Predicting relapses in ANCA associated vasculitis

PROEFSCHRIFT

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Introduction

"Διαιτήμασι τε χρήσομαι ἐπί ὑφελείη καμνόντων κατὰ δύναμιν καὶ κρίσιν ἐμήν, ἐπὶ δηλήσει δὲ καὶ ἀδικίη εἰρξειν.”

"I will apply, for the benefit of the sick, all measures which are required, avoiding those twin traps of overtreatment and therapeutic nihilism.”

The quote mentioned above is part of a modern translation of the oath of Hippocrates (460 BC – 370 BC). Even though the original Hippocratic oath has been highly debated throughout the years, in particular regarding abortion and euthanasia,¹ it is unlikely that any physician will disagree with the moral value behind the highlighted quote. This quote implies that the outcome of patients is continuously monitored and new measures that benefit the sick are proactively investigated. In this thesis, we investigate new measures to accurately monitor patients with ANCA associated vasculitis for disease activity during follow-up. These findings may in the future be used to guide maintenance therapy in order to reduce overtreatment and therapeutic nihilism. Before we continue to recent findings and new insights regarding ANCA associated vasculitis, we shall first describe the history of research in the field of ANCA associated vasculitis in order to appreciate our findings in the correct context.
A historical perspective

Periarteritis nodosa

In 1866, Kussmaul and Maier reported a 27-year old journeyman tailor who developed fever, myalgia, mononeuritis multiplex, abdominal pain and proteinuria. The disease progressed rapidly and he died 5 weeks later. At autopsy the involvement of medium-sized vessels was observed with the naked eye: “Peculiar mostly nodular thickening of countless arteries of and below the calibre of the liver artery and the major branches of the coronary arteries of the heart, principally in the bowel, stomach, kidneys, spleen, heart and voluntary muscles, and, to a lesser extent, also in the liver, subcutaneous cell tissue, and in the bronchial and phrenic arteries.”

According to Kussmaul and Maier, the vascular abnormalities originated from foci of fibrinoid necrosis and inflammation in the perivascular sheaths and resulted in small aneurysms along the course of the vessel wall. Hence, they described their observations as periarteritis nodosa. Ferrari later observed that the necrotizing vascular lesions were transmural and not only limited to the perivascular sheaths and reasoned that the term polyarteritis [acuta] nodosa was more appropriate.

After the original report of Kussmaul and Maier of polyarteritis nodosa, defined by the gross macroscopic abnormalities of the vasculature, several other cases were reported with large heterogeneity in clinical and histopathologic features. In fact, by 1938 an estimated 395 cases were reported according to Boyd. Some authors argued that ‘the term [periarteritis nodosa] had gradually encompassed a wide variety of vascular conditions, some of which had little in common with those lesions for which the name was originally coined.”

Several cases of subvariants of periarteritis nodosa are highlighted below that formed the basis of the nomenclature that we use in the present time.

Microscopic Polyangiitis

The involvement of various sizes of arteries and venules was subject to debate. While macroscopic nodular abnormalities along the course of the wall of arteries were observed in most reported cases with polyarteritis nodosa, in some patients vascular inflammation was detectable only by microscope. In his article ‘Uber die nur mikroskopisch erkennbare form der periarteritiis nodosa’, Wohlwill introduced the term microscopic periarteriitis nodosa. Davson et al. highlighted the renal lesions of
segmental necrotizing glomerulonephritis as a form of microscopic periarteritis nodosa. At that time, microscopic necrotizing arteritis was increasingly recognized as a manifestation of hypersensitivity. Similar lesions of microscopic necrotizing arteritis were sometimes observed after administration of the antibiotic drug sulphonamide, as well as in patients with serum sickness. In addition, it was claimed by several authors that similar lesions could be induced in animal models by administration of various substances, including horse serum, egg white, bacterial toxin and other non-specific proteins. Zeek argued that patients with microscopic necrotizing arteritis should be distinguished from the classic periarteritis nodosa and categorized them as hypersensitivity angiitis because she assumed that the lesions were most likely induced by a foreign agent, such as sulphonamide. In addition, she reasoned that the term angiitis would be more appropriate because abnormalities were observed in arteries as well as venules.

Allergic Granulomatosis

Jacob Churg and Lotte Strauss had read the same cases as Zeek regarding the causal role of hypersensitivity with polyarteritis nodosa. However, they were most intrigued by the relationship between allergy, such as severe asthma, and the development of polyarteritis nodosa, as had been reported among others by Rackemann and Greene in 1939 and later emphasized by Harkavy. In their seminal paper in 1951, 13 cases of polyarteritis nodosa were presented with severe asthma which they named Allergic Granulomatosis. Other symptoms included fever, hypereosinophilia, cardiac involvement, in particular pericarditis, peripheral neuropathy and all patients had signs of renal damage in the form of hematuria and albuminuria. At autopsy, macroscopic nodular abnormalities along the course of arteries were observed in the majority of patients. In addition, extravascular lesions in the connective tissue were present in the form of necrotizing granulomas associated with or replacing an inflammatory exudate. While this was also observed in cases with Wegener’s Granulomatosis, the common denominator in the presented cases was the presence of eosinophils in the exudate as well as in the necrotic core of the granuloma (which they subsequently called the allergic granuloma). Different stages could be identified from the composition of the exudate:

“In the acute stage the predominant cell was the eosinophilic leukocyte, which constituted as much as 70 or 80% of the exudate. The number of eosinophils decreased as the acute
inflammation subsided, but they were seldom entirely absent. Further characteristic elements were macrophages and giant cells [...], these became more prominent as the process tended towards the subacute and chronic stages. In addition, there were varying numbers of plasma cells, lymphocytes and neutrophils.\textsuperscript{12}

While renal involvement was observed in most cases, histologic evidence frequently consisted of diffuse or focal interstitial nephritis with influx of eosinophils. Glomerular lesions were seen in most of the cases, but mostly focal and rarely affecting the majority of glomeruli. Churg and Strauss hypothesized that the presence of the allergic granuloma was a separate histopathologic entity, while the presence of angiitis was its most malignant expression.\textsuperscript{12}

Churg and Strauss agreed with Zeek that hypersensitivity angiitis most likely was a separate disease entity as compared to the classic polyarteritis nodosa. Interestingly, one of the patients presented by Zeek as hypersensitivity angiitis had severe asthma, similar to the cases of Churg and Strauss. Churg and Strauss argued that an allergic etiology of necrotizing arteritis was clearly proven in their case series and that it also should be regarded as a separate disease entity. The terms allergic and hypersensitivity have later been abandoned by others because even though a sensitivity phenomenon is involved, there is no unequivocal evidence of hypersensitivity or allergy in all cases.\textsuperscript{13-15}

\textbf{Wegener’s Granulomatosis}

Another variant of periarteritis nodosa of importance was first described by Klinger in 1931. He reported a patient who presented with severe sinusitis with inflammatory upper respiratory tract lesions, proptosis and arthralgia and eventually died due to renal failure.\textsuperscript{16}

Thereafter, Wegener reported several patients with similar disease manifestations and proposed that they form an entity separable from the usual forms of periarteritis nodosa on the basis of clinical and histopathological evidence.\textsuperscript{17,18}

Histologic evaluation was characterized by necrotizing angiitis and glomerulonephritis, but also by granuloma formation in the upper respiratory tract, often necrotic and usually containing giant cells. The term Wegener’s Granulomatosis was first coined by Godman and Churg, who formulated the following criteria:\textsuperscript{14}

"(1) necrotizing granulomatous lesions in the upper air passages, lower respiratory tract or both, (2) generalized focal necrotizing vasculitis involving both arteries and veins, almost always present in the lungs and more or less widely
disseminated in other sites, and (3) glomerulitis, characterized by necrosis and thrombosis of loops or lobes of the capillary tuft, capsular adhesions and evolution as a granulomatous lesion.”

Similar to the diagnosis of polyarteritis nodosa, the prognosis of these patients was morbid:19

“The duration is brief, six months on the average, and the outcome in recognized cases would appear to be fatal. At times the illness runs an intermittent or subacute course for two or more years.”

Making sense of nomenclature

Differences in histopathology have led to considerable nosological confusion.15 A particular definition could describe such a heterogeneous group of features (e.g. periarteritis nodosa), while a particular feature was described by various definitions (e.g. hypersensitivity and microscopic angiitis). The following comment of Fienberg captured the associated frustration strikingly:

“It can hardly be doubted that this plethora of terms has added to the confusion regarding this disease.”15

Several attempts were made to classify patients in different forms of vasculitis with unconvincing results, which led to even more classification schemes. This is not surprising, as stated by Lie: “For a group of conditions that lack pathognomonic clinical features, diagnostic laboratory tests, or a uniform histopathologic expression, and with unknown or incompletely understood etiology and pathogenesis for all of them save few, the classification of vasculitis according to a schema with universal acceptance is virtually an impossible task.”13

Lack of standardisation in diagnostic terms and definitions was a major problem in order to establish the clinical significance of the histopathologic features. Therefore, a group of clinicians sought consensus during the Chapel Hill Conference (CHCC) in 1994 regarding the nomenclature.20 Small vessel vasculitis was divided in Wegener’s Granulomatosis if granulomatous inflammation and necrotizing vasculitis was observed, Churg-Strauss Syndrome if tissue eosinophilia, hypereosinophilia or asthma was also present and Microscopic Polyangiitis (MPA) if necrotizing vasculitis was observed but not granulomatous inflammation. The CHCC nomenclature was revised during the next Chapel Hill Consensus Conference in 2012.21 In particular, there was a gradual shift from naming diseases in honour of the first person(s) who described them in greater detail, to a terminology based on pathology or etiology. In addition, evidence was found that Friedrich
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Wegener was a member of the Nazi party before and during the Second World War. Therefore, Wegener’s Granulomatosis was renamed to Gr
anulomatosis with Polyangiitis (GPA) and Churg Strauss Syndrome to Eosinophilic Granulomatosis with Polyangiitis (EGPA)(see Table 1-1).

<table>
<thead>
<tr>
<th>Name</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulomatosis with Polyangiitis (Wegener’s)</td>
<td>Necrotizing granulomatous inflammation usually involving the upper and lower respiratory tract, and necrotizing vasculitis affecting predominantly small to medium vessels (e.g., capillaries, venules, arterioles, arteries and veins). Necrotizing glomerulonephritis is common.</td>
</tr>
<tr>
<td>Microscopic Polyangiitis</td>
<td>Necrotizing vasculitis, with few or no immune deposits, predominantly affecting small vessels (i.e., capillaries, venules, or arterioles). Necrotizing arteritis involving small and medium arteries may be present. Necrotizing glomerulonephritis is very common. Pulmonary capillaritis often occurs. Granulomatous inflammation is absent</td>
</tr>
<tr>
<td>Eosinophilic Granulomatosis with Polyangiitis (Churg Strauss)</td>
<td>Eosinophil-rich and necrotizing granulomatous inflammation often involving the respiratory tract, and necrotizing vasculitis predominantly affecting small to medium vessels, and associated with asthma and eosinophilia. ANCA is more frequent when glomerulonephritis is present.</td>
</tr>
</tbody>
</table>

Table 1-1. Definitions for small vessel vasculitis adopted by the 2012 International Chapel Hill Consensus Conference (CHCC2012).
The double-edged sword known as cyclophosphamide

Most of the clinicians involved in the diseases described above were of the opinion that the etiology was most likely related to hypersensitivity.\textsuperscript{14,15} Hence, several immunosuppressive therapies were tried, such as corticosteroids, azathioprine, methotrexate and cyclophosphamide.\textsuperscript{23-27} The effect of cyclophosphamide in particular seemed promising, as reported by several case reports in the 1970s.\textsuperscript{26-29} A single-centre cohort study showed that cyclophosphamide therapy resulted in the complete resolution of symptoms of active disease in 79 of 85 (93\%) patients.\textsuperscript{29} As a result, cyclophosphamide became the cornerstone of induction therapy in patients with Granulomatosis with Polyangiitis. At that time, the treatment protocol consisted of cyclophosphamide, 2mg/kg bodyweight, and prednisone, 1mg/kg bodyweight. Prednisone regimen was gradually tapered, starting with an alternate-day regimen after 1 to 2 months, while cyclophosphamide was continued for at least one full year after the patient was in complete remission. In the initial cohort study, 23 (27\%) patients were completely off therapy. However, 25 (29\%) patients who had initially achieved remission experienced symptoms related to active disease during follow-up, in particular when cyclophosphamide was tapered. Immunosuppressive therapy was restarted in these patients and symptoms quickly resolved.\textsuperscript{30} Unfortunately, immunosuppressive therapy, in particular cyclophosphamide, was associated with serious side-effects; patients were at risk of leukopenia and susceptible to potentially life-threatening infections.\textsuperscript{31} Gonadal dysfunction was invariably linked to the treatment, as well as haemorrhagic cystitis.\textsuperscript{30,32,33} In addition, long-term effects were sobering; patients treated with cyclophosphamide had a 2.4-fold overall increase in malignancies, in particular an increased risk for bladder cancer and lymphomas.\textsuperscript{34} Immunosuppressive therapy was effective against active disease, but not against vasculitic damage. As such, almost all patients suffered from morbidity related to the disease and/or treatment, including end-stage renal failure.\textsuperscript{34} These results ushered in a new era in which patients are no longer diagnosed with a fatal disease, but they suffer from a chronic disease instead. In this new era, clinicians were looking for strategies to decrease treatment-related side-effects, while keeping the disease activity under control.
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A spectrum of disease severities

After the establishment of cyclophosphamide induction therapy, it was hypothesized that immunosuppressive therapy could be reduced in patients with less severe disease activity in order to avoid treatment-related side-effects. At the time of the first classification schemes, the insidious onset of Granulomatosis with Polyangiitis was already appreciated. In 1956, Leggat and Walton presented an illustrative case of a 42-year old women with necrotizing granulomas that were limited to the respiratory tract and no signs of renal involvement. During follow-up, 7 months after initial presentation, hematuria and albuminuria was observed and her condition deteriorated shortly thereafter. At autopsy, glomerulonephritis was uncovered amongst other findings of Granulomatosis with Polyangiitis. It was postulated that a diagnosis in the early stage of the disease was possible in which the lesions are confided to the respiratory tract. In addition, a diagnosis of Granulomatosis with Polyangiitis was debated in several cases with pulmonal lesions with similar histopathology and no renal involvement, but the diagnosis of Granulomatosis with Polyangiitis was at that time rejected because they did not fulfil the full triad of criteria as described by Godman and Churg.

In 1966, Carrington and Liebow presented 16 patients with pulmonary lesions identical to Granulomatosis with Polyangiitis, but with absent or limited lesions elsewhere. Importantly, no signs of glomerulonephritis were observed. The authors proposed that these patients suffered from a limited form of Granulomatosis with Polyangiitis as there was no means at the time to distinguish them at the onset from the classic disease. The outcome in these patients was more favourable but nevertheless grave as approximately one third died within a year. Promisingly, several patients responded to corticosteroid monotherapy, which was not the case in patients with generalized forms of Granulomatosis with Polyangiitis.

Not everyone agreed with the sentiment to reduce treatment in patients with limited forms of the disease. Fienberg observed the development of renal failure and mutilation of the face in a series of 12 patients because cytotoxic therapy was not started at the onset of symptoms, often because of a lack of proper interpretation of histologic details. He described the earliest lesions that should warn pathologists for Granulomatosis with Polyangiitis as:
“Very small foci consisting of a granular type of necrosis or fibrinoid degeneration without neutrophils or vascular participation. These foci at first [...] possessed few or no palisading histiocytes at the periphery, but as they became older and larger they developed well defined palisading margins. In some older foci of fibrinoid degeneration the affected collagen became smudged and then disintegrated, but occasionally necrotic bundles remained intact within the margin of palisaded cells.”

Interestingly, necrotizing angiitis was absent in 4 of the 12 cases. As such, Fienberg was reluctant to define limited forms of vasculitis as a separate disease entity but at the same time refuted the necessity of a full triad of criteria as described by Godman and Churg.

Nevertheless, the European Vasculitis Society (EUVAS) set out in the nineties to harmonize treatment strategies for the different stages of disease. It was agreed that the immunosuppressive treatment should reflect the severity of the disease. The EUVAS group arbitrarily categorized patients in localised, early systemic, generalised, renal severe or refractory disease. This classification tries to reflect the postulated (natural) progress of the disease, although refractory disease is included as well and it is clear that patients cannot be classified as such at presentation.

Among others and not limited to the following examples, the efforts of the EUVAS group has resulted in randomized clinical trials that investigated the use of methotrexate in early systemic disease (NORAM), oral versus intravenous cyclophosphamide as induction therapy (CYCLOPS) and azathioprine (CYCAZAREM) or Mycophenolate mofetil (IMPROVE) as maintenance therapy. Most recently, an induction therapy based primarily on Rituximab, a monoclonal antibody targeted against B-cells, was found to be non-inferior as compared to an induction therapy based on cyclophosphamide (RITUXVAS). The Wegener’s Granulomatosis Etanercept Trial (WGET) group later classified their patients as "limited" in the absence of disease features that pose immediate threats to either a critical individual organ or to the patient’s life and as "severe" otherwise. The WGET group classified patients based on the need of directly initiating maximal therapy, which may be more pragmatic for the clinician who needs to decide between aggressive (i.e. cyclophosphamide or Rituximab) or a milder form of treatment (i.e. methotrexate).
The discovery of anti-neutrophil cytoplasmic antibodies

The occurrence of antibodies directed against neutrophils had already been described in the 1960s. In those early days, granulocyte-specific antinuclear antibodies (GS-ANA) were defined as antinuclear antibodies reacting specifically with granulocyte nuclei or when reactivity with granulocytes was higher than reactivity with other cell nuclei (by at least two dilution steps). These antibodies were most often found in patients with rheumatoid arthritis and/or Felty’s syndrome. Unfortunately, attempts to discover the antigen failed repeatedly. In 1982, the occurrence of anti-neutrophil cytoplasmic antibodies in 8 patients with segmental necrotizing glomerulonephritis was described by Davies et al. ANCA were detected by the staining of neutrophil leukocytes after formalin fixation by indirect immunofluorescence (IIF), which resulted in a cytoplasmic pattern (C-ANCA). Thereafter, it was recognized that ANCA was observed in GPA and that the target antigen of ANCA in these patients was proteinase 3. Furthermore, MPO was found to be the target antigen in patients with PR3-ANCA negative GPA, MPA and/or EGPA. Indirect immunofluorescence using ethanol dissolves the myeloperoxidase from the granules, which subsequently attaches to the cell nucleus, resulting in a perinuclear staining pattern (P-ANCA). This is not the case with proteinase 3, which remains cytoplasmic after ethanol fixation and thereby keeping the C-ANCA pattern as a result. Importantly, patients with necrotizing glomerulonephritis are nearly always ANCA positive, while ANCA are not always found in a subgroup of patients with more limited disease.

The pathogenicity of MPO-ANCA was confirmed using a mouse model in 2002 by Xiao et al. In this study, antibodies against MPO were first produced in MPO-deficient mice that were immunized by mouse MPO. These antibodies subsequently induced focal necrotizing crescentic glomerulonephritis one week after injection into wild-type C57BL/6 mice. Vasculitis has, subsequently, been induced by MPO-ANCA in several other mouse models, thereby emphasizing the pathogenicity of the autoantibody. Induction of vasculitis by PR3-ANCA in mice has proven to be difficult, in part because proteinase 3 is not expressed on peripheral blood leukocytes in mice. Immunisation with chimeric human/mouse proteinase 3 led to an autoantibody response but not to gross pathological abnormalities in rats and mice. Little et al. circumvented the lack of proteinase 3 on mice neutrophils by using chimeric mice that were generated by injecting human
haematopoietic stem cells into irradiated mice. Injection of human IgG from patients with PR3-ANCA in these mice subsequently led to mild proliferative glomerulonephritis and punctate bleeding on the surface of lungs. Importantly, vasculitis has readily been induced by ANCA in mice but not granuloma formation. It has been suggested but not proven that “[MPO-ANCA] can cause severe extravascular granulomatous inflammation in mice if they have predisposing characteristics of their innate immune system and synergistic inflammation.”

Furthermore, results from animal studies have led to new insights in the pathophysiology of ANCA associated vasculitis. The priming of neutrophils by bacterial lipopolysaccharide is required for anti-proteinase-3 antibody-induced alveolar haemorrhage and glomerulonephritis and aggravates anti-myeloperoxidase antibody-induced glomerulonephritis. These findings have resulted in the “second hit” hypothesis, which postulates that ANCA-induced (severe) disease reactivation is enabled by or aggravated by a “second hit”, such as a bacterial infection. Next to microorganisms, several other candidates for a second hit have been postulated, such as environmental factors and/or other autoantibodies.

The presence of PR3- or MPO-ANCA in ANCA-associated vasculitis has since been undisputed and has been incorporated in the current classification schemes. The association between ANCA and the diagnosis of GPA, MPA or EGPA was not yet fully elucidated at the time of the first Chapel Hill Consensus Conference and was therefore not included. The revised CHCC in 2012 has incorporated the presence of PR3- or MPO-ANCA in small vessel vasculitis, but still requires histological evidence. The European Medical Agency developed and validated another classification scheme that replaces the need for histology if positive serology for PR3- or MPO-ANCA is present and surrogate markers for GPA or renal vasculitis are present.

The association between serial ANCA measurements and disease activity

Soon after the discovery of ANCA as a diagnostic marker for GPA, it was recognized that ANCA titer rises were associated with disease relapses during follow-up. In one of the initial studies, 35 patients with GPA were prospectively followed. Patients were evaluated every month for signs and symptoms attributable to disease activity and blood was drawn for ANCA testing using IIF. Most importantly, physicians were blinded to the results of the ANCA tests.
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Relapses were subdivided in minor or major, depending on whether the relapse was potentially organ- or life threatening or not. Eight major relapses and 9 minor relapses were observed during the study period of 16 months. All relapses were preceded by an ANCA rise. Only one patient demonstrated ANCA rises that were not followed by relapses.\textsuperscript{71} This observation led to a multitude of studies to investigate the value of serial ANCA measurements to predict disease activity.\textsuperscript{72,73} If an ANCA rise is indeed followed by a relapse of disease activity, then immunosuppressive therapy may be pre-emptively started to prevent the relapse to occur.\textsuperscript{74} However, such a strategy is hampered by potential unnecessary exposure to treatment-related side-effects in patients in whom no relapse would occur after an ANCA rise. Therefore, the use of serial ANCA measurements to guide therapy has been controversial and subject to debate.\textsuperscript{72,73}
The present state of ANCA associated vasculitis

The prognosis of patients with ANCA associated vasculitis has improved dramatically over the years. This is not only related to the introduction of immunosuppressive therapy in the 1960s, as survival rates increased even further in the next decades. Results from a single centre inception cohort of 181 patients from the Limburg Renal Registry have shown that patients diagnosed in the period 1979-1989 had a 3.9 times higher mortality rate as compared to patients diagnosed in the period 2001-2009. An analysis on the long term outcome of a combined cohort of EUVAS trials observed a 1-, 2- and 5-year survival of 88%, 85% and 78%, respectively. The major prognostic factors for mortality at the time of diagnosis are advanced age, worse kidney function and higher disease activity using the Birmingham Vasculitis Activity Score (BVAS) at presentation. Most deaths occur in the first year after the initiation of treatment and are most often caused by treatment-related side-effects, such as infections (50%), rather than active vasculitis (14%). After the first year, mortality rates markedly improve but remain slightly higher than the general population and are most often caused by cardiovascular events, malignancy and infection. Cardiovascular disease remains an important issue during follow-up, since patients with ANCA associated vasculitis have a two- to fourfold increased risk of coronary artery disease as compared to the general population, independent of classic cardiovascular risk factors.

Multiple factors may have resulted in the markedly improved survival rate in patients with ANCA associated vasculitis. First, discovery of ANCA has significantly helped to establish the correct diagnosis. This is evident in the reduction in the delay between the diagnosis and the first onset of symptoms. A Finnish study reported a decrease in diagnostic delay from a median of 17 months in the period of 1981-1985 to only 4 months in the period of 1996-2000. An earlier survey amongst GPA in North America observed similar results and observed a decrease in number of physicians seen by the patient prior to the diagnosis from 5.5 in the 1970s to 4.4 in the 1990s, a number that is still in need of improvement. Second, clinical trials from the last decades have resulted in treatment strategies that requires less cyclophosphamide to induce remission. This is most evident considering the reported cyclophosphamide exposure was an average of 40 months in the original study of Fauci et al., while the cyclophosphamide exposure is currently kept to a minimum of 3 to 6 months. The reduction in
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cyclophosphamide has subsequently resulted in a decrease of treatment-related side-effects, in particular the incidence of malignancy. While a recent meta-analysis concluded that patients treated with cyclophosphamide are still at increased risk of late-occurring malignancies, malignancy risk reduces significantly with lower exposure to cyclophosphamide. Most importantly, patients treated with rituximab do not have an increased risk of a malignancy as compared to the general population.

Relapses were less of a concern at the time of the introduction of cyclophosphamide. The reduction in mortality was a major improvement and patients were simply given more cyclophosphamide when symptoms returned. However, with the advent of immunosuppressive sparing treatment protocols, relapses have become a major concern. A systematic review in 2008 observed a relapse rate of 18 to 40% at 24 months and a time to first relapse of 15 to 29 months. The combined cohort of EUVAS trials reported an overall relapse risk of 38% at 5 years. Relapses during follow-up are associated with the occurrence of irreversible damage. While most damage occurs at initial presentation, patients are still at risk of developing further damage at a subsequent relapse. The reduction of damage occurring at relapse versus diagnosis can most likely be accredited to the vigilance of expert physicians, since most patients are referred to a centre of excellence once the diagnosis has been made. A further analysis of occurring relapses show that relapses with renal involvement lead to more damage, in particular renal failure. Damage during long term follow-up most often consists of disease related damage caused by relapses, e.g. reduced renal function, nasal blockage and hearing loss, although treatment-related damage also occurs, such as osteoporosis, malignancy and diabetes. These observations have led to the proposal to classify patients based on relapse risk rather than the histopathology, disease severity at diagnosis or serology. However, no consensus has been reached yet regarding such a classification system. Moreover, as we will further describe in this thesis, these classification systems do not yet fully incorporate the biologic behavior of the disease and are in need of improvement. Such a classification system may in the future be used to guide maintenance therapy in patients with ANCA associated vasculitis.
**Aim of the current thesis**

From the historical perspective it is apparent that physicians have continuously searched for the optimal strategy to avoid those twin traps of overtreatment and therapeutic nihilism. However, evidence from the present state of ANCA associated vasculitis emphasizes the need for further improvement on this regard. The aim of the current thesis is to provide physicians tools to predict future disease activity in order to guide maintenance therapy in patients with ANCA associated vasculitis. These tools may in the future be used to prevent morbidity and mortality related to active disease, while minimizing treatment-related side-effects.

