Treatment outcomes in ANCA-associated vasculitis

Determinants of efficacy and toxicity

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Determinants of efficacy and toxicity
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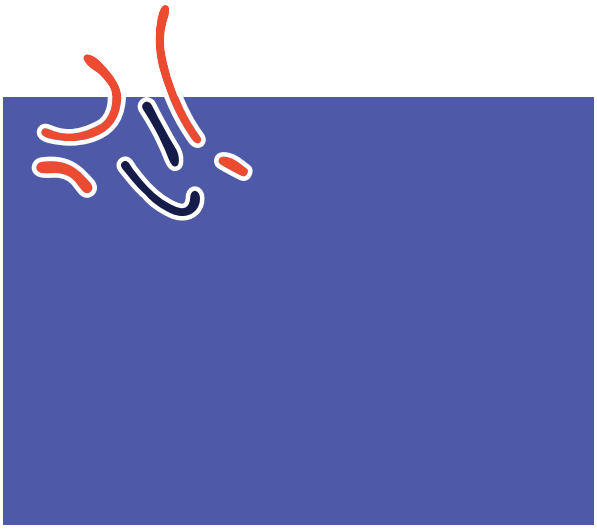
01 Chapter

Introduction: Current challenges in the treatment of ANCA-associated vasculitis



ANCA-associated vasculitis

ANCA-associated vasculitides (AAV) constitute a group of auto-immune diseases associated with inflammation of mainly small blood vessels. In the majority of patients, antibodies directed against myeloperoxidase (MPO) or proteinase 3 (PR3) are present, while some AAV patients are ANCA-negative [1], or have atypical types such as bactericidal permeability-increasing protein (BPI)- or human neutrophil elastase (HNE)-ANCA [2].

Several subtypes are distinguished based on clinical characteristics. These are granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg-Strauss) and single-organ AAV (most commonly renal-limited AAV) [1]. Renal-limited AAV, or necrotizing and crescentic glomerulonephritis (NCGN), is often regarded as a subtype of MPA.

Clinical characteristics of AAV

Although AAV can affect any organ, some organs are more frequently affected than others (**Figure 1**). The typical characteristics per subtype of AAV are discussed below and are summarized in **Table 1**.

Microscopic polyangiitis (MPA)

MPA is characterized by necrotizing vasculitis with few or no immune deposits (i.e., pauci-immune). This disease is not associated with granulomatous inflammation. The most common manifestations are necrotizing crescentic glomerulonephritis (NCGN) and pulmonary capillaritis presenting as hemoptysis, dyspnea or even respiratory failure [1]. Other common manifestations are skin symptoms, most commonly purpura, and mononeuritis multiplex.

Granulomatosis with polyangiitis (GPA)

GPA is characterized by neutrophil-rich granulomatous inflammation of the upper (e.g., nasal crusting, epistaxis, hearing loss, subglottic stenosis) and lower respiratory tract (e.g., pulmonary nodules or cavities). NCGN is also common. Other frequent manifestations include ocular vasculitis (e.g., conjunctivitis, (epi-)scleritis), purpura, mononeuritis multiplex and pulmonary capillaritis with hemorrhage. Limited forms of GPA exist with only involvement of the eyes or the upper or lower airways. Limited GPA usually requires less intensive treatment [1].

Eosinophilic granulomatosis with polyangiitis (EGPA)

EGPA is characterized by eosinophil-rich granulomatous inflammation of the upper and lower respiratory tract. The most characteristic manifestations are eosinophilia, asthma and nasal polyps. Other common manifestations include eosinophil-rich inflammation of the myocardium and gastrointestinal tract. Most patients are ANCA-negative. ANCA-positive EGPA patients more frequently have vasculitis symptoms such as alveolar hemorrhage, glomerulonephritis and peripheral neuropathy, while ANCA-negative patients have a higher risk of cardiovascular involvement and eosinophilic tissue infiltration [3]. EGPA also has a limited form with only involvement of the upper or lower respiratory tract [1].

Figure 1. Possible symptoms of ANCA-associated vasculitis.

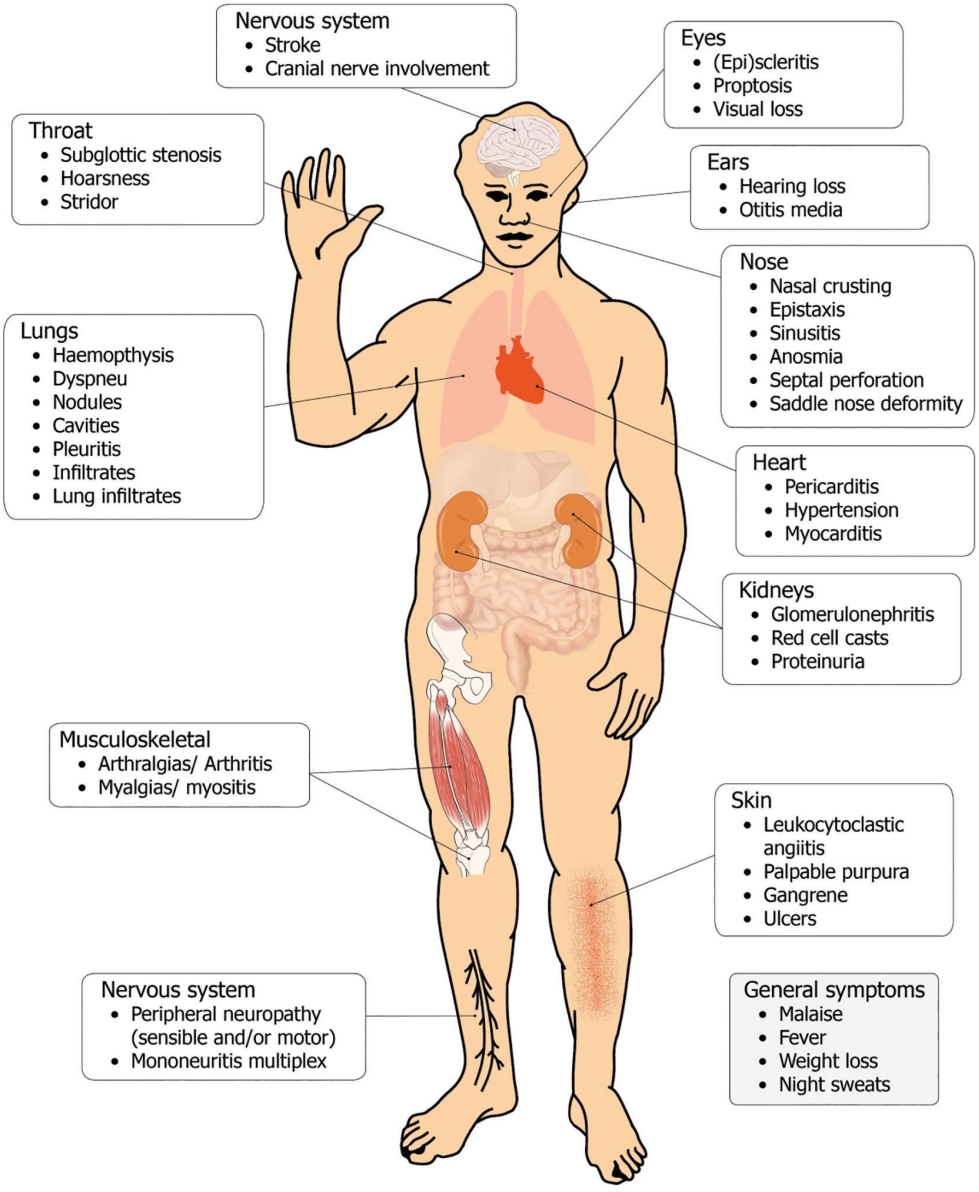


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Table 1. Clinical phenotypes of ANCA-associated vasculitis.

Disease	Incidence	Typical involvement	ANCA	Histological hallmarks	5-year outcome
GPA	±10/million	Ears, nose, upper airways Lung nodules/ cavities Kidneys	PR3-ANCA >> MPO-ANCA	Granulomas Necrotizing vasculitis NCGN	Relapse rate >50% Mortality <10%
MPA	±10/million	Kidneys Pulmonary hemorrhage	MPO-ANCA > PR3-ANCA	Necrotizing vasculitis NCGN	Relapse rate 20% Mortality 10-20%
EGPA	±1/million	Allergic rhinitis Nasal polyps Asthma + eosinophilia ANCA-positive: kidneys	MPO-ANCA / negative	Eosinophil granulomas Necrotizing vasculitis NCGN	Relapse rate >50% Mortality a<10%

EGPA eosinophilic granulomatosis with polyangiitis; GPA granulomatosis with polyangiitis; MPA microscopic polyangiitis; NCGN necrotizing and crescentic glomerulonephritis. MPA includes patients with renal-limited ANCA-associated vasculitis.

ANCA specificity to categorize patients

Increasing evidence suggests that ANCA specificity may be a better way of classifying AAV patients than the previously mentioned clinical subtypes [4]. In a genome-wide association study (GWAS) investigating the genetic basis for AAV, ANCA specificity showed stronger genetic associations than clinical subtype [5]. It is also a better predictor of relapse than clinical subtype, with PR3-ANCA positive patients having a higher risk of disease relapse [6,7]. Some studies suggest that MPO-ANCA positive patients have a higher risk of mortality and end-stage renal disease [8,9], while other studies show no predictive value of ANCA specificity for either outcome [6].

Epidemiology and risk factors of AAV

The annual incidence of AAV is low. In Europe, it is estimated to be 13-20/million. The prevalence of AAV is approximately 46-184/million. Slightly more males than females are affected. While it can occur at any age, the highest incidence of AAV is in the 6th and 7th decades [10].

Geographic differences exist regarding the distribution of ANCA specificities. MPO-ANCA is more common in Japan and China, while PR3-ANCA is more common in Northern Europe, the Middle East and India. MPO-ANCA and PR3-ANCA occur in similar frequency in Caucasian Americans and Southern Europeans [11].

Genetic and environmental factors both have a potential role in AAV pathogenesis. In the previously mentioned GWAS, genetic associations were found with major-histocompatibility complex (MHC) and non-MHC loci, which differed depending on ANCA specificity (e.g., HLA-DP and genes encoding α1-antitrypsin (SERPINA1) and proteinase 3 (PRTN3) for PR3-ANCA; HLA-DQ for MPO-ANCA). Smaller associations were found with factors such as interleukin-10 (IL10) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4). All factors make only a small contribution to disease risk, but may help identify targets for therapy [5].

Several findings suggest a role for the environment in AAV pathogenesis. First, some studies suggest a role for infections in AAV pathogenesis. In particular, Staphylococcus aureus has been implied to play a role in the pathogenesis of GPA [12]. This is supported by the effectiveness of the antimicrobial drug co-trimoxazole for maintenance of remission in GPA [13]. Second, GPA and EGPA incidence increase with higher geographic altitudes and lower ambient UV exposure; this association was not found for MPA patients [14]. Last, silica exposure has been suggested as a risk factor for AAV [15].
In conclusion, several genetic and environmental factors make a limited contribution to risk of developing AAV. In most cases, the specific cause of disease is unknown. Importantly, AAV is not a genetic disease in the narrow sense, as genetic factors alone are not sufficient to trigger disease.

Current treatment of AAV

History of AAV treatment

Over the past decades, AAV has developed from a deadly disease with a first-year mortality rate of over 80% to a chronic relapsing-remitting disease with a remission rate of over 90% and a 5-year survival rate of over 75% [8]. This results in a large part from the introduction of immunosuppressive therapy with glucocorticoids and oral cyclophosphamide in the 1960s [16,17].

Unfortunately, a high cumulative dose of cyclophosphamide is associated with severe adverse effects such as hemorrhagic cystitis, neutropenia, infections and (hematological and urinary) malignancies [18]. The CYCAZAREM trial, published in 2003, showed that cyclophosphamide therapy can be safely shortened by switching to the less toxic drug azathioprine after attaining disease remission for three consecutive months [19]. In a further attempt to reduce cumulative cyclophosphamide exposure, the CYCLOPS trial was conducted, comparing oral cyclophosphamide to pulsed intravenous cyclophospha-

mide with lower cumulative cyclophosphamide dose. The results of this study showed that pulsed intravenous cyclophosphamide was as effective as oral cyclophosphamide for inducing remission [20], but was associated with a higher long-term risk of relapse [21].

In a search for safer alternatives to cyclophosphamide, the RAVE and RITUXVAS studies were conducted and published in 2010. These studies showed that the anti-CD20 monoclonal antibody rituximab had non-inferior efficacy compared to cyclophosphamide [22,23]. In the short-term results of the RAVE trial, rituximab was even superior to cyclophosphamide for treatment of relapsing patients [22]. On the other hand, rituximab was no longer superior at long-term follow-up, although this might be due to a lack of maintenance therapy in the rituximab group [24]. Also, rituximab treatment was not associated with fewer (infectious) adverse events [22,23].

Induction therapy

The EULAR/ERA-EDTA recommendations published in 2016 make a distinction between organ- or life-threatening disease and non-organ threatening disease [25]. For organ- or life-threatening disease, cyclophosphamide or rituximab are combined with high-dose glucocorticoids, which are slowly tapered starting after six weeks. In non-organ threatening disease, treatment with less toxic drugs such as methotrexate or mycophenolate mofetil may be given together with glucocorticoids. In patients with rapidly progressive renal failure (serum creatinine >500 µmol/l and/or dialysis dependency) or diffuse alveolar hemorrhage, additional treatment with plasma exchange (PLEX) is advised [25]. Although PLEX for severe renal AAV showed short-term efficacy in the MEPEX trial [26], long-term results showed no reduction of end-stage renal disease or mortality [27]. In a presentation at the 55th ERA-EDTA congress in 2018, preliminary results from the completed PEXIVAS trial (ISRCTN07757494; clinicaltrials.gov NCT00987389), that enrolled over 700 patients with a follow-up of up to 7 years, suggested no effect of PLEX.

Maintenance therapy

After three months of stable remission on induction therapy, patients switch to a maintenance therapy using azathioprine, rituximab, methotrexate or mycophenolate mofetil combined with low-dose glucocorticoids [25]. Based on the results of the IMPROVE trial, azathioprine is preferred over mycophenolate mofetil for maintenance therapy [28]. Methotrexate is another effective drug for maintenance of remission [29] and is seen as an equivalent option to azathioprine [25]. The results of the MAINRITSAN trial, conducted by the French Vasculitis Study Group, suggest that rituximab may be superior to azathioprine as remission maintenance therapy following induction therapy of pulsed intravenous cyclophosphamide and glucocorticoids, even up to 60 months of follow-up [30,31]. However, long-term toxic effects of rituximab use are largely unknown. An important long-term adverse effect is hypogammaglobulinemia, which results in a higher risk of severe infections [32].

The subsequently performed MAINRITSAN2 trial indicated that exposure to rituximab maintenance therapy could be reduced safely by 1-3 infusions (out of 5) when only infusing rituximab upon a return of CD19+ B lymphocytes or a rise in ANCA titer [33].

General consensus is that maintenance therapy should be continued for 24 months [25]. Recent data on the optimal duration is contradictory. One study concludes that extending maintenance therapy with azathioprine and low-dose prednisolone to 48 months reduces relapse risk and increases renal survival [34], while another suggests that continuing for more than 18 months does not further reduce relapse risk [35]. These different results might be explained by the duration of low-dose prednisolone therapy, as longer courses of glucocorticoids likely protect against relapse [36]. This should be weighed against the cumulative adverse effects of glucocorticoids [37].

Treatment of EGPA patients

Because of the different clinical picture of EGPA compared to GPA and MPA patients [1], especially due to frequent exacerbations of asthma and rhinosinusitis, treatment in EGPA differs in some aspects. Patients with life- and/or organ-threatening disease are treated similarly to other types of AAV, with a lower priority to rituximab because of limited experience with the drug in EGPA [38]. Patients without these manifestations may be treated with glucocorticoid monotherapy [38], although more recent recommendations advise full induction and maintenance therapy in all EGPA patients [25]. Besides vasculitis treatment, EGPA patients require therapy for asthma and rhinosinusitis [3].

New developments in treatment

Recently, research focus has shifted towards precision medicine, specifically targeting pathophysiologic pathways in AAV [4]. This approach aims to reduce cumulative exposure to the currently used drugs and their toxic effects by (partly) replacing them with drugs targeting specific inflammatory pathways involved in AAV pathogenesis. For example, the CLEAR trial investigates the complement C5a receptor inhibitor avacopan as a possible replacement for glucocorticoids [39]. Also, in a randomized clinical trial, adding anti-interleukin-5 monoclonal antibody mepolizumab to standard therapy in patients with relapsing of refractory EGPA increased duration of remission and resulted in a modest reduction of required prednisolone dose [40].

Current outcomes in AAV

Relapse

Despite effective treatment for AAV, still many patients (35% within 5 years [41]; 25-59% depending on the number of risk factors [7]) experience disease relapses that result in damage and require renewed immunosuppressive therapy. Several predictors of relapse have been identified, including PR3-ANCA positivity, pulmonary and cardiovascular involvement of AAV.[7,42] A higher serum creatinine at baseline was found to be protective against relapse [7].

Mortality

As mentioned previously, survival of AAV has drastically improved after the introduction of immunosuppressive therapy. Still, AAV patients have a reduced overall survival compared to the general population [8]. In untreated AAV, disease activity was the main cause of death.

Nowadays, adverse effects of therapy, in particular infections, account for 60% of deaths in the first year. Approximately 15-20% of early deaths are still caused by active vasculitis

[8,43]. During long-term follow-up, causes of death are at least partly treatment-related, namely cardiovascular disease (26%), malignancy (22%) and infection (20%) [8]. Importantly, renal function after induction of remission is a main predictor of both adverse events and overall survival [8,43]. This stresses the importance of limiting both disease activity and treatment toxicity, which results in a delicate balance.

Challenges in AAV treatment

Now that disease activity can be effectively treated and survival has drastically improved, research focus shifts to new challenges in AAV treatment. One of them is accumulation of damage from disease activity and treatment. Another is a reduced quality of life despite successful treatment of disease activity.

Damage from disease and treatment

Over the course of their disease, AAV patients accumulate damage from disease activity and treatment. At long-term follow-up, approximately 90% of patients will have some form of damage, with 34% of patients having at least five items from the Vasculitis Damage Index (VDI) [44]. Of note, a major limitation of the VDI is that it is a cumulative scoring system taking into account any damage that exists for at least three consecutive months. It does not make a distinction between temporary and permanent damage [45]. Frequent disease-related damage items for MPA/MPO-ANCA patients include proteinuria and reduced renal function (GFR <50mL/min), while frequent damage items for GPA/PR3-ANCA patients include hearing loss and nasal crusting [44]. At long-term follow-up, approximately 67% of patients have VDI items that are potentially treatment-related, most commonly hypertension, osteoporosis, malignancy and diabetes [44]. Older age, elevated serum creatinine, higher disease activity score, higher number of relapses and higher cumulative glucocorticoid use are all independent risk factors for increased damage [37].

Quality of life

Quality of life (QoL), especially physical QoL, is reduced in AAV [46,47]. This is true even after successful achievement of remission. Several predictors of QoL have been identified. Predictors of poor physical QoL include older age, prednisolone use and nervous system involvement [46,48]. Predictors of poor mental QoL include fatigue and psychological variables such as depression and anxiety [46,49]. The relation of QoL with damage as measured by the VDI is unclear, as some studies report associations of VDI score with physical QoL [50,51], while others did not find any association between the outcomes [46,47,52].

Respiratory and quadriceps muscle strengths are reduced in AAV. Both seem to contribute to a reduced exercise capacity. Leg fatigue is the main reason reported by patients to prematurely stop an exercise. Reduced exercise tolerance, in turn, contributes to poor physical QoL [53]. Based on this data and the reported effects of interventions in conditions such as chronic obstructive pulmonary disease and rheumatoid arthritis [54,55], exercise training might be beneficial for QoL in AAV.

AAV patients report increased fatigue compared to controls. The extent of fatigue is associated with damage from disease and treatment [56]. Fatigue is negatively correlated

to QoL [49,56]. Interestingly, the lower measured quadriceps force in AAV seems to result from higher perceived exertion rather than reduced muscle mass or function [56]. The most important predictors of fatigue are depression, anxiety, pain and sleep disturbance [49,56]. These results point out that a rehabilitation program for AAV patients should address psychosocial factors in addition to exercise capacity. The recently developed patient-reported outcomes questionnaire (AAV-PRO) may assist in monitoring such a program [57].

Aims of this thesis

In order to improve the balance between inflammation and treatment toxicity in AAV and give treatment tailored to the individual, determinants of efficacy and toxicity first have to be identified. The aim of this thesis is therefore to identify predictors of treatment efficacy and toxicity that can be used to optimize therapy of AAV.

In the first part, we investigate genetic factors that may predict efficacy and toxicity of AAV treatment. In **Chapter 2**, we review the literature for genetic polymorphisms associated with outcomes of current treatment in AAV. In **Chapter 3**, we investigate genetic variants and activity levels of the enzyme thiopurine methyltransferase (TPMT) in relation to efficacy and toxicity of azathioprine maintenance therapy. In **Chapter 4**, we investigate whether haplotypes of the glucocorticoid receptor (GR) and a single-nucleotide polymorphism of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) affect disease outcomes in AAV patients treated with standard immunosuppressive therapy. Though we report some interesting associations of gene variants with clinical outcomes, we conclude that clinical application of these variants is still several steps away.

The second part of this thesis focuses on characterization of treatment outcomes in AAV. In **Chapter 5**, we investigate differences in clinical presentation and outcomes of AAV between Brazil, China and the Netherlands, and whether these may (partly) be explained by differences in ANCA-specificity. In **Chapter 6,** we discuss the azathioprine hypersensitivity syndrome and its characteristics in an observational cohort of AAV patients. In **Chapter 7**, we investigate whether steroid myopathy and reduced physical activity might be part of the explanation for reduced physical quality of life in AAV. With this part, we hope to raise awareness of differences in treatment response between countries, the high frequency of azathioprine hypersensitivity and the associations of reduced muscle strength and physical activity with reduced quality of life in AAV.

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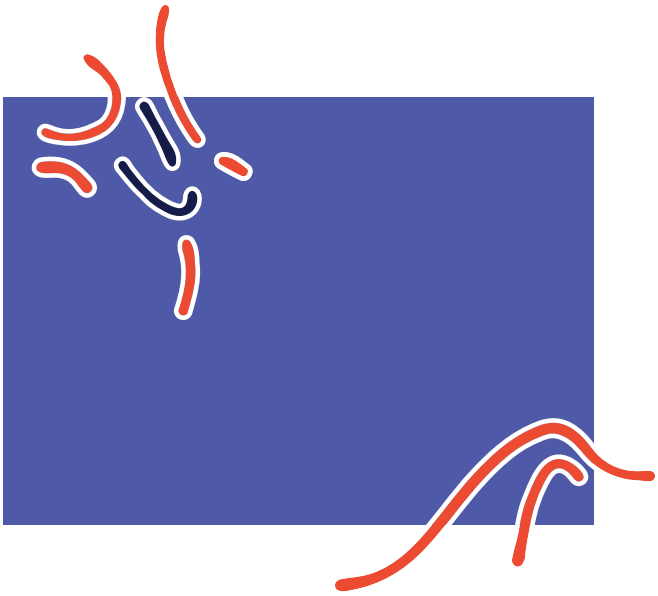
Part 1

Pharmacogenetics

02 Chapter

Review: Gene variants and treatment outcomes in antineutrophil cytoplasmic antibody-associated vasculitis



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ABSTRACT

The introduction of immunosuppressive therapy for ANCA-associated vasculitis (AAV) has greatly improved outcomes, though patients now accumulate damage from vasculitis activity and adverse effects of treatment. Prediction of treatment outcomes using gene variants might help reduce this damage by allowing for personalized treatment. Several studies have studied genetic polymorphisms in relation to treatment outcomes of AAV. This review gives an overview of these studies, discussing both disease modifiers (influencing disease outcomes such as activity, severity and relapse risk) and pharmacogenetics (influencing drug metabolism and/or drug response). Subsequently, potential benefits of testing genetic variants for AAV and the steps needed for its implementation in clinical practice are discussed. The conclusion of this review is that measurement of most polymorphisms is currently not indicated in clinical practice, with the possible exception of thiopurine methyltransferase (TPMT), since homozygous carriers of TPMT variants have a strongly increased risk of severe bone marrow depression.

