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## Disease-activity in ANCA-associated vasculitis

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## Summary and discussion

## SUMMARY

In this thesis disease activity in ANCA-associated vasculitis (AAV) was explored. **Part I** focused on prediction and occurrence of relapses in AAV. Subsequently, **part II** of the thesis studied renal production of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) in AAV in relation to disease activity and damage.

In **chapter 2** risk factors for relapse in AAV were reviewed. Several factors have been associated with the occurrence of relapses. First, relapses occur more often in patients diagnosed WG than MPA or NCGN [1]. Additional identified factors related to the occurrence of relapses are ANCA-status, level of T cell activation, therapy related factors, renal function, genetic factors, and microbiological factors. ANCA status, T cell activation and therapy related factors have been subjects of studies described in this thesis.

In **chapter 3** we studied the relation between ANCA-status during follow-up and relapse. We assessed whether C-ANCA titers as measured by IIF and PR3-ANCA levels measured by ELISA, at diagnosis and following immunosuppressive treatment, are predictive for relapse of AAV. In this retrospective analysis we evaluated 87 PR3-ANCA positive patients during five years follow-up. Patients who became and stayed negative for C-ANCA (IIF) (n=16) or PR3-ANCA (ELISA) (n=7) had a lower risk to experience relapse. Positive C-ANCA (IIF) titers at 3, 12, 18 and 24 months after diagnosis and increased PR3-ANCA levels at 12, 18 and 24 months after diagnosis were significantly associated with relapse within 5 years after diagnosis.

In **chapter 4** we evaluated whether T cell activation as reflected by levels of sIL-2R, sCD30, IL-10 and B cell activator of the TNF family (BAFF) at diagnosis and during follow-up is predictive for ANCA-positivity and clinical relapse in PR3-AAV. Plasma levels of sIL-2R, sCD30, and BAFF were higher in patients than in healthy controls at all time points. Plasma levels of sIL-2R, sCD30 and IL-10 were higher at diagnosis and relapse than during remission. At 18 months, sCD30 and sIL-2R levels were significantly higher in patients positive for PR3-ANCA than in PR3-ANCA negative patients. Thus we found indications for increased T cell activation in patients with ANCA-associated vasculitis in remission during and after immunosuppressive treatment. T cell activation was associated with persistent or renewed ANCA positivity. However, we did not find a significant relation between levels of T cell activation markers and the occurrence of relapse.

Previously, the CYCAZAREM study showed that cyclophosphamide can be safely replaced by azathioprine after induction of remission without increased relapse rate [2]. However, this study was limited by follow-up up to 18 months and did not show any

short-term significant differences between cyclophosphamide and azathioprine toxicity. We retrospectively studied outcome of all patients diagnosed with AAV from 1990 at our center. Initially, from 1990 until 1996, these patients were treated with cyclophosphamide both for induction and maintenance. From 1996, after induction of stable remission on cyclophosphamide, treatment was switched to azathioprine maintenance. In 2003, studying 136 patients, we found a trend towards increased relapses in the azathioprine maintenance cohort (**chapter 5**). However, as described in **chapter 6**, with longer follow-up including 247 patients, we did not find a difference in relapse rate between patients on cyclophosphamide versus azathioprine maintenance treatment. Additionally, we did not find differences in infectious complications. These infectious complications primarily occurred in the first three months after diagnosis, when both groups were treated with cyclophosphamide and most leukopenic episodes occurred. We did not detect a difference in damage scores between the cyclophosphamide and azathioprine group. In conclusion, also during long-term follow-up azathioprine maintenance is not inferior to cyclophosphamide maintenance in prevention of relapses.

