCA positive patients compared to PR3-ANCA positive patients after adjusting for sex, age, and serum creatinine level at diagnosis.[58] Similarly, a study including 735 patients who participated in one of the EUVAS clinical trials found that patients with MPA are more likely to develop ESRD than patients with GPA.[59]

In addition to clinical parameters, the prognostic importance of histopathological parameters in renal biopsies of patients with AAGN has been demonstrated in a number of studies. In 1996, a scoring form was designed by a group of pathologists called the Renal Histology (RENHIS) group in order to quantify the histopathological features in AAGN.[60] This scoring form became standard for evaluating the diagnostic renal biopsies of patients in the EUVAS trials. Several studies investigated the prognostic significance of the scored parameters in biopsies from patients in the EUVAS clinical trials.[61-63] These studies stressed the importance of the percentage of normal glomeruli (i.e. glomeruli without light microscopic lesions) as this parameter was most strongly associated with renal function during follow-up. This finding was also reported in other studies which were not conducted by the EUVAS.[64,65] In 2010, as a result of these and other studies investigating the renal biopsy in AAGN, Berden *et al*. proposed a histopathological classification in order to predict renal outcome.[47] This classification system is based on glomerular lesions and distinguishes four classes: focal, crescentic, mixed, and sclerotic class biopsies (Figure 3). To validate this classification, Berden *et al*. analyzed biopsies of 100 patients and found that the classes correlated to the degree of renal function at 1- and 5-year follow-up. In multivariate analysis, eGFR at baseline and the classification were the only significant predictors of eGFR at 1- and 5-year follow-up. Moreover, renal survival was also associated with the classes; the 5-year renal survival rate was 93% for the focal class, 76% for the crescentic class, 61% for the mixed class, and 50% for the sclerotic class. The addition of tubulointerstitial parameters did not meaningfully increase the predictive value of the classification and only increased its complexity. Subsequently, centers from different continents validated the histopathological classification of AAGN.[66] The favorable outcome in the focal class and the poor outcome in sclerotic class were recognized in all studies. However, differences between studies were noticed on the outcome in crescentic and mixed classes. Some studies suggested that the inclusion of

tubulointerstitial parameters could solve this discrepancy.[67,68] In order to improve the histopathological classification, we performed a large international validation study and a meta-analysis. The results are presented in this thesis (chapter 3).

Renal survival in patients with anti-GBM glomerulonephritis is much poorer compared to that of patients with AAGN; over half of the patients with anti-GBM glomerulonephritis require dialysis at the time of presentation, and approximately two-third of patients develop ESRD during follow-up.[69] The development of ESRD is strongly associated with renal function at presentation. Histopathologically, a high percentage of crescents is associated with a poor renal outcome in anti-GBM glomerulonephritis.[70] To a large extent, the histopathological parameters in anti-GBM glomerulonephritis are similar to AAGN. However, crescent formation is usually more extensive in anti-GBM glomerulonephritis.[71] A histopathological classification predicting renal outcome does not exist in anti-GBM glomerulonephritis. In chapter 4 of this thesis, renal biopsies of patients with anti-GBM glomerulonephritis were evaluated by the application of the histopathological classification of Berden *et al*.



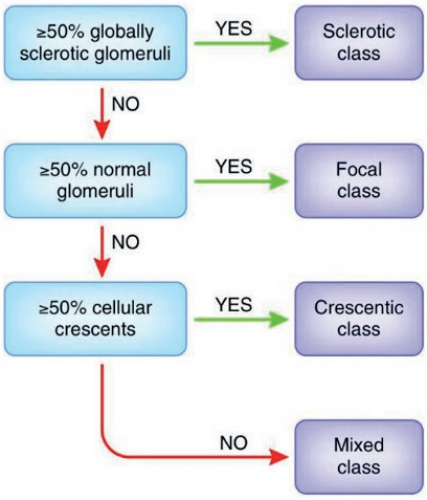


**Figure 3.** The histopathological classification of ANCA-associated glomerulonephritis.[47]

## Relapses

Despite the advances made in recent years, the risk of relapse has not changed in patients with AAV.[51] Relapses are generally described as the recurrence of signs or symptoms of active vasculitis in any organ system after the achievement of remission.[72] However, the precise definition of a relapse varies across studies. Some studies defined relapse as reappearance of BVAS≥0, others as reappearance of BVAS≥1. Relapse has also been defined as reappearance of the disease requiring immunosuppressive therapy, or as a substantial rise in ANCA titer.[73] The risk of relapse differed enormously between studies,

ranging from 10 to 60%.[72-74] This might be due to the differences in patient cohorts, treatments, but also in definitions of relapses. Identifying risk factors associated with the occurrence of relapses is extremely important; patients at high risk may need more intensive maintenance therapy, while patients at low risk may be spared from exposure to immunosuppressive agents. PR3-ANCA positivity, lung involvement, and upper respiratory tract involvement were found to be associated with an increase in relapse risk.[72] As these features are more often present in patients with GPA, the risk of relapse is higher in patients with GPA compared to patients with MPA.[74] Moreover, once a patient has experienced a relapse, the risk to develop another relapse is greater than in a patient who has never relapsed. Nasal carriage of Staphylococcus Aureus was also found to be a risk factor for disease relapse.[75] It has been reported that a rise in ANCA titer can predict the occurrence of a relapse; however, the titer does not consistently predict relapse and is therefore limited in defining the therapeutic strategy.[76] Regarding histopathological parameters in diagnostic renal biopsies, sclerotic class and absence of interstitial infiltrates were shown to increase the risk of a renal relapse.[77]

### Adverse events

The occurrence of adverse events is another aspect of prognosis in addition to patient survival, renal survival, and relapses. Adverse events can be related to the aggressive therapy that patients with AAV usually receive. Cyclophosphamide has been first choice for induction therapy for decades, but comes at a cost of worrisome adverse events, including infections, alopecia, reproductive abnormalities, and malignancies. The risk of malignancy in patients with AAV has been extensively investigated in retrospective cohort studies. All these studies showed an increased risk of malignancy in patients with AAV compared to the general population.[78] In the early 1990s, the risk of bladder carcinoma was reported to be 33-fold increased, and the risk of lymphoma was reported to be 11-fold increased.[16] In later studies, the malignancy risk was demonstrated to be cyclophosphamide dose-dependent.[79,80] In 2003, the CYCAZAREM trial was published, leading to widespread use of lower cumulative cyclophosphamide doses.[39] Consequently, it was demonstrated that the malignancy risk after 2003 was no longer increased except for non-melanoma skin cancers.[81] In 2016, rituximab was added as first choice option to induce remission in patients with AAV. The follow-up data from the RAVE and RITUXVAS clinical trials indicated similar rates of adverse events between cyclophosphamide- and rituximab-treated patients.[82,83] However, long-term follow-up data on patients with rituximab is lacking, and therefore, risks of late occurring adverse events such as malignancies are mainly unknown. The malignancy risk in rituximab-treated patients is investigated in chapter 6 of this thesis.

It has also been suggested that the increased malignancy risk in patients with AAV is caused by an intrinsic higher risk to develop a malignancy, regardless of therapy. This could possibly be explained by a shared pathogenetic pathway in malignancy and AAV.

In order to investigate this hypothesis, some studies investigated the malignancy risk of patients with AAV prior to or simultaneous with their diagnosis of AAV.[84-86] Some studies found an increased malignancy risk prior to AAV diagnosis, whereas another study did not. Therefore, this question required further investigation and is studied in this thesis (chapter 7).

## THESIS OUTLINE

The work performed by the RENHIS group has extended our knowledge of the predictive value of the diagnostic renal biopsy in patients with AAV. The course and results of their work in the past 25 years are summarized in **chapter 2** of this thesis. The studies performed by, among others, the RENHIS group have resulted in the establishment of a histopathological classification of AAGN. In **chapter 3**, an analysis is described including 145 patients to validate this classification system in an international cohort. Moreover, this chapter includes a meta-analysis comparing the outcomes in crescentic and mixed class. The predictive value of the histopathological classification was also investigated in 123 patients with anti-GBM glomerulonephritis. The results of this study are reported in **chapter 4**. Previous studies showed that proteinuria is a prognostic marker in AAGN. In **chapter 5**, we investigated podocyte foot process effacement and podocyte number in relation to proteinuria at baseline and during follow-up. In **chapter 6**, the malignancy risk after the diagnosis of AAV was investigated in relation to therapeutic regimen, i.e. cyclophosphamide and rituximab. Finally, the malignancy risk of patients with AAV prior to the diagnosis of AAV was explored in **chapter 7**, with the aim to further elaborate on the putative association between these two conditions. The results of the studies described are summarized and discussed in **chapter 8**.

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# CHAPTER 2

# Twenty-five years of RENHIS: a history of histopathological studies within EUVAS

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## ABSTRACT

In the early 1990s, an international working group of experienced renal pathologists, the Renal Histology group, set up a scoring system for biopsies with anti-neutrophil cytoplasmic autoantibody (ANCA)-associated glomerulonephritis. This scoring system subdivided glomerular, interstitial and vascular lesions and served as a tool for the evaluation of all renal biopsies from studies of the European Vasculitis Study Group (EUVAS). Histopathological studies gave new insights into the prediction of renal outcome in patients with ANCA-associated glomerulonephritis. Percentage of normal glomeruli and a selected number of interstitial parameters were reliable predictors of long-term follow-up glomerular filtration rate in all studies. Out of these results, a histopathological classification distinguishing focal, crescentic, mixed and sclerotic classes of ANCA-associated glomerulonephritis was developed. Until today, 13 studies have validated this classification system. Future studies will try to determine if and how renal histology could be helpful in guiding treatment of ANCA-associated glomerulonephritis.

## EARLY BEGINNINGS

While the European Community/Bureau Centrale de Référence (EC/BCR) study was being conducted by Fokko van der Woude at Leiden University Medical Centre, a medical student with an interest in renal pathology was invited to assemble renal biopsies obtained from patients enrolled in this study. Ingeborg Bajema, supervised by Jan Anthonie Bruijn and Chris Hagen, took this task upon her in an era where there was no e-mail, just phones and faxes. She got in touch with the 12 European centers from which patients were enrolled and eventually collected 193 renal biopsy specimens. In the meantime, a group of pathologists among whom were Franco Ferrario, Laure-Hélène Noël, Rüdiger Waldherr and Jan Anthonie Bruijn [today known as the Renal Histology (RENHIS) group] came together in order to set up a scoring system for biopsies with anti-neutrophil cytoplasmic autoantibody (ANCA)-associated glomerulonephritis. Anecdotically, it is often mentioned that the first issue of debate concerned the total number of glomeruli on which there was considerable disagreement. Should incomplete glomeruli be scored, and how incomplete could they be in order to be counted? Should multiple levels and stainings be taken into consideration to establish the total number of glomeruli, or should an encircled specimen be scored by all pathologists disregarding other levels? A form was devised subdividing glomerular, interstitial and vascular lesions, with the possibility to score separate lesions in a quantitative manner. This form, together with a study on the inter- and intraobserver variability among the pathologists, was published in 1996.[1] Results showed that overall, interobserver agreement was good, but pathologists were doing better when they had to deal with quantitative data than when they had to score dichotomous data, i.e. deciding for a specific lesion whether it was present or absent. The explanation given for this difference is that for quantitative data, which usually

referred to glomerular lesions that were scored per glomerulus, pathologists were forced to systematically evaluate each glomerulus in the biopsy. Obviously, this would bring their scores closer together than in the case of dichotomous data (typically used for interstitial lesions), which were scored only once. The fact that definitions for lesions and how to score their extensiveness are important here is obvious, and over the past 25 years, the scoring form and the definitions for various renal lesions were fine-tuned many times. Still, it was decided that all renal biopsies enrolled in the various studies would have to be scored by two pathologists followed by consensus meetings in which the final score would have to be decided upon. In this way, all renal biopsies from the European Vasculitis Study Group (EUVAS) studies obtained a standardized evaluation based on the consensus of experienced renal pathologists.

The first study for which the results from the RENHIS group were used in a clinicopathological analysis was the EC/BCR study.[2] An important finding, which would later be reproduced in many other studies, was that the percentage of normal glomeruli in the renal biopsies correlated most significantly with renal function, both at the time of biopsy and during follow-up.[3-5] Moreover, the amount of glomerulosclerosis predicted for renal function at 1 year after the biopsy was taken.[2] These ends of the spectrum, representing the preserved and the irreversibly chronically affected parts of the kidney, turned out to be important parameters for patients' prognosis in ANCA-associated glomerulonephritis, in part irrespective of the characteristic glomerular lesions such as crescents and fibrinoid necrosis. Furthermore, in a spin-off study from the main clinicopathological study, it was shown that the renal granuloma was a relatively infrequent finding in the renal biopsy (only present in 16 out of 157 patients) not related to renal function and very diverse in its histomorphology.[6] The aforementioned results lie at the basis of the later established classification of ANCA-associated glomerulonephritis, in which the focal and the sclerotic classes represent both ends of the spectrum.[7] Apart from glomerular lesions, diffuse interstitial infiltrates and tubular lesions (necrosis and atrophy) were also related to renal outcome.[8,9] Today, it is debated if and how interstitial lesions should be incorporated into the histopathological classification system for ANCA-associated glomerulonephritis.



## RENAL HISTOLOGY STUDIES FROM CYCAZAREM, MEPEX AND RITUXVAS

Treatment regimens in the EC/BCR study were not standardized and, therefore, an analysis of the effect of therapy on renal outcome in combination with the histopathological data was not performed. In subsequent studies from which renal biopsies were evaluated, standardized treatment, mostly in two treatment arms, was given. Two studies following the EC/BCR study in which treatment was standardized were the CYCAZAREM and the MEPEX studies.[10,11] These studies were conducted in parallel. The inclusion criterion for

the CYCAZAREM study was mainly based on a serum creatinine level of <500 µmol/L at entry, whereas the MEPEX study included patients with a serum creatinine level of >500 µmol/L. Consequently, the whole spectrum of renal involvement is represented in patients from these two studies, 292 in total, of whom 269 representative renal biopsies were collected. A number of clinical histopathological studies emerged from these two studies, with Hauer and De Lind van Wijngaarden in the lead.

The predictive value of clinical and renal histological features for renal outcome from 96 patients in CYCAZAREM, of whom renal biopsies were available, was reported first.[3] End points included renal function at 18 months and the occurrence of relapse or death. Results showed that renal function at the time of biopsy and predominantly chronic renal lesions (glomerulosclerosis and interstitial fibrosis and tubular atrophy) were potent predictors of renal function at 18 months after study entry. Parameters that most strongly correlated with estimated glomerular filtration rate (eGFR) at 18 months after correction for the baseline GFR were segmental and cellular crescents and fibrinoid necrosis. Interestingly, none of the clinical and histological features predicted the occurrence of relapse or death.

The predictive value of clinical and renal histological features for renal outcome from 100 patients in MEPEX, of whom renal biopsies were available, was reported secondly.[8] End points included renal function at the time of diagnosis, 12 months after diagnosis, dialysis at entry and 12 months after diagnosis, and death. Normal glomeruli were a positive predictor of dialysis independence and improved renal function after 12 months, indicating that the unaffected part of the kidney is vital in determining renal outcome. Both chronic and acute tubulointerstitial lesions predicted eGFR at 12 months. Fibrous crescents were predictive of dialysis at entry. No parameter predicted death. In a substudy investigating in more detail the chances of recovery for those patients who are dialysis dependent at diagnosis, it was found that even with ominous histologic findings, the chance of renal recovery exceeded the chance of therapy-related death when patients were treated with plasma exchange as adjunctive therapy.[12]

In a spin-off study, data from the CYCAZAREM and MEPEX trials were used to gain more insight into the pathogenesis of vasculitis. Investigating histological differences between patients with MPO-ANCA and PR3-ANCA, Hauer *et al*. found that chronic lesions were more abundantly present in MPO-ANCA-positive patients than in PR3-ANCA-positive patients, favoring the idea of different pathways in the pathogenesis of MPO-ANCA and PR3-ANCA vasculitides.[13] Besides gaining insights into the pathogenesis of ANCA-associated glomerulonephritis, MPO- and PR3-ANCA can be important outcome predictors. MPO-ANCA-positive patients present with worse renal function and have less recovery of renal function than PR3-ANCA-positive patients. Therefore, MPO-ANCA-positive patients more often reach end-stage renal disease (ESRD).[14]

More recently, the association between renal outcome and histopathological lesions was studied in 30 patients from the RITUXVAS trial.[9] In this trial, patients were treated in

the induction phase with a rituximab-based regimen instead of the standard treatment with cyclophosphamide. In rituximab-treated patients, tubular atrophy and T cell tubulitis were the most important predictors of eGFR at 12 months.

## TOWARD A HISTOPATHOLOGICAL CLASSIFICATION FOR ANCA-ASSOCIATED GLOMERULONEPHRITIS

Combining the results from previous EUVAS studies, a first attempt was made towards a formula representing the most robust parameters to predict for renal outcome 1 year after disease onset. Vergunst *et al*. incorporated data of 160 patients from MEPEX and CYCAZAREM and established the following formula to calculate GFR at 1 year after the biopsy: 36.96 + 0.65 × (GFR at t = 0) + 10.52 (if normal glomeruli present) + 7.72 (if fibrinoid necrosis present) − 0.42 × age.[15] This formula was based on data showing that renal function at the time of biopsy was the best predictor for renal function 1 year later, but combining renal function with percentage of normal glomeruli, the amount of fibrinoid necrosis and patient's age could effectively explain for 60% of the variation in renal function 1 year after disease onset. ANCA subset was no independent predictor for renal outcome.

To the purpose of summarizing the most important data from the renal biopsy more succinctly, in order to give an indication about long-term renal outcome, a histopathological classification for ANCA-associated glomerulonephritis was launched in 2010.[7] Four classes were distinguished, named the focal, crescentic, mixed and sclerotic classes. Renal survival during long-term follow-up could be predictive on the basis of these four categories, since the order of categories (focal, crescentic, mixed and sclerotic) corresponded to the severity of renal function loss.[7] Since 2010, 13 studies have validated the classification system. These studies came from Japan, China, Australia, the United States, the Netherlands, Turkey, Canada, the United Kingdom and India[16-28] and confirmed that the classification system is of predictive value for renal outcome.[29] Slightly conflicting outcomes are noticed with regard to crescentic and mixed class biopsies. In some studies,[16-20,22] the outcome of patients with a crescentic class renal biopsy is worse or similar to that of patients with mixed class renal biopsies, which is in contrast to the original validation study.[7] An overview of the differences between the validation studies and the primary study is provided in Table 1. Although further studies are needed to address these differences in more detail, one suggestion is that interobserver variability in the evaluation of the crescentic lesion may account in part for this discrepancy. Crescentic class ANCA-associated glomerulonephritis is defined by a majority of glomeruli with cellular crescents, leaving out those crescentic lesions that are fibrocellular or fibrous. Given the relatively low interobserver agreement amongst pathologists for evaluating ANCA-associated glomerulonephritis as recently reported by Ford *et al*.,[21] it is possible that the difficulty to distinguish between cellular crescents

(characteristic of a crescentic class) and fibrocellular and fibrous crescents (which would more likely be found in the mixed class) has led to these discrepant results.

**Table 1.** Outcomes differences between validation studies and the primary study by Berden et al.[7]

| Study | Population | Outcome difference |
|---|---|---|
| Iwakiri *et al.*[16] | 102 Japanese patients, mostly MPO-ANCA positive | No significant differences in eGFR at 1-year among crescentic, mixed and sclerotic class. No significant difference in probability of progressing to ESRD between crescentic and mixed class. |
| Togashi *et al.*[17] | 54 Japanese patients, all MPO-ANCA positive | No significant difference in eGFR at entry and follow-up between crescentic and mixed class. Higher probability of progressing to ESRD in crescentic than mixed class. |
| Muso *et al.*[18] | 87 Japanese patients, mostly MPO-ANCA positive | Slightly better renal survival in mixed than crescentic class. |
| Chang *et al.*[19] | 121 Chinese patients, mostly MPO-ANCA positive | Higher probability of progressing to ESRD in crescentic than mixed class. |
| | | No difference in eGFR at follow-up and 5-year renal survival between crescentic and mixed class. Subdividing these classes on the basis of % normal glomeruli showed that patients with >25% normal glomeruli had a significantly better renal survival. |
| Hilhorst *et al.*[20] | 164 Dutch patients, only 1 biopsy classified as sclerotic | No difference in eGFR at follow-up and 5-year renal survival between crescentic and mixed class. Subdividing showed that patients with >25% normal glomeruli had a significantly better renal survival. |
| Ford *et al.*[21] | 120 Australian patients | No significant difference in eGFR at 1-year and probability of progressing to ESRD among focal, crescentic and mixed class. |
| Ellis *et al*.[22] | 76 American patients | No significant difference in eGFR at 1-year between crescentic and mixed class. No significant difference in renal survival at 1-year between classes. |
| Unlu *et al*.[23] | 141 Turkish patients | Classification predicted dialysis requirement in the log-rank test, but not in the Cox regression model. |
| Nohr *et al.*[24] | 67 Canadian patients | No difference with primary study. |
| Quintana *et al*.[25] | 136 Spanish and British patients | No significant difference in ESRD between crescentic and mixed class. |
| Tanna *et al*.[26] | 104 British patients | No significant difference in outcome between mixed and crescentic class. No significant differences in renal function at follow-up among classes in multivariate analysis. |
| Naidu *et al.*[27] | 73 Indian patients, much lower mean age | No significant differences in improvement rates and probability of progressing to ESRD among classes. |
| Noone *et al*.[28] | 40 Canadian children, mixed and crescentic class were combined for analysis | No difference with primary study. |

## CONTEMPLATIONS

In the majority of our studies, we investigated renal outcome of ANCA-associated vasculitis with respect to histopathological findings of the renal biopsy at disease onset, mostly in relation to clinical parameters at onset. We would like to conclude with a number of considerations on the merits of our studies and make recommendations for future investigations. With respect to the data derived from the renal biopsies, it can be stated that these were in the vast majority of cases not influenced by therapy initiated before the biopsy was taken and therefore reflected the actual state of the disease at study entry. To what extent duration of disease influenced the amount of acute and chronic lesions in the biopsy remains an interesting point of discussion and one that cannot be solved easily by human studies. Especially, in the case of ANCA-associated vasculitis, it is practically impossible to determine the duration of disease before a diagnosis is made because the early symptoms can be non-specific and regarded both by the patient and by the physician as relatively benign. Consequently, in many patients, no further diagnostic measurements are taken until the moment at which more serious symptoms present themselves such as hearing loss, recurrent otitis media, persistent (bloody) rhinorrhea, dyspnea, hemoptysis and hematuria.[30] Although it is likely that duration of disease before diagnosis is highly variable among patients, and therefore, an important contributor to the findings in the renal biopsy, it cannot be ruled out that other factors may be of equal or even greater importance here. To illustrate this point, we refer to recent findings of Xiao *et al*., showing that the genetic makeup in mice greatly determines the percentage of glomeruli with crescents in a model of ANCA-associated glomerulonephritis.[31]

Over the years, it was discussed many times how many glomeruli a biopsy should contain in order to be diagnostic or to be of prognostic relevance. According to a statistical analysis into the predictive value of the renal biopsy,[32] only biopsies with 20 glomeruli or more start to predict with moderate accuracy for the state of the entire organ. But 20 glomeruli is fairly high, and for practical purposes also decisions have to be made on biopsies that are less generous in their material. For some our studies, we set the minimum at 7,[33] for others at 10.[7] In most of the validation studies on the ANCA-associated glomerulonephritis classification, the question of whether to include tubulointerstitial parameters or not has been raised.[18-21,25] Tubular atrophy and tubulitis predict eGFR at 12 months in ANCA-associated glomerulonephritis patients treated with a rituximab regimen.[9] However, adding these parameters to the classification had no significant effect on the prognostic value and were therefore not included in the current classification.[7] The validation study performed by Quintana *et al*. suggested that tubulointerstitial fibrosis enhances the prognostic value of the classification, but this was not analyzed in a multivariate regression analysis.[25] The variability in histologic features that have been found to be of prognostic significance in ANCA-associated glomerulonephritis may be due

to differences in patient demographics or different treatment regimens,[24] and therefore, a worldwide study is needed to solve this issue.

As for prognosis, we ponder upon a number of fundamental questions. First, it is uncertain over which period of time findings from the renal biopsy may be expected to have clinical significance. In our studies, we took various time points for renal function: 1 year, 2 years and for the classification of histopathological lesions even 5 years and more. A graph in which histological class is set out against renal survival[7] seems to show that even during long-term follow-up, renal histology remains to play a role in the prediction of outcome. It has been argued that because of the numerous events that may occur in the meantime, e.g. disease relapses and toxicity of treatment, the predictive values of renal biopsy findings should not be overestimated and confined to 1 or 2 years, but our results seem to be in favor of the counterargument. Second, it may be questioned for which clinical outcome we actually want to predict. Most studies focus on renal outcome in terms of renal function, but some new studies are currently being conducted investigating histology in relation to renal relapse, development of proteinuria and cardiovascular events. For each of these outcome parameters, we should always question the likeness and then the weight of the renal biopsy parameters at entry playing a role.

It is evident that ultimately, we do not only want to be able to predict renal outcome to inform our patients about the future of their health status but also to adjust therapy according to these findings. Given the severe and life-threatening complications of immunosuppressive therapy, it would, for instance, be helpful if in some cases where chronic lesions dominate the histological picture, we could advise that a milder therapy regime could suffice because of the relatively low chances of renal recovery in relation to the risk of complications. This has not been investigated thus far. In fact, our study on histopathological determinants of renal outcome in patients who had severely disturbed renal function at entry showed that age, normal glomeruli, tubular atrophy, intraepithelial infiltrate and GFR at baseline are predictive of eGFR at 12 months. To our knowledge, there are no studies yet that investigate how histology-guided treatment in human native renal diseases could be approached. This is opposed to the setting of renal transplantation, where renal transplant biopsy findings seem to have more direct consequences of the therapeutic regime than in native renal diseases. The relatively low threshold of taking multiple biopsies in renal transplant patients over time probably plays an important role in this issue. It should be realized that because of the abundance of protocolized and for cause biopsies in renal transplantation patients, we have gained a tremendous amount of knowledge on the development of lesions in renal grafts, and we know far more about, e.g. the reversibility of lesions and of their grumbling nature than in many native diseases. Only few studies have reported on repeat biopsies in ANCA-associated vasculitis; in a recent study, it was shown that protocolized biopsies taken ca. 1 year after disease onset overall showed an increase of chronic lesions while some acute lesions were still present, resulting in 11 out of 17 patients in a higher class

of the histopathological classification of ANCA-associated glomerulonephritis in the repeat biopsy in comparison to the biopsy at disease onset.[34] Another study by Hauer *et al*. emphasized the stability of the amount of unaffected glomeruli in repeat biopsies taken for cause.[35] With reference to the genetic study by Xiao *et al*.,[31] it is interesting to speculate that also in humans, genetic background may influence the severity of disease in ANCA-associated glomerulonephritis. However, many clinicians are reluctant to take repeat biopsies in ANCA-associated glomerulonephritis, which is an obstacle for our further understanding of the development of lesions and the possibility of histology-guided treatment in these patients.

## THE PRESENT AND THE FUTURE



Currently, the RENHIS group is evaluating renal biopsies from the recently conducted MYCYC trial and from the ongoing PEXIVAS trial. A website was launched this year that makes it possible for the renal pathologists to score scanned slides and transmit data electronically. These new logistics also facilitate the scoring of biopsies in parallel, glass slides no longer need to be distributed by regular mail and the biopsies will be returned to the participating centers much more rapidly than in the past.

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# CHAPTER 3

# The international validation study of the histopathological classification for ANCA-associated glomerulonephritis: a retrospective cohort study and meta-analysis

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*Submitted*

## ABSTRACT

### Background

In the original validation study for the histopathological classification of ANCA-associated glomerulonephritis, the order of classes from focal to sclerotic corresponded to increasing severity of renal function loss. Subsequent validation studies disagreed on outcomes in crescentic and mixed class. We here present an international validation study driven by the original investigators in combination with a meta-analysis.

### Methods

A total of 145 patients were included from 10 centers worldwide. A group of seven pathologists scored the renal biopsies of these patients according to the histopathological classification for ANCA-associated glomerulonephritis. Tubulointerstitial parameters were evaluated as well. Renal outcome was expressed as eGFR or ESRD. The results from previous validation studies were used for a meta-analysis.

### Results

In the validation study, renal outcome was most favorable in the focal class and worst in sclerotic class. No significant difference was observed in renal outcome between crescentic and mixed class. In multivariable analysis, eGFR at baseline, the histopathological classification, and age were independently associated with eGFR after 5 years. The meta-analysis showed a similar risk of ESRD between crescentic and mixed class.

### Conclusions

The prognostic value of the crescentic and mixed class may be considered the same. The addition of tubulointerstitial parameters did not increase the prognostic value of the histopathological classification. Future research will elucidate whether biopsies that are not showing a focal or sclerotic class, should hither on be regarded as one or whether subclasses are clinically relevant.

## INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) represent a spectrum of diseases characterized by inflammation of small- to medium-sized blood vessels in the absence or paucity of immune deposits.[1] The most common entities of AAV are granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA), while renal-limited vasculitis (RLV) and eosinophilic granulomatosis with polyangiitis (EGPA) occur less frequently.[1] Renal involvement is a common and severe disease manifestation in patients with AAV[2] and usually presents as rapidly progressive glomerulonephritis. Renal disease in AAV is confirmed by the classical feature of pauci-immune necrotizing glomerulonephritis on renal biopsy.[1] Approximately 20 to 40% of patients with renal involvement progress to end-stage renal disease (ESRD).[3-5] The development of ESRD can be prevented by timely initiation of immunosuppressive therapy. However, this therapy is associated with serious side-effects, and some patients experience more disadvantages than benefits. Estimation of the risk of ESRD is important in the selection of the optimal immunosuppressive regimen; thereby improving patient-tailored therapy.



Several studies found associations between histopathological parameters in the diagnostic renal biopsy and renal outcomes such as ESRD. The most consistent findings are the associations between percentage of normal glomeruli and favorable renal outcome, and between percentage of sclerotic glomeruli and worse renal outcome.[6-9] Moreover, the presence of active lesions such as cellular crescents increases the probability of renal recovery under immunosuppressive therapy.[6, 7] These results were incorporated in the proposal of a histopathological classification for ANCA-associated glomerulonephritis (AAGN) by an international working group of renal pathologists in 2010.[10] The devised algorithm distinguishes four classes: sclerotic, focal, crescentic and mixed class. The original study by Berden *et al*. showed that this classification predicted renal outcomes at 1- and 5-year follow-up in 100 patients. The order of focal, crescentic, mixed, and sclerotic class corresponded to increasing severity of renal function loss.[10] The ultimate goals of the classification are uniform histopathological reporting and to improve individualized treatment.

Twenty studies validated the histopathological classification in adults and were performed in Asia,[11-18] North-America,[19-21] Australia,[22] and Europe.[23-30] Moreover, four studies validated the classification in pediatric patients.[17, 31-33] The results from some of these studies were recently analyzed in a meta-analysis.[16] In summary, all validation studies reported best renal outcomes in the focal class and worst renal outcomes in the sclerotic class.[34, 35] However, in some studies, renal outcomes in crescentic and mixed class were similar[16] or even significantly better in mixed class compared to crescentic class.[26] Given these conflicting results, a large international validation study is called for. The present study is the result of international collaboration between 10 centers and is driven by the original investigators. Additionally, the outcomes in crescentic and mixed class

were compared in a meta-analysis including results from performed validation studies. The presented results will aid in optimizing the histopathological classification for AAGN.

## METHODS

### Study cohort

In this study, patients were enrolled from 10 centers worldwide (Europe, North-America, and Asia). Patients with histopathologically proven AAGN who underwent a diagnostic renal biopsy and who had been followed up for at least 3 years (including patients who developed ESRD or died within the first 3 years) were included. Exclusion criteria were: age below 18 years, overlap syndrome (such as AAGN in combination with anti-glomerular basement membrane disease), and participation in previous validation studies.

### Diagnostic renal biopsies

Biopsy slides were assembled at Leiden University Medical Center. The original study proposed the requirement of a minimum of 10 glomeruli for defining the class.[10] However, a recent study showed that the prognostic capability of the classification was also valid in biopsies containing three to nine glomeruli.[29] The current study included biopsies with a minimum of five glomeruli. The biopsies were scanned with the Philips Image Manager System (IMS) scanner by magnification of 40x. The scanned slides were placed on a secured website where a group of six pathologists (FF, KJ, YO, SW, LHN, and IMB) scored the biopsies, blinded to the clinical data. The website scoring form (Supplementary Document 1) was a simplified version of the original scoring tool for AAGN that was published in 1996.[36] Among others, histopathological class, inflammatory infiltrate, interstitial fibrosis and tubular atrophy (IFTA), and tubulitis were determined for each case. For analytic purposes, tubulointerstitial scores from the two pathologists were averaged and categorized: inflammatory infiltrate <25% or ≥25% of unscarred parenchyma; IFTA <25% or ≥25% of cortical area; tubulitis foci with <5 or ≥5 cells/tubular cross section. Each case was scored by two pathologists and in case of disagreement, a third pathologist (IMB or JAB) made the final decision on the case.

### Clinical data

Patient demographics, type of diagnosis (GPA, MPA, EGPA or RLV), serum and urine laboratory values, and details on induction and maintenance therapy were retrieved from the clinical records at the different participating centers. Renal function was expressed as estimated glomerular filtration rate (eGFR) calculated by the Chronic Disease Epidemiology Collaboration (CKD-EPI) equation adjusted for race/ethnicity (Caucasian/Asian or other).[37-39] The eGFR was calculated at the time of biopsy ($eGFR_0$) and at 1- and 5-year follow-up ($eGFR_1$ and $eGFR_5$). To investigate whether the classification has an independent predictive effect on eGFR at follow-up, eGFR was corrected for $eGFR_0$

($COReGFR_1$ and $COReGFR_5$). The corrected eGFR at a time point was defined as the difference between the observed eGFR at that time point and its linear prediction on the basis of baseline eGFR.[40, 41] Calculation of eGFR was omitted when patients reached ESRD; in that case, eGFR was considered 0 for analytic purposes. ESRD was defined as need for renal replacement therapy (dialysis for at least three months or transplantation) or as eGFR below 15 mL/min for at least three months.[42] Renal survival was expressed as time between diagnosis and ESRD.

### Literature search

A trained librarian performed a literature search on previously published validation studies for the meta-analysis in June 2016 and updated the findings in March 2017. Web of Science and Google Scholar were searched for articles referring to the original study. Additionally, a search in PubMed and Embase was created for validation studies on AAGN by using combinations of the following words or part of the following words: "biopsy", "histopathological", "classification", "ANCA", "antineutrophil cytoplasmic antibody", "validation". The abstracts found in this search were evaluated, and only those studies that associated histopathological class to renal outcome in patients with AAGN were selected. The proportion of patients who had developed ESRD at the end of follow-up, and the adjusted hazard ratios (HRs) were extracted from the included validation studies. Differences between crescentic and mixed class were analyzed.



### Statistical analyses

Continuous variables are expressed as means±SD and were compared between groups using the student's t-test or one-way analysis of variance. Categorical variables are expressed as numbers (%), and differences were assessed with the Fisher's exact test or chi-square test. Renal survival was analyzed using the Kaplan Meier method and log-rank test. Pearson's correlation coefficients were calculated to identify predictors of $eGFR_5$. Stepwise multiple linear regression analysis was performed to find the best model to predict $eGFR_5$. Interobserver agreement was investigated by calculating kappa (κ) for the classification, and intraclass correlation coefficients (ICC) for tubulointerstitial parameters. A κ or ICC of >0.75 indicated excellent agreement, a κ or ICC of 0.40-0.75 was considered fair to good agreement, and a κ or ICC <0.40 showed poor agreement.[43, 44] All analyses in the validation study were performed in SPSS version 23 (IBM Corp., Armonk, N.Y., USA.). The meta-analysis used random effects models and was performed in ReviewManager version 5.3. The meta-analysis calculated relative risks (RR) of the development of ESRD. The variation across studies due to heterogeneity beyond chance was estimated by $I^2$. An $I^2$ of 25% was considered as low, 50% as moderate, and 75% as high heterogeneity.[45] In all analyses, *P* values <0.05 were considered significant.

# RESULTS

## Patient characteristics

The histopathological and clinical data of 157 patients were assembled. Twelve cases were excluded due to missing clinical data or insufficient number of glomeruli. Patients were diagnosed between 1991 and 2011. The characteristics of the 145 included patients are summarized in Table 1.

**Table 1.** Characteristics of the total cohort

| | **Total (*n*=145)** |
|---|---|
| **Age at biopsy, year, mean±SD** | 61.2±12.7 |
| **Male (%)** | 83 (57.2) |
| **Diagnosis (%)**[a] | |
| GPA | 63 (45.0) |
| MPA | 71 (50.7) |
| EGPA | 2 (1.4) |
| RLV | 4 (2.9) |
| **Diagnostic delay, months, mean±SD** | 3.1±9.0 |
| **ANCA specificity (%)**[b] | |
| PR3 | 50 (37.0) |
| MPO | 73 (54.1) |
| Negative | 6 (4.4) |
| Double positive | 6 (4.4) |
| **Center** | |
| Cochin Hospital, Paris | 6 (4.1) |
| General University Hospital in Prague | 38 (26.2) |
| Leiden University Medical Center, Leiden | 36 (24.8) |
| Medical University of Innsbruck | 7 (4.8) |
| Medical University of Vienna | 9 (6.2) |
| Necker Hospital, Paris | 4 (2.8) |
| Rigshospitalet Copenhagen | 7 (4.8) |
| Teinekeijnkai Hospital Sapporo | 7 (4.8) |
| JCHO Sendai Hospital, Sendai | 24 (16.6) |
| Weill Cornell Medic al College New York | 7 (4.8) |

[a] The AAGN diagnosis was not further specified in 5 patients.

[b] ELISA test results were available in 135 patients.

## Histopathological classes and clinical parameters

In the final evaluation, 52 (35.9%) biopsies were scored as focal, 37 (25.5%) as crescentic, 39 (26.9%) as mixed, and 17 (11.7%) as sclerotic class (Table 2). Clinical diagnosis differed significantly between classes; GPA predominated in the focal class, MPA in the mixed class, and GPA and MPA were found in a similar number of patients in the crescentic

and sclerotic class. When comparing MPO- versus PR3-ANCA positivity, MPO-ANCA was found most frequently in the crescentic and mixed class, PR3-ANCA was found slightly more often in the focal class, and MPO- and PR3-ANCA were found in equal numbers in the sclerotic class (*P*=0.04). The mean diagnostic delay in the focal class was 4.7±12.9 months, which was non-significantly higher compared to the other classes (Table 2).

**Table 2.** Patient characteristics according to histopathological class

| | Focal (*n*=52) | Crescentic (*n*=37) | Mixed (*n*=39) | Sclerotic (*n*=17) | *P* value |
|---|---|---|---|---|---|
| **Age at biopsy, year, mean±SD** | 59.7±12.6 | 61.6±11.4 | 60.6±14.4 | 65.9±11.7 | 0.37 |
| **Male (%)** | 33 (63.5) | 24 (64.9) | 18 (46.2) | 8 (47.1) | 0.22 |
| **Diagnosis (%)**[a] | | | | | 0.005 |
| GPA | 33 (63.5) | 14 (40.0) | 8 (21.6) | 8 (50.0) | |
| MPA | 17 (32.7) | 19 (54.3) | 27 (73.0) | 8 (50.0) | |
| EGPA | 1 (1.9) | 0 (0.0) | 1 (2.7) | 0 (0.0) | |
| RLV | 1 (1.9) | 2 (5.7) | 1 (2.7) | 0 (0.0) | |
| **ANCA specificity (%)**[b] | | | | | 0.13 |
| PR3 | 23 (47.9) | 13 (37.1) | 7 (19.4) | 7 (43.8) | |
| MPO | 19 (39.6) | 21 (60.0) | 25 (69.4) | 8 (50.0) | |
| Negative | 3 (6.3) | 0 (0.0) | 3 (8.3) | 0 (0.0) | |
| Double positive | 3 (6.3) | 1 (2.9) | 1 (2.8) | 1 (6.3) | |
| **Diagnostic delay, months, mean±SD** | 4.7±12.9 | 1.4±2.7 | 2.4±3.2 | 3.6±11.5 | 0.39 |
| **eGFR$_0$, ml/min/1.73 m2, mean±SD** | 49.9±29.3 | 18.0±15.7 | 26.7±18.6 | 19.4±11.8 | <0.001 |
| **Proteinuria class at biopsy (%)**[c] | | | | | 0.005 |
| Normal | 4 (8.7) | 1 (3.1) | 2 (5.3) | 0 (0.0) | |
| Moderately increased | 18 (39.1) | 4 (12.5) | 3 (7.9) | 3 (18.8) | |
| Severely increased | 24 (52.2) | 27 (84.4) | 33 (86.8) | 13 (81.3) | |
| **eGFR$_1$, ml/min/1.73 m2, mean±SD** | 61.4±23.9 | 37.3±20.6 | 37.7±21.1 | 20.3±16.0 | <0.001 |
| **COReGFR$_1$, ml/min/1.73 m2, mean±SD**[d] | 4.2±17.5 | 4.4±18.5 | -3.0±14.4 | -15.1±9.3 | <0.001 |
| **eGFR$_5$, ml/min/1.73 m2, mean±SD** | 59.7±21.1 | 34.9±20.4 | 37.3±23.7 | 19.3±20.0 | <0.001 |
| **COReGFR$_5$, ml/min/1.73 m2, mean±SD**[d] | 5.7±16.8 | 2.7±20.9 | -2.0±19.0 | -15.3±15.1 | 0.004 |
| **Renal relapse (%)** | 20 (40.0) | 14 (40.0) | 11 (28.9) | 3 (18.8) | 0.33 |
| **ESRD (%)** | 1 (1.9) | 9 (24.3) | 6 (15.4) | 8 (47.1) | <0.001 |
| **Death (%)** | 15 (28.8) | 15 (40.5) | 9 (23.1) | 6 (35.3) | 0.40 |

[a] The AAGN diagnosis was not further specified in 5 patients.

[b] ELISA test results were available in 135 patients.

[c] In accordance with the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines, normal level of proteinuria was defined as protein excretion of <0.15 g/day, or as a negative protein dipstick test; moderately increased proteinuria was defined as a protein excretion rate of 0.15–0.50 g/day, or as trace or + on protein dipstick test; severely increased proteinuria was defined as total protein excretion >0.50 g/day, or as + or more on protein dipstick. The proteinuria class could be determined in 132 patients.

[d] The corrected eGFR at a time point was defined as the difference between the observed eGFR at that time point and its linear prediction on the basis of baseline eGFR.

## Histopathological classes and renal function

The focal class had the highest $eGFR_0$ (Table 2). At 1- and 5-year follow-up, focal class remained to have the highest eGFR, whereas sclerotic class had the lowest eGFR. This was also true for corrected eGFR values. $EGFR_0$, $eGFR_1$, $COReGFR_1$, $eGFR_5$, and $COReGFR_5$ were not significantly different between crescentic and mixed class. The amount of proteinuria at biopsy was lowest in the focal class, but was comparable between crescentic, mixed and sclerotic class. During a mean duration of follow-up of 8.0±5.4 years, 24 (16.6%) patients developed ESRD, and 45 (31.0%) patients died. Seven (4.8%) patients underwent renal transplantation. Renal survival at 10 year follow-up was significantly different between the four histopathological classes, but not between crescentic and mixed class (Figure 1).

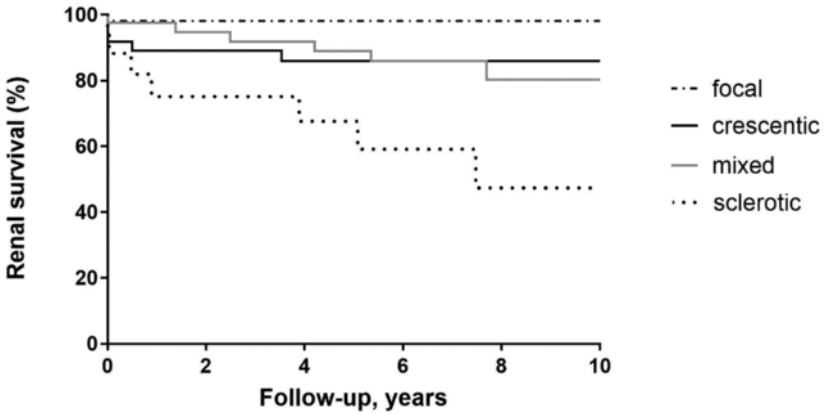


**Figure 1. Renal survival according to histopathological class**. At 10-year follow-up, renal survival was different between the four classes (*P*<0.001), but not between crescentic and mixed class (*P*=0.98).

## Predictors of renal function during follow-up

The following variables were significantly associated with $eGFR_5$: age, clinical diagnosis (MPA or GPA), ANCA serology (MPO- or PR3-ANCA), $eGFR_0$, the histopathological classification, the extent of interstitial infiltrate, the amount of IFTA, and the amount of tubulitis. In stepwise multivariate regression analysis, the variables $eGFR_0$, histopathological classification, and age composed the best model to predict $eGFR_5$ (Table 3).

## Treatment

The majority of patients was treated with corticosteroids and cyclophosphamide for induction of remission (106 [74.1%] patients; Supplementary Table 1). Patients treated with corticosteroids and cyclophosphamide had a similar 10-year renal survival rate

compared to patients treated with other regimens (*P*=0.17). Maintenance therapy consisted most frequently of corticosteroids in combination with azathioprine and/or mycophenolate mofetil (83 [61.5%] patients). Patients receiving no or minimal maintenance therapy (only corticosteroids) had a similar 10-year renal survival rate compared to patients who received corticosteroids in combination with another immunosuppressive drug (*P*=0.37). Patients from the crescentic class received plasma exchange more frequently compared to patients from other classes (24.3% versus 10.4%, *P*=0.04). Within the crescentic class, no difference in renal survival at 10 years was observed between patients receiving plasma exchange and patients not receiving plasma exchange (*P*=0.34). Patients from the sclerotic class received treatment with corticosteroids alone more frequently (Supplementary Table 1).

**Table 3.** Multivariable prediction models of $eGFR_5$

| Variable | Model 1[a] | | Model 2[b] | | Model 3[c] | |
|---|---|---|---|---|---|---|
| | β | P-value | β | P-Value | β | P-value |
| $eGFR_0$ | 0.64 | <0.001 | 0.55 | <0.001 | 0.48 | <0.001 |
| Histopathological classification | | | -0.27 | 0.002 | -0.27 | 0.001 |
| Age | | | | | -0.23 | 0.006 |

[a] Model 1 includes $eGFR_0$; $R^2 = 0.41$.

[b] Model 2 includes $eGFR_0$ and histopathological classification; $R^2 = 0.48$.

[c] Model 3 includes $eGFR_0$, histopathological classification and age; $R^2 = 0.52$.

## Interobserver agreement

Agreement on histopathological class among the first two pathologists was observed in 99 (68.3%) cases, corresponding to a κ of 0.56 which indicates moderate agreement (Figure 2). Complete disagreement among three pathologists occurred in 7 cases. During re-evaluation of the cases with disagreement (*n*=46), we distinguished three types of possible causes of disagreement. First, differences between stainings, different number of glomeruli, and/or approximation of 50% were probably the cause of disagreement, and this occurred in 21 cases. A second cause was differences in interpretation (*n*=21). Lastly, miscalculations, incomplete scoring, and/or wrong use of the algorithm were the most likely causes of disagreement (*n*=4). The ICC for interstitial infiltrate between two pathologists was 0.57, the ICC for IFTA was 0.46, and the ICC for tubulitis was 0.36.

## Meta-analyses

Twenty studies validated the histopathological classification in adult patients, excluding the current study and the original study by Berden *et al*.[10] (Figure 3).The studies by Ford *et al*. and Andreiana *et al*. were excluded from the ESRD-event rate meta-analysis, because of the way they handled with death in their analyses.[22, 30] The risk of ESRD in the crescentic

and mixed class was similar between the two classes (RR 1.19; 95% CI, 0.97-1.46) (Figure 4A). Only five studies reported adjusted HRs between crescentic and mixed class; the pooled risk ratio was 1.08 (95% CI, 0.53-2.22) (Figure 4B). Four studies investigated the prognostic value of the histopathological classification in pediatric patients (aged <18 years). The study by Sacri *et al*. combined the sclerotic and mixed class, and the focal and crescentic class; therefore, it was excluded from the meta-analysis. The risk of ESRD in pediatric patients of the crescentic and mixed class was similar (RR 0.67; 95% CI, 0.25-1.80) (Figure 4C). Heterogeneity was low in all three meta-analyses.

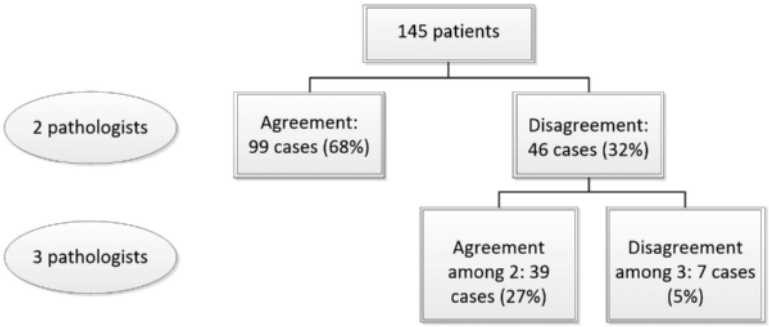


**Figure 2.** Interobserver agreement on histopathological class

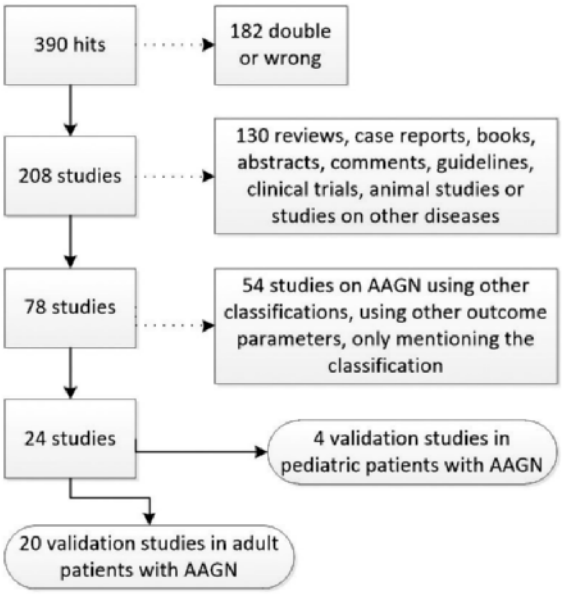


**Figure 3.** Flowchart illustrating how validation studies were selected for the meta-analyses

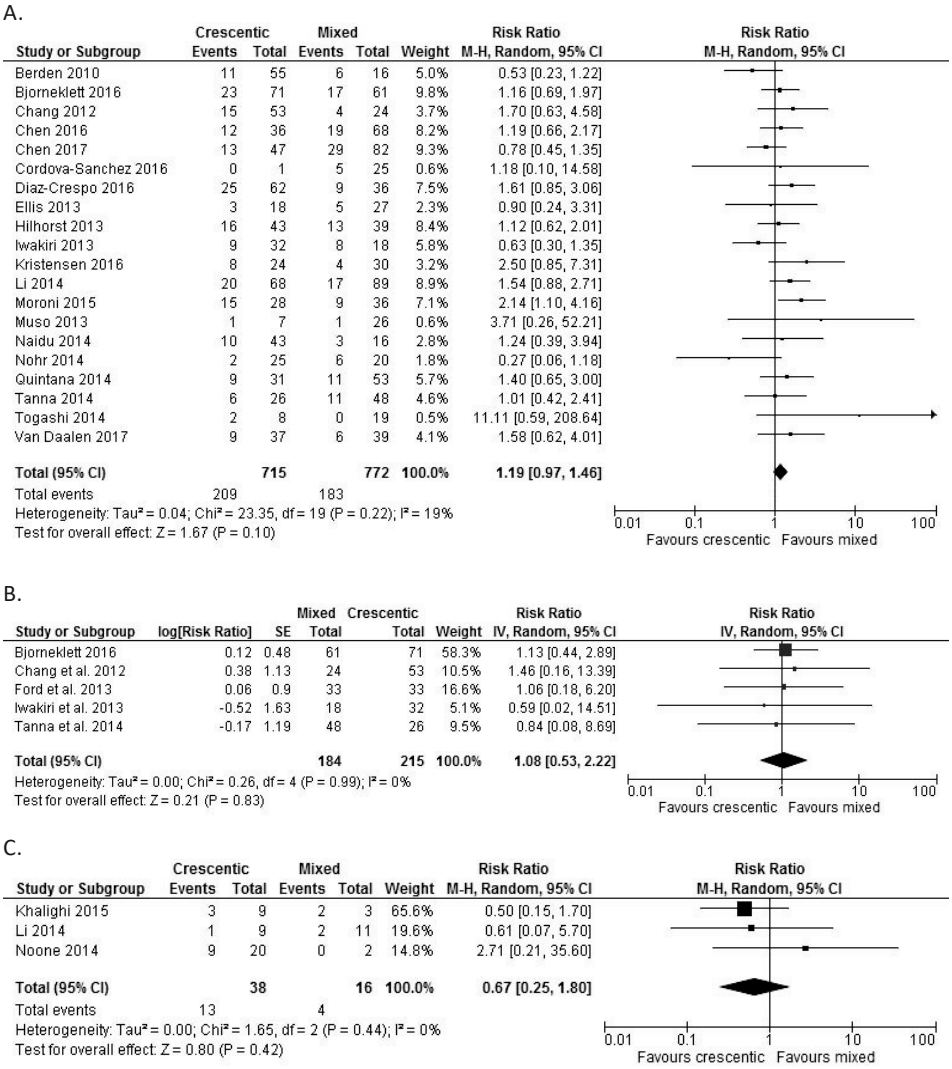


**Figure 4.** Forest plots of risk ratios comparing crescentic and mixed class. (A) Using ESRD-event rates in adult patients. (B) Using adjusted hazard ratios in adult patients. (C) Using ESRD-event rates in pediatric patients.

## DISCUSSION

This multicenter, international study was performed to validate the histopathological classification for AAGN. In line with previous validation studies, we found a favorable outcome in the focal class and a poor outcome in the sclerotic class. In contrast with the original study by Berden *et al.*,[10] we found no significant difference in renal survival

between the crescentic and mixed class. Recently, Chen *et al*. performed a meta-analysis of previous validation studies, also showing a similar renal survival in the crescentic and mixed class.[16] We increased the number of validation studies with seven in our meta-analysis, but still, there was no significant difference in the occurrence of ESRD between crescentic and mixed class.

In line with the study by Berden *et al.*, eGFR values at 1- and 5-year follow-up corrected for eGFR at baseline were significantly associated with the histopathological classification in the order of focal-crescentic-mixed-sclerotic. However, the differences between crescentic and mixed class were not significant. Nevertheless, these results show substantial renal function recovery in the crescentic class, a finding which is in line with previous studies.[6, 7] Therefore, the percentage of cellular crescents is important for predicting a potential reversibility of renal impairment during follow-up and consequently, may be important for therapeutic purposes, especially for intensified immunosuppressive treatment (i.e. addition of plasma exchange). In contrast to the association between cellular crescents and renal function recovery, fibrous crescents have a negative effect on long-term renal outcome. Fibrous crescents rarely occur in >50% of the glomeruli in one biopsy; therefore, it was not included in the histopathological classification of AAGN. However, perhaps the predictive value of fibrous crescents has been underestimated.

Previously, three causes have been proposed for the conflicting results regarding crescentic and mixed class: differences in patient populations, differences in treatment, and moderate interobserver agreement. Based on the results in the meta-analysis, the first explanation becomes unlikely; in Figure 4A, it is shown that there are no trends in different outcomes between countries or continents. Moreover, similar results were obtained in this study when only Caucasian patients were included (data not shown). It was also suggested that differences in treatment play a role in the conflicting results. In our study, treatment modality was taken into account, and indeed, it was found that patients from the crescentic class received plasma exchange more frequently compared to other classes. However, renal survival did not differ between patients in the crescentic class receiving plasma exchange and those not receiving plasma exchange. Therefore, we are not able to draw firm conclusions on the effect of therapy on outcomes in addition to the histopathological classification. Further studies with more details on therapy, such as doses and duration, are required to investigate this issue. Another possible cause to account for the conflicting results of the previous validation studies - moderate interobserver agreement - was carefully taken into account in the present study, with seven pathologists scoring renal biopsies and a protocol for coming to consensus agreement. Given the control we had in this study on the three possible causes for the conflicting results from previous validation studies, and the outcome of the present study that crescentic and mixed class have similar renal outcomes, we are inclined to regard our results as definite. Since these data are different from data reported in our original study, an adjustment to the histopathological classification of AAGN is called for.

The current histopathological classification only includes glomerular lesions, as Berden *et al*. did not find a significant increase in predictive value of the classification by including tubulointerstitial parameters. It has been suggested that adding tubulointerstitial parameters might improve the

classification.[13, 22, 24, 25] Ford *et al*. and Quintana *et al*. found that patients with severe IFTA had a poor renal outcome.[22, 24] Tanna *et al*. found an association between tubular atrophy and renal outcome. In their study, the histopathological classification did not significantly predict eGFR and ESRD in multivariate analyses including tubular atrophy, baseline eGFR and age.[25] In the present study, the extent of interstitial infiltrate, the amount of IFTA, and the amount of tubulitis were significantly associated with $eGFR_5$. However, in multivariable analysis, $eGFR_0$, histopathological classification, and age remained significantly associated with $eGFR_5$. Therefore, we could not find evidence for improvement of the histopathological classification through addition of tubulointerstitial infiltrate. A more detailed scoring system for tubulointerstitial parameters may be required to give a conclusive answer on their additive value. However, given the relatively low interobserver agreement that we found in the current study, adding tubulointerstitial parameters may reduce the reproducibility of the classification.