Serial ANCA measurements have been at the centre of attention in predicting disease activity since the discovery of ANCA, as reviewed in Chapter 3. However, the predictive value of an ANCA rise for a relapse could not be confirmed in several other studies, possibly due to considerable methodological and clinical heterogeneity.\(^{72,73}\) History has shown ANCA associated vasculitis consists of a spectrum of diseases based on differences in histopathology, disease severity and laboratory test results. Moreover, animal studies have shown that ANCA can readily induce vasculitis but not granulomatous disease. In addition, ANCA are nearly always found in severe disease, but not always in limited disease. Therefore, we hypothesized in Chapter 1 that ANCA rises may be highly associated with a relapse of capillaritis, in particular necrotizing glomerulonephritis, but not with a relapse of granulomatous disease.

While several risk factors for a relapse have been described in the literature at the time of remission, no analyses have been performed at the time of an ANCA rise. In Chapter 2, we set out to investigate risk factors for a relapse at the time of an ANCA rise. In particular, we hypothesize that a second hit may be required to create a pro-inflammatory environment that enables the ANCA to become pathogenic. In the “second hit” hypothesis, ANCA-induced (severe) disease reactivation is enabled by or aggravated by an as yet unidentified “second hit”.

Previous studies have only looked at the quantity of ANCA in relation to disease activity. We hypothesize that the pathogenicity of an ANCA rise is modulated by the quality of the autoantibody. Several characteristics of the autoantibody are worth mentioning. First, antibodies are separated in isotypes, of which immunoglobulin G is the most abundant. Immunoglobulin G consists of four subclasses, ranging from IgG1
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to IgG4, based on the heavy-chain structure, resulting in different effector functions.\textsuperscript{100} In particular immunoglobulin G3 is effective in the induction of effector functions. Therefore, a prospective study investigated whether serial ANCA testing of the IgG3 subtype was more predictive of a relapse, but this could not be confirmed.\textsuperscript{101} Second, not every ANCA targeted against PR3 or MPO reacts to the same epitope of PR3 or MPO, respectively. Antibodies may react to different epitopes of the antigen, ranging from a linear sequence of amino acids to a discontinuous complex of amino acids that come together in a three-dimensional conformation. In patients with ANCA associated vasculitis, it has been demonstrated that a substantial diversity of MPO-ANCA epitopes is present and some epitopes are exclusive to active disease.\textsuperscript{102} Third, antibodies and antigen bind together to form an antigen-antibody complex. This complex is reversible and the strength of the complex may vary between antibody and antigens, which is expressed as the avidity. In Chapter 4, we explore whether the avidity of PR3-ANCA changes during follow-up. Importantly, we hypothesize that the avidity of the autoantibody is higher in relapsing patients compared to non-relapsing patients.

Fourth, the effector function of the antibody is dependent on the glycan structure attached to the fragment crystallizable region. The glycosylation profile of the crystallizable fragment (Fc) of the immunoglobulin G (IgG) is characterized by a single N-linked glycan attached to each heavy chain at the asparagine-297.\textsuperscript{103} In Chapter 5, we investigate the predictive value of the glycosylation profile at the PR3-ANCA rise for a relapse.
Chapter 1. ANCA as a predictor of a relapse: useful in patients with renal involvement but not in patients with non-renal disease

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Abstract

The value of measuring anti-neutrophil cytoplasmic antibodies (ANCA) during follow-up to predict a relapse is controversial. Based on recently obtained pathophysiological insights, we postulated that measuring ANCA in patients with renal involvement is useful but less valuable in patient with non-renal disease. One-hundred-and-sixty-six consecutive patients with ANCA-associated vasculitis, positive for either PR3- or MPO-ANCA were included in our study, followed at regular intervals, and tested for PR3- and MPO-ANCA. One-hundred-and-four patients had renal involvement (72 PR3-ANCA, 32 MPO-ANCA) and 62 patients had non-renal disease (36 PR3-ANCA, 26 MPO-ANCA). During an average (±SD) follow-up of 49±33 months and 19±14 ANCA measurements, 89 ANCA rises and 74 relapses were recorded. An ANCA rise is related with relapses in patients who present with renal involvement (HR 11.09 [95%-CI 5.01-24.55]), while ANCA rises are only weakly associated with relapses in patients who present with non-renal disease (HR 2.79 [95%-CI 1.30-5.98]). In conclusion, longitudinal ANCA measurements are useful in patients with renal involvement but less valuable in patients with non-renal disease.
Anti-neutrophil cytoplasmic antibodies (ANCA) play an important role in the pathophysiology of ANCA-associated vasculitis (AAV). The value of measuring ANCA to predict disease activity, however, is controversial. A recent meta-analysis calculated a pooled positive likelihood ratio of 2.8 (95% CI 1.7-4.9), concluding that serial ANCA measurements are of limited value. The interpretation of this meta-analysis is, however, difficult since the published studies are very heterogeneous and prospective and retrospective studies are lumped together. Based on pathophysiological insights, we hypothesize that serial measuring ANCA is useful in patients with renal involvement but less valuable in patients with non-renal disease. In mouse models it has been demonstrated that ANCA induce glomerulonephritis but require more modification to induce granulomatous lesions. Furthermore, ANCA is nearly always found in patients with renal involvement whereas a subgroup of patients with non-renal disease are clearly ANCA negative. Therefore, we investigated the relationship of an ANCA rise with a relapse in our cohort and divided our patients in those with and without renal involvement.

Between January 1, 2000 and November 1, 2011, 201 patients visited our University hospital with a diagnosis of ANCA-associated vasculitis and were positive for either MPO- or PR3-ANCA. Twenty-eight patients were excluded because we could not obtain follow-up data. Seven patients were refractory to treatment and did not reach stable clinical remission; these patients were also excluded. Of the remaining 166 patients, 74 (44.6%) patients did not experience a relapse during an average (±SD) follow-up of 7.5±5.9 years. All other patients had one or more relapses: 1, 2, 3, 4, or 5 relapses in 42 (25.3%), 31 (18.7%), 7 (4.2%), 4 (2.4%) and 8 (4.8%) patients, respectively. The relapse frequency was on average 0.17±0.21 per year for patients with PR3-ANCA and 0.11±0.18 for patients with MPO-ANCA (p=0.06).
## ANCA as a predictor of a relapse

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Renal</th>
<th>Non-renal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td>166</td>
<td>104</td>
<td>62</td>
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<tr>
<td><strong>Characteristics at time of previous disease activity</strong></td>
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<tr>
<td>Age in years</td>
<td>56.7±14.5</td>
<td>56.2±14.8</td>
<td>57.5±13.9</td>
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<tr>
<td>Men/women</td>
<td>99/67</td>
<td>71/33</td>
<td>28/34</td>
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<tr>
<td>PR3/MPO</td>
<td>108/58</td>
<td>72/32</td>
<td>36/26</td>
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<tr>
<td>GPA/MPA/EGPA</td>
<td>126/23/17</td>
<td>83/19/2</td>
<td>43/4/15</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>60.5 [15-144]</td>
<td>60 [17-144]</td>
<td>64 [12-135]</td>
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<td><strong>Organ involvement</strong></td>
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<tr>
<td>Arthralgia</td>
<td>76</td>
<td>49</td>
<td>27</td>
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<tr>
<td>Cutaneous</td>
<td>41</td>
<td>31</td>
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<tr>
<td>Eyes</td>
<td>30</td>
<td>20</td>
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<tr>
<td>Ear, nose, throat</td>
<td>122</td>
<td>75</td>
<td>47</td>
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<tr>
<td>Lung</td>
<td>124</td>
<td>74</td>
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<tr>
<td>Peripheral nervous system</td>
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<td>16</td>
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<tr>
<td><strong>Induction treatment resulting in remission</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide + GC</td>
<td>111</td>
<td>77</td>
<td>34</td>
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<tr>
<td>Rituximab + GC</td>
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<tr>
<td>Methotrexate + GC</td>
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<td>6</td>
<td>7</td>
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<tr>
<td>Mofetil mycophenolate + GC</td>
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<td>2</td>
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<tr>
<td>Azathioprine + GC</td>
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<td>13</td>
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<td>49.6±13.7</td>
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<td>43</td>
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<tr>
<td>ANCA rise</td>
<td>89</td>
<td>60</td>
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</tr>
<tr>
<td>Major relapse</td>
<td>26</td>
<td>21</td>
<td>5</td>
</tr>
<tr>
<td>Minor relapse</td>
<td>48</td>
<td>23</td>
<td>25</td>
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Table 1-1 (previous page). Patient characteristics. Values are expressed as mean ± SD or median [inter-quartile range, IQR] or as the total. GC, glucocorticoids; PR3, proteinase-3; MPO, myeloperoxidase; GPA, granulomatosis with polyangiitis; MPA, microscopic polyangiitis; EGPA, eosinophilic GPA.

To study the association of an ANCA rise with a relapse, the 166 patients were included when they entered their first remission after visiting our Hospital. Hundred and twenty-six patients (75.9%) were included during remission after diagnosis and 40 patients during remission after a relapse. Baseline patient characteristics are presented in Table 1-1. During an average follow-up of 49±33 months and an average of 18±14 ANCA measurements, 74 patients relapsed: 26 major and 48 minor. Eighteen patients included after a relapse and 74 patients included after diagnosis remained in remission. Seven patients died after a median follow-up of 3.3 year (1.2-5.3), causes of death were unknown (n=4), cardiovascular (2), malignancy (2), infection (1) or cerebral (1). Five patients were lost to follow-up after a median follow-up of 3.3 years (1.8-4.1). During follow-up, 89 ANCA rises were identified. These rises were observed after an average of 20±17 months since the previous period of disease activity. After an ANCA rise, a relapse was diagnosed in 2 (2.2%), 26 (29.2%), 35 (39.3%) and 45 (50.6%) patients after <1, 6, 12 and 18 months since the rise, respectively. The median (IQR) time elapsed between the ANCA rise and last follow-up visit in the patients that did not relapse was 41.5 months (30.3-51.0).

At the time of relapse, all 26 patients with a major relapse were ANCA positive. In 24 (92%) patients the relapse was preceded by an ANCA rise. In contrast, only 32 of 48 patients (67%) with a minor relapse were ANCA positive at the time of relapse and in only 28 (58%) patients the relapse was preceded by an ANCA rise. In patients who became ANCA negative during follow-up, all major relapses were preceded by an ANCA rise (see Figure 1-1).
In the entire cohort, an ANCA rise is significantly associated with a relapse (HR 5.84 [95%-CI 3.44-9.92], p<0.001). A rise is strongly related to a relapse in patients with renal involvement (HR 11.09 [95%-CI 5.01-24.55], p<0.001), while an ANCA rise is only weakly associated with a relapse in patients with non-renal disease (HR 2.79 [95%-CI 1.30-5.98], p=0.008). Also, a rise is strongly related with a relapse in patients with non-renal severe disease (4 relapses in 16 patients, HR 13.08 [95%-CI 1.03-166.7], p=0.043), but not in patients with non-renal limited disease (26 relapses in 46 patients, HR 2.09 [95%-CI 0.92-4.78], p=0.078). Sampling interval (categorized as ≥4 measurements per year or fewer) and ANCA pattern (categorized as persistently ANCA positive or not) were additional factors that influenced the relationship of an ANCA rise with a relapse (see Figure 1-2 and supplementary data).
Figure 1-2. The predictive value of an ANCA rise as determined by the antigen-specific solid-phase ANCA method in the entire cohort and subgroups of patients with AAV. A differentiation was made in renal involvement, sampling interval (<4 versus ≥4 per year), ANCA pattern (persistently positive or non-persistently positive) and ANCA serotype (MPO-ANCA versus PR3-ANCA). Hazard ratios are shown with 95% confidence intervals. The area up to a hazard ratio of 1 marks the border of significance.

How can our results be translated to daily practice? Firstly, in patients who became ANCA negative during follow-up, the risk to develop a major relapse is extremely low as long as no rise in ANCA level occurs. Secondly, an ANCA rise in a patient with previous renal involvement should warn the clinician for a relapse since the probability of a relapse occurring after the rise is more than eleven times higher compared to the period before the rise. Thirdly, measuring serial ANCA levels in patients without renal involvement, especially when severe vasculitic manifestations are lacking, is of limited value. An ANCA rise should, however, not be used to preemptively treat the patient, since only less than half of the patients with an ANCA rise will experience a relapse within a year. Serial ANCA measurement is an important factor, but not the only factor that should be considered during follow-up in patients with ANCA-associated vasculitis.
In 2006, a meta-analysis on the value of ANCA measurements to predict relapses concluded that no firm conclusions could be drawn because of considerable methodological heterogeneity in the different studies.\(^7^2\) In contrast, another meta-analysis based on a more selected subgroup of these studies concluded that there is limited use to serial ANCA measurements.\(^7^3\) We found that the proportion of patients with renal involvement dictates the conclusion whether ANCA measurements are of value during follow-up. Furthermore, we found that the association of an ANCA rise with a relapse was influenced by the ANCA method (see supplementary data), sampling interval and persistence of ANCA. Interestingly, we did not find a difference between MPO-ANCA and PR3-ANCA as previously suggested.\(^7^3,1^0^9,1^1^1,1^1^2\) We analyzed the articles that were in the latest meta-analysis and specified the study-level variables that may have influenced the results (**Table 3-1, page 65**).\(^7^3\) Studies with a high prevalence of renal or 'severe' disease\(^4^8\) tended to have higher likelihood ratios, although variation in other study-level variables such as the ANCA method used or the definition of an ANCA rise may obscure this association. This suggests that spectrum bias is involved.\(^1^1^3\)

The presence of ANCA is, however, not the only factor that is required for disease re-activation, since many rises are not followed by a relapse.\(^7^1,1^0^1,1^1^0\) This suggests that other qualities, such as epitope specificity and the sialylation ratio, may determine the pathogenicity of ANCA.\(^1^0^2,1^0^5,1^1^4\) Otherwise, the association may be stronger when newer generation ANCA methods are used.\(^1^0^5,1^0^6,1^1^5\) Lastly, a second hit may be required after the occurrence of an ANCA rise before the patient truly develops a relapse. The importance of a second hit has been demonstrated in a mouse model of MPO-ANCA\(^6^4\), but remains elusive in humans. Possible candidates are microorganisms\(^1^1^6\), environmental factors,\(^6^6,6^7\) and/or other auto-antibodies.\(^6^8\)

Our study suffers from several limitations. In daily practice,\(^7^1,1^0^1\) time between ANCA measurements varies as was found in our study. We resolved this issue by using the slope of a rise, thereby taking into account the time between measurements. In our current study, physicians were not blinded to ANCA results\(^7^1,1^0^1\), which could have had an effect in diagnosing relapses. This may have influenced the association between an ANCA rise and a relapse in the entire cohort but could not explain the marked difference between patients with and without renal involvement. A minority of patients suffered from MPA or EGPA, making any conclusion on
ANCA testing in these diseases less valid. Renal involvement may be a proxy for severe vasculitic disease as we found in non-renal patients with severe disease that an ANCA rise was also associated with a relapse in this small subgroup of patients. Finally, 5 of 62 patients who were initially diagnosed with non-renal disease developed a renal relapse, suggesting that we should probably focus on markers of the biologic behavior of the disease in patients that initially present with non-renal disease in order to predict the value of serial ANCA measurements in this subgroup of patients.\textsuperscript{117}

In summary, we have demonstrated in the largest published cohort with longitudinal ANCA measurements that an ANCA rise is related to a relapse in patients with renal involvement, but less in patients with non-renal disease.
**Concise methods**

Renal involvement was determined by a renal biopsy showing pauci-immune necrotizing glomerulonephritis. However, the presence of hematuria in combination with red cell casts, dysmorphic erythrocytes (>10) and/or proteinuria sufficed (a renal biopsy was not performed in twelve patients with renal involvement). Disease activity was scored using the BVAS v3. Patients were treated according to the European League Against Rheumatism (EULAR) guidelines as previously described. Maintenance therapy was given for at least 18 months and tapered within 24 months according to protocol in newly diagnosed patients. Patients who previously relapsed and/or relapsed during our study while using maintenance therapy were offered long term maintenance therapy. Remission was defined as absence of disease activity attributable to active disease during maintenance immunosuppressive therapy of a prednisone dosage of 7.5mg or lower. A relapse was defined as reoccurrence or new onset of disease attributable to active disease combined with an increase or addition of immunosuppressive treatment. Relapses were further subdivided in minor or major, depending on whether the relapse was potentially organ- or life threatening or not. For a renal relapse, the (re)occurrence of hematuria in combination with a rise in serum creatinine of 25% was required to be characterized as a major relapse.

Solid-phase ANCA tests were used to detect and quantify PR3-ANCA and MPO-ANCA. For quantitation of MPO- and PR3-ANCA the samples were diluted 1:50 in PBS as instructed by the manufacturer. Results were calculated in arbitrary units by a standard curve. Patient samples with results above the highest standard were further tested in two-fold dilutions as appropriate. For the detection of an ANCA rise, the ANCA value was compared with the value measured in the samples obtained in the 6 months prior to the current sample. We defined a rise using the slope of an increase, for which a receiver operating characteristics (ROC) curve was calculated to determine the optimal cut-off value (see supplementary data). The association between an ANCA rise and relapse was investigated using a Cox regression model with an ANCA rise included as a time-dependent, binary, nonreversible predictor.
Chapter 2. Seasonal influence on the risk for a relapse at a rise of anti-neutrophil cytoplasmic antibodies in vasculitis patients with renal involvement.

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Jan Willem Cohen Tervaert
Kelly Broen
Sjoerd Timmermans
Pieter van Paassen
Jan Damoiseaux

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Chapter 3. Maintaining remission in patients with Granulomatosis with Polyangiitis or Microscopic Polyangiitis: the role of ANCA

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Pieter van Paassen
Jan Damoiseaux
Jan Willem Cohen Tervaert

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Abstract

Introduction

Granulomatosis with Polyangiitis (GPA; formerly Wegener's) and Microscopic Polyangiitis (MPA) are inflammatory disease entities affecting small to medium vessels. They are characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) and are frequently grouped together with Eosinophilic Granulomatosis with Polyangiitis (EGPA; formerly Churg Strauss Syndrome) under the term ANCA-associated Vasculitis (AAV). Due to effective immunosuppressive therapy that was introduced more than 45 years ago, AAV has become a chronic disease in which relapses occur frequently during maintenance treatment or later on after all immunosuppressive therapy is stopped. Since relapses are associated with morbidity and mortality, early detection and prevention of relapses is of great importance.

Areas covered

This review focuses on the potential strategies to guide maintenance therapy in patients with GPA or MPA, with an emphasis on patient classifications and the role of serial ANCA measurements.

Expert opinion

Risk factors for relapses, e.g., genetic background of the patient, have been studied during the last three decades. Only few of these risk factors directly influence the management of patients. One potentially important factor that may influence therapeutic decisions in AAV patients is serial measurement of ANCA. Recently, it became clear that serial ANCA measurement is an attractive approach in AAV patients with (a history of) capillaritis, e.g., renal involvement or alveolar haemorrhage, but not in patients with more limited disease. Whether ANCA rises can be used to guide therapy and –hence- to prevent relapses has been a great debate during the last three decades. At present, we conclude that small studies do support such an approach but that these findings require further validation in larger randomized clinical trials.
**Introduction**

Granulomatosis with Polyangiitis (GPA; formerly Wegener's) and Microscopic Polyangiitis (MPA) are inflammatory disease entities affecting small to medium vessels.\(^{104}\) They are characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) and are frequently grouped together with Eosinophilic Granulomatosis with Polyangiitis (EGPA; formerly Churg Strauss Syndrome) under the term ANCA-associated Vasculitis (AAV). ANCA can be detected using indirect immunofluorescence (IIF), upon which a perinuclear pattern (P-ANCA) or a cytoplasmic pattern (C-ANCA) can be observed.\(^{105}\) In addition, antigen-specific tests are available for myeloperoxidase (MPO), which is typically found with a perinuclear pattern, and proteinase 3 (PR3), which is associated with a cytoplasmic pattern.\(^{105}\)

The paradigm of AAV changed from a fatal diagnosis to a chronic disease after the introduction of immunosuppressive therapeutics.\(^{29}\) The majority of patients, however, experience relapses during treatment or at remission. Since relapses are associated with morbidity\(^{89}\) and mortality, early detection and prevention of relapses is of great importance. The therapeutic agents used to maintain remission and the clinical evidence thereof have been extensively reviewed elsewhere.\(^{144-146}\)

In this review, we will focus on potential strategies to guide maintenance therapy in patients with GPA or MPA, with an emphasis on patient classifications and the role of serial ANCA measurements.
The classification of patients to guide maintenance therapy

**Patient classifications – the old-fashioned way**

Godman and Churg described in their seminal paper in 1954 the triad of pathological features that characterized patients with GPA: systemic necrotizing angiitis, necrotizing granulomatous inflammation of the respiratory tract and necrotizing glomerulonephritis.\(^{14}\) Thereafter, several systems have been described to classify patients based on clinical characteristics, histopathological and serological evidence.\(^{13}\) The American College of Rheumatology developed criteria based on clinical and histopathological evidence in 1990 to classify patients as GPA, EGPA or polyarteritis nodosa (PAN). At that time, the association of ANCA to necrotizing vasculitis was not yet widely accepted in the USA\(^{147}\) and was therefore not included as a classification criterion.\(^{70}\)

In 1994, the Chapel Hill Consensus Conference (CHCC) aimed to standardize the nomenclature of the various individual vasculitic diseases and focused mainly on the histopathological evidence.\(^{20}\) The typical histopathological finding of AAV is necrotizing vasculitis of small to medium sized vessels with few or no immune deposits, wherein GPA is distinguished by granulomatous inflammation (vascular and/or extravascular), while EGPA is characterized by eosinophil-rich inflammation.\(^{21}\) The size of the involved vessels dictated the classification of patients according to the 1994 CHCC nomenclature, while in the revised CHCC criteria of 2012 overlap of vessel involvement was acknowledged in the various disease entities.\(^{21}\) Novel findings using 2-[18F]fluoro-D-glucose (FDG) positron emission tomography (PET) scanning support this notion, since AAV patients with renal involvement have similar values of aortic FDG uptake as patients with large vessel vasculitis, reflecting aortic inflammation.\(^{148}\) The CHCC also formulated definitions for MPA and single-organ vasculitis, thereby acknowledging the possibility of necrotizing angiitis without the occurrence of granuloma formation, or vasculitis in a particular organ without systemic involvement, such as renal-limited vasculitis.\(^{54}\)

In 2007, the European Medicines Agency (EMA) developed a classification system that combined the CHCC and ACR criteria. Importantly, seropositivity for PR3- or MPO-ANCA was included in this algorithm. This allows physicians to classify patients who do not (yet) have biopsy-proven vasculitis and do not fulfill the ACR criteria. When these patients are seropositive for PR3-ANCA or MPO-ANCA and surrogate markers for AAV are available they can be classified as well as either GPA,
MPA, or EGPA. Importantly, it is acknowledged in the EMA classification scheme that histopathological evidence is not always available in clinical practice. It should be noted, however, that the described classification criteria and nomenclature were developed to enable comparison between different disease groups in epidemiological studies and that these criteria are, in general, more stringent diagnostic criteria.\(^{21,70}\)

In addition, patients within clinical entities such as GPA and MPA have been categorized based on the severity of disease activity.\(^{117}\) Organ involvement at the time of active disease and the severity thereof has been summarized in the Birmingham vasculitis activity score (BVAS),\(^{119}\) while the extent of the disease is summarized by the disease extent index (DEI).\(^{149}\) The European Vasculitis Society (EUVAS) classified patients as having localized, early systemic, generalized, renal severe or refractory disease.\(^{41}\) Otherwise, the Wegener’s Granulomatosis Etanercept Trial (WGET) group classified patients as having severe or limited disease, depending on whether there are disease features present that pose immediate threats either to a critical organ or the patient’s life.\(^{48}\) The purpose of these classification systems is to provide a framework to tailor induction therapy based on the severity of the disease at the initial presentation.\(^{41,47}\) During the last decade it became clear that these systems are of limited prognostic value since they only reflect the severity of disease at initial presentation and do not take the clinical follow-up into account.\(^{117}\) Therefore, these classification systems are limited in the guidance of maintenance therapy.\(^{123}\)

**Definitions of remission and relapse**

Remission and relapse are arbitrary definitions that reflect the disease-specific outcome of vasculitis. In order to harmonize these definitions, the European League against Rheumatism (EULAR) analysed the various definitions used in previously published studies and formulated definitions based on expert opinion that should be used in future studies.\(^{121}\) Patients are in remission when there is complete absence of active clinical disease. In addition, the EULAR proposed that patients may still use a glucocorticoid dose of less than or equal to 7.5mg per day. A relapse was defined as the reoccurrence or new onset of disease activity attributable to active inflammation. Moreover, a differentiation was made between major and minor relapses, depending on whether the relapse is potentially organ- or life-threatening.