INTRODUCTION

Over the years, treatment of ANCA associated vasculitis (AAV) has greatly improved. After the introduction of combined therapy using cyclophosphamide (CYC) and high doses of glucocorticoids, it has turned from a lethal disease to a chronic relapsing-remitting condition with much improved patient survival [1,2]. Treatment toxicity was reduced by shortening CYC exposure and switching to azathioprine (AZA) maintenance therapy after stable induction of remission [3]. More recently, the monoclonal anti-CD20 antibody rituximab (RTX) was introduced, both as an alternative to CYC for induction therapy [4], as well as an alternative to AZA for maintenance therapy [5,6].

Despite these advances, patients still accumulate damage. This damage not only results from vasculitis activity, but also from adverse effects of treatment [7,8]. The balance between disease activity and treatment toxicity could potentially be improved using personalized therapy. Studying genetic variation in relation to disease and treatment outcomes might be an interesting approach to achieve this [9]. Some genetic factors predict individual handling and/or sensitivity to a drug (i.e., pharmacogenetics), possibly allowing for individual dosing to achieve maximum efficacy and minimum toxicity. Other genetic factors affect inflammatory pathways and may indirectly affect drug response. These factors might help in guiding drug choice [10].

This review will discuss gene polymorphisms studied in relation to outcomes of currently used treatment in AAV. Some of these polymorphisms are disease modifiers, as they are mainly involved with disease outcomes such as disease activity, severity and risk of relapse. Other polymorphisms are associated with pharmacogenetics, since they affect drug metabolism and/or response to drugs. Subsequently, the likelihood of and steps needed for practical usage of these polymorphisms to improve AAV treatment will be discussed.

Cyclophosphamide (CYC)

CYC has been the main drug used for induction therapy of AAV since its first introduction [1], and is still one of the primary options advocated for active AAV [6]. Unfortunately, CYC use is associated with adverse events such as leukopenia, infections, haemorrhagic cystitis and development of infertility and malignancies.

CYC is a prodrug that requires activation by cytochrome P (CYP)450 enzymes in the liver into the active metabolites 4-hydroxycyclophosphamide and aldophosphamide. Catalysts for this conversion include CYP2B6, CYP2C9, CYP2C19 and CYP3A4 [11]. Several studies performed in systemic lupus erythematosus patients found that the genetic variant CYP2C19*2 (681G>A; rs4244285) was associated with a reduced risk of ovarian toxicity [12-14], possibly at the cost of a reduced clinical response [12]. Two studies have investigated the clinical implications of CYP450 gene variants for ANCA associated vasculitis.

Schirmer et al. studied several gene variants of CYP2C9 and CYP2C19 in relation to treatment outcomes of 196 mostly Caucasian AAV patients treated with CYC. The presence of genetic variant CYP2C9*2 (430C>T; rs1799583) or CYP2C9*3 (1075A>C; rs1057910), as

was the case in 65 (33%) patients, was associated with a higher risk of leukopenia. This difference existed only in patients treated with oral (not intravenous) CYC, and carriers of a CYP2C9 variant (CYP2C9*2 or CYP2C9*3) treated with oral CYC also showed a trend towards a lower risk of refractory disease. CYP2C9*2 and CYP2C9*3 have been shown to result in a slower conversion of CYC to active metabolites [15,16]. This slower activation might result in prolonged exposure to active metabolites, with increased CYC efficacy and toxicity as a result. CYP2C19*2 (681G>A; rs4244285), present in 55 (28%) patients, was not related to clinical endpoints in this study [17].

In a study by Cartin-Ceba et al. based on data from the RAVE trial, including 93 patients treated with CYC, most frequently of Caucasian ethnicity, no associations were found for SNPs of CYP2B6 (1459C>T, rs3211371), present in 16 (17%) patients, or CYP2C19 (681G>A, rs4244285), present in 18 (19%) patients, with time to complete remission. Unfortunately, due to a relatively small sample size, effects of more uncommon SNPs (e.g., CYP2C9) could not be addressed in this study [18].
In conclusion, genetic variants of CYP2C9 may be associated with clinically relevant differences in efficacy and toxicity of CYC. Theoretically, CYP2C9*2 and CYP2C9*3 carriers should receive lower doses of oral cyclophosphamide to compensate for prolonged exposure to its active metabolites.

Azathioprine (AZA)
AZA is currently the main drug used for maintenance therapy of AAV, after disease remission has been attained [6]. The CYCAZAREM trial, published in 2003, showed that switching to AZA after induction of remission with CYC was equally effective as continued CYC therapy for prevention of relapse, allowing for reduced exposure to the cumulative toxic effects of CYC [3].

AZA is a prodrug that is converted enzymatically into 6-mercaptopurine (6-MP). Subsequently, it is converted into 6-thioguanine nucleotides (6-TGN), the active metabolite responsible for the immunosuppressive effects, by the enzyme hypoxanthine phosphoribosyltransferase (HPRT). Two competitive pathways exist that instead convert 6-MP to inactive metabolites. The most well-known is the enzyme thiopurine methyltransferase (TPMT), that converts 6-MP into 6-methylmercaptopurine (6-MMP). The other pathway, Xanthine Oxidase (XO), converts it into thiouric acid (TUA) instead [19]. Several SNPs of TPMT have been described, resulting in reduced activity of the enzyme. Reduced TPMT activity indirectly results in higher levels of 6-TGN [19,20]. Because of a strongly increased risk of myelosuppression resulting from these increased 6-TGN levels, it has been advised that patients homozygous for genetic variants of TPMT either start with a 10-fold reduced dose of AZA or use a different immunosuppressive drug [20]. Patients heterozygous for gene variants of TPMT are advised to start with a 30-70% reduced dose of AZA [20]. In an RCT performed in 2015, dose reduction in patients heterozygous for TPMT variants resulted in a strong reduction of adverse effects in this subgroup of inflammatory bowel disease patients, while maintaining efficacy. Because of the low frequency of TPMT variant carriers, with only 10% of patients in the intervention group requiring dose adjustment, no effect was shown in intention-to-treat analysis [21].

Two studies on TPMT genotype have been performed in AAV patients. Neither study found a significant association of TPMT genotype with adverse effects or efficacy of AZA therapy [22,23]. This indicates that TPMT pretesting might not be as useful for AAV patients as it is for other populations. However, the most recent study showed a trend towards a higher sensitivity to leukocytopenia in patients with lower TPMT activity, as well as a trend towards better relapse-free survival in patients with lower TPMT activity [23]. This suggests that there might be a small effect of TPMT genotype on AZA efficacy and toxicity, and that the sample size of these studies might simply be too small to detect these effects, in particular because of the low frequency (6-10%) of TPMT variant carriers. Of note, neither of these studies included patients that were homozygous for TPMT variants (prevalence approximately 0.3%) [22,23].

In conclusion, adjustment of initial AZA dose may be less relevant for AAV patients carrying one gene variant of TPMT, as long as dose is adjusted based on frequent blood count measurements. This is not to say that TPMT pretesting is irrelevant in AAV, since homozygous variant carriers (1 in 178 to 1 in 3,736 patients) have a strongly increased risk of severe myelosuppression from AZA [20].

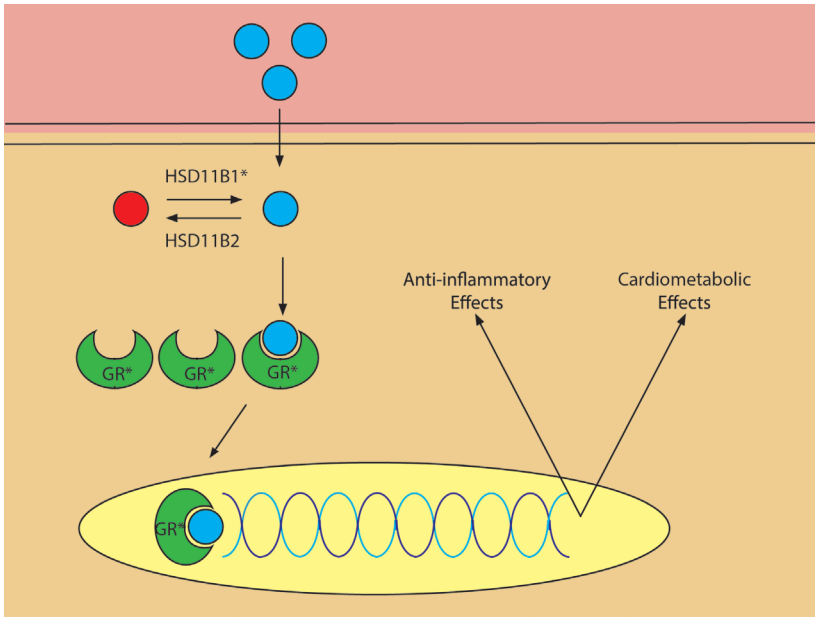
Prednisolone (PRED)

Glucocorticoids, usually PRED, form a standard part of induction and maintenance therapy for AAV [6]. Although effective, PRED treatment in AAV is associated with a wide range of adverse effects affecting, among others, inflammation, the cardiovascular system and glucose, lipid and bone metabolism. Some of these adverse effects contribute to treatment-related mortality [24-26]. A simplified scheme of glucocorticoid action is shown in **Figure 1**. Several gene polymorphisms have been described of the glucocorticoid receptor (GR) and 11β-Hydroxysteroid dehydrogenase type 1 (HSD11B1). To our knowledge, only one observational cohort study has been performed that related gene polymorphisms affecting glucocorticoid sensitivity to relevant clinical outcomes in AAV [27].

Glucocorticoid receptor (GR)

PRED, as well as the endogenous glucocorticoid cortisol, exert most of their effects through interaction with the GR, a member of the nuclear receptor family. This receptor binds its ligand in the cytoplasm and subsequently moves to the nucleus, where it affects gene expression. This results in a multitude of cardiovascular, metabolic and inflammatory effects [28]. Several functional variants of the gene encoding the GR have been identified. Some of them, including N363S (rs6195) and BclI (rs41423247), are associated with increased glucocorticoid sensitivity. Others, such as ER22/23EK (rs6189 and rs6190) and 9β (rs6198), are associated with glucocorticoid resistance [28]. Lastly, TthIII1 (rs10052957) is associated with glucocorticoid resistance mainly through linkage disequilibrium with ER22/23EK [29]. Haplotypes of the GR have been defined based on frequent combinations of SNPs [30]. Genetic variation in the GR has been linked to various cardiometabolic and inflammatory outcomes in the general population [31,32], as well as to disease severity and PRED response in inflammatory conditions such as multiple sclerosis and RA [33-37].

Figure 1. Simplified scheme of glucocorticoid action.



Cortisol/prednisolone (blue) enters a cell through the cell membrane. Subsequently, it can be converted to inactive cortisone/prednisone (red) by hydroxysteroid dehydrogenase 11β type 2 (HSD11B2), or reactivated by hydroxysteroid dehydrogenase 11β type 1 (HSD11B1). Cortisol/prednisolone binds the glucocorticoid receptor (GR) in the cytoplasm. After binding, the complex moves to the nucleus where it affects expression of genes related to inflammation, volume homeostasis and carbohydrate, lipid and protein metabolism. Gene polymorphisms of proteins marked with "" are discussed in this review.*

In an observational cohort study involving 241 patients from our center, two haplotypes of the GR were related to relevant clinical outcomes in AAV. Haplotype 4 (ER22/23EK+9β+TthIII1), present in 6% of patients, was associated with an increased risk of end-stage renal disease as well as an increased risk of mortality, suggesting that patients with this haplotype have a more severe disease phenotype. Homozygous carriers of haplotype 1 (BclI), entailing 6% of patients, had an increased risk of developing dyslipidemia, suggesting a less favourable metabolic phenotype in these patients. These differences existed despite similar glucocorticoid exposure in all haplotypes [27].

11β-Hydroxysteroid dehydrogenase type 1 (HSD11B1)

HSD11B1 is an enzyme present in most cells of the body that regulates local glucocorticoid levels. In vivo, it mainly converts cortisone to its active metabolite cortisol [38]. Based on findings from an earlier study, the A variant of rs11119328 SNP was hypothesized to be associated with reduced expression of HSD11B1 compared to the C variant [39].

In a cohort study of 241 AAV patients, the A variant of rs11119328 was associated with an increased risk of relapse in AAV despite similar glucocorticoid exposure to non-carriers of this variant, but only in non-carriers of Haplotype 1 of the GR. This combination exists in 19% of patients. These findings suggest that reduced local activation of glucocorticoids as a result of rs11119328 A results in a pro-inflammatory phenotype, but can be compensated for by increased sensitivity of the GR. Interestingly, glucocorticoid exposure did not differ between carriers and non-carriers of rs11119328 [27].

Overall conclusions on SNPs in PRED treatment

Two haplotypes of the GR (Haplotypes 1 and 4) and one SNP of HSD11B1 (rs11119328) were associated with relevant clinical outcomes of AAV in one study. After confirmation of the effects in an independent cohort, it might be interesting to investigate adjustment of treatment. For homozygous Haplotype 1 carriers this would entail reduction of glucocorticoid exposure, since these patients are apparently at increased risk of developing metabolic adverse events from PRED. For Haplotype 4 carriers, research on early aggressive treatment to prevent end-stage renal disease and subsequent mortality might be interesting. Carriers of rs11119328 without Haplotype 1 might benefit from prolonged maintenance therapy of glucocorticoids with or without another immunosuppressive drug, to compensate for the increased relapse risk when receiving standard treatment.

Rituximab (RTX)

RTX is a chimeric immunoglobulin 1 (IgG1) monoclonal antibody targeting CD20, present on B-cells. It is a relatively new drug for remission induction in AAV. The Rituximab in ANCA-Associated Vasculitis (RAVE) trial, published in 2010, showed it to be non-inferior to CYC [4,40]. More recently, the results of the Maintenance of Remission using Rituximab in Systemic ANCA-associated Vasculitis (MAINRITSAN) trial indicated that RTX might be superior to AZA for maintenance of remission after CYC induction [5,41].

As RTX is a monoclonal antibody targeting a specific inflammatory pathway, response to RTX is mainly influenced by genetic variation in its effector pathways rather than its metabolic pathway. Three studies have been performed investigating disease-modifying gene variants and their association with outcome of RTX treatment [18,42,43].

Fcγ receptor (FcγR)

RTX is capable of depleting B-cells through complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC) by binding of its Fc fragment to Fcγ receptors (FcγR). The affinity of FcγR is affected by genetic variation and potentially influences RTX response. An FcγRIIIa SNP has previously been associated with clinical response to RTX in rheumatoid arthritis (RA) patients [44,45], as well as other auto-immune diseases [46,47].

In a study using data from the RAVE trial, which included 96 mostly Caucasian patients receiving RTX treatment, the 27 (28%) homozygous carriers of the FcγRIIa 519 AA genotype (rs1801274), associated with increased receptor affinity for IgG3 and capability of binding IgG2, had a shorter time to complete remission compared to the GG and GA variants. Interestingly, this association was independent of the induction treatment used, as it was also present in the CYC-treated group. The combination of FcγRIIa 519

(rs1801274) AA and FcγRIIIa 559 (rs396991) GG genotypes was associated with a higher frequency of disease remission, again irrespective of induction treatment used [18]. These results were in line with an earlier study showing that the combination FcγRIIa 519 GG + FcγRIIIa 559 TT was associated with an increased risk of relapse in GPA [48]. Based on the results of these studies, the FcγRs seem to play a role in AAV pathogenesis. One hypothesis is that genetic variation in FcγRIIa and FcγRIIIa affects the binding of ANCA to these receptors, resulting in increased ANCA clearance in those carrying FcγRIIa 519 GG + FcγRIIIa 559 TT [18,48]. Another hypothesis is that FcγRIIa 519 GG + FcγRIIIa 559 TT reduces clearance of staphylococcus aureus, resulting in chronic nasal carriage as a risk factor for relapse [48,49]. Alberici et al. did not find a relation of rs1801274 or rs396991 with treatment response, although they did not analyse homozygous variant carriers separately [42].

B cell activator of the tumor necrosis factor family (BAFF)

B cell activator of the tumor necrosis factor family (BAFF) is a cytokine relevant for B-cell development and survival. In auto-immune diseases, it is thought to promote inflammation by stimulating survival and proliferation of autoreactive B cells [50]. BAFF levels in AAV patients are increased compared to healthy controls. In contrast to other auto-immune diseases, BAFF levels showed no positive correlation with auto-antibody levels in AAV [51-53]. The rs9514828 SNP of BAFF has previously been associated with RTX response rate in RA [54].

In a European cohort of 213 AAV patients investing a panel of 18 candidate SNPs potentially associated with response to RTX, the B-cell activating factor (BAFF) SNP rs3759467 was associated with shorter time to relapse within 12 months [42]. This finding was confirmed in a replication cohort of 109 patients from the United Kingdom, but existed only in homozygous carriers of the C variant allele (n=7, or 2% of combined cohort). Besides a shorter time to relapse within 12 months after first RTX infusion, patients with the CC genotype of rs3759467 had a higher rate of detectable peripheral B-cells and less reduction of IgM levels compared to TC and TT genotypes. The association was not present in MPO-ANCA positive patients, although this might be due to the limited sample size of the study and the low frequency of homozygous variant carriers. The study did not show any association of other BAFF-related SNPs such as rs9514828 with clinical response in AAV. The exact mechanism behind the effects of the rs3759467 SNP are not yet clear. Most likely, the SNP affects B-cell survival, possibly through an increase in BAFF levels [42]. Hypothetically, patients with the rs3759467 SNP might benefit from addition of the monoclonal antibody belimumab, which blocks binding of BAFF to B-cell receptors [55].

Interleukin-6

Interleukin-6 (IL-6) is a cytokine with a wide range of functions. In the immune system, it is important for inducing the acute phase response and has a role in B-cell maturation and plasma cell proliferation. In auto-immune disease, it contributes to on-going inflammation through stimulation of immune cell proliferation and activation of lymphocytes. It also skews development of naïve T-cells towards T helper (T_H)17 cells rather than forkhead box protein 3 positive regulatory T (FOXP3+ T_{reg}) cells [56]. In RA, being homozygous for the -174 (rs1800795) C variant of the IL-6 promoter region has been associated with poor response to RTX [57].

In 2012, Robledo et al. conducted an observational study investigating the -174 (rs1800795) SNP in a Spanish Caucasian cohort of 144 patients with varying systemic auto-immune diseases, including 16 AAV patients. They found that homozygous carriers of the C variant of this SNP (9% of patients) had a significantly higher risk of non-response to RTX compared to carriers of CG and GG variants [43]. Alberici et al. did not find such an association in their study of 213 European AAV patients, although they did not analyse homozygous carriers of the C variant of rs1800795 separately [42].
The functional relation between this IL-6 SNP and RTX response is not entirely clear. A feasible hypothesis is that the reduced efficacy of RTX in -174 C homozygotes results from improved B-cell survival mediated by IL-6. An association of -174 genotype with serum IL-6 levels was not found, although a great inter-individual variation was noted [57]. Theoretically, -174 C homozygotes might benefit from addition tocilizumab to treatment, as this drug blocks the IL-6 receptor [58].

IL-2–IL-21 region

The rs6822844 SNP in the IL-2–IL-21 region on chromosome 4 has previously been associated with risk of autoimmunity and response to rituximab therapy [59]. IL-2 induces T-cell proliferation and promotes differentiation of regulatory T-cells while inhibiting Th17 differentiation and inducing activation-induced cell death [60]. IL-21 has a broad range of effects on the immune system, including both stimulatory and regulatory effects. Several auto-immune diseases have been associated with increased levels of IL-21 [61].

When specifically analysing MPO-ANCA positive patients(n=29), Alberici et al. found an association of SNP rs6822844 in the IL-2–IL-21 area (minor allele frequency 0.127) with risk of relapse within 6 months and time to relapse within 12 months [42]. The mechanism behind this might be that the G variant of rs6822844 promotes ADCC through IL2-mediated stimulation of natural killer (NK) cell proliferation and cytotoxicity [59,60]. The finding was not confirmed in a smaller replication cohort (n=19), although this could be due to a lack of statistical power, as there was a trend towards a better treatment response in carriers of the G variant of rs6822844 [42].

Overall conclusion on SNPs in RTX treatment

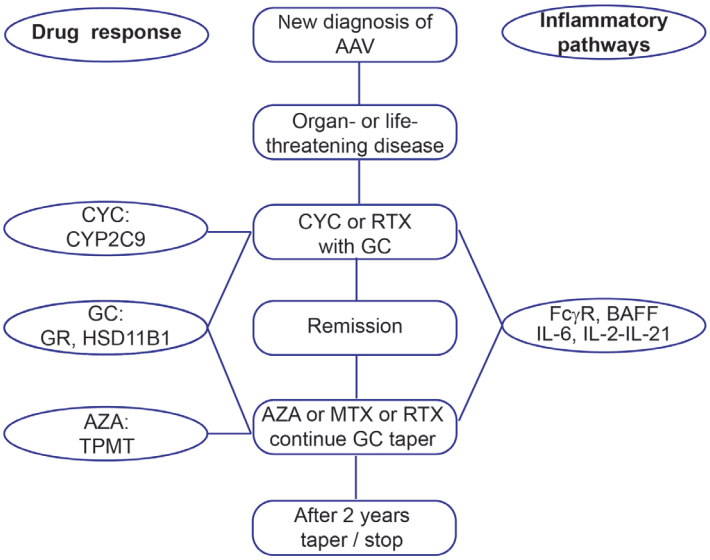
In conclusion, the SNPs rs1801274 (FcγRIIa), rs396991 (FcγRIIIa), rs3759467 (BAFF), rs1800795 (IL-6) and rs6822844 (IL-2 – IL-21) all appear to affect response to RTX treatment. All studied polymorphisms are related to effector pathways rather than drug metabolism, contrary to the gene variants studied in relation to CYC and AZA treatment. Therefore, these effects are not specific to RTX treatment and affect treatment efficacy more than toxicity. In some cases, such as SNPs related to BAFF and IL-6, it might be interesting to study whether patients with specific gene variants might benefit from addition of therapy specifically targeting the respective factor, such as belimumab (targeting BAFF signalling) and tocilizumab (targeting the IL-6 receptor). This would first require confirmation of increased BAFF or IL-6 signalling in patients with the respective genotypes.

Other genetic associations with outcomes of ANCA-associated vasculitis
To our knowledge, no studies have been performed in AAV patients of gene variants in relation to efficacy and toxicity of methotrexate or mycophenolate mofetil. Several other genetic variants, not related to a specific treatment, have been associated with clinical outcomes of AAV. Examples include gene polymorphisms of monocyte chemoattractant protein-1 (MCP-1) [62], alpha-1 antitrypsin [63] and human leukocyte antigen (HLA)-DPB1 [64]. Except for TPMT, none of the gene variants currently has practical relevance in AAV treatment.

DISCUSSION

Several observational studies have been performed regarding genetic factors associated with response to cyclophosphamide (CYC), rituximab (RTX), prednisolone (PRED) and azathioprine (AZA) therapy in AAV. An overview of proteins encoded by potentially relevant SNPs per drug used in AAV treatment is shown in **Figure 2**. The main results per study are summarized in **Table 1** and **Table 2**.

Figure 2. Treatment of ANCA-associated vasculitis and potential targets for personalized treatment



AZA azathioprine; BAFF B cell activator of the tumor necrosis factor family; CYC cyclophosphamide; CYP cytochrome P; FcγR Fcγ receptor; GR glucocorticoid receptor; HSD11B1 11β-hydroxysteroid dehydrogenase type 1; IL interleukin; MMF mycophenolate mofetil; MTX methotrexate; PRED prednisolone; RTX rituximab.

Table 1. Overview of drug metabolism-related gene polymorphisms in relation to clinical outcomes in ANCA-associated vasculitis

Drug/gene	Participants (ethnicity)	MAF	Efficacy	Toxicity	Reference
CYC					
CYP2C9	196 (96% Caucasian)	0.171	Trend: less refractory disease in carriers	Higher risk of leukopenia in carriers	[17]
CYP2C19	196 (96% Caucasian)	0.153	NS	NS	[17]
	93 (97% Caucasian)	0.108	NS	NS	[18]
CYP2B6	93 (97% Caucasian)	0.091	NS	NS	[18]
AZA					
TPMT	108 (Caucasian)	0.032	NS	NS	[22]
	207 (Caucasian)	0.046	NS	NS	[23]
PRED					
GR Haplotype 1	241 (Caucasian)	0.247	No increased relapse risk from HSD11B1	Homozygous: more dyslipidaemia	[27]
GR Haplotype 4	241 (Caucasian)	0.031	Higher risk of ESRD and mortality	NS	[27]
HSD11B1	241 (Caucasian)	0.190	Higher risk of relapse in carriers	NS	[27]

AZA azathioprine; CYC cyclophosphamide; CYP Cytochrome P; ESRD end-stage renal disease; GR glucocorticoid receptor; MAF minor allele frequency; NA not analysed; NS no significant differences; PRED Prednisolone; TPMT thiopurine methyltransferase.