The strength of this study is the international patient cohort; only one previous validation study included patients from two different European countries.[24] Moreover, this is the first validation study in which a large group of pathologists scored the renal biopsies. The international character of the study also has limitations; for instance, the variety of therapeutic regimens to some extent hinders the performance of statistical analyses with respect to effects of therapy. Another limitation of the study is its retrospective design, hampering the collection of complete clinical data, although missing data with respect to renal function, diagnosis, serology and therapy were less than 7% for all categories.

In conclusion, our study highlights the need for adjusting the histopathological classification. For the moment, we here report that in relation to prognostic value, the crescentic and mixed class of the histopathological classification for AAGN may be considered the same. Most importantly, it has to be investigated whether biopsies that are not showing a focal or sclerotic class, should hither on be regarded as one or whether subclasses are clinically relevant. Therefore, we shall perform a study in which both crescents and interstitial parameters will be scored in more detail. Results from this study will determine which adjustments need to be made to the histopathological classification for AAGN.

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**Supplementary File 1.** Scoring questionnaire

**Overall**
**1** Total number of glomeruli
**2** AAGN class
a) Focal
b) Crescentic
c) Mixed
d) Sclerotic

**Inflammatory infiltrate present in:**
**3** Infiltrates
a) <10% of unscarred parenchyma
b) 10 to 25% of unscarred parenchyma
c) 26 to 50% of unscarred parenchyma
d) >50% of unscarred parenchyma
**4** Dominant cell type of infiltrate
a) Neutrophils
b) Mononuclear cells
c) Eosinophils
**5** Interstitial fibrosis and tubular atrophy
a) No interstitial fibrosis and tubular atrophy
b) Mild interstitial fibrosis and tubular atrophy (<25% of cortical area)
c) Moderate interstitial fibrosis and tubular atrophy (26-50% of cortical area)
d) Severe interstitial fibrosis and tubular atrophy/loss (>50% of cortical area)
**6** Intra-epithelial infiltrate
a) No mononuclear cells in tubules
b) Foci with 1 to 4 cells/tubular cross section or 10 tubular cells
c) Foci with 5 to 10 cells/tubular cross section
d) Foci with >10 cells/tubular cross section

**Vessels**
**7** Is vasculitis present in the small vessels (arterioles and/or arteries)?
a) Yes
b) No
**8** Are large vessels present in the biopsy?
a) Yes (please answer question 9)
b) No (proceed to question 10)
**9** Is vasculitis present in the large vessels?
a) Yes
b) No

**Granulomas**
**10** Are granulomas present?
a) Yes
b) No

**Conclusion**
**11** Do you have any comments?

**Supplementary Table 1.** Treatment according to histopathological class

| Induction therapy | Total (*n*=143)[a] | Focal class (*n*=51) | Crescentic class (*n*=37) | Mixed class (*n*=39) | Sclerotic class (*n*=16) |
|---|---|---|---|---|---|
| **Plasma exchange** | 20 (14.0) | 5 (9.8) | 9 (24.3) | 4 (10.3) | 2 (12.5) |
| **Corticosteroids only** | 19 (13.3) | 4 (7.8) | 4 (10.8) | 6 (15.4) | 5 (31.3) |
| **Corticosteroids and cyclophosphamide** | 106 (74.1) | 43 (84.3) | 30 (81.1) | 23 (59.0) | 10 (62.5) |
| **Corticosteroids and azathioprine or MMF** | 8 (5.6) | 2 (3.9) | 1 (2.7) | 4 (10.3) | 1 (6.3) |
| **Corticosteroids and mizoribine** | 5 (3.5) | 1 (2.0) | 0 (0.0) | 4 (10.3) | 0 (0.0) |
| **Corticosteroids and rituximab**[b] | 5 (3.5) | 1 (2.0) | 2 (5.4) | 2 (5.2) | 0 (0.0) |

| Maintenance therapy | Total (*n*=136)[c] | Focal class (*n*=49) | Crescentic class (*n*=35) | Mixed class (*n*=36) | Sclerotic class (*n*=16) |
|---|---|---|---|---|---|
| **Initially none** | 5 (3.7) | 2 (4.1) | 1 (2.9) | 2 (5.6) | 0 (0.0) |
| **Corticosteroids only** | 27 (19.9) | 8 (16.3) | 4 (11.4) | 7 (19.4) | 8 (50.0) |
| **Corticosteroids and cyclophosphamide** | 8 (5.9) | 5 (10.2) | 1 (2.9) | 2 (5.6) | 0 (0.0) |
| **Corticosteroids and azathioprine or MMF** | 83 (61.0) | 29 (59.2) | 25 (71.4) | 21 (58.3) | 8 (50.0) |
| **Azathioprine or MMF** | 5 (3.7) | 3 (6.1) | 2 (5.7) | 0 (0.0) | 0 (0.0) |
| **Corticosteroids and mizoribine** | 8 (5.9) | 2 (4.1) | 2 (5.7) | 4 (11.1) | 0 (0.0) |

MMF, mycophenolate mofetil.

[a] Data on induction therapy was missing in 2 patients.

[b] One of these patients also received 2 doses of intravenous cyclophosphamide.

[c] Data on maintenance therapy was available in 142 patients. Six patients did not receive maintenance therapy due to death or dialysis dependency.

# CHAPTER 4

# Predicting outcome in patients with anti-GBM glomerulonephritis

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## ABSTRACT

### Background and objectives

Large studies on long-term kidney outcome in patients with anti-glomerular basement membrane (anti-GBM) GN are lacking. This study aimed to identify clinical and histopathologic parameters that predict kidney outcome in these patients.

### Design, setting, participants, & measurements

This retrospective analysis included a total of 123 patients with anti-GBM GN between 1986 and 2015 from six centers worldwide. Their kidney biopsy samples were classified according to the histopathologic classification for ANCA-associated GN. Clinical data such as details of treatment were retrieved from clinical records. The primary outcome parameter was the occurrence of ESRD. Kidney survival was analyzed using the log-rank test and Cox regression analyses.

### Results

The 5-year kidney survival rate was 34%, with an improved rate observed among patients diagnosed after 2007 ($P$=0.01). In patients with anti-GBM GN, histopathologic class and kidney survival were associated ($P$<0.001). Only one of 15 patients with a focal class biopsy sample (≥50% normal glomeruli) developed ESRD. Patients with a sclerotic class biopsy sample (≥50% globally sclerotic glomeruli) and patients with 100% cellular crescents did not recover from dialysis dependency at presentation. In multivariable analysis, dialysis dependency at presentation (hazard ratio [HR], 3.17; 95% confidence interval [95% CI], 1.59 to 6.32), percentage of normal glomeruli (HR, 0.97; 95% CI, 0.95 to 0.99), and extent of interstitial infiltrate (HR, 2.02; 95% CI, 1.17 to 3.50) were predictors of ESRD during follow-up.

### Conclusions

Dialysis dependency, low percentage of normal glomeruli, and large extent of interstitial infiltrate are associated with poor kidney outcome in anti-GBM GN. Kidney outcome has improved during recent years; the success rate doubled after 2007.

## INTRODUCTION

Anti-glomerular basement membrane (anti-GBM) disease is an aggressive autoimmune disease,[1] with an estimated incidence of 1–2 cases per million population per year.[2] The disease affects glomerular capillaries, which usually leads to rapidly progressive GN, and pulmonary capillaries, possibly manifesting as alveolar hemorrhage. Kidney disease predominates in the majority of patients and is characterized by glomerular fibrinoid necrosis and crescent formation.[3] To distinguish anti-GBM GN from other kidney diseases, immunofluorescence appearances are decisive, showing linear Ig deposits along the glomerular basement membrane (GBM).[4] In most cases, these deposits signify binding of circulating IgG autoantibodies with the NC1 domain of the α-3 chain of type IV collagen in the GBM.[5,6] Anti-GBM autoantibodies can be detected in the serum of most patients, serving as a diagnostic tool. Besides anti-GBM antibody positivity, approximately one third of patients have ANCA. [3,7]

The discovery of anti-GBM antibodies and their pathogenicity provide the rationale for treatment with plasma exchange and immunosuppressive agents, particularly cyclophosphamide and corticosteroids.[8] This therapy combined with better supportive care and earlier diagnosis has improved the extremely poor outcome of patients with anti-GBM GN over the past decades.[9-12] Despite these improvements, the rapid deterioration caused by the disease still causes ESRD, requiring dialysis at presentation in approximately 55% of patients.[9] At 1-year follow-up, reported kidney survival rates range between 20% and 40%.[10,12–15] Kidney outcome has been correlated with the severity of kidney failure at presentation and the percentage of crescents on kidney biopsy. [9,11–16]

In a landmark paper from 2001, patients with anti-GBM GN who were dialysis dependent at presentation and had 100% crescents on their kidney biopsy did not recover kidney function.[9] On the basis of this study, the Kidney Disease: Improving Global Outcomes guidelines currently recommend that patients who present with dialysis dependency and have 100% cellular crescents in an adequate biopsy sample, and who are without lung hemorrhage, can refrain from intensive treatment with plasma exchange, corticosteroids, and cyclophosphamide.[17] However, a recent study suggested that all patients with anti-GBM GN should receive intensive therapy because the combination of plasma exchange, corticosteroids, and cyclophosphamide had a favorable effect even on patients who presented with clinical and/or histologic parameters indicative of a poor prognosis.[11] In contrast, another recent study found that oligo-anuric patients who received intensive treatment and those who received minimal treatment had similarly poor patient and kidney survival.[16] Because none of these patients recovered kidney function, a kidney biopsy may not be essential for predicting the prognosis of oligo-anuric patients.[16]

Given the controversies regarding the predictive value of the kidney biopsy and the utility of intensive therapy in patients with anti-GBM GN, large studies on long-term kidney outcome are highly warranted to further improve patient-tailored therapy.

Therefore, we investigated the clinical and histopathologic predictors of long-term kidney outcome in a large, international cohort of patients with anti-GBM GN.

## MATERIALS AND METHODS

### Study Cohort

Patients were eligible for this study if they had a clinical presentation compatible with anti-GBM GN in combination with positive (>1+) glomerular linear IgG staining on immunofluorescence and/or positive serum anti-GBM antibodies. Entry criterion for this study was the availability of a kidney biopsy sample, which was collected from a pathology department at one of the following centers: the University of North Carolina at Chapel Hill (n=34); Imperial College London (n=26); Addenbrooke's Hospital, Cambridge (n=25); LabPlus, Auckland (n=16); Leiden University Medical Center (n=15); and University Medical Center Utrecht (n=7). The cohort was divided according to year of diagnosis from which we created three groups of similar sizes in advance, which led to the following categorization: 1986–2000, 2001–2006, and 2007–2015. Patients with double positivity for anti-GBM antibodies and ANCA were included in the study, whereas patients with any other coexisting kidney disease were excluded. This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

### Biopsy Specimen Evaluation

A diagnostic kidney specimen was available for light microscopic evaluation in 118 patients. Biopsy specimens were evaluated independently by one of two experienced nephropathologists (I.M.B. or J.C.J.) who were blinded to the clinical data. The Berden classification for ANCA-associated GN was used to score the biopsy samples; focal class contained biopsy samples with ≥50% normal glomeruli, crescentic class contained biopsy samples with ≥50% glomeruli with cellular crescents, sclerotic class contained biopsy samples with ≥50% glomeruli with global sclerosis, and the remaining biopsy samples were designated as mixed class.[18] Moreover, tubulointerstitial parameters were evaluated; interstitial fibrosis and tubular atrophy and interstitial infiltrate were scored semiquantitatively (scale from 0 to 3), and tubulitis was scored as absent or present (0 or 1). Kidney biopsy samples from five patients could not be retrieved, but pathology reports had sufficient information to classify the biopsy samples according to the Berden classification, and medical files comprised complete clinical data. Results from immunofluorescence were assessed from the pathology report in all patients.

### Clinical Data

Clinical data were retrieved from medical records at the participating centers. Kidney function at baseline (before start of dialysis) was expressed as serum creatinine (milligrams per deciliter) and was classified as serum creatinine <5.7 or ≥5.7 mg/dl as

reported in previous studies. [9,12,16] Dialysis dependency at presentation was defined as need for acute RRT during the first hospital admission. The outcome of ESRD was defined as dialysis dependency during follow-up or kidney transplantation. Kidney survival was expressed as time to ESRD, and patient survival as time to death. Standard therapy was similar between centers and consisted of plasma exchange (minimum of seven exchanges or until anti-GBM antibodies were negative), oral and/or intravenous cyclophosphamide for 3–6 months, and oral and/or intravenous corticosteroids. Missing data are reported in the legends of tables, and percentages of the total of nonmissing data are presented. Each analysis included patients with complete data on the variable of interest.

### Statistical Analyses

Continuous variables are expressed as means±SD or medians (interquartile range), and were compared between groups using the t test, one-way ANOVA, Mann-Whitney U test, or Kruskal-Wallis H test, as appropriate. Categoric variables are expressed as numbers (%), and differences were assessed with the chi-squared test or chi-squared trend test. Kidney and patient survival was analyzed using the Kaplan-Meier method and log-rank test. Univariable and multivariable Cox regression analyses were performed to identify predictors of kidney survival. Results are expressed as hazard ratio (HR) with 95% confidence interval (95% CI). SPSS version 23 (IBM Corp., Armonk, NY) was used for all analyses, and *P* values below 0.05 were considered statistically significant.



## RESULTS

### Patient Characteristics

The characteristics of the total 123 patients are depicted in Table 1. Males in the cohort (54%) were significantly younger than females (47±20 versus 55±18 years; *P*=0.02). Data on serology were available for 108 patients; 49% were positive for anti-GBM antibodies only (“single positive”); 32% were positive for anti-GBM antibodies and ANCA (“double positive”); 6% had detectable ANCA only; and 13% had no detectable anti-GBM antibodies or ANCA. Double-positive patients were older than single-positive patients (*P*=0.003; Table 2). Patients who had negative serology test results were significantly younger compared with patients with positive serology test results (i.e., anti-GBM antibody and/or ANCA positivity) (*P*=0.02; Supplementary Table 1). Median duration of follow-up was 3.9 (1.3-6.1) years. Five patients were lost to follow-up within 1 year. The 5-year kidney survival rate was 34%, and the 5-year patient survival rate was 83%. Kidney survival at 5 years was 50% in patients who were diagnosed after 2007 (Figure 1).

**Table 1.** Characteristics of the study population according to the year of diagnosis

| | Total (*n*=123) | 1986 to 2000 (*n*=41) | 2001 to 2006 (*n*=38) | 2007 to 2015 (*n*=44) |
|---|---|---|---|---|
| **Baseline (*n*=123)** | | | | |
| Age, years | 51±19 | 54±19 | 46±21 | 53±17 |
| Males | 66 (54) | 22 (54) | 24 (63) | 20 (46) |
| Anti-GBM antibodies positive[a] | 92 (82) | 31 (87) | 28 (78) | 33 (81) |
| Anti-GBM antibodies, ANCA negative | 53 (60) | 12 (44) | 20 (71) | 21 (64) |
| Anti-GBM antibodies, ANCA positive | 35 (40) | 15 (56) | 8 (27) | 12 (36) |
| ANCA type[b] | 23 (85) | 9 (90) | 7 (100) | 7 (70) |
| Anti-MPO | 4 (15) | 1 (10) | 0 (0) | 3 (30) |
| Anti-PR3 | | | | |
| Lung involvement[c] | 41 (35) | 14 (37) | 12 (33) | 15 (35) |
| Serum creatinine, mg/dl** | 7.0 (3.5–10.3) | 9.0 (5.8–13.9) | 7.3 (4.5-11.4) | 4.6 (1.9–7.0) |
| Dialysis dependent** | 69 (56) | 28 (68) | 27 (71) | 14 (32) |
| **Follow-up (*n*=123)** | | | | |
| Duration of follow-up, years** | 4.6±4.0 | 5.3±5.1 | 5.9±3.5 | 2.8±2.2 |
| ESRD at end of follow-up* | 82 (67) | 31 (76) | 29 (76) | 22 (50) |
| Transplantation | 32 (26) | 9 (22) | 15 (40) | 8 (18) |
| Death* | 34 (28) | 17 (42) | 11 (29) | 6 (14) |
| **Treatment (*n*=110)** | | | | |
| Plasma exchange | 91 (83) | 24 (71) | 31 (89) | 38 (88) |
| Median number of exchanges | 7 (6-12) | 6 (5-10) | 10 (7-10) | 7 (6-14) |
| Cyclophosphamide* | 88 (80) | 21 (62) | 29 (88) | 38 (88) |
| Oral[d] | 43 (60) | 13 (68) | 14 (67) | 16 (50) |
| Intravenous[d] | 25 (35) | 6 (32) | 6 (28) | 13 (41) |
| Both | 4 (5) | 0 (0) | 1 (5) | 3 (9) |
| Corticosteroids | 106 (96) | 33 (94) | 32 (97) | 42 (98) |
| Azathioprine | 21 (19) | 5 (15) | 6 (18) | 10 (23) |
| Mycophenolate mofetil | 12 (11) | 4 (12) | 4 (12) | 4 (9) |
| Rituximab** | 10 (9) | 0 (0) | 0 (0) | 10 (23) |

Data are presented as numbers (%), means±SD, or medians (interquartile range).

ANCA, anti-neutrophil cytoplasmic antibodies; GBM, glomerular basement membrane; MPO, myeloperoxidase; PR3, proteinase 3.

* Different between subgroups with *P* value <0.05.

** Different between subgroups with *P* value <0.001.

[a] Results from anti-GBM antibody enzyme-linked immunosorbent assay (ELISA) were available in 112 patients. Results from ANCA ELISA were available in 114 patients. Both tests were performed in 108 patients; four patients with positivity for anti-GBM antibodies had no available results from ANCA ELISA.

[b] Thirty-five patients were anti-GBM antibodies and ANCA positive. Of these, ANCA type (anti-MPO or anti-PR3) was known in 27 patients.

[c] Total, *n*=117 patients

[d] Route of administration of cyclophosphamide was known for 72 patients. The mean oral cyclophosphamide dose at the start of therapy was 118±49 g (*n*=30), and the mean intravenous dose was 1113±460 g (*n*=8).

**Table 2.** Differences between single- and double-positive patients

| | Single positive[a] (*n*=53) | Double positive[a] (*n*=35) | *P* value |
|---|---|---|---|
| Age, years | 47±19 | 59±17 | 0.003 |
| Males | 27 (51) | 19 (54) | 0.76 |
| Lung involvement[b] | 15 (28) | 13 (39) | 0.29 |
| Serum creatinine at presentation, mg/dl | 7.2 (3.5–10.0) | 7.2 (3.9–10.1) | 0.92 |
| Dialysis dependent at presentation | 32 (60) | 20 (57) | 0.76 |
| Year of diagnosis | | | 0.11 |
| 1986 to 2000 | 12 (23) | 15 (43) | |
| 2001 to 2006 | 20 (38) | 8 (23) | |
| 2007 to 2015 | 21 (39) | 12 (34) | |
| ESRD at end of follow-up | 36 (68) | 25 (71) | 0.73 |
| Histopathological class | | | 0.03 |
| Focal | 7 (13) | 4 (11) | |
| Crescentic | 37 (70) | 18 (51) | |
| Mixed | 9 (17) | 8 (23) | |
| Sclerotic | 0 (0) | 5 (14) | |
| Normal glomeruli, percentage[c] | 0 (0–26) | 0 (0–13) | 0.40 |
| Cellular crescents, percentage[c] | 61±32 | 58±32 | 0.63 |
| Sclerotic glomeruli, percentage[c] | 2 (0–16) | 13 (0–28) | 0.07 |
| Interstitial fibrosis and tubular atrophy [c] | | | 0.07 |
| 0-1 | 36 (72) | 18 (53) | |
| 2-3 | 14 (28) | 16 (47) | |
| Interstitial infiltrate[c] | | | 0.10 |
| 0-1 | 22 (44) | 9 (27) | |
| 2-3 | 28 (56) | 25 (73) | |
| Tubulitis[c] | | | 0.20 |
| 0 | 18 (36) | 17 (50) | |
| 1 | 32 (64) | 17 (50) | |
| Intensive treatment[d] | 34 (71) | 14 (45) | 0.02 |
| Maintenance therapy with azathioprine or mycophenolate mofetil[e] | 7 (14) | 13 (38) | 0.01 |

Data are presented as numbers (%), means±SD, or medians (interquartile range).

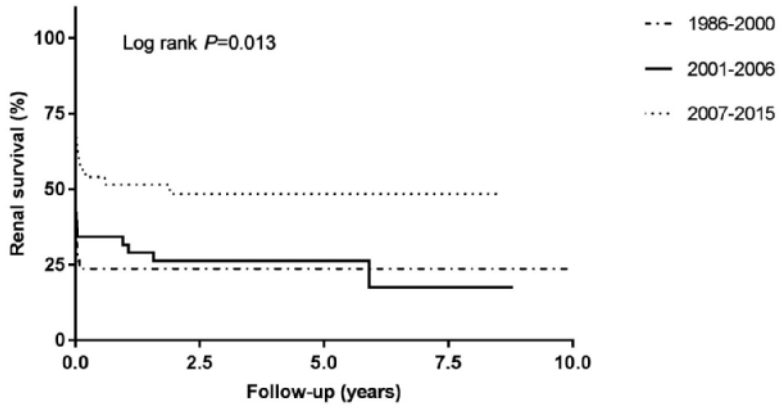
[a] Single-positive patients were positive for serum anti-glomerular basement membrane (GBM) antibodies and negative for anti-neutrophil cytoplasmic antibodies (ANCA). Double-positive patients were positive for anti-GBM antibodies and positive for ANCA.

[b] Total, *n*=86 patients

[c] Total, *n*=84 patients

[d] Intensive treatment consisted of at least 7 plasma exchanges, corticosteroids, and cyclophosphamide, mycophenolate mofetil, or rituximab. Excluding patients without intensive treatment and relatively preserved renal function at presentation. Total, *n*=79 patients

[e] The minimal duration of maintenance therapy was three months. Total, *n*=84 patients




| *N* at risk | 0.0 | 2.5 | 5.0 | 7.5 | 10.0 |
|---|---|---|---|---|---|
| 1986 to 2000 | 41 | 8 | 7 | 5 | 2 |
| 2001 to 2006 | 38 | 6 | 4 | 2 | 0 |
| 2007 to 2015 | 44 | 11 | 6 | 2 | 0 |

**Figure 1.** Renal survival according to year of diagnosis

## Evaluation of Diagnostic Renal Biopsy Specimens

Immunofluorescence was performed successfully in 117 patients and was positive for linear IgG in all of them; six specimens for immunofluorescence did not contain assessable glomeruli. Ten biopsy samples were classified as sclerotic class, 15 biopsy samples as focal class, 72 biopsy samples as crescentic class, and the remaining 26 biopsy samples as mixed class (Table 3). The focal group had a younger mean age than the other three classes together (41±19 versus 52±19 years; *P*=0.03). Serum anti-GBM antibodies were more frequently detected in patients from the crescentic class than in those from the other classes. All patients from the sclerotic class who were positive for anti-GBM antibodies and who were tested for ANCA showed double positivity for both antibodies. The amount of interstitial fibrosis and tubular atrophy differed significantly between classes, but the extent of interstitial infiltrate did not (Table 4). Double-positive patients tended to have a higher percentage of sclerotic glomeruli and a higher interstitial fibrosis and tubular atrophy score compared with single-positive patients (*P*=0.07 for both parameters; Table 2). The minimum number of glomeruli was set at six in this study, whereas the Berden classification set a minimum of ten glomeruli. The predictive value of glomerular lesions was greater in biopsy samples with ten or more glomeruli (*n*=101) compared with biopsy samples with 6-10 glomeruli (*n*=22). However, including biopsy samples with 6-10 glomeruli did not change the predictive value of the glomerular lesions (Supplementary Table 2); therefore, they were included. No differences in treatment were observed between histopathologic classes, except for cyclophosphamide; patients from the focal and mixed class received cyclophosphamide more frequently than patients from the crescentic and sclerotic class (Table 5).

**Table 3.** Characteristics stratified by histopathologic class: Baseline and follow-up characteristics

| | Focal (*n*=15) | Crescentic (*n*=72) | Mixed (*n*=26) | Sclerotic (*n*=10) | *P* value |
|---|---|---|---|---|---|
| Age, years | 41±19 | 53±19 | 53±16 | 50±25 | 0.17 |
| Males | 9 (60) | 41 (57) | 10 (39) | 6 (60) | 0.37 |
| Anti-GBM antibodies positive[a] | 11 (79) | 58 (91) | 17 (71) | 6 (60) | 0.02 |
| Anti-GBM antibodies, ANCA negative | 7 (64) | 37 (67) | 9 (53) | 0 (0) | 0.03 |
| Anti-GBM antibodies, ANCA positive | 4 (36) | 18 (33) | 8 (47) | 5 (100) | |
| ANCA type[b] | | | | | 0.001 |
| Anti-MPO | 0 (0) | 14 (100) | 7 (87) | 2 (100) | |
| Anti-PR3 | 3 (100) | 0 (0) | 1 (13) | 0 (0) | |
| Lung involvement[c] | 7 (50) | 26 (37) | 4 (16) | 4 (57) | 0.06 |
| Serum creatinine at presentation, mg/dl | 1.4 (1.0–1.7) | 7.8 (6.4–12.6) | 4.2 (2.6–7.2) | 6.6 (4.5–15.2) | <0.001 |
| Dialysis dependent at presentation | 0 (0) | 51 (71) | 10 (39) | 8 (80) | <0.001 |
| Year of diagnosis | | | | | |
| 1986 to 2000 | 3 (20) | 28 (39) | 4 (15) | 6 (60) | 0.007 |
| 2001 to 2006 | 2 (13) | 26 (36) | 8 (31) | 2 (20) | |
| 2007 to 2015 | 10 (67) | 18 (25) | 14 (54) | 2 (20) | |
| Duration of follow-up, years | 4.7±3.7 | 4.9±4.2 | 4.3±3.8 | 3.4±3.6 | 0.72 |
| ESRD at end of follow-up | 1 (7) | 57 (79) | 15 (58) | 9 (90) | <0.001 |
| Transplantation | 0 (0) | 25 (35) | 4 (15) | 3 (30) | 0.01 |
| Death | 2 (13) | 24 (33) | 5 (19) | 3 (30) | 0.32 |

Data are number (%), mean±SD, or median (interquartile range). GBM, glomerular basement membrane; MPO, myeloperoxidase; PR3, proteinase 3.

[a] Results from anti-GBM antibody ELISA were available in 112 patients. Results from ANCA ELISA were available in 114 patients. Both tests were performed in 108 patients.

[b] Thirty-five patients were anti-GBM antibodies and ANCA positive. Of these, ANCA type (anti-MPO or anti-PR3) was known in 27 patients.

[c] Total, *n*=117 patients.

**Table 4.** Characteristics stratified by histopathologic class: Biopsy specimen characteristics

| | Focal (*n*=15) | Crescentic (*n*=68) | Mixed (*n*=25) | Sclerotic (*n*=10) | *P* value |
|---|---|---|---|---|---|
| Total number of glomeruli | 22±12 | 17±9 | 19±9 | 19±9 | 0.35 |
| Normal glomeruli, percentage | 71 (62–90) | 0 (0–8) | 9 (0–31) | 0 (0–0) | <0.001 |
| Cellular crescents, percentage | 12±10 | 81±16 | 32±15 | 19±16 | <0.001 |
| Sclerotic glomeruli, percentage | 0 (0–5) | 0 (0–14) | 17 (8–30) | 63 (58–70) | <0.001 |
| Interstitial fibrosis and tubular atrophy | | | | | <0.001 |
| 0 | 8 (53) | 16 (24) | 1 (4) | 0 (0) | |
| 1 | 7 (47) | 30 (44) | 11 (44) | 1 (10) | |
| 2 | 0 (0) | 19 (28) | 10 (40) | 3 (30) | |
| 3 | 0 (0) | 3 (4) | 3 (12) | 6 (60) | |
| Interstitial infiltrate | | | | | 0.16 |
| 0 | 5 (33) | 5 (8) | 1 (4) | 1 (10) | |
| 1 | 9 (60) | 17 (25) | 12 (48) | 2 (20) | |
| 2 | 1 (7) | 22 (32) | 6 (24) | 7 (70) | |
| 3 | 0 (0) | 24 (35) | 6 (24) | 0 (0) | |
| Tubulitis | | | | | 0.02 |
| 0 | 12 (80) | 27 (40) | 15 (60) | 6 (60) | |
| 1 | 3 (20) | 41 (60) | 10 (40) | 4 (40) | |

Data are number (%), mean±SD, or median (interquartile range). Excluding five patients without kidney biopsy samples available for re-evaluation. Total, *n*=118 patients.