The EULAR recommendations allow us to compare results obtained during
Maintaining remission in GPA and MPA patients

clinical trials. At present, however, many differences still remain making comparisons between trials and published cohort studies difficult.\textsuperscript{150} For example, both the RITUXVAS and the RAVE trial studied the efficacy of rituximab to induce remission in patients with AAV.\textsuperscript{46,151} However, sustained remission was defined by the RITUXVAS trial as BVAS = 0 maintained for at least 6 months, while the RAVE trial defined complete remission as BVAS/WG = 0 and being off glucocorticoid therapy.\textsuperscript{46,151} Moreover, the definitions by the EULAR are still open to subjective interpretation. For example, it can be difficult to differentiate minor symptoms, such as fever or arthralgia that are caused by vasculitis from symptoms that are caused by an infection. Therefore, in our studies, a minor relapse was diagnosed only if symptoms were refractory to appropriate antibiotic therapy.\textsuperscript{124} In addition, a relapse was defined as reoccurrence or new onset of disease attributable to active disease combined with an increase or addition of immunosuppressive treatment. The increase of glucocorticoid therapy almost always consisted of at least 20mg per day. This is not always the case in other studies, in which relapses can occur that were treated with an increase of glucocorticoid therapy of only 2.5mg per day.\textsuperscript{152}

Risk factors for a relapse

Several studies have investigated potential risk factors for a relapse. Positivity for PR3-ANCA has repeatedly been identified as an independent risk factor for a relapse during follow-up.\textsuperscript{75,88,96,120,151,153-155} A diagnosis of GPA, as well as lung involvement or upper respiratory tract involvement is related to a higher relapse risk.\textsuperscript{97,153,154,156,157} Patients with a high serum creatinine level (e.g. >200 $\mu$mol/l) tend to relapse less often than patients with a lower serum creatinine level.\textsuperscript{88,157} Cardiovascular involvement has been identified as a predictor of a relapse.\textsuperscript{88} In a small study, infection has been reported to be a risk factor for GPA.\textsuperscript{158} In a larger study, however, we could not confirm this.\textsuperscript{157} Nasal carriage of staphylococcus aureus has been associated with a higher relapse rate and the addition of co-trimoxazole protects against relapses in patients with (limited) GPA.\textsuperscript{137,157} The assessment of chronic nasal carriage of s. aureus is, however, at present in clinical practice hindered by the use of antibiotics such as used for prophylaxis against pneumocystis pneumonia. Another risk factor for relapses is the presence of an increased waist circumference or the presence of metabolic syndrome.\textsuperscript{159} Whether exposure to silica is also a risk factor for a relapse is at present not well studied.\textsuperscript{160} Finally, several
genetic factors may predispose to relapses.  

**Patient classifications - based on the chance to relapse**

Given previously described risk factors for a relapse, several attempts have been made to generate a classification system that stratifies patients according to their chance of a relapse. Using a cluster analysis approach in a population of 673 patients, five subgroups of patients were identified with distinct differences in relapse rate: cardiovascular AAV (highest relapse risk), non-renal AAV, renal AAV with PR3-ANCA, renal AAV without PR3-ANCA and gastrointestinal AAV (lowest risk). However, studies concerning cardiovascular involvement are hampered due to differences in applied diagnostic methods and lack of a clear definition of cardiovascular involvement due to vasculitis. In a prospective study with extensive cardiac screening including cardiac magnetic resonance imaging, it has been shown that 61% of patients with GPA have cardiac involvement, independent of clinical symptoms. In a study with 502 patients, it was shown that ANCA specificity predicted relapses better than clinical diagnosis based on CHCC or EMA classification. Importantly, no difference in the relapse risk between GPA and MPA was observed if patients were stratified according to their ANCA specificity. These findings, in addition to distinct genetic differences between PR3-ANCA and MPO-ANCA positive patients but less between patients with GPA and MPA, lead to the suggestion that patients should be classified based on ANCA specificity and not the disease entity. Differences between PR3-ANCA and MPO-ANCA associated vasculitis have been extensively reviewed elsewhere. The risk to develop a relapse is not only associated with clinical or serological phenotypes, differences in the genetic background may also play a role. Carriage of HLA-DPB1*04:01 (DPB4) has recently been identified as a strong independent risk factor for a relapse. Patients that are homozygous for DPB4 relapsed more often than heterozygous patients, while noncarriers relapsed less often compared to homozygous or heterozygous patients. PR3-ANCA positive patients were more often carrier of DPB4 (55.7% homozygous, 34.5% heterozygous) compared to MPO-ANCA positive patients (25.3% homozygous, 41.3% heterozygous), as was previously observed in a genome-wide association study in patients with AAV. Most importantly, carriage of DPB4 was associated with a relapse independent of ANCA specificity. Therefore, DPB4 homozygous patients are prone to
relapse also when they are MPO-ANCA positive. The incorporation of these findings into a classification system may lead to a classification that is focused on the biologic behavior of the disease and may in the future be used to guide maintenance therapy in patients with AAV.¹¹⁷

Serial ANCA measurements to guide maintenance therapy

Serial ANCA measurements – a historical perspective

The occurrence of antibodies directed against neutrophils had already been described in the Sixties.⁴⁹ In those early days, granulocyte-specific antinuclear antibodies (GS-ANA) were defined as antinuclear antibodies reacting specifically with granulocyte nuclei or when reactivity with granulocytes was higher than reactivity with other cell nuclei (by at least two dilution steps).⁵¹ These antibodies were most often found in patients with rheumatoid arthritis and/or Felty’s syndrome. Unfortunately, attempts to discover the antigen failed repeatedly.⁵¹ In 1982, the occurrence of anti-neutrophil cytoplasmic antibodies in 8 patients with segmental necrotizing glomerulonephritis was described by Davies et al. ⁵² Thereafter, it was recognized that ANCA was observed in GPA⁵³ and that the target antigen of ANCA in these patients was proteinase 3. Furthermore, MPO was found to be the target antigen in patients with PR3-ANCA negative GPA, MPA and/or EGPA.⁵⁴-⁵⁶ Importantly, patients with necrotizing glomerulonephritis are nearly always ANCA positive⁵⁴,⁵⁶ while ANCA are not always found in a subgroup of patients with more limited disease.⁵⁷,⁵⁸ The presence of PR3- or MPO-ANCA in ANCA-associated vasculitis has been undisputed and has been incorporated in the current classification schemes.²¹,⁷⁰

Soon after the discovery of ANCA as a diagnostic marker for GPA, it was recognized that ANCA titer rises were associated with disease relapses during follow-up.⁷¹ In one of the initial studies, 35 patients with GPA were prospectively followed. Patients were evaluated every month for signs and symptoms attributable to disease activity and blood was drawn for ANCA testing using IIF. Eight major relapses and 9 minor relapses were observed during the study period of 16 months. All relapses were preceded by an ANCA rise. Only one patient demonstrated ANCA rises that were not followed by relapses.⁷¹ This observation led to a multitude of studies to investigate the value of serial ANCA measurements to predict disease activity.⁷²,⁷³ If an ANCA rise is indeed followed by a relapse of disease activity, then immunosuppressive therapy may be
pre-emptively started to prevent the relapse to occur. However, such a strategy is hampered by potential unnecessary exposure to treatment-related side-effects in patients in whom no relapse would occur after an ANCA rise. Therefore, the use of serial ANCA measurements to guide therapy has been controversial and subject to debate.

Persistence of ANCA positivity

Several characteristics can be derived from longitudinal ANCA measurements. Although the ANCA levels usually decrease from the start of induction therapy to the moment of sustained remission, this is not always the case. A reduction or complete disappearance of ANCA is therefore not required to achieve sustained remission. Several studies have investigated a potential increased risk of relapse in patients who are persistently ANCA positive, with various results. A meta-analysis observed a relatively low but significant positive likelihood ratio of a relapse of 1.97 (95% confidence interval [CI] 1.43-2.70). A different approach was undertaken by Slot et al, who investigated the risk for a relapse in patients who were ANCA positive or ANCA negative when cyclophosphamide was switched to azathioprine. In this study, patients who were C-ANCA positive at the time of switch had a significantly higher chance for a relapse compared to patients who were ANCA negative (relative risk 2.6 [95% CI 1.1-8.0]). Recently, however, these results could not be reproduced in a prospective study.

The predictive value of an ANCA rise for a relapse

In 2006, a meta-analysis concluded that the presence of considerable methodological heterogeneity precludes firm conclusions concerning serial ANCA measurements for monitoring disease activity from the available literature. Another meta-analysis, which included several newly published studies and excluded multiple other studies, concluded that there is limited use to serial ANCA measurements to guide maintenance therapy. An overview of published studies regarding the association between an ANCA rise and disease activity is presented in Table 3-1 and Table 3-2. Several studies were excluded from this table either because the predictive value of a rise in ANCA was not investigated, or because treatment changes were based on ANCA titer, because of inclusion of patients with PAN or various other reasons.
Maintaining remission in GPA and MPA patients

<table>
<thead>
<tr>
<th>Article</th>
<th>Total (severe)</th>
<th>Relapse (major)</th>
<th>Design</th>
<th>Relapses per patient</th>
<th>Statistics</th>
<th>Interval</th>
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<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Tervaert (1989)\textsuperscript{71}</td>
<td>35 (100%)</td>
<td>17 (47%)</td>
<td>pro</td>
<td>Multiple</td>
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</tr>
<tr>
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<td>4 (NA)</td>
<td>retro</td>
<td>Single</td>
<td>Cross tabulation</td>
<td>NA</td>
</tr>
<tr>
<td>Egner (1990)\textsuperscript{180}</td>
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<td>6 (NA)</td>
<td>pro</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>NA</td>
</tr>
<tr>
<td>Halma (1990)\textsuperscript{181}</td>
<td>23 (87%)</td>
<td>3 (NA)</td>
<td>retro</td>
<td>Single</td>
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</tr>
<tr>
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<td>6 (100%)</td>
<td>retro</td>
<td>Single</td>
<td>Cross tabulation</td>
<td>2</td>
</tr>
<tr>
<td>Kerr (1993)\textsuperscript{182}</td>
<td>68 (~63%)</td>
<td>45 (NA)</td>
<td>pro</td>
<td>Single</td>
<td>Cross tabulation</td>
<td>1 to 3</td>
</tr>
<tr>
<td>Chan (1993)\textsuperscript{183}</td>
<td>10 (~70%)</td>
<td>11 (NA)</td>
<td>retro</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>&lt;1 to 4</td>
</tr>
<tr>
<td>Davenport (1995)\textsuperscript{184}</td>
<td>37 (NA)</td>
<td>44 (NA)</td>
<td>retro</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>2.4 (median)</td>
</tr>
<tr>
<td>De’Oliviera (1995)\textsuperscript{185}</td>
<td>56</td>
<td>27</td>
<td>retro</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>NA</td>
</tr>
<tr>
<td>Jayne (1995)\textsuperscript{170}</td>
<td>60 (~65%)</td>
<td>23 (~43%)</td>
<td>retro</td>
<td>Single</td>
<td>Cross tabulation</td>
<td>1</td>
</tr>
<tr>
<td>Ara (1999)\textsuperscript{186}</td>
<td>25 (100%)</td>
<td>4 (100%)</td>
<td>NA</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>4</td>
</tr>
<tr>
<td>Kyndt (1999)\textsuperscript{171}</td>
<td>43 (~72%)</td>
<td>52 (NA)</td>
<td>pro</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>1 to 3</td>
</tr>
<tr>
<td>Boomsma (2000)\textsuperscript{101}</td>
<td>100 (NA)</td>
<td>37 (73%)</td>
<td>pro</td>
<td>Single</td>
<td>Cox regression (time-dependent)</td>
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</tr>
<tr>
<td>Girard (2001)\textsuperscript{172}</td>
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<td>23 (NA)</td>
<td>pro</td>
<td>Multiple</td>
<td>Cross tabulation</td>
<td>1 to 4</td>
</tr>
</tbody>
</table>
Table 3-1. An analysis of published studies that investigate the predictive value of an ANCA rise for a relapse. The severity of the included cohort and/or relapses is preceded by a tilde if the exact proportion was not described in the article, but an indication could be derived (from organ involvement for example). If multiple ANCA rises and/or relapses from one patient were included, the total amount of ANCA rises and relapses are shown. § References 106,115 analyze different ANCA methods on the same cohort. Retro, retrospective; pro, prospective; NA, Not available.
Serial ANCA measurements – heterogeneity between studies

Several differences between published studies should be taken into consideration when interpreting the results. First, differences in the definition between relapse and remission may be present. Second, the method of ANCA testing may influence the usefulness of serial ANCA measurements.\textsuperscript{105,106} Indirect immunofluorescence was mostly used in the initial studies, a technique that is at present infrequently used for quantification of ANCA levels. Subsequently, most studies were performed using a direct ELISA (a so-called first-generation antigen-specific test). At present, second (capture ELISA) and third (anchor ELISA) generation tests are available.\textsuperscript{105} For serial ANCA measurements, an ANCA rise as measured by ELISA is a stronger predictor of a relapse compared to a rise as measured by IIF.\textsuperscript{101,124} One study found that a combination of a rise of PR3-ANCA and C-ANCA was most predictive of a relapse.\textsuperscript{101} However, this was not confirmed by a later study.\textsuperscript{124} Preliminary results suggest that the second and third generation tests predict relapses better than IF tests and/or first generation tests.\textsuperscript{105,115} Monitoring ANCA of the IgG3 subtype does not warrant additional benefits,\textsuperscript{101,173} nor does the detection of ANCA targeted against the proenzyme of PR3.\textsuperscript{122} Third, more frequent sampling may result in a higher association of an ANCA rise with a relapse.\textsuperscript{73} A higher association was observed in patients who were tested at least 4 times per year (HR 6.40 [95% CI 3.45-11.88]) compared to patients who were tested less frequently (HR 2.13 [95% CI 0.67-6.77]).\textsuperscript{124} However, more frequent sampling may not always be feasible.\textsuperscript{192} Fourth, large variations exist between the definitions used for an ANCA rise. Initial studies used a relatively high cut-off value, such as quadrupling of the previous result.\textsuperscript{71} Other studies have used a lower cut-off value that may be less specific for a relapse. A definition that is only determined by the relative increase of an ANCA titer compared to the previous sample(s) is unreliable if there is a large variability in the time between samples. To resolve this issue, ANCA rises have previously been defined using the slope of the rise (e.g. doubling of the ANCA rise in 3 months).\textsuperscript{124,192} Another study has retrospectively calculated the peak of the ANCA rise, which cannot be used in a prospective study.\textsuperscript{141} Several studies have used a receiver operating characteristic curve to define the most optimal cut-off value.\textsuperscript{101,115,124} Usually, the cut-off value closest to the upper left corner is chosen (the cut-off value closest to 100% sensitivity and 100% specificity). A different approach that is not yet applied is to use two
different cut-off values for an ANCA rise: one definition with a low cut-off value that is highly sensitive and one definition with a high cut-off value that is highly specific. Whether such an approach offers additional value should be investigated in future studies.

Fifth, there are differences in the interpretation of the clinical usefulness of an ANCA rise that is detected concomitant to clinical symptoms of a relapse. Since the intention of serial ANCA measurements is to prevent relapses, an ANCA rise concomitant to a relapse is of limited value. Therefore, some researchers define these ANCA rises as false-negative results.\textsuperscript{191} However, this occurrence may be caused by a lack of frequent ANCA sampling. Moreover, these ANCA rises may still be of value in order to differentiate a relapse from an infection. The concomitant occurrence of a relapse and an ANCA rise underline the tight relationship of ANCA with disease activity. It should be further investigated why some ANCA rises are directly followed by a relapse while other ANCA rises are not. The pathogenicity of an ANCA rise may be determined by other qualities of the autoantibody, such as the avidity, glycosylation profile or epitope specificity.\textsuperscript{102,193-195}

Sixth, some studies include multiple relapses from a single patient, while other studies only include the first relapse of a patient. The results of the first type of studies are prone to be biased since the association between an ANCA rise and a relapse may be specific to a particular subset of patients. Therefore, one patient that frequently relapses shortly after several ANCA rises may therefore (incorrectly) skew the results of the study toward a high association between ANCA rises and a relapse.

Seventh, different statistical methods have been used. Serial ANCA measurements can be approached with cross tabulation, after which the sensitivity, specificity and positive and negative likelihood ratios can be derived.\textsuperscript{196} This method increases the clinical usefulness of ANCA testing to diagnose AAV\textsuperscript{197}, but has several limitations when used for serial ANCA measurements. First, this method does not take the duration of follow-up into account. A second limitation of this approach is that the association of a negative ANCA titer with sustained remission is negated if an ANCA rise occurs. Another limitation of this method is that the method is unable to deal with patients that are lost to follow-up. A different approach is using the Cox regression with an ANCA rise as a time-dependent, binary, non-reversible predictor.\textsuperscript{122,124,191,198} This approach elegantly deals with the limitations of the previous approach but is more difficult to apply. A major drawback of the Cox regression is that a high
hazard ratio does not necessarily mean that a relapse will shortly occur after an ANCA rise. For example, the hazard ratio will also be high if no relapses occur in patients in whom no ANCA rise has been detected. As such, the Cox regression investigates the association of an ANCA rise with a relapse and not the predictive value of an ANCA rise for a relapse. Therefore, a combination of both approaches will give the most optimal representation of the clinical usefulness of serial ANCA measurements.

Last, it is important to note that a prospective study design and blinding of the physicians to the ANCA titers is present in only a minority of previously published studies\textsuperscript{71,74,101} which are essential requirements to ensure strong internal validity.\textsuperscript{72}

\textbf{Table 3-2 (next page).} The results of published studies that investigate the predictive value of an ANCA rise for a relapse. Positive likelihood ratios are shown only if self-reported or sensitivity and specificity were reported. *The cut-off value was determined using a receiver operating characteristic curve. ± The ELISA was not specific for PR3 or MPO, instead the antigen was obtained by lysing granules of normal donor granulocytes.\textsuperscript{181} HR, hazard ratio; LR+, positive likelihood ratio; NA, Not available.
<table>
<thead>
<tr>
<th>Article</th>
<th>Method</th>
<th>Definition</th>
<th>True positive</th>
<th>False positive</th>
<th>False negative</th>
<th>HR or LR+</th>
</tr>
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<td>Tervaert (1989)71</td>
<td>IIF</td>
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<td>4</td>
<td>3</td>
<td>0</td>
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</tr>
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<td>6</td>
<td>2</td>
<td>0</td>
<td></td>
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<tr>
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<td>IIF</td>
<td>800%</td>
<td>3</td>
<td>10</td>
<td>3</td>
<td></td>
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<td>NA</td>
<td>3</td>
<td></td>
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<td>appearance</td>
<td>2</td>
<td>1</td>
<td>4</td>
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</tr>
<tr>
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<td>9</td>
<td>6</td>
<td>30</td>
<td></td>
</tr>
<tr>
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<td>&gt;10 units</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td></td>
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<td>63</td>
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<td>9</td>
<td>2</td>
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<td>10</td>
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<td>0</td>
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<td>8</td>
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</tr>
<tr>
<td></td>
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<td>21</td>
<td>10</td>
<td>NA</td>
<td></td>
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<td>7</td>
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<td>Method</td>
<td>Definition</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>% n n n</td>
<td></td>
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<tr>
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<td>150% 13 NA 0</td>
<td></td>
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<tr>
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<td>IIF</td>
<td>200%* 15 9 8 1.7</td>
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<td></td>
<td>FEIA</td>
<td>200%* 18 8 5 2.3</td>
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<tr>
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<td>2.56 %/day* 52 37 22 5.84</td>
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<td>Fussner (2016)\textsuperscript{191}</td>
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<td>200%, ≥40 if negative 39 19 16 2.24</td>
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<td>Capture ELISA</td>
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An ANCA rise is highly associated with a relapse in a subgroup of patients

Patients with renal involvement are almost always ANCA positive, whereas a positive ANCA is not always present in patients with localized disease.\(^{57,105}\) Also, animal models have shown that necrotizing vasculitic lesions are readily induced by ANCA, while granulomas are more difficult to induce.\(^{65,99}\) These observations led to the hypothesis and the ensuing observation that serial ANCA measurements are highly associated with a relapse in patients with renal involvement (HR 11.09 [95% CI 5.01-24.55]) but not in patients with non-renal disease (HR 2.79 [95% CI 1.30-5.98]).\(^{124}\) Renal involvement may be a proxy for severe vasculitic activity, since an ANCA rise was also highly associated with a relapse in patients with non-renal severe disease (HR 13.08 [95% CI 1.03-166.7]). This observation was confirmed in the RAVE trial, wherein an ANCA rise was highly associated with a severe relapse in patients with renal involvement (HR 7.94 [95% CI 2.72-29.18]) or alveolar haemorrhage (HR 24.19 [95% CI 3.05-447.2]).\(^{191}\) Whether ANCA rises are indeed highly associated with vasculitic disease but not granulomatous disease should be further investigated in future studies. The classification of granulomatous versus vasculitic disease is difficult, since both types of lesions can be simultaneously present.\(^{199}\) The study by Fussner et al. also observed a good association between an ANCA rise with a severe relapse in patients that were treated with Rituximab (HR 5.80 [95% CI 2.06-19.77]), but not in patients that were treated with cyclophosphamide (HR 2.84 [94%-CI 0.87-11.40]).\(^{191}\) This finding should be further investigated in other cohorts.

Therapy based on ANCA levels

Few studies have investigated the therapeutic potential to guide therapy based on serial ANCA measurements.\(^{74,108,176,178}\) In the initial study performed in the 1980s, 57 patients were followed and ANCA was monthly measured.\(^{74}\) Nine of a total of 20 patients with ANCA rises were randomly assigned to receive a 9-month course of cyclophosphamide and a short course of prednisolone at the time of an ANCA rise, while no changes in immunosuppressive therapy were made in the remaining 11 patients. Nine of 11 untreated patients with ANCA rises relapsed, while all patients who were pre-emptively treated remained in remission. Importantly, the cumulative cyclophosphamide and prednisolone dose was higher in initially untreated patients at the time of the ANCA rise compared to the patients who were pre-emptively treated.\(^{74}\) A similar - but nonrandomized – study was
maintaining remission in GPA and MPA patients

performed in 2003, in which all 8 patients relapsed in whom no treatment changes were made after a quadrupling of the ANCA titer, while only 2 of 11 patients relapsed if immunosuppressive therapy was preemptively increased at the time of the ANCA rise (azathioprine was increased in 7 patients, cyclophosphamide increased in 2 patients, azathioprine was switched to cyclophosphamide in one patient and the prednisone dose was increased in all patients with a mean of 10mg).\textsuperscript{108} In a third study, only one of 22 patients relapsed that were preemptively treated with glucocorticoids monotherapy after a MPO-ANCA rise, while 29 of 35 patients relapsed in whom no change in immunosuppressive therapy was made.\textsuperscript{178} These studies were all performed using the traditional therapeutic regimens using cyclophosphamide induction therapy and azathioprine maintenance therapy. Recent studies, however, have demonstrated that a rituximab-based regimen is non-inferior to a cyclophosphamide-azathioprine-based regimen for remission induction and maintenance.\textsuperscript{46,151,192,200} As the duration of response to a cycle of Rituximab is, however, variable among patients, the timing of repeating Rituximab cycles is at present unknown and is being investigated in the MAINRITSAN2 trial (ClinicalTrials.gov Identifier: NCT01731561), in which individually timed retreatment based on ANCA rise or B cell reconstitution is compared with fixed interval dosing of Rituximab. Finally, in another clinical trial patients were randomized when they were C-ANCA positive at the time of switch of therapy from cyclophosphamide to azathioprine to receive either an extended (i.e. up to 48 months after diagnosis) or standard (i.e. up to 24 months after diagnosis) period of azathioprine maintenance therapy in order to prevent relapses. It was concluded that extended azathioprine maintenance therapy does not result in a longer period of sustained remission.\textsuperscript{176}
Conclusion

AAV has become a chronic disease in which relapses occur frequently during maintenance treatment or later on after all immunosuppressive therapy is stopped. Since relapses are associated with morbidity and mortality, early detection and prevention of relapses is of great importance. Risk factors for relapses, for example genetic background of the patient, have been studied during the last three decades. Only few of these risk factors directly influence the management of patients. One potentially important factor that may influence therapeutic decisions in AAV patients is serial measurement of ANCA. Recently, it became clear that serial ANCA measurement is an attractive approach in AAV patients with (a history of) capillaritis, e.g. renal involvement or alveolar haemorrhage, but not in patients with more limited disease. Whether ANCA rises can be used to guide therapy and hence to prevent relapses has been a great debate during the last three decades. At present, we conclude that small studies do support such an approach but that these findings require further validation in larger randomized clinical trials.
The ultimate goal regarding the guidance of maintenance therapy in ANCA-associated vasculitis is to reduce the incidence of relapses and concomitantly reduce the exposure to immunosuppressive therapy, thereby reducing the incidence of vasculitic damage and the mortality rate, while minimizing treatment-related side-effects. Another goal, which is equally important, yet often overlooked, is to increase the quality of life of patients suffering from ANCA-associated vasculitis during follow-up.\textsuperscript{201} Especially the latter should get more attention amongst researchers and clinicians in the future. In addition, the active involvement of the patient may result in a better prediction model for disease reactivation, as simply asking the patient how active he believes his disease has been in the past month is predictive of a future relapse.\textsuperscript{202} Current research is hampered due to the heterogeneity between studies in the study protocol, used definitions and methodology as described above. Clinical research in ANCA-associated vasculitis can therefore be strengthened by the harmonization and standardization of the used methodology, ranging from autoantibody testing to the definitions used to define relapse and remission. Indeed, the standardization of autoantibody testing may be the most difficult challenge to achieve,\textsuperscript{105,203}, although significant steps have been made.\textsuperscript{204} The methodology behind serial ANCA measurements may also be further fine-tuned, including an in-depth analysis regarding the definition used to characterize an ANCA rise.\textsuperscript{192} Major limitation of current research, however, remains the limited number of included patients in trials, which is inherent to the low incidence of the disease. Previous collaborations, such as the EUVAS and the French Vasculitis Study Group (FVSG) have been highly effective to solve this issue. We expect that intensive collaboration between research groups resulting in multicenter studies will most likely generate a large body of data that can be used to better predict relapses during follow-up. Even though a large number of studies have already been published in which the presence of ANCA to predict relapses was studied, the role of serial ANCA measurements during follow-up remains at present uncertain. In recent years, studies that have been published showed that an ANCA rise is closely associated with a relapse in specific subsets of patients, such as patients with necrotizing glomerulonephritis or alveolar haemorrhage. These findings suggest a close association between ANCA and vasculitic but not with granulomatous disease activity. However, this hypothesis needs to be
tested in future prospective studies that are not only highly powered but are also capable to accurately define granulomatous and vasculitic disease activity. The latter prerequisite may in particular be difficult since it is unpractical (and unethical) to obtain histological evidence of every lesion. A first step toward a better classification in granulomatous and vasculitic lesions, however, has recently been made. Such studies may better specify the subsets of patients in whom serial ANCA measurements can help guide maintenance therapy during follow-up.