Table 2. Overview of inflammation-related gene polymorphisms in relation to clinical outcomes in ANCA-associated vasculitis

Drug/gene	Participants (ethnicity)	MAF	Efficacy	Toxicity	Reference
BAFF	213 (Europe), 109 (UK)	0.172	Homozygous: higher risk of and shorter time to relapse	NA	[41]
Complement C1QA	213 (Europe), 109 (UK)	0.380	NS	NA	[41]
FCγR IIA	96 (93% Caucasian)	0.500	Shorter time to complete remission in homozygous	NA	[18]
	213 (Europe), 109 (UK)	0.495	NS	NA	[41]
FCγR IIB	213 (Europe), 109 (UK)	0.110	NS	NA	[41]
	96 (93% Caucasian)	0.104			[18]
FCγR IIIA	213 (Europe), 109 (UK)	0.439	NS	NA	[41]
	96 (93% Caucasian)	0.280	NS	NA	
IL-2-IL-21	213 (Europe), 109 (UK)	0.127	MPO-AAV: less relapse, longer time to relapse (not in UK cohort)	NA	[41]
IL-6	213 (Europe), 109 (UK)	0.359	NS	NA	[41]
	144 (Caucasian), 16 AAV	0.306	More non-response in homozygous carriers	NA	[42]
NFKB1	213 (Europe), 109 (UK)	0.383	NS	NA	[41]
TGFB1	213 (Europe), 109 (UK)	0.090	NS	NA	[41]

BAFF B cell activator of the tumor necrosis factor family; FcγR Fcγ receptor; IL-6 Interleukin-6; NA not analysed; NS no significant differences

While pharmacogenetics in the case of synthetic immunosuppressive drugs such as CYC, AZA and PRED involves mainly genetic variation in metabolic or functional pathways of these drugs, potentially allowing individual dosing of these drugs, genetic variants studied in relation to RTX response are mainly involved in inflammatory pathways. The latter may not necessarily be specific to RTX-treatment, as was seen for gene variants of the FcγR [18,48]. On the other hand, the inflammatory pathways identified might be interesting for targeted therapy, especially considering the large variety of targeted drugs currently available.

Theoretically, pretesting SNPs will help improve prediction of efficacy and toxicity of drugs used for AAV treatment. Treatment dose or modality could be adjusted in carriers of a genetic variant in order to optimize treatment outcomes for these patients. As a result, toxicity and healthcare costs would be reduced for these patients, especially considering the reduction of genotyping costs over recent years. Ideally, treatment modality and dose for the individual patient would become partly based on a panel of relevant SNPs.

Despite the theoretical benefits of applying genetic testing, several factors hinder (immediate) application of the reviewed findings to clinical practice.

Firstly, except for one study [42], none of the studies have used replication cohorts. Also, most of the studies do not mention correction for multiple comparisons. Therefore, in order to exclude type I errors (i.e., false-positive findings), the study results should be confirmed in replication cohorts.

Secondly, most of these studies have been performed in Caucasian patients. The frequency of genetic polymorphisms is known to vary greatly between ethnicities. Therefore, an important and frequent SNP in one population might be much less relevant in another, limiting the external validity of pharmacogenetics studies to the ethnic group in which the studies were performed. To illustrate, Black and Hispanic lupus nephritis patients respond better to mycophenolate mofetil (MMF) than CYC, while MMF and CYC are equally effective in Asian and Caucasian patients [65].

Thirdly, the studies deal with genetic variants that occur only in a minority of patients. This means that large sample sizes will be required to detect relevant differences in clinical endpoints associated with these variants. Furthermore, all patients need to be genotyped to find the few patients that might benefit from adjustment of therapy. As AAV is a rare disease group, the required sample size will often be difficult to achieve, especially in a single-center setting.

Lastly, all of these studies were small observational cohort studies. In order to find the right dose adjustment or alternative drug and to confirm that this will achieve the desired improvement in treatment outcomes, randomized clinical trials (RCTs) should be performed. These studies should use relevant clinical outcomes to evaluate adjustment of therapy based on gene variants. Again, these studies require large sample sizes because of the large number of patients needed to genotype to find a patient requiring adjustment of therapy and the even larger number needed in to prevent occurrence of

an adverse effect. To illustrate, a large multi-center RCT investigating thiopurine dose reduction in inflammatory bowel disease patients carrying TPMT gene variants (the TOPIC trial) did not find a reduction of thiopurine toxicity for the intervention group in intention-to-treat analysis, even though a large reduction of toxicity was found within TPMT variant carriers. This is most likely because the majority of patients (90%) had a normal TPMT genotype and received a normal thiopurine dose even in the intervention group [21]. Because of the 10-fold increased risk of severe myelosuppression in the occasional homozygous variant carrier [20], TPMT pretesting may be relevant regardless. Because of small effects per genetic variant, combined with the low prevalence of both these gene variants and AAV, it might be most feasible to design one RCT in which multiple gene variants are measured simultaneously to inform treatment adjustment in the intervention group.

The studies reviewed in this paper have identified some interesting genetic variants that might be used to improve treatment outcomes after the previously mentioned hurdles have been overcome. On the other hand, based on the strength of current data, further confirmation of findings is needed before an RCT should be performed. Additional useful SNPs might be discovered by using data from genome-wide association studies, such as the one performed by Lyons et al. [66]. To illustrate, a GWAS study performed in RA patients identified several genetic variants associated with response to anti-TNF therapy [67]. The association of these gene variants with relevant clinical outcomes may be tested by genotyping samples from previously performed multi-center clinical trials. The on-going PEXIVAS trial, for example, may provide interesting samples and data to this end [68].

In summary, several studies have been performed regarding genetic polymorphisms in relation to efficacy and toxicity of CYC, RTX, PRED and AZA for treatment of AAV. Several challenges will need to be overcome for clinical application of these results, including independent replication of findings, identification of relevant SNPs per ethnic group, performing large enough multi-center studies to detect relevant clinical effects of SNPs that are present in a minority of patients, and performing large multi-center RCTs to evaluate personalized therapy based simultaneously on multiple SNPs to improve outcomes in this small group. Additional polymorphisms may be identified using GWAS data or samples from previously performed multi-center clinical trials.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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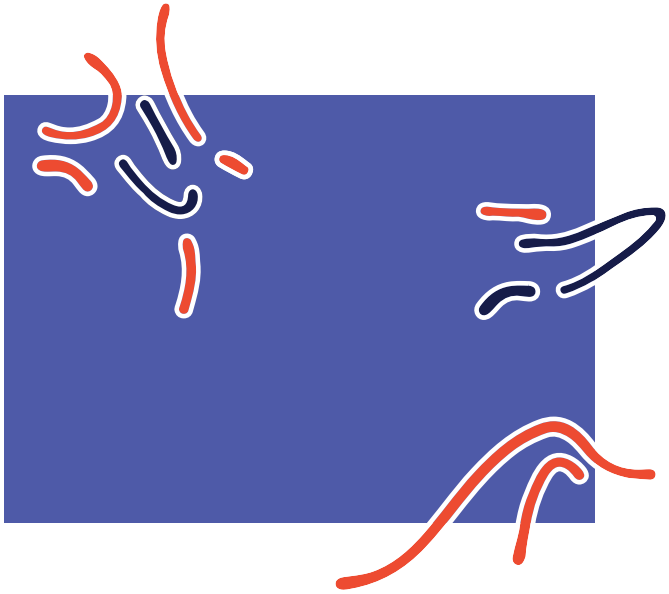
03 Chapter

Thiopurine methyltransferase genotype and activity cannot predict outcomes of azathioprine maintenance therapy for anti-neutrophil cytoplasmic antibody-associated vasculitis: a retrospective cohort study



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ABSTRACT

Objective
Azathioprine is a widely used immunosuppressive drug. Genetic polymorphisms and activity of the enzyme thiopurine methyltransferase (TPMT) have been associated with azathioprine efficacy and toxicity in several populations. We investigated whether these associations also exist for ANCA associated vasculitis (AAV) patients, who receive azathioprine maintenance therapy after remission induction with cyclophosphamide.

Methods
207 AAV patients treated with cyclophosphamide induction and azathioprine maintenance therapy were included and followed for 60 months. TPMT genotype and tertiles of TPMT activity were compared to relapse free survival and occurrence of adverse events, particularly leukopenia. Multivariable regression was performed to account for confounders.

Results
In univariable analysis, relapse free survival was not significantly associated with TPMT genotype (P = 0.41) or TPMT activity (P = 0.07), although it tended to be longer in lower tertiles of TPMT activity. There was no significant association of TPMT genotype and activity with occurrence of any adverse event. In multiple regression, leukocyte counts at the end of cyclophosphamide induction were related to risk of leukopenia during azathioprine therapy [P<0.001; OR 0.54 (95% CI 0.43±0.68)] and risk of relapse during follow-up [P = 0.001; HR 1.17 (95% CI 1.07±1.29)] irrespective of TMPT genotype or activity.

Conclusion
TPMT genotype and activity were not independent predictors of relapse, and could not predict leukopenia or other adverse effects from azathioprine. Leukocyte counts after cyclophosphamide induction were related to both outcomes, implying a greater influence of cyclophosphamide response compared to azathioprine and TPMT in AAV patients.

INTRODUCTION

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) refers to a group of primary small-vessel vasculitides. The most common forms are granulomatosis with polyangiitis (GPA, formerly Wegener's Granulomatosis) and microscopic polyangiitis (MPA).[1] Induction treatment with cyclophosphamide, rituximab or mycophenolate mofetil combined with corticosteroids can achieve remission in most AAV patients and reduce mortality, but is associated with considerable toxicity.[2-4] For this reason, patients switch to less toxic maintenance therapy after achieving remission, most frequently azathioprine.[3-5] Even with azathioprine maintenance therapy, there is a risk of potentially severe adverse effects, most frequently leukopenia and infection.[5,6] In recent years, there has been increasing interest in personalised medicine whereby treatment is adjusted based upon characteristics of an individual patient, thereby optimizing efficacy and reducing toxicity.

Azathioprine and its metabolite 6-mercaptopurine are converted via several enzymatic steps into 6-thioguanine nucleotides (6-TGN), the active metabolites responsible for the immunosuppresive effect and myelotoxicity. The enzyme thiopurine methyltransferase (TPMT) methylates several metabolites along the enzymatic pathway, thereby reducing the amount of 6-TGN formed.[7,8] Several polymorphisms of the gene encoding TPMT have been identified, each resulting in decreased activity of the enzyme.[7-9] Approximately 89% of Caucasians are homozygous for wildtype TPMT alleles corresponding with normal or high activity.11% carry a wildtype and a variant allele corresponding with intermediate TPMT activity. Very few individuals (0.3%) are homozygous or compound heterozygous for variant alleles resulting in absence of TPMT activity.[8,10]

Studies in several populations, mainly inflammatory bowel disease (IBD), have shown that TPMT variant alleles and lower TPMT activity are associated with a higher risk of bone marrow toxicity.[7,11-13] Patients carrying two variant TPMT alleles are especially at risk for severe myelotoxicity[11] and require either a 10-fold lower dose or alternative therapy (e.g. methotrexate).[6,8,14] For patients with intermediate TPMT activity carrying one variant allele, more controversy exists. Several meta-analyses have shown an increased risk of myelotoxicity in these patients.[12,15] While clinical trials did not find a significant reduction of toxicity when adjusting azathioprine or 6-mercaptopurine dose on TPMT genotype or activity, [14,16,17] post-hoc analysis showed a significant reduction of myelotoxicity within carriers of a variant allele.[14]

The aforementioned studies mainly involve patients with IBD, who receive azathioprine as their main treatment drug. Since AAV patients receive azathioprine after an induction phase with cyclophosphamide,[4] the influence of TPMT might be smaller and less relevant in this population.

The aim of this study was to see whether TPMT genotype and activity are associated with bone marrow toxicity and risk of relapse in AAV patients treated with azathioprine maintenance therapy. We expanded on an earlier study in our population[18] by taking into account the influence of cyclophosphamide induction therapy on these outcomes.

PATIENTS AND METHODS

Patients

For this retrospective cohort study, 377 patients, diagnosed with GPA, MPA or Renal Limited Vasculitis (RLV) between September 1984 and August 2013 in the University Medical Center Groningen (UMCG) and treated with oral cyclophosphamide following diagnosis, were considered for inclusion. Patients were included if they switched to azathioprine after induction of remission and had a follow-up of at least a year. All patients have given written informed consent according to the Declaration of Helsinki for participation in a large cohort study investigating biomarkers (including TPMT) in relation to disease outcome in AAV. Ethical approval for the study was granted by the local Medical Ethical Committee of the University Medical Center Groningen (NL29354.042.10).

Treatment protocol

Following diagnosis, all patients were treated with oral cyclophosphamide (1.5±2.0 mg/kg/ day) combined with prednisolone (1mg/kg/day, max 60mg/day). Prednisolone dose was reduced according to a standard schedule (**S1 Table**). After 3 months of stable remission, all patients switched to maintenance therapy with azathioprine. The starting dose was a conversion from cyclophosphamide dose to the same azathioprine dose. The target azathioprine dose was 1.5±2.0mg/kg/day. Starting 12 months after diagnosis, azathioprine dose was reduced by 25 mg/day every 3 months. Leukocyte counts were measured 1 week after starting azathioprine and at least every 4 weeks thereafter. During treatment,cyclophosphamide and azathioprine dose were adjusted based on leukocyte counts (goal: leukocytes $\geq 4.0*10^9$/l) in accordance with the CYCAZAREM protocol,[5] and occurrence of infections.

Data collection

All information was collected from the patients' records. For all patients, demographic, disease and treatment characteristics, as well as clinical outcome data were registered. Diagnosis was based on the 2012 Chapel Hill Consensus Conference definitions. [1] Disease activity at diagnosis was scored using the Birmingham Vasculitis Activity Score 1 (BVAS-1).[19] Patients were screened for the presence of ANCA using indirect immune fluorescence (IIF), and ANCA-specificity was determined using ELISA.

The primary endpoints of the study were relapse-free survival in months and leukopenia. Relapse was defined as new or worsening disease activity requiring dose increase or switch of immunosuppressive medication. Leukopenia was defined as leukocyte count $<4.0*10^9$/l.[20] Secondary categorical endpoints were moderate leukopenia (leukocyte count $<3.0*10^9$/l), [20] macrocytic anemia (Hb <7.5 for females and <8.0 for males; MCV>96fl), hepatotoxicity (ASAT and/or ALAT >2x upper limit of normal, or AF >125 U/l), infection (requiring hospitalisation and/or antibiotics, or opportunistic e.g. CMV, VZV, HSV, and/or pneumocystis jirovecii pneumonia). These endpoints were scored if they occurred at any time during azathioprine therapy. Secondary continuous endpoints were leukocyte counts 3,6,9 and 12 months after switch to azathioprine, and the [leukocyte($*10^9$/l)]*[azathioprine(mg/kg/d)] product 3,6,9 and 12 months after switch as a measure of sensitivity for azathioprine-induced bone mar-

row depression.[18] Diagnosis, ANCA specificity, age at diagnosis, baseline serum creatinine, co-trimoxazole use at switch to azathioprine (none, prophylactic or therapeutic dose), leukocyte count at switch and duration of azathioprine therapy were registered for their potential influence on relapse. Factors registered for their potential influence on risk of leukopenia include prednisolone dose at switch, cyclophosphamide dose at switch, leukocyte count at switch and azathioprine dose at switch. Prednisolone dose during azathioprine therapy was registered to account for its influence on leukocyte counts.

Measurement of TPMT genotype and TPMT activity

Four variants of the TPMT gene, located on chromosome 6, were determined using PCR, as described by Yates et al.[9] The genetic variants were TPMT*2 (G→C translocation at nucleotide 238), TPMT*3A (460G→A and 719A→G), TPMT*3B (460G→A), and TPMT*3C (719A→G).

TPMT activity was determined by adding 6-thioguanine to human erythrocytes in vitro, and measuring the amount of 6-methylthioguanine formed (TPMT catalyses this reaction), expressed in nmol 6-methylthioguanine formed per gram haemoglobin per hour (nmol/gHb/ hr).[21] In the majority of patients (67%), TPMT genotype and activity were measured after starting azathioprine treatment. In some patients (33%), these were measured before starting azathioprine. The date of blood withdrawal for TPMT measurement was registered for all patients.

Statistics

Statistical analysis was done using SPSS Statistics 22 (IBM Corporation, New York, US).

Data are shown as median + interquartile range (IQR) or number + percentage. A twosided $P<0.05$ was considered statistically significant. Univariate analysis was performed for TPMT genotypes and tertiles of TPMT activity (tertiles determined based on equal numbers of patients per group) using a Log Rank test for relapse free survival (up to 60 months after diagnosis), Fisher's exact test or Chi Square test for risk of adverse events, and Mann-Whitney or Kruskal-Wallis test for leukocyte count and [leukocyte]*[azathioprine] product. Multivariate analysis was performed with relapse-free survival, risk of leukopenia and leukocyte counts as outcome variable, using Cox regression, logistic regression and linear regression, respectively. Possible predictors in the analysis were TPMT genotypes, tertiles of TPMT activity and potential influencing factors for the respective outcomes mentioned under `data collection'. A forward stepwise model was used, where variables were included as covariates based on a univariate $P<0.05$ and excluded on a multivariate P-value >0.10. Non-proportional hazards for predictors in Cox regression were accounted for by adding a time-by-predictor interaction variable to the model.[22]

RESULTS

Patients and TPMT genotype and activity

207 patients were included in the analysis.(**Figure 1**) Demographic and disease characteristics, as well as distribution of TPMT genotypes and TPMT activity, are shown in **Table 1**.

Figure 1. Flow-chart of patient selection

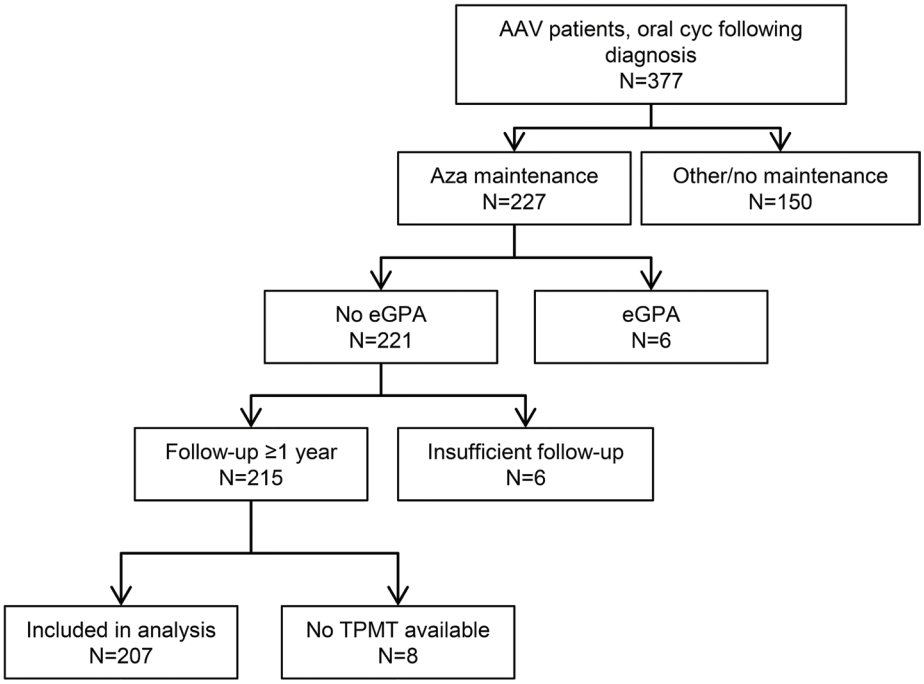


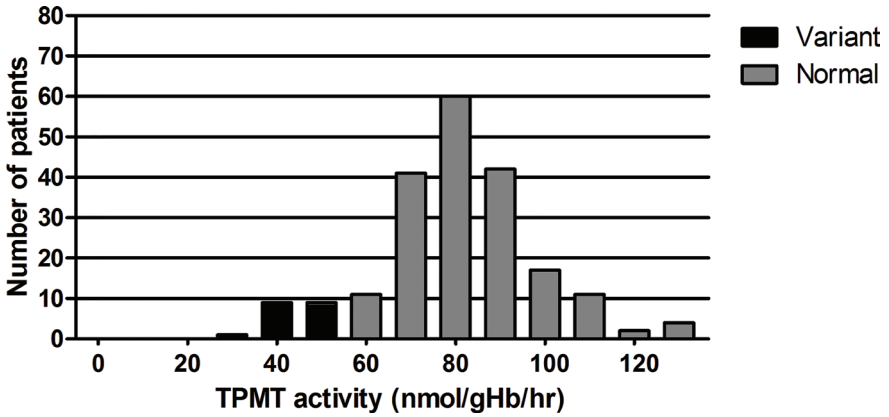
Table 1. Patient Characteristics

Characteristics	**N (%)/mean (SD)/ median (IQR)**
Female	94 (45%)
Age at diagnosis (years)	57 (46-66)
Diagnosis GPA	152 (73%)
PR3-ANCA	150 (73%)
BVAS at diagnosis	18 (13-24)
Serum creatinine at baseline (mg/dl) (n=182/207)	1.24 (0.89-2.84)
Leukocyte count at switch (*10 9 /l) (n=187/207)	6.6 (5.5-8.3)
Co-trimoxaxole use at switch (n=194/207)	
• None	41 (21%)
• Prophylactic dose (480 mg/day)	130 (67%)
• Therapeutic dose (1920 mg/day)	23 (12%)
Cyc start dose (mg/kg/day) (n=191/207)	1.7 (0.4)
Duration of cyc therapy (months) (n=206/207)	5 (4-6)
Prednisolone switch dose (mg/day)(n=188/207)	12.5 (8.1-20.0)
Azathioprine switch dose (mg/kg/day) (n=195/207)	1.4 (0.5)
Duration of azathioprine therapy (months) (n=204/207)	17 (7-24)
Follow-up time (months)	54 (32-60)
TPMT genotype	
• No variant (*1/*1)	188 (91%)
• TPMT *1/*3A	16 (8%)
• TPMT *1/*3C	3 (1%)
TPMT activity (nmol/gHb/hr)	80.0 (17.9)

GPA granulomatosis with polyangiitis; MPA microscopic polyangiitis; RLV renal limited vasculitis; PR3 proteinase 3; MPO myeloperoxidase; BVAS Birmingham Vasculitic Activity Score; Cyc cyclophosphamide; TPMT thiopurine methyltransferase

TPMT activity approximated a Gaussian distribution (**Figure 2**). TPMT activity was significantly lower in carriers of TPMT*3A (43.9; IQR 40.6±49.5 nmol/gHb/hr) and TPMT*3C (43.5 nmol/gHb/hr) compared to patients with a homozygous normal genotype (81.4; IQR 73.5± 92.2) nmol/gHb/hr)(P<0.001). TPMT activity was divided in tertiles based on the number of patients. The lowest tertile (T1) contains patients with TPMT activity ≤74.5, the second tertile (T2) contains patients with TPMT activity 74.6±86.4 and the highest tertile (T3) contains patients with TPMT activity ≥86.5 nmol/gHb/hr. None of the characteristics differed between TPMT genotypes or tertiles of TPMT activity, except azathioprine dose at switch, which was significantly lower in patients with heterozygous TPMT genotype (1.0, IQR 0.7±1.4 mg/kg/ day) compared to patients with normal genotype (1.5, IQR 1.1±1.8 mg/kg/day) (P = 0.001). Azathioprine starting dose was not significantly related to measurement of TPMT status prior to (n = 68) or after (n = 139) start of azathioprine therapy (P = 0.92), even when specifically analyzing patients with a heterozygous TPMT genotype (P = 0.28). As expected from the treatment protocol, azathioprine starting dose showed a strong positive correlation with cyclophosphamide dose at switch (Rho = 0.70, P<0.001). No patients were treated with 6-mercaptopurin.