**Table 5.** Characteristics stratified by histopathologic class: Treatment

| | Focal (*n*=14) | Crescentic (*n*=65) | Mixed (*n*=23) | Sclerotic (*n*=8) | *P* value |
|---|---|---|---|---|---|
| Intensive treatment[a] | 8 (57) | 33 (51) | 16 (70) | 4 (50) | 0.46 |
| Plasma exchange | 12 (86) | 53 (82) | 21 (91) | 5 (63) | 0.32 |
| Cyclophosphamide | 13 (93) | 46 (71) | 23 (100) | 6 (75) | 0.005 |
| Corticosteroids | 14 (100) | 62 (95) | 23 (100) | 7 (88) | 0.33 |
| Azathioprine | 4 (29) | 8 (12) | 6 (26) | 3 (38) | 0.11 |
| Mycophenolate mofetil | 2 (14) | 7 (11) | 2 (9) | 1 (13) | 0.92 |
| Rituximab | 3 (21) | 3 (5) | 4 (17) | 0 (0) | 0.06 |

Data are number (%), mean±SD, or median (interquartile range). Total, *n*=110 patients.

[a] Intensive treatment was defined as treatment consisting of at least seven plasma exchanges, corticosteroids, and cyclophosphamide, mycophenolate mofetil or rituximab.

## Kidney Function According to Histopathologic Class

Kidney function at baseline in terms of serum creatinine was significantly better in the focal class compared with the other three classes (Table 3). No patient from the focal class required dialysis at presentation, and only one developed ESRD during follow-up (Figure 2). Most patients (90%) from the sclerotic class developed ESRD at some time point, whereas the outcome in crescentic and mixed classes was more variable (Figures 2 and

3A). When patients were categorized according to the percentage of normal glomeruli, kidney survival decreased with each lower percentage category of normal glomeruli (Figure 3B). Patient survival did not differ between histopathologic classes (Figure 3C).

### Predictors of ESRD

In Kaplan–Meier analysis, double-positive patients had a similar kidney survival to single-positive patients (*P*=0.75; Supplementary Figure 1A). Kidney survival was also similar in patients with negative and positive serology (*P*=0.69; Supplementary Figure 1B). Age, serum creatinine level, dialysis dependency at presentation, histopathologic classification, percentage of normal glomeruli, percentage of cellular crescents, extent of interstitial fibrosis and tubular atrophy, and extent of interstitial infiltrate were associated with ESRD in univariable Cox regression analyses (Table 6). Dialysis dependency at presentation was a discontinuous variable that predicted ESRD most significantly. Therefore, each parameter that was significant in univariable analysis was evaluated separately in a multivariable Cox regression analysis that included dialysis dependency at presentation. The percentage of normal glomeruli and the extent of interstitial infiltrate remained significant predictors of ESRD in multivariable analysis. A multivariable analysis, including dialysis dependency, percentage of normal glomeruli, and extent of interstitial infiltrate, showed that these parameters predicted ESRD independently (Table 6).



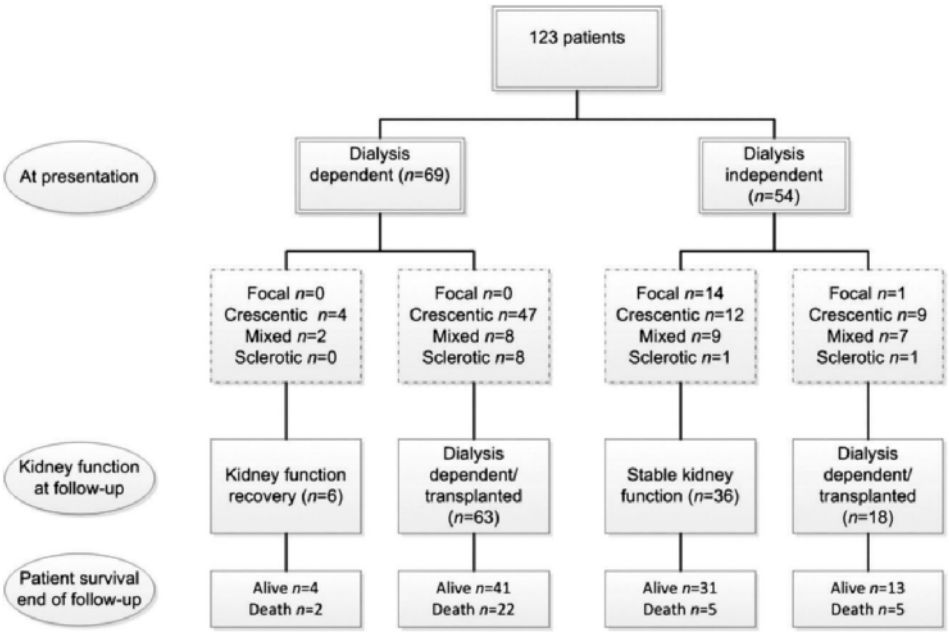



**Figure 2.** The flowchart shows a favorable outcome in the focal class, a poor outcome in the sclerotic class, and variable outcomes in the mixed and crescentic class.

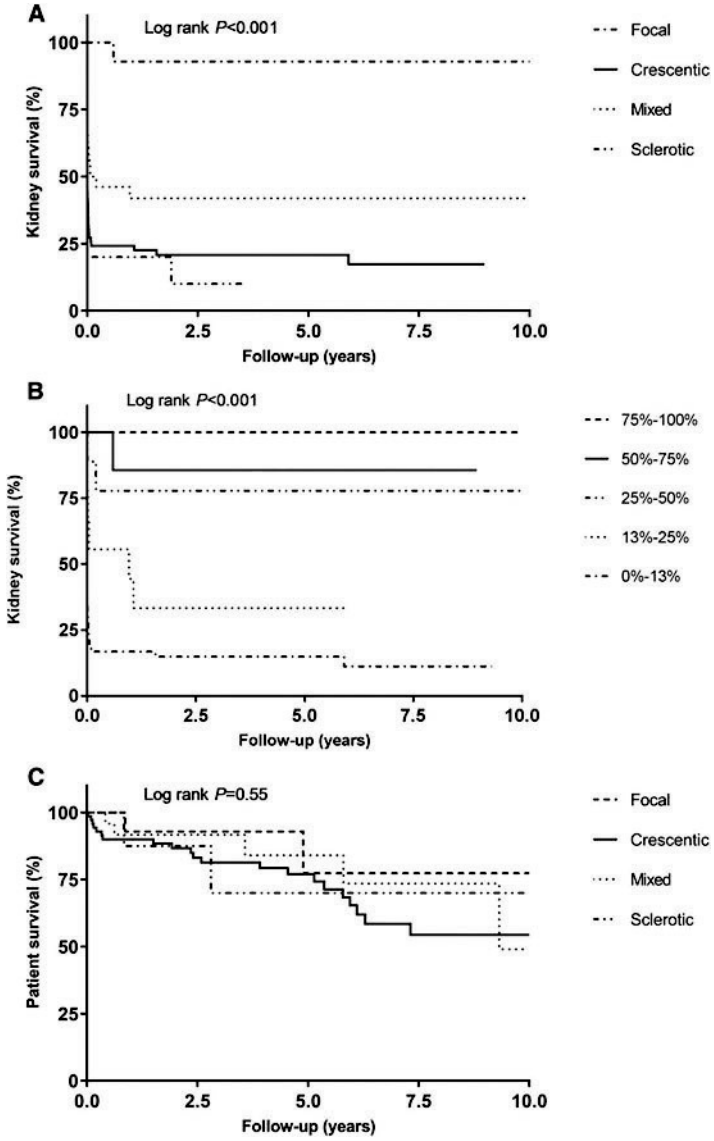



**Figure 3.** Kidney survival is highest in patients with a focal class biopsy, and patient survival is similar in all histopathological classes. (A) Kidney survival according to histopathologic class. (B) Kidney survival according to percentage of normal glomeruli. (C) Patient survival according to histopathologic class.

**Table 6.** Prognostic significance of clinical and histopathologic parameters on ESRD

| | Univariate analysis | | Multivariate analysis[a] | | Multivariate analysis[b] | |
|---|---|---|---|---|---|---|
| | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** |
| Age, years | 1.02 (1.01-1.03) | 0.005 | 1.01 (1.00-1.03) | 0.06 | | |
| Males | 1.19 (0.77-1.84) | 0.44 | | | | |
| Lung involvement | 1.19 (0.75-1.88) | 0.47 | | | | |
| Double positivity | 1.07 (0.64-1.78) | 0.80 | | | | |
| Serum creatinine at presentation ≥5.7 mg/dl | 4.83 (2.75-8.47) | <0.001 | 1.98 (0.96-4.10) | 0.07 | | |
| Dialysis dependency at presentation | 6.50 (3.68-11.47) | <0.001 | [a] | [a] | 3.2 (1.6-6.3) | 0.001 |
| Histopathological classification | | 0.005 | | 0.15 | | |
| Focal (reference) | | | | | | |
| Crescentic | 20.29 (2.80-147.26) | | 8.38 (1.10-63.87) | | | |
| Mixed | 11.67 (1.54-88.47) | | 6.77 (0.87-52.65) | | | |
| Sclerotic | 25.72 (3.24-204.51) | | 11.17 (1.34-3.13) | | | |
| Percentage normal glomeruli | 0.95 (0.93-0.98) | <0.001 | 0.97 (0.95-0.99) | 0.007 | 0.97 (0.95-0.99) | 0.01 |
| Percentage cellular crescents | 1.02 (1.01-1.02) | <0.001 | 1.02 (1.00-1.05) | 0.09 | | |
| Percentage globally sclerotic glomeruli | 1.01 (1.00-1.02) | 0.20 | | | | |
| Interstitial fibrosis and tubular atrophy, score 2-3 | 1.88 (1.20-2.95) | 0.006 | 1.38 (0.87-2.18) | 0.17 | | |
| Interstitial infiltrate, score 2-3 | 2.35 (1.44-3.83) | 0.001 | 1.90 (1.16-3.13) | 0.01 | 2.02 (1.2-3.5) | 0.01 |
| Tubulitis, present | 1.51 (0.96-2.35) | 0.07 | | | | |

HR, hazard ratio; 95% CI, 95% confidence interval.

[a] Each parameter that was significant in univariable analysis, was analyzed in a multivariable analysis including dialysis dependency at presentation. Dialysis dependency at presentation remained significant in each multivariable analysis with P values ≤0.002.

[b] Parameters that were significant in the multivariable analysis were included in this multivariate analysis.

### Treatment

Data on therapy were complete for 110 patients. Intensive treatment consisting of at least seven plasma exchanges[19], corticosteroids, and cyclophosphamide, mycophenolate mofetil, or rituximab was given to 61 patients. Of the 49 remaining patients, 25 patients were treated with plasma exchange but not with cyclophosphamide and 24 with cyclophosphamide but no or less than seven plasma exchanges. Five of these 49 patients had relatively preserved kidney function at presentation (serum creatinine ≤1.4 mg/dl); therefore, they were not included in the analysis comparing intensive versus mild treatment (Supplementary Table 3). Older patients, double-positive patients, and patients with severe kidney failure at presentation were less likely to receive intensive treatment. Moreover, patients with a lower percentage of normal glomeruli and a higher percentage of crescentic glomeruli were treated less intensively. Over time, there was a significant trend toward increased use of an intensive treatment regimen (Supplementary Table 3). Twenty-one patients received azathioprine, and 12 patients received mycophenolate mofetil for induction and/or remission therapy. Double-positive patients received maintenance therapy with azathioprine or mycophenolate mofetil more frequently than single-positive patients (Table 2). Ten patients were treated with rituximab; nine of these patients also received cyclophosphamide concurrently or before treatment with rituximab.

## DISCUSSION

In one of the largest studies to date, we investigated the long-term outcome of 123 patients with anti-GBM GN from six centers worldwide. We analyzed the predictive value of the kidney biopsy in anti-GBM GN by applying the histopathologic classification for ANCA-associated GN.[18] The histopathologic classification was a significant predictor of kidney survival in univariable analysis, but not in multivariable analysis including dialysis dependency at presentation. However, the percentage of normal glomeruli and the extent of interstitial infiltrate remained significant predictors in multivariable analysis. We also found that patients with ≥50% globally sclerotic glomeruli did not recover from the need for acute dialysis. The crescentic and mixed classes, as defined for ANCA-associated GN, seemed less important in predicting the outcome of anti-GBM GN because their kidney outcome was variable. In line with the study by Levy *et al.*,[9] we found that patients who were dialysis dependent at presentation and had 100% cellular crescents at biopsy did not recover kidney function.

Age seemed to be an important denominator in determining the therapeutic strategy and in outcome. Younger patients more often had a focal class biopsy specimen, were more likely to stay dialysis independent, and received intensive treatment more frequently. However, when we performed multivariable analyses, age was no longer significantly associated with ESRD. From our data, we can conclude that histopathologic

parameters, i.e., percentage of normal glomeruli and extent of interstitial infiltrate, are more predictive of outcome. Nevertheless, we feel that it is important to take age into account when defining the optimal individual treatment strategy. Interestingly, older age and double positivity were significantly associated, but double positivity was not associated with a worse outcome compared with single positivity.

The 5-year kidney survival rate in this study was 34%, which is higher than reported by Cui *et al*.[11] but in line with results from Levy *et al*.[9] Patients who were diagnosed after 2007 had a two-fold higher kidney survival rate compared with patients who were diagnosed before 2007. Interestingly, most patients with a focal class biopsy sample were diagnosed recently (2007–2015), suggesting earlier detection of the disease or increased awareness of the heterogeneity of the disease.[20,21] The favorable outcome of this focal group can partially explain the observation of improved kidney outcome since 2007. The other contributing factor to this improvement is possibly the significant trend toward increased use of intensive therapy. From 1986 to 2000, 36% of the patients received intensive therapy, whereas 64% of the patients diagnosed after 2007 were treated intensively.

The heterogeneity of anti-GBM disease has recently been described by Nasr *et al.*[21] They described an atypic variant of anti-GBM disease, characterized by linear GBM staining for Igs, negative serum anti-GBM antibodies, absence of a crescentic phenotype, mild renal insufficiency, and absence of pulmonary hemorrhage. In our study, 20 patients had linear IgG deposits, but no detectable serum anti-GBM antibodies; three of them fulfilled the description of atypic anti-GBM disease. Therefore, the group of patients without detectable serum anti-GBM antibodies, but with linear IgG deposits, is rather heterogeneous.

Forty-five percent of our patients did not receive intensive treatment and the reason for refraining from intensive therapy was not always reported. However, some medical reports stated that treatment was withdrawn because of the results of kidney biopsy (i.e., a high percentage of affected glomeruli), therapy-related complications such as serious infections, or lack of improvement under therapy. Five patients who did not receive intensive treatment presented with relatively preserved kidney function, which was probably the reason for not initiating the full treatment regimen. Our data suggest that patients who are dialysis dependent at presentation and have either ≥50% globally sclerotic glomeruli or 100% cellular crescents do not benefit from intensive therapy and could therefore avoid the risk of immunosuppression and follow a conservative regimen. Other patients, including those with alveolar hemorrhage, should be treated intensively.

In our cohort, ten patients were treated with rituximab. In one center, rituximab along with cyclophosphamide has become standard therapy in patients who are double positive for anti-GBM antibodies and ANCA. Other indications for rituximab were found in three patients. In one patient, the kidney biopsy specimen showed a large infiltrate of CD20 positive B cells, providing a rationale to start anti-CD20 therapy (i.e., rituximab).

Another patient did not tolerate azathioprine; therefore, rituximab was given instead. A third patient received rituximab, because the side-effects of cyclophosphamide were likely to cause problems. Because only nine patients were treated with rituximab and cyclophosphamide, conclusions on the response to rituximab could not be drawn.

This study included 123 patients with anti-GBM GN and is therefore the largest study evaluating kidney biopsy samples in relation to clinical outcome in this rare disease. Another strength of the study is the relatively long duration of follow-up (median 3.9 years). We consider the possibility of the development of ESRD after the last date of follow-up unlikely, because progression to ESRD usually occurs in the initial phase of this disease.[9] Because of the rapid onset of anti-GBM disease, patient or referral delay have probably not influenced our findings. A limitation of the study is the possibility of selection bias; we only included patients with a diagnostic kidney biopsy performed, thereby excluding patients with a contraindication for a kidney biopsy (e.g., frail patients). Moreover, treatment was a potential confounder in our study; therefore, we have investigated treatment in detail. Future studies on histopathology in anti-GBM GN with patients who are treated similarly are highly warranted.

In summary, dialysis independency at presentation, a high percentage of normal glomeruli, and a lower extent of interstitial infiltrate are associated with a favorable outcome in patients with anti-GBM GN. Dialysis-dependent patients with 100% cellular crescents or ≥50% sclerotic glomeruli are very unlikely to recover, and may be refrained from intensive therapy. Kidney survival has improved during recent years, doubling the success rate after 2007. This is possibly the result of earlier detection accompanied by the increased use of intensive therapy in anti-GBM GN.

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**Supplementary Table 1.** Differences between patients with negative and positive serology

| | Patients with negative serology[a] (*n*=14) | Patients with positive serology[a] (*n*=94) | *P* value |
|---|---|---|---|
| Age, years | 39±19 | 53±19 | 0.02 |
| Males | 10 (71) | 50 (53) | 0.20 |
| Lung involvement[b] | 6 (46) | 31 (34) | 0.54 |
| Serum creatinine at presentation, mg/dl | 4.2 (1.7–10.3) | 7.1 (3.6–10.0) | 0.25 |
| Dialysis dependent at presentation | 8 (57) | 54 (57) | 0.98 |
| Year of diagnosis | | | 1.00 |
| 1986 to 2000 | 4 (28) | 27 (29) | |
| 2001 to 2006 | 5 (36) | 31 (33) | |
| 2007 to 2015 | 5 (36) | 36 (38) | |
| ESRD at end of follow-up | 9 (64) | 65 (69) | 0.76 |
| Histopathological class | | | 0.14 |
| Focal | 2 (14) | 12 (13) | |
| Crescentic | 5 (36) | 56 (60) | |
| Mixed | 4 (29) | 20 (21) | |
| Sclerotic | 3 (21) | 6 (6) | |
| Normal glomeruli, percentage[c] | 7 (0–79) | 0 (0–17) | 0.46 |
| Percentage of cellular crescents[c] | 41±32 | 58±33 | 0.08 |
| Sclerotic glomeruli, percentage[c] | 7 (0–47) | 6 (0–20) | 0.44 |
| Interstitial fibrosis and tubular atrophy [c] | | | 1.00 |
| 0-1 | 8 (61) | 56 (62) | |
| 2-3 | 5 (39) | 34 (38) | |
| Interstitial infiltrate[c] | | | 0.27 |
| 0-1 | 7 (54) | 34 (38) | |
| 2-3 | 6 (46) | 56 (62) | |
| Tubulitis[c] | | | 0.58 |
| 0 | 7 (54) | 41 (46) | |
| 1 | 6 (46) | 49 (54) | |
| Intensive treatment[d] | 7 (50) | 52 (62) | 0.34 |

Data are presented as numbers (%), means±SD, or medians (interquartile range).

[a] Patients with negative serology were negative for serum anti-glomerular basement membrane (GBM) antibodies and anti-neutrophil cytoplasmic antibodies (ANCA). Patients with positive serology were positive for anti-GBM antibodies and/or positive for ANCA.

[b] Total, *n*=104 patients

[c] Total, *n*=103 patients

[d] Intensive treatment consisted of at least 7 plasma exchanges, corticosteroids, and cyclophosphamide, mycophenolate mofetil, or rituximab. Excluding patients without intensive treatment and relatively preserved renal function at presentation. Total, *n*=98 patients

**Supplementary Table 2.** Number of glomeruli and predictive value of glomerular lesions

| | Biopsies with 6-10 glomeruli (*n*=22) | | Biopsies with ≥10 glomeruli (*n*=101) | | Biopsies with ≥6 glomeruli (*n*=145) | |
|---|---|---|---|---|---|---|
| | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** | **HR (95% CI)** | ***P* value** |
| Percentage normal glomeruli | 0.93 (0.87-1.01) | 0.07 | 0.96 (0.94-0.98) | <0.001 | 0.95 (0.93-0.98) | <0.001 |
| Percentage cellular crescents | 1.02 (1.00-1.04) | 0.04 | 1.02 (1.01-1.03) | <0.001 | 1.02 (1.01-1.02) | <0.001 |
| Percentage globally sclerotic glomeruli | 1.01 (0.96-1.05) | 0.82 | 1.01 (1.00-1.02) | 0.21 | 1.01 (1.00-1.02) | 0.20 |

HR, hazard ratio to develop ESRD.

The predictive value of the glomerular parameters are similar in the group of biopsies containing more than 10 glomeruli and the group of biopsies containing more than 6 glomeruli.

**Supplementary Table 3.** Differences between patients receiving minimal and intensive treatment

| | No intensive treatment[a] (*n*=44 [41%]) | Intensive treatment[a] (*n*=61 [59%]) | ***P* value** |
|---|---|---|---|
| Age, years | 56±20 | 46±19 | 0.009 |
| Males | 26 (45) | 32 (55) | 0.50 |
| Anti-GBM antibodies positive[b] | 33 (41) | 48 (59) | 0.91 |
| Anti-GBM antibodies, ANCA negative | 14 (29) | 34 (71) | 0.02 |
| Anti-GBM antibodies, ANCA positive | 17 (55) | 14 (45) | |
| Lung involvement[c] | 13 (36) | 23 (64) | 0.35 |
| Serum creatinine at presentation, mg/dl | 8.1 (6.7–14.6) | 15.2 (3.2–7.7) | <0.001 |
| Dialysis dependent at diagnosis | 33 (55) | 27 (45) | 0.002 |
| Year of diagnosis | | | 0.007 |
| 1986 to 2000 | 21 (64) | 12 (36) | |
| 2001 to 2006 | 9 (27) | 24 (73) | |
| 2007 to 2015 | 14 (36) | 25 (64) | |
| ESRD at end of follow-up | 37 (51) | 36 (49) | 0.006 |
| Normal glomeruli, percentage[d] | 0 (0–5) | 8 (0–27) | 0.005 |
| Cellular crescents, percentage[d] | 70±29 | 51±32 | 0.003 |
| Patients with 100% crescents[d] | 12 (80) | 3 (20) | 0.001 |
| Sclerotic glomeruli, percentage[d] | 6 (0–21) | 7 (0–22) | 0.92 |

Data are presented as numbers (% in rows), means±SD, or medians (interquartile range).

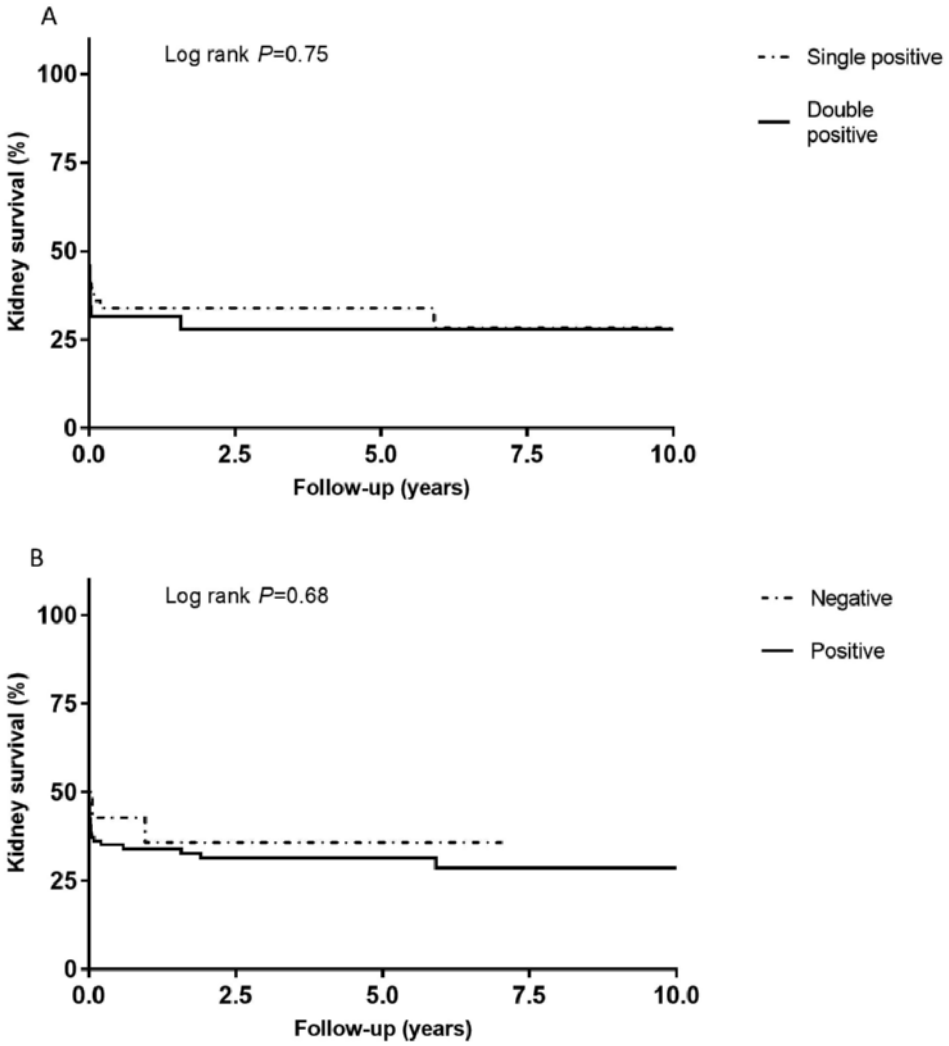
ANCA, anti-neutrophil cytoplasmic antibodies; GBM, glomerular basement membrane.

[a] Intensive treatment is defined as minimal 7 plasma exchanges, corticosteroids, and either cyclophosphamide, mycophenolate mofetil or rituximab. Data on therapy was complete for 110 patients (89%). Five patients with preserved renal function at baseline did not receive intensive treatment, and they were excluded from this analysis; therefore, total number in this analysis was 105 patients (85%).

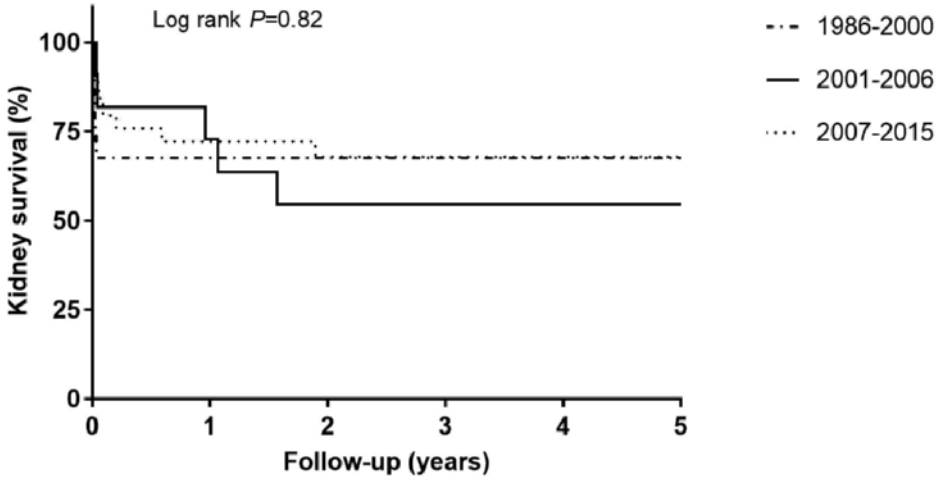
[b] Total, *n*=100 patients

[c] Total, *n*=104 patients

[d] Total, *n*=101 patients




**Supplementary Figure 1. Kidney survival according to serology**
(A) Kidney survival in single- and double-positive patients. Single-positive patients were positive for serum anti-glomerular basement membrane (GBM) antibodies and negative for anti-neutrophil cytoplasmic antibodies (ANCA). Double-positive patients were positive for anti-GBM antibodies and positive for ANCA. (B) Kidney survival in patients with negative anti-GBM antibodies and ANCA ELISA tests compared to patients with positive anti-GBM antibodies and/or ANCA ELISA test(s).




**Supplementary Figure 2.** Kidney survival of patients with dialysis independency at presentation

# CHAPTER 5

# Loss of WT-1 positive podocytes in ANCA-associated glomerulonephritis

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## ABSTRACT

Proteinuria has been identified as prognosticator of renal outcome in patients with ANCA-associated glomerulonephritis (AAGN), but whether proteinuria is related to podocyte abnormalities in these patients is largely unknown. We investigated podocyte foot process width (FPW) and number of podocytes positive for the podocyte marker WT-1 in renal biopsies of 25 Caucasian patients with AAGN in relation to proteinuria. Control tissue was used from pre-transplantation donor kidney biopsies. Proteinuria at 10 weeks follow-up correlated significantly with FPW ($P = 0.04$). Biopsies with FPW ≥600 nm belonged more often to the crescentic or mixed class, whereas biopsies with a FPW <600 nm were most often categorized as focal class ($P = 0.03$). The mean number of podocytes based upon expression of WT-1 was significantly lower in patients compared to controls (15 versus 34 podocytes per glomerulus; $P < 0.0001$). In conclusion, the significant decrease in expression of the podocyte WT-1 marker in AAGN was considered indicative of actual podocyte loss or at least, of a loss of functionality. Furthermore, our study indicates that podocyte FPW at baseline is indicative for proteinuria at short term follow up. For prognostic purposes, we therefore suggest to include a description of the FPW in the diagnostic report of a biopsy with AAGN.