In part 2 of the thesis we focused on renal markers of disease activity and damage. In **chapter 7** we analysed renal expression of MMPs, tissue inhibitor of metalloproteinase-1 (TIMP-1) and markers of neutrophil and monocyte infiltration in renal biopsies of patients with active ANCA-associated glomerulonephritis. MMP-2, -3, -9, and TIMP-1 positive cells were detected in ANCA-associated glomerulonephritis in glomeruli with active inflammation (cellular crescents or fibrinoid necrosis), only occasionally in normal appearing glomeruli, and not in sclerotic glomeruli and in the tubulo-interstitium. MMPs and TIMP-1 were predominantly expressed by infiltrating neutrophils and monocytes. So, we detected increased glomerular and interstitial expression of MMP-2, -3, -9, and TIMP-1 in active ANCA-associated glomerulonephritis and this expression co-localized and correlated with inflammatory activity. This supports the hypothesis that MMP-2, -3 and -9 have a role in the pathogenesis of ANCA-associated vasculitis. Subsequently, we questioned whether local expression of these inflammatory markers might be reflected by plasma and urine levels. In **chapter 8** we studied whether urinary and plasma levels of MMP-2, -9, and TIMP-1 reflect renal expression of these proteins and renal disease-activity in ANCA-associated vasculitis. However, urinary activity of MMP-2 and MMP-9 did not correlate with renal MMP expression or plasma levels. Urinary MMP activity correlated negatively with glomerular inflammation, but positively with fibrous crescents. Urinary MMP-2 and TIMP-1 levels showed a positive correlation with tubulointerstitial damage and a negative correlation with creatinine clearance.

## DISCUSSION

With the introduction of cyclophosphamide AAV have become chronic relapsing diseases, although still with considerable early mortality [1]. Nowadays, relapses of disease activity are a major problem associated with increased morbidity and mortality, and subsequent increased exposure to cytotoxic therapy. Active disease and treatment during relapse is directly associated with mortality [3-6]. Additionally, active disease and treatment during relapse lead to increased damage [5, 7], and increased damage is associated with mortality. Finally, the number of relapses is the primary predictor of long-term renal survival in proteinase-3 ANCA-associated vasculitis (PR3-AAV) [8]. So identification of patients at risk and, if possible, prevention of relapses is of major importance.

### Risk factors for relapse

Increased relapse risk in patients with WG is in part associated with chronic nasal carriage of *S. Aureus* [9]. Furthermore, a proportion of patients with limited WG can be successfully treated with monotherapy with trimethoprim-sulfamethoxazole only. Additionally, maintenance treatment with trimethoprim-sulfamethoxazole upon standard treatment during two years reduced relapse risk by 60% [10]. However, most of the associations between infection and relapse have been descriptive and causality has not been proven. Recently, new evidence was provided of a mechanism by which infection could induce autoantibody mediated vasculitis. An immune response against adhesin FimH present on Gram-negative bacteria induced a cross-reactive auto-immune response towards lysosomal-associated membrane protein-2 (LAMP-2) [11]. Auto-antibodies against LAMP-2 were present in almost all studied patients with AAV. When injected into rats these antibodies to LAMP-2 induced pauci-immune focal necrotizing glomerulonephritis. Thus, auto-immunity might be induced by an immune response against an exogenous, bacterial antigen.

Previously, it was feared that vaccination against influenza might be able to induce immune activation and subsequent relapses of disease. However, in a recent retrospective study of 230 patients with AAV we did not find an increase of relapses after vaccination against influenza [12].

### ANCA-status and relapse

In particular PR3-ANCA positive patients have, compared to MPO-ANCA positive patients, a significantly increased risk to experience relapse (RR 3.7; 95% CI 1.6-4.1) [13]. This increased relapse risk based on ANCA specificity can also be found within the different

disease entities composing AAV. In addition, ANCA-status during follow-up has been linked to relapse. Previously, Slot et al showed that C-ANCA positivity after induction of remission is associated with the occurrence of relapse [14]. Also other smaller previous studies showed a relationship between disappearance of C-ANCA and relapse-free survival [9, 15-18]. In this thesis we confirmed the relation between persistence of C-ANCA positivity, during and after induction of remission, and the occurrence of relapse in patients with PR3-AAV. For a definitive answer regarding the clinical value of C-ANCA status at stable remission we will have to await the results of a multicenter prospective clinical trial that we are currently undertaking. In this trial therapy is adapted according to C-ANCA status at switch from azathioprine to cyclophosphamide in PR3-ANCA positive patients. Patients who are C-ANCA positive at stable remission are randomized to standard azathioprine therapy, with tapering from 12 months after diagnosis, and long-term azathioprine therapy which is continued until 48 months after diagnosis. Patients who are C-ANCA negative receive standard azathioprine therapy with tapering from 12 months after diagnosis. In this trial C-ANCA titers are measured centrally by IIF in the laboratory of clinical immunology of the University Medical Center Groningen in order to avoid the assumed inter-laboratory variation in ANCA-titers which is a disadvantage of this method when compared with quantitative measurements by ELISA. However, the study described in this thesis showed a better predictive value of C-ANCA by IIF as compared to levels of PR3-ANCA measured by ELISA. Additionally, a recent multicenter study from the USA did not find a relationship between PR3-ANCA levels and the occurrence of relapse in WG [19]. In this study PR3-ANCA levels from 156 patients collected approximately every 3 months were measured centrally by capture ELISA. Limitations of the study included differences in follow-up duration and long intervals between PR3-ANCA measurement in some patients, in 17 patients more than 6 months.