Figure 2. Distribution of TPMT activity

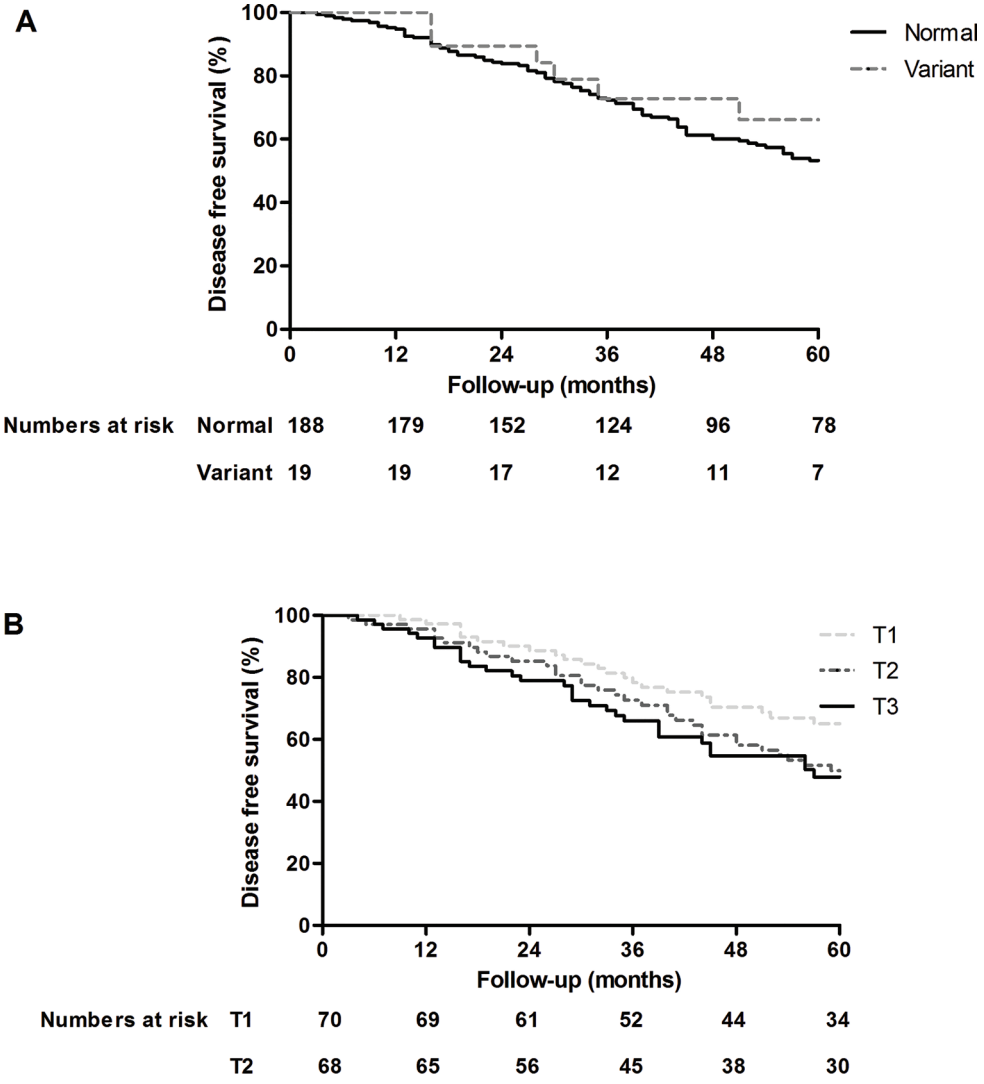


TPMT activity in nmol/gHb/hr for patients with normal (gray) and heterozygous (black) TPMT genotype.

Relapse free survival

Within 5 years after diagnosis, 6 of 19 patients (32%) with heterozygous TPMT genotype experienced a relapse, compared to 84 of 188 patients (45%) with normal TPMT genotype. There was no significant difference in relapse-free survival (P = 0.30) between TPMT genotypes (**Figure 3A**). Tertiles of TPMT activity showed a negative trend with relapse-free survival (P = 0.07) (**Figure 3B**). In the lowest tertile (T1), 34% of patients experienced relapse within 5 years, compared to 47% in the middle tertile (T2) and 50% in the highest tertile (T3). Relapse free survival was still not significantly related to TPMT genotype (P = 0.39) and tertiles of TPMT activity (P = 0.21) after exclusion of patients intolerant to azathioprine.

Figure 3. Relapse free survival for TPMT genotypes and tertiles of TPMT activity



Relapse free survival after start of cyclophosphamide induction therapy. Upper graph (3A): Variant = heterozygous TPMT variant carrier, Normal = normal genotype (wildtype TPMT). Lower graph (3B): T1 = lowest tertile, T2 = middle tertile, T3 = highest tertile of TPMT activity.

In Cox regression, TPMT genotypes (P = 0.39), tertiles of TPMT activity (P = 0.24), co-trimoxazole use (P = 0.15), age (P = 0.69) and diagnosis (P = 0.94) were not significantly related to the occurrence of relapse. ANCA specificity (P = 0.003), duration of azathioprine therapy (P<0.001), serum creatinine at baseline (P = 0.007) and leukocyte count at

switch (P = 0.001) were significantly associated with relapse. Risk of relapse was higher for PR3-ANCA positive patients (Hazard Ratio (HR)3.1; 95% CI 1.5±6.6), and for patients with a higher leukocyte count after cyclophosphamide induction (HR 1.17; 95% CI 1.07±1.29). Risk of relapse was lower for patients with a longer duration of azathioprine maintenance (HR 0.91; 95%CI 0.87± 0.96), and patients with a baseline creatinine >1.24 mg/dl (HR 0.5, 95%CI 0.3±0.8). The interaction [azathioprine duration]*[time] was significant (P = 0.008) and indicated a declining protective effect of azathioprine duration over time (HR 1.002, 95%CI 1.000±1.003). The same variables remained significant after exclusion of patients intolerant to azathioprine (**S2 Table**).

Adverse events

In total, 35 patients (16%) were intolerant to azathioprine. 17 patients (8%) had gastro-intestinal complaints, 17 (8%) had a febrile hypersensitivity reaction, and 1 patient (1%) had a rash. Intolerance to azathioprine was not related to TPMT genotype (P = 0.11) or tertiles of TPMT activity (P = 0.39). There was no significant difference between TPMT genotypes in occurrence of mild or moderate leukopenia (**Table 2**). Occurrence of mild or moderate leukopenia also did not significantly differ between tertiles of TPMT activity (**Table 3**).

Table 2. Adverse events in relation to TPMT genotype

Adverse events	All n(%)	Variant n(%)	Normal n(%)	P*
All azathioprine tolerant patients	172	15	157	
Missing data on leukopenia	8	0	8	
Leukopenia	75 (46)	6 (40)	69 (46)	0.79
• Mild leukopenia (<4*10 ^{9} /l)	54 (33)	4 (27)	50 (34)	
• Moderate leukopenia (<3*10 ^{9} /l)	21 (13)	2 (13)	19 (13)	>0.99
Missing data on macrocytic anemia	10	0	10	
Macrocytic anemia	74 (46)	9 (60)	65 (44)	0.28
Missing data on hepatotoxicity	8	0	8	
Hepatotoxicity	26 (16)	4 (27)	22 (15)	0.26
Missing data on infections	11	1	10	
Infection	62 (39)	7 (50)	55 (37)	0.40

*Number of patients experiencing adverse events. All analyses (except for intolerance) have been done only in patients who were not intolerant to azathioprine (n=172). All= all patients. Variant= heterozygous TPMT variant carrier, Normal=normal TPMT genotype. *Compared between TPMT genotypes.*

Table 3. Adverse events in relation to TPMT activity

Adverse events	All n(%)	T1 n(%)	T2 n (%)	T3 n (%)	P*
All azathioprine tolerant patients	172	57	55	60	
Missing data on leukopenia	8	1	1	6	
Leukopenia	75 (46)	27 (48)	23 (43)	25 (46)	0.82
• Mild leukopenia (<4*10 ^{9} /l)	54 (33)	17 (31)	20 (37)	17 (31)	
• Moderate leukopenia (<3*10 ^{9} /l)	21 (13)	10 (18)	3 (6)	8 (15)	0.13
Missing data on macrocytic anemia	10	2	2	6	
Macrocytic anemia	74 (46)	29 (53)	18 (34)	27 (50)	0.11
Missing data on hepatotoxicity	8	1	1	6	
Hepatotoxicity	26 (16)	11 (20)	6 (11)	9 (17)	0.48
Missing data on infections	11	3	1	7	
Infection	62 (39)	22 (41)	21 (39)	19 (36)	0.90

*Number of patients experiencing adverse events. All analyses (except for intolerance) have been done only in patients who were not intolerant to azathioprine (n=172). All= all patients. T1= first tertile, T2= second tertile, T3= third tertile of TPMT activity. *Compared between tertiles of TPMT activity.*

The lowest measured leukocyte count was 1.4*10^{9}/l. Two patients had concomitant infections (PCP pneumonia and CMV antigenemia, candida stomatitis and PCP pneumonia, respectively). In 12 patients with moderate leukopenia, azathioprine dose was reduced and in 9 patients azathioprine was (temporarily) discontinued. In all except 3 patients, moderate leukopenia was incidental. In the others durations were 5, 7 and 35 days before leukocyte counts were >3.0*10^{9}/l.

Azathioprine starting dose was not significantly different between patients with (median 1.6; IQR 1.2±1.8 mg/kg) and without (median 1.4; IQR 0.9±1.8 mg/kg) mild leukopenia (P = 0.08), neither between patients with (median 1.5; IQR 1.1±1.7 mg/kg) or without (median 1.5; IQR 1.1±1.8) moderate leukopenia (P = 0.65). The same goes for patients with and without macrocytic anemia (P = 0.09), liver toxicity (P = 0.26) and infections (P = 0.69).

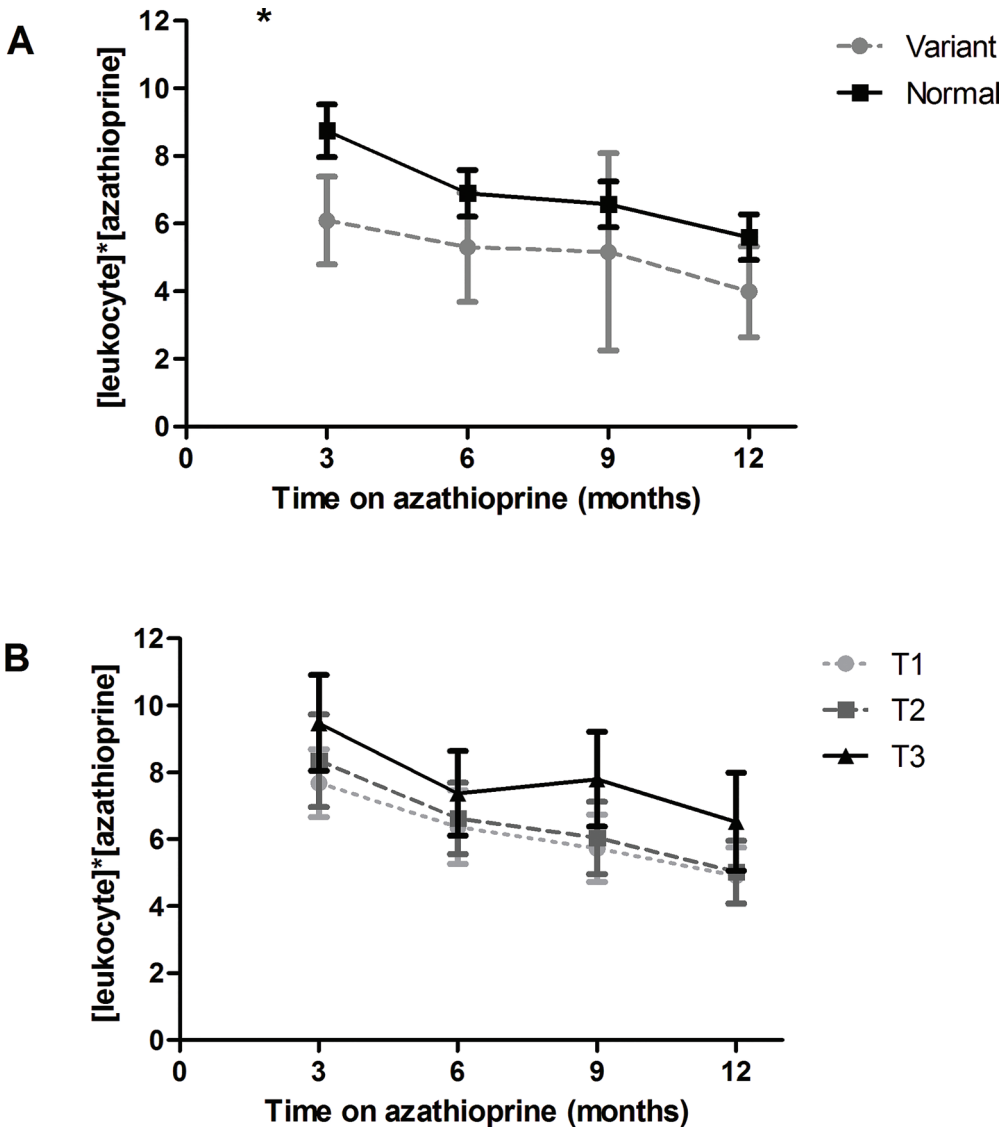
In logistic regression, TPMT genotype and activity were not significantly related to leukopenia during azathioprine therapy. Prednisolone dose and cyclophosphamide dose at switch were also not significant. Leukocyte count at switch (i.e. at the end of induction therapy with cyclophosphamide) remained in the model as a significant predictor of leukopenia ($P<0.001$), as well as azathioprine dose at switch ($P = 0.04$) with a higher risk of leukopenia during azathioprine therapy in patients with a lower leukocyte count at the end of cyclophosphamide therapy (Odds Ratio (OR) 0.54; 95% CI 0.43±0.68), and for patients with a higher starting dose of azathioprine (OR 2.2; 95% CI 1.0±4.6). See also **S3 Table**.

Leukocyte counts 3, 6, 9 and 12 months after switch to azathioprine were not significantly different between TPMT genotypes or tertiles of TPMT activity. The [leukocyte]*[azathioprine] product was significantly lower for heterozygous patients 3 months after switch ($P = 0.03$) but not at later time points (**Figure 4A**). The [leukocyte]*[azathioprine] product did not significantly differ between tertiles of TPMT activity (**Figure 4B**).

In multivariate linear regression, after correction for prednisolone dose, TPMT genotype was still not a significant predictor of leukocyte count or [leukocyte]*[azathioprine] product at any time point. Tertiles of TPMT activity, on the other hand, were positively related to leukocyte counts 3 months ($P<0.001$; b (regression coefficient of predictor) = 0.74; 95% CI 0.35± 1.14) and 9 months after switch ($P = 0.04$; b = 0.36; 95% CI 0.02±0.70) after correction for prednisolone dose. Multivariate linear regression also showed a significant association of TPMT activity tertiles with the [leukocyte]*[azathioprine] product 3 ($P = 0.003$; b = 1.20; 95% CI 0.41±2.00), 9 ($P = 0.01$; b = 0.94; 95% CI 0.20±1.69) and 12 months after switch ($P = 0.02$; b = 0.76; 95% CI 0.12±1.41). This indicates higher [leukocyte]*[azathioprine] product and therefore lower sensitivity for azathioprine-induced leukopenia in patients with higher TPMT activity. Prednisolone dose showed a significant positive association with leukocyte counts and the [leukocyte]*[azathioprine] product at every time point ($P\leq0.001$).

TPMT genotype was not related to occurrence of macrocytic anemia, liver toxicity, infection, or intolerance to azathioprine (**Table 2**). Occurrence of these adverse events was also not significantly different between tertiles of TPMT activity (**Table 3**).

Figure 4. [leuko]*[aza] product over time for TPMT genotypes and tertiles of TPMT activity



[leukocyte][azathioprine] product 3,6,9 and 12 months after switch to azathioprine. *P<0.05. Upper graph (4A): Variant = heterozygous TPMT variant carrier; Normal = normal genotype (wildtype TPMT). Lower graph (4B): T1 = lowest tertile, T2 = middle tertile, T3 = highest tertile of TPMT activity.*

DISCUSSION

In this study, we found no significant association of TPMT genotype and TPMT activity with relapse free survival. TPMT genotype and activity were not related to occurrence of azathioprine related adverse events. Leukocyte counts at the end of cyclophosphamide induction therapy were significantly associated with both relapse free survival and occurrence of leukopenia during azathioprine maintenance therapy.

Because azathioprine therapy is associated with a risk of potentially severe adverse events such as bone marrow toxicity, several studies have focused on TPMT genotypes and activity as predictors of these adverse events.[8,15] It has been established mainly in IBD patient populations that patients with a heterozygous TPMT genotype and lower TPMT activity are at increased risk of developing adverse events,[8] and that pretesting for TPMT has a beneficial effect specifically in the group of patients with one or several TPMT variants.[14] Although we found that a lower TPMT activity was associated with a lower [leukocyte]*[azathioprine] product, indicating an increased sensitivity to azathioprine-induced leukopenia, we did not find an association of TPMT genotype and activity with bone marrow toxicity in our population of AAV patients. This might be explained by the fact that AAV patients do not receive azathioprine as the main treatment drug, but as maintenance therapy after induction therapy with cyclophosphamide.[4] The effect of cyclophosphamide on bone marrow toxicity during azathioprine therapy may be greater than the effect of TPMT, as evidenced by the strong association of leukocyte counts after cyclophosphamide treatment with both relapse free survival and leukopenia during azathioprine therapy in this study, and the lack of a significant difference in azathioprine starting dose between patients with and without leukopenia. Another explanation may be that leukocyte counts after cyclophosphamide therapy reflect an overall bone marrow susceptibility to the effects of both drugs.

The frequency of leukopenia in or population was relatively high compared to previous reports, such as in the CYCAZAREM trial (30%).[5] The first reason for this is that patients with leukopenia at the start of azathioprine therapy were also scored as having leukopenia during azathioprine therapy. The second reason is that any leukopenia during the full duration of azathioprine therapy was scored, compared to only the first 15 months after switch in the CYCAZAREM trial.[5] When counting only patients that developed leukopenia within 15 months after start of azathioprine, the frequency of leukopenia ($<4.0*10^9$/l) in our population was 31%, similar to the frequency previously reported.[5]

Theoretically, patients with lower TPMT activity can achieve a higher efficacy of azathioprine.[8,23] Some studies indeed found an association of TPMT activity with clinical response. [24,25] In a recently published RCT, adjusting azathioprine dose based on TPMT genotype did not result in a difference in treatment response between intervention and control groups.[14] Although we found that patients with TPMT variant alleles and patients with lower TPMT activity had a higher relapse free survival, these differences were not significant, especially when taking other predictors of relapse into account. Interestingly, higher leukocyte counts after cyclophosphamide therapy were a strong predictor of relapse. This indicates that response to cyclophosphamide may be a stronger predictor of clinical efficacy than TPMT. This study has several limitations. First, although 207

patients is an impressive number for a single center study on a rare disease such as AAV, the sample size may be insufficient to detect relevant associations with sufficient power. This is especially true for the analyses on TPMT genotype, since there are only 19 patients with a heterozygous TPMT genotype in our study. Second, treating physicians were not blinded to a patient's TPMT genotype and activity. On the other hand, adjustment of azathioprine dose based on TPMT status was not included in the treatment protocol, and the initial azathioprine dose of patients with heterozygous TPMT genotype did not differ between patients whether their TPMT genotype and activity were measured before or after azathioprine therapy. Third, the study was conducted in a tertiary referral center, with some patients receiving part of their follow-up elsewhere. This resulted in missing values on adverse effects of 11 patients. The baseline characteristics and induction treatment of these patients did not significantly differ from those of patients with follow-up during azathioprine therapy. Lastly, the ethnicity for patients was not registered, although over 95% of patients in our study population are estimated to be Caucasian. As we only genotyped for TPMT variants common in Caucasians, some non-Caucasian patients might have reduced TPMT activity resulting from an untyped TPMT variant. This could theoretically result in underestimation of the effects from TPMT genotype.

The study also has several strengths. It was performed in a single center and all patients were treated according to the same protocol, thereby eliminating between-center differences in treatment and followup measurements. Also, compared to an earlier study on TPMT genotype and activity in AAV patients from our population,[18] this study has a larger sample size (207 compared to 108), has a longer duration of follow-up and includes multivariable analyses to account for induction treatment and other factors influencing disease free survival and risk of adverse events.

In conclusion, TPMT genotype and activity were not related to azathioprine efficacy and toxicity in our retrospective cohort of AAV patients receiving azathioprine maintenance therapy. Response to cyclophosphamide, on the other hand, may have a stronger predictive value on these outcomes. This should be confirmed in a sufficiently large multicenter study.

SUPPORTING INFORMATION

S1 Table. Tapering scheme for prednisolone

Time from start of therapy (weeks)	Prednisolone daily dose (mg)
0 – 6†	60
6 – 12	Reduce 10 mg per 2 weeks up to 30 mg
12 – 18	Reduce 5 mg per 2 weeks up to 15 mg
18 – 28	Reduce 2,5 mg per 2 weeks up to 0
> 28	Stop

† Start tapering earlier when in full remission for 2weeks, <6wks of therapy.

S2 Table. Cox regression for 5 year relapse free survival

Variable	P-value	HR + 95% CI
Included in final model		
Creatinine level at baseline (<=110 or >110 umol/l)	0.01 (*)	0.5 (0.3-0.9)
Leukocyte count at switch	0.001 (**)	1,18 (1,07-1,31)
ANCA (PR3 vs MPO/other/negative)	0.007 (**)	3.1 (1.4-6.9)
Duration of azathioprine therapy (months)	<0.001 (***)	0.86 (0.79-0.93)
Time * duration of azathioprine therapy	0.002 (**)	1.003 (1.001-1.005)
Not included in final model		
TPMT genotype	0.66	-
Tertiles of TPMT activity	0.41	-
Age	0.91	-
Diagnosis (GPA/MPA/NCGN)	0.69	-
Co-trimoxazole dose at switch (high/low/none)	0.38	-

*Cox regression analysis for 5 year/ 60 month relapse free survival for non-azathioprine intolerant patients (n = 172). Variables for the final model were selected using a forward stepwise method (inclusion if univariate P<0.05, exclusion if multivariate P>0.1). ANCA specificity, duration of azathioprine therapy, creatinine at baseline and leukocyte count after cyclophosphamide induction therapy were significantly associated with risk of relapse. *P<0.05; **P<0.01; ***P<0.001.*

S3 Table. Logistic regression for risk of leukopenia

Variable	P-value	OR + 95% CI
Included in final model		
Azathioprine switch dose	0.04(*)	2.2 (1.0-4.6)
Leukocyte count at switch	<0.001 (***)	0.54 (0.43-0.68)
Not included in final model		
TPMT genotype	0.85	-
Tertiles of TPMT activity	0.83	-
Cyclophosphamide switch dose	0.44	-
Prednisolone switch dose	0.14	-

*Logistic regression for risk of leukopenia (leukocyte count <4.0*10^9/l) for non-intolerant patients (n = 172). Variables for the final model were selected using a forward stepwise method (inclusion if univariate P<0.05, exclusion if multivariate P>0.1). A higher leukocyte count after cyclophosphamide induction therapy was associated with a lower risk of leukopenia, and a higher starting dose of azathioprine was associated with a higher risk of leukopenia during azathioprine therapy. *P<0.05; **P<0.01; ***P<0.001.*

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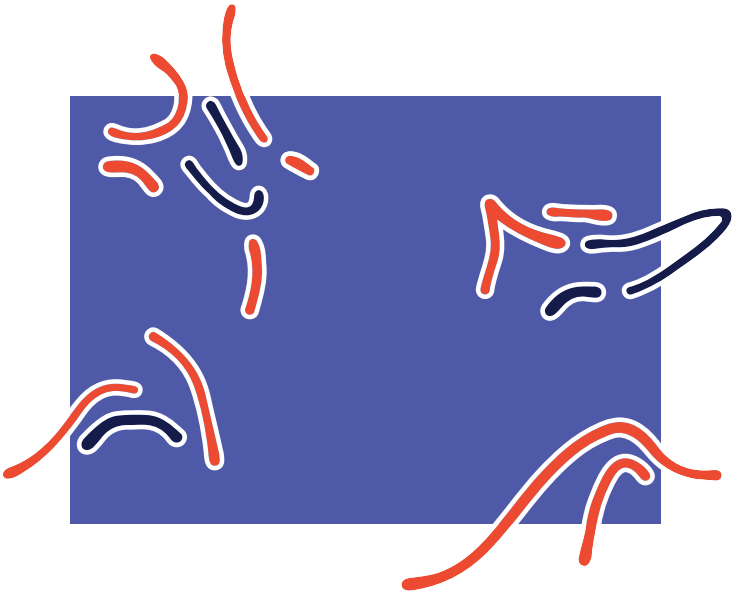
04 Chapter

Clinical outcome in anti-neutrophil cytoplasmic antibody-associated vasculitis and gene variants of 11β-hydroxysteroid dehydrogenase type 1 and the glucocorticoid receptor



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Key messages

-The 11β-hydroxysteroid dehydrogenase type 1 genotype affects the risk of relapse in ANCA-associated vasculitis.