## INTRODUCTION

In the patient care and research of anti-neutrophil cytoplasmic antibody (ANCA-) associated glomerulonephritis (AAGN), proteinuria is a subject matter which so far received relatively little attention. Studies on AAGN have mainly focused on renal function deterioration in combination with findings in the urine sediment. However, there are some data indicating that the degree of proteinuria at diagnosis is associated with renal outcome in patients with AAGN.[1-3] Also, preliminary data combined from three European Vasculitis Society (EUVAS) clinical trials show that the level of proteinuria during follow-up is a prognostic marker of chronic kidney disease progression.[4] At disease presentation, the majority of patients with AAGN have proteinuria, the amount of which is quite variable.[5]

In general, the presence of proteinuria in kidney diseases is associated with changes in podocyte morphology.[6,7] Podocytes are highly specialized epithelial cells that, together with the glomerular basement membrane (GBM) and glomerular endothelial cells, constitute the filtration barrier of the glomerular capillary wall. The notion that podocytes react to injury by effacement is generally accepted, but exactly how this reactive change relates to the level of proteinuria, remains a matter of debate.[8] Two recent studies investigating foot process effacement in different human glomerulopathies suggested that the amount of foot process effacement is related to the type of glomerulopathy rather than to the amount of proteinuria; for example, patients with IgA nephropathy and minimal change nephrotic syndrome had similar proteinuria levels at diagnosis, but foot processes were significantly more effaced in minimal change nephrotic syndrome.[9,10]

To study podocyte morphology, images at high magnification with electron microscopy (EM) of the podocytes are required. In most centers, EM is not routinely performed in AAGN, because the characteristic findings by light microscopy (LM) and the pauci-immune pattern by immunofluorescence are usually diagnostic. A number of studies investigated EM samples from patients with AAGN,[11-15] but only one described the podocyte morphology in detail.[16] This was a recent study from China showing that foot process width (FPW) was significantly higher and that podocyte density was significantly lower in an Asian group of patients with AAGN compared to healthy controls. In the current study, we investigate the podocyte morphology and number in renal biopsies of a Caucasian population of patients with AAGN. We analyzed whether and how these parameters were related to proteinuria at baseline and during follow-up.

## MATERIALS AND METHODS

### Study population

Patients with histopathologically proven AAGN were retrieved from the Pathology database at Leiden University Medical Center, the Netherlands. Patients had to fulfil the criteria for ANCA-associated vasculitis as specified in the 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.[17] Only patients with

available samples for EM could be included. Samples were either retrieved from tissue obtained by renal biopsy that had previously been stored in glutaraldehyde, or from the paraffin blocks in case of which the quality for EM had to be sufficient for the evaluation of podocyte morphology. Control human renal tissue was used from five pre-transplantation donor kidney biopsies, which showed no abnormalities by LM and from which it was known that the donors were non-proteinuric at time of donation. This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

### Clinical data

Medical records were used to retrieve data on sex, age, diagnosis (granulomatosis with polyangiitis or microscopic polyangiitis), serology (proteinase 3-[PR3-] or myeloperoxidase-[MPO-]ANCA), and laboratory results (serum creatinine and proteinuria). The estimated glomerular filtration rate (eGFR) at time of biopsy and during follow-up was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[18] Proteinuria was expressed as total protein excretion in 24-hour urine. In case this value was missing, proteinuria by dipstick measurement (scale from negative to +++) was used. All patients were classified as having either moderately or severely increased proteinuria according to the Kidney Disease: Improving Global Outcomes (KDIGO) clinical guidelines: moderately increased proteinuria was defined as a protein excretion rate of 0.15 – 0.50 g/day, or as trace or + on protein dipstick test; severely increased proteinuria was defined as total protein excretion over 0.50 g/day, or as + or more on protein dipstick.[19] Proteinuria levels were assessed at least twice during follow-up: at 10 weeks and 1 year, which were regular moments of outpatient visits for all patients.

### Histopathological parameters

Renal biopsies were re-evaluated and classified as either focal, crescentic, mixed, or sclerotic class, following the Berden classification.[20] Moreover, inflammatory infiltrate (<10%, 10-25%, 26-50%, or >50% of unscarred parenchyma), interstitial infiltrate and tubular atrophy (IFTA [0%, <25%, 26-50%, or >50% of cortical area]), and tubulitis (no mononuclear cells in tubules, foci with 1-4 cells/tubular cross section, foci with 5-10 cells/tubular cross section, or foci with >10 cells/tubular cross section) were determined for each case, according to the Banff classification for allograft pathology.[21]

### Measurement of foot process effacement

Renal specimens were fixed in 1.5% GA / 1.0% PF fixative or formalin, post-fixed in osmium tetroxide, and embedded in epon (LADD Research Industries Inc., USA). EM sections were stained with uranyl acetate and lead citrate. For each patient and control, 15 pictures were taken with a JEM-1011 electron microscope (JEOL USA, Inc.) at 10.000-fold magnification. As a measure of foot process effacement, FPW was calculated

using the formula π/4*(ΣGBM length)/(Σfoot processes), where ∑foot processes is the total number of foot processes, ∑GBM length is the total length of GBM, and π/4 is a correction factor for random variation in the angle of section relative to the long axis of the podocyte.[9] The total length of GBM in each picture was measured by ImageJ 1.46r software (National Institutes of Health, rsb.info.nih.gov/ij). The number of foot processes was manually counted.

### Measurement of podocyte number

We used immunohistochemistry to identify and count podocytes based on staining for WT-1, a podocyte-specific transcription factor.[22] Paraffin sections (4-µm thickness) were stained with rabbit anti-human WT-1 (sc-192, Santa Cruz Biotechnology, Dallas, TX, USA), followed by goat anti-rabbit EnVision-HRP conjugate (Dako, Glostrup, Denmark) with diaminobenzidine as the chromogen. The sections were counterstained with hematoxylin. The number of WT-1 positive nuclei per glomerular tuft (referred to as number of podocytes) was counted in three glomeruli unaffected by light microscopic lesions per patient. In the control group, six glomeruli per biopsy were analyzed. The number of podocytes was expressed as number of WT-1 positive nuclei per glomerulus. In the same glomeruli, all nuclei and the surface area of the glomerular tuft were quantified. The software used to count podocytes and nuclei and to measure glomerular surface areas was IMS viewer (Philips Digital Pathology Solution).



### Statistical analysis

Means were compared between groups by using the Student's t-test or one-way ANOVA. Categorical data were compared by using the χ2 test or Fisher's exact test. FPW was correlated to demographic and clinical parameters with Pearson correlation coefficients. All analyses were performed with SPSS statistical software, version 23 (IBM Corp., Armonk, NY, USA). *P* values less than 0.05 were considered significant.

## RESULTS

### Patient characteristics

A total of 25 patients were included in this study. The mean ± SD age at biopsy was 55.4 ± 13.5 years, which was similar to the mean age in the control group (47.2 ± 17.3; p = 0.24). The 24-hour proteinuria at baseline (proteinuria$_0$) was available in 23 patients; the mean was 1.6 ± 1.9 g/day (Table 1). The two patients whose 24-hour proteinuria$_0$ was unavailable had a positive dipstick (+ and ++ respectively). The mean eGFR at baseline (eGFR$_0$) was 42.3 ± 28.6 ml/min/1.73 m2. The level of proteinuria$_0$ and eGFR$_0$ did not correlate (r = 0.07; *P* = 0.75), similar to the level of proteinuria$_0$ and eGFR at 1 year (eGFR$_{1year}$) (r = 0.17; *P* = 0.48). Treatment regimens were as follows: all patients were treated with prednisone; 24 patients received cyclophosphamide, which was switched to

maintenance therapy with azathioprine in 17 patients. Six patients received angiotensin converting enzyme – inhibitor (ACE-I) therapy before or after the diagnosis of AAGN; their level of proteinuria$_0$ was non-significantly higher than the level in patients who did not receive ACE-I therapy (2.3 ± 2.9 vs. 1.3 ± 1.5 g/day; *P* = 0.45). After 10 weeks of follow-up, the level of proteinuria (proteinuria$_{10weeks}$) was similar in patients receiving ACE-I therapy and patients not receiving ACE-I therapy (1.6 ± 0.9 vs. 1.4 ± 1.6; *P* = 0.76). The levels of proteinuria at 1-year follow-up (proteinuria$_{1year}$) were again lower in patients treated with ACE-I and patients who did not receive this treatment (0.9 ± 0.8 vs. 0.6 ± 0.9; *P* = 0.58).

**Table 1.** Characteristics of the study cohort and according to FPW

| | **All patients (*n* = 25)** | **Patients with FPW <600 nm (*n* = 11)[a]** | **Patients with FPW ≥600 nm (*n* = 10)[a]** | ***p* value[b]** |
|---|---|---|---|---|
| **Male** | 15 (60) | 6 (55) | 6 (60) | 1.00 |
| **Age, yr** | 55.4 ± 13.5 | 51.3 ± 14.4 | 60.4 ± 13.1 | 0.15 |
| **Diagnosis** | | | | 0.39 |
| GPA | 16 (64) | 8 (73) | 5 (50) | |
| MPA | 9 (36) | 3 (27) | 5 (50) | |
| **ANCA serotype** | | | | 0.43 |
| PR3-ANCA | 13 (52) | 7 (64) | 4 (40) | |
| MPO-ANCA | 9 (36) | 4 (36) | 4 (40) | |
| Double positive | 2 (8) | 0 (0) | 1 (10) | |
| Negative | 1 (4) | 0 (0) | 1 (10) | |
| **Histopathological class** | | | | 0.03 |
| Focal | 13 (54) | 9 (82) | 3 (33) | |
| Crescentic/mixed | 11 (46) | 2 (18) | 6 (67) | |
| **Podocytes/glomerulus** | 15.0 ± 6.5 | 15.8 ± 6.6 | 13.4 ± 6.4 | 0.49 |
| **eGFR$_0$, mL/min/1.73m$^2$** | 42.3 ± 28.6 | 49.4 ± 33.9 | 38.1 ± 21.4 | 0.38 |
| **eGFR$_{1year}$, mL/min/1.73m$^2$** | 59.1 ± 23.4 | 68.4 ± 19.1 | 57.3 ± 22.5 | 0.31 |
| **Proteinuria$_0$, g/day** | 1.6 ± 1.9 | 0.9 ± 0.5 | 2.4 ± 2.7 | 0.14 |
| **Proteinuria$_{10weeks}$, g/day** | 1.4 ± 1.4 | 1.0 ± 1.1 | 2.0 ± 2.0 | 0.21 |
| **Proteinuria$_{1year}$, g/day** | 0.7 ± 0.9 | 0.7 ± 1.0 | 1.0 ± 0.9 | 0.58 |
| **ESRD[c]** | 3 (12.0) | 0 (0.0) | 1 (12.5) | 0.44 |

Values are reported as number (%) or mean ± SD.

[a] FPW could not be measured in four patients, because of insufficient EM material.

[b] Indicating differences between patients with FPW<600 nm and ≥600 nm.

[c] Missing data for 2 patients due to limited follow-up.

eGFR = estimated glomerular filtration rate; ESRD = end-stage renal disease; FPW = foot process width; GPA = granulomatosis with polyangiitis; MPA = microscopic polyangiitis; PR3-ANCA = proteinase 3 ANCA; MPO-ANCA = myeloperoxidase ANCA.

### Glomerular and tubulointerstitial parameters

Thirteen biopsies were scored as focal, five as crescentic, six as mixed, and one could not be classified due to insufficient number of glomeruli (i.e., less than 7). Patients with a biopsy categorized as focal class had the lowest level of proteinuria$_0$ (0.9 ± 0.5 g/day), followed by mixed class (1.2 ± 1.1 g/day), and crescentic class (3.4 ± 3.1 g/day; $P$ = 0.02). Proteinuria$_{10weeks}$ did not differ between classes ($P$ = 0.39), similar to the level of proteinuria$_{1year}$ ($P$ = 0.35). Inflammatory infiltrate, IFTA, and tubulitis were not associated to the level of proteinuria at baseline or during follow-up.

### Foot process width

Figure 1 shows examples of EM pictures from the patient and control group. EM material turned out to be insufficient in four patients. The mean FPW in renal biopsies of 21 patients with AAGN was 603 ± 66 nm. In the control group (biopsies from five living donors), mean FPW was 571 ± 35 nm, which is in accordance with the normal range in previous studies.[7,9,10,16] The mean FPW in patients was not significantly different from the FPW in controls ($P$ = 0.31), but the three patients presenting with nephrotic range proteinuria (i.e., >3 g/day) did have a higher FPW compared to controls (657 ± 35 nm; $P$ = 0.02). Characteristics were compared between patients with a FPW <600 nm and ≥600 nm; this cut-off point was chosen because the highest FPW in the control group was 602 nm. Biopsies from patients with a FPW <600 nm were most often categorized as focal class, whereas biopsies with FPW ≥600 nm belonged more often to the crescentic or mixed class ($P$ = 0.03; Table 1). Tubulointerstitial parameters were not different between the two groups of FPW. The mean level of proteinuria$_0$ was not significantly higher in patients with FPW ≥600 nm compared to patients with FPW <600 nm (2.4 ± 2.7 versus 0.9 ± 0.5 g/day; $P$ = 0.14; Table 1). Figure 2 shows proteinuria levels during follow-up of individual patients according to FPW subgroups. Proteinuria$_0$ did not correlate with FPW (r = 0.33; $P$ = 0.16). Proteinuria$_{10weeks}$ correlated significantly with FPW (r = 0.50; $P$ = 0.04). At 1-year follow-up, the correlation between proteinuria and the FPW at biopsy was lost (r = 0.22; $P$ = 0.40). A correlation of borderline significance was found between FPW and age at biopsy (r = 0.43; $P$ = 0.05). No significant correlation was observed between FPW and eGFR at baseline and during follow-up.

### Number of podocytes

Material for immunohistochemistry was available in 19 patients, of which four were excluded due to the absence of glomeruli without light microscopic lesions. The remaining 15 patients had a mean of 15 ± 7 podocytes per glomerulus. The mean number of podocytes was 34 ± 4 per glomerulus in the control group, which was significantly higher compared to the patients with AAGN (Figure 3; $P$ < 0.0001). The mean surface area of the glomerular tuft was not significantly different in patients versus controls (0.019 ± 0.006 mm2 and 0.025 ± 0.012 mm2 respectively; $P$ = 0.12); also the total number of

nuclei per glomerulus was not significantly different between patients and controls (84 ± 24 and 98 ± 12 respectively; *P* = 0.30). The percentage nuclei positive for WT-1 of the total number of nuclei was significantly lower in patients compared to controls (19.4 ± 9.0% vs. 34.3 ± 1.1%; *P* < 0.001). The number of podocytes per glomerulus in patients with AAGN did not correlate with FPW (r = -0.190; *P* = 0.52) or any of the clinical parameters. No significant differences were observed between patients with less and more than the median of 18 podocytes per glomerulus (Table 2).

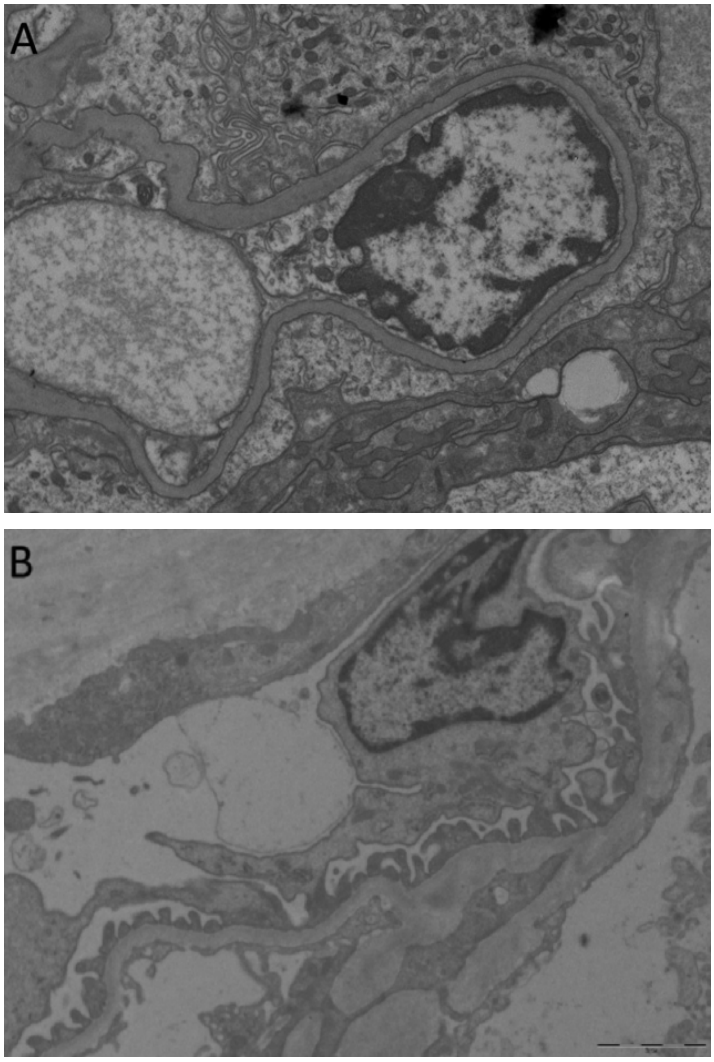


**Figure 1.** Examples of EM pictures used to calculate FPW (magnification 10.000-fold). (A) EM picture of a patient with AAGN showing foot process effacement. (B) EM picture of a control with normal foot processes.

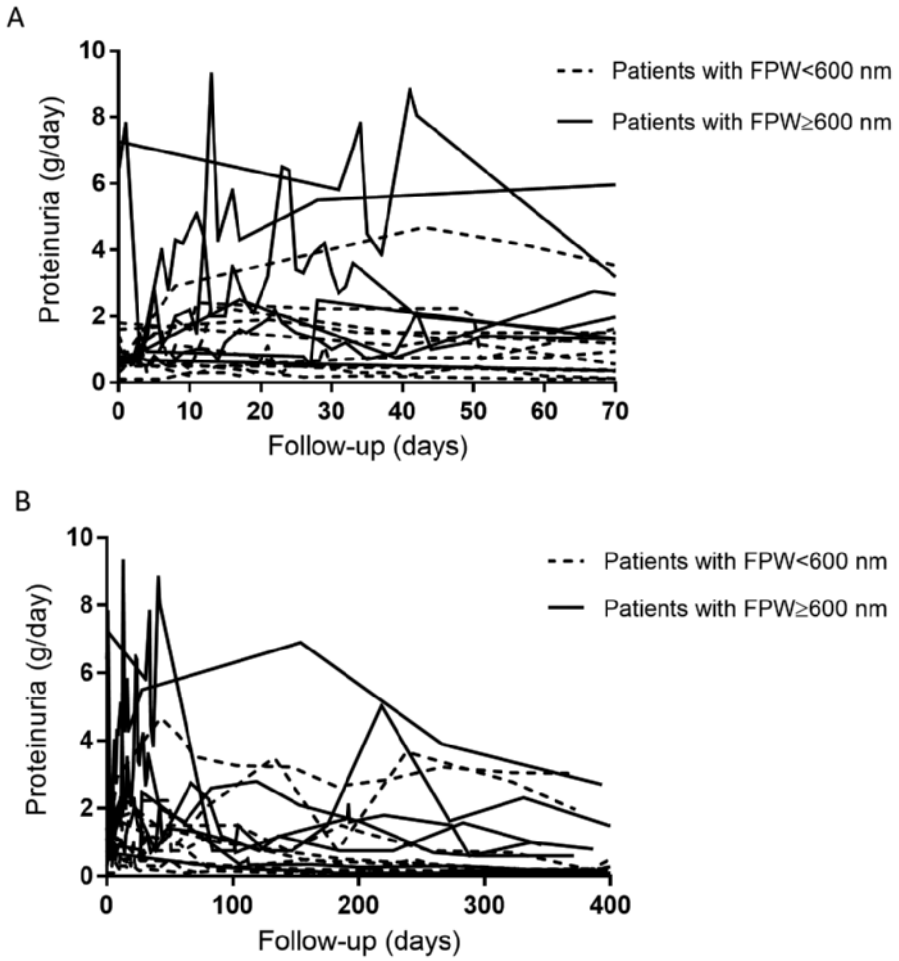


**Figure 2.** Course of patients' individual 24-hours proteinuria levels during follow-up. (A) Proteinuria levels during 10 weeks of follow-up. (B) Proteinuria levels during 400 days of follow-up.

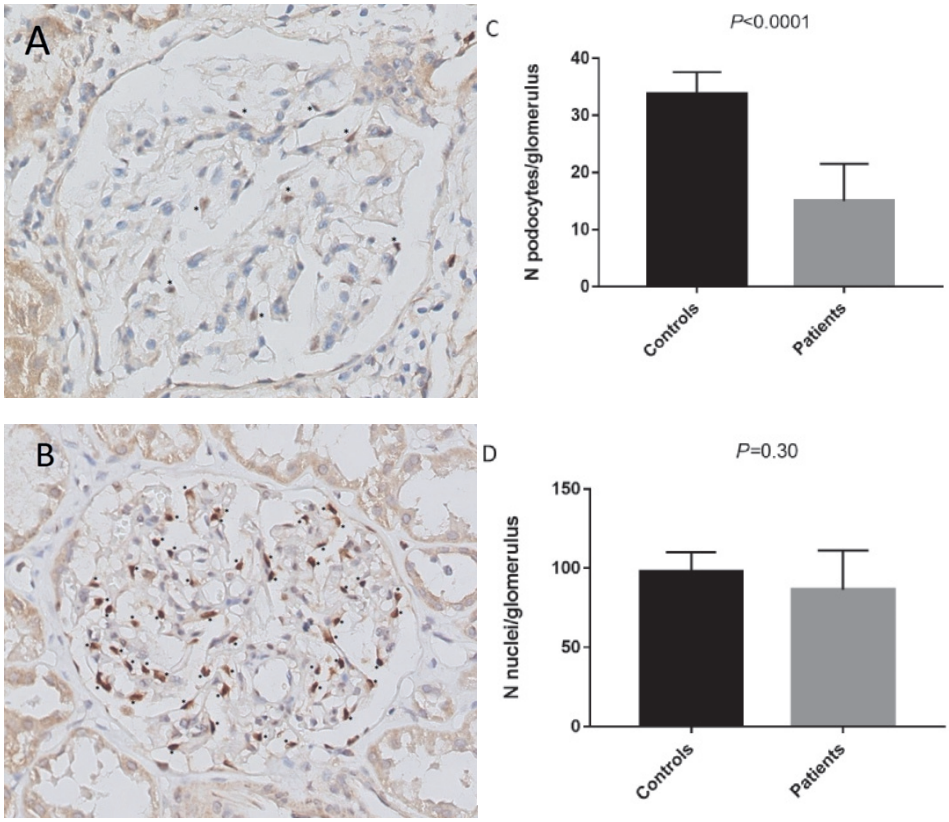


**Figure 3.** Podocytes positive for WT-1. (A) WT-1 staining in a glomerulus of a patient with AAGN. (B) WT-1 staining in a glomerulus of a control. Asterisks (*) indicate a podocyte positive for WT-1. (C) Number of podocytes per glomerulus in controls and in patients ($P < 0.0001$). (D) Number of nuclei per glomerulus in controls and in patients.

**Table 2.** Characteristics according to number of podocytes

| | Patients with <18 podocytes/glomerulus (*n* = 9)[a] | Patients with ≥18 podocytes/glomerulus (*n* = 6)[a] | *P* value |
|---|---|---|---|
| **Male** | 5 (55.6) | 4 (66.7) | 1.00 |
| **Age, yr** | 54.2 ± 19.4 | 58.0 ± 8.4 | 0.62 |
| **Diagnosis** | | | 1.00 |
| GPA | 5 (55.6) | 4 (66.7) | |
| MPA | 4 (44.4) | 2 (33.3) | |
| **ANCA serotype** | | | 0.61 |
| PR3-ANCA | 4 (44.4) | 4 (66.7) | |
| MPO-ANCA | 5 (55.6) | 2 (33.3) | |
| **Histopathological class** | | | 0.59 |
| Focal | 3 (37.5) | 4 (66.7) | |
| Crescentic/mixed | 5 (62.5) | 2 (33.3) | |
| **$eGFR_0$, mL/min/1.73m** | 34.4 ± 18.7 | 48.6 ± 14.8 | 0.14 |
| **$eGFR_{1year}$, mL/min/1.73m** | 56.3 ± 18.9 | 59.6 ± 7.2 | 0.75 |
| **$Proteinuria_0$, g/day** | 2.4 ± 2.7 | 1.6 ± 1.7 | 0.59 |
| **$Proteinuria_{10weeks}$, g/day** | 1.7 ± 1.9 | 0.9 ± 0.9 | 0.46 |
| **$Proteinuria_{1year}$, g/day** | 0.9 ± 0.9 | 0.3 ± 0.1 | 0.21 |
| **ESRD[b]** | 0 (0.0) | 1 (16.7) | 0.46 |

Values are reported as number (%) or mean ± SD.

[a] Material for immunohistochemistry was available in 19 patients, of which four were excluded due to the absence of glomeruli without light microscopic lesions.

[b] Missing data for 2 patients due to limited follow-up.

eGFR = estimated glomerular filtration rate; ESRD = end-stage renal disease; GPA = granulomatosis with polyangiitis; MPA = microscopic polyangiitis; PR3-ANCA = proteinase 3 ANCA; MPO-ANCA = myeloperoxidase ANCA.



## DISCUSSION

Previous studies have underlined the importance of proteinuria as a prognostic marker in patients with AAGN.[1-4] Since proteinuria has been associated with podocyte abnormalities, we here investigated the structural changes in podocytes in Caucasian patients presenting with AAGN. Although the FPW in patients was not statistically different from the mean FPW in healthy controls, we did identify an interesting association with clinical data as FPW correlated with the level of proteinuria 10 weeks after diagnosis. During these 10 weeks, the level of proteinuria increased in particular in patients whose FPW ≥600 nm (Figure 2b). Therefore, studying podocyte morphology in patients with AAGN may be indicative of whether or not patients will have an increase of proteinuria at short-term follow-up. At 1 year, the correlation between FPW and proteinuria was lost. The anti-inflammatory effect of immunosuppressive therapy may reduce the altered permeability of the glomerular capillary wall, thereby reducing the leak of proteins.[23] Moreover, in

vitro experiments have demonstrated a direct effect of corticosteroids on podocytes, enhancing their survival and promoting their repair.[24,25] Therefore, in addition to reducing inflammation, it could be hypothesized that corticosteroids cause podocytes to regain their normal morphology, leading to the observed decrease in level of proteinuria during 1-year follow-up in our patients with AAGN (Figure 2a). Only by performing EM on repeat protocolized biopsies, which were unavailable in the current study, more insights in this process could be obtained.

The exact relationship between foot process effacement and level of proteinuria is a topic of debate; some studies on glomerular diseases found a correlation between the degree of foot process effacement and amount of proteinuria,[6,7] whereas others did not.[9,10] In our study, FPW did not correlate with the amount of proteinuria at baseline, but we observed severely increased levels of proteinuria at baseline in all patients with a FPW ≥600 nm. Moreover, the three patients presenting with nephrotic range proteinuria had a FPW of 627 nm, 648 nm, and 696 nm; all higher than the highest reported value of 602 nm in controls. These data suggest that foot process effacement and proteinuria are related in patients with AAGN; however, a firm association could not be established.

In contrast to our results, the study by Zou *et al*. reported a mean FPW of 1269 nm in patients with AAGN, which was significantly higher than the mean FPW of 586 nm they measured in controls.[16] In their study, the FPW was higher in patients with elevated serum creatinine (>133 µmol/L). However, they did not find a correlation between FPW and proteinuria at baseline, and did not report on proteinuria during follow-up. Although the mean FPW in our patients was much lower than in the Zou study, FPW in our study did correlate to the proteinuria level at 10 weeks. The different results in the study by Zou *et al.* and ours could have arisen from the differences in study cohorts: 96% of patients from the Zou study were positive for ANCA directed against MPO-ANCA, vs. 36% in our study; and the mean level of proteinuria was higher in their study (2.6 g/day vs. 1.6 g/day in our study). Another explanation could be that the patients in the Zou study had a greater patient and/or diagnostic delay, resulting in more severe renal damage at presentation. The results from the study by Zou *et al.* and our study underline the differences between Asian and Caucasian patients with AAGN.[26,27] Whether FPW differs between other populations should be the focus of future studies.