In addition, studies that characterize patients that relapse while ANCA are not detectable may give new insights regarding the role of ANCA in disease reactivation. To our knowledge, no studies have been published that investigated these ANCA negative relapses in previously ANCA positive patients. In our own experience, these ANCA negative relapses predominantly occur in patients with non-renal disease and are characterized by granulomatous inflammation of the respiratory tract, but this should be further investigated.

Future studies that are focused on novel biomarkers that better predict disease (re)activation may also yield new findings that can be used to guide maintenance therapy during follow-up (e.g. urinary soluble CD163 in active renal vasculitis). Obvious targets to study more intensively concerning ANCA as a predictor of relapse are the avidity, glycosylation profile or epitope specificity of ANCA. In addition, biomarkers that are not (necessarily) related to ANCA may also be relevant, such as markers reflecting inflammation, injury and repair. Multiple studies have already shown an association of such a biomarker, but the number of included patients restrict the analysis to the predictive value of a single biomarker. Future collaborations may increase the number of included patients and will enable us to analyze the predictive value of a panel of biomarkers to better predict relapses. As previously stated, these results may subsequently lead to a classification system that is focused on the biologic behavior of the disease and may in the future be used to guide maintenance therapy in patients with AAV.
Chapter 4. The avidity of PR3-ANCA in patients with Granulomatosis with Polyangiitis during follow-up

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Jan Damoiseaux
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Abstract

Objective

The objective of this study is to investigate whether the avidity of PR3-ANCA changes during follow-up in different subgroups of patients with Granulomatosis with Polyangiitis (GPA).

Methods

We selected 10 patients with renal relapsing GPA, 10 patients with renal non-relapsing GPA and 10 patients with non-renal relapsing GPA. In all patients, an ANCA rise occurred during remission. The avidity was measured using a chaotropic approach at the time of an ANCA rise and at the time of a relapse in relapsing patients or time-matched during remission in non-relapsing patients.

Results

No difference was observed in the avidity at the ANCA rise between renal relapsing patients (26.2% [15.5-47.5]), renal patients without a relapse (39.6% [21.2-63.4]) and non-renal relapsing patients (34.2% [21.6-59.5]). In renal relapsing patients, the avidity significantly increased from the moment of the ANCA rise to the relapse (difference 6.4% [0.0–17.1], p=0.0273). The avidity did not increase after an ANCA rise in renal non-relapsing patients (difference 3.5 [-6.0–10.1], p=0.6250) or in non-renal relapsing patients (difference -3.1% [-8.0–5.0], p=0.5703).

Conclusion

The avidity of PR3-ANCA increases after an ANCA rise during follow-up in renal relapsing patients but not after an ANCA rise in renal patients who remain in remission or in non-renal relapsing patients.
**Introduction**

Granulomatosis with Polyangiitis (GPA; formerly Wegener's), Microscopic Polyangiitis (MPA) and Eosinophilic Granulomatosis with Polyangiitis (EGPA; Churg Strauss Syndrome) are inflammatory disease entities affecting small to medium vessels. They are characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) against myeloperoxidase (MPO) or proteinase-3 (PR3) and are frequently grouped together under the term ANCA-associated vasculitis (AAV). Since the eighties it has been advocated that ANCA rises predict disease reactivation. However, the relation between ANCA rise and relapse of the disease is far from absolute since many ANCA rises are not followed by a relapse and relapses may occur without a preceding ANCA rise. Recently, we have demonstrated that longitudinal ANCA measurements are highly predictive for disease activity in patients with renal involvement, but not in patients with non-renal disease. At present, it is clear that not every ANCA rise is pathogenic, since only a subset of patients who have an ANCA rise will experience disease reactivation within a period of 12 months. Our hypothesis is that the pathogenicity of an ANCA rise is determined by the quality of the autoantibodies, such as the avidity, glycosylation profile or epitope specificity. The clinical relevance of the avidity of an autoantibody has been demonstrated in patients with antiphospholipid syndrome (APS), in which patients with high avidity anti-β2-glycoprotein I antibodies (anti-β2-GPI) have a higher risk of thrombosis compared to patients with low or heterogeneous avidity anti-β2-GPI. In systemic lupus erythematosus (SLE), high avidity anti-dsDNA antibodies are more closely associated with renal involvement and/or disease activity than low or intermediate avidity anti-dsDNA antibodies. Lastly, determination of the avidity of antiviral antibodies is useful to differentiate a primary infection from reactivation. Several published studies investigated the avidity of MPO-ANCA. The avidity from natural auto-antibodies against MPO in healthy controls is lower compared to the avidity of MPO-ANCA in patients with primary AAV. The avidity of MPO-ANCA antibodies reduces during remission in patients with vasculitis induced by propylthiouracil (PTU), but remains constant in primary ANCA-associated vasculitis. Patients with high avidity MPO-ANCA generally suffer from severe vasculitis disease activity, while patients with low avidity more often suffer from mild vasculitis activity. To our knowledge, no studies have currently
The avidity of PR3-ANCA during follow-up

been published on the avidity of PR3-ANCA.
The primary objective of this study is to evaluate whether the avidity of PR3-ANCA changes during follow-up in patients with GPA. The secondary objective is to determine whether the avidity of PR3-ANCA differs in patients with renal involvement compared to patients without renal involvement during follow-up.
Materials and methods

Patient inclusion

In our cohort of patients with GPA\textsuperscript{124}, we defined three different subgroups and we selected 10 patients from each subgroup: 10 patients with renal involvement with a relapse during follow-up (i.e., ‘renal relapsing’), 10 patients without renal involvement with a relapse during follow-up (i.e., ‘non-renal relapsing’) and 10 patients with renal involvement without a relapse during follow-up (i.e., ‘renal non-relapsing’).

All selected patients fulfilled the following criteria: (1) patients were diagnosed with biopsy-proven GPA according to the EMA classification system\textsuperscript{70}, (2) patients were positive for PR3-ANCA\textsuperscript{106}, (3) remission was induced after the initiation of immunosuppressive induction therapy\textsuperscript{123}, (4) an ANCA rise occurred during follow-up\textsuperscript{124} and (5) serum was available as described below. Serum was obtained for clinical purpose and patients did not object to the (anonymous) use of surplus serum for the purpose of research. Therefore, the requirement of ethical approval was waived according to the Dutch law.

Classification of patients

Renal involvement was determined by a renal biopsy showing pauci-immune necrotizing glomerulonephritis.\textsuperscript{118} Disease activity was scored using the BVAS v3.\textsuperscript{119} Patients were treated according to the European League against Rheumatism (EULAR) guidelines as previously described.\textsuperscript{118,123} Remission was defined as absence of disease activity attributable to active disease during maintenance immunosuppressive therapy of a prednisone dosage of 7.5 mg or lower.\textsuperscript{121}

Follow-up

Patients were routinely screened for potential symptoms of a relapse and blood was drawn.\textsuperscript{124} Antigen-specific solid-phase ANCA tests were performed for the detection and quantification of PR3-ANCA. An ANCA rise was defined as described previously.\textsuperscript{124} A relapse was defined as reoccurrence or new onset of disease attributable to active disease combined with an increase or addition of immunosuppressive treatment.\textsuperscript{74,101,120,121,124}

Serum selection

In all patients, a serum sample (‘T1’) was selected at the time of an ANCA rise.\textsuperscript{124} In renal relapsing and non-renal relapsing patients, a second serum sample (‘T2rel’) was selected prior to the start of the immunosuppressive induction therapy at the time of the relapse. In renal
non-relapsing patients, a second serum sample (‘T2rem’) was selected after the ANCA rise whereas the time since the ANCA rise was matched with the time between the ANCA rise and the relapse of the renal relapsing patients. If available, we also included a diagnostic sample prior to the start of the immunosuppressive induction therapy at diagnosis.

**Determination of the avidity of PR3-ANCA**

Sera were tested using the PR3-hn-hr ELISA (Euroimmun AG, Luebeck, Germany). Briefly, 100 µl of serum diluted 1:101 in sample buffer was added to each of three wells coated with PR3 antigen. After incubation for 30 min at room temperature (+18 °C to +25 °C) and washing, wells were exposed to 200 µl of either a 5 M urea solution, a 3 M urea solution or phosphate buffered saline (PBS) for 10 min. After washing three times, wells were incubated with 100 µl peroxidase-labeled anti-human IgG for 30 min at room temperature, followed again by three washing cycles. 100 µl of a chromogenic substrate solution was added, and the reactions were stopped after 15 min by the addition of 100 µl stop solution per well. The reactions were read immediately at a wavelength of 450 nm using a reference wavelength of 650 nm. A highly positive index patient serum was used to generate a standard curve consisting of three calibrators (2, 20 and 200 relative units (RU)/ml). RU were calculated by this standard curve. A relative avidity index (RAI) was calculated for each specimen and was expressed as the percentage of reactivity remaining in the urea-treated wells. The inter- and intra-assay coefficients of variability of the PR3-hn-hr ELISA were 3.4% and 2.1% when using PBS, 4.4% and 2.9% when using 3 M urea and 4.2% and 4.3% when using 5 M urea.

**Statistics**

Numerical variables were expressed as median [interquartile range] and categorical variables as numbers (percentages). Continuous variables were correlated with the Spearman test. Three unpaired variables were compared with the Kruskal-Wallis H test and post-hoc with the Mann Whitney U test. Two paired variables were compared with the Wilcoxon matched-pairs signed rank test. For the main research question regarding the change of avidity over time, we also applied a sensitivity analysis using a linear mixed model with time (1=ANCA rise, 2=relapse or time-matched during remission), group (1=renal relapsing, 2=renal non-relapsing and 3=non-renal relapsing) and time x group as fixed factor and
an unstructured covariance structure for the repeated measurements. All statistical analyses were performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla California USA) and SPSS statistics for Windows, version 23.0 (IBM, Armonk, NY). A p-value <0.05 was considered significant.

Figure 4-1 (a; upper left). The correlation of the relative avidity index as measured with a urea concentration of 5 M and 3 M. (b; upper right) The correlation of the ANCA level as measured in PBS with the relative avidity index as measured with a urea concentration of 3 M. (c; below) The correlation of the ANCA level as measured in PBS with the relative avidity index as measured with a urea concentration of 5 M.
The avidity of PR3-ANCA during follow-up

Results

Description of the cohort
Thirty patients were included: 10 renal relapsing patients, 10 renal non-relapsing patients and 10 non-renal relapsing patients (see Table 4-1).

Avidity as measured with different urea concentrations
The median PR3-ANCA level as measured with PBS was 142 [82-247] RU/ml. One sample in a non-renal relapsing patient at the time of a minor relapse tested negative for PR3-ANCA and this sample was excluded for the evaluation of avidity. The relative avidity index (RAI) as measured with 3 M and 5 M urea was 59.3% [45.8-73.3] and 35.6% [24.5-49.9], respectively. A strong correlation between the relative avidity index as measured with 3 M and the relative avidity index as measured with 5 M existed (R=0.951, p<0.001, see Figure 4-1a). In addition, the PR3-ANCA level as measured with PBS correlated with the relative avidity index as measured with 3 M and 5 M (R=0.432, p<0.001 and R=0.296, p<0.001, respectively, see Figure 4-1b and Figure 4-1c). The relative avidity index as measured with 5M urea was used in all further analyses.

Avidity at the time of the ANCA rise
No difference was observed in the avidity of PR3-ANCA if obtained at the time of an ANCA rise between renal relapsing patients (26.2% [15.5-47.5] Kruskal-Wallis p=0.4756), renal patients without a relapse (39.6% [21.2-63.4]) and non-renal relapsing patients (34.2% [21.6-59.5], see Figure 4-2).

Table 4-1 (next page). Patient characteristics. Values are expressed as median [inter-quartile range, IQR] or as the total. GC, glucocorticoids; PR3, proteinase-3; MPO, myeloperoxidase.
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<th>Renal relapse</th>
<th>Renal remission</th>
<th>Non-renal relapse</th>
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<tr>
<td>Patients</td>
<td>10</td>
<td>10</td>
<td>10</td>
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**Characteristics at time of previous disease activity**

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<th>Renal relapse</th>
<th>Renal remission</th>
<th>Non-renal relapse</th>
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<tr>
<td>Included at diagnosis</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Age in years</td>
<td>58 [52-72]</td>
<td>50 [31-54]</td>
<td>56 [49-63]</td>
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<tr>
<td>Women</td>
<td>0</td>
<td>4</td>
<td>5</td>
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**Organ involvement at previous disease activity**

| Arthralgia                                             | 8             | 6               | 7                 |
| Cutaneous                                             | 3             | 2               | 1                 |
| Eyes                                                  | 2             | 3               | 2                 |
| Ear, nose, throat                                     | 9             | 8               | 8                 |
| Lung                                                  | 8             | 9               | 8                 |
| Cardiovascular                                        | 1             | 0               | 0                 |
| Renal                                                 | 10            | 10              | 0                 |
| Central nervous system                                | 1             | 0               | 0                 |
| Peripheral nervous system                             | 1             | 2               | 0                 |

**Induction treatment resulting in remission**

| Cyclophosphamide + GC                                  | 7             | 7               | 5                 |
| Rituximab + GC                                         | 1             | 2               | 0                 |
| Methotrexate + GC                                      | 0             | 0               | 3                 |
| Mofetil mycophenolate + GC                            | 0             | 1               | 0                 |
| Gusperimus + GC                                        | 2             | 0               | 1                 |
| GC monotherapy                                         | 0             | 0               | 1                 |

**Characteristics during follow-up**

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<th>Characteristics during follow-up</th>
<th>Renal relapse</th>
<th>Renal remission</th>
<th>Non-renal relapse</th>
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<td>Time between ANCA rise and relapse/remission in months</td>
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<td>7.4 [2.8-12.9]</td>
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<td>Major relapse</td>
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<td>3</td>
</tr>
<tr>
<td>Minor relapse</td>
<td>2</td>
<td>0</td>
<td>7</td>
</tr>
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</table>
Changes in avidity during follow-up

In renal relapsing patients, the avidity significantly increased from the moment of the ANCA rise to the relapse (difference 6.4% [0.0–17.1], p=0.0273). This was not the case in renal non-relapsing patients, in whom the avidity was similar at the ANCA rise and time-matched during remission (difference 3.5 [-6.0–10.1], p=0.6250) or in non-renal relapsing patients, in whom the avidity was similar at the ANCA rise and at the relapse (difference -3.1% [-8.0–5.0], p=0.5703, see Figure 4-3). The sensitivity analysis using a linear mixed model gave similar results as the Wilcoxon matched-pairs signed rank test (p=0.012, p=0.662 and p=0.453 for renal relapsing, renal non-relapsing and non-renal relapsing patients). Concomitantly, in renal relapsing patients the ANCA level significantly increased from the moment of the ANCA rise (140 [102-201]) to the relapse (222 [123-340], p=0.0488), while ANCA levels did not increase in renal non-relapsing patients and in non-renal relapsing patients (data not shown).

No significant differences were observed at the relapse or time-matched in remission in the avidity of PR3-ANCA between renal relapsing patients (35.8% [22.2-70.5], Kruskal-Wallis p=0.5912), renal patients without a relapse (41.1% [31.3-51.2], post-hoc p=0.6706 versus renal relapsing patients) and/or non-renal relapsing patients (27.2% [23.3-54.6], post-hoc p=0.6447 versus renal relapsing patients).

Avidity at the time of diagnosis

Finally, we examined the avidity at the time of diagnosis to investigate whether there are differences in avidity in the early stages of disease process. A diagnostic sample was available in 7 renal relapsing patients, 9 renal non-relapsing patients and 7 non-renal relapsing patients. No significant differences were observed in the avidity at the diagnosis between renal relapsing patients (49.1% [29.3-60.2], Kruskal-Wallis p=0.1653), renal patients without a relapse (34.5% [15.2-43.9], post-hoc p=0.0712 versus renal relapsing patients) and non-renal relapsing patients (37.1% [28.9-61.6], post-hoc p=0.6894 versus renal relapsing patients).
Figure 4-2. The relative avidity index in renal relapsing patients, renal non-relapsing patients and non-renal relapsing patients at the ANCA rise (T1). Post-hoc p-values from the Mann Whitney U test are shown.
The avidity of PR3-ANCA during follow-up

Figure 4-3. The relative avidity index during follow-up in renal relapsing patients in renal patients who remain in remission and in non-renal relapsing patients. P-values of the Wilcoxon matched-pairs signed rank test are shown.
**Discussion**

In this study, we report the results of avidity measurement of PR3-ANCA in patients with GPA. Most importantly, the avidity increases in patients with renal involvement who relapse during follow-up, while it remains constant in renal patients who do not relapse and in non-renal patients who relapse during follow-up. We did not find a difference in the avidity between patients with renal involvement compared to patients with non-renal disease at the time of the ANCA rise and/or the time of diagnosis.

The affinity of an antibody is defined as the binding energy of a monovalent antibody with a single epitope of the target antigen, while the avidity of an antibody represents the binding energy of the antibody with all available epitopes. The avidity of an antibody can be determined using two distinct approaches. The chaotropic method is based on the chemical dissociation of the antigen-antibody complex by a chaotropic agent, such as urea. Another approach is based on competitive inhibition of the binding site of the antibody. The results of the distinct approaches are similar, although assays employing a chaotropic agent may more often detect antibodies with inappropriately low avidity. The applied method to determine the avidity in this study has been extensively validated with large panels of characterized samples in the field of infectious serology.

Interestingly, the avidity increases in patients with renal involvement who relapse during follow-up, while it remains constant in renal patients who do not relapse and non-renal patients who relapse during follow-up. Similar results were found with an additional sensitivity analysis, thereby strengthening the observation. These changes in the avidity of PR3-ANCA may be linked to the fluctuating presence of the antigen source, as has been suggested in patients with MPO-ANCA. In addition, the immune system in these patients may be skewed to a pro-inflammatory state, e.g. due to a chronic upper respiratory infection, which may contribute towards avidity maturation.

This study was designed as a pilot study to gain insight in the avidity of PR3-ANCA during follow-up in patients with renal involvement and patients with non-renal disease. Importantly, we did not find a difference in the avidity level at the ANCA rise of patients with renal involvement compared to patients with non-renal disease. Therefore, we conclude that the avidity of PR3-ANCA does not explain why ANCA rises are highly associated with a relapse in patients with renal involvement but
The avidity of PR3-ANCA during follow-up

not in patients with non-renal disease.\textsuperscript{124} The avidity index may be correlated with the ANCA level due to several reasons. First, low ANCA levels and low avidity may represent natural autoantibodies, while high avidity PR3-ANCA production may result in high ANCA levels that are pathogenic.\textsuperscript{216} Alternatively, the observed correlation between the avidity index and the ANCA level may be related to the method of avidity testing. Avidity assays have been shown to be less sensitive to changes in avidity in samples with a high concentration of antibodies.\textsuperscript{221} Moreover, using a different assay but utilizing a similar test principle, Dangel et al. have shown that the avidity index varied between various dilutions of the same sample.\textsuperscript{227}

Our study suffers from several limitations. First, we tested only a small group of highly selected patients. The strength of our study is that patients are well characterized and the avidity of PR3-ANCA was determined at several moments during follow-up that are clinically relevant.\textsuperscript{124} Moreover, the avidity was determined using a method that has been extensively validated.\textsuperscript{214,215}

In conclusion, the avidity of PR3-ANCA increases during follow-up in renal relapsing patients but not in renal patients who remain in remission or in non-renal relapsing patients. Whether the avidity of PR3-ANCA is associated with more severe vasculitic disease activity at diagnosis should be further studied, as has been demonstrated for MPO-ANCA.
Chapter 5. Galactosylation and sialylation levels of IgG predict relapse in patients with PR3-ANCA associated vasculitis

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Published in EBioMedicine, 2017

* Authors contributed equally; should be considered shared first author
± Authors contributed equally; should be considered shared last author
Abstract

Objective

The objective of our study is to investigate the Fc glycosylation profiles of both antigen-specific IgG targeted against proteinase 3 (PR3-ANCA) and total IgG as prognostic markers of relapse in patients with Granulomatosis with Polyangiitis (GPA).

Methods

Seventy-five patients with GPA and a PR3-ANCA rise during follow-up were included, of whom 43 patients relapsed within a median period of 8 (2-16) months. The N-glycan at Asn297 of affinity-purified and denatured total IgG and PR3-ANCA was determined by mass spectrometry of glycopeptides in samples obtained at the time of the PR3-ANCA rise and at the time of the relapse or time-matched during remission.

Results

Patients with total IgG1 exhibiting low galactosylation or low sialylation were highly prone to relapse after an ANCA rise (HR 3.46 [95%-CI 1.73-6.96], p<0.0001 and HR 3.22 [95%-CI 1.52-6.83], p=0.002, respectively).

In relapsing patients, total IgG1 galactosylation, sialylation and bisection significantly decreased and fucosylation significantly increased from the time of the PR3-ANCA rise to the relapse (p<0.0001, p=0.0087, p<0.0001 and p=0.0025), while the glycosylation profile remained similar in non-relapsing patients. PR3-ANCA IgG1 galactosylation, sialylation and fucosylation of PR3-ANCA IgG1 decreased in relapsing patients (p=0.0073, p=0.0049 and p=0.0205), but also in non-relapsing patients (p=0.0007, p=0.0114 and p=0.0002), while bisection increased only in non-relapsing patients (P<0.0001).

Conclusion

While Fc glycosylation profiles have been associated with clinically manifest autoimmune diseases, in the present study we show that low galactosylation and sialylation in total IgG1 but not PR3-ANCA IgG1 predicts disease reactivation in patients with GPA who experience an ANCA rise during follow-up. We postulate that glycosylation profiles may be useful in pre-emptive therapy studies using ANCA rises as guideline.
Introduction

Granulomatosis with Polyangiitis (GPA; Wegener's), Microscopic Polyangiitis (MPA) and Eosinophilic Granulomatosis with Polyangiitis (EGPA; Churg Strauss Syndrome) are inflammatory disease entities affecting small to medium vessels. They are characterized by the presence of anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase-3 (PR3) or myeloperoxidase (MPO) and are frequently grouped together under the term ANCA-associated vasculitis (AAV). The pathogenic potential of ANCA to cause necrotizing glomerulonephritis (NCGN) is well established in mouse models. Patients with severe NCGN are almost always positive for either PR3- or MPO-ANCA, while ANCA are less often detected in patients with localized forms of vasculitis. After remission induction, a rise in the ANCA titer is detected in some patients and disease reactivation may occur shortly thereafter. However, the relation between longitudinal ANCA measurements and disease reactivation is far from absolute since many ANCA rises are not followed by a relapse and relapses may occur without a preceding ANCA rise. Recently, it has been shown that ANCA rises are highly predictive for disease activity in patients with severe vasculitic disease, e.g. NCGN or alveolar haemorrhage, but not in patients with limited granulomatous disease. Our current hypothesis is that the pathogenicity of an ANCA rise is modulated by the quality of the auto-antibody, for which the glycosylation profile is an important factor. The glycosylation profile of the crystallizable fragment (Fc) of the immunoglobulin G (IgG) is characterized by a single N-linked glycan attached to each heavy chain at the asparagine-297. The Fc N-glycan composition affects Fcy receptor (FcyR) affinity and can influence complement activation. The lack of a core fucose, N-acetylneuraminic (sialic) acids and galactose residues on the Fc N-glycan have been found to increase the inflammatory capacity of IgG, at least in mice. The Fc N-glycan is essential for the pathogenicity of the antibody, since deglycosylation of MPO-ANCA significantly attenuates ANCA-mediated neutrophil activation and reduces glomerular crescent formation in a mouse model. Already in the early eighties, it was recognized that total IgG in patients with rheumatoid arthritis (RA) contained less galactose and sialic acid at the non-reducing termini compared to healthy controls. A difference in the glycosylation profile of total IgG has since been demonstrated in patients with various
Glycosylation profile of PR3-ANCA

other auto-immune diseases when compared to healthy controls, including systemic lupus erythematosus, inflammatory bowel disease, myasthenic gravis, ankylosing spondylitis, primary Sjögren’s syndrome, psoriatic arthritis and multiple sclerosis.\textsuperscript{237-241} Furthermore, significant differences have been observed in the glycosylation profile of total IgG and specific auto-antibodies, such as anti-citrullinated protein antibodies (ACPA), anti-\(\beta\)2GP1 and anti-histone IgG.\textsuperscript{242-245} Finally, Fc glycosylation of auto-antibodies may change during disease development. For example, the glycosylation profile of ACPA changes prior to the onset of RA towards a more inflammation-associated phenotype.\textsuperscript{243,246} In patients with PR3-ANCA, it was previously shown with lectin assays that total IgG exhibits a lower degree of galactosylation when compared to healthy controls.\textsuperscript{247,248} In addition, the degree of sialylation of PR3-ANCA is lower during active disease compared to inactive disease.\textsuperscript{114} Recently, it has been shown with mass spectrometric IgG Fc glycosylation analysis that total IgG Fc of patients with severe AAV exhibits lower levels of galactosylation, sialylation and bisecting \(N\)-acetylglucosamine (GlcNAc) compared to healthy controls.\textsuperscript{193} This finding was more pronounced for PR3-ANCA compared to total IgG.\textsuperscript{193} Correlations were observed between the glycosylation profile of PR3-ANCA and several cytokine concentrations, suggesting that the glycosylation of ANCA may be driven by T-cell activation in an antigen-specific manner.\textsuperscript{193} Potential differences and similarities in the glycosylation profile of total IgG and antigen-specific IgG between patients with PR3-ANCA and patients with MPO-ANCA associated vasculitis have not yet been investigated.