-The glucocorticoid receptor haplotype affects renal survival, mortality and the development of dyslipidaemia in ANCA-associated vasculitis.

ABSTRACT

Objectives
We aimed to investigate whether five potential functional haplotypes of the glucocorticoid receptor (GR) gene and a single-nucleotide polymorphism of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) are associated with clinical outcome in ANCA-associated vasculitis.

Methods
Patients diagnosed with ANCA-associated vasculitis (n = 241) were genotyped for five polymorphisms of the GR gene and one polymorphism of the HSD11B1 gene. GR gene haplotypes were predicted based on genotyping results. Relapse-free survival, mortality, renal survival, metabolic adverse events and infections were compared between carriers and non-carriers of GR haplotypes and the HSD11B1 genotype.

Results
Carriers of haplotype 4 (ER22/23EK + 9β+TthIII1) of GR had a significantly higher 5-year mortality risk [hazard ratio (HR) 4.5 (95% CI 1.6, 12.8)] and had a higher risk of developing end-stage renal disease [HR 7.4 (95% CI 1.9, 28.7)]. Carriers of a minor variant of HSD11B1 more frequently experienced relapse [HR 2.5 (95% CI 1.5, 4.1)] except if they also carried haplotype 1 (BclI) of GR. Homozygous carriers of haplotype 1 had a higher risk of developing dyslipidaemia [HR 4.1 (95% CI 1.8, 9.6)]. The occurrence of infections did not differ between GR haplotypes and HSD11B1 genotypes.

Conclusion
Haplotypes 1 and 4 of GR and a polymorphism of the HSD11B1 gene were associated with clinically relevant inflammatory and metabolic outcomes in ANCA-associated vasculitis.

INTRODUCTION

ANCA-associated vasculitis (AAV) is a group of autoimmune diseases associated with inflammation of small and medium-sized blood vessels. Granulomatosis with polyangiitis, microscopic polyangiitis and eosinophilic granulomatosis with polyangiitis are diagnoses within this group [1].

Regardless of further induction and maintenance therapy, AAV patients are treated with high initial doses (up to 1 mg/kg/d) of the exogenous glucocorticoid (GC) prednisolone, because of its effectiveness in inducing disease remission [2]. Unfortunately, high-dose GCs are associated with many immunological, cardiometabolic and other adverse effects [3-5]. Furthermore, GC sensitivity differs widely between patients [6], which may affect treatment efficacy and toxicity in individual patients.

In recent years, personalized medicine has received more attention in AAV research. One approach has been to identify disease subsets and to target specific pathways involved in the pathogenesis of AAV [7]. Examples include the identification of different genetic variants associated with either PR3- or MPO-ANCA-positive patients [8] and a clinical trial for a drug that targets the C5a receptor in the alternative complement pathway [9]. Another approach to personalized medicine is pharmacogenetics (i.e. identification of genetic polymorphisms related to drug efficacy and/or toxicity) [10]. Examples for AAV include cytochrome P450 polymorphisms for CYC [11] and Fcγ receptor polymorphisms for rituximab therapy [12].

Hydroxysteroid dehydrogenase 11β type 1 (HSD11B1) is an enzyme involved in the regulation of local GC levels. In vivo, it predominantly converts inactive cortisone into active cortisol [13]. Due to disadvantageous cardiometabolic effects of cortisol, inhibition of HSD11B1 has been investigated as a potential treatment for diabetes and obesity [14]. In contrast, HSD11B1 deficiency may worsen inflammatory diseases or hinder their resolution [14, 15]. The latter has been demonstrated in animal studies but not yet in a clinical setting [14-16]. A common genetic polymorphism of HSD11B1, rs11119328, is hypothesized to result in reduced expression of the enzyme [17]. The influence of rs11119328 on inflammation has not yet been studied.

Cortisol and prednisolone bind to the glucocorticoid receptor (GR) in the cytoplasm. After binding, the resulting complex moves to the nucleus where it affects gene expression; these genomic effects influence cardiometabolic effects and inflammation [6]. Several polymorphisms have been described for the GR encoding gene. Some of these polymorphisms, such as N363S (rs6195) and BclI (rs41423247) are associated with an increased sensitivity to GCs. Other polymorphisms, such as ER22/23EK (rs6189 and rs6190) and 9β (rs6198), are associated with relative GC resistance [6]. The polymorphism TthIII1 (rs10052957) is thought to have no influence on GC sensitivity by itself but is strongly associated with the simultaneous presence of the ER22/23EK polymorphism [18].

In the general population, GR polymorphisms have been associated with distinct clinical phenotypes. The N363S and BclI polymorphisms have been associated with obesity, insulin resistance and a reduced risk of inflammatory diseases such as RA [6, 19, 20]. In

contrast, ER22/23EK has been associated with higher insulin sensitivity, lean mass and muscle strength and the ER22/23EK and 9β polymorphisms have been associated with an increased risk of RA [6, 19, 20]. In multiple sclerosis and RA, the ER22/23EK polymorphism in particular has been associated with a more severe disease phenotype [21, 22]. Several studies have suggested associations between GR polymorphisms and GC treatment response [20, 23-26].

The aim of this study was to explore the influence of five GR polymorphisms and one polymorphism of HSD11B1 (rs11119328) on the efficacy and toxicity of high-dose prednisolone therapy in AAV. Given their functional consequences, we hypothesized that N363S and BclI would be associated with a higher risk of cardiometabolic adverse events and that ER22/23EK, 9β and rs11119328 would be associated with more severe disease activity and a higher risk of relapse.

METHODS

Study population

All 421 consecutive AAV patients diagnosed from 1990 to 2015 receiving treatment and follow-up at the University Medical Center Groningen (UMCG) were considered for inclusion. Patients were included if they received treatment with GCs combined with another immunosuppressive drug as initial treatment, according to the EULAR/European Renal Association-European Dialysis and Transplant Association recommendations [27]. Prednisolone was slowly tapered according to the local protocol of the UMCG starting 6 weeks after initiation of treatment. Eosinophilic granulomatosis with polyangiitis (formerly Churg-Strauss syndrome) patients were excluded because they have a different clinical course, with frequent asthma and ENT exacerbations often requiring chronic GC therapy [28]. This study is part of a large cohort study investigating biomarkers in relation to clinical outcomes in AAV. All patients gave written informed consent. The protocol for the biomarker study was approved by the local medical ethical committee (NL29354.042.10) and adheres to the principles of the Declaration of Helsinki.

Data collection

Clinical data were collected from the patients' medical records. Disease activity at diagnosis and first relapse were scored using the BVAS version 1 [29]. Comorbidity at diagnosis relevant for mortality risk was scored using the Charlson comorbidity index [30]. The primary endpoint of the study was 10 year relapse-free survival. Secondary endpoints were 10 year mortality, 1 year renal survival, risk of infections (overall, severe, opportunistic) in the first year after diagnosis and occurrence of hypertension, diabetes and dyslipidaemia. Definitions of study endpoints are included as **S1 Table**, available at Rheumatology online. Data were collected up to October 2017.

Genotyping and determination of haplotypes

Genotyping has been performed at the Laboratory of Clinical and Experimental Endocrinology, Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands. All genotypes were determined in one batch. The following five GR

polymorphisms on chromosome 5 were determined using TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA): BclI (rs41423247), Tth111I (rs10052957), 9β(rs6198), N363S (rs6195) and ER22/23EK (rs6189 and rs6190). Additionally, the presence of the HSD11B1 (rs11119328) polymorphism on chromosome 1 was determined. Haplotypes were determined based on combinations of GR polymorphisms using the computer programme PHASE [31, 32]. The haplotypes were defined as follows: haplotype 0 (reference)—major variant of all polymorphisms; haplotype 1—minor variant of the BclI polymorphism; haplotype 2—minor variant of the Tth111I and BclI polymorphisms; haplotype 3—minor variant of the 9β and Tth111I polymorphisms; haplotype 4—minor variant of the ER22/23EK, 9β and Tth111 polymorphisms; haplotype 5—minor variant of the N363S polymorphism. Detailed methods are described elsewhere [33]. As rs11119328 was the only gene polymorphism from chromosome 1 included in this study, it was not analysed as part of a haplotype. In the results, it will be called HSD11B1 minor for simplicity.

Statistical analysis

Data were analysed using SPSS Statistics version 23 (IBM, Armonk, NY, USA). The proportionality assumption for Cox regression was tested in R version 3.2.3 (R Project for Statistical Computing, Vienna, Austria) using scaled Schoenfeld residuals [34]. Correction for multiple comparisons was performed in R using the Benjamin-Hochberg (BH) procedure with a false discovery rate of 0.05 [35]. BH-adjusted P-values were calculated, with P-values <0.05 indicating statistical significance. Missing data were handled using complete case analysis. Baseline characteristics were compared between carriers and non-carriers of each haplotype and HSD11B1 minor using Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. Deviation from Hardy-Weinberg equilibrium was tested using a χ^2 test [36]. Univariable survival analysis was performed for relapse-free, overall, renal and adverse event-free survival using the log rank test. In case of significant differences between GR haplotypes or HSD11B1 genotypes, Cox regression was performed to quantify the differences. Multivariable relapse-free and overall survival analyses were performed using Cox regression with adjustment for previously reported risk factors of relapse [37, 38] and mortality [5, 39], respectively. Interactions between GR haplotypes and HSD11B1 minor were checked by adding the product of both variables as an interaction term to the regression model. Lastly, differences regarding the occurrence of infections in the first year after diagnosis were analysed using Fisher's exact test.

RESULTS

Baseline results

Baseline characteristics of the 241 patients in the study are shown in **Table 1**, as well as differences between carriers and non-carriers of the different haplotypes of the GR and the rs11119328 polymorphism of HSD11B1 (HSD11B1 minor). Patients received a median initial prednisolone dose of 60 mg/d [interquartile range (IQR) 60-60]. After 6 months the median dose was 7.5 mg/d (IQR 5-10 mg/d). Discontinuation of prednisolone within 1 year after diagnosis was achieved for 60% of patients. GC exposure did not differ between GR haplotypes or between carriers and non-carriers of HSD11B1 minor. A flowchart of inclusion is shown in **S1 Figure** available at *Rheumatology* online.

Table 1. Baseline characteristics.

Variable	All (n=241)	HT 1 (n=104)	HT 2 (n=70)	HT 3 (n=60)	HT 4 (n=15)	HT 5 (n=22)	rs11119328 (n=80) [a]
Male (%)	56	58	49	60	60	55	65
Age at diagnosis median (IOR), years	56 (44-66)	57 (44-66)	53 (43-65)	55 (44-64)	59 (50-77)	62 (46-71)	55 (44-65)
Follow-up, median (IOR), years	10 (6-15)	10 (6-14)	9 (6-15)	10 (5-17)	12 (3-13)	10 (8-17)	9 (6-19)
GPA (%)	75	73	79	72	72	69	75
PR3 ANCA (%)	73	71	80	70	73	64	74
CYC induction (%)	88	88	87	92	100	82	89
Plasmapheresis (%)	19	26	16	13	40	14	16
Hemodialysis (%)	8	11	9	7	13	9	10
AZA maintenance	60	64	63	58	60	64	51
ENT activity (%)	63	64	67	60	53	41	63
Pulmonary activity (%)	46	52	40	38	33	50	50
Renal activity (%)	69	70	73	70	80	73	70
Serum creatinine median (IOR), µmol/l	100 (79-227)	106 (77-247)	112 (72-234)	105 (80-209)	180 (100-350)	101 (87-215)	106 (81-236)
BVAS at diagnosis, median (IOR)	18 (12-24)	19 (12-25)	20 (13-25)	16 (12-22)	18 (13-20)	19 (15-24)	18 (13-26)
Charlson index score	1 (0-3)	2 (0-3)	1 (0-3)	1 (0-3)	2 (1-4)	2 (0-3)	2 (0-3)

Variables reported as % or median (IQR). [a] Available for 227 of 241 patients.
GPA granulomatosis with polyangiitis. HT haplotype.

Genotype and haplotype frequencies

The minor allele frequencies of the GR gene and the HSD11B1 gene were the following: 40% for BclI, 32% for Tth111I, 17% for 9β, 5% for N363S, 4% for ER22/23EK and 19% for rs11119328 (HSD11B1 minor). These were comparable to minor allele frequencies found in the general population [6].

Haplotypes were derived for the GR gene polymorphisms. The haplotype frequency was 38.6% for haplotype 0 (all major), 24.7% for haplotype 1 (minor BclI), 15.8% for haplotype 2 (minor Tth + BclI), 13.1% for haplotype 3 (minor Tth + 9β), 3.1% for haplotype 4 (minor ER22 + 9β + Tth), and 4.8% for haplotype 5 (minor N363S). This is comparable to haplotype frequencies reported by Wester et al. [40] based on a large general population cohort.

Relapse-free survival

In total, 129 of 241 (54%) patients experienced a relapse within 10 years after diagnosis. Cumulative relapse-free survival was 92% after 1 year, 53% after 5 years and 38% after 10 years. The median BVAS at relapse was 12 (IQR 7-16). The most frequent organ manifestations were kidneys (56%), ENT (53%) and systemic (84%). Patients most frequently received CYC induction therapy (68%) and AZA maintenance therapy (47%) in addition to prednisolone in a high initial dose. Plasmapheresis was required for 7% of patients and 5% needed (temporary) haemodialysis therapy.

When accounting for multiple comparisons, there was no statistically significant difference in the risk of relapse between carriers and non-carriers of rs11119328 (BH adjusted P=0.18; Fig. 1), haplotype 1 (P=0.97), haplotype 2 (P=0.68), haplotype 3 (P=0.91), haplotype 4 (P=0.77) and haplotype 5 of GR (P=0.77).

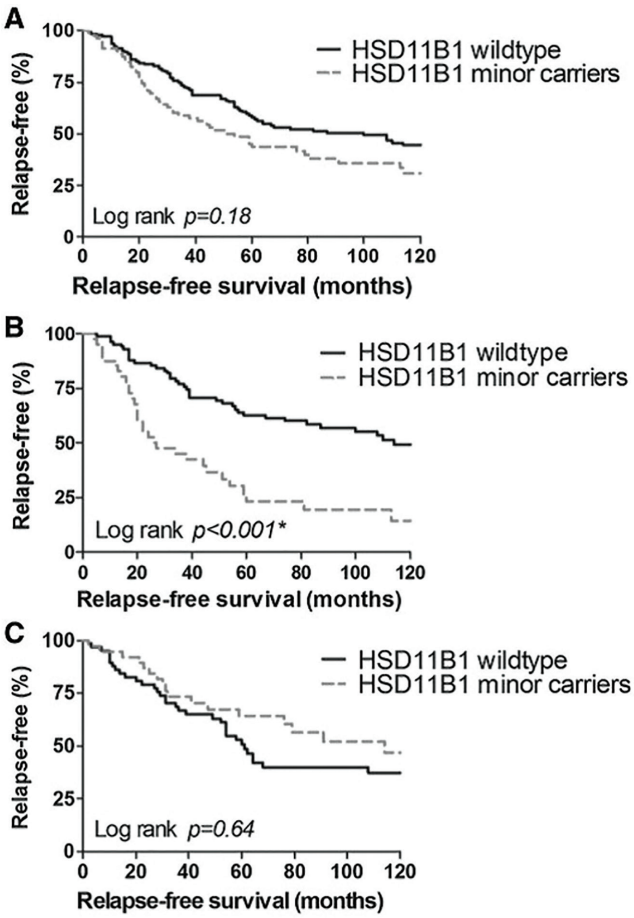
In the Cox regression, the HSD11B1 genotype showed a significant interaction with haplotype 1. Carriers of HSD11B1 minor without a concomitant haplotype 1 [n = 42/227 (19% of patients)] had a significantly higher risk of relapse [hazard ratio (HR) 2.5], while this risk was compensated (HR 1.0) by the simultaneous presence of haplotype 1 [n = 38/227 (17% of patients)]. This remained true after correction for age, gender, serum creatinine, ENT and pulmonary involvement, ANCA specificity and AZA maintenance therapy (**Table 2, Fig. 1**).

Table 2. Cox regression analysis for 10 year relapse-free survival

	Model 1		**Model 2[a]**	
Variable	**HR (95% CI)**	**BH-adjusted P-value**	**HR (95% CI)**	**BH-adjusted P-value**
HSD11B1 minor carrier	2.9 (1.8 to 4.7)	<0.001*	2.5 (1.5 to 4.1)	0.005*
HT 1 carrier	1.5 (0.9 to 2.4)	0.42	1.4 (0.8 to 2.2)	0.60
HT 1 * HSD11B1 minor	0.2 (0.1 to 0.5)	0.005*	0.3 (0.1 to 0.6)	0.02*

*Cox regression analyses of the HSD11B1 genotype, Haplotype 1 (BclI) of the glucocorticoid receptor and their interaction for 10 year relapse-free survival. * significant after adjustment for multiple comparisons. [a] adjusted for age, gender, serum creatinine, ear-nose-throat and pulmonary involvement, ANCA specificity and azathioprine maintenance therapy. BH Benjamini-Hochberg. HR Hazard Ratio. HT Haplotype.*

Figure 1. Relapse-free survival per genotype of HSD11B1



*Relapse-free survival for carriers (grey dashed line) and non-carriers (black solid line) of the HSD11B1 minor allele. P-values shown are BH adjusted. (A) Overall relapse-free survival. (B) Non-carriers of haplotype 1 (glucocorticoid receptor). (C) Carriers of haplotype 1. *Significant after adjustment for multiple comparisons.*

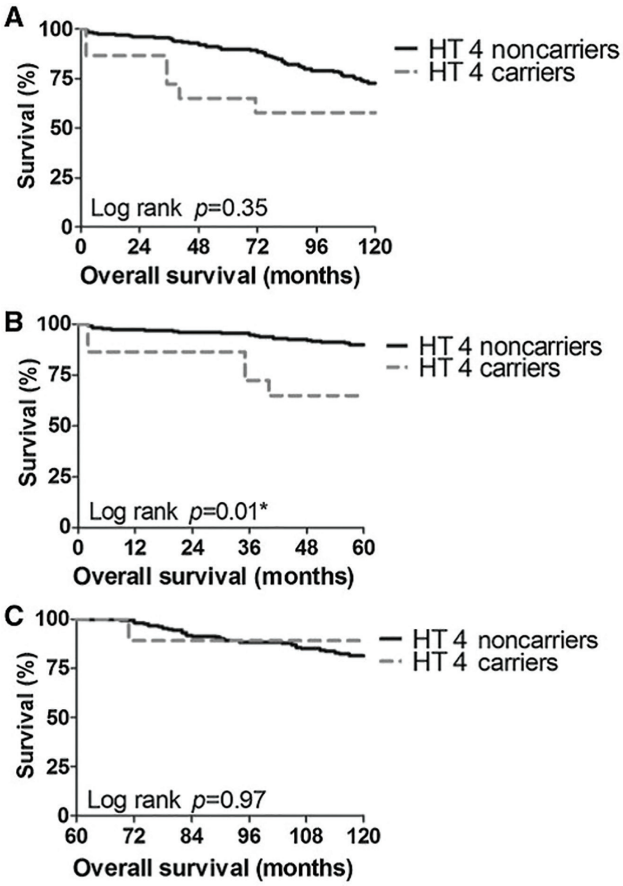
Mortality
In total, 58 of 241 (24%) patients died within 10 years of diagnosis. Cumulative survival was 97% after 1 year (n at risk = 241), 88% after 5 years (n = 209) and 72% after 10 years (n = 136). The log rank test showed no significant differences in the risk of mortality between carriers and non-carriers of haplotype 1 (BH adjusted P=0.97), haplotype 2 (P=0.97), haplotype 3 (P>0.99), haplotype 4 (P=0.35) and haplotype 5 (P=0.99) or the HSD11B1 genotype (P=0.42). In univariable Cox regression, haplotype 4 did not meet the proportionality assumption. After stratification by time, haplotype 4 was a significant predictor of mortality only in the first 5 years following diagnosis. In multivariable Cox regression, after adjusting for age, gender, serum creatinine, BVAS, requirement of plasmapheresis, AZA maintenance therapy and ANCA specificity, haplotype 4 remained a significant predictor of 5 year mortality (**Table 3, Figure 2**).

Table 3. Cox regression analysis for 10 year overall survival

	Model 1		Model 2[a]	
Variable	**HR (95% CI)**	**BH-adjusted P-value**	**HR (95% CI)**	**BH-adjusted P-value**
Haplotype 4 (0-5 years)	4.3 (1.6 to 11.3)	0.03*	4.5 (1.6 to 12.8)	0.03*
Haplotype 4 (5-10 years)	0.6 (0.1 to 4.5)	0.97	1.0 (0.1 to 7.7)	>0.99

*Cox regression analyses of Haplotype 4 (ER22/23EK+9β+TthIII1) of the glucocorticoid receptor, stratified by time, for 10 year overall survival. * significant after adjustment for multiple comparisons. [a] adjusted for age, gender, serum creatinine, BVAS score, requirement of plasmapheresis, azathioprine maintenance therapy and ANCA type. BH Benjamini- Hochberg. HR Hazard Ratio.*

Figure 2. Overall survival for carriers and non-carriers of haplotype 4 (ER22 + 9β+Tth)



*Kaplan-Meier curve for overall survival of carriers (grey dashed line) and non-carriers (black solid line) of haplotype 4 of GR. P-values shown are BH adjusted. (A) 10 year mortality. (B) 5 year mortality. (C) Mortality between 5 and 10 years of follow-up. *Significant after adjustment for multiple comparisons. HT: haplotype.*

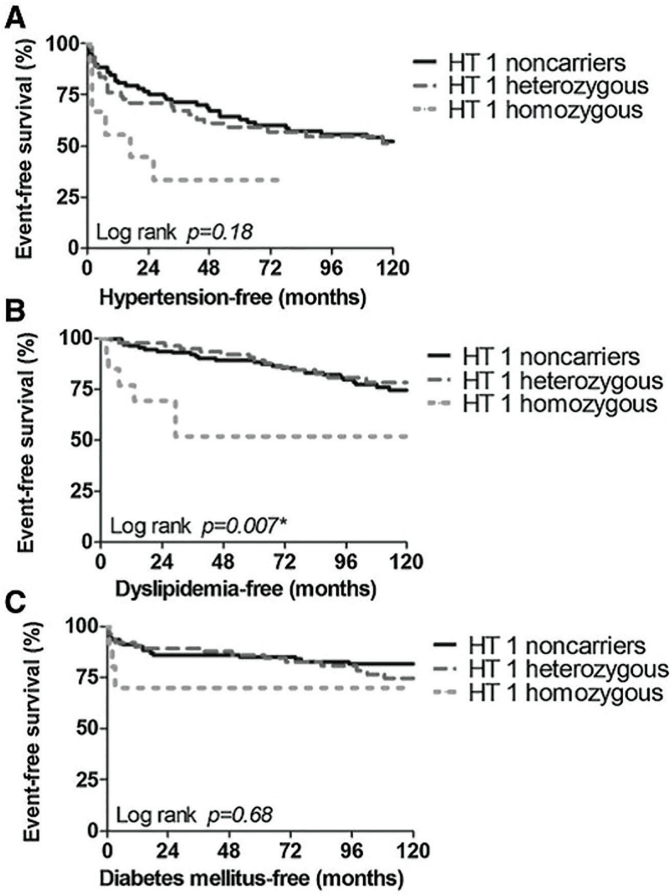
Renal survival
In total, 26 of 241 patients (11%) developed end-stage renal disease within 10 years after diagnosis. Relatively many events (10/26) of end-stage renal disease occurred in the first year after diagnosis. Carriers of haplotype 4, even after correction for multiple comparisons, had a significantly worse 1 year renal survival compared with noncarriers [HR 7.4 (95% CI 1.9, 28.7), BH adjusted P=0.007] (see **S2 Figure** available at Rheumatology online). One-year renal survival did not differ significantly between carriers and non-carriers of haplotype 1 (BH adjusted P=0.68), haplotype 2 (P=0.96), haplotype 3 (P=0.97) or haplotype 5 (P=0.60), nor between carriers and non-carriers of a minor variant of HSD11B1 (P=0.97). Because of the limited number of events, multivariable Cox regression was not performed.