In the current study, we found that biopsies containing a relatively high amount of lesions characteristic for AAGN (i.e., crescentic or mixed class) more often had a FPW ≥600 nm than biopsies with a small number of lesions (i.e., focal class). This is in line with the findings by Zou *et al.*, showing a correlation between FPW and percentage of crescents.[16] It has been suggested that podocytes have an active role in crescent formation; in the early stages before crescent formation, they form bridges between the tuft and Bowman's capsule.[28] In a later stage, they constitute a component of the crescent, and during the transformation to crescentic cells, they lose podocyte-specific antigens, such as WT-1.[29,30] In line with this hypothesis, we found a 50% decrease in

podocytes positive for WT-1 compared to healthy controls, probably reflecting either loss of podocytes or changes in functionality of the podocyte. Our finding of similar numbers of nuclei in glomeruli of patients and controls is suggestive for the latter explanation, and given the diminishment of proteinuria during follow-up this change may be reversible.

The current study has limitations, of which sample size is the major issue. However, EM material of patients with AAGN is scarce, and data on proteinuria are often not routinely documented. Therefore, we decided to focus on quality, rather than quantity of data. Still, we acknowledge that larger studies are required to study podocyte morphology in AAGN into more detail, especially in different populations. Another limitation is that we could not investigate changes in podocyte morphology during follow-up, since repeated biopsy sampling is not part of the standard protocol in AAGN.

In conclusion, we here firstly describe the details of podocyte morphology in Caucasian patients with AAGN. In renal biopsies with AAGN a significant decrease of the podocyte WT-1 marker was found that could be indicative of actual podocyte loss or at least, of a loss of functionality. Patients had variable amounts of FPW, and in particular biopsies with a crescentic or mixed class had the highest FPW. These findings together merit further studies into the morphology and functionality of the podocyte in AAGN. In the meantime, our study indicates that podocyte FPW at baseline is indicative for proteinuria at short term follow up. Therefore, it would be valuable for prognostic purposes to include a description of the FPW in the diagnostic report of a biopsy with AAGN.

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# CHAPTER 6

# Effect of rituximab on malignancy risk in patients with ANCA-associated vasculitis

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## ABSTRACT

### Objectives

Patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) treated with cyclophosphamide have an increased malignancy risk compared with the general population. We investigated whether treatment with rituximab instead of cyclophosphamide has decreased the malignancy risk in patients with AAV.

### Methods

The study included patients with AAV treated at a tertiary vasculitis referral center between 2000 and 2014. The malignancy incidence in these patients was compared with the incidence in the general population by calculating standardized incidence ratios (SIRs), adjusted for sex, age and calendar year. Malignancy incidence was compared between rituximab-treated and cyclophosphamide-treated patients.

### Results

Of the 323 included patients, 33 developed a total of 45 malignancies during a mean follow-up of 5.6 years. This represented a 1.89-fold increased (95% CI 1.38 to 2.53) malignancy risk, and a non-significantly increased risk if non-melanoma skin cancer was excluded (SIR, 1.09; 95% CI 0.67 to 1.69). The risk of non-melanoma skin cancer was 4.58-fold increased (95% CI 2.96 to 6.76). Cyclophosphamide-treated patients had an increased malignancy risk compared with the general population (SIR, 3.10; 95% CI 2.06 to 4.48). In contrast, rituximab-treated patients had a malignancy risk similar to the general population (SIR, 0.67; 95% CI 0.08 to 2.43). The malignancy risk in cyclophosphamide-treated patients was 4.61-fold higher (95% CI 1.16 to 39.98) than in rituximab-treated patients.

### Conclusions

The malignancy risk in patients with AAV was lower in rituximab-treated patients than in cyclophosphamide-treated patients. Notably, rituximab treatment was not associated with an increased malignancy risk compared with the general population. Rituximab could therefore be a safe alternative to cyclophosphamide in the treatment of AAV.

## INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a systemic autoimmune disease that affects small-sized to medium-sized blood vessels in multiple organs. AAV comprises granulomatosis with polyangiitis (formerly Wegener's granulomatosis), microscopic polyangiitis and eosinophilic granulomatosis with polyangiitis (formerly Churg-Strauss syndrome).[1] Autoantibodies against proteinase 3 (PR3-ANCA) and myeloperoxidase (MPO-ANCA) assist in the diagnosis of AAV, but patients can also be negative for ANCA.[2] Although the introduction of cyclophosphamide therapy for AAV has improved patient survival considerably,[3,4] the carcinogenic effects of cyclophosphamide put patients at increased risk of developing malignancies. Several studies have reported increased malignancy risks in patients with AAV who were treated with cyclophosphamide compared with the general population, especially for non-melanoma skin cancer, bladder cancer, malignant lymphoma and leukaemia.[5-12] Moreover, two studies found a dose–response association between cyclophosphamide and malignancy risk.[8,13] These results are restricted to patients with granulomatosis with polyangiitis and microscopic polyangiitis, and the malignancy risk in patients with eosinophilic granulomatosis with polyangiitis has not been investigated in detail before.

International efforts have been devoted to find less cytotoxic regimens for the treatment of AAV. In particular, the cumulative cyclophosphamide doses have been lowered,[14,15] and rituximab has emerged as a promising substitute for cyclophosphamide.[16,17] The initial findings from randomized controlled trials showed similar treatment efficacy in patients treated with either cyclophosphamide or rituximab.[18-20] However, concerns were raised about a possible higher malignancy rate in patients treated with rituximab.[21,22] Notably, the trials focused on treatment efficacy; thus, their results regarding malignancy incidence should be interpreted in light of their small sample sizes and the short follow-up of a maximum of 24 months.

This study investigated the long-term malignancy risk in 323 patients with AAV. This is, to our knowledge, the first study to compare the long-term malignancy risks between patients treated with rituximab and patients treated with cyclophosphamide.

## METHODS

### Study population

The study included patients with AAV (granulomatosis with polyangiitis, microscopic polyangiitis or eosinophilic granulomatosis with polyangiitis) who were treated at the Vasculitis and Lupus Clinic at Addenbrooke's Hospital, Cambridge, UK, between 2000 and 2014. The diagnosis was established according to the European Medicines Agency algorithm.[23] Follow-up began on the date of diagnosis and ended on the date of death, the date the patient was lost to follow-up or on 1 July 2015, whichever occurred first. Follow-

up surveillance was performed at Addenbrooke's Hospital. This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

### Clinical data

The following data were obtained from the medical records of the patients: demographic characteristics, diagnosis, date of diagnosis, ANCA serotype, organ involvement, therapy regimen, renal transplantation and the occurrence of malignancies. Patients with incomplete or missing medical records were excluded from further analyses. The cumulative doses of cyclophosphamide and rituximab during follow-up were determined. For subgroup analysis, patients were categorized according to their cyclophosphamide and/or rituximab exposure into the following categories: patients treated only with cyclophosphamide, patients treated only with rituximab, patients treated with both cyclophosphamide and rituximab, or patients who were not treated with either cyclophosphamide or rituximab. In all categories, the treatment may also have included other immunosuppressive agents, such as glucocorticoids, azathioprine, mycophenolate mofetil, methotrexate and/or tumor necrosis factor (TNF)-α inhibitors.

### Standardized incidence ratio calculations

Standardized incidence ratios (SIRs) were calculated to compare the malignancy incidence between the study cohort and the general UK population, expressing the malignancy risk relative to the general population and matching for sex, age and calendar year. The SIR is the observed number of malignancies divided by the expected number of malignancies. The observed number of malignancies was the total number of primary invasive malignancies. The expected number of malignancies was the number of person-years at risk in our cohort multiplied by the malignancy incidence rates in the general UK population as obtained from the Office for National Statistics and matched for sex, 5-year age group and 1-year calendar time period.[24] Since the malignancy incidence rates were available until 2013, the malignancy incidence rate in 2013 was extrapolated to 2014 and 2015. The SIR was calculated for malignancies at all sites, for all malignancies except non-melanoma skin cancers and for each malignancy site as reported in the study population. SIRs were stratified by sex, age category at diagnosis (younger than the median age of 59 years vs 59 years or older), clinical diagnosis, ANCA serotype, renal transplantation and follow-up duration. Moreover, SIRs were compared in different treatment categories and according to the cumulative doses of cyclophosphamide and rituximab.

### Statistical analyses

Student's t-test, the $\chi 2$ test, Fisher's exact test and the one-way analysis of variance (ANOVA) test were used to compare the characteristics of different subgroups (SPSS statistical software, V.23). SIR values were compared between subgroups by calculating relative risks (RRs). Exact Poisson regression analysis was used to calculate 95% CIs for

the SIR and RR values assuming a Poisson distribution of the observed number of cases (SAS software, V.9.3; SAS Institute).[25-27] *P* values less than 0.05 were considered significant in all analyses.

## RESULTS

### Patient characteristics

The characteristics of the 323 patients with AAV included in this study are shown in Table 1. The mean (SD) age at diagnosis was 56.4 (16.1) years, and the mean follow-up was 5.6 (3.2) years (1802 person-years). A total of 160 (49%) patients were diagnosed with microscopic polyangiitis; 109 patients (34%) were diagnosed with granulomatosis with polyangiitis; and 54 patients (17%) were diagnosed with eosinophilic granulomatosis with polyangiitis. Finally, 12 patients (4%) underwent renal transplantation, and 39 patients (12%) died during follow-up.

### Malignancy occurrence

Of the 323 patients, 33 developed a total of 45 malignancies during follow-up. The sex, age and calendar year-adjusted malignancy risk was 1.89-fold higher in the patients with AAV than in the general population (95% CI 1.38 to 2.53) (Table 2). There were 13 different malignancy types, with non-melanoma skin cancer occurring most frequently (10 basal cell carcinomas and 15 squamous cell carcinomas). The SIR for non-melanoma skin cancer was significantly increased (SIR, 4.58; 95% CI 2.96 to 6.76), while the risk for all malignancies excluding non-melanoma skin cancer was comparable to that of the general population (SIR, 1.09; 95% CI 0.67 to 1.69) (Table 2).



### Malignancy occurrence in the subgroups

The SIR for overall malignancy risk was stratified by gender, age, clinical diagnosis, ANCA serotype, renal transplantation and follow-up duration (Supplementary Table 1). Patients with eosinophilic granulomatosis with polyangiitis had the highest malignancy risk (SIR, 2.75; 95% CI 1.19 to 5.40), followed by those with granulomatosis with polyangiitis (SIR, 2.20; 95% CI 1.20 to 3.68) and those with microscopic polyangiitis (SIR, 1.59; 95% CI 1.01 to 2.38). Transplanted patients had a higher malignancy risk (SIR, 4.31; 95% CI 1.17 to 11.04) than patients who did not undergo renal transplantation (SIR, 1.79; 95% CI 1.29 to 2.43). The treatment duration and cumulative doses of cyclophosphamide and rituximab in subgroups are shown in Supplementary Table 2.

### Effects of cyclophosphamide and rituximab on malignancy risk

Patients treated only with cyclophosphamide had a 3.10-fold higher (95% CI 2.06 to 4.48) malignancy risk than the general population (Table 3), and a 1.14-fold higher (95% CI 0.49

**Table 1.** Characteristics of the patients with ANCA-associated vasculitis who were included in this study[a]

| | All patients (n=323) | No malignancy occurrence (n=290) | Malignancy occurrence (n=33) | *P*[b] |
|---|---|---|---|---|
| **Age (years) at diagnosis, mean (SD)** | 56.4 (16.1) | 55.9 (16.3) | 61.3 (12.7) | 0.03 |
| **Follow-up (years), mean (SD)** | 5.6 (3.2) | 5.5 (3.2) | 6.3 (3.2) | 0.20 |
| **Male, n (%)** | 149 (46) | 135 (47) | 14 (42) | 0.65 |
| **Clinical diagnosis, n (%)** | | | | 0.64 |
| MPA | 160 (49) | 146 (50) | 14 (42) | |
| GPA | 109 (34) | 97 (33) | 12 (36) | |
| EGPA | 54 (17) | 47 (16) | 7 (21) | |
| **ANCA serotype, n (%)**[c] | | | | 0.89 |
| MPO | 110 (34) | 99 (34) | 11 (33) | |
| PR3 | 152 (47) | 136 (47) | 16 (49) | |
| **Organ involvement, mean (SD)** | 2.3 (1.5) | 2.3 (1.5) | 2.2 (1.2) | 0.85 |
| **Deaths, n (%)** | 39 (12) | 30 (10) | 9 (27) | 0.01 |
| **Relapsing disease, n (%)** | 86 (28) | 79 (28) | 7 (22) | 0.54 |
| **Renal transplantation, n (%)** | 12 (4) | 11 (4) | 1 (3) | 1.00 |
| **Treatment, n (%)** | | | | |
| Glucocorticoids | 318 (99) | 286 (99) | 32 (97) | 0.33 |
| Cyclophosphamide | 233 (72) | 207 (72) | 26 (79) | 0.38 |
| Rituximab | 155 (48) | 144 (50) | 11 (33) | 0.07 |
| Cyclophosphamide and rituximab | 114 (35) | 105 (36) | 9 (27) | 0.31 |
| Azathioprine | 218 (68) | 196 (68) | 22 (67) | 0.89 |
| Mycophenolate mofetil | 154 (48) | 141 (50) | 13 (39) | 0.31 |
| Methotrexate | 39 (12) | 35 (12) | 4 (12) | 1.00 |
| TNF-α inhibitors | 19 (6) | 15 (5) | 4 (12)[d] | 0.12 |

[a] Values are reported as means (SD) or as numbers (%).

[b] p Values were calculated using Student's t-test, χ2 test or Fisher's exact test.

[c] ANCA serotype data were not available for 61 patients.

[d] Four of the 19 patients (21%) who received TNF-α inhibitors developed, in total, two basal cell carcinomas, one breast carcinoma and one prostate carcinoma. All four patients were also treated with cyclophosphamide, and one was treated with rituximab. Malignancy risk was similar in patients treated with and without a TNF-α inhibitor.

ANCA, antineutrophil cytoplasmic antibody; MPO-ANCA, myeloperoxidase-ANCA; PR3-ANCA, proteinase 3 ANCA; TNF, tumor necrosis factor.

**Table 2.** SIR for malignancies overall and per observed malignancy site[a]

| Malignancy or malignancy site | Observed malignancies (n) | Expected malignancies (n) | SIR (95% CI)[b] | *P*[b] |
|---|---|---|---|---|
| **All sites** | 45 | 23.80 | 1.89 (1.38- 2.53) | <0.001 |
| **NMSC** | 25 | 5.46 | 4.58 (2.96-6.67) | <0.001 |
| **All malignancies excl. NMSC** | 20 | 18.33 | 1.09 (0.67-1.69) | 0.76 |
| **Lung** | 4 | 2.61 | 1.53 (0.42-3.92) | 0.53 |
| **Breast** | 3 | 2.82 | 1.06 (0.22-3.11) | 1.00 |
| **Colon or rectum** | 3 | 1.98 | 1.52 (0.31-4.44) | 0.63 |
| **Prostate** | 2 | 2.74 | 0.73 (0.09-2.64) | 0.97 |
| **Bladder** | 1 | 0.65 | 1.53 (0.04-8.57) | 0.96 |
| **Pancreas** | 1 | 0.52 | 1.94 (0.05-10.81) | 0.81 |
| **Testis** | 1 | 0.04 | 22.66 (0.62-137.41) | 0.08 |
| **Ovary** | 1 | 0.39 | 2.54 (0.06-14.14) | 0.65 |
| **Melanoma** | 1 | 0.66 | 1.52 (0.04-8.49) | 0.96 |
| **Tongue** | 1 | 0.07 | 13.70 (0.35-76.34) | 0.14 |
| **CNS** | 1 | 0.25 | 3.94 (0.10-21.95) | 0.45 |
| **Kidney** | 1 | 0.49 | 2.03 (0.05-11.32) | 0.78 |

[a] SIR is the ratio of the observed to expected malignancies adjusted for sex, age (per 5-year age group) and calendar time period (per 1-year calendar time period).

[b] Calculated by exact Poisson regression analysis.

CNS, central nervous system; NMSC, non-melanoma skin cancer; SIR, standardized incidence ratio.



to 2.25) malignancy risk if non-melanoma skin cancer was excluded. Patients treated only with rituximab had no increased malignancy risk compared with the general population (SIR, 0.67; 95% CI 0.08 to 2.43), which was similar if non-melanoma skin cancer was excluded (SIR, 0.88; 95% CI 0.11 to 3.19). The malignancy risk inpatients treated only with cyclophosphamide was 4.61-fold higher (95% CI 1.16 to 39.98) than in patients treated only with rituximab and was 3.05-fold higher (95% CI 1.40 to 7.35) than in patients treated with both cyclophosphamide and rituximab (Table 4). The mean cumulative cyclophosphamide dose was lower in patients treated only with cyclophosphamide than in patients treated with both cyclophosphamide and rituximab (7.3 g vs 11.1 g; *P*=0.002). The duration of follow-up was longer for patients who received rituximab than for patients who did not receive rituximab (*P*<0.001). In terms of mean organ involvement, the disease extent did not differ between the treatment groups (*P*=0.07) (Table 3). Patients treated with cyclophosphamide received azathioprine maintenance therapy more frequently than those treated with rituximab (81% vs 42%; *P*<0.001). The SIR of malignancy for patients receiving a combination of cyclophosphamide and azathioprine was 3.20 (95% CI 2.05 to 4.76; *P*<0.001), whereas patients receiving a combination of rituximab and azathioprine

expressed a comparable malignancy risk to that of the general population (SIR, 1.52; 95% CI 0.18 to 5.50; *P*=0.38).

**Table 3.** SIR stratified according to treatment category[a]

| Treatment[b] | Patients (n) | SIR (95% CI)[c] | *P*[c] | CYC dose (g)[d] | Follow-up[e] (years) | Organ involvement[f] |
|---|---|---|---|---|---|---|
| **Only CYC** | 119 | 3.10 (2.06-4.48) | <0.001 | 7.26 (4.94) | 4.92 (3.10) | 2.11 (1.49) |
| **Only RTX** | 41 | 0.67 (0.08-2.43) | 0.86 | 0.00 | 6.34 (3.56) | 2.35 (1.09) |
| **Both** | 114 | 1.01 (0.46-1.93) | 1.00 | 11.05 (11.63) | 6.60 (2.84) | 2.56 (1.63) |
| **None** | 48 | 2.10 (0.77-4.56) | 0.14 | 0.00 | 4.20 (2.94) | 1.96 (1.44) |

[a] Values are reported as means (SD) unless otherwise indicated. The SIR is the ratio of the observed to expected malignancies adjusted for sex, age (per 5-year age group) and calendar time period (per 1-year calendar time period).

[b] The 'only CYC' group was treated with cyclophosphamide but not with rituximab. The 'only RTX' group was treated with rituximab but not with cyclophosphamide. 'Both' indicates a group that received cyclophosphamide and rituximab. 'None' indicates a patient group that neither received cyclophosphamide nor rituximab, but instead had various heterogeneous treatments including glucocorticoids, azathioprine, mycophenolate mofetil and methotrexate. Other immunosuppressive drugs were also administered in all of the groups.

[c] Calculated by exact Poisson regression analysis.

[d] The mean cumulative cyclophosphamide dose differed between the 'only cyclophosphamide' and 'both' groups (Student's t-test, *P*=0.002).

[e] The mean follow-up duration differed between groups (ANOVA, *P*<0.001). The mean follow-up duration also differed when the 'only rituximab' and 'both group' were compared with the 'only cyclophosphamide' and 'none' group (Student's t-test, *P*<0.001).

[f] The mean organ involvement did not differ between groups (ANOVA, *P*=0.07).

ANOVA, analysis of variance; CYC, cyclophosphamide; RTX, rituximab; SIR, standardized incidence ratio.

**Table 4.** Relative risks (RR) according to treatment category

| Treatment[a] | RR (95% CI)[b] | *P*[b] |
|---|---|---|
| **Only CYC vs only RTX** | 4.61 (1.16-39.98) | 0.03 |
| **Only CYC vs both** | 3.05 (1.40-7.35) | 0.003 |
| **Only CYC vs none** | 1.48 (0.60-4.36) | 0.52 |

[a] The 'only CYC' group was treated with cyclophosphamide but not with rituximab. The 'only RTX' group was treated with rituximab but not with cyclophosphamide. 'Both' indicates a group that received cyclophosphamide and rituximab. 'None' indicates a group that did not receive cyclophosphamide or rituximab. Other immunosuppressive drugs were also administered in all of the groups.

[b] RR represents the risk of malignancy compared with the reference group. Calculated by exact Poisson regression analysis.

CYC, cyclophosphamide; RR, relative risk; RTX, rituximab.

### Effects of cumulative cyclophosphamide and rituximab doses on malignancy risk

The mean (SD) cumulative cyclophosphamide and rituximab doses were 9.1 (9.0) g and 5.9 (3.4) g, respectively. The highest cyclophosphamide dose was 108 g, given intermittently for 7.6 years, during a follow-up period of 8.1 years, in which the patient experienced no relapses. The highest rituximab dose was 18 g, given intermittently over 6.1 years, during a follow-up period of 9.1 years, in which one relapse occurred. A positive dose–response relationship was found between cyclophosphamide therapy and the overall malignancy risk (Table 5), and between cyclophosphamide therapy and the risk of non-melanoma skin cancer (Supplementary Table 3). The opposite relationship was found for patients treated with rituximab: the higher the cumulative rituximab dose, the lower the overall malignancy risk (Table 5), and the lower the risk of non-melanoma skin cancer (Supplementary Table 3). Patients who did not receive rituximab had a 2.86-fold higher (95% CI 1.98 to 3.99) malignancy risk than the general population. No increased risk was observed when patients had a cumulative rituximab dose below 6.0 g (SIR, 1.41; 95% CI 0.57 to 2.90). A total of 83 patients received more than 6.0 g rituximab, and these patients had a non-significantly lower malignancy risk than the general population (SIR, 0.45; 95% CI 0.09 to 1.32) and a 6.32-fold lower (95% CI 1.99 to 32.15) malignancy risk than patients who did not receive rituximab (Table 5). The cumulative cyclophosphamide and rituximab doses individually received by the patients who developed a malignancy during follow-up are shown in Supplementary Table 4.



**Table 5.** SIR stratified according to cumulative cyclophosphamide and rituximab doses[a]

| Cumulative dose (g) | Patients (n) | N observed malignancies | SIR (95% CI)[b] | *P*[b] | RR (95% CI)[b] | *P*[b] |
|---|---|---|---|---|---|---|
| **CYC** | | | | | | |
| 0 | 89 | 8 | 1.37 (0.59-2.70) | 0.47 | 1 (reference) | |
| 0.1-20 | 207 | 31 | 1.91 (1.30-2.71) | 0.001 | 1.39 (0.63-3.50) | 0.52 |
| 20-108 | 16 | 5 | 5.06 (1.64-11.82) | 0.007 | 3.69 (0.95-12.78) | 0.06 |
| **RTX** | | | | | | |
| 0 | 167 | 34 | 2.86 (1.98-3.99) | <0.001 | 1 (reference) | |
| 0.1-6 | 70 | 7 | <0.001 | 0.47 | 0.49 (0.18-1.13) | 0.11 |
| 6-18 | 83 | 3 | 0.45 (0.09-1.32) | 0.10 | 0.16 (0.03-0.50) | |

[a] SIR is the ratio of the observed to expected malignancies adjusted for sex, age (per 5-year age group) and calendar time period (per 1-year calendar time period). SIR represents the malignancy risk compared with the general population, and the RR represents the malignancy risk compared with the reference group.

[b] Calculated by exact Poisson regression analysis.

CYC, cyclophosphamide; RR, relative risk; RTX, rituximab; SIR, standardized incidence ratio.

## DISCUSSION

This study compared the malignancy risks in patients with AAV treated with rituximab versus cyclophosphamide. Strikingly, patients treated with cyclophosphamide had a 4.61-fold higher risk than those treated with rituximab. In patients treated with cyclophosphamide, the malignancy risk was 3.10-fold higher than in the general population; in contrast, patients treated with rituximab did not show an increased risk compared with the general population. Patients treated with both rituximab and cyclophosphamide (*n*=114) had a lower malignancy risk than those treated with only cyclophosphamide, even though the mean cyclophosphamide dose was lower in the latter group. In addition, there was a non-significant trend towards an inverse dose–response relationship between the cumulative rituximab dose and malignancy risk: the more rituximab a patient received, the lower the malignancy risk, with the risk actually falling below the risk in the general population if more than a cumulative dose of 6.0 g was given. The relative risk for developing a malignancy was more than six times lower in patients who had received a cumulative dose of rituximab of more than 6.0 g than in patients who had not received rituximab at all.

Interestingly, our findings—although the number of patients was relatively low—may point towards the possibility that rituximab has a protective role in the development of malignancies. This hypothesis is underlined by data showing a trend of an inverse dose–response relationship, and by the difference in malignancy development of the combined treatment group (i.e., patients receiving both cyclophosphamide and rituximab). Depletion of B cells due to rituximab may increase antitumor immunity, as was demonstrated in mouse models in which B-cell-deficient mice are resistant to the development of certain malignancies.[28,29] The enhanced antitumor immune response in these mice is probably caused by decreased IL-10 production by B cells, leading to enhancement of the antitumor effects of cytotoxic T cells.[28] There is emerging evidence that regulatory B cells are the main mediators of this mechanism.[30] In humans, the hypothesis that rituximab enhances the antitumor immune response is supported by the trend towards a lower risk of developing a second primary malignancy in patients with non-Hodgkin's lymphoma treated with rituximab-containing chemotherapy compared with patients treated with chemotherapy that does not include rituximab.[31,32] However, clarification of the effects of B-cell depletion on antitumor immunity in humans requires further investigation.

The increased risks of bladder and hematological malignancies that have been previously reported for patients treated with cyclophosphamide did not materialize in this study, possibly reflecting the ongoing efforts to reduce cumulative cyclophosphamide doses.[11] In accordance with two recent studies, only the risk of non-melanoma skin cancer was increased in the current study.[9,11] To prevent the development of these lesions, all patients were given written information concerning the risks of non-melanoma skin cancer. Moreover, they were advised to avoid ultraviolet radiation, to use sunscreens

and to promptly report skin lesions. Of the patients who developed non-melanoma skin cancer despite these preventative measures, the majority had received azathioprine as maintenance therapy before the occurrence of this malignancy. Therefore, the previously reported association between non-melanoma skin cancer and azathioprine exposure is confirmed in our study.[33-37] However, in our study, only the combination of cyclophosphamide and azathioprine treatment was associated with an increased malignancy risk. In contrast, patients treated with rituximab and azathioprine had a malignancy risk similar to the general population. Lowering cyclophosphamide and azathioprine exposure will most likely decrease the malignancy risk. For patients with AAV who receive azathioprine, especially those who received cyclophosphamide as induction therapy, regular skin cancer screening should be started to control and prevent the development of non-melanoma skin cancers. Moreover, patients should be advised as to how to protect themselves against ultraviolet radiation.[38]

Previous studies that investigated the malignancy risk in patients with AAV were restricted to patients with granulomatosis with polyangiitis and microscopic polyangiitis, and did not include patients with eosinophilic granulomatosis with polyangiitis. Eosinophilic granulomatosis with polyangiitis has a lower incidence than granulomatosis with polyangiitis and microscopic polyangiitis, and it is treated similarly.[39] The 54 patients with eosinophilic granulomatosis with polyangiitis who were included in this study had a 2.75-fold increased malignancy risk compared with the general population. We therefore recommend that clinicians monitor patients with eosinophilic granulomatosis with polyangiitis for malignancies as carefully as patients with granulomatosis with polyangiitis and microscopic polyangiitis.



One limitation of this study is its retrospective design. However, it excluded patients with unclear or missing data. A second limitation is the relative short follow-up, with a mean of 5.6 years. Longer follow-up studies are now required to validate our findings. A third limitation of this study is the relatively small number of patients, particularly in the subgroup analyses. This could explain the non-significance of the inverse dose–response relationship between rituximab and malignancy risk. This relationship merits further investigation in larger studies. Finally, the study involved just one medical center, so the findings may not be generalizable to other settings. One strength of this study is the large study population, in which, for the first time, the malignancy risk was evaluated in patients treated with rituximab during long-term follow-up. This is also the first study to analyze the malignancy risk in patients with eosinophilic granulomatosis with polyangiitis. Another strength of our study is the calculation of cumulative cyclophosphamide and rituximab doses. Finally, the calculation of sex, age and calendar-year period-matched SIRs ensured reliable comparisons between our cohort and the general population.

In conclusion, we demonstrated that patients with AAV who are treated with rituximab have a decreased burden of malignancy, which surpasses expectations from clinical trials data.[18,19] Moreover, our results suggest that rituximab may protect against

the occurrence of malignancies, a possibility that should be explored in further detail using larger cohort populations. Patients with AAV treated with rituximab had a strikingly lower malignancy risk than those treated with cyclophosphamide and no increased malignancy risk compared with the general population. Therefore, the rituximab dose currently used in clinical practice could be a safe alternative to cyclophosphamide in the treatment of AAV.