The objective of our study is to investigate differences in the glycosylation profile of both PR3-ANCA and total IgG and the prognostic value at the time of a rise in PR3-ANCA in patients with GPA. The primary question is whether patients with a particular glycosylation profile are more prone to relapse. Furthermore, upon an ANCA rise, changes in glycosylation profiles associated with a relapse are examined. To increase the homogeneity of our cohort, we included only GPA patients that are PR3-ANCA positive, the disease subgroup that is most prevalent in our area.
Materials and methods

Inclusion criteria
All patients who visited the clinic at the Maastricht University Medical Center (MUMC) between January 1, 2000 and November 1, 2011 were evaluated. Inclusion criteria were a diagnosis of GPA according to the EMA classification system and a rise in PR3-ANCA during remission.\textsuperscript{70, 121, 124} Clinical characteristics were recorded in all subjects according to the Dutch law on Medical Treatment Act (WGBO), the Personal Data Protection Act (Wbp) and the Code of Conduct for Health Research (Federa).\textsuperscript{134} Ethics approval was waived by our local ethics committee.

Classification of patients
Renal involvement was preferably determined by a kidney biopsy showing pauci-immune necrotizing glomerulonephritis.\textsuperscript{118, 131} However, surrogate markers such as hematuria in combination with red cell casts, dysmorphic erythrocytes (>10) and/or proteinuria sufficed.\textsuperscript{70} All patients have been treated according to the European League against Rheumatism (EULAR) guidelines.\textsuperscript{118, 123} The definitions recommended by the EULAR of 2007 were applied to define disease activity states.\textsuperscript{121} Remission was defined as absence of disease activity attributable to active disease during maintenance immunosuppressive therapy of a prednisone dosage of 7.5mg or lower. A relapse was defined as re-occurrence or new onset of disease attributable to active disease combined with an increase or addition of immunosuppressive treatment. Relapses were further subdivided in 'major' or 'minor' depending on whether the relapse was potentially organ- or life-threatening or not.\textsuperscript{121}

ANCA measurements
Patients were routinely evaluated during follow-up, generally every three months during the first two years after diagnosis and/or a relapse and 2-3 per year later on. At every visit, patients were screened for potential symptoms of a relapse\textsuperscript{121} and blood was drawn. Antigen-specific solid-phase ANCA tests were performed for the detection and quantification of PR3-ANCA. Initially, commercially available direct PR3-ANCA enzyme linked immunosorbent assays (ELISA) were used (Euro Diagnostica, Malmö, Sweden).\textsuperscript{133} On October 1, 2005, this assay was replaced by a fluorescent-enzyme immune-assay (FEIA) for PR3-ANCA (EliA, Thermo Fisher, Freiburg, Germany).\textsuperscript{106} During the transition, ANCA measurements were performed using both methods.
**Definition of an ANCA rise**

For the detection of an ANCA rise, the ANCA titer was compared to all measurements made with the same assay in the past 6 months. We defined a rise using the slope of an increase as previously described\textsuperscript{124}, thereby taking into account the relative increase (in %) and the time between measurements (in days). A receiver operating characteristics (ROC) curve was calculated to determine the optimal cut-off value of the slope. The chosen cut-off values as determined by the ELISA and FEIA method were 2.56 and 2.25 %/day, respectively. This is equivalent to a relative increase of 78% and 68% over one month or 233% and 205% over three months.

To ensure that small elevations were above the intra-assay coefficient of variation, a rise had to constitute to a relative increase of at least 25% and an absolute increase equivalent to a doubling of the lowest value of a borderline result (at least 10 AU for the ELISA and 5 U/ml for the FEIA). Because our analysis is focused on patients in remission, only serum samples drawn at least 3 months after the previous disease activity were eligible for detection of an ANCA rise.\textsuperscript{124}

**Serum selection**

In all 75 patients, a serum sample was selected at the time of an ANCA rise (‘T1’).\textsuperscript{124} In relapsing patients, either renal or non-renal, a second serum sample was selected prior to the start of the immunosuppressive induction therapy at the time of the relapse (‘T2rel’). In non-relapsing patients, a second serum sample was selected after the ANCA rise during remission (‘T2rem’), of which the time between the first and second sample was matched with the time between the first and second sample in the renal relapsing patients. For 4 relapsing patients no serum sample was available at the time of the relapse, and therefore only 71 T2 serum samples were analyzed.

**Total IgG purification**

For total IgG purification, the wells of two filter plates (Multiscreen filter plates with Durapore membrane, pore size 0.65 µm; Merck Millipore, Darmstadt, Germany) were filled with 15 µl of Protein G Sepharose 4 Fast Flow beads (GE Healthcare, Uppsala, Sweden) in 200 µl PBS, followed by the addition of 2 µl of the serum samples. Furthermore, 10 of the serum samples were purified in duplicate. Fifteen wells were filled with 2 µl Milli-Q-purified water to serve as negative control and 20 wells were filled with 2 µl of the serum of a healthy donor to serve as positive control. The plates were incubated on a shaker at room temperature for 1 hour. The samples were then washed
on a vacuum manifold with 4x 200 µl PBS and 3x 200 µl Milli-Q-purified water, followed by the addition of 100 µl 100 mM formic acid (Fluka, Steinheim, Germany) for elution into a V-bottom 96-well plate (Greiner Bio-One, Frickenhausen, Germany), which has been shown to result in near-complete denaturation of IgG. Eluates were dried in a centrifugal vacuum concentrator (Eppendorf, Hamburg, Germany) at 60 °C for approximately 2 hours.

PR3-ANCA purification

An ELISA kit (Wieslab PR3-ANCA; Euro Diagnostica) was used for purification of PR3-ANCA. Eighty µl diluent containing PBS (Wieslab kit) and 20 µl of the serum samples were added to two PR3-coated 96-well plates. Ten of the serum samples were purified in duplicate in separate wells. Fifteen wells were filled with 20 µl Milli-Q-purified water to serve as negative control and 20 wells were filled with 20 µl of the serum of a healthy donor to serve as a further negative control. The samples were incubated on a shaker at room temperature for 1 hour, followed by washing 4x with 250 µl wash buffer (Wieslab kit) and 1x with 150 µl 50 mM ammonium bicarbonate buffer (Sigma-Aldrich, St. Louis, MO). The PR3-ANCA was eluted by adding 100 µl 100 mM formic acid to the wells and collected into a V-bottom 96-well plate. The samples were then dried in a centrifugal vacuum concentrator at 60°C for approximately 2 hours.

Trypsin digestion

The dried total IgG and PR3-ANCA samples were resuspended in 20 µl 50 mM ammonium bicarbonate and incubated on a shaker at room temperature for 15 minutes. Twenty µl of 0.01 µg/µl trypsin (sequencing grade modified trypsin, Promega, Madison, WI) was then added to each well. The samples were again incubated on a shaker at room temperature for 15 minutes, followed by overnight incubation at 37°C. Samples were stored at -20°C.
Figure 5-1. LC-MS spectra showing tryptic IgG1 glycopeptides for total IgG and PR3-ANCA belonging to an AAV patient (#9; details in Supplemental Table 1) at the time of relapse. The peaks denoted with an asterisk belong to a co-enriched contaminant (an apolipoprotein O-glycopeptide). Pep=peptide.

**LC-MS/MS analysis**

The digested samples were analyzed by nanoLC-reversed phase (RP)-electrospray ionization (ESI) – quadrupole time-of-flight (qTOF)-MS on an Ultimate 3000 HPLC system (Dionex/Thermo Scientific, Sunnyvale, CA) coupled to a MaXis Impact (Bruker Daltonics, Bremen, Germany). The samples were concentrated on a Dionex Acclaim PepMap100 C18 trap column (particle size 5 µm, internal diameter 300 µm, length 5 mm) and separated on an Ascentis Express C18 nano column (2.7 µm HALO fused core particles, internal diameter 75 µm, length 50 mm; Supelco, Bellefonte, PA). The following linear gradient was applied, with solvent A consisting of 0.1% trifluoroacetic acid (TFA; Fluka) and solvent B of 95% acetonitrile (Biosolve, Valkenswaard, The Netherlands): t=0 min, 3% solvent B; t=2, 6%; t=4.5, 18%; t=5, 30%; t=7, 30%; t=8, 1%; t=10.9, 1%. The sample was ionized in positive ion
mode with a CaptiveSprayer (Bruker Daltonics) at 1100 V. A nanoBooster (Bruker Daltonics) was used to enrich the nitrogen gas with acetonitrile to enhance the ionization efficacy. A mass spectrum was acquired every second (frequency of 1 Hz), with the ion detection window set at m/z 550-1800. In between every 12 measurements an external IgG standard was run.

**Data processing**

Mass spectrometric identification and processing of tryptic IgG glycopeptide data was done as described previously\textsuperscript{250}, with a few differences as stated below, and was performed blindly in order to prevent bias. The LC-MS data files were examined in Compass Data Analysis 4.2 software (Bruker Daltonics). Negative controls were inspected and found to contain no significant intensity of IgG signals. First, the m/z axis of the mass spectra was calibrated internally using several known IgG glycopeptides masses. Next, the files were converted to the .mzXML data format using the MSconvert program from the ProteoWizard 3.0 suite.\textsuperscript{251} The m/z and retention times of all the tryptic IgG glycopeptides were manually determined using MZmine 2.1\textsuperscript{0252} and can be found in supplemental table S1. The time axes were aligned based on a list of the most prominent IgG glycopeptide peaks and their retention times, using an alignment tool designed in-house.\textsuperscript{253} The intensity of the first three isotopes of every analyte was extracted using a window of ± 0.04 Thomson and ± 10 s around the manually determined retention time. This was achieved using the in-house developed 3D Total Extractor (3DTE) program (software code can be found in the supplemental data). A list of analytes is provided to 3DTE, which then uses a binary search to find the retention time and mass region around each analyte. Afterwards, the maximum intensity of each analyte is determined per mass spectrum that falls within the retention time window. The value that is reported for each analyte by 3DTE is the sum of the highest intensity values from the individual mass spectra. Lastly, the program generates a tab-separated output file that lists all the compositions and their respective total intensities. Background correction was optimized to minimize intensity biases in the determination of glycopeptide ratios. In Excel 14.0, the background-corrected signal intensities of the three isotopic peaks in both 2+ and 3+ charge state were summed to obtain a single value for each glycopeptide. IgG1 G2 and G2S2 were excluded because of overlap with contaminant peaks. Isotopic peaks for several other IgG1 glycopeptides were also excluded due to peak overlap, and these values were
replaced by an estimate based on the remaining isotopic peaks and the theoretical isotopic pattern (these isotopic peaks account for less than 3% of the total glycopeptide signal intensity). Afucosylated IgG4 glycopeptides were excluded due to peak overlap with IgG1 glycopeptides. In order to obtain a percentage value for each glycopeptide, this value was then divided by the sum of all signal intensities of glycopeptides, and this was done separately for IgG1, IgG2/3 and IgG4. In Caucasian populations the tryptic glycopeptide of IgG3 generally has the same peptide sequence as IgG2. Therefore it was not possible to distinguish between IgG2 and IgG3 glycopeptides with the methods we used, and these glycopeptides will be referred to as IgG2/3. The IgG percentage data can be seen in supplemental table S2. Several glycosylation features were calculated from this data: fucosylation (% of glycans bearing a core fucose), bisection (% of glycans with a bisecting GlcNAc), galactosylation (% of antennae carrying a galactose) and sialylation (% of antennae carrying a sialic acid, see supplemental data for the exact definitions).

In order to guard the quality of the acquired data, an intensity threshold was set. If the sum of the signal intensities of G0F, G1F and G2F in triple charge state for an IgG subclass did not exceed this threshold, all data for that subclass was excluded. Additionally, data was excluded if the signal/background of the highest peak per subclass did not exceed 20. Finally, because of the chance of overlap between IgG4 glycopeptide peaks and later eluting IgG2/3 peaks, IgG2/3 data was excluded if more IgG4 was present than IgG2/3 (as determined by G0F+G1F+G2F (3+) signals). Lastly, we determined the average relative standard deviation (RSD) of eight prominent glycans (G0F, G1F, G2F, G0FN, G1FN, G2FN, G1FS and G2FS) to further examine the quality of the data.

**Statistical analysis**

Numerical variables were expressed as median (interquartile range [IQR]) and categorical variables as relative abundances (percentages). Two unpaired variables were compared with the Mann Whitney U test. Two paired variables were compared with the Wilcoxon signed rank test. Continuous variables were correlated with the Spearman test. Receiver operating characteristic (ROC) curves were constructed for galactosylation, sialylation, fucosylation and bisection at the time of an ANCA rise to discriminate relapsing patients from patients who remained in remission. If a trend towards significance was observed in an ROC curve, an optimal cut-off value was derived that was closest to the upper left corner (the cut-off value
with the highest sum of sensitivity and specificity). Patients were subsequently categorized as high (above or equal to the cut-off) or low (below cut-off) level for the concerning glycosylation trait. Differences in the time to relapse between high and low were assessed using the log-rank test. The time to relapse was estimated using the Kaplan-Meier method. An event was defined as a relapse at the time of the start or increase of immunosuppressive treatment. Subjects were censored at the time of last follow-up. Hazard ratios (HR) with the 95% confidence interval were derived using the Cox regression analysis, adjusted for age and sex. The proportional hazards assumption was assessed by visually inspecting log-log plots.

All statistical analyses were performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, California, USA) and SPSS statistics for Windows, version 23.0 (IBM, Armonk, NY, USA). Bonferroni corrections were applied to the statistical testing, in which a p-value of <0.0125 was considered significant and <0.05 was considered as a trend.

**Table 5-1 (next page).** Patient characteristics. The organ involvement refers the organ involvement during any previous periods of disease activity in the past (at the diagnosis or any previous relapse). The induction treatment refers to the treatment regimen that was used to induce remission during the most recent period of disease activity only. Abbreviations: SD, standard deviation. GC, glucocorticosteroid therapy.

*This patient was included after a minor relapse which was treated with GC monotherapy, while remission was induced after diagnosis using cyclophosphamide + GC. ±The follow-up time refers to the time from the most recent disease activity to the endpoint of the study (at the time of relapse or at the last time of follow-up).
<table>
<thead>
<tr>
<th>Demographics</th>
<th>Renal (n=31)</th>
<th>Non-renal (n=12)</th>
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</thead>
<tbody>
<tr>
<td>Age (in years + SD)</td>
<td>58 (14)</td>
<td>55 (14)</td>
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<tr>
<td>Male</td>
<td>23 (74%)</td>
<td>13 (65%)</td>
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<tr>
<th>Organ involvement at previous disease activities</th>
<th>Renal (n=31)</th>
<th>Non-renal (n=12)</th>
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<tbody>
<tr>
<td>Arthralgia</td>
<td>19 (61%)</td>
<td>10 (50%)</td>
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<tr>
<td>Cutaneous</td>
<td>13 (42%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Eyes</td>
<td>8 (226%)</td>
<td>5 (25%)</td>
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<tr>
<td>Ear, nose, throat</td>
<td>26 (83%)</td>
<td>17 (85%)</td>
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<tr>
<td>Lung</td>
<td>23 (74%)</td>
<td>14 (70%)</td>
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<tr>
<td>Cardiovascular</td>
<td>1 (3%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Renal</td>
<td>31 (100%)</td>
<td>20 (100%)</td>
</tr>
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<td>Central nervous system</td>
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<tr>
<td>Peripheral nervous system</td>
<td>7 (23%)</td>
<td>4 (20%)</td>
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<th>Induction treatment at previous disease activity resulting in remission</th>
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<th>Non-renal (n=12)</th>
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<tr>
<td>Cyclophosphamide + GC</td>
<td>19 (61%)</td>
<td>14 (70%)</td>
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<tr>
<td>Rituximab + GC</td>
<td>1 (3%)</td>
<td>3 (15%)</td>
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<tr>
<td>Methotrexate + GC</td>
<td>5 (16%)</td>
<td>0 (0%)</td>
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<tr>
<td>Mofetil mycophenolate + GC</td>
<td>3 (10%)</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Gusperimus + GC</td>
<td>2 (6%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>GC monotherapy</td>
<td>1 (3%)*</td>
<td>0 (0%)</td>
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<th>Renal (n=31)</th>
<th>Non-renal (n=12)</th>
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<tr>
<td>Immunosuppressive therapy</td>
<td>27 (87%)</td>
<td>16 (80%)</td>
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<td>None</td>
<td>4 (13%)</td>
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<th>Follow-up</th>
<th>Renal (n=31)</th>
<th>Non-renal (n=12)</th>
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<tr>
<td>Follow-up time (in months + SD)</td>
<td>13 (20)</td>
<td>42 (21)</td>
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<tr>
<td>Persistently ANCA positive</td>
<td>10 (32%)</td>
<td>6 (30%)</td>
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<th>Relapse</th>
<th>Renal (n=31)</th>
<th>Non-renal (n=12)</th>
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<tr>
<td>Major</td>
<td>18 (58%)</td>
<td>-</td>
</tr>
<tr>
<td>Minor</td>
<td>13 (42%)</td>
<td>-</td>
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</table>
Results

Patient characteristics

Seventy-five patients with Granulomatosis with Polyangiitis positive for PR3-ANCA were included in this study, of whom 51 patients had renal involvement in the past and 24 patients did not (see Table 5-1). Forty-three (57.3%) patients relapsed within a median time of 8 (2-16) months since the ANCA rise. Out of 156 samples total (75 at the time of ANCA rise, 71 at a second time point, and 10 duplicates), PR3-ANCA IgG1 data was excluded in 16 samples, total IgG1 data in 0 samples, total IgG2/3 data in 14 samples and total IgG4 in 20 samples. Because the signal intensity of PR3-ANCA IgG2/3 and IgG4 in the majority of samples did not surpass the threshold, we excluded this data group altogether. For 8 prominent glycoforms, the external IgG standard run in between every 12 measurements showed an average RSD of 4.2% for IgG1, 4.0% for IgG2/3 and 7.1% for IgG4. The on-plate healthy donor serum-derived total IgG samples exhibited an average RSD of 4.0% for IgG1, 3.0% for IgG2/3 and 3.5% for IgG4. Finally, for the aforementioned 8 glycoforms, the 10 ANCA serum samples which were processed in duplicate showed an average RSD of 2.3%, 1.5% and 1.3% for total IgG1, IgG2/3 and IgG4, and 3.1% for PR3-ANCA IgG1. Two LC-MS spectra of total IgG and PR3-ANCA IgG, belonging to an AAV patient at the time of relapse, can be seen in Figure 5-1.

For better readability, the results of total IgG1 and PR3-ANCA IgG1 are presented in the manuscript, while the results of total IgG2/3 and total IgG4, which exhibit the same general trend as total IgG1, can be found in the supplemental data.

IgG1 Fc glycosylation of total IgG and PR3-ANCA at the time of the ANCA rise

At the time of the ANCA rise, we analyzed whether there were differences in the glycosylation profile of total IgG1 and PR3-ANCA IgG1. The degree of galactosylation, sialylation and fucosylation of total IgG1 was lower compared to PR3-ANCA IgG1 (46.0% [39.1-49.7%] versus 47.4% [40.4-57.5%], p=0.0175; 6.2% [5.2-7.1%] versus 6.9 [5.1-9.0%]; p=0.0289 and 93.3% [89.9-95.5%] versus 98.4% [97.3-99.0%], p<0.0001, respectively). In contrast, the level of bisection of total IgG1 was higher compared to PR3-ANCA IgG1 (19.0 [15.9-21.7%] versus 11.4% [10.1-13.9%], p<0.0001, see figure S1 in the supplemental data).
Figure 5-2. Time to relapse after an ANCA rise, (a) according to the degree of galactosylation of total IgG 1 Fc and (b) according to the degree of sialylation of total IgG 1 Fc.
IgG1 Fc glycosylation as a predictor of a relapse at the time of an ANCA rise

Thereafter, we investigated whether patients with a particular IgG1 Fc glycosylation profile at the time of an ANCA rise are more prone to relapse after an ANCA rise. The ROC curve of total IgG1 galactosylation and sialylation were found to differentiate relapsing patients from non-relapsing patients (supplemental figure S2; AUC 0.6533, p=0.02385 and AUC 0.6563, p=0.02131, respectively). Total IgG1 bisect and fucosylation did not yield significant discrimination between relapsing and non-relapsing patients (p=0.9488 and p=0.9402, respectively; data not shown). The ROC curve of PR3-ANCA IgG1 was not significant for any of the glycosylation traits (data not shown). An optimal cut-off value was derived from the significant ROC curves, and patients were classified as ‘low galactosylation’ if the galactosylation rate of total IgG1 was lower than 46.1% and as ‘low sialylation’ if the sialylation rate of total IgG1 was lower than 6.9% (see figure S2 in the supplemental data).

Forty-one of 75 (54.7%) patients were classified as low galactosylation, of whom 30 (73.2%) patients relapsed within a median of 6 (1-15) months after the ANCA rise. In comparison, only 13 of 34 (38.2%) patients who were classified as high galactosylation relapsed in a median of 10 (2.5-17.5) months. Thus, patients with low galactosylation total IgG1 were highly prone to relapse after an ANCA rise (HR 3.46 [95%-CI 1.73-6.96], p<0.0001, adjusted for age and sex, see Figure 5-2a).

Similar results were found for the degree of sialylation. Forty-seven of 75 (62.7%) patients were classified as low sialylation, of whom 34 (72.3%) patients relapsed within a median of 8 (1-17) months after the ANCA rise. In comparison, only 9 of 28 (32.1%) patients with a high sialylation relapsed in a median of 7 (2-12) months. Low sialylation patients were highly prone to relapse after an ANCA rise (HR 3.22 [95%-CI 1.52-6.83], p=0.002, adjusted for age and sex; see Figure 5-2b).

Because the degree of sialylation and galactosylation are highly correlated (terminal N-acetylneuraminic acid is attached to galactose), we in addition calculated the sialic acid per galactose. No association was observed between the sialic acid per galactose and the time to relapse (see figure S3 and S4 in the supplemental data).

Changes in IgG1 Fc glycosylation during follow-up

Next, we looked at potential changes of the glycosylation profile during follow-up. With regards to the total IgG1, the degree of galactosylation, sialylation and bisect significantly decreased and fucosylation...
Glycosylation profile of PR3-ANCA

significantly increased in relapsing patients \( (p<0.0001, \ p=0.0087, \ p<0.0001 \ \text{and} \ p=0.0025, \ \text{respectively}) \), while the glycosylation profile remained similar in patients who remained in remission (see Figure 5-3 and figure S5 and S6 in the supplemental data).

With regard to PR3-ANCA IgG1, in relapsing patients, a significant reduction in the degree of galactosylation and sialylation and a trend towards a reduction in fucosylation were observed from the ANCA rise to the time of the relapse \( (p=0.0073, \ p=0.0049 \ \text{and} \ p=0.0205, \ \text{respectively}, \ \text{see Figure 5-4}) \). Similarly, in patients who remained in remission, a significant reduction in the degree of galactosylation, sialylation and fucosylation was observed from the ANCA rise to the time of the second sample \( (p=0.0007, \ p=0.0114 \ \text{and} \ p=0.0002, \ \text{respectively}) \). Moreover, the proportion of bisection of PR3-ANCA IgG1 significantly increased from the ANCA rise to the time-matched sample during remission \( (p<0.0001) \).

Figure 5-3 (next page). The glycosylation profile of total IgG1 Fc at the time of an ANCA rise \( (T1) \) and at the time of a relapse \( (T2\text{rel}) \) in relapsing patients (black dots) and time-matched during remission \( (T2\text{rem}) \) in patients who remain in remission (gray dots). Dots represent individual patients, lines indicate corresponding pairs. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Wilcoxon signed rank test, P-values are shown if <0.10 and in bold if <0.0125.
IgG1 Fc glycosylation at the time of the relapse or time-matched during remission

The changes in the glycosylation profile over time lead to significant differences between patients in relapse or in remission at the second time point. With regard to total IgG1, a significantly lower degree of galactosylation and sialylation and a trend towards a lower degree of bisection was observed in relapsing patients compared to patients who remain in remission \( (p=0.0015, \ p=0.0120 \) and \( p=0.0443, \) respectively, see Figure 5-5 and figure S7 in the supplemental data). Conversely, the glycosylation profile of PR3-ANCA was only significantly different with regard to bisection, which was lower in relapsing patients compared to patients who remained in remission \( (p=0.0009). \)

Figure 5-4 (next page). The glycosylation profile of antigen specific PR3-ANCA IgG1 Fc at the time of an ANCA rise (T1) and at the time of a relapse (T2rel) in relapsing patients (black dots) and time-matched during remission (T2rem) in patients who remain in remission (gray dots). Dots represent individual patients, lines indicate corresponding pairs. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Wilcoxon signed rank test, \( P \)-values are shown if \( <0.10 \) and in bold if \( <0.0125. \)
Fc IgG1 glycosylation of PR3-ANCA during follow-up

Relapsing patients

Galactosylation (%)

P = 0.0073

Sialylation (%)

P = 0.0049

Bisection (%)

P = 0.0205

Fucosylation (%)

P = 0.0002

Non-relapsing patients

Galactosylation (%)

P = 0.0007

Sialylation (%)

P = 0.0114

Bisection (%)

P < 0.0001

Fucosylation (%)

P = 0.0002

ANCA rise
n = 32

Relapse
n = 32

ANCA rise
n = 27

Remission
n = 27
Discussion

In this study we investigate with mass spectrometry the changes in Fc glycosylation over time in both total IgG and PR3-ANCA of patients with AAV. In patients who were in clinical remission, a first serum sample was taken at the time of an ANCA rise, whereas a second sample was acquired either after relapse but before therapy was started, or for patients who remained in remission, after a similar length of time. Analysis of the two longitudinal samples revealed a significant reduction in galactosylation, sialylation and bisection and an increase in fucosylation in total IgG Fc of relapsing patients, while these glycosylation traits did not change significantly in patients who remain in remission. Most importantly, the Fc glycosylation profile of total IgG at the time of an ANCA rise predicts a relapse. Namely, patients with low galactosylation or low sialylation in total IgG are more prone to relapse compared to patients with high galactosylation or sialylation in total IgG. In addition, we observed that the level of sialylation and galactosylation of PR3-ANCA is decreased over time, both in relapsing and non-relapsing patients. Therefore, it appears that PR3-ANCA gains a more inflammation-associated phenotype during follow-up in both groups of patients, independent of disease course.