Infection
Overall, 103/241 (43%) patients had an infection requiring antimicrobial treatment during the first year after diagnosis. A severe infection occurred in 48 (20%) patients, an opportunistic infection in 49 (20%), varicella zoster in 9 (4%) and CMV in 21 patients (9%). There were no significant differences between GR haplotypes or HSD11B1 genotypes regarding the occurrence of any severe or opportunistic infections in the first year after diagnosis.

Metabolic adverse events
There were no significant differences between haplotypes of the GR or HSD11B1 genotypes for prevalence of diabetes or hypertension at diagnosis, although homozygous carriers of haplotype 1 tended to have diabetes more frequently at baseline [4/15 (27%)] compared with heterozygous [6/89 (7%)] and non-carriers [10/135 (7%)].

After 10 years of follow-up, 156 patients (67%) had hypertension (77 new onset), 75 (31%) had diabetes (57 new onset) and 68 (29%) had dyslipidaemia (49 new onset). When accounting for multiple comparisons, haplotype 1 was significantly related to the development of new-onset dyslipidaemia (BH adjusted log rank P = 0.007), but not hypertension (P = 0.18) or type 2 diabetes (P = 0.68). Homozygous carriers of haplotype 1 [HR 4.1 (95% CI 1.8, 9.6)], but not heterozygous carriers [HR 0.9 (95% CI 0.5, 1.6)], had an increased risk of new-onset dyslipidaemia compared with non-carriers (see **Figure 3**). There were no significant differences in the risks of new onset hypertension, diabetes or dyslipidaemia between other GR haplotypes or HSD11B1 genotypes.

Figure 3. Adverse event-free survival and haplotype 1 (BclI)



*Kaplan-Meier curves for 10 year event-free survival vs haplotype 1 of the GR. P-values shown are BH adjusted. (A) Hypertension-free survival. (B) Dyslipidaemia-free survival. (C) Diabetes-free survival. *Significant after adjustment for multiple comparisons. HT: haplotype.*

DISCUSSION

In this retrospective study in a large cohort of AAV patients, we found that haplotype 4 of the GR gene (ER22/23EK + 9β + Tth111I), related to relative GC resistance, was associated with an increased risk of 5 year mortality and the development of end-stage renal disease. Furthermore, the rs11119328 polymorphisms of the gene encoding HSD11B1, hypothesized to be associated with reduced local GC activation, in the absence of haplotype 1 (BclI) of the GR gene, was associated with an increased risk of disease relapse. Lastly, homozygous carriers of haplotype 1, previously shown to be associated with increased GC sensitivity, had an increased risk of developing dyslipidaemia after diagnosis of vasculitis.

GC resistance due to haplotype 4 seems to be a two-sided coin. In the general population, it is associated with improved overall survival [41]. This might be mediated by beneficial metabolic effects of GC resistance (i.e. higher insulin sensitivity, lower cholesterol levels) [42]. In case of an inflammatory disease, haplotype 4 is potentially disadvantageous. This has been shown for RA [21], multiple sclerosis [22] and now for AAV. In RA patients, patients with the ER22/23EK polymorphism more frequently had erosive disease and more frequently required anti-TNF therapy [21]. This is in line with our finding that haplotype 4 carriers more frequently developed end-stage renal disease and had a higher risk of 5 year mortality, suggesting more severe inflammation in these patients. Contradictory to these results, we found no association of haplotype 4 with relapse-free survival. This could be explained, at least in part, by the higher risk of severe renal disease in haplotype 4 carriers, which has been associated with a lower risk of relapse in AAV [37]. This might counteract an increased risk of relapse due to GC resistance in haplotype 4, resulting in no net effect on relapse. Also, as haplotype 4 is relatively uncommon (n = 15) and is associated with an increased risk of mortality in our population, the number of haplotype 4 carriers with long-term data on relapse might not be sufficient to detect an association.

To our knowledge, we are the first to report the relation between a genetic polymorphism of HSD11B1 and clinical outcomes of an autoimmune inflammatory disease in humans. While GR gene polymorphisms have also been studied for RA and multiple sclerosis patients [21, 22], polymorphisms of HSD11B1 have not previously been studied in human patients with an inflammatory disease. In a mouse model of inflammatory arthritis, HSD11B1-deficient mice had earlier onset and later resolution of inflammation [16]. This is in accordance with the increased risk of relapse of patients with an HSD11B1 minor(rs11119328) genotype in our study, which suggests a pro-inflammatory phenotype due to reduced local activation of cortisone in carriers of this polymorphism. Interestingly, we found that the increased risk of relapse by the minor variant of HSD11B1, which would theoretically decrease local availability of cortisol to bind GR [15], can be compensated by the simultaneous presence of haplotype 1, which is associated with increased sensitivity of GR to active GC [43]. Nevertheless, 19% of the study population belonged to the group with increased risk (i.e. rs11119328 and no haplotype 1), which is a considerable number of patients.

Our finding of an increased risk of dyslipidaemia in homozygous carriers of haplotype 1 is in accordance with similar findings in the literature [19, 44, 45], although associations of the BclI polymorphism with metabolic outcomes were not consistently found in all studies [20]. Based on results from this study and the aforementioned previous studies, two copies of haplotype 1 are required for significant effects on metabolic adverse events (i.e. heterozygous carriers did not have an increased risk of developing dyslipidaemia).

This study has several limitations. First, this retrospective study spans more than a decade. Therefore differences in treatment exist between patients according to each patient's time of diagnosis. In particular, some patients were diagnosed before the introduction of AZA maintenance therapy. Although we studied a cohort of >200 AAV patients from a single centre, the cohort size might be too small for some analyses, especially for comparing heterozygous vs homozygous carriers of and testing for interactions with relatively uncommon haplotypes (i.e. haplotypes 4 and 5). Furthermore, we did not include a validation cohort to confirm our findings. Therefore the results should be verified in other cohort studies. Nevertheless, our analyses were based on pre-specified hypotheses, correction was performed for multiple comparisons and our findings are consistent with previous literature.

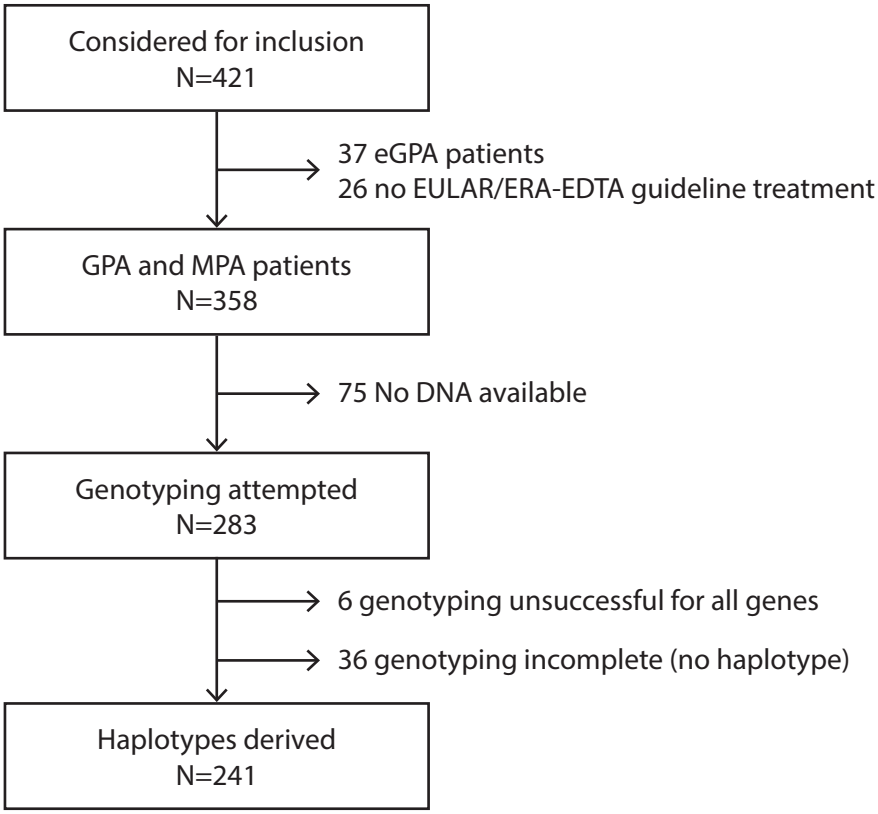
The findings of this study provide insight into the influence of GC sensitivity on clinical outcomes in AAV. They indicate proneness to relapse in carriers of a minor variant of HSD11B1, more severe renal disease and mortality in carriers of haplotype 4 and an increased risk of dyslipidaemia in homozygous carriers of haplotype 1 of GR. This might prove useful in guiding treatment for individual patients. Examples of potential applications, after appropriate validation of these results, include the use of more intensive induction therapy for carriers of haplotype 4 with renal disease activity, longer duration of maintenance therapy for carriers of an HSD11B1 variant without haplotype 1 or more emphasis on GC-sparing treatment in homozygous carriers of haplotype 1.

SUPPORTING INFORMATION

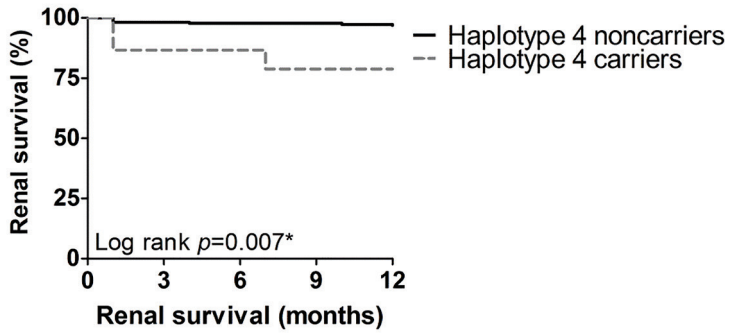
S1 Table. Definitions of study outcomes

Outcome	Definition
Relapse	Re-occurrence of active vasculitis that required a restart or a dose increase of immunosuppressive therapy
Relapse-free survival	Time in months between diagnosis and first relapse, or the last known follow-up visit up to 10 years after diagnosis if this event did not occur
Overall survival	Time in months between diagnosis and death, or the last known follow-up visit up to 10 years after diagnosis if this event did not occur
End-stage renal disease	Requirement of renal replacement therapy or transplantation or eGFR <15 for at least three consecutive months
Renal survival	Time in months between diagnosis end-stage renal disease, or the last known follow-up visit up to 1 year after diagnosis if this event did not occur
Hypertension	Systolic blood pressure >140 mmHg or use of antihypertensive drugs
Diabetes mellitus	Random blood glucose >11mmol/l, HbA1c >53 mmol/l and/or use of antidiabetic treatment
Dyslipidemia	Total cholesterol >6.5 mmol/l or use of lipid lowering drugs
Infections	Scored if they required treatment with antimicrobial drugs and/or hospital admission, and/or were opportunistic
Severe infections	Infections requiring hospital admission and/or prolonged hospital stay
Opportunistic infections	Infections by pathogens associated with an immunocompromised status, such as pneumocystis carinii, herpes zoster or cytomegalovirus. Varicella Zoster virus (VZV) and Cytomegalovirus (CMV) infections were scored separately

S1 Figure. Flowchart of patient selection



S2 Figure. Renal survival for carriers and non-carriers of Haplotype 4



Kaplan Meier curve for 1-year renal survival of carriers (grey dashed line) and non-carriers (black solid line) of Haplotype 4 (ER22/23EK+9β+TthIII1) of the glucocorticoid receptor. The p-value shown is Benjamini-Hochberg adjusted.

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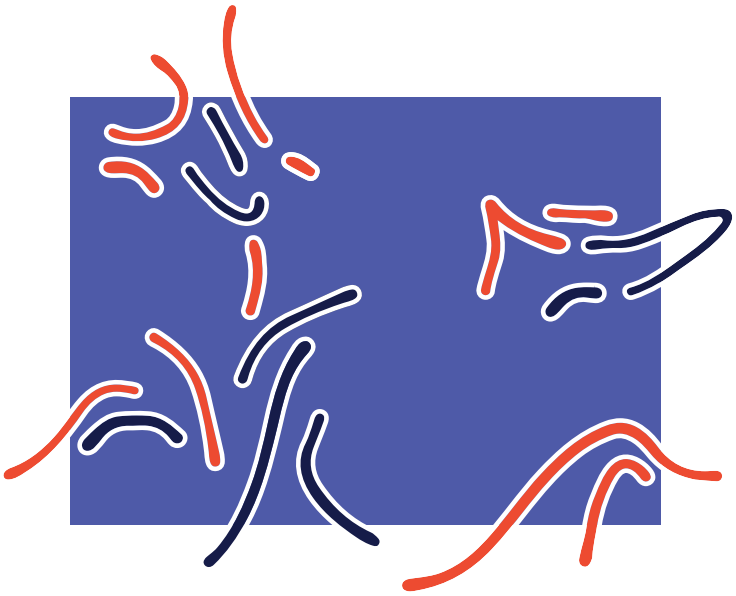
Part 2

Characterizing treatment outcomes

05 Chapter

Geographic differences in clinical presentation and outcome of antineutrophil cytoplasmic antibody-associated vasculitis: role of antibody specificity



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ABSTRACT

Objective
Clinical characteristics of ANCA-associated vasculitis (AAV) differ between geographic regions and ethnicities. Since ANCA-specificity varies between geographic regions and has been associated with differences in clinical picture of AAV, our objective was to investigate whether regional differences in clinical manifestations and outcomes might (partly) be explained by differences in ANCA-specificity.

Methods
ANCA specificity, organ manifestations at diagnosis, relapse-free survival and overall survival were compared between AAV patients from Dutch (n=264), Chinese (n=411) and Brazilian (n=97) observational cohorts.

Results
Frequencies of disease manifestations differed between countries. Mucosa/eye and otolaryngeal involvement were both associated with the presence of PR3-ANCA, irrespective of country. The differences of other organ manifestations between countries were independent of ANCA-specificity. In Cox regression, after correction for ANCA-specificity and organ manifestations associated with relapse risk, Chinese patients had an increased risk of relapse compared to patients from the Netherlands and Brazil (HR 1.9, 95% CI 1.3 to 2.8; Bonferroni-corrected P=0.03). Chinese patients had an increased mortality rate compared to patients from the Netherlands and Brazil (HR 15.5, 95% CI 6.7 to 36.0; P<0.001).

Conclusion
The lower frequencies of mucosa/eye and otolaryngeal involvement in China can be explained by a lower frequency of PR3-ANCA specificity. Chinese patients have a higher relapse risk than expected from lower frequencies of PR3-ANCA, GPA, and otolaryngeal involvement, and more frequent renal involvement. They also have a higher risk of mortality even after correction for baseline characteristics and treatment. This suggests that additional risk factors for relapse and mortality are present in this population.

INTRODUCTION

Clinical characteristics of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) differ between geographic regions and different ethnicities. Most importantly, while granulomatosis with polyangiitis (GPA) and PR3-ANCA specificity are most common in Northern Europe, microscopic polyangiitis (MPA) and MPO-ANCA specificity are more common in Southern Europe, Japan and China [1].

Previous studies found that PR3-ANCA specificity is a risk factor for relapse [2,3]. Also, PR3-ANCA positive patients had different genetic associations compared to MPO-ANCA positive patients in a Genome-Wide Association Study [4]. Therefore, differences in clinical characteristics and outcomes between populations might in part be explained by ANCA specificity. In two studies comparing the UK to Japan, ANCA type explained most phenotypic differences between GPA patients of both countries [5], while several population differences in organ manifestations of MPA patients could not be explained by ANCA specificity [6].

In this study, we sought to investigate the differences in disease characteristics and clinical outcome between a Brazilian, Chinese and Dutch cohort of AAV patients, spanning multiple continents and including long-term follow-up. Secondly, we aimed to investigate whether these differences might be explained by population differences in ANCA specificity.

PATIENTS AND METHODS

Study populations

For this retrospective cohort study, 264 patients were recruited from the departments of Internal Medicine/Nephrology and Rheumatology of the University Medical Center Groningen in the Netherlands, 411 from the Institute of Nephrology, Peking University First Hospital in China, and 97 from Rheumatology divisions of the following centers in Brazil: Hospital Federal dos Servidores do Estado do Rio de Janeiro (HFSE-RJ), Universidade do Estado do Rio de Janeiro (UERJ), State University of São Paulo, and. Universidade Federal de São Paulo - Escola Paulista de Medicina. Consecutive GPA and MPA patients diagnosed between 1987 and 2015 (Brazil), between 1996 and 2012 (China), and between 1990 to 2015 (Netherlands), respectively, were considered for inclusion in the study. Patients were classified according to the 2012 updated Chapel Hill Consensus Conference definitions [7]. Patients were treated according to previously described guidelines used in the respective countries [8-10]. All patients signed informed consent for collection of their data for the study. In the Netherlands, approval was given by the local Medical Ethical Committee of the University Medical Center Groningen (METc 2010.057). In China, approval was provided by the Ethics Committee of the Peking University First Hospital (IRB00001052-16049). In Brazil, approval was granted by the Ethics Committee on Research (process nr. 0147/2016). The research was conducted according to the principles from the declaration of Helsinki.

Data collection

Baseline patient characteristics were age, sex and geographic origin. The following disease characteristics at baseline were collected: diagnosis (GPA, MPA or renal limited vasculitis), disease activity and organ involvement using the Birmingham Vasculitis Activity Score (BVAS) [11], and ANCA status and specificity by Indirect Immune Fluorescence (IIF) or ELISA, if available. Modality of induction therapy was collected as treatment information. As follow-up data, relapses (new onset of disease activity attributable to vasculitis [12]) and mortality were collected for all centers.

Statistical analysis

Statistical analyses were performed using SPSS version 23 (IBM) and R version 3.4.2. A two-sided P-value <0.05 was considered statistically significant. Data are presented as mean (SD) or median (IQR) for continuous variables as appropriate, and as n (%) for categorical variables. Univariable analyses were performed to compare baseline variables between geographic origins. These were Kruskall Wallis test for continuous variables and Fisher's Exact test for categorical variables. In case of significant group differences, post-hoc tests were performed. Multivariable Cox regression was performed for relapse-free survival using geographic origin and previously reported factors associated with relapse-free survival as predictors [2,13]. Geographic origin, in addition to previously reported factors associated with mortality in AAV [14], were used as predictors in multivariable Cox regression analysis of overall survival. The proportional hazards assumption was tested using the scaled Schoenfeld residuals test. Due to the large number of tests performed in this study, Bonferroni-corrected P-values were calculated by multiplying all P-values by the number of tests performed (i.e., 74). P-values greater than 0.99 after Bonferroni correction are shown as P>0.99. A Bonferroni-corrected P-value <0.05 (corresponding to an unadjusted P-value of $6.8*10^{-4}$) was considered statistically significant.

RESULTS

Baseline differences

Several differences existed between patients from the three countries. Chinese patients were significantly older at diagnosis than Dutch and Brazilian patients. Chinese patients were less frequently PR3-ANCA positive and less frequently diagnosed with GPA compared to Dutch and Brazilian patients. See **Table 1**.

Disease characteristics per population are also shown in **Table 1**. Chinese patients, compared to both Dutch and Brazilian patients, less frequently had eye/mucosa (i.e., mouth ulcers, episcleritis) and ENT involvement (i.e., nasal complaints, sinusitis, otitis media) and more frequently had renal involvement (i.e., proteinuria, hematuria, elevated serum creatinine). Dutch patients less frequently had pulmonary involvement (i.e., nodules on chest imaging) compared to patients from other countries, and less frequently had abdominal involvement (i.e., abdominal pain/bloody diarrhea) compared to Chinese patients. Brazilian patients more frequently had skin involvement (i.e., ulcers, purpura) compared to Chinese patients, and less frequently had systemic involvement (i.e., malaise) compared to both other countries.

Table 1. Baseline characteristics.

Variable	NL (n=264)	CN (n=411)	BR (n=97)	Overall P†	P CN vs NL†	P BR vs NL†	P CN vs BR.†
Male	138 (52)	194 (47)	43 (44)	>0.99			
Age (years)	52 (40-63)	66 (53-73)	44 (36-57)	**<0.001**	**<0.001**	0.19	**<0.001**
GPA	208 (79)	97 (24)	88 (91)	**<0.001**	**<0.001**	0.91	**<0.001**
PR3-ANCA (ANCA-pos.)	191/248 (77)	38/409 (9)	47/56 (84) ‡	**<0.001**	**<0.001**	>0.99	**<0.001**
Creatinine >125 µmol/l	94 (36)	282 (69)	34 (35)	**<0.001**	**<0.001**	>0.99	**<0.001**
Induction				**<0.001**	**<0.001**	**<0.001**	**<0.001**
Oral CYC	156 (71)	67 (16)	40 (42)				
Pulsed CYC	3 (1)	248 (60)	32 (33)				
Other	62 (28)	96 (23)	24 (25)				
Disease activity	17 (11-23)	20 (15-23)	17 (12-22)	**0.001**	**0.001**	>0.99	0.20
Systemic	216 (82)	373 (91)	62 (64)	**<0.001**	0.06	**0.05**	**<0.001**
Cutaneous	46 (17)	49 (12)	33 (34)	**<0.001**	>0.99	0.11	**<0.001**
Mucosa/eyes	64 (24)	47 (11)	27 (28)	**<0.001**	**0.001**	>0.99	**0.01**
Otolaryngeal	179 (68)	150 (36)	71 (73)	**<0.001**	**<0.001**	>0.99	**<0.001**
Chest	121 (46)	294 (72)	67 (69)	**<0.001**	**<0.001**	**0.006**	>0.99
Cardiovascular	7 (3)	13 (3)	2 (2)	>0.99			
Abdominal	4 (2)	66 (16)	6 (6)	**<0.001**	**<0.001**	>0.99	0.71
Renal	161 (61)	387 (94)	60 (62)	**<0.001**	**<0.001**	>0.99	**<0.001**
Neurological	61 (23)	76 (18)	14 (14)	>0.99			

Variables shown as N (%) or median (IQR). BR Brazil; CN China; CYC cyclophosphamide; GPA granulomatosis with polyangiitis; NL Netherlands. † Bonferroni corrected. ‡21 patients PR3-ANCA positive ELISA; 26 patients c-ANCA on IIF, no ELISA.

In multivariable logistic regression, country remained significantly associated with systemic, chest, abdominal and renal involvement of AAV independent of ANCA-specificity. Presence of PR3-ANCA was the main predictor of mucosa/eye and ENT involvement. Results are shown in **Table 2**.