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**Supplementary Table 1.** Subgroup analysis

| | N patients | N observed malignancies | SIR (95% CI)[a] | SIR *P* value[a] | RR (95% CI)[a] | RR *P* value[a] |
|---|---|---|---|---|---|---|
| **Gender** | | | | | | |
| Male | 149 | 20 | 1.58 (0.96–2.44) | 0.07 | 1 (reference) | |
| Female | 174 | 25 | 2.25 (1.45–3.32) | <0.001 | 1.43 (0.76–2.71) | 0.30 |
| **Age at diagnosis** | | | | | | |
| ≥59 years | 159 | 29 | 1.60 (1.07–2.29) | 0.02 | 1 (reference) | |
| <59 years | 164 | 16 | 2.84 (1.62–4.61) | <0.001 | 1.78 (0.90–3.38) | 0.10 |
| **Clinical diagnosis** | | | | | | |
| Microscopic polyangiitis | 160 | 23 | 1.59 (1.01–2.38) | 0.05 | 1 (reference) | |
| Granulomatosis with polyangiitis | 109 | 14 | 2.20 (1.20–3.68) | 0.01 | 1.39 (0.66–2.81) | 0.43 |
| Eosinophilic granulomatosis with polyangiitis | 54 | 8 | 2.75 (1.19–5.41) | 0.02 | 1.73 (0.67–4.02) | 0.27 |
| **ANCA serotype[b]** | | | | | | |
| MPO-ANCA | 110 | 15 | 1.56 (0.87–2.58) | 0.13 | 1 (reference) | |
| PR3-ANCA | 152 | 24 | 2.18 (1.40–3.25) | <0.001 | 1.40 (0.70–2.87) | 0.39 |
| **Renal transplantation** | | | | | | |
| No | 311 | 41 | 1.79 (1.29–2.43) | <0.001 | 1 (reference) | |
| Yes | 12 | 4 | 4.31 (1.17–11.04) | 0.03 | 2.40 (0.62–6.62) | 0.20 |
| **Follow-up** | | | | | | |
| 0–5 years | 156 | 16 | 2.38 (1.36–3.86) | <0.001 | 1 (reference) | |
| 5–10 years | 135 | 23 | 1.81 (1.15–2.72) | 0.01 | 0.76 (0.39–1.54) | 0.50 |
| >10 years | 32 | 6 | 1.38 (0.51 – 3.00) | 0.55 | 0.58 (0.19–1.56) | 0.35 |

[a] The standard incidence ratio (SIR) is the ratio of observed to expected malignancies and represents the malignancy risk compared to the general population, and the relative risk (RR) represents the malignancy risk compared to the reference group. Calculated by exact Poisson regression analysis.

[b] Unknown for 61 patients.

MPO-ANCA, myeloperoxidase ANCA; PR3-ANCA, proteinase 3 ANCA.

**Supplementary Table 2.** Cyclophosphamide and rituximab treatment in the subgroups

| | N patients treated with cyclophos-phamide | Duration of cyclophos-phamide treatment, months (SD) | Mean cumulative cyclophos-phamide dose, g (SD) | N patients treated with rituximab | Duration of rituximab treatment, months (SD) | Mean cumulative rituximab dose, g (SD) |
|---|---|---|---|---|---|---|
| **Gender** | | | | | | |
| Male | 107 | 5.5 (5.5) | 9.9 (6.5) | 68 | 22.1 (14.9) | 5.8 (3.4) |
| Female | 116 | 6.4 (14.4) | 8.4 (10.8) | 85 | 20.9 (14.4) | 5.9 (3.4) |
| **Age at diagnosis** | | | | | | |
| ≥59 years | 114 | 6.5 (13.8) | 6.8 (3.9) | 63 | 20.2 (14.2) | 5.2 (2.9) |
| <59 years | 109 | 5.1 (4.2) | 11.5 (11.8) | 90 | 22.5 (14.9) | 6.3 (3.6) |
| **Clinical diagnosis** | | | | | | |
| Microscopic polyangiitis | 116 | 6.3 (12.6) | 8.1 (10.5) | 65 | 18.7 (13.3) | 5.2 (2.6) |
| Granulomatosis with polyangiitis | 88 | 5.4 (4.4) | 10.7 (7.3) | 66 | 27.7 (15.0) | 6.7 (4.0) |
| Eosinophilic granulomatosis with polyangiitis | 19 | 4.5 (2.8) | 7.8 (4.2) | 22 | 22.6 (15.7) | 5.3 (2.9) |
| **ANCA serotype**[a] | | | | | | |
| MPO-ANCA | 72 | 7.4 (16.1) | 7.2 (4.7) | 43 | 20.7 (16.9) | 5.1 (2.9) |
| PR3-ANCA | 121 | 5.0 (4.2) | 9.2 (6.5) | 82 | 20.9 (13.0) | 6.1 (3.3) |
| **Renal transplantation** | | | | | | |
| No | 213 | 5.2 (4.9) | 9.1 (9.0) | 149 | 21.6 (14.4) | 5.9 (3.4) |
| Yes | 10 | 15.6 (37.3) | 8.5 (8.6) | 4 | 16.7 (19.4) | 4.5 (2.4) |
| **Follow-up** | | | | | | |
| 0–5 years | 102 | 4.7 (3.5) | 7.0 (4.6) | 52 | 17.5 (10.9) | 4.6 (2.8) |
| 5–10 years | 101 | 7.2 (15.5) | 10.8 (11.9) | 82 | 23.5 (15.2) | 6.3 (3.6) |
| >10 years | 20 | 5.4 (5.1) | 10.9 (6.8) | 19 | 24.6 (19.6) | 7.4 (3.1) |

[a] Unknown for 61 patients.

MPO-ANCA, myeloperoxidase ANCA; PR3-ANCA, proteinase 3 ANCA.

**Supplementary Table 3.** SIR for non-melanoma skin cancer according to cumulative cyclophosphamide and rituximab dose[a]

| Cumulative dose (g) | N patients | N observed non-melanoma skin cancer | SIR (95% CI)[b] | SIR *P* value[b] |
|---|---|---|---|---|
| **Cyclophosphamide** | | | | |
| 0 | 89 | 3 | 2.17 (0.45 – 6.34) | 0.16 |
| 0.1–20 | 207 | 18 | 4.89 (2.90 – 7.72) | <0.001 |
| 20–108 | 16 | 3 | 11.72 (2.42 – 34.25) | 0.002 |
| **Rituximab** | | | | |
| 0 | 167 | 23 | 8.47 (5.37 – 12.71) | <0.001 |
| 0.1–6 | 70 | 1 | 0.83 (0.02 – 4.64) | 0.66 |
| 6–18 | 83 | 0 | 0 (0 – 2.47) | 0.23 |

[a] SIR, standardised incidence ratio; the SIR is the ratio of the observed to expected malignancies adjusted for sex, age (per 5-year age group), and calendar time period (per 1-year calendar time period).

[b] Calculated by exact Poisson regression analysis.

**Supplementary Table 4.** Cumulative cyclophosphamide and rituximab dose of each patient with a malignancy

| Malignancy or malignancy site | N observed malignancies | Cumulative cyclophosphamide dose (g)[a] | Cumulative rituximab dose (g)[a] | Time to malignancy (years)[b] |
|---|---|---|---|---|
| Lung | 4 | 36.0; 21.1; 4.9; 0.0 | 0.0; 7.0; 0.0; 5.0 | 3.7; 4.8; 0.5; 8.5 |
| Breast | 3 | 18.0; 6.0; 3.4 | 0; 4.0; 8.0 | 0.9; 1.1; 4.0 |
| Colon or rectum | 3 | 13.8; 9.5; 4.0 | 0.0; 0.0; 0.0 | 2.4; 4.5; 4.0 |
| Prostate | 2 | 10.0; 0.0 | 0.0; 0.0 | 7.4; 1.2 |
| Bladder | 1 | 0.0 | 1.0 | 2.4 |
| Pancreas | 1 | 0.0 | 0.0 | 1.4 |
| Testis | 1 | 7.0 | 5.0 | 4.6 |
| Ovary | 1 | 3.0 | 6.6 | 2.4 |
| Melanoma | 1 | 3.3 | 0.0 | 1.8 |
| Tongue | 1 | 0.0 | 0.0 | 4.5 |
| Central nervous system | 1 | 2.0 | 5.6 | 3.2 |
| Kidney | 1 | 7.0 | 4.0 | 2.4 |

[a] The cumulative doses of each patient with a malignancy is given. Patients with a cumulative dose of 0.0 did not receive the treatment. When more cases of the malignancy were observed, the first reported cumulative cyclophosphamide dose corresponds to the first reported cumulative rituximab dose, and to the first reported time to malignancy.

[b] This is the time between the date of diagnosis of ANCA-associated vasculitis and the date of diagnosis of the malignancy.

CHAPTER 7

# Incidence of malignancy prior to antineutrophil cytoplasmic antibody-associated vasculitis compared to the general population

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## ABSTRACT

### Objectives

Previous studies have reported an increased malignancy risk preceding antineutrophil cytoplasmic antibody–associated vasculitis (AAV), suggesting common pathogenic pathways in these 2 entities. However, the study results were conflicting and often limited to patients with granulomatosis with polyangiitis (GPA). Here, we study the malignancy risk prior to AAV diagnosis [either GPA or microscopic polyangiitis (MPA)] to elaborate on the putative association between malignancy and AAV.

### Methods

A total of 203 patients were selected for the current study. Malignancies prior to AAV diagnosis were identified using a nationwide pathology database, and their occurrence was verified by reviewing the medical files of 145 patients (71.4%). The malignancy incidence was compared to the general population by calculation of standardized incidence ratios (SIR), matching for sex, age, and time period. SIR were calculated for 2 intervals: < 2 years and ≥ 2 years prior to AAV diagnosis. Separate analyses were performed for GPA and MPA.

### Results

The overall risk for malignancy prior to AAV diagnosis was similar to that of the general population (SIR 0.96, 95% CI 0.55–1.57), as was true when risks were analyzed by malignancy type, including skin, bladder, kidney, lung, stomach, rectum, and uterus (SIR ranged from 1.64 to 4.14). We found no significant difference in malignancy risk between patients with GPA and MPA.

### Conclusion

Our findings do not support the hypothesis that preceding malignancies and AAV have a causal relationship or shared pathogenic pathways.

## INTRODUCTION

Antineutrophil cytoplasmic antibodies (ANCA)–associated vasculitis (AAV) is a spectrum of diseases that includes granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA). AAV is a relatively rare systemic autoimmune disease that primarily affects the kidneys and respiratory tract.[1] The presence of autoantibodies against proteinase 3 (PR3-ANCA) and myeloperoxidase (MPO-ANCA) is an important criterion for establishing a diagnosis of AAV, although some patients are negative for both PR3-ANCA and MPO-ANCA.[2]

The precise pathogenesis of AAV is currently unknown; however, both genetic factors and environmental factors (e.g., infection, occupational and lifestyle factors, and specific medications) may be involved.[3] Based on several case reports, malignancies have been suggested to trigger the onset of AAV,[4-10] possibly accounting for the etiology in a small subset of patients.[11] The association between a preceding malignancy and the onset of AAV has also been investigated in larger studies, including retrospective case-control studies, which reported a 4.8%–10% prevalence of prior malignancies in patients with AAV.[12-14] One such study suggested that malignancy should be included in the differential diagnosis of patients presenting with renal vasculitis.[13]

One possible explanation for the putative association between malignancy and AAV is that these conditions have common pathogenic pathways. Two studies have hypothesized on the common pathways in AAV and malignancies.[12,14] Tatsis *et al*. hypothesized that PR3 expression in malignant tissues may initiate the formation of autoantibodies, thereby leading to GPA. They searched for, but did not detect, the PR3 antigen in malignant tissues that developed before the diagnosis of GPA.[12] Faurschou *et al.* hypothesized that the increased prevalence of non-melanoma skin cancer (NMSC) preceding GPA could be due to an imbalance in immune status; they stated that it would be tempting to speculate that a state of acquired immunological dysfunction predisposes to both conditions, given the well-established association between immunosuppression and development of NMSC.[14] This immune imbalance may also contribute to the higher incidence of malignancies after a diagnosis of AAV, in addition to the effects of some immunosuppressive agents.[11] However, because AAV is generally treated with immunosuppressive therapy, investigating the relationship between AAV and malignancy becomes complicated once the patient has been diagnosed with AAV.

Previous studies have yielded inconsistent results regarding the putative association between malignancy and subsequent AAV. These controversies should be resolved before researchers can investigate the relationship between these two conditions in further detail. Here, we calculated malignancy risk using standardized incidence ratios (SIR) based upon histopathologically confirmed malignancy diagnoses. Moreover, we included patients with MPA in our analysis, thereby adding to the limited data available regarding malignancy risk prior to MPA diagnosis.

## MATERIALS AND METHODS

### Study population

For our study, a single cohort was created by selecting patients from two previous studies in which patients were diagnosed with AAV from 1989 through 2015.[15,16] The study by Rahmattulla *et al.* included patients with histopathologically confirmed AAV.[15] In the study by Göçeroğlu *et al.*, diagnosis was based on a clinical presentation that was consistent with ANCA-associated glomerulonephritis in combination with positive ANCA serology and/or histology.[16] The second selection criterion was based on identifying patients in PALGA (the Pathological Anatomy National Automated Archive, a non-profit organization that archives histopathology reports from throughout the Netherlands).[17] A total of 203 patients were included in our study.

### Identification of malignancies

Histopathologically confirmed malignancies that occurred between 1989 and the diagnosis of AAV were identified from the PALGA database. The PALGA database has complete coverage of malignancies since 1991, whereas the coverage is < 100% for 1989 and 1990. Therefore, medical records were used to identify additional malignancies in 145 patients (71.4%). Medical records were not available for 58 patients (28.6%), because they were diagnosed and treated at various medical centers. Medical file review did not reveal other malignancies than those found by PALGA, indicating a relatively small chance of missing malignancies. Only primary invasive malignancies were included in the analysis. In cases in which a patient developed several NMSC, only the first NMSC was included in the analysis, in accordance with guidelines established by the Netherlands Cancer Registry database. Retrospective patient file research is not covered by Dutch legislation on medical research involving human subjects, therefore review by a Research Ethics Board was omitted. The study was conducted in compliance with the Declaration of Helsinki.

### Calculation of SIR

Sex, date of birth, AAV diagnosis (GPA or MPA), date of diagnosis, and ANCA serotype (PR3-ANCA and/or MPO-ANCA) were obtained from the medical records. The incidence of malignancies in our cohort was compared to the general Dutch population by calculating the SIR (the observed number of malignancies divided by the expected number of malignancies). The expected number of malignancies was calculated using the cancer incidence rate obtained from the Netherlands Cancer Registry database. These incidence numbers were stratified according to sex, 5-year age categories, and 1-year time periods. For each patient, the expected incidences of all malignancies and of each malignancy type were calculated according to these stratifications. Malignancy incidence rates were available from 1989; therefore, for each patient, the observation time was from 1989 until the date of AAV diagnosis. A subgroup analysis was performed to calculate SIR for

the following two periods: 0–2 years prior to AAV diagnosis and ≥ 2 years prior to AAV diagnosis. The SIR values were calculated separately for GPA and MPA cases, and the relative incidence between these two groups was compared by calculating relative risk (RR).

### Statistical analysis

To compare the baseline characteristics between the patients with malignancy prior to AAV diagnosis and patients without a preceding malignancy, the Student's t-test or chi-square test was used, where appropriate (SPSS version 23.0; IBM Corp.). Exact Poisson regression analysis (SAS version 9.3; SAS Institute) was used to calculate the 95% CI of the SIR and RR values, assuming a Poisson distribution of the observed cases.[18,19] In all analyses, differences with a *P* value < 0.05 were considered significant.

## RESULTS

### Cohort characteristics

Data were collected from 203 patients (65.0% male, 35.0% female) with AAV. The mean (± SD) age at AAV diagnosis was 55.7 years (16.5) and the mean time of patient observation was 12.1 years (6.6; 2418.3 person-yrs). In 21 patients (10.3%) there was a histologically confirmed diagnosis of AAV and the history of malignancies was known, but because clinical data and/or serological data were missing, a subdivision in GPA and MPA could not be made. Clinical diagnoses were available for 182 patients; GPA and MPA were confirmed in 120 (65.9%) and 62 (34.1%), respectively. Data regarding the ANCA serotype (PR3-ANCA and/or MPO-ANCA positivity) were available for 180 patients; 79 patients (43.8%) were PR3-ANCA-positive, 84 (46.7%) were MPO-ANCA-positive, five (2.8%) were double-positive, and 12 patients (6.7%) were ANCA-negative. The baseline characteristics are summarized in Table 1.



### Observed malignancies

In our cohort, 16 patients had developed a total of 21 malignancies during a mean observation time of 14.3 years (6.5; range 6.5–26.7 yrs; 228.4 person-yrs) prior to their diagnosis of AAV. The types of malignancies are listed in Table 2. Two patients developed multiple basal cell carcinomas. The mean time between the diagnosis of malignancy and the diagnosis of AAV was 6.1 years (6.7; range 0.1–20.0 yrs). Neither the clinical diagnosis nor the ANCA serotype differed significantly between patients with a pre-AAV malignancy and patients without a pre-AAV malignancy. Patients with a preceding malignancy were significantly older at the time of AAV diagnosis (Table 1). Five patients with a malignancy prior to AAV diagnosis were also diagnosed with one or more malignancies after their diagnosis of AAV.

**Table 1.** Cohort characteristics

| | Total cohort (*n* = 203) | No preceding malignancy (*n* = 187) | Preceding malignancy (*n* = 16) | *P*[a] |
|---|---|---|---|---|
| **Age at diagnosis, yrs (mean ± SD)** | 55.7 ± 16.5 | 54.6 ± 16.7 | 68.4 ± 5.6 | <0.001 |
| **Male, n (%)** | 132 (65.0) | 118 (63.1) | 14 (87.5) | 0.05 |
| **Follow-up, yrs (mean ± SD)** | 12.1 ± 6.6 | 11.9 ± 6.5 | 14.3 ± 6.5 | 0.17 |
| **Diagnosis, n (%)** | | | | 0.53[b] |
| GPA | 120 (59.1) | 109 (58.3) | 11 (68.8) | |
| MPA | 62 (30.6) | 58 (31.0) | 4 (25.0) | |
| Unknown | 21 (10.3) | 20 (10.7) | 1 (6.2) | |
| **ANCA serotype, n (%)** | | | | 0.69[c] |
| PR3-ANCA | 79 (38.9) | 74 (39.6) | 5 (31.4) | |
| MPO-ANCA | 84 (41.4) | 76 (40.6) | 8 (50.0) | |
| ANCA-neg | 12 (5.9) | 11 (5.9) | 1 (6.2) | |
| Double-pos | 5 (2.5) | 4 (2.1) | 1 (6.2) | |
| Unknown | 23 (11.3) | 22 (11.8) | 1 (6.2) | |

[a] Patients with a preceding malignancy versus patients without a preceding malignancy. Preceding malignancy was defined as any primary invasive, histopathologically confirmed malignancy that occurred between 1989 and the diagnosis of AAV.

[b] *P* value refers to the comparison between GPA and MPA subgroups.

[c] *P* value refers to the comparison between PR3-ANCA and MPO-ANCA positive subgroups.

GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; neg, negative; pos, positive; PR3-ANCA, proteinase 3-ANCA; MPO-ANCA, myeloperoxidase-ANCA.

**Table 2.** SIR values for all malignancies and by malignancy type

| | Malignancies, *n* | SIR (95% CI) | *P* |
|---|---|---|---|
| **All malignancies**[a] | 16 | 0.96 (0.55-1.57) | 1.00 |
| All malignancies excluding NMSC | 9 | 0.70 (0.32-1.34) | 0.36 |
| NMSC | 7 | 1.83 (0.73-3.76) | 0.19 |
| **By malignancy site** | | | |
| Lung carcinoma | 2 | 3.10 (0.38-11.21) | 0.27 |
| Bladder carcinoma | 2 | 3.47 (0.42-12.55) | 0.23 |
| Melanoma | 1 | 1.95 (0.05-10.84) | 0.80 |
| Gastric carcinoma | 1 | 2.37 (0.06-13.20) | 0.69 |
| Rectal carcinoma | 1 | 1.64 (0.04-9.13) | 0.91 |
| Renal cell carcinoma | 1 | 2.36 (0.06-13.13) | 0.35 |
| Uterine carcinoma | 1 | 4.14 (0.10-23.09) | 0.43 |

[a] In cases in which the patient developed several NMSC, only the first NMSC was included in the analysis, in accordance with guidelines established by the Netherlands Cancer Registry database. Therefore, of the 21 malignancies observed, only 16 were included in the analyses.

SIR, standardized incidence ratio; NMSC, non-melanoma skin cancer.

### Malignancy risk

Overall, the risk of malignancy prior to AAV diagnosis was similar to that of the general population (SIR 0.96, 95% CI 0.55–1.57). We also found that the risk of each malignancy type was similar between our cohort and the general population (Table 2). Given the limited numbers of malignancies at specific sites, we calculated SIR based on the time period for all malignancies and for NMSC. In the period between 1989 and ≥ 2 years prior to AAV diagnosis, a total of 10 malignancies were observed (SIR 0.78, 95% CI 0.38–1.44). During this period, five NMSC occurred, corresponding to a SIR of 1.72 (95% CI 0.56–4.02). Six malignancies were observed < 2 years prior to AAV diagnosis, resulting in a SIR of 1.56 (95% CI 0.57–3.40); of these, two were NMSC (SIR 2.10, 95% CI 0.25–7.60). A separate analysis revealed that prior to AAV diagnosis, patients with GPA had a higher malignancy risk (SIR 1.38, 95% CI 0.69–2.48) than patients with MPA (SIR 0.74, 95% CI 0.20–1.90); however, although the RR was high (RR 1.86, 95% CI 0.55–8.03), it was not statistically significant.

## DISCUSSION

In this study we found no relationship between AAV diagnosis and preceding malignancies. Three previously published studies revealed slightly different results, as summarized in Table 3. Our finding that overall malignancy risk prior to the diagnosis of AAV in 203 patients was similar to the sex-, age-, and period-matched general population is in contrast with the study by Pankhurst *et al*.[13] They reported that overall malignancy risk was 6-fold higher prior to AAV in 200 patients compared to healthy controls. Moreover, malignancy risk in the Pankhurst study was higher in patients with AAV than in patients with Henoch-Schönlein purpura (HSP) or systemic lupus erythematosus (SLE).[13] Tatsis *et al*. did not find an increase in overall malignancy risk;[12] however, they did find an 18-fold increase in the risk of simultaneous (i.e., within 3 mos) occurrence of malignancy and GPA compared to the risk of simultaneous occurrence of malignancy and rheumatoid arthritis (RA). In separate analyses according to malignancy site, an increased risk of renal cell carcinoma was observed in their study.[12] Our cohort did include one case of renal cell carcinoma, but the incidence was similar to the general population. Moreover, our case of renal cell carcinoma was a patient with MPA, whereas the previous study suggested that renal cell carcinoma occurred more frequently in patients with GPA.[12] More recently, Faurschou *et al.* also found no increased risk of preceding malignancy, but they did find a significant, 4-fold increase in the prevalence of NMSC < 2 years before GPA diagnosis.[14] Our cohort had only a 2-fold increase in the incidence of NMSC < 2 years prior to AAV compared to the general population; this increase was not statistically significant. Moreover, Faurschou *et al*. observed a significantly increased risk of testicular cancer, which they attributed to chance finding.[14] Our study did not include a case of testicular carcinoma.

**Table 3.** Design and results from previous studies and the current study

| | Tatsis *et al.* 1999[12] | Pankhurst *et al.* 2004[13] | Faurschou *et al.* 2009[14] | Van Daalen *et al.* |
|---|---|---|---|---|
| **Study period** | 1989-1993 | 1982-2002 | 1973-1999 | 1989-2015 |
| **Study area** | Germany | United Kingdom | Denmark | The Netherlands |
| **Cohort** | 477 patients with GPA | 78 patients with GPA and patients with 122 MPA | 293 patients with GPA | 203 patients with AAV: 120 patients with GPA and 62 patients with MPA |
| **Controls** | 479 patients with RA (unmatched) | 129 patients with HSP (unmatched), 333 patients with SLE (unmatched), and incidence rates from the general population (matched for sex and age) | 2930 controls from the general population (matched for sex and year of birth) | Incidence rates from the general population (matched for sex, age, and time period) |
| **Main results** | OR for all malignancies: 1.79 (95% CI: 0.92-3.48)<br>OR for simultaneous occurrence of GPA and malignancy: 18.00 (95% CI: 2.30-140.67)<br>OR for renal cell carcinoma: 8.73 (95% CI: 1.04-73.69) | RR (compared to HSP): 0.85 (95% CI: 0.69-1.05)<br>RR (compared to SLE): 0.31 (95% CI: 0.14-0.7)<br>RR (compared to the general population): 6.02 (95% CI: 3.72-9.74) | OR for all malignancies: 1.4 (95% CI: 0.9-2.2)<br>OR for testicular carcinoma: 6.4 (95% CI: 1.1- 38)<br>OR for NMSC occurring <2 years before GPA: 4.0 (95% CI: 1.4-12) | SIR for all malignancies: 0.96 (95% CI: 0.55-1.57)<br>SIR for NMSC occurring <2 years before AAV: 2.1 (95% CI: 0.25-7.60)<br>RR for GPA (compared to MPA): 1.86 (0.55-8.03) |
| **Patients with preceding malignancy (%)** | 23 (4.8) | 20 (10.0) | 26 (8.9) | 18 (8.9) |
| **Specific malignancies (number)** | Renal cell carcinoma (7)<br>Bladder carcinoma (1) | Renal cell carcinoma (1)<br>NMSC (1) | Renal cell carcinoma (2)<br>Bladder carcinoma (1)<br>NMSC (7)<br>Testicular carcinoma (2) | Renal cell carcinoma (1)<br>Bladder carcinoma (2)<br>NMSC (7) |

AAV: ANCA-associated vasculitis; GPA: granulomatosis with polyangiitis; MPA: microscopic polyangiitis; RA: rheumatoid arthritis; HSP: Henoch-Schönlein purpura; SLE: systemic lupus erythematosus; OR: odds ratio; 95% CI: 95% confidence interval; RR: relative risk; NMSC: non-melanoma skin cancer.

Differences in study design may explain at least some of the discrepancies between our study and previous studies. For example, we compared the risk of malignancy between patients with AAV and the sex-, age-, and period-matched general population; in contrast, other groups compared patients with AAV to non-matched control groups of patients with RA[12] or patients with HSP or SLE.[13] Moreover, one study excluded NMSC from the analysis, using the general population as reference group.[13] These differences in study designs hamper the execution of a meta-analysis, which could give a more definite answer on whether there is an association between AAV and preceding malignancies. None of the other studies used a comparison by SIR. In the current study, two malignancies (one basal cell carcinoma and one dermatofibrosarcoma protuberans) occurred before 1989 and were excluded from our SIR calculations. Including these malignancies increases the total number of patients with a pre-AAV malignancy to 18. As a result, 8.9% of the patients in our cohort had a malignancy prior to AAV diagnosis, the same percentage reported by Faurschou *et al*. and similar to the prevalence reported by Pankhurst *et al*. (10.0%) and Tatsis *et al*. (4.8%; Table 3).

A dose-response relationship was reported between the increased risk of malignancy after AAV diagnosis and exposure to cyclophosphamide.[20,21] Moreover, other immunosuppressive agents such as azathioprine and tumor necrosis factor-α inhibitors have been associated with an increased risk of certain malignancies.[22,23] Immunosuppressive therapy, therefore, seems to be an important factor in the development of malignancies after AAV diagnosis. Together with the current data showing no relationship between malignancies and the development of AAV, the hypothesis of common pathogenic pathways in malignancies and AAV becomes unlikely, as suggested by Faurschou *et al*.[14] We tentatively conclude that AAV and malignancies are not necessarily related and that the increased malignancy risk after AAV should be considered as a side effect of certain immunosuppressive agents.

An important strength of our study is the inclusion of patients with MPA. Only one other study included this patient population;[13] the two other studies included only patients with GPA.[12,14] Moreover, our data provide the first comparison of malignancy risk between patients with MPA and patients with GPA. An additional strength of our study is that we calculated SIR that were matched for sex, age, and a 1-year time period. Finally, our study was strengthened by the thorough documentation of histopathology by PALGA. This is a clear advantage over large population database studies, in which diagnostic accuracy is often a concern. On the other hand, PALGA had incomplete coverage in 1989 and 1990, leading to a relatively small chance that malignancies were missed. To minimize this possibility, we reviewed the medical records of 145 patients (71.4%); this analysis did not lead to the identification of any malignancies other than those identified by PALGA. Lastly, the relatively small sample size was a limitation of our study, caused by the low incidence of AAV. Therefore, the low numbers of malignancies in the subgroups

may have contributed to a lack of statistical power. Future studies should include larger numbers to address this issue.