While IgG with a low degree of galactosylation has repeatedly been found to be associated with pro-inflammatory autoimmune responses, the underlying mechanisms are still largely elusive. In contrast, highly galactosylated IgG may confer anti-inflammatory activities through the association with the inhibiting receptor FcyRIIb and the C-type lectin-like receptor Dectin-1 in mice. This latter pathway has been shown to block C5a effector functions in vitro and C5a-dependent inflammatory responses in animal mouse models. This is highly relevant in AAV, since C5a plays a pivotal role in the pathophysiology. Mouse models have shown that the blocking of C5a or C5a receptor (C5aR) ameliorates anti-MPO induced necrotizing glomerulonephritis. The safety and efficacy in the treatment of non-life-threatening AAV with CCX168, a C5aR inhibitor, is currently being tested in a phase 2 study (EudraCT Number: 2011-001222-15). The addition of galactose to the glycan structure also no longer enables the interaction of MBL with IgG, and may thereby block the lectin pathway. The clinical relevance of the lectin pathway in AAV is questionable however, since mannose-binding lectin (MBL) deposition in the kidney was found in only a minority of patients and complement activation in
AAV occurs predominantly via the alternative pathway.\textsuperscript{259}

Sialylated IgG has been reported to have anti-inflammatory properties likely mediated through interaction with the murine C-type lectin receptor SIGN-R1 (a homologue of the human DC-SIGN) and FcγRIIb.\textsuperscript{234,262} In our study, we found that total IgG preserved an anti-inflammatory glycosylation profile in patients who remained in remission, but changed to an inflammation-associated phenotype in relapsing patients. It remains unclear whether this change merely represent a bystander acute-phase reaction or whether the loss of anti-inflammatory effector function of total IgG enabled PR3-ANCA to induce disease reactivation. Notably, low galactosylation and sialylation at the ANCA rise predicts disease reactivation, suggesting that the change of total IgG towards an inflammation-associated phenotype precedes the onset of disease reactivation. We hypothesize that these anti-inflammatory mechanisms of total IgG are involved in the suppression of active disease in our AAV patients who remain in remission.

One may wonder what causes the change of the glycosylation profile of total IgG from an anti-inflammatory towards a pro-inflammatory phenotype. One study observed an average reduction of total IgG sialylation by 40% upon antigenic challenge in a mouse model.\textsuperscript{234} Based on our findings, we speculate that a second hit that is not related to the presence of PR3-ANCA nor the glycosylation profile of PR3-ANCA is required for a relapse after an ANCA rise (the “first hit”). Several candidates for such a second hit have been postulated, such as microorganisms\textsuperscript{116}, environmental factors\textsuperscript{66,67}, and/or other auto-antibodies.\textsuperscript{68} No functional data are currently available regarding the role of the glycosylation profile of total IgG and/or antigen-specific IgG in the capacity of PR3-ANCA to induce inflammation and this should be further studied.

**Figure 5-5 (next page).** The glycosylation profile at the time of a relapse in relapsing patients and time-matched during remission in patients who remain in remission (gray dots). IgG1 Fc glycosylation of total IgG (left side, white background) and antigen specific PR3-ANCA (right side, yellow background) is shown. Dots represent individual patients. The box represents the median with interquartile range, the whiskers delineate the min-max range. Significant differences were evaluated using the Mann Whitney U test, P-values are shown if <0.10 and in bold if <0.0125.
Glycosylation profile of PR3-ANCA

**Fc IgG1 glycosylation at the relapse or time-matched during remission (T2)**

<table>
<thead>
<tr>
<th>Glycosylation (%)</th>
<th>Total IgG</th>
<th>PR3-ANCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P = 0.0015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sialylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P = 0.0120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P = 0.0443</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucosylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P = 0.0009</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relapsers: n = 39  Non-relapsers: n = 32  Relapsers: n = 35  Non-relapsers: n = 28
A recent study reported a novel correlation between bisection of PR3-ANCA and disease state.\(^{193}\) In our study we find a correlation between bisection and relapse/remission. The level of bisection of total IgG decreases significantly in relapsing patients, while it does not change in non-relapsing patients. In PR3-ANCA the level of bisection remains stable in relapsing patients, while it increases in non-relapsing patients. While the effect of a bisecting GlcNAc on IgG effector functions is minor compared to that of the other glycosylation features, it has been reported that bisection can enhance antibody-dependent cellular cytotoxicity (ADCC) through increased FcyRIIIa affinity.\(^{263,264}\) Since a reduction in bisection is seen during AAV relapse, it is likely that the minor anti-inflammatory effect of decreased bisection is overshadowed by effects of other changes in glycosylation and that ADCC may only play a minor role in the pathogenesis of AAV.\(^{265}\) A decrease in IgG bisection has not been reported for any other autoimmune disorders, while an increase in bisection has been observed for Lambert-Eaton Myasthenic Syndrome (LEMS).\(^{241}\) A slight increase in fucosylation over time was seen in total IgG of relapsing patients. In contrast, PR3-ANCA showed a minor decrease in fucosylation for both relapsing and non-relapsing patients. While the absence of a core fucose can greatly enhance the inflammatory properties of IgG through increased FcyRIIIa affinity, the differences in fucosylation in our study cohort are likely too small to be of much influence.

Our findings regarding the glycosylation profile of total IgG compared to antigen-specific PR3 markedly differ from those of a previous study, which reported a reduction of galactosylation, sialylation and bisection of PR3-ANCA as compared to total IgG, while we observe that bisection, but not galactosylation and sialylation is reduced in PR3-ANCA. These differences may be largely caused by the pronounced differences in study design.\(^{193}\) First, we included patients with severe AAV as well as patients with more limited forms of AAV, while the previous report only included patients with severe AAV.\(^{193}\) Second, our patients were in remission at the time of sampling, while the patients of Wuhrer et al. were sampled at the time of active disease.\(^{193}\) Interestingly, both studies observe a lower degree of bisection in PR3-ANCA compared to total IgG.

Our study is limited by the number of included patients, hence only the N-glycan analysis was included in the statistical evaluation. Future validation in other, larger study cohorts is warranted in which the predictive value of a multitude of
factors, in particular the IgG N-glycan analysis, should be evaluated using multivariate techniques, including principle components analysis. A strong aspect of our study is that our patients are highly characterized and we only included patients with GPA positive for PR3-ANCA. Yet differences still remain in organ involvement and immunosuppressive therapy. Our findings, however, may not apply to patients with MPA or patients positive for MPO-ANCA and this should be further investigated.

Most importantly, we addressed the clinical value of the aberrant glycosylation which we observe in AAV patients. Changes in IgG glycosylation, especially galactosylation and sialylation, might be useful to screen patients for their risk of relapse. Our data indicates that analysis of total IgG would be sufficient for this purpose. Already in the first serum sample, acquired a median time of 8 months before the time of relapse, we could identify patients that are at risk for a future disease relapse. Differences in the glycosylation profile between relapsing and non-relapsing patients becomes more pronounced as the time of relapse approaches. Longitudinal acquisition of serum samples taken every few months would reveal changes in the personal glycosylation profile of each patient that could possibly help as a guide when to start treatment in these patients. It remains to be studied, however, whether treatment based on this information will be able to minimize tissue and organ damage.
General discussion

In this thesis, we set out to provide physicians tools to predict future disease activity in order to guide maintenance therapy in patients with ANCA associated vasculitis. In the first section of the general discussion, we summarize the main findings as described in this thesis. Most importantly, our studies have further substantiated the evidence behind a classification system of patients based on the biologic behaviour of the disease to guide maintenance therapy in ANCA associated vasculitis. Our studies suffer from several limitations however, which are highlighted in the next paragraph. Furthermore, we propose recommendations for future research to realize a prediction model to accurately identify patients that are at risk of a relapse. We hypothesize that such a model can be constructed if a vast amount of patient data is collected in a standardized manner to allow for the application of artificial intelligence. While our studies have outlined factors that should be incorporated in the future model, other factors should be further investigated, which are highlighted in the last section. Ultimately, our recommendations will result in a prediction model that may in the future be used to prevent morbidity and mortality related to active disease, while minimizing treatment-related side-effects.
Summary of our findings

To accomplish our objectives, we analysed a cohort of patients with ANCA associated vasculitis that were closely monitored during follow-up in a tertiary referral hospital. In Chapter 1, we analysed the association of serial ANCA measurements with disease activity during follow-up. In this study, we observed that ANCA rises are highly associated with a future relapse in patients with renal involvement, but not in patients with non-renal disease. Renal involvement may be a proxy for severe vasculitic disease as we found in a small subgroup of non-renal patients with severe disease were prone to relapse after an ANCA rise. This hypothesis is further substantiated by a later study in which ANCA rises were highly associated with disease activity in patients with a history of alveolar haemorrhage, a life-threatening vasculitic manifestation. Not every ANCA rise leads to disease activation, however, since only 43.3% of our patients with renal involvement relapsed within a year after the ANCA rise. Therefore, we set out to identify additional risk factors for a relapse at the time of an ANCA rise in Chapter 2. We included all patients with renal involvement in whom an ANCA rise was detected during follow-up. We identified three independent risk factors for a relapse: previous induction therapy lacking cyclophosphamide or rituximab, an ANCA rise during the fall season and a perpetuating ANCA rise. An ANCA rise during the fall season in particular increased the proportion of relapsing patients within a year to 84.6%. We hypothesize that the seasonal influence may be a proxy for an increased risk of infections, which may serve as a second hit. Alternatively, during follow-up we observed lower levels of vitamin D, an important immunodulator, in relapsing patients, but not in patients who remained in remission. In Chapter 3, we reviewed the current literature regarding potential strategies to guide maintenance therapy in patients with GPA or MPA, with an emphasis on patient classifications and the role of serial ANCA measurements. In this review, we hypothesize that a classification model that is focused on the biologic behavior of the disease may in the future be used to guide maintenance therapy in patients with AAV. To substantiate this hypothesis, we analysed additional biomarkers in serum samples that were previously obtained for clinical purposes during follow-up. To reduce heterogeneity in our cohort, we limited our studies to patients with Granulomatosis with Polyangiitis that were PR3-ANCA positive. First, we investigated whether the avidity of PR3-ANCA changes during follow-up in different subgroups of
patients with Granulomatosis with Polyangiitis. In Chapter 4, we observed that avidity of PR3-ANCA increases after an ANCA rise during follow-up in renal relapsing patients but not after an ANCA rise in renal patients who remain in remission or in non-renal relapsing patients. The findings of our pilot study warrant a prospective study to investigate whether the avidity of PR3-ANCA at the time of an ANCA rise identifies patients that are at a risk of a relapse. In Chapter 5, we investigated the Fc glycosylation profiles of total IgG and PR3-ANCA as prognostic markers of a relapse. Most importantly, at the time of the ANCA rise, we observed that patients with low galactosylation or low sialylation in total IgG are more prone to relapse compared to patients with high galactosylation or sialylation in total IgG. Furthermore, we analysed a second sample after the ANCA rise taken at the time of the next disease activity in relapsing patients or time-matched in patients that remain in remission. The longitudinal analysis revealed a reduction in galactosylation, sialylation and bisection and an increase in fucosylation in total IgG of relapsing patients, while these glycosylation traits did not change significantly in patients who remain in remission. Our findings suggest that the glycosylation profile of total IgG during follow-up may be a useful marker to guide maintenance therapy in the future.

Limitations of our studies

Limitations of our studies have been extensively reported in the discussion of previous chapters and will be summarized next. Most importantly, our studies were performed in a single-centre cohort at a tertiary referral hospital using data from clinical practice that has been obtained in a retrospective manner. While such a cohort offers advantages, it is also associated with several limitations. As such, our cohort is limited in sample size and consists of a highly specific subset of patients as compared to cohorts originating from secondary care. Patient management in our cohort, while performed in accordance with appropriate guidelines, is more heterogeneous as compared to patient management in a randomized clinical trial. Our physicians were not blinded to the ANCA values. Also, retrospective observational studies lag behind on new developments. Most of our patients are therefore treated with cyclophosphamide, which will continue to be relevant, yet patients will more often receive rituximab in the future. Whether our
findings are applicable in patients treated with rituximab should be investigated. Last, our data are less reliable as compared to data obtained in a prospective manner. This is most evident when looking at the collection of serial ANCA measurements. In prospective trials, serum samples are collected at a strict time interval (e.g. 1 month) and all samples are measured with the same ANCA method. This is in sharp contrast to clinical practice, in which the time interval is tailored to the preference of the patient and the method of ANCA testing may change throughout the years. In our studies, we had to implement several additional procedures to account for these limitations. Most importantly, we took the time between samples into consideration when defining the ANCA rise. In addition, samples were measured with both ANCA methods during the period in which the ANCA method was changed.

**Recommendations for future research**

Future studies should take the limitations that have been described above into account. Most importantly, our findings need to be replicated in a prospective multi-centre cohort study with sufficient power. Because serial ANCA measurements are performed in daily practice, population-based observational studies are ideal to further investigate the clinical usefulness of serial ANCA measurements. However, the quality of data in this kind of studies are often low, as relevant data are not obtained in a standardized and well-defined manner. In the following paragraphs, we will discuss how standardized data collection in daily practice may lead to high quality observational studies regarding the prediction of relapses. The results of these studies can be directly implemented in clinical practice using clinical decision support systems. In addition, we will discuss how patient data may be automatically evaluated using machine learning. The transformation of electronic medical record systems by standardized data collection, clinical decision support and machine learning may in the future lead to a new approach in predicting relapses in ANCA associated vasculitis.

**Standardized data collection**

Electronic medical record (EMR) systems have been highly debated since their introduction. This is most apparent after the rejection of a legislative proposal to implement a national EMR by the Senate on the 5th of April, 2011, due to concerns regarding the security and privacy protection (‘voorstel tot Wijziging van
de Wet gebruik burgerservicenummer in de zorg in verband met de elektronische informatieuitwisseling in de zorg). Nevertheless, hospitals have independently been working on local EMRs. In the current state, however, these EMRs offer insufficient possibilities to adapt the database to a specific patient group such as ANCA associated vasculitis. EMRs often only provide one text field in which the clinician can write his findings, which does not allow for automated processing and analysis of the patient data. Instead, patient data should be stored in a standardized manner and data should be well defined. Examples of patient input are clinical characteristics, such as the Birmingham Vasculitis Activity Score and the Vasculitis Damage Index, outcome registration such as relapses, adverse events (e.g. infections, malignancies and thrombosis) and patient reported outcomes such as the 36-item Short Form Health Survey.

Active involvement of the patient should also be encouraged, as it has been shown that his own assessment is useful in the prediction of disease reactivation.

The usefulness of standardized data collection has been shown by the advent of population-based observational studies. While randomized controlled trials are the cornerstone to investigate new interventions, observational studies are essential in the evaluation of interventions and diagnostic approaches that have already been introduced in clinical healthcare. Population-based observational studies offer large datasets unrivalled in size by randomized clinical trials that are capable to detect associations that were previously unidentified. In addition, they are capable of monitoring adverse events that rarely occur. These datasets make precision medicine possible, a strategy that takes individual variability into account with regards to the prevention and treatment of diseases. Oncology in particular has seen a lot of interest in precision medicine, due to insights regarding the clinical impact of biological characteristics of the malignancy that have become available in the -omics era (e.g. genomics, metabolomics and glycomics). Precision medicine may also be possible in the field of ANCA associated vasculitis, as we have shown that genomics and glycomics (see Chapter 5) provide useful information regarding the prediction of disease reactivation. While oncology is ahead in the implementation of standardized data collection, other specialties are picking up the pace. For instance, a workgroup statement has been issued regarding the optimization of administrative datasets to make registry-based observation studies
regarding acute kidney injury possible.\textsuperscript{272} The pivotal role of the integration of datasets has been appreciated and recommended in a consensus report for any form of biomedical research in the 21st-century to facilitate a paradigm shift towards human biology-based models.\textsuperscript{273} In the field of ANCA associated vasculitis, several attempts have been made that can be regarded as standardized data collection.\textsuperscript{121,274-276} Recently, a case-finding algorithm has been developed to accurately identify AAV patients in a large healthcare administrative database.\textsuperscript{275} While such an algorithm may in the future facilitate large observation studies, especially with regards to epidemiology, the possibilities are limited without standardized data collection.\textsuperscript{275}

Harmonization of definitions
Standardized data collection may also help alleviate differences in definitions that are currently being used. The development of classification schemes have led to better comparability between different cohorts of patients with ANCA associated vasculitis, as described in the introduction of this thesis.\textsuperscript{21} Nevertheless, important differences are present between cohorts that should be taken into account when interpreting the results of the different studies.\textsuperscript{150} Most importantly, the definitions used for a relapse as the endpoint of studies are controversial. For example, the RAVE trial defines the primary endpoint as a relapse based on a BVAS score >0 without the added requirement of a treatment change.\textsuperscript{151} This is in contrast to the MAINRITSAN trial, in which the primary endpoint is a major relapse and the secondary endpoint is a minor relapse, defined as a BVAS score >0 and, in addition, requiring mild treatment intensification.\textsuperscript{200}

Standardisation of ANCA detection
So far, we have only discussed the opportunities to standardize data collection and the harmonization of definitions with regards to the clinical characteristics of the patient. In addition, ANCA detection may also greatly benefit from the standardization of immunoassays for PR3- and MPO-ANCA.\textsuperscript{105,204,277} Currently, large differences are present between available antigen-specific ELISAs with regards to their reference values, cut-off values and performance.\textsuperscript{278-281} In a recent multi-centre study, the concordance between eight different immunoassays in controls, GPA and MPA was respectively 94\%, 89\% and 91\% for PR3-ANCA and respectively 94\%, 93\% and 77\% for MPO-ANCA.\textsuperscript{58} While these concordance rates are relatively high, they are not absolute. In addition, only the qualitative results of the different immunoassays were compared (e.g. positive versus negative) and not the quantitative
value (e.g. 10 or 100 arbitrary units). While the qualitative result is of interest for diagnostic purposes, the quantitative result is of importance during follow-up.\textsuperscript{141,282} The comparability of the quantitative results is lacking, as demonstrated by Trevisin et al.\textsuperscript{281} Trevisin analysed a PR3-ANCA positive serum standard, with a reported value of 101 U/ml, using 8 different commercial ELISAs and observed values ranging from 4 to 102 U/ml.\textsuperscript{281} The authors note differences in the used testing dilutions, methods of PR3 purification, binding properties of the ELISA plates, buffers used for coating and incubation, negative and positive controls and methods used to construct standard curves and calculate PR3-ANCA binding.\textsuperscript{281} Importantly, these methodological differences could be standardized to align test results between different ELISA-based immunoassays. Importantly, it was shown that this objective could be achieved by using a commutable reference material for calibration.\textsuperscript{283} Therefore, the Institute for Reference Materials and Measurements (IRMM), in collaboration with the Working Group Harmonisation of Autoantibody Tests (WG-HAT), developed a serum Certified Reference Material (CRM) that can be used as a common calibrant for immunoassays targeted against IgG MPO-ANCA.\textsuperscript{284} The standardisation of immunoassays is not only important to detect MPO-ANCA, but is also for other autoantibodies, which will be developed in the future.\textsuperscript{204,284}

The benefits from standardisation are highlighted by the success of the EU-supported EuroFlow Consortium, which has developed laboratory protocols and software tools to standardize flow cytometric immunophenotyping of leukocytes.\textsuperscript{285} The lack of standardisation of ANCA testing is evident in our studies, as the method of ANCA detection was replaced during follow-up and the results of the new method could not be compared with the old method (see Chapter 1). The impact of the standardisation of immunoassays on the predictive value of serial ANCA measurements for disease reactivation will be of great interest in the future.

Integration of data sources
Next to standardized data collection, the underlying database infrastructure should be unified across healthcare providers to allow for straightforward exchange of patient data upon patient’s consent to facilitate second opinions. Currently, the electronic record of a patient is printed at the secondary hospital and faxed to the tertiary hospital, which subsequently scans the paper records to add the data to their EMR. This process is highly inefficient, time-consuming and prone to mistakes.
The importance of integration between healthcare providers is most evident in the field of ANCA associated vasculitis since most patients are treated in expert centres after referral from primary or secondary healthcare. Seamless integration of patient data may in the future promote management collaboration between primary, secondary and tertiary healthcare providers. In such a collaboration, patients could be seen more often by their local specialist or even using any digital device at home, while expert centres continue to supervise the management plan to provide the most optimal healthcare. Integration of anonymized datasets for research purposes should not only be limited to national healthcare providers but should be done globally. This is essential in orphan diseases such as ANCA associated vasculitis.

The integration of datasets on a global level can be achieved by one universal database, or by the use of common templates and universal ontologies that allow for automated integration when future research questions are formulated. The first approach would face several difficulties to implement, which includes aligning the vision of collaborators, overcoming differences in cross-border legislation and assuring the publication rights of contributing collaborators. These obstacles are problematic in the entire field of medicine and are often discussed in the renowned *New England Journal of Medicine*. In the field of ANCA associated vasculitis, most research databases are currently maintained by individual expert centres, with recent efforts to expand to a national level. In this fragmented environment, with a limited number of patients and restricted financial possibilities, the latter approach may be more practical to implement.

Clinical decision support

Clinical decision support (CDS), also known as computer-assisted medical decision making or artificial assistance, utilizes computer generated data to assist the physician to provide optimal healthcare. Systems that apply CDS have become available after the introduction of EMR systems, which enable automated processing of patient data. While several definitions for CDS systems have been described, the term generally refer to "any electronic system designed to aid directly in clinical decision making, in which characteristics of individual patients are used to generate patient specific assessments or recommendations that are then presented to clinicians for consideration." Importantly, no medical decision is made independently by the system, the physician remains responsible for the decision. Interest in CDS systems was
sparked by the observation that about 28% of adverse drug events among hospitalized patients were preventable.\textsuperscript{297} A British study observed that 11% of admitted patients experience adverse events, of which 8% were fatal and 48% were retrospectively judged to be preventable.\textsuperscript{298} In the United States, physicians and hospitals are obliged to implement CDS in order to demonstrate meaningful use of EMR systems according to the 2009 Health Information Technology for Economic and Clinical Health (HITECH) Act. While the clinical rules that form the basis of CDS systems are available in clinical practice guidelines, the adoption and adherence to these guidelines are far from perfect.\textsuperscript{299,300} This is not surprising, since physicians would require at least 21.7 hours per day if they wish to adhere to these recommendations!\textsuperscript{301} Several barriers are present regarding the implementation and adherence thereof, which may in part be resolved by the use of CDS systems.\textsuperscript{299,302}

\textit{Examples of clinical decision support}

The implementation of CDS can range from simple calculations to adjust the medication dose based on the patient’s age or weight, providing likelihood ratios with test results,\textsuperscript{197} to complex algorithms that combine a multitude of patient characteristics.\textsuperscript{303} The principle can be implemented on clinical features to accurately diagnose pre-defined diseases, as has been proven in the case of sepsis\textsuperscript{303} and urticaria.\textsuperscript{304} Another well-known example of artificial assistance is the evaluation of newly prescribed medicine for potential drug-drug interactions with the patient’s current medication, which notifies the physicians when a potentially life-threatening interaction may occur.\textsuperscript{305,306} Artificial assistance has also been applied to prevent venous thromboembolism to correctly identify patients that require thromboprophylaxis.\textsuperscript{307,308} In a different approach, physicians were assisted in the decision whether blood transfusion was necessary based on haemoglobin values in order to significantly reduce inappropriate blood transfusions.\textsuperscript{309} A systematic review in 2012 described CDS systems in topics that addressed pharmacotherapy, diagnosis, chronic disease management, laboratory test ordering and interpretation,\textsuperscript{310} preventive care, immunizations and initiating discussions with patients.\textsuperscript{296} The review concluded that CDS systems improve health care process outcomes, e.g. adherence of physicians to local recommendations\textsuperscript{296}, and a slight reduction in morbidity was noted.\textsuperscript{296,311} No evidence was observed that suggests that CDS systems directly reduce mortality, although included studies were heterogenous in methodology and
underpowered to show improvements in this outcome, as this was not the primary outcome. Currently, efforts to implement CDS are often not well-regarded as physicians become alert fatigue by an overflow of alerts that are incorrect or of low clinical importance, a shortcoming that can be addressed in future iterations of CDS systems.296,311

Clinical decision support in ANCA associated vasculitis
Clinical management in patients with ANCA associated vasculitis may in particular benefit from clinical decision support systems due to the complexity of patient care and the low incidence of the disease. A simple implementation would be the standardisation and automation of induction protocols according to the published trials. Currently, each separate medication and dose is manually prescribed, which is both time-consuming and prone to mistakes. This is also the case with regard to the monitoring of side-effects during follow-up, as each laboratory test needs to be manually ordered. Automation of prescribing induction protocols could potentially reduce the risk of unnecessary exposure to immunosuppressive therapy and ensure that prophylaxis therapy is prescribed if applicable (e.g. osteoporosis prophylaxis during corticosteroid therapy313,314 and Pneumocystis pneumonia prophylaxis during cyclophosphamide therapy). Importantly, physicians will save time that was otherwise spent on manually prescribing and ordering standard protocols, so they can focus on the patient instead.