Table 2. Multivariable logistic regression of organ manifestations per country

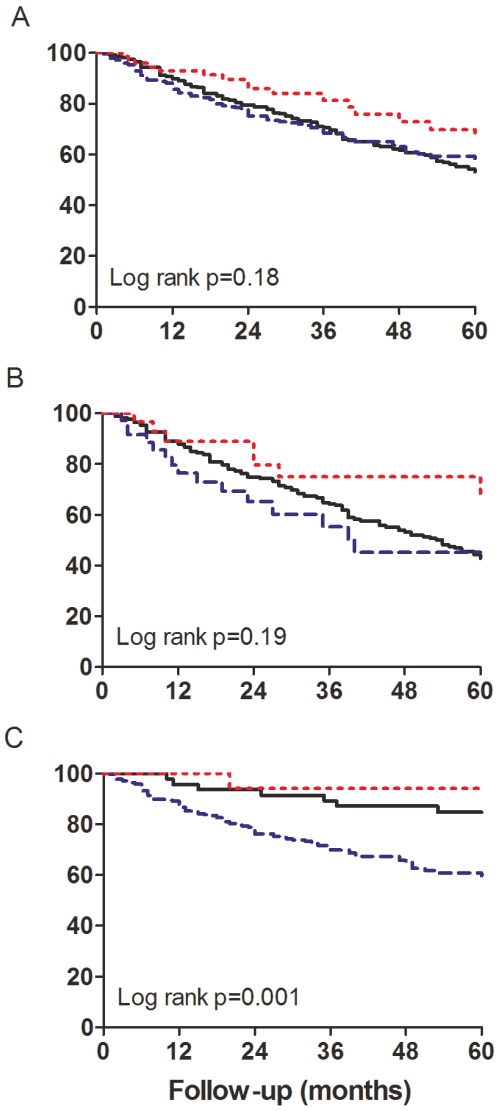
Variable		OR (95% CI)	P-value†
Systemic	China (vs Netherlands)	3.8 (2.1 to 6.9)	<0.001*
	Brazil (vs Netherlands)	0.3 (0.1 to 0.5)	
	PR3-ANCA (vs MPO-ANCA)	3.3 (1.8 to 6.2)	0.009*
Skin	China (vs Netherlands)	0.8 (0.5 to 1.5)	0.14
	Brazil (vs Netherlands)	2.8 (1.5 to 5.2)	
	PR3-ANCA (vs MPO-ANCA)	1.6 (0.9 to 2.8)	>0.99
Eye/mucosa	China (vs Netherlands)	1.1 (0.6 to 1.9)	>0.99
	Brazil (vs Netherlands)	1.1 (0.6 to 2.2)	
	PR3-ANCA (vs MPO-ANCA)	4.4 (2.4 to 7.8)	<0.001*
Otolaryngeal	China (vs Netherlands)	0.8 (0.5 to 1.2)	>0.99
	Brazil (vs Netherlands)	1.0 (0.5 to 2.0)	
	PR3-ANCA (vs MPO-ANCA)	5.3 (3.4 to 8.4)	<0.001*
Chest	China (vs Netherlands)	3.6 (2.3 to 5.7)	<0.001*
	Brazil (vs Netherlands)	2.7 (1.4 to 5.1)	
	PR3-ANCA (vs MPO-ANCA)	1.5 (0.9 to 2.3)	>0.99
Abdominal	China (vs Netherlands)	17.4 (5.5 to 55.4)	<0.001*
	Brazil (vs Netherlands)	7.1 (1.9 to 26.1)	
	PR3-ANCA (vs MPO-ANCA)	1.8 (0.9 to 3.8)	>0.99
Renal	China (vs Netherlands)	7.7 (4.2 to 14.1)	<0.001*
	Brazil (vs Netherlands)	1.1 (0.6 to 2.0)	
	PR3-ANCA (vs MPO-ANCA)	0.7 (0.4 to 1.3)	>0.99

*Country and ANCA specificity were entered simultaneously as predictors of organ involvement. * Statistically significant. † Bonferroni corrected.*

Relapse-free survival

Using a log rank test, relapse-free survival did not differ between countries (Bonferroni-corrected P>0.99). Chinese MPO-ANCA positive patients had a higher risk of relapse, but only before Bonferroni correction (**Figure 1**). Relapse free survival was not affected by induction therapy (corrected P>0.99). In multivariable Cox regression, after correction for ANCA-specificity, otolaryngeal and chest involvement, as well as serum creatinine >125 µmol/l at diagnosis, Chinese patients (HR 1.9, 95% CI 1.3 to 2.8), but not Brazilian patients (HR 0.5, 95% CI 0.2 to 1.1), had an increased risk of relapse compared to Dutch patients (Bonferroni-corrected P=0.03).

Figure 1. 60-month relapse free survival per country stratified by ANCA type



Relapse-free survival (%) for Brazilian (red line), Chinese (blue line) and Dutch (black line) patients. A: overall, B: PR3-ANCA positive, C: MPO-ANCA positive. P-values shown are unadjusted.

Mortality

In log rank analysis, Chinese patients had a significantly higher 5-year mortality rate than Brazilian and Dutch patients (Bonferroni-corrected P<0.001). This was true for both MPO-ANCA and PR3-ANCA positive patients (**S1 Figure**). In multivariable Cox regression with adjustment for age, serum creatinine >125 μmol/l at diagnosis, pulmonary involvement and induction treatment, patients from China still had a higher risk of mortality (Bonferroni-corrected P<0.001) compared to Dutch patients (HR 15.5, 95% CI 6.7 to 36.0). Brazilian patients also had an increased risk of mortality (HR 3.5, 95% CI 1.1 to 11.6), although this difference was no longer statistically significant after correction for multiple comparisons.

DISCUSSION

In this study, we found differences in distribution of ANCA-specificity, clinical characteristics and clinical outcome between Brazil, China and The Netherlands. Except for eye/mucosa and ENT involvement, both of which were mainly associated with PR3-ANCA positivity, inter-regional differences in clinical manifestations persisted after correction for ANCA specificity.

After correction for ANCA specificity, otolaryngeal involvement, chest involvement and serum creatinine >125 μmol/l at diagnosis, relapse risk was significantly higher for Chinese patients compared to Dutch and Brazilian patients. Based on a lower frequency of PR3-ANCA positivity and patients with otolaryngeal involvement [13], as well as a higher frequency of patients with elevated creatinine levels at diagnosis [2], Chinese patients were expected to have a lower risk of relapse compared to Dutch and Brazilian patients. However, despite these disease characteristics, they had a similar risk of relapse. This indicates an additional risk factor for relapse in Chinese patients that is not, or to a lesser extent, present in Dutch and Brazilian patients. As a relatively large number of Chinese patients used IV cyclophosphamide, which has been associated with an increased risk of relapse compared to oral cyclophosphamide [15], we hypothesized that the more frequent use of IV cyclophosphamide in China might explain the higher-than-expected risk of relapse for this group. However, the type of induction therapy was not associated with relapse-free survival in this study, possibly due to the Chinese treatment protocol dictating longer duration of IV cyclophosphamide (6-9 months) compared to oral cyclophosphamide (3-4 months) induction therapy [8].

Chinese patients had a higher risk of mortality regardless of ANCA type, even after correction for factors associated with mortality such as age, elevated serum creatinine at diagnosis, pulmonary involvement and type of induction treatment. This again indicates that AAV in Chinese patients behaves differently during follow-up than in comparable Brazilian and Dutch patients. One explanation might be a worse renal function in Chinese AAV patients, most likely because the Chinese cohort being derived from a tertiary Nephrology referral center. Alternatively, unmeasured differences in treatment or follow-up could be an explanation, although reported adherence to the treatment protocol is strong in all countries.

In line with previous studies, PR3-ANCA positivity, as well as eye/mucosa and ENT involvement, were less common in China compared to Brazil and the Netherlands [1]. The high frequency of Chinese patients with kidney involvement and elevated serum creatinine in this study is most likely due to the participating center in China being a tertiary nephrology referral center. Most other differences in disease characteristics between patients from China versus the other two countries can be explained by a lower frequency of PR3-ANCA positive patients in China. An exception is the higher frequency of abdominal pain/bloody diarrhea in China compared to the Netherlands.

Striking differences between Brazil and the other countries were a higher frequency of skin manifestations (ulcers and purpura) compared to Chinese patients and a lower frequency of systemic AAV manifestations (malaise) compared to both other countries. We did not find a clear explanation for these differences in the present study. The difference might result from genetic or environmental factors. Alternatively, the differences might be due to the Rheumatology department being the main source of patients from the Brazilian cohort, while Dutch and Chinese patients were partly or fully included from Nephrology departments.

Dutch patients have a relatively low frequency of pulmonary nodules. A possible explanation might be less frequent pulmonary imaging in the Netherlands, considering that imaging may still show pulmonary abnormalities in asymptomatic patients. Unfortunately, data on whether or not imaging was performed was not collected in the database.

A major strength of this study is the availability of long-term follow-up data, allowing for comparison of treatment outcomes between countries. Also, Chinese and Dutch patients were both recruited from one center, each using their own standardized treatment protocol. This results in less variation in treatment of patients from the respective countries. Another strength is the strict correction for multiple comparisons, which increases confidence in the relevance of statistically significant findings in this study.

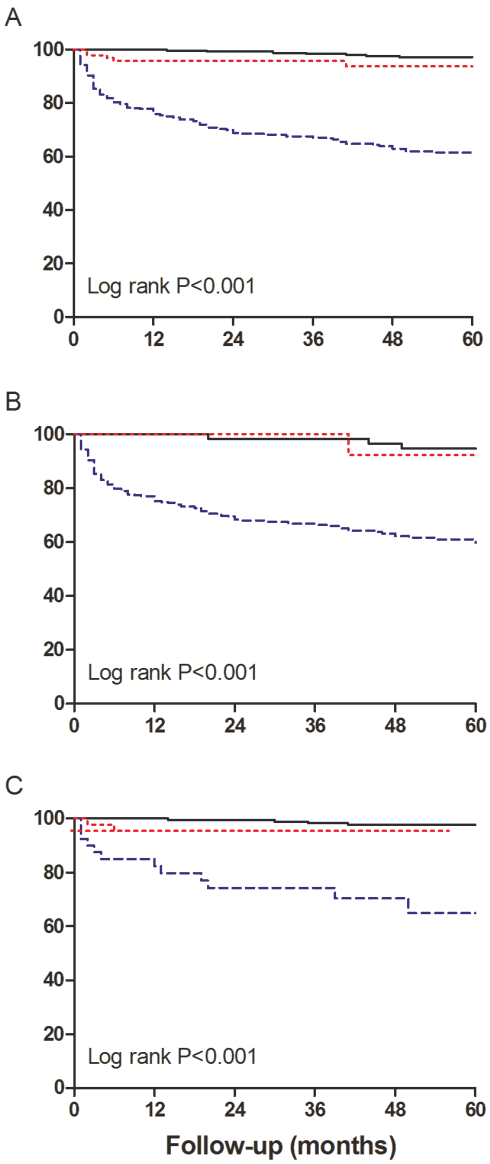
The study also has several limitations. First, data for each study has been collected in different clinical centers. This results in different treatments per country, as well as possible differences in disease assessment, although the same criteria were used to classify patients and the same BVAS version was used to score disease manifestations. Second, differences in distribution of recruiting specialties could have resulted in over- or underrepresentation of patients with certain organ manifestations in a country. For example, the frequency of renal involvement may be overestimated in Chinese patients of this study, because the recruiting center was a nephrology specialty referral hospital. Lastly, exact serum creatinine levels at diagnosis were not available for all cohorts. Also, insufficient data about renal function and dialysis dependence over time was available to include them in the study. These data would have been especially relevant in relation to overall survival in the different cohorts.

In conclusion, differences in mucosa/eye and ENT involvement between Chinese patients versus Dutch and Brazilian patients could be explained by the lower frequency of PR3-ANCA in China. Other differences in organ manifestations between countries could

not be explained by differences in ANCA-specificity. Chinese patients have a similar risk of relapse to Dutch and Brazilian patients despite a theoretically lower risk of relapse based on disease characteristics and ANCA-specificity, as well as a higher risk of mortality, suggesting the presence of additional risk factors for relapse and mortality in the Chinese population.

SUPPORTING INFORMATION

S1 Figure. 60-month overall survival per country stratified by ANCA type



Overall survival (%) for Brazilian (red line), Chinese (blue line) and Dutch (black line) patients. A: overall, B: MPO-ANCA positive, C: PR3-ANCA positive. P-values shown are unadjusted.

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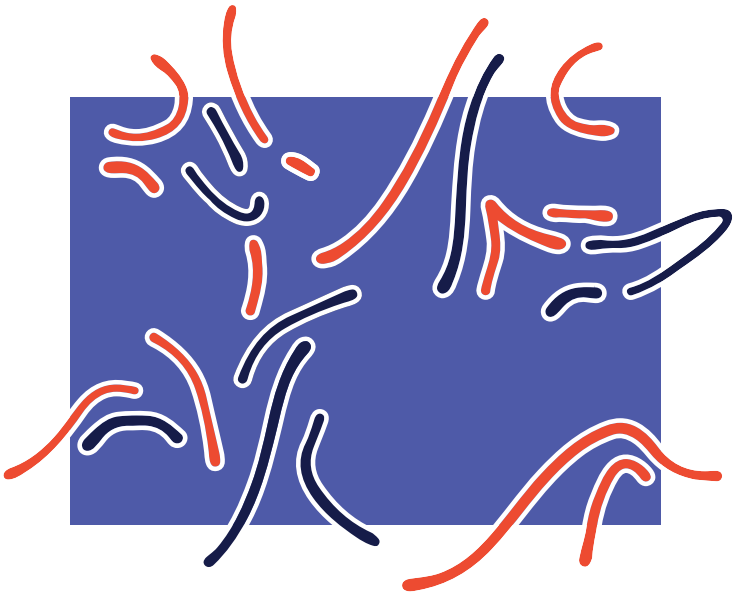
06 Chapter

Azathioprine hypersensitivity syndrome in a cohort of antineutrophil cytoplasmic antibody-associated vasculitis patients



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What is already known about the topic?
Azathioprine hypersensitivity syndrome is a rare adverse effect of azathioprine therapy associated with fever, nausea, arthralgia, and cutaneous eruptions. It should be separated from other drug-related side effects, such as hepatotoxicity and leukocytopenia.

What does this article add to our knowledge?
Although earlier studies were mostly case reports/series or literature overviews, this study gives more accurate estimations of the incidence of azathioprine hypersensitivity syndrome and its characteristics within an observational cohort. This helps in its identification.

How does this study impact current management guidelines?
Its frequency (9%) warrants more awareness of azathioprine hypersensitivity as a cause of systemic inflammation and skin eruptions. It should be an important differential diagnosis besides infection or relapse for clinical deterioration after starting azathioprine.

ABSTRACT

Background
Azathioprine hypersensitivity syndrome is a rare complication of azathioprine therapy. Its symptoms resemble infection or relapse of inflammatory disease, hindering correct diagnosis. Current literature is limited to sporadic case reports and reviews.

Objective
To estimate the incidence of azathioprine hypersensitivity syndrome and describe its characteristics in the context of an observational cohort of patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Also, to facilitate early recognition and awareness among clinicians.

Methods
Within a cohort of 290 patients with ANCA-associated vasculitis receiving azathioprine maintenance therapy, frequency of azathioprine hypersensitivity was described and characteristics were compared between hypersensitive and non-hypersensitive patients. Clinical picture, laboratory abnormalities, and concurrent medication of patients with azathioprine hypersensitivity were described.

Results
Of 290 patients, 25 (9%) experienced azathioprine hypersensitivity after a median of 14 (interquartile range [IQR] 12-18) days. Frequent symptoms were fever (100%), malaise (60%), arthralgia (36%), and rash (32%). All patients used prednisolone (median 10 mg/day, IQR 9.4-16.3 mg/day) at the time of the hypersensitivity reaction. Most patients had a rise in C-reactive protein (CRP), leukocyte counts, and neutrophil counts, but no eosinophilia. Thiopurine S-methyltransferase (TPMT) activity was significantly lower in hypersensitive patients (median 74.4 [IQR 58.0-80.1] nmol/gHb/L) compared with controls (median 81.4 [71.9-90.5] nmol/gHb/L), P=.01. Hypersensitive patients had a higher risk of relapse (hazard ratio 2.2, 95%confidence interval 1.2-4.2; P=.01).

Conclusions
Azathioprine hypersensitivity syndrome is strikingly common in ANCA-associated vasculitis, might be associated with reduced TPMT activity, is accompanied by an increase in neutrophil counts, and may occur even during concomitant prednisolone therapy. Proper recognition may prevent unnecessary hospital procedures and damage to the patient.

INTRODUCTION

Azathioprine is widely used for the treatment of inflammatory conditions including inflammatory bowel disease (IBD) and multiple sclerosis (MS). In antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), a group of autoimmune diseases comprising granulomatosis with polyangiitis (GPA), microscopic polyangiitis, nephritic crescentic glomerulonephritis, and eosinophilic granulomatosis with polyangiitis (EGPA),[1] azathioprine is used as maintenance therapy after remission induction, usually with cyclophosphamide or rituximab.[2]

A relatively uncommon adverse effect of azathioprine is a hypersensitivity syndrome characterized by systemic symptoms such as fever, arthralgia, abdominal pain, and nausea, with or without cutaneous symptoms.[3] Because of the clinical picture of the hypersensitivity reaction and accompanying laboratory abnormalities, it can be mistaken for an infection or relapse of vasculitis activity.[4] Although usually self-limiting on termination of azathioprine therapy, the azathioprine hypersensitivity syndrome can be life-threatening with shock and acute renal insufficiency as possible features.[5,6]

The pathogenesis of azathioprine hypersensitivity syndrome has not been fully elucidated. A type III (immune complex-mediated) and type IV (T-cell-mediated) reaction are possible underlying types of hypersensitivity.[3,7] As neutrophilia is frequently seen in patients with azathioprine hypersensitivity, neutrophils might also play a role in the pathogenesis.[7]

Azathioprine is a prodrug. It is first enzymatically converted into 6-mercaptopurine (6-MP), which is further converted through 1 of 3 competing pathways. Conversion of 6-MP by hypoxanthine phosphoribosyltransferase results in 6-thioguanine nucleotides (6-TGN), the active metabolites responsible for the cytotoxicity of azathioprine. By contrast, conversion by xanthine oxidase results in formation of inactive thiouric acid, whereas conversion by thiopurine S-methyltransferase (TPMT) results in formation of inactive 6-methylmercaptopurine.[8]

The gene encoding TPMT is localized on chromosome 6.[8] Several single nucleotide polymorphisms have been described for this gene. The most common variants in Caucasians, besides wild-type (TPMT*1), are TPMT*2 (C>G at rs1800462), TPMT*3A (C>T at rs1800460 and T>C at rs1142345), TPMT*3B (C>T at rs1800460), and TPMT*3C (T>C at rs1142345). All of these are nonfunctional variants causing reduced TPMT activity.[9] This indirectly results in increased levels of 6-TGN and, therefore, increased myelotoxicity.[8,10] In previously published cases of azathioprine hypersensitivity, patients had a normal TPMT genotype and activity. Therefore, TPMT is not considered to be related to the occurrence of azathioprine hypersensitivity.[3]

The treatment of azathioprine hypersensitivity syndrome is generally not required, as symptoms usually resolve within a few days after discontinuation of azathioprine.[3,7] Switching from azathioprine to 6-MP and vice versa was successful in a minority of cases.[3,11] Also, several cases of successful desensitization have been reported.[11] In case of AAV, most patients switch to other maintenance therapy, usually mycophenolate mofetil, methotrexate, or rituximab, in accordance with treatment guidelines.[2]

So far, studies on azathioprine hypersensitivity were mostly case reports, case series, or literature overviews of such studies. This does not allow for an adequate estimation of the incidence and distribution of symptoms of the azathioprine hypersensitivity syndrome. This study describes the incidence of azathioprine hypersensitivity, distribution of symptoms, associated laboratory abnormalities, and concomitant medication use within an observational cohort of patients with ANCA-associated vasculitis treated with azathioprine maintenance therapy according to a local AAV treatment protocol.

METHODS

Participants

The study is part of a single-center observational cohort study investigating potential biomarkers related to disease outcomes in patients with ANCA-associated vasculitis, diagnosed and treated in
the Vasculitis Expertise Center of the University Medical Center Groningen between 1972 and 2017. Recruitment for the present study took place between July 2010 and April 2017. During this period, 12 of 359 patients approached refused participation in the observational cohort. Of the remaining 347 patients, 57 were excluded because they never used azathioprine during follow-up. The remaining 290 patients with ANCA-associated vasculitis were included for analysis. All patients provided written informed consent for participation in the cohort study. In addition, 1 patient provided written informed consent to publish his clinical photographs, made at the time of azathioprine hypersensitivity, in a scientific journal. The study was approved by the local medical ethical committee of the University Medical Center Groningen (METc 2010/057) and was conducted according to the principles outlined in the Declaration of Helsinki (Fortaleza, Brazil, October 2013).

Patients with documented fever (temperature >38ºC) and/or elevated C-reactive protein (CRP >5 mg/L) and/or skin manifestations and/or reoccurrence of the same symptoms within a day after rechallenge, attributed to azathioprine and resolving within a week after stopping the drug, were labeled as cases. All other patients were labeled as controls. All patients were approached and asked consent by their physician for a rechallenge with a small dose (25 mg) of azathioprine. A positive rechallenge of azathioprine hypersensitivity was defined as reoccurrence of fever and/or other symptoms attributed to azathioprine hypersensitivity after rechallenge with azathioprine, which improved within days after discontinuation of azathioprine.

Data collection

All data were retrieved from the patients' medical records. Follow-up data were collected until May 2018. Demographic and disease characteristics as well as any relapses within 60 months after start of therapy, or until the last visit if follow-up was shorter than 60 months, were collected for all patients. A relapse of vasculitis was defined as any disease activity requiring new immunosuppressive therapy or intensification of current treatment, in accordance with European League Against Rheumatism (EULAR) recommendations. [12] For cases with azathioprine hypersensitivity, if present, symptoms of

azathioprine hypersensitivity, laboratory values before start of azathioprine and during azathioprine hypersensitivity, as well as concurrent medication use were collected.

Statistics

Statistical analysis was performed using SPSS Statistics 23 (IBM Corporation, Armonk, NY). A 2-sided P value <.05 was considered statistically significant. Missing data were handled using pairwise deletion. Continuous variables were described as median (interquartile range [IQR]); categorical variables were described as n (%). A 95% confidence interval (CI) around the proportion of patients with azathioprine hypersensitivity was calculated using the Wilson procedure with continuity correction.[13] Demographic and disease characteristics were compared between cases and controls using the Mann-Whitney U test for continuous variables and the Fisher exact test for categorical variables. Subsequently, time intervals, symptoms, and laboratory findings were described for cases of febrile azathioprine hypersensitivity. Finally, the risk of relapse up to 5 years after the start of induction therapy was compared between cases and controls using Cox proportional hazards analysis. The proportionality assumption of Cox regression was tested using scaled Schoenfeld residuals (R version 3.4.2). After univariable survival analysis, multivariable Cox regression was performed with correction for TPMT activity and ANCA specificity (proteinase 3 vs myeloperoxidase/other/negative) because of their known relation with relapse/efficacy of azathioprine therapy and the baseline differences observed in this study.

RESULTS

Patients

Of the 290 patients included, 25 (8.6% [95% CI 5.8% to 12.6%]) had a febrile hypersensitivity reaction, of whom 16 were confirmed through rechallenge; 9 patients refused rechallenge. Several differences were found between patients with and without azathioprine hypersensitivity. First, patients with azathioprine hypersensitivity had a significantly lower TPMT activity (median 74.4 [IQR 58.0-80.1] nmol/gHb/hour) compared with controls (median 81.4 [IQR 71.9-90.5] nmol/gHb/hour; P = .01). This translates into a higher frequency of patients with reduced (≤52 nmol/gHb/hour) TPMT activity (5 [20%]) in cases compared with controls (19 [7%]; P = .05). Second, they had a higher frequency of variant TPMT carriers (5 [20%]) compared with controls (22 [9%]; P = .05). Finally, there was a trend toward a lower frequency of patients classified as GPA in the hypersensitive group (13 [52%]) compared with the control group (198 [75%]; P = .06). Baseline results are summarized in **Table 1**.

Symptoms of azathioprine hypersensitivity

Besides fever (diagnostic criterion in this study), the most frequent symptoms noted in the group of 25 hypersensitive patients were malaise, chills, arthralgia, myalgia, skin involvement, and gastrointestinal complaints (see **Table 2**). Four patients (16%) experienced acute kidney injury and 1 patient (4%) experienced circulatory shock. Median interval between the start of azathioprine and complaints was 14 days (IQR 12-18, range 7-37 days). All 16 rechallenged patients had a recurrence of symptoms within hours. The

symptoms of patients with rechallenge confirmed (definite) hypersensitivity were similar in type and frequency to those of patients without rechallenge. All symptoms including frequencies are summarized in **Table 2**. An example of skin involvement is shown in **Figure 1**.