Our observations support previously published data indicating that routine screening for an underlying malignancy is not necessary for patients with newly diagnosed AAV.[14] Most importantly, our findings suggest that malignancies and AAV do not have common pathogenic pathways.

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# CHAPTER 8

# Summary and general discussion

## Summary and general discussion

Antineutrophil cytoplasmic autoantibody (ANCA-) associated vasculitis (AAV) is a systemic autoimmune disease which is associated with increased risk of mortality and morbidity. The outcomes of patients with AAV have improved substantially as a consequence of earlier detection of the disease and more sophisticated therapy regimens. These improvements have been illustrated in the cases described in the introduction of this thesis. Despite these advances, the disease influences the lives of patients and of their relatives and intimates substantially. Therefore, this thesis focused on prognosis and outcomes with the aim to further improve the outcomes of patients with AAV.

### Renal histopathology

The renal biopsy is a useful tool to predict renal outcome in patients with ANCA-associated glomerulonephritis (AAGN). The review in chapter 2 summarizes the work performed by a group of experienced renal pathologists that has increased our knowledge on the predictive value of the renal biopsy in this disease. This group of six pathologists (the RENHIS group) developed a systematic scoring system for renal biopsies of patients with AAGN.[1] Their scoring format became standard for evaluating the renal biopsies of patients enrolled in the EUVAS trials. Several studies investigated the prognostic value of the scored parameters. Hauer *et al*. showed that the amount of glomerulosclerosis, the severity of interstitial fibrosis and tubular atrophy, the presence of segmental and cellular crescents, the amount of fibrinoid necrosis, and the percentage of normal glomeruli correlated with renal function at 18 months after diagnosis.[2] As a result of this study and many others, a histopathological classification for AAGN was proposed in 2010 within the collaboration of the EUVAS.[3] This classification system distinguishes four classes based on the predominant glomerular lesion: focal (≥50% normal glomeruli), crescentic (≥50% cellular crescents), mixed (no predominant lesion), and sclerotic class (≥50% globally sclerotic glomeruli). The phenotypical order of focal, crescentic, mixed, and sclerotic corresponded to increasing loss of renal function during follow-up in the original study by Berden *et al*.[3] Between 2010 and 2017, 24 studies have validated the classification system in adults and/or children. The overall conclusion from these studies was that patients with a focal class biopsy have the most favorable renal outcome, whereas patients with a sclerotic class biopsy have the worst renal outcome. However, crescentic and mixed classes seemed to have similar outcomes. These conflicting results with regards to the original study required further investigation and were therefore studied in chapter 3. This chapter describes the results of an international validation study and a meta-analysis investigating the prognostic value of the histopathological classification with a focus on crescentic and mixed class. Both the validation study and meta-analysis showed no significant differences between the crescentic and mixed class regarding renal outcome. These results call for an adjustment of the histopathological classification. Possible improvements may be the addition of tubulointerstitial parameters or the division of

mixed class according to the glomerular lesion that is most frequent; for example, a patient with mixed class renal biopsy and 40% sclerotic glomeruli would have a mixed-sclerotic type biopsy, and a patient with a mixed class biopsy and 40% normal glomeruli would have a mixed-focal type biopsy. Besides improving the classification itself, the study in chapter 3 also stressed the importance of improving the use of the classification, as we observed only moderate interobserver agreement in the group of six pathologists. Therefore, sharpening definitions for histopathological lesions is required.

### Anti-glomerular basement membrane glomerulonephritis

A histopathological classification may aid in prognostication and conceivably, in guidance of treatment. Several renal diseases, such as diabetic nephropathy and systemic lupus erythematosus have histopathological classifications, but no histopathological classification for anti-glomerular basement membrane (anti-GBM) glomerulonephritis has been proposed yet. Given the histopathological similarities between anti-GBM glomerulonephritis and AAGN, we applied the histopathological classification for AAGN to patients with anti-GBM glomerulonephritis in chapter 4. Including 123 patients, this study is the largest to date that investigated renal biopsies of patients with anti-GBM glomerulonephritis. Similar to patients with AAGN, patients with anti-GBM glomerulonephritis and a focal class biopsy had a favorable renal outcome, and patients with a sclerotic class biopsy had a poor renal outcome. The renal outcomes of patients with crescentic and mixed classes were variable. Another finding in this study was the increased renal survival rate in patients with anti-GBM glomerulonephritis after 2007; the success rate doubled after this year. We hypothesize that this is due to earlier detection of the disease and the increased use of intensive therapy regimen in anti-GBM glomerulonephritis. Our study and other recent studies demonstrated the clinical and histopathological heterogeneity in anti-GBM disease;[4,5] therefore, it may be useful to develop a classification for this disease. Since renal function at presentation, percentage of normal glomeruli, and extent of interstitial infiltrate were independently associated with the occurrence of ESRD in our study, these parameters should be included in a classification for anti-GBM glomerulonephritis. Further research on anti-GBM glomerulonephritis should focus on the development of such a classification system and should prospectively evaluate the indication for intensive therapy with plasma exchange and immunosuppression in these patients.

### Podocytes

The studies described in chapters 3 and 4 of this thesis investigated the prognostic value of generally known histopathological characteristics. In contrast, chapter 5 sheds light on a largely unknown feature in AAGN, namely damage of podocytes. Podocytes have an important function in maintaining the glomerular filtration barrier, and damage of these cells can result in proteinuria. In 25 patients with AAGN, we investigated foot

process effacement by measuring the foot process width (FPW) with electron microscopy and calculated the number of podocytes positive for Wilms Tumor 1 (WT-1). FPW in patients was not significantly higher compared to healthy controls; however, the three patients presenting with nephrotic range proteinuria did have a significantly higher FPW compared to healthy controls. Recently, Zou *et al*. investigated FPW in Chinese patients with AAGN and found that FPW was almost two times higher in patients compared to healthy controls.[6] Given these different results, foot process effacement requires further investigation in different ethnic patient groups with AAGN. In line with the Zou study, we found a lower number of podocytes in AAGN; the amount of podocytes positive for WT-1 was 50% lower in patients compared to controls. Since patients and controls had similar numbers of total nuclei, we hypothesize that the functionality of podocytes may change during the disease process; therefore, specific podocyte markers such as WT-1 may be lost. However, the WT-1 marker reduction may also be due to a true loss of podocytes. This hypothesis is strengthened by preliminary results that indicate the presence of podocyturia in patients with AAGN.[7] In conclusion, podocyte abnormalities in patients with AAGN are reported in chapter 5. To further understand this pathology of the podocyte, studies using other podocyte markers and repeat biopsy samples are warranted. From a clinical point of view, we consider it useful to measure FPW in biopsies with AAGN, as FPW was correlated with proteinuria level at 10 weeks of follow-up. This finding requires validation in other cohorts, but could be helpful in determining a therapeutic strategy with or without ACE-inhibition for patients with AAGN.

### Malignancies

Proteinuria and other clinical manifestations of AAV may normalize under appropriate therapy. The cornerstone of therapy for AAV has consisted of cyclophosphamide and corticosteroids since the 1980s. Unfortunately, these agents are associated with adverse events. For example, cyclophosphamide was demonstrated to increase the malignancy risk in a dose-response relationship in patients with AAV.[8,9] Obviously, treatment with a less toxic agent is preferred; therefore, several randomized clinical trials evaluated the efficacy and adverse events of different treatment strategies. Two clinical trials reported very promising results with the monoclonal antibody rituximab, resulting in the approval of rituximab as an alternative for cyclophosphamide in patients with AAV.[10] Because it was largely unknown whether treatment with rituximab was associated with an increased malignancy risk, we investigated the malignancy risk of patients with AAV in relation to therapy in chapter 6. Patients who were treated with cyclophosphamide had a 3.1-fold increased malignancy risk compared to the general population. In contrast, patients treated with rituximab had a similar malignancy risk compared to the general population. Strikingly, patients treated with cyclophosphamide had a 4.6-fold increased malignancy risk compared to patients treated with rituximab. Another interesting finding was that patients who received both cyclophosphamide and rituximab had a similar

malignancy risk compared to the general population. Moreover, a trend towards an inverse dose-response relationship between malignancy risk and rituximab was observed. The latter two findings suggest that rituximab may protect against the development of malignancies; an interesting suggestion which clearly needs further investigation. In conclusion, rituximab could be a safe alternative for cyclophosphamide in the treatment of AAV with regards to malignancies.

It could be hypothesized that the increased malignancy risk that has previously been reported in patients with AAV is not only related to therapy. Two studies found an increased malignancy risk in patients before they were diagnosed with AAV,[11,12] suggesting that these patients have an intrinsic higher risk to develop malignancies. This led to the hypothesis that malignancies and AAV might have common pathogenetic pathways. However, this was disputed by another retrospective study and the study described in chapter 7;[13] these studies did not find an increased malignancy risk prior to the diagnosis of AAV. Although the last mentioned studies suggested that malignancies and AAV do not share pathogenetic pathways, both diseases are characterized by dysregulation of the immune system. Moreover, some therapeutic agents that are used for AAV, are also used in the treatment of certain malignancies, such as lymphomas. Possibly, research in autoimmunity can acquire and apply relevant findings from oncology research, and vice versa.

### Improving prognosis: future perspectives

This thesis describes several aspects of the prognosis of patients with small vessel vasculitis. The prognosis has ameliorated substantially during the last decades as a result of earlier detection and improved therapy. Nevertheless, clinical practice and research are still challenged to further improve the outcomes of patients with AAV. Ultimately, we would like to cure this disease. A fully elucidated pathogenesis can help to find cures. Therefore, studies on molecular and cellular mechanisms in AAV are ongoing. Until we have found a cure for AAV, we have to improve disease control. Therefore, patients should receive tailor-made therapy, i.e. therapy that is most appropriate for a particular patient, aiming to maximize disease control and minimize adverse events. Four key factors play a major role in the process of establishing patient-tailored therapy, which will be discussed in the next paragraphs.

Firstly, there is a need for clear-cut severity scores. One of the most important severity score that is lacking, is a detailed definition for relapses. Currently, centers use different definitions for relapses, making it impossible to compare studies investigating relapses. In the presence of clear-cut severity scores, patients may be better classified according to severity and treated accordingly. In addition to objective severity scores, subjective patient severity scores are needed. Patients differ in their experience of the disease and their experience of side-effects, which should be taken into account in determining the therapeutic strategy.

Secondly, the classification of AAV requires refinement. The clinical and genetic differences between PR3-ANCA positive and MPO-ANCA positive patients have shown to be greater than between patients with GPA and patients with MPA.[14,15] Moreover, PR3-ANCA positive patients achieve remission more frequently after induction treatment with rituximab than after induction treatment with cyclophosphamide.[16] Therefore, ANCA serotype may play a role in treatment decision making, substantiating the suggestion to add ANCA serotype as prefix to the diagnosis.[17]

A third factor to improve patient-tailored therapy is the identification of prognostic markers. In this thesis, we described the histopathological classification as prognosticator in AAGN. After refining this classification, research should point out whether it could be useful to guide therapy based on the histopathological classes. Thus, renal function loss in AAGN can be predicted, but the occurrence of relapses in AAV seems to be difficult to predict. Whether ANCA titer can predict relapses is a matter of debate;[18] therefore, other prognostic markers for relapses are highly warranted.

The fourth factor – genetic markers - overlaps with the third factor. Genetic markers may be useful as a prognostic marker, but also as determinant of relapses or of response to treatment. Large genetic studies are required to investigate the utility of genetic markers, and whether they can be used as guidance of treatment.

Besides the improvement of the patient-tailored aspect of therapy, therapy itself can also be further improved. An increasing number of biotherapies are becoming available, which may extend the therapeutic options for AAV and may increase disease control. For a number of existing therapies, optimal dose, use, and monitoring still need to be determined. Importantly, the optimal strategy should not take into account therapeutic efficacy only, but also the risks of adverse events. Malignancy risks have decreased by using less cyclophosphamide and could be further reduced by using rituximab. Next to reducing the number of malignancies, a decrease in the numbers of infections and cardiovascular events is also highly desired. Therefore, corticosteroid-sparing treatment regimens are currently being investigated.

As described in this thesis, research has extended our knowledge on small vessel vasculitis tremendously. However, many questions remain unanswered, and the ultimate goal of curing the disease is not within reach. Therefore, controlling the disease and minimizing adverse events are today's focus. Cooperation between centers and between patients and physicians are essential in climbing the ladder towards improved prognosis.

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# CHAPTER 9

# Nederlandse samenvatting

In dit proefschrift staat de prognose van patiënten met antineutrofiele cytoplasmatische antilichamen (ANCA-) geassocieerde vasculitis (AAV) centraal. AAV is een systemische auto-immuunziekte die de kleine bloedvaten aantast en omvat verschillende ziekte-entiteiten, waaronder granulomatosis met polyangiitis (GPA; voorheen Wegener's granulomatosis), microscopische polyangiitis (MPA) en eosinofiele granulomatosis met polyangiitis (EGPA; voorheen Churg-Strauss syndroom). Het is meestal moeilijk om de diagnose AAV in een vroeg stadium te stellen, aangezien de eerste presentatie van de ziekte bestaat uit aspecifieke klachten, zoals koorts, hoofdpijn en verminderde eetlust. Later in het ziekteproces worden de klachten meer specifiek, waarbij nieren, luchtwegen, oren, neus, keel, huid, spieren en zenuwen kunnen zijn aangedaan. Met name de nieren zijn frequent aangedaan: bij circa 85% van de patiënten met GPA en MPA zijn de nieren betrokken in het ziekteproces.

AAV is erg zeldzaam: de jaarlijkse incidentie in Europa en Noord-Amerika wordt geschat op 20 nieuwe patiënten per miljoen inwoners. GPA/MPA komt net iets vaker voor bij mannen en het risico om de ziekte te krijgen stijgt met de leeftijd. De pathogenese moet voor een groot deel nog ontrafeld worden. Er wordt verondersteld dat de boosdoeners de ANCA zijn: dit zijn auto-antilichamen gericht tegen een component in de kernen van neutrofiele granulocyten. Hoe de ANCA-productie tot stand komt, is nog niet duidelijk. Waarschijnlijk gebeurt dit door een samenspel van genetische en omgevingsfactoren.

Het is lastig om de diagnose AAV te stellen, aangezien het klachtenpatroon erg divers kan zijn. Aan het diagnostisch proces kan de bepaling van ANCA in het bloed een grote bijdrage leveren. Echter, niet alle patiënten met AAV zijn positief voor ANCA, vanwege een tot nu toe onbekende oorzaak. Vice versa, een positieve ANCA test wil niet direct zeggen dat een patiënt AAV heeft, omdat ANCA ook positief kunnen zijn bij andere ziekten en bij gezonde personen. Het biopt heeft een grotere diagnostische waarde dan de ANCA test en is daarom de gouden standaard bij het stellen van de diagnose. Bij patiënten met AAV en nierbetrokkenheid (de ziekte heet dan ook wel ANCA-geassocieerde glomerulonefritis, AAGN) worden celdood en crescents in het nierbiopt aangetroffen. Crescents zijn halvemaanvormige afwijkingen in de glomeruli, welke zijn ontstaan door ophoping van verschillende cellen. Celdood en crescents zorgen er mede voor dat de nieren niet meer goed kunnen functioneren en er kans op eindstadium nierfalen is. In dit stadium is de schade irreversibel en zal de patiënt dialyse of een niertransplantaat nodig hebben.

## De histopathologische classificatie

Om een nierbiopt van een patiënt met AAGN te evalueren, heeft een groep van vijf nefropathologen een scoringsformulier opgesteld. In **hoofdstuk 2** van dit proefschrift wordt samengevat hoe deze nefropathologen, de RENHIS groep genaamd, te werk zijn gegaan en wat uit hun werk is voortgekomen. Het scoringsformulier werd als

standaard gebruikt voor het beoordelen van nierbiopten van patiënten die met klinische onderzoeken van de European Vasculitis Society (EUVAS) meededen. Studies van deze nierbiopten lieten onder andere zien dat het percentage onaangetaste glomeruli een belangrijke voorspeller is voor de nierfunctie op de langere termijn. Deze bevinding en andere bevindingen in histopathologische studies hebben uiteindelijk geleid tot een voorstel van een histopathologische classificatie voor AAGN in 2010. Deze classificatie heeft als doel om o.a. de nierfunctie te voorspellen aan de hand van histopathologische kenmerken – kenmerken van het nierweefsel die onder een microscoop zichtbaar zijn. De classificatie onderscheidt vier klassen: de "focal class" waarin ≥50% van de glomeruli niet aangetast zijn, de "crescentic class" waarin ≥50% van de glomeruli halvemaanvormige afwijkingen toont, de "mixed class" waarin geen dominante afwijking te vinden is, en de "sclerotic class" waarin ≥50% van de glomeruli verlittekening tonen. Samen met het voorstel van deze classificatie werd een studie gepubliceerd waarin de classificatie werd getest op 100 nierbiopten van patiënten met AAGN. Hieruit bleek dat de klassen in de orde van focal-crescentic-mixed-sclerotic gecorreleerd waren met het verlies van nierfunctie dat optrad in de jaren na het vaststellen van de diagnose. Bijvoorbeeld: een patiënt met een focal class biopt had een betere nierfunctie over 5 jaar dan een patiënt met een mixed class biopt. Deze correlatie kan bijdragen aan de inschatting van de prognose van een patiënt en kan daarmee erg belangrijk zijn voor het bepalen van een juiste therapeutische strategie.

Circa 24 studies hebben gekeken of deze volgorde van klassen correleert met uitkomst in hun eigen groep patiënten. Alle 24 studies valideren dat de focal class de beste uitkomst voor wat betreft nierfunctie heeft, en de sclerotic class de slechtste. Echter, er zijn verschillende uitkomsten in de crescentic en mixed class. Daarom hebben we een grote, internationale validatiestudie gedaan om dit verschil onder de loep te leggen. In **hoofdstuk 3** worden de resultaten van deze studie beschreven, die geen verschil laten zien in uitkomsten tussen de crescentic en mixed class. Voor wat betreft het voorspellen van de nierfunctie op langere termijn is een onderscheid maken tussen crescentic en mixed class dus niet zinvol. In de toekomst gaan we kijken of deze groepen op een ander histopathologisch kenmerk, zoals type crescent of tubulointerstitiële parameters, onderscheiden kunnen worden. Deze studie zal dan bepalend zijn voor de aanpassing van de histopathologische classificatie voor AAGN.

In **hoofdstuk 4** passen we de histopathologische classificatie voor AAGN toe op een ander type kleine vaten vasculitis, namelijk op anti-glomerulaire basaalmembraan glomerulonefritis (anti-GBM ziekte). Dit is een auto-immuunziekte waarbij autoantilichamen gemaakt worden tegen cellen in de filtratiebarrière in de glomeruli, waardoor de glomeruli beschadigen en de nierfunctie achteruit gaat. Bij anti-GBM ziekte gaat dit proces veel sneller dan bij AAGN en daarom wordt de ziekte vaak gediagnosticeerd op het moment dat er al veel (irreversibele) schade is. Daarom heeft een groot deel van de patiënten met anti-GBM ziekte dialyse of een niertransplantaat

nodig. De histopathologische kenmerken van anti-GBM ziekte komen voor een groot deel overeen met de kenmerken van AAGN, maar er is geen histopathologische classificatie voor anti-GBM ziekte. Toen wij de classificatie voor AAGN toepasten op patiënten met anti-GBM ziekte, zagen we dat 59% van de biopten een crescentic class betrof. Opmerkelijk was dat we in 12% van de patiënten een focal class biopt troffen. Dit hadden we gezien de agressiviteit van de ziekte niet verwacht. Deze patiënten hadden allen op één na een goede nierfunctie gedurende de tijd dat ze vervolgd werden. Een andere interessante bevinding was dat patiënten die na 2007 gediagnosticeerd waren 50% minder vaak eindstadium nierfalen ontwikkelden ten opzichte van patiënten die tussen 1986 en 2007 gediagnosticeerd waren. Kortom, patiënten met anti-GBM ziekte hebben vandaag de dag een beter behoud van hun nierfunctie, wat mogelijk het resultaat is van vroegere opsporing van de ziekte en/of verbeteringen in therapie.

In **hoofdstuk 5** gaan we terug naar het nierbiopt en de prognose van patiënten met AAGN. De meeste patiënten met AAGN hebben eiwit in de urine. Normaal gesproken houden de glomeruli eiwitten tegen, zodat ze in het lichaam blijven. Maar als bepaalde structuren in de nier kapot gaan, dan gaan de eiwitten verloren. Eén van die structuren is de podocyt: een cel die vast zit aan de basaalmembraan in de glomeruli en een belangrijke functie heeft in het filtratiesysteem. Podocyten kunnen gezien worden onder een elektronenmicroscoop, waarmee het beeld van een stukje weefsel zeer sterk kan worden vergroot en dus kleine structuren zichtbaar worden. In verschillende nierziekten is gevonden dat vormverandering, namelijk afplatting, van podocyten samen gaat met het verlies van eiwit. Het was grotendeels nog onbekend of deze afplatting ook in nierbiopten van patiënten met AAGN te zien was. Daarom hebben wij dat in dit hoofdstuk onderzocht. We vonden geen significant verschil in mate van afplatting van podocyten tussen biopten van patiënten met AAGN en van patiënten zonder nierziekte. De mate van afplatting zou wel klinisch relevant kunnen zijn, omdat we een correlatie vonden tussen de mate van deze afplatting en de hoeveelheid eiwitverlies 10 weken na het biopt. Naast de vormverandering keken we ook naar het aantal podocyten. Ten opzichte van gezonde nieren, hadden de nieren van patiënten met AAGN bijna 60% minder podocyten, wat betekent dat er verlies van podocyten heeft plaatsgevonden. Echter zou het ook kunnen betekenen dat met behoud van de aanwezigheid de podocyten de functie van de podocyten is aangetast.

## Behandeling

Onbehandeld is AAV een dodelijke ziekte, maar sinds de introductie van het geneesmiddel cyclofosfamide zijn de uitkomsten van patiënten met AAV drastisch verbeterd. Een nadeel van dit geneesmiddel is echter wel dat het ernstige bijwerkingen heeft, waaronder onvruchtbaarheid, infecties en het ontwikkelen van maligniteiten. Daarom hebben verschillende klinische studies onderzoek gedaan naar verkorting van de duur van therapie met cyclofosfamide en verlaging van de dosis cyclofosfamide. Deze

studies hebben ertoe geleid dat patiënten zo min mogelijk worden blootgesteld aan cyclofosfamide en dus minder bijwerkingen ervaren. Daarnaast werden alternatieve geneesmiddelen voor cyclofosfamide gevonden, waaronder rituximab. Twee studies hebben gevonden dat rituximab even effectief is als cyclofosfamide. Echter rezen er zorgen over een mogelijk verhoogd risico op maligniteiten in patiënten die behandeld waren met rituximab ten opzichte van mensen in de algehele bevolking.

In **hoofdstuk 6** vergeleken we het risico op maligniteiten bij patiënten die behandeld waren met cyclofosfamide, met rituximab, met beiden of met geen van beiden. Het risico op maligniteiten in de groep behandeld met cyclofosfamide was 4.6 keer zo groot ten opzichte van de groep behandeld met rituximab. In tegenstelling tot cyclofosfamide was rituximab niet geassocieerd met een verhoogd risico op maligniteiten ten opzichte van de algehele bevolking. We vonden zelfs dat hoe hoger de dosis rituximab, des te lager het maligniteitenrisico was. Daarnaast vonden we dat patiënten die zowel cyclofosfamide als rituximab kregen, geen verhoogd risico op maligniteiten hadden. Deze laatste twee bevindingen zouden kunnen wijzen op een beschermend effect van rituximab op het ontwikkelen van maligniteiten. Aangezien het aantal patiënten in de studie relatief klein was, zou deze hypothese verder onderzocht moeten worden in grotere patiëntengroepen. Wat we wel met zekerheid kunnen concluderen is dat rituximab een veilig alternatief is voor cyclofosfamide in de behandeling voor AAV voor wat betreft het ontwikkelen van maligniteiten.

In **hoofdstuk 7** onderzochten we de hypothese of patiënten met AAV ook al een verhoogd risico op maligniteiten hebben vóór hun ziekte AAV. Het verhoogde risico op maligniteiten ná de ziekte zou in dat geval niet alleen veroorzaakt worden door therapeutische effecten, maar ook doordat patiënten met AAV al een intrinsiek verhoogd risico hebben. Mogelijk zouden maligniteiten en AAV eenzelfde pathogenetische route kunnen hebben, waardoor ze vaak samen voorkomen. Dit was eerder gesuggereerd door onderzoekers die een verhoogd risico op maligniteiten hadden gevonden vóór de diagnose AAV. Echter, het risico van maligniteiten vóór de diagnose AAV was in onze studie even groot bij patiënten als in de algehele bevolking. Wij vonden dus geen aanwijzing voor eenzelfde pathogenetische route in maligniteiten en AAV.



## Toekomstplannen

Het terugkerend thema in dit proefschrift is de prognose van patiënten met AAV. Kernvragen zijn: Hoe kunnen we het nierbiopt gebruiken in de voorspelling van uitkomsten? En hoe kunnen we therapie zo aanpassen dat een patiënt minder bijwerkingen ervaart? Dit proefschrift geeft antwoorden op deze vragen en geeft ruimte tot verdere verbetering van de prognose. Toch blijft meer kennis en onderzoek nodig, waar de laatste alinea ideeën voor geeft.

Aangezien genezing van AAV momenteel niet binnen handbereik ligt, moet de focus liggen op het onder controle houden van de ziekte om de prognose te verbeteren.

Hiervoor is het belangrijk dat arts en patiënt nauw samenwerken om een individueel therapeutisch plan vast te stellen, dat rekening houdt met de ernst van de ziekte en de wensen van de patiënt. Dit wordt ook wel “patient-tailored therapy” genoemd. Om patient-tailored therapy te bereiken voor AAV, is een aantal verbeteringen nodig. Allereerst zijn scores om de ernst van de ziekte weer te geven niet scherp genoeg en wordt er in deze scores geen rekening gehouden met de subjectieve ervaringen van een patiënt. Ten tweede zou de classificatie voor AAV aangepast moeten worden. Dit is het geval voor de histopathologische classificatie, maar ook voor de klinische classificatie. De klinische subgroepen die nu worden beschreven, hebben namelijk veel overlap. Als derde zou patient-tailored therapy verbeterd kunnen worden door onderzoek naar meerdere prognostische markers, zowel klinische als histopathologische parameters. De vierde factor hangt hiermee samen, namelijk genetische markers. Recent onderzoek levert steeds meer aanwijzingen op voor een sterke genetische rol in de ontwikkeling van AAV, maar ook in de reactie op bepaalde therapieën. Om al deze verbeteringen te kunnen bewerkstelligen, is internationale samenwerking en samenwerking tussen patiënt en arts noodzakelijk.

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## Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 2 april 1992 te ’s-Hertogenbosch. In 2010 behaalde zij haar gymnasiumdiploma cum laude (Natuur & Gezondheid en Natuur & Techniek) aan het Gymnasium Beekvliet te Sint-Michielsgestel. In datzelfde jaar begon zij met de opleiding Geneeskunde aan de Universiteit Leiden. Na het behalen van het bachelordiploma volgde zij een half jaar psychologievakken aan the University of British Columbia in Vancouver. Na terugkomst in Nederland begon zij een wetenschappelijke stage op de afdeling Pathologie van het Leids Universitair Medisch Centrum (hoofd: prof. dr. V.T.H.B.M. Smit). In haar project naar ANCA-geassocieerde vasculitis werd zij begeleid door prof. dr. J.A. Bruijn, dr. I.M. Bajema en dr. C. Rahmattulla. Deze stage werd uitgebreid naar een fulltime PhD-onderzoek, dat aanving in oktober 2014. Zij kreeg drie jaar lang de Kolff Student Researcher Grant van de Nierstichting. In mei 2016 is zij begonnen met coschappen lopen en zal naar verwachting in het najaar van 2019 haar artsendiploma behalen.

Tijdens haar periode als PhD-student kreeg zij verschillende keren de kans om congressen bij te wonen. Zo mocht zij een oral presentation geven op de 18th International Vasculitis and ANCA Workshop in Tokio en heeft zij posters gepresenteerd op de ASN Kidney Week 2015 in San Diego, de ASN Kidney Week 2016 in Chicago en de Nederlandse Nefrologiedagen 2016. Voor deze congressen ontving zij verscheidene beurzen van de Vasculitis Stichting, de KNAW Van Walree beurs en de ASN Kidney Stars beurs. Ook is zij lid van de European Vasculitis Society, waarvan zij de jaarlijkse vergaderingen bijwoont. Zij is vrijwilligster bij de Vasculitis Stichting en was gedurende twee jaar redacteur van hun tijdschrift Vascuzine.

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