Diagnostic delay, a glaring problem despite the introduction of ANCA testing, may be challenged by implementing a CDS system which alerts physicians on the possibility of orphan diseases based on clinical features. For instance, in patients with bloody nasal discharge and persisting pulmonary infiltrates or nodules on x-ray despite appropriate antibiotic therapy, the physician may be advised to refer the patient to a centre with expertise in ANCA management, especially if surrogate markers for renal involvement are present. Such a system would mostly assist general practitioners who would generally only see an AAV patient once in their career, thereby alleviating disparities in healthcare outcomes based on the individual physicians’ knowledge.

In this thesis, we report findings that can be implemented as clinical decision support to assist physicians in the maintenance of remission in patients with ANCA associated vasculitis. In Chapter 1, we report an algorithm that alerts physician about the occurrence of an ANCA rise. The algorithm automatically calculates whether a fluctuation in ANCA values fulfils the definition of an ANCA rise.
The most optimal cut-off value of an ANCA rise is derived from an ROC curve on previous patient data to increase sensitivity and specificity for a relapse. In addition, the algorithm is capable of presenting the relapse risk based on clinical features at the time of the ANCA rise, as reported in Chapter 2. These risk factors are realtime updated based on clinical data of all included patients with available serial ANCA measurements in an anonymized manner. The system can be further extended by including other risk factors, such as patient characteristics at the time of diagnosis, genetics and previous therapeutic interventions. Whether the implementation of such a system decreases the occurrence of vasculitic damage and/or therapeutic complications and increases the quality of life of patients with ANCA associated vasculitis should be further investigated.

**Machine learning**

Standardized data collection in healthcare will lead to vast amounts of structured and well-defined data, which ushers in a new era of big data. However, big data will be of little use if we do not have advanced analytical tools that can translate the data to useful findings that can impact clinical healthcare. Due to recent advances in processing power of computers, machines are now capable of learning from large datasets, also known as machine learning. Using intricate statistical methods, machines are capable of deriving patterns and rules from these datasets, with which they can subsequently solve complex tasks with minimal human intervention. There are two approaches in machine learning: (1) supervised learning is possible in data that is labelled by a gold standard (e.g. a tumor is malignant or benign), and (2) unsupervised learning in data sets that are not labelled. These approaches have different purposes, wherein a supervised learning algorithm is capable of classifying and predicting new instances of data, while an unsupervised learning algorithm is capable of finding patterns in data that were previously unidentified.

Machine learning has become popular due to the successes it has achieved in mastering (video) games, such as the defeat of world chess champion Gary Kasparov in 1997 (*Deep Blue*, IBM), the mastering of classic Atari 2600 computer games (*Google Deepmind*) and the recent victories in Go (*AlphaGo*, Google Deepmind) and no-limit Texas hold'em poker (*Libratus*, Carnegie Mellon University). However, it has also been successful in the field of healthcare. Recently it has been shown that the prognosis of patients with non-small
cell lung cancer can be accurately predicted based on microscopic pathology image features that are processed by a machine learning algorithm.\textsuperscript{319} Importantly, the algorithm that was used in this study can also be applied to other specimens and diseases, provided that a sufficient number of digital images is available. In another setting, it is also capable of diagnosing skin cancer from a picture taken with a mobile phone with similar accuracy as expert dermatologists, who even had a slight edge because they were allowed to use dermatoscopes.\textsuperscript{320} Such an algorithm has also been applied to retinal fundus photographs to detect diabetic retinopathy and diabetic macular edema.\textsuperscript{321} Machines can also learn from radiology images\textsuperscript{322}, for example to diagnose and characterize thyroid nodules on ultrasound.\textsuperscript{323} Impressively, machines can learn from intracortically recorded signals to restore cortical control of functional movement in quadriplegia.\textsuperscript{324}

In ANCA associated vasculitis, machine learning can be implemented in various ways. First, it can be implemented to enhance pattern recognition in indirect immunofluorescence. Second, it can be utilized to diagnose patients on the basis of clinical characteristics, including serology, imaging and histology. In addition, such a system may better predict the prognosis on the basis of these characteristics as compared to conventional classification models. The main advantage of machine learning is the ability to evaluate an unprecedented amount of data that is impossible with conventional statistical methods. Therefore, a predictive model of an ANCA rise for a relapse that is created using conventional methods (see \textit{Chapter 1} and \textit{Chapter 2}) will in the future most likely be improved by a model that is created using machine learning.

However, current implementations of machine learning suffer from several limitations.\textsuperscript{325} For instance, current algorithms are not yet capable of survival analysis.\textsuperscript{325} It should also be emphasized that the effectiveness of machine learning is dependent on the quality of the data ("garbage in is garbage out"). Therefore, standardized data collection is of utmost importance. Moreover, machine learning can only be functional if it can learn from large datasets, which is difficult to obtain in a disease that seldom occurs. For instance, the algorithm that predicts the prognosis of non-small cell lung cancer required thousands of histopathology whole-slide images\textsuperscript{325}, while renal biopsy registries are limited to merely hundreds of cases of crescentic glomerulonephritis.\textsuperscript{118,131,326}
further emphasizes the need of integration of data on a global manner in orphan diseases such as ANCA associated vasculitis. Last, the rules and patterns that have been learned by machine learning appear as a black box to the physician. While machine learning may indeed predict more accurately the prognosis of a patient with non-small cell lung cancer, a physician and decision makers may want to know on what features the prognosis was made. These algorithms analyze images of histology in far greater detail than humanly possible, but at the same time the translation to the pathophysiology is lost, nor can it infer causality in the correlations it has observed. Therefore, machine learning will complement but not replace current research methods.

Toward a model that predicts disease activity

As described in the introduction, classification models have had different purposes throughout the years. The initial reports sought similarities in patients to make research regarding potential treatment options possible. After the successful introduction of cyclophosphamide, classification schemes focused on patient characteristics that stratified the need for immunosuppressive induction therapy according to the disease severity at the time of diagnosis. Due to the success of these classification schemes, ANCA associated vasculitis is no longer a fatal diagnosis and several treatment modalities with different side-effect profiles are available to effectively induce remission. This success has lead researchers to focus on the maintenance of remission, an aspect that was previously unimaginable. Researchers are now looking for strategies to prevent comorbidities from active disease while reducing treatment-related side-effects by personalizing maintenance therapy. Personalized maintenance therapy would entail increasing therapy in patients who are at risk of a relapse, while reducing therapy for those who are not. However, current classification schemes are of limited use to guide maintenance therapy because the current schemes are based on patient characteristics at the time of diagnosis and do not take follow-up into account. Therefore, in Chapter 3 we propose a new model that is focused on the biologic behavior of the disease that may in the future be used to guide maintenance therapy in patients with AAV.

Which factors should be included in such a prediction model? In Chapter 1 and Chapter 2, we investigate the role of serial ANCA measurements in such a model. In Chapter 4 and Chapter 5
we analyse whether other qualities of the autoantibody may contribute to the predictive value of serial ANCA measurements. While it is tempting to conclude that these factors should all be included in a future model, it should be emphasized that our findings need to be replicated in other cohorts. These studies should be of sufficient power to not only test the predictive value of the factors that we have identified, they may also identify new factors that were previously undetected. In addition, these studies require well-defined and high-quality data, which often cannot be extracted from retrospective data and should therefore be prospectively gathered. Prospective studies should include the systematic collection of samples (e.g. serum and urine samples, but also plasma dialysate during plasmapheresis and histologic samples) to make genomic, proteinomic and glycomic studies possible. The samples should be gathered according to standardized protocols at the time of active disease and during follow-up to study the onset of disease activity. Given the association with disease activity, ANCA rises may guide the collection of these samples. For instance, samples could be collected every (two) month(s) after an ANCA rise until the relapse occurs.

While reading the seminal papers in the field of ANCA associated vasculitis (see *A historical perspective*), the high quality of these investigations is evident by the level of detail of the description of patient characteristics and histopathologic features. Interestingly, even the patient initials were reported, which would not be accepted today due to privacy issues. The current classification schemes have led to greater homogeneity in patient cohorts, but as a consequence they have also led to a diminished attention to detail. In order to realize the full potential of personalized medicine, we will most likely have to establish a similar level of detail as the seminal papers. Not only should the clinical and histologic characteristics be included in greater detail, several other biomarkers could be included, which we will discuss hereafter.

**New targets for biomarkers**

In this thesis, several novel markers were investigated for their potential predictive value for disease reactivation. A biomarker should indicate a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In particular, we focused on several qualities of the autoantibody. Our studies have shown that these qualities offer important prognostic information and should be further
investigated, as extensively discussed in the appropriate chapters. Most importantly, a prospective study should analyse the combined predictive value of a panel of these qualities at the time of an ANCA rise. In addition, in vitro studies should be performed to demonstrate the pathogenic process of these qualities of the autoantibody. In vitro studies include the ability of ANCA to prime neutrophils, consecutively leading to respiratory burst, increased adhesion and injury to endothelial cells and subsequent migration of neutrophils, as well as the ability to induce neutrophil extracellular traps.\textsuperscript{329-334} Furthermore, our hypothesis that ANCA rises are associated with vasculitic and not granulomatous disease activity should be further investigated. A consensus classification of disease manifestations into “granulomatous” or “vasculitic” without the need of histologic evidence\textsuperscript{199} could be utilized to further substantiate our hypothesis. However, research regarding biomarkers that objectively measure disease activity should not only be limited to ANCA. In the next paragraphs, we will discuss potential biomarkers that could be of value to predict relapses in ANCA-associated vasculitis.

Assessing disease activity with a panel of protein markers

Using serum samples that were systematically obtained by the RAVE trial, a panel of 28 markers reflecting inflammation, injury and repair were tested to discriminate active disease from remission.\textsuperscript{206} The authors concluded that three biomarkers in particular were of interest for future studies: matrix metalloproteinase-3 (MMP-3), tissue inhibitor of metalloproteinases-1 (TIMP-1) and chemokine CXCL13 (BCA-1). These biomarkers did not only distinguish active vasculitis from remission, but they were also capable of differentiating remission from grumbling disease. In a different study, it was shown that MMP-3 levels are higher in patients with renal involvement compared to patients with non-renal disease.\textsuperscript{335} In addition, MMP-3 and TIMP-1 levels were higher in patients with AAV compared to healthy controls, reflecting increased systemic inflammation and enhanced vascular remodeling.\textsuperscript{336,337} Given the promising results of these biomarkers, they are merited as promising candidates for future studies in staging vasculitis activity and predicting the risk of relapse. Another marker that has shown significant potential to predict relapses is serum calprotectin (S100A8/A9).\textsuperscript{207,338} In 2013, Pepper et al. identified high levels of calprotectin at 1 and 6 months after
treatment as a highly predictive risk factor for a relapse (a positive likelihood ratio of 10.3 at month 1). More recently, using samples from the RAVE trial, it was shown that higher levels of calprotectin at 2 months after the induction therapy was predictive for a relapse in PR3-ANCA positive patients, but not in MPO-ANCA positive patients.

**Urinary biomarkers of renal vasculitis**

A multi-centre cohort study with systematic collection of samples will allow for the investigation of the predictive value of a complete panel of biomarkers in serum or urine samples. Such an investigation has already been performed in (juvenile) lupus nephritis, which revealed a good correlation of several urinary markers, such as monocyte chemoattractant protein 1 (MCP-1), kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL), with disease activity determined by a kidney biopsy.

This lead to the development of a novel renal activity index of lupus nephritis, which accurately identifies active lupus nephritis without the need of a biopsy. In ANCA associated vasculitis, a similar study has been performed with a limited number of patients. While these results are less convincing than the results in lupus nephritis, they warrant further investigation, especially regarding the discriminatory capacity of urinary MCP-1 to assess active disease. Using a different approach, a metabolomics profile that reflects renal disease activity was delineated by inducing MPO-ANCA associated vasculitis in Wistar-Kyoto rats and analysing urinary metabolites using nuclear magnetic resonance spectroscopy. This metabolomics profile, with a prominent role for the myo-inositol/citrate ratio, was then applied on a retrospective cohort of patients and accurately identified patients with active renal disease. The diagnostic accuracy of existing markers (CRP, eGFR and protein to creatinine ratio) was increased by adding the results of metabolomics profile (from 89.6% to 95.8%, respectively).

Another urinary marker that has recently received a lot of interest is soluble CD163. This marker, shed by monocytes and macrophages, is highly specific for active renal disease in AAV patients (positive likelihood ratio of 20.8) and has been confirmed in internal and external validation cohorts.

**Leukocyte subsets**

After the successful introduction of rituximab, B-cell subsets have received a lot of attention once they return after B-cell depletion. In particular, the return of B-cells with a low proportion of anti-inflammatory IL-10+ regulatory B cells, CD5+ regulatory B-cells and naïve B-
cells may predict a future relapse. The impact of the presence or absence of specific subsets of leukocytes is not only limited to B-cells. T-cells also play an important role, including CD8+ T-effector cells, CD4+ T-helper cells, IL-17-secreting T-cells, regulatory CD4+ T-cells and CD4+ effector memory T-cells, especially when expressing CD134 and GITR. Last, low-density granulocyte and hypogranular granulocyte subsets may also prove to be effective biomarkers to assess disease activity in patients with ANCA associated vasculitis.

Infections and vasculitis – in search for the second hit

Since the first description of cases with Granulomatosis with Polyangiitis, the aetiology of the disease was associated with infections. In fact, one of the first descriptions of ANCA were linked to a possible Ross River virus infection. Nasal carriage of staphylococcus aureus is a risk factor for a relapse and maintenance therapy with high-dose co-trimoxazole decreases the risk of a relapse. In animal studies, lipopolysaccharide aggravates MPO-ANCA induced vasculitis and is required for PR3-ANCA induced vasculitis. Immunisation of rats with bacterial protein from Staphylococcus ureus and Escherichia coli lead to a positive C-ANCA titer, but not PR3- or MPO-ANCA positivity, and some evidence of vasculitis, but not convincing. In a recent study, mice were immunized with a trypsin-like protease of Saccharomonospora viridis, which shares 30% homology with proteinase 3. PR3-ANCA positivity was induced, as well as granulomatous inflammation, although the latter can also be attributed to the use of complete Freund’s adjuvant. Also, a LAMP-2 epitope shares 100% homology with an amino-acid sequence from FimH, an adhesion molecule found in gram-negative pathogens such as Escherichia coli, Klebsiella pneumoniae and Proteus mirabilis. Rats immunized with FimH develop antibodies to LAMP-2 and subsequent necrotizing glomerulonephritis via a mechanism named molecular mimicry. In another model, PR3-ANCA but no signs of vasculitis were induced in mice immunized with recombinant complementary (anti-sense) PR3, a protein that may be derived from exogenous DNA or RNA brought in by pathogens. However, this mechanism has neither been completely elucidated nor confirmed. With regard to serial ANCA measurements, infections have been suggested as a potential second hit that creates a pro-inflammatory environment that enables the ANCA to become pathogenic. In the “second hit” hypothesis, ANCA-induced
(severe) disease reactivation is enabled by or aggravated by an as yet unidentified “second hit”. As described above, infections have been hinted as a potential candidate of a second hit, but this has never been fully elucidated, nor has it been thoroughly investigated. Therefore, signs of infections should be prospectively monitored during follow-up and the association with serial ANCA measurements and disease activity should be investigated. In addition, the impact of the pro-inflammatory stimulus of an influenza vaccination could also be of interest to investigate in relation to the presence of ANCA. While an influenza vaccination does not increase relapse rate\cite{143}, it has previously been shown that it leads to a pro-inflammatory glycosylation profile of anti-vaccine IgG but not of total IgG.\cite{363} Whether a potential spill-over effect occurs in antibodies targeted against MPO or PR3 could be further investigated.

Research regarding infections and active vasculitis is not only warranted in order to understand the role of infections as an aggravator of vasculitis, it is also important in order to better differentiate symptoms caused by infections from symptoms caused by active vasculitis. This differentiation can in particular be difficult during induction immunosuppressive therapy, in which patients are prone to infections and conventional diagnostic methods may sometimes be inconclusive. Procalcitonin has been investigated as a potential marker to differentiate infections from active disease.\cite{364} In this study, the receiver operating characteristics curve for procalcitonin to differentiate AAV patients with an intercurrent bacterial infection (n=10) from AAV patients with a relapse (n=36) was 0.75 [95% CI 0.55-0.94], slightly higher than C-reactive protein (0.67 [95% CI 0.46-0.88]).\cite{364} Whether other markers are valuable in differentiating infection from active vasculitis should be prospectively studied.

**Fine-tuning maintenance therapy**

A prediction model of disease activity, as proposed above, is only of value if the results have clinical consequences. Therefore, future prospective studies should investigate the ability of such a prediction model to guide maintenance therapy. While mortality and the occurrence of (major) relapses should be the primary research question, secondary questions should include the prevention of vasculitic or treatment-related damage, a reduction of cumulative treatment exposure and quality of life indicators, as well as the economic impact in terms of hospitalization rate and a possible cost reduction.
Several strategies of maintenance therapy guided by ANCA rises have been investigated, as described in Chapter 3. Most importantly, cyclophosphamide at the time of an ANCA rise has proven to be effective in the prevention of relapses, but this strategy is heavily debated because cyclophosphamide is a potentially harmful drug.\textsuperscript{74,365} Lengthening the duration of azathioprine therapy based on the ANCA titer at the time that induction therapy is switched to maintenance therapy does not seem to protect against relapses, nor does it lower the severity of relapses that occur.\textsuperscript{176} Pre-emptive treatment at the time of an ANCA rise with azathioprine is not sufficient (unpublished data).\textsuperscript{365} Strategies that guide corticosteroid therapy based on serial ANCA measurements require further investigation.\textsuperscript{178} With the advent of rituximab therapy\textsuperscript{46,151,192,200}, novel strategies are investigated to guide rituximab maintenance therapy. In particular, the timing of repeating Rituximab cycles is at present unknown and is being investigated in the MAINRITSAN2 trial (ClinicalTrials.gov Identifier: NCT01731561), in which individually timed retreatment based on ANCA rise or B cell reconstitution is compared with fixed interval dosing of Rituximab. Future studies that utilize advanced prediction models to guide maintenance therapy will in the future harbour exciting results that will further reduce the burden of the disease in patients with ANCA associated vasculitis.

In summary, we have shown the predictive value of several biomarkers in this thesis. We propose a new prediction model that is focused on the biologic behavior of the disease that may in the future be used to guide maintenance therapy in patients with AAV. An effective method to achieve this objective is using data obtained from clinical healthcare once high-quality data is ensured by standardized data collection. Several markers have been proposed that may be of added value to accurately predict disease activity, which should also be analysed according to standardized protocols. Promising markers include a panel of protein markers, urinary markers, serial ANCA measurements, including characteristics of ANCA, as well as an increased vigilance for infections as a potential second hit. Future research regarding these biomarkers will potentially lead to a prediction model of disease activity that can be used to guide maintenance therapy to reduce the morbidity caused by treatment-related and vasculitic damage. Clinical decision support will ensure that novel findings will be successfully implemented in clinical healthcare. Last, machine learning could in the future be introduced to further improve the accuracy of the model.
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Het afweersysteem

Het afweersysteem beschermt de mens tegen de gevaren van de buitenwereld. Zo werken de verschillende cellen van dit systeem nauw samen om schadelijke bacteriën en virussen te vernietigen. Daarnaast controleert het afweersysteem, ook wel het immuun systeem genoemd, of onze eigen cellen niet ongeremd vermenigvuldigen om kanker te voorkomen. Het afweersysteem is niet alleen in staat om ons te beschermen tegen nieuwe infecties, het kan ook een herinfectie goed voorkomen. Dit doet het door eiwitten genaamd “antilichamen” te maken tegen specifieke herkenningspunten van deze bedreigingen, zogenaamde “antigenen”. Deze antilichamen circuleren rond in ons bloed en plakken aan de antigenen bij een herinfecctie, waardoor het afweersysteem direct in actie komt. Dit onderdeel van het immuun systeem wordt onder andere gebruikt om toekomstige infecties te voorkomen door middel van vaccinaties. Bij een vaccinatie worden antigenen van een virus of bacterie aangeboden aan het afweersysteem, waarop het antilichamen aanmaakt die een toekomstige infectie ongevaarlijk maken.

Vasculitis – ontstekingen van de vaten

Ontsteking aan de vaten ("vasculitis" in het Latijn) werd al in de achttiende eeuw opgemerkt bij patiënten die met een grote verscheidenheid aan klachten bij de dokter kwamen. Deze klachten verergerden na verloop van tijd en vrijwel allen overleden binnen een jaar. Destijds werden bij de eerste onderzoeken afwijkingen van de grote vaten gezien met het blote oog. De vaten waren hierbij op verschillende plaatsen verwijd en verlittekend. Later zagen onderzoekers met behulp van een microscoop dat ook de kleine bloedvaten aangetast waren door een actieve ontsteking. Doordat de kleine vaten overal in het lichaam aanwezig zijn, kan het allerlei klachten veroorzaken – van de huid, de neus en de ogen tot en met de longen, de zenuwen en de nieren. Veel patiënten overleden uiteindelijk aan het uitvallen van dit laatste orgaan, aangezien de nierfunctie vervangende behandelingen nog niet beschikbaar waren.

Midden in de negentiende eeuw werd duidelijk dat afweerremmende medicijnen verbetering gaven voor deze patiënten. Deze medicatie wordt ook wel immunosuppressiva genoemd. Dit was destijds een grote doorbraak aangezien patiënten niet meer stierven door deze aandoening. De patiënten hadden na behandeling geen klachten meer en zijn dan "in remissie". De afweerremmende medicatie, zoals cyclofosfamide en prednison, zijn echter niet zonder bijwerkingen. Doordat deze medicijnen het afweersysteem onderdrukken kunnen de patiënten zich minder goed beschermen tegen infecties. Daarnaast ontwikkelden patiënten na verloop van tijd vaker kanker bij langdurig gebruik. Volledig afbouwen van de medicatie is echter ook niet zonder risico’s, aangezien de aandoening vervolgens weer terugkomt in een groot gedeelte van de patiënten. Meerdere termen worden gebruikt voor de terugkeer van de ziekte, zoals een "relapse", een "flare", of een "chute". De voor- en nadelen van de behandeling moeten dus tegen elkaar worden afgewogen. De perfecte balans is echter tot op heden niet duidelijk.

In de jaren zestig werd een tweede belangrijke ontdekking gedaan. Destijds werden er auto-antilichamen ontdekt die vastplakten op neutrofielen, lichaamseigen cellen van de mens die onderdeel zijn van ons afweersysteem. Deze autoantilichamen werden alleen gevonden in het bloed van patiënten met vasculitis van de allerkleinste vaten en niet in het bloed van gezonde mensen. Onder de microscoop was te zien dat deze auto-antilichamen vastplakten binnen de cel van de neutrofiel, in het zogenaamde cytoplasma (zie Figure 5-6). Na deze bevinding werden de gevonden auto-
antilichamen dan ook omgedoopt tot ‘anti-neutrofiel cytoplasmatische antilichamen’, afgekort ANCA. De term ANCA geassocieerde vasculitis wordt vervolgens gebruikt bij patiënten met ontstekingen rondom de kleine vaten bij wie deze auto-antilichamen gedetecteerd worden. Verder onderzoek liet zien dat deze antilichamen specifiek zijn gericht op de eiwitten proteinase 3 (PR3) en myeloperoxidase (MPO). In een klein gedeeltje van de patiënten worden deze auto-antistoffen niet aangetroffen maar is er wel sprake van vasculitis in de kleine vaten. De ziekte in deze ANCA negatieve patiënten is meestal gelimiteerd tot de neus en longen, terwijl vrijwel alle patiënten met betrokkenheid van de nieren ANCA positief zijn.

**Figure 5-6.** Auto-antilichamen gericht op de neutrofiel kunnen zichtbaar gemaakt worden onder de microscoop (licht groene kleur). Aan de linkerkant is te zien hoe ze vastplakken rondom de celkern, terwijl ze aan de rechterkant vastplakken in het cytoplasma.

Wat betekenen deze auto-antilichamen? Onderzoekers hebben aangetoond dat het toedienen van ANCA in muizen leidt tot vasculitis in de nieren en longen. Dit onderzoek laat zien dat de auto-antilichamen gevaarlijk kunnen zijn in muizen, hoe zit dit echter in de mens? In het bloed van patiënten met ANCA geassocieerde vasculitis werd gezien dat de ANCA in hoge mate aanwezig waren op het moment dat de ziekte voor het eerst tot uiting kwam (bij diagnose). De hoeveelheid ANCA nam na de behandeling met afweerremmende medicatie af en verdween zelfs volledig in een deel van de patiënten. Bij sommige patiënten kwamen echter de ANCA weer terug in het bloed. Hoewel deze patiënten op het moment van zo’n ANCA stijging klachtenvrij waren,
kregen zij na verloop van tijd weer terugkeer van de klachten. Zo’n ANCA stijging werd dus gezien als een alarmsignaal voor een toekomstige relapse (zie Figure 5-7). Op basis van bovenstaande bevindingen werd daarom beredeneerd dat de afweerremmende medicatie gegeven kon worden op geleide van de ANCA waarden. Zo zou de medicatie volledig afgebouwd kunnen worden bij patiënten waarbij de ANCA niet meer detecteerbaar is en zou de medicatie voortijdig opgehoogd kunnen worden bij een ANCA stijging. Het verband tussen een stijging van de ANCA waarde en terugkeer van de ziekte is echter niet absoluut, aangezien niet elke patiënt terugkeer van de ziekte krijgt na een ANCA stijging en de ANCA niet in elke patiënt volledig verdwijnt wanneer zij langdurig geen klachten meer hebben. Tot op heden wordt daarom niet geadviseerd om de medicatie aan te passen op basis van de ANCA waarden.