Thus, there is an imperfect relation between persistent ANCA positivity or re-occurrence of ANCA and occurrence of relapse. Apparently, more is needed in a chain of immunologic events to induce relapse. Data suggest qualitative differences between ANCA in addition to quantitative differences. For instance, epitope specificity and subclass of ANCA might explain discrepancies between ANCA levels and clinical status [20]. We assume that ANCA positive patients have a persistent state of immune activation and an ongoing smouldering autoimmune response.

### T cells and disease activity

Patients with WG are lymphopenic which might result from a combined effect of therapy and disease [21]. This lymphopenia has been associated with a lower incidence of relapse [22]. Previously, in WG increased surface expression of T cell activation markers was detected in patients with active disease as compared to healthy controls, and this finding persists during remission [23]. In this thesis we confirmed increased T cell activation in patients with ANCA-associated vasculitis in remission during and after immunosuppressive treatment. Additionally, T cell activation was associated with persistent or renewed ANCA positivity. However, we did not find a significant relation between levels of T cell activation markers and the occurrence of relapse.

The role of T cells has been more appreciated and elucidated in recent years [24]. It was shown that the distribution of T cell populations in patients with AAV differs from normal. Persistent T cell stimulation leads to a decrease in circulating naïve T cells and expansion of CD4<sup>+</sup> T-effector memory cells during remission [25]. Subsequently, during active disease a significant decrease in circulating CD4<sup>+</sup> T-effector memory cells was observed as compared to remission, suggesting migration of these cells to sites of inflammation [25]. Additionally, increased numbers of T-effector memory cells have been found in urine sediments of patients with active WG [26]. This might thus be a new marker for active renal vasculitis. Furthermore, a new class of T cells has been identified. These Th17 cells produce IL-17 and are thought to be pivotal in a range of human auto-immune disorders [27-29]. In line with these findings, a significant increase in percentages of Th17 cells was found in WG patients as compared to healthy controls when peripheral blood cells were stimulated *in vitro* [30]. In ANCA-positive patients an increased frequency of PR3-specific Th17 cells was found as compared to ANCA-negative patients. Although data in AAV are scarce, Th17 cells might be important effector cells in AAV [26].

### Therapy and relapse

Several studies have shown a relation between type, dosage and duration of induction and maintenance therapy and risk the for relapse. Up till now no alternative for induction with cyclophosphamide has been proven to have similar efficacy with reduced toxicity. Toxicity of cyclophosphamide is well known and includes myelosuppression, infectious complications, and carcinogenesis, in particular bladder cancer. However, a dosage of <10 gram of cyclophosphamide in the first 6 months was associated with an increased relapse rate [31]. Recently, the multicenter EUVAS CYCLOPS study found that pulse cyclophosphamide induced remission of AAV as effective as the oral regimen at a reduced cumulative

cyclophosphamide dose (8.18 versus 15.75 gram) and at a reduced incidence of leukopenia [32]. Within a median follow-up of 18 months 14.5% of patients experienced a relapse, with 13 relapses in the pulse group and 6 in the daily oral group (HR 2.01 (95% CI 0.77-5.30)). Although not significant, this finding confirms an earlier meta-analysis of 3 randomized controlled trials by De Groot et al which also suggested a trend towards increased relapses in groups treated with three different pulse regimens as compared to the oral group (RR 1.79; 95% CI 0.85-3.75) [33]. However, the CYCLOPS study was powered and designed to compare induction of remission and not to evaluate relapse frequency. Thus, we should consider pulse therapy in patients without immediate life-threatening disease to be equal to oral cyclophosphamide in terms of efficacy for induction of remission. However, the search for alternative induction strategies continues. We are coordinating a multi-center study comparing mycophenolate mofetil (MMF) as a less toxic but non-inferior induction therapy with cyclophosphamide in patients with a relapse of AAV. One previous smaller randomized trial on renal vasculitis found similar induction rates between mycophenolate mofetil and cyclophosphamide [34]. Other efforts include a multinational multi-center study comparing anti-CD20 therapy (rituximab) with cyclophosphamide for induction of remission in AAV (RAVE) [35].