**Dit proefschrift**

Het doel van dit proefschrift is om de terugkeer van ziekte te voorspellen in patiënten met ANCA geassocieerde vasculitis die in remissie zijn. De relatie tussen de ANCA waarden in het bloed en de ziekte activiteit speelt hierbij een centrale rol. Waarom leidt een ANCA stijging in de ene patiënt wel tot terugkeer van de ziekte, maar niet in een ander? Kunnen wij op het moment van zo’n ANCA stijging voorspellen wie wel een relapse krijgt en wie niet? Indien wij nauwkeurig kunnen voorspellen wie een relapse krijgt, kunnen wij namelijk preventief beginnen met het ophogen van afweerremmende medicatie om de terugkeer van de ziekte te voorkomen.
Figure 5-7. Een voorbeeld van het beloop van een denkbeeldige patiënt met ANCA geassocieerde vasculitis. Bij het vaststellen van de ziekte wordt afweerremmende medicatie gestart, waarna de klachten van de patiënt verdwijnen. Vanwege de bijwerkingen van de medicatie wordt de medicatie langzaam afgebouwd. Echter, sommige patiënten krijgen daarna weer terugkeer van de klachten. De ANCA waarde is bij diagnose hoog en neemt af na het starten van de medicatie. In sommige patiënten stijgt de ANCA waarde voordat de klachten terugkomen. Indien dit het geval is, kan medicatie preventief gestart worden om een toekomstige relapse te voorkomen.

Om dit doel te bereiken, onderzochten wij alle patiënten met ANCA geassocieerde vasculitis die behandeld werden bij de afdeling immunologie van het Maastricht University Medical Center. Dit centrum is gespecialiseerd in zeldzame aandoeningen van het afweersysteem en er worden daardoor veel patiënten met vasculitis doorverwezen door andere specialisten uit de zuidelijke regio van Nederland.

In de periode van januari 2000 tot en met november 2011 werden 166 patiënten behandeld voor ANCA geassocieerde vasculitis in Maastricht die na behandeling in remissie kwamen. Van deze 166 patiënten hadden er 104 patiënten ook betrokkenheid van de nieren in het verleden. In het beloop van de studie zijn 7 patiënten komen te overlijden, voornamelijk aan de gevolgen van hart- en vaatziekten, kanker of infectie. Bij 74 patiënten vond er een relapse plaats tijdens het beloop van de studie, wat inhoud dat de kans op een relapse gemiddeld 17% per jaar is. In 26 gevallen was de relapse ernstig en levensbedreigend, bijvoorbeeld doordat de nieren...
betrokken waren. In de overige 48 relapses waren de klachten mild, bijvoorbeeld doordat de klachten gelimiteerd waren tot de gewrichten, de neus of de longen.

Tijdens de studie verdween de ANCA in 116 patiënten. In totaal werd een ANCA stijging gezien in 89 patiënten. Een belangrijke bevinding is dat geen van onze patiënten die ANCA negatief bleven een ernstige relapse kregen tijdens onze studie. In andere woorden, als de ANCA verdwijnt, dan is de kans op een levensbedreigende terugkeer van de ziekte minimaal. Tweeënvijftig van de 89 patiënten met een ANCA stijging kregen uiteindelijk terugkeer van de ziekte (58.4%). Echter, deze relapse vond slechts in 35 patiënten (39.5%) plaats binnen een half jaar na de ANCA stijging.

Wij hadden het vermoeden dat een ANCA stijging wel gerelateerd was met terugkeer van de ziekte in patiënten met nierbetrokkenheid, maar niet in patiënten waarbij dit niet het geval was (Chapter 1). Bij de ziekte worden namelijk twee verschillende beelden gezien onder de microscoop: ten eerste ontsteking van de vaten (vasculitis) en ten tweede een opeenstapeling van ontstekingscellen. Dit laatste wordt ook wel granulomateuze ontsteking genoemd (zie Figure 5-8). In de nieren is vrijwel altijd sprake van vasculitis en maar zelden van granulomateuze ontsteking. Vasculitis wordt ook gezien bij andere levensbedreigende uitingen van de ziekte, zoals bloedingen in de longen en zenuwbetrokkenheid. Granulomateuze ziekte is vaak zichtbaar in de neus, de luchtpijp en als knobbels in de longen. Over het algemeen zijn uitingen van de granulomateuze ziekte minder ernstig, maar het kan nog steeds levensbedreigend zijn. Bij patiënten waarin vasculitis wordt gezien onder de microscoop, wordt bijna altijd een ANCA waarde in het bloed gevonden. Dit in tegenstelling tot patiënten met enkel granulomateuze ontsteking, waarbij net iets meer dan de helft ANCA positief zijn. In muizen zijn onderzoekers enkel in staat om vasculitis uit te lokken na de toediening van ANCA, terwijl granulomateuze ontsteking zelden wordt gezien.

Om ons vermoeden te bevestigen, verdeelden wij onze groep patiënten in twee groepen: een groep met nierbetrokkenheid en een groep zonder nierbetrokkenheid. Om de relatie tussen een ANCA stijging en een relapse te meten, maakten wij gebruik van een wiskundige analyse. Deze analyse geeft een waarde aan het verband tussen een ANCA stijging en een relapse, genaamd de hazard ratio (HR). Hoe hoger de HR, hoe groter de kans op een relapse na de ANCA stijging, terwijl een HR van 1
aangeeft dat de kans op een relapse niet veranderd. In de gehele groep van patiënten is de hazard ratio 5.8. De kans op een relapse is dus 5.8 keer groter nadat een ANCA stijging heeft plaatsgevonden. In de groep van patiënten met nierbetrokkkenheid is de hazard ratio 11.1, terwijl deze slechts 2.8 is in de groep met patiënten zonder nierbetrokkkenheid. Met andere woorden, de kans op een relapse is sterk verhoogd na een ANCA stijging in patiënten wanneer de nieren betrokken zijn. Dit in tegenstelling tot patiënten met gelimiteerde ziekte, waarbij een ANCA stijging weinig vertelt over een toekomstige relapse.

**Figure 5-8.** Twee verschillende beelden onder de microscoop. Aan de linkerkant is een biopt getoond van een nier van een patiënt met ANCA geassocieerde vasculitis. Door de uitgebreide ontsteking in de vaten is het nierfilter volledig verwoest. Aan de rechterkant is een voorbeeld van een granuloom te zien. Dit granuloom bestaat uit immuuncellen die bij een langdurige ontsteking opeenhopen.

Na de bevestiging dat de ANCA waarden gerelateerd zijn met ziekteactiviteit in patiënten met nierbetrokkkenheid en niet in andere patiënten, gingen wij op zoek naar andere factoren die voorspellend zijn voor een relapse ten tijde van een ANCA stijging (*Chapter 2*). Om dit te onderzoeken, analyseerden wij alle patiënten met nierbetrokkkenheid en een ANCA stijging. Dit waren 60 patiënten in totaal, waarvan 36 patiënten een relapse kregen na verloop van tijd (60%). Zestien patiënten daarvan hadden een relapse binnen een half jaar (31.7%). In totaal vonden wij drie risicofactoren voor een relapse ten tijde van een ANCA stijging. Ten eerste hebben patiënten die behandeld zijn met milde afweerremmende medicatie een grotere kans op een relapse vergeleken met patiënten die
behandeld werden met zwaardere medicatie, zoals cyclofosfamide en rituximab. Ten tweede hebben patiënten een hogere kans op een relapse wanneer de ANCA door blijft stijgen na de eerste ANCA stijging. Patiënten waarbij de ANCA waarde weer daalt of stabiel blijft na de ANCA stijging, krijgen dus minder snel een relapse. Als laatste vonden wij dat een ANCA stijging tijdens de herfst vaker wordt gevolgd door een relapse dan tijdens de andere seizoenen. Wij vermoeden dat dit komt doordat er in de periode na de herfst vaker een infectie plaatsvindt ten opzichte van de lente en de zomer. Wij denken dat een infectie ervoor zorgt dat het afweersysteem geactiveerd wordt en zo de ANCA in staat stelt om schade te doen. Bovendien daalt de vitamine D waarde in de herfst en winter, een vitamine die wordt aangemaakt in de huid bij zonlicht en een belangrijke sturende werking heeft op het afweersysteem.

Slechts 16 van de 60 patiënten hadden (31.7%) een relapse binnen een half jaar na een ANCA stijging. Van alle patiënten die een ANCA stijging in de herfst hadden, hadden 9 van de 13 patiënten een relapse (69.2%). De kans voor een relapse binnen een half jaar is nog groter wanneer de ANCA waarde blijft stijgen na de ANCA stijging (77.8%). In patiënten met deze risicofactoren is de kans op een relapse zo groot, dat de voordelen van preventieve ophoging van afweerremmende medicatie mogelijk opweken ten opzichte van de nadelen.

In een uitgebreide analyse van alle voorgaande onderzoeken naar de relatie tussen een ANCA stijging en een relapse (Chapter 3), concluderen wij dat er ruimte is voor een behandeling op basis van de ANCA waarden in een specifieke groep van patiënten bij specifieke omstandigheden. Wij stellen voor dat patiënten ingedeeld behoren te worden op basis van risicofactoren voor een relapse. De afweerremmende medicatie kan op basis van deze indeling gedoseerd worden. Zo kan de medicatie gepersonaliseerd worden naar elke individuele patiënt. De risicofactoren die wij hebben gevonden (Chapter 1 en Chapter 2), kunnen hierbij een rol spelen. Deze factoren moeten echter ook door andere onderzoekers onderzocht worden om onze bevindingen te bevestigen. Daarnaast verwachten wij dat een aantal andere factoren ook een rol kunnen spelen. Een aantal factoren hebben wij in de volgende hoofdstukken onderzocht.

Wij hadden het vermoeden dat niet elke auto-antilichaam hetzelfde is als het ander. Zo verwachten wij dat bepaalde ANCA minder snel leiden tot een relapse dan andere ANCA. Auto-antilichamen kunnen onderscheiden worden op bepaalde kwaliteiten. Een
van die kwaliteiten is de mate waarop de auto-antilichaaam blijft vastplakken aan het antigeen. Waarschijnlijk zal een ANCA die beter vastplakt vaker leiden tot een relapse dan een ANCA die nauwelijks vastplakt. De sterkte van deze binding wordt ook wel de aviditeit genoemd; hoe hoger de aviditeit, hoe sterker het auto-antilichaaam vastplakt aan het antigeen. Om de invloed van de aviditeit te onderzoeken, analyseerde wij de aviditeit van PR3-ANCA in 30 patiënten met een ANCA stijging (Chapter 4). Wij konden niet bevestigen dat ANCA met een hoge aviditeit vaker leidde tot een relapse dan ANCA die minder goed vastplakten. Wij vonden echter wel dat de aviditeit van ANCA na verloop van tijd hoger werd in patiënten met nierbetrokkenheid die een relapse kregen. Dit was niet het geval in patiënten zonder nierbetrokkenheid met een relapse. Ook in patiënten met nierbetrokkenheid die in remissie bleven, bleef de aviditeit stabiel.


Om de invloed van het glycosylatie profiel op de kans van een relapse te bepalen, analyseerden wij het profiel in alle PR3-ANCA positieve patiënten met een ANCA stijging (Chapter 5). Uit de resultaten van de analyse bleek dat het glycosylatie profiel inderdaad voorspellend is voor een relapse. Op het moment van de ANCA stijging hebben patiënten met weinig galactose of siaal groepen aan de antilichamen een hogere kans op terugkeer van de ziekte. Met andere woorden, patiënten met antilichamen met veel galactose of siaal groepen aan de antilichamen zijn beschermd tegen een relapse. Na de ANCA stijging neemt de hoeveelheid galactose en siaal groepen verder af in patiënten die een relapse krijgen en niet in patiënten die in remissie blijven. Deze bevinding ondersteunt
onze conclusie dat het glycosylatie profiel voorspellend is voor een relapse. In de toekomst kan deze analyse gebruikt worden om de behandeling aan te passen aan het risico op een relapse.


Conclusie en discussie

In dit proefschrift hebben wij aangetoond dat een ANCA stijging voorspellend is voor een relapse in patiënten met nierbetrokkenheid maar niet in patiënten zonder betrokkenheid van de nieren. In patiënten met nierbetrokkenheid is de kans op een relapse 11 keer groter nadat een ANCA stijging heeft plaatsgevonden. Daarnaast is de kans op een relapse 11 keer groter nadat een ANCA stijging heeft plaatsgevonden. Drie risico factoren voor een relapse op het moment van een ANCA stijging werden geïdentificeerd: (1) voorgaande behandeling met milde afweerremmende medicatie, (2) wanneer de ANCA blijft doorstijgen na de initiele ANCA stijging en (3) wanneer de ANCA stijging plaatsvindt tijdens de herfst.

Daarnaast onderzochten wij de invloed van de eigenschappen van het antilichaam. De kracht van de binding tussen het antilichaam en het antigeen neemt toe in patiënten met nierbetrokkenheid die een relapse krijgen, maar niet in andere subgroepen. Een andere eigenschap van het antilichaam is het glycosylatie profiel. Uit ons onderzoek bleek dat patiënten met weinig galactose of siaal groepen aan antilichamen meer risico lopen op een relapse ten tijde van een ANCA stijging.
Wij concluderen uit ons onderzoek dat er voldoende mogelijkheden zijn om ziekte activiteit te voorspellen in patiënten met ANCA geassocieerde vasculitis. Wij stellen een model voor die de kans op een relapse voorspelt ten tijde van remissie. Dit model geeft op basis van de eigenschappen van de patiënt de kans aan op een relapse. Middels het voorgestelde model kan de afweerremmende medicatie gepersonaliseerd worden voor elke individuele patiënt. Zo kan de medicatie opgehoogd worden in patiënten met een hoog risico, om toekomstige ziekte activiteit te voorkomen. In patiënten met een lage kans op ziekte activiteit zou de medicatie juist gestaakt kunnen worden, om bijwerkingen van de medicatie te voorkomen.

Uit ons onderzoek zijn meerdere risicofactoren geïdentificeerd die opgenomen kunnen worden in dit model. Echter, onze resultaten zijn gebaseerd op de patiënten die behandeld zijn in Maastricht. Onze bevindingen dienen getoetst te worden in andere onderzoeksgroepen om onze conclusies te bevestigen.

Wij verwachten dat het voorgestelde model het beste gerealiseerd kan worden door een grote onderzoekspopulatie te analyseren. Dit kan mogelijk gemaakt worden door gebruik te maken van de data van alle patiënten die klinisch behandeld worden. Door data op gestandardiseerde wijze te verzamelen zijn complexe wiskundige methoden mogelijk, zoals het gebruik van kunstmatige intelligentie. Door de rekenkracht van de huidige computers te gebruiken, kan de arts in staat gesteld worden om de behandeling te personaliseren voor elke individuele patiënt. ANCA geassocieerde vasculitis komt zelden voor, het is daarom van cruciaal belang dat er op internationaal gebied samengewerkt wordt om dit doel te bereiken.
Valorisation addendum

Relevantie

ANCA geassocieerde vasculitis komt zelden voor en is door het European Medicines Agency (EMA) aangewezen als weesziekte. Per definitie is onderzoek in ANCA geassocieerde vasculitis daardoor gelimiteerd om kennis beschikbaar te maken voor maatschappelijke benutting ten opzichte van onderzoek in meer prevalentie aandoeningen. Het klein aantal patiënten met de aandoening leidt ertoe dat er een minimale financiële prikkel is om onderzoek in ANCA geassocieerde vasculitis mogelijk te maken. Het onderzoek is echter niet minder relevant, aangezien het een grote impact kan hebben voor de specifieke doelgroep. Een overzicht van het onderzoek naar ANCA geassocieerde vasculitis van de afgelopen eeuw bevestigd de aanzienlijke impact, zoals beschreven in de introductie. Aan het begin van de 20ste eeuw overleed elke patiënt binnen een jaar na het vaststellen van de diagnose, terwijl de aandoening tegenwoordig goed behandeld kan worden. Ook het onderzoek van de laatste decennia heeft een grote impact gehad op de mortaliteit, zoals geconstateerd uit een cohortonderzoek van Maastricht University. Uit dit onderzoek is gebleken dat patiënten behandeld in het eerste decennium van de 21ste eeuw 4 keer minder vaak kwamen te overlijden dan patiënten behandeld in de tachtiger jaren.

De aandoening wordt tegenwoordig gezien als een chronische ziekte in plaats van een fatale diagnose. Toekomstig onderzoek richt zich daarom voornamelijk op de lange termijn. Een belangrijk aandachtspunt is de optimale onderhoudsbehandeling om terugkeer van de ziekte te voorkomen en tegelijkertijd de kans op bijwerkingen door de medicatie te minimaliseren. Het onderzoek beschreven in dit proefschrift sluit goed aan bij de onderzoeksagenda’s opgesteld door internationale verbanden zoals de European Vasculitis Society (EUVAS) en de OMERACT Vasculitis Working Group.

Een belangrijk onderdeel van de valorisatie traject is de minimale kosten van het onderzoek dat is beschreven in dit proefschrift. Dit onderzoek is tot stand gekomen door de klinische data van patiënten die reeds behandeld werden in het academisch ziekenhuis in Maastricht op structurele wijze te verzamelen om statistische analyses mogelijk te
Valorisatie addendum

maken. Dit onderzoek is verwezenlijkt zonder structurele, noch substantiële financiering en heeft desondanks meerdere wetenschappelijke publicaties geproduceerd in vooraanstaande medische vakbladen.

**Innovatie**

Het onderzoek uit dit proefschrift is innoverend in meerdere opzichten. In *Chapter 1* werden nieuwe inzichten gegeven in de vraag of het systematisch meten van de ANCA waarden na de diagnose van ANCA geassocieerde vasculitis voorspellend is voor ziekte activiteit. Deze onderzoeksvraag is sinds de introductie van ANCA relevant is gebleven, zoals uiteengezet in *Chapter 3*. De vraagstelling werd op een wiskundige manier benaderd om rekening te houden met zowel de stijging van de ANCA waarde als de tijd tussen de ANCA bepalingen. Het onderzoek heeft nieuwe inzichten gegeven in de relevantie van serieele ANCA bepalingen door het verband met bepaalde histologische beelden te leggen. Het resultaat draagt hierdoor bij aan het paradigma dat het verschil tussen granulomateuze en vasculitis activiteit relevant is, ook al ligt de nadruk de laatste jaren voornamelijk op het verschil in de serologische klassificatie tussen PR3- en MPO-ANCA. Dit onderzoek heeft tevens laten zien dat er geen toegevoegde waarde is om de ANCA te bepalen met zowel de antigeen specifieke ELISA’s als de indirecte immunofluorescentie, waardoor onnodige kosten bespaard kan worden.

Tot dusver werd alleen gekeken naar de eerste ANCA stijging, zonder rekening te houden met de veranderingen na de ANCA stijging. In *Chapter 2* onderzoeken wij als eerste onderzoeksgroep wat risicofactoren voor terugkeer van de ziekte zijn op het moment van de ANCA stijging. Een verdere stijging van de ANCA waarde zorgt voor een verhoogde kans op ziekte activiteit, daarnaast wordt er een associatie gevonden met seizoensgebondenheid. We leveren hiermee een belangrijke bijdrage aan het paradigma dat een tweede ‘hit’ nodig is, welke waarschijnlijk seizoensgebonden is. Dit onderzoek laat zien dat het mogelijk is om een voorspellend model te ontwikkelen om terugkeer van de ziekte te voorspellen.

Na het beschrijven en het analyseren van het cohort van patiënten met ANCA geassocieerde vasculitis, zijn de daaropvolgende onderzoeken gericht op het identificeren van nieuwe biomarkers die voorspellend zijn voor een relapse op het moment van de ANCA stijging. In *Chapter 4* onderzochten wij het beloop van de bindingskracht van het antilichaam
tijdens de followup. Hoewel de aviditeit van het antilichaam op theoretische gronden van belang kan zijn, zijn tot op heden nog weinig indicaties om de aviditeit te bepalen in een klinische setting. De aviditeit van PR3-ANCA stijgt in patiënten met renale betrokkenheid waarbij de ziekte in de toekomst terugkeert. In Chapter 5 onderzochten wij de voorspellende waarde van het glycosylatie profiel van PR3-ANCA voor een relapse. Het onderzoek naar het glycosylatie profiel is een veelbelovend onderzoeksved, ook wel glycomics genoemd. Tot nu toe zijn er echter enkel associaties gevonden met bepaalde aandoeningen, zoals de ernst van de ziekte activiteit in reumatoïde arthritis. Ons onderzoek heeft aangetoond dat het glycosylatie profiel ten tijde van een PR3-ANCA stijging voorspellend is voor de terugkeer van de ziekte in ANCA geassocieerde vasculitis. Deze bevindingen tonen voor het eerst de klinische relevantie aan van het bepalen van het glycosylatie profiel.

Planning en realisatie

Onze bevindingen dienen bevestigd te worden in andere cohorten voordat ze toegepast kunnen worden in de kliniek. De risicofactoren die zijn geïdentificeerd in onze studies dienen in een prospectieve studie onderzocht worden in een cohort met een groot aantal patiënten. Om dit te verwezenlijken is een database intern ontwikkeld waarmee de klinische data op systematische en gestandaardiseerde wijze verzameld kan worden om automatische analyse mogelijk te maken. Om een voldoende aantal patiënten te analyseren dient daarnaast samengewerkt te worden in een internationaal verband om genoeg power te hebben om het individuele effect van alle risicofactoren te bepalen. Op basis van de resultant van zo’n toekomstige studie kan een predictie model ontwikkeld worden toekomstige ziekte activiteit accuraat te voorspellen. Wanneer dit predictie model is ontwikkeld, kan vervolgens onderzocht worden of het geleiden van de onderhoudsbehandeling op basis van dit model leidt tot het voorkomen van zowel toekomstige ziekteactiviteit als vasculitis schade als bijwerkingen van de medicatie. Daarnaast dient in een toekomstige studie onderzocht te worden of zo’n strategie leidt tot een verbetering van de kwaliteit van leven.
Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.

**The road not taken**
Robert Frost (1874 – 1963)
Dankwoord

Dit boekje is het resultaat van een consortium filantropisten die mijn naïeve ambitie hebben geleid in een turbulente omgeving met bodemloze potentie. Waar mijn intenties enkel waren om mij vast te bijten op een nieuwe uitdaging, heeft de tomeloze inzet van iedereen om mij heen geleid tot dit eindresultaat. Ik wil dan ook iedereen bedanken die dit mogelijk heeft gemaakt!

Het avontuur begon als onwetende 19-jarige geneeskunde student in de collegebanken die teveel tijd over had nadat het wedstrijdseizoen roeien tot een einde was gekomen. Of het nu de compositie van Isaac Israëls was of de heldere lezing over de immunologie, ik was verkocht aan het onderwerp. Ik besloot dan ook om het gesprek aan te gaan met de lezinggever, waarvan ik nooit gedacht had dat hij mijn promotor zou worden. Jan Willem, ik wil je bedanken dat je deze jongen destijds een kans hebt gegeven en later het geduld hebt behouden om deze hardleerse pupil te begeleiden tot het einde.

Elke promotie kent zijn unieke beloop, zo ook het traject dat aan dit boekje vooraf is gegaan. De omstandigheden hebben het niet makkelijk gemaakt, zo is het mij nog steeds niet duidelijk welk(e) department(en) ik bij de affiliaties had moeten opgeven. Ik heb het mijzelf ook niet makkelijk gemaakt door mijn passie te volgen in plaats van te kiezen voor de gespreide bedjes die mij werden aangeboden. Ik wil dan ook mijn ouders bedanken die altijd onvoorwaardelijk achter mij hebben gestaan en tegelijkertijd mijn overwegingen voortdurend uitgedaagd hebben. Zonder jullie ondersteuning was ik hier nooit gekomen. Dan zal ik ook maar meteen mijn broer bedanken, die de lat torenhoog heeft gelegd. Als kleine broertje wil je dat natuurlijk overtreffen en je was nooit te beroerd om achterom te kijken. Later besef je pas hoe weinig relevant het eerste is en hoe veelbetekenend het laatste.

Ditzelfde kan gezegd worden over de vele vrienden die mij altijd ondersteund hebben. Binnen de studenten-roeivereniging en later ook de INKOM organisatie heb ik altijd kunnen genieten van (een overdosis) aan sociale activiteiten. De
keuze om zoveel van mij vrije tijd te stoppen in een uit de hand gelopen vrijwilligersproject zal voor menig tweedejaars student als bizar overkomen, voor wie het concept van promotie onderzoek nog een ver-van-je-bed show is.

Iedere geneeskunde student begint met een romantisch beeld van de gezondheidszorg, waarna de roze bril al snel verloren raakt wanneer ze kniehoog in de modder staan te ploeteren. Soms kom je echter een briljante diamant tegen die desondanks vast blijft houden aan dat romantische beeld. Pieter, ik heb ongekend veel respect voor je onuitputtelijke inzet en je uitzonderlijke kennis, niet alleen op het gebied van de klinische immunologie maar ook over Ben van Gelder en Dostojevski. Het is bewonderenswaardig hoe, ondanks dat je destijds veel andere zaken aan je hoofd had, je deze eigenzinnige student onder je hoede hebt genomen om mij de praktijk te laten zien.

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Publications


Michael Joseph Kemna werd op 2 maart 1991 geboren in Wierden. Hij behaalde zijn VWO diploma cum laude in 2009 aan het Newman college te Breda, waarna hij begon met de studie Geneeskunde aan de universiteit van Maastricht. Na een jaar wedstrijdroeien kreeg Michael in het tweede jaar van de bachelor de mogelijkheid om onderzoek te doen bij de afdeling Klinische en Experimentele Immunologie, onder leiding van Prof. Dr. Cohen Tervaert. Michael voerde naast zijn studie meerdere onderzoeksprojecten uit onder begeleiding van zijn promotor en co-promotoren Dr. Damoiseaux en Dr. Van Paassen. Deze onderzoeken werden mogelijk gemaakt door een zelf ontwikkeld database systeem die gestandardiseerde data verzameling, geautomatiseerde analyse en het systematisch toepassen van klinische regels mogelijk maakt. Middels deze database werden de klinische data geanalyseerd van patiënten die behandeld werden bij de afdeling Klinische Immunologie van het Maastricht University Medical Center, waar Michael zijn laatste klinische stage van zijn master studie heeft gevolgd.
Michael studeert sinds september 2017 Technische Informatica aan de Technische Universiteit in Delft.