Previously, the CYCAZAREM study showed that cyclophosphamide can be safely replaced by azathioprine after induction of remission without increased relapse rate [2]. However, this study was limited by a follow-up of 18 months only, and did not show any short-term significant differences between cyclophosphamide and azathioprine toxicity. We retrospectively studied outcome of all patients diagnosed with AAV from 1990 at our center. Initially, from 1990 until 1996, these patients were treated with cyclophosphamide both for induction and maintenance. From 1996 on, after induction of stable remission on cyclophosphamide, treatment was switched to azathioprine maintenance. Also during long-term follow-up we found that azathioprine maintenance is not inferior to cyclophosphamide maintenance in prevention of relapses. As we also did not detect differences in infectious complications, malignancies and damage score, so far the preference of azathioprine over cyclophosphamide in AAV is not supported by head to head comparisons. Also methotrexate (MTX) has been proven effective as maintenance therapy. A recent study comparing side-effects of MTX and azathioprine maintenance did not find significant differences in treatment toxicity and showed similar relapse rates [36]. Thus, azathioprine and MTX seem to have similar efficacy as maintenance treatment in AAV, but MTX is contra-indicated in patients with renal failure. One should take into account that a considerable proportion of patients will develop an adverse reaction to azathioprine or MTX. The active metabolite



of azathioprine is 6-mercaptopurine, which levels are dependent on the enzyme thiopurine methyltransferase (TPMT). Previously, Stassen et al did not find a relation between TPMT activity and the occurrence of relapses and adverse effects, in particular hepatotoxicity and leukopenia [37]. For patients intolerant to azathioprine or MTX mycophenolate mofetil might be an alternative [38]. So far, no prospective studies have evaluated optimal duration of maintenance therapy with azathioprine or MTX. Most relapses occur after therapy has been stopped, so prolongation might be an effective means to prevent relapses. Nevertheless, also during low-dose maintenance with azathioprine patients can experience relapses. Long-term studies comparing maintenance regimens are eagerly awaited, and individualization of immunosuppressive regimens according to cumulative risk-factors for relapse might be the way to go. Currently, we are undertaking a prospective randomized multi-center trial adapting therapy according to C-ANCA status at switch from azathioprine to cyclophosphamide in PR3-ANCA positive patients. Patients who are C-ANCA positive at stable remission are randomized to standard azathioprine therapy, with tapering of dosage from 12 months after diagnosis, and long-term azathioprine therapy which is continued until 48 months after diagnosis.

### **MMPs in renal vasculitis**

In part 2 of the thesis we focused on renal markers of disease activity and damage. Expression of matrix metalloproteinases (MMPs) by infiltrating and intrinsic renal cells is increased in inflammatory conditions, and may correlate with disease activity of glomerulonephritis [39, 40]. The gelatinases (MMP-2 and MMP-9) are a subfamily of MMP that are able to degrade basement membrane type IV and V collagen, aggrecan, elastin and gelatins [39]. However, substrate specificity is not completely equal, with MMP-9 being significantly more active against basement membrane type IV and V collagen. Furthermore, expression differs as MMP-9 is tightly regulated by many cytokines and growth factors, and, in contrast, MMP-2 is constitutively expressed [41].

Previously, Urushihara et al detected that MMP-9 expression was increased and corresponded to the level of glomerular cell proliferative changes in renal biopsies of patients with mesangial proliferative glomerulonephritis (IgA nephritis, Henoch-Schönlein nephritis, mesangial proliferative GN and class II lupus nephritis). In contrast, MMP-2 was not detected in normal renal tissue or in renal biopsies of patients with IgA nephropathy, mesangial proliferative glomerulonephritis and lupus nephritis [42]. We detected increased glomerular and interstitial expression of MMP-2, -3, -9, and TIMP-1 in renal biopsies of patients with active ANCA-associated glomerulonephritis and their expression co-localized and correlated with inflammatory activity.

The critical targets of MMPs in acute kidney injury have still not been elucidated. Because MMP-2 and MMP-9 degrade basement membrane compounds, they can cause damage to the glomerular basement membrane. MMPs influence the behavior of glomerular cells, either directly or by regulating growth factors [43]. *In vitro* production of MMP-2 by mesangial cells resulted in a proliferative, highly inflammatory phenotype, whereas latent MMP-2 or MMP-9 showed no effect on mesangial cells [44]. Additionally, data from ischemia-reperfusion animal models show that MMPs mediate acute kidney injury via changes in the vascular endothelium and tubular epithelial cells [45]. In experimental glomerulonephritis increased expression of MMPs is associated with disease activity and infiltration by inflammatory cells. Inhibition of MMPs in Thy 1.1 nephritis in rats reduces glomerular lesions, proteinuria and matrix accumulation [46]. However, in a model of anti-GBM nephritis, MMP-9 deficient mice showed more severe disease than control mice, implying that MMP-9 can have anti-inflammatory properties [47].

Our observation that hardly any MMP-activity was present in normal glomeruli is consistent with the correlation between normal glomeruli in renal biopsies of patients with AAV at diagnosis and subsequent renal recovery. Also active renal lesions like fibrinoid necrosis and cellular crescents correlated with GFR 18 months after diagnosis in mild to moderate renal involvement [48]. However, in more severe renal involvement these active glomerular lesions had no predictive value for GFR 18 months after diagnosis [49]. In our study glomerular MMP-9 expression was significantly higher in PR3-ANCA positive than MPO-ANCA positive patients which might reflect underlying differences in presentation and course of the disease [50].

Several previous studies examined renal, urinary and plasma levels of cytokines, among others MCP-1 and IL-8, but not MMPs and TIMPs [51]. Here we studied plasma and urinary levels of MMP-2, MMP-9 and TIMP-1. We hypothesized that plasma and urine levels of these inflammatory markers reflect local expression. However, locally increased MMP-2, -9 and TIMP-1 were not reflected by increased urinary levels, although urinary MMP-2 and TIMP-1 reflected tubular damage. As the tubulo-interstitium is the major renal compartment this might explain the correlation between tubule-interstitial damage and urinary levels of MMP-2 and TIMP-1. As discussed previously MMPs have been shown to release or activate several growth factors that have been associated with tubular cell proliferation, and renal fibrosis, such as pro-transforming growth factor- $\beta$  [52]. In several other renal disorders a role for MMP-2 has been shown in the pathway leading to chronic renal disease [53, 54]. *In vitro*, MMP-2 is necessary and sufficient for the transformation of renal tubular epithelium to the myofibroblastic phenotype, an essential step in the process leading to interstitial fibrosis [55].

Thus, instead of a reflection of acute glomerular inflammation, elevated MMP-2 and TIMP-1 in the urine of patients with AAV appear to be markers of the common pathway leading to chronic renal damage. In the near future new and combined markers of inflammation and damage might provide more detailed information about both glomerular and interstitial inflammation and fibrosis in the different stages of disease. However, for now the renal biopsy remains pivotal both at diagnosis and during follow-up when detailed information about disease development in the different renal compartments is considered necessary.

## CONCLUSIONS

In conclusion, AAV are nowadays chronic diseases with a major disease burden. Although treatment has progressed over the last decades and is considered less toxic, both therapy and disease related morbidity and mortality have not improved significantly (chapter 6). A major determinant regarding outcome is the occurrence of relapse (chapter 2 and 6). However, the prediction of relapses in AAV is still imperfect. Several strong predictors have been identified and confirmed, among others PR3-ANCA positivity at diagnosis (chapter 2). In addition we report that patients with PR3-ANCA-associated vasculitis who remain C-ANCA positive during and after induction of remission are at increased risk for relapse (chapter 3). This might reflect ongoing smoldering immune activation including T cell activity (chapter 4). The challenge ahead is whether adaptation of treatment according to risk factors will result in a better outcome. Part two of the thesis focused on monitoring kidney disease in AAV. We report the increased presence of MMP-2, -3, -9 and TIMP-1 in patients with renal AAV (chapter 7). However, locally increased MMP-2, -9 and TIMP-1 were not reflected in increased urinary levels, although urinary MMP-2 and TIMP-1 reflected tubular damage (chapter 8). So, we and others are still searching for better and more specific markers of renal inflammation and damage.

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