Table 1. Patient characteristics

Characteristic	All (n=290)	Hypersensitive (n=25)	Control (n=265)	P-value
Age at AAV diagnosis (y)	54 (42-63)	56 (45-66)	53 (41-63)	0.22
Duration of follow-up (m)	48 (27-60)	49 (28-60)	44 (16-60)	0.31
Female, n (%)	140 (48)	8 (32)	132 (50)	0.10
Diagnosis				0.06
GPA	211 (72.8)	13 (52)	198 (75)	
MPA	42 (14.5)	6 (24)	36 (14)	
NCGN	13 (4.5)	2 (8)	11 (4)	
EGPA	24 (8.3)	4 (16)	20 (8)	
ANCA specificity				0.22
PR3	200 (69.0)	13 (52)	187 (71)	
MPO	62 (21.4)	8 (32)	54 (20)	
Other	4 (1.3)	0 (0)	4 (1)	
Negative	24 (8.3)	4 (16)	20 (8)	
TPMT genotype	N=280/290	N=25/25	N=255/265	0.05*
*1/*1	253 (90)	20 (80)	233 (91)	
*1/*3A	22 (8)	3 (12)	19 (8)	
*1/*3C	5 (2)	2 (8)	3 (1)	
TPMT activity (nmol/gHb/h) (n=283/290)	80.4 (70.2-90.4)	74.4 (58.0-80.1)	81.4 (71.9-90.5)	0.01*
Low TPMT activity (≤52.0 nmol/gHb/h)	N=283/290 24 (9%)	N=25/25 5 (20%)	N=258/265 19 (7%)	0.05*

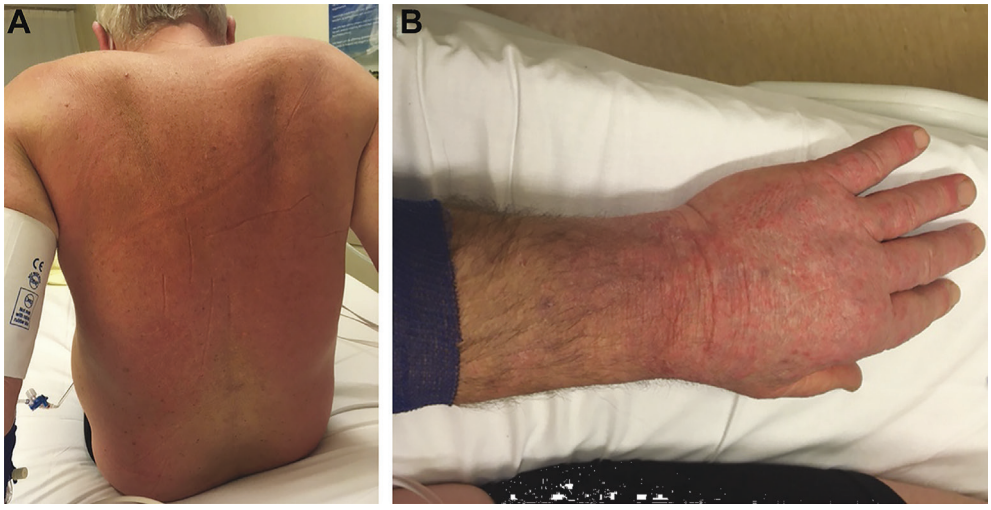
Baseline characteristics for all included patients, split out for patients with azathioprine hypersensitivity and azathioprine tolerant patients. Data described as n (%) or median (IQR). P<0.05.*

Table 2. Symptoms of azathioprine hypersensitivity syndrome

Symptom	All hypersensitive (n=25)	Rechallenge confirmed (n=16)
Patient reported		
Fever*	25 (100)	16 (100)
Malaise	15 (60)	10 (63)
Arthralgia, myalgia	9 (36)	6 (38)
Chills	5 (20)	5 (31)
Gastrointestinal (pain, nausea, vomiting)	5 (20)	4 (25)
Objective		
Skin involvement	8 (32)	4 (25)
Acute kidney injury	4 (16)	2 (13)
Hypotension/circulatory shock	1 (4)	1 (6)
Hepatotoxicity	0 (0)	0 (0)

*Overview of frequency of azathioprine hypersensitivity symptoms in the cohort shown as n (%). *Objective confirmation in 22 of 25 patients (14 of 16 rechallenged patients).*

Figure 1. Picture of skin involvement in azathioprine hypersensitvity



Skin eruption in a 65-year-old patient with GPA with azathioprine hypersensitivity. He devel-

oped malaise, fever, nausea, and vomiting 2.5 weeks after starting azathioprine. On physical examination, a maculopapular exanthema was seen (A), which was most pronounced on the backs of his hands and feet (B). The pathology report described superficial lymphocytic and neutrophilic granulomatous inflammation, without micro-organisms or vasculitis. GPA, Granulomatosis with polyangiitis.

Laboratory values of hypersensitive patients

In total, 12 patients had laboratory evaluation at the time of azathioprine hypersensitivity. Most patients had a rise in CRP and neutrophil counts compared with the values before starting azathioprine (see **Table 3**). During the hypersensitivity reaction, the most frequent laboratory abnormalities were elevated CRP, present in all 12 patients, and neutrophilia, present in 7 of 9 measured patients (78%). No eosinophilia was observed.

Table 3. *Laboratory findings of patients with azathioprine hypersensitivity*

Patient no., sex, age (y)	Diagnosis, ANCA type	CRP (mg/l)		Leukocytes (10^9/l)		Neutrophils (10^9/l)		Eosinophils (10^9/l)	
		Before reaction	With reaction	Before reaction	With reaction	Before reaction	With reaction	Before reaction	With reaction
1,M,45	MPA, MPO	↑7	↑315	4.8	9.8	3.66	↑9.10	0.13	0.03
2,M,57	GPA, PR3	↑11	↑222	5.9	9.4	NA	NA	NA	NA
3,F,69	eGPA, MPO	↑31	↑112	5.5	6.6	3.97	5.16	↑0.44	0.40
4,M,56	MPA, MPO	↑40	↑390	8.5	↑17.0	7.18	↑16.48	0.00	0.00
5,M,45	GPA, PR3	<5	↑327	9.6	9.7	↑8.34	↑8.38	0.03	0.03
6,M,65	MPA, MPO	↑21	↑225	3.8	8.9	2.67	↑7.55	0.00	0.01
7,M,49	GPA, PR3	<5	↑125	6.3	↑11.5	4.87	↑10.19	0.03	0.08
8,F,74	MPA, MPO	<5	↑194	5.0	8.7	4.33	↑8.00	0.00	0.03
9,M,73	GPA, PR3	↑20	↑166	8.5	7.7	7.07	6.15	0.03	0.19
10,M,47	GPA, PR3	<5	↑25	5.6	3.8	5.21	NA	0.02	NA
11,M,46	GPA, PR3	<5	↑22	9.9	9.4	↑8.83	NA	0.00	NA
12,F,59	MPA, MPO	<5	↑34	↑10.8	↑10.1	↑9.36	↑8.61	0.14	0.43

↑ elevated according to local reference values; eGPA eosinophilic granulomatosis with polyangiitis; GPA granulomatosis with polyangiitis; MPA microscopic polyangiitis; MPO myeloperoxidase; NA not available; NCGN nephritic crescentic glomerulonephritis; Pre before start of azathioprine; PR3 proteinase 3

Concurrent medication
Median azathioprine dose of hypersensitive patients was 100 mg/day (IQR 100-150 mg/day). All patients used prednisolone (median 10 mg/day, IQR 9.4-16.3 mg/day) at the time of the hypersensitivity reaction.

Clinical course after azathioprine hypersensitivity
Of all 25 hypersensitive patients, 13 (52%) switched to mycophenolate mofetil, 7 (28%) to cyclophosphamide, 2 (8%) to methotrexate, and 3 (12%) received no therapy after azathioprine hypersensitivity. Relapse data were available for 24 of 25 hypersensitive patients and 257 of 265 nonhypersensitive controls. In total, 12 (50%) hypersensitive cases and 104 (40%) controls experienced a relapse of vasculitis within 5 years. In univariable Cox regression, azathioprine hypersensitivity was not associated with risk of relapse (hazard ratio [HR] 1.4, 95% CI 0.8-2.6; P = .25). In multivariable Cox regression, after correction for ANCA specificity and TPMT activity, azathioprine hypersensitivity was a statistically significant risk factor of relapse (HR 1.9, 95% CI 1.0-3.6; P = .04).

DISCUSSION

In this description of azathioprine hypersensitivity cases within our single-center cohort of 347 patients with ANCA-associated vasculitis, there were several unexpected findings.

First, febrile azathioprine hypersensitivity occurred in 9% (95% CI 6% to 13%) of azathioprine users, which is more frequent than the 2% (95% CI 1% to 4%) frequently mentioned based on a cohort study of patients with IBD using 6-MP.[11] On the other hand, similar frequencies of febrile azathioprine hypersensitivity were found in patients with MS (10% [95% CI 4% to 23%]) and IBD (6% [95% CI 3% to 11%]) using azathioprine.[14,15] Indeed, some previously reported patients have successfully switched from 6-MP to azathioprine,[11] or vice versa,[16,17] indicating that the imidazole group of azathioprine is an additional epitope capable of inducing azathioprine hypersensitivity, besides epitopes from 6-MP and/or its metabolites. This might explain a higher frequency of hypersensitivity in azathioprine-treated populations compared with 6-MP-treated populations. The high frequency of azathioprine hypersensitivity in this population is given more relevance by our finding that febrile azathioprine hypersensitivity is an independent risk factor of relapse, most likely due to the necessity of a switch to less effective maintenance therapy.[2] The interval between the start of azathioprine and onset of symptoms (median 14 days), and the most frequently occurring symptoms (fever, malaise, arthralgia, skin eruption) were similar to those described previously.[3]

Second, hypersensitive patients had a significantly lower TPMT activity compared with controls, with 20% of hypersensitive patients having reduced TPMT activity (≤52 nmol/gHb/hour) compared with 7% of controls. Earlier studies did not find an association with TPMT status, but were limited by incomplete reporting of TPMT status and lack of a control group.[3] As only 20% of hypersensitive patients had reduced TPMT activity, TPMT deficiency is not required for the development of azathioprine hypersensitivity. More likely, reduced TPMT activity is a susceptibility factor resulting in prolonged exposure

of azathioprine, 6-MP, or another metabolite responsible for the response, to T-cells. Importantly, TPMT deficiency by itself is not sufficient to develop azathioprine hypersensitivity.

Finally, all patients with azathioprine hypersensitivity were using prednisolone at the time of their hypersensitivity reaction, meaning that low- to medium-dose prednisolone use (up to 30 mg/day in this study) does not (sufficiently) protect against the occurrence of this clinical syndrome.

The mechanism of azathioprine hypersensitivity has not yet been elucidated. Based on the low dose (25 mg) required for rechallenge and normal TPMT in most (80% of) patients, it is most likely dose independent (type B).[18] Based on the time course at the first exposure and rechallenge and the frequent presence of neutrophilia, it is most likely either a type IVd hypersensitivity reaction (T-cell mediated, with involvement of neutrophils)[19] or a direct pharmacologic interaction of the drug or a metabolite to an immune receptor (p-i reaction).[18]

In this study, we describe azathioprine hypersensitivity in a large observational cohort, resulting in more accurate estimation of the frequency of azathioprine hypersensitivity and distribution of symptoms compared with previous studies. Also, 16 (64% of) patients had a confirmation of azathioprine hypersensitivity through rechallenge with a low dose of azathioprine, allowing for a more reliable description of symptoms. The similar distribution of symptoms in patients with and without rechallenge indicates that patients with azathioprine hypersensitivity have been adequately selected in this study.

This study also has some limitations. First, not all patients received an in-hospital laboratory evaluation during their hypersensitivity reaction, making it difficult to draw solid conclusions regarding laboratory abnormalities associated with azathioprine hypersensitivity. Second, descriptions of azathioprine hypersensitivity symptoms were collected retrospectively, making them dependent on reporting in the electronic patient records. Third, the frequency of azathioprine hypersensitivity in this cohort might be underestimated due to the strict definition requiring fever or due to misdiagnosis as infection or disease relapse. Fourth, no biopsies were performed and descriptions were limited for most hypersensitive patients with skin rash, making it difficult to accurately classify the skin reactions in this cohort. Finally, we did not perform an lymphocyte transformation test with azathioprine to strengthen our hypothesis of a type IV hypersensitivity reaction.

In conclusion, azathioprine hypersensitivity is more frequent than previously mentioned and results in less effective maintenance of disease remission, at least in ANCA-associated vasculitis. Symptoms of azathioprine hypersensitivity reflect systemic inflammation and must be distinguished from disease relapse and infection. Many patients experience an increase in neutrophil counts. The reaction can occur despite (low- to medium-dose) prednisolone therapy.

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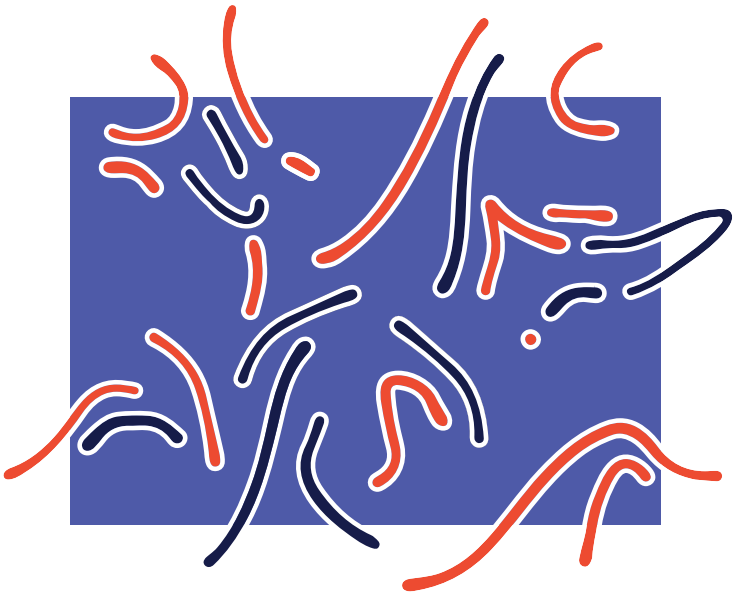
07 Chapter

Leg muscle strength is reduced and is associated with physical quality of life in antineutrophil cytoplasmic antibody-associated vasculitis



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ABSTRACT

Objective
Physical quality of life is reduced in ANCA-associated vasculitis (AAV). This study aims to investigate whether this may be explained by reduced muscle strength and physical activity resulting from disease damage and steroid myopathy.

Methods
Forty-eight AAV patients were sequentially included from the outpatient clinic. Patients in different stages of disease and treatment underwent measurements of muscle strength and anthropometric parameters. Patients filled in physical activity (Baecke) and quality of life questionnaires (RAND-36) and carried an accelerometer for a week. Muscle strength and physical activity were compared to quality of life, prednisolone use and disease duration.

Results
Most AAV patients had lower knee extension (76%) and elbow flexion (67%) forces than expected based on healthy norms. Also, physical ($P<0.001$) and mental ($P=0.01$) quality of life were significantly reduced compared to healthy norm values. Lower knee extension force ($P=0.009$), younger age <70 ($P<0.001$) and relapse of vasculitis ($P=0.003$) were associated with lower age-adjusted physical quality of life. Lower Baecke index ($P=0.006$), higher prednisolone dose ($P=0.005$) and ENT involvement ($P=0.006$) were associated with lower age-adjusted mental quality of life. Leg muscle strength showed no association with current or cumulative prednisolone use. Disease duration was longer in patients with knee extension force below healthy norms ($P=0.006$).

Conclusion
Knee extension force and physical activity are positively associated with quality of life in AAV. Knee extension force decreases with longer disease duration, suggesting that disease- and treatment-related damage have a cumulative negative effect on muscle strength.

INTRODUCTION

ANCA-associated vasculitis (AAV) is a group of primary vasculitides associated with inflammation of the small and medium sized blood vessels. The most frequent forms are Granulomatosis with Polyangiitis (GPA, formerly Wegener's Granulomatosis) and Microscopic Polyangiitis (MPA) [1].

Mortality has drastically decreased after introduction of immunosuppressive therapy and most patients can now be brought into remission. Unfortunately, the disease and its treatment are associated with damage that accumulates with prolonged disease duration, recurrent disease episodes and treatment exposure [2,3].

Quality of Life (QoL), especially physical QoL, is reduced in AAV patients compared to the general population [4-6]. It is important to identify modifiable factors associated with health-related QoL [5,7], as these factors will guide development of new treatments that improve the outcome.

One of the factors previously associated with reduced physical QoL is prednisolone use [4,5]. A well-known adverse effect of glucocorticoids (GCs) such as prednisolone is skeletal muscle atrophy [8]. GC-induced skeletal muscle atrophy results from a combination of reduced protein synthesis and increased muscle proteolysis [8,9]. Mainly fast-twitch (type II) muscle fibres are affected [8]. Proximal muscles are more severely affected than distal and cranial muscles [9]. GC-induced skeletal muscle atrophy develops after approximately 4 weeks of therapy, and is most frequently seen with higher doses of GCs (prednisolone 40-60mg/d or equivalent doses of other GC) [9].

GC-induced skeletal muscle atrophy might partly explain the relation between prednisolone use and impaired physical QoL in AAV. In our clinical experience, many patients with AAV suffer from a significant loss of leg muscle strength during prednisolone treatment. They report difficulties rising from a chair and walking stairs. In several studies that focused on patient perspectives, patients reported muscle weakness as an important disease burden [10,11]. In a study by Newall et al, AAV patients had a reduced exercise capacity, which correlated with quadriceps force [12]. These findings suggest an impact of leg muscle force on exercise performance in AAV patients, which might in turn affect QoL.

Muscle strength can be improved through exercise [13]. Therefore, reduced muscle strength and an association of muscle strength with QoL in AAV patients would warrant intervention studies regarding exercise programs or improvement of muscle strength for this population.

The first goal of this cross-sectional study performed at the outpatient clinic of the Vasculitis Expertise Center Groningen was to investigate the relation between leg muscle force, physical activity and health-related QoL in AAV patients. The second goal was to study the relation of leg muscle force with disease duration and treatment exposure, especially GC treatment.

PATIENTS AND METHODS

Study population

GPA and MPA patients were recruited from the outpatient clinic of the University Medical Center Groningen (UMCG) between July 2015 and October 2017. Patients were eligible for inclusion if they met the following inclusion criteria: age ≥18 years, diagnosis of Granulomatosis with Polyangiitis (GPA) or Microscopic Polyangiitis (MPA) according to the Chapel Hill criteria [1], first diagnosis or most recent relapse of disease activity within 3 years prior to inclusion, and treatment with a high dose of prednisolone (1mg/kg/day, tapered according to local protocol) and an additional immunosuppressive drug (e.g., cyclophosphamide, rituximab) as induction therapy. Patients with comorbidity causing reduced mobility or muscle strength and patients with active disease were excluded. More specifically, patients with neurological disease (cerebrovascular accident, hernia nuclei pulposi, peripheral motor nerve damage, critical illness neuropathy), pulmonary disease (dyspnea and activity limitations due to pulmonary involvement of AAV, chronic obstructive pulmonary disease, restrictive pulmonary disease), cachexia and fractures (vertebral fractures, radial fracture) were excluded. All patients signed written informed consent for participation in the study. The study was performed according to the principles of the Declaration of Helsinki and has received ethical approval by the local Medical Ethical Committee of the University Medical Center Groningen (METc 2015/184).

Design

All patients received an initial cross-sectional measurement after a regular visit to the outpatient clinic. Patients with a prednisolone dose ≥30mg/d at the first study visit were approached and asked informed consent for a follow-up measurement at a later time point, at a low prednisolone dose (≤2.5mg/d), or after discontinuation of prednisolone.

MEASUREMENTS

Demographic and clinical characteristics

Demographic data and clinical characteristics were collected from the patients' medical records. The following data were collected: age, sex, diagnosis (GPA or MPA), ANCA specificity (PR3, MPO, negative), disease activity at most recent disease episode as measured by the Birmingham Vasculitis Activity Score (BVAS) version 3 [14,15], ear-nose-throat (ENT) and neurological vasculitis activity (derived from BVAS), drug used for induction therapy, drug used for maintenance therapy, prednisolone use (initial dose (mg/d), current dose (mg/d), cumulative dose of last six months (g)), C-reactive protein (CRP) levels (mg/l) and 24-hour urinary creatinine excretion (mmol/24h).

Measurement of muscle strength

Isometric knee extension, elbow flexion and hip flexion strength were measured using a handheld dynamometer (CIT Technics, Groningen, The Netherlands) and expressed in Newton (N), as described by Van der Ploeg et al [16]. Test positions for the muscle strength measurements are the same as described by Bohannon [17] and shown by Douma et al [18]. The 'break' method was used, in which the researcher slightly overcomes the maximum force of the subject [19]. The measurements were performed by four operators. All operators received instructions and training before performing measurements for the study. Hand-held dynamometry of hip and knee force has previously been shown to have

a good to excellent (ICC ≥0.75) intra- and inter-rater reliability as well as a good to excellent concurrent validity compared to gold-standard measurement using an isokinetic dynamometer [20]. The measurements were performed twice on each side, alternating measurements between sides. The highest measured value per muscle group was used as the maximum muscle force of that muscle group. Expected elbow flexion and knee extension forces for each patient were calculated based on age, sex, height and weight using a regression equation derived from a Dutch healthy population sample [18]. Elbow flexion and knee extension force were expressed as a percentage of this expected value.

Bioelectric Impedance Analysis

Bioelectric Impedance Analysis (BIA) was performed using electrodes on the right hand and foot using the Bodystat Quadscan 4000 (Bodystat Ltd, British Isles). The fat free mass index (kg/m^2) was calculated using a built-in formula of the Bodystat.

Physical activity

The Baecke questionnaire was used as generic (i.e., not disease-specific) self-report measure of physical activity. It has been validated for use in the general Dutch population [21,22]. The Baecke questionnaire measures physical activity at work, leisure time and sports. The scores are summarised into work, sports and leisure time indices which are added into a total score. Calculations have been described by Baecke et al [21]. The intensity levels of sports were derived from the Ainsworth compendium [23,24].

The Actiwatch 7 (Camntech, Papworth Everard, United Kingdom) was used as an objective measure of physical activity. Accelerometry is a reliable method for measurement of physical activity in patients with rheumatic disease [25]. Participants were instructed to wear the accelerometer day and night on the non-dominant wrist, except for activities involving water, such as swimming or taking a bath. The accelerometer output was expressed as the average kilo-counts per waking day.

Health-related quality of life

Health-related quality of life (HRQoL) was assessed using the RAND-36 questionnaire. This questionnaire contains eight subscales with scores ranging from 0 to 100. These can be further summarised into a physical component summary (PCS) and mental component summary score (MCS), which are calculated in such a way that the reference population has a mean of 50 and a standard deviation of 10 [26]. Age-adjusted reference values used for calculating the PCS and MCS were derived from a Dutch general population sample [27]. Background information on the questionnaire and its subscales can be found elsewhere [28].

Statistics

All analyses were performed using SPSS Statistics version 23 (IBM). A two-sided P-value <0.05 was considered statistically significant. Variables were presented as n (percent [%]) or median (interquartile range [IQR]). Reliability of muscle strength measurements was assessed by comparing repeated muscle strength measurements using intraclass correlation coefficients (ICC) with an absolute agreement definition. Point estimates of ICC were assessed as poor (<0.50), moderate (0.50-0.74), good (0.75-0.89) or excellent (≥0.90) as described previously [20]. Muscle strength and physical activity were compared with

all subscales of the RAND-36, prednisolone dose and disease duration using Spearman Rank correlation. Disease duration was compared between patients with muscle strength below and above 100% of their predicted value[18]. For patients who received paired measurements, muscle strength (N) and self-reported physical activity were compared between the first (prednisolone dose >30mg/d) and second (prednisolone dose ≤2.5mg/d) visits using the Wilcoxon signed rank test.

Finally, linear regression was performed with the age-adjusted PCS and MCS of the RAND-36 as dependent variables and previously reported factors associated with these scores in AAV [5,7], as well as muscle strength and physical activity, as independent variables in univariable analysis. Subsequently, a multivariable model was built for age-adjusted PCS and MCS with statistically significant factors from univariable analysis, as well as previously reported potential confounders [5,7], included as independent variables in the model. For age-adjusted PCS, these potential confounders were: age >69 years, sex, current prednisolone dose >5mg/d, nervous system involvement and previous vasculitis relapse. For age-adjusted MCS, potential confounders were: age >69 years, sex, current prednisolone dose >5mg/d, ENT involvement and previous vasculitis relapse.

RESULTS

In total, 92 patients were considered for inclusion. A flowchart of inclusion is shown in **Figure 1**. Characteristics of the 48 patients included in the study are shown in **Table 1**. At the day of measurement, 12 patients (25%) had an elevated CRP (≥5mg/l). Incomplete variables include fat free mass index (present for 37/48, 77%), urinary creatinine excretion (present for 43/48, 90%) and total accelerometer score (present for 28/48, 58%).
In total, seven patients have received paired measurements. Median prednisolone dose of these patients was 40mg/d (IQR 30-60) at the first measurement, versus 0mg (IQR 0-2.5) at follow-up. Median time between measurements was 6 months (IQR 4 - 25).

Figure 1. Flowchart of patient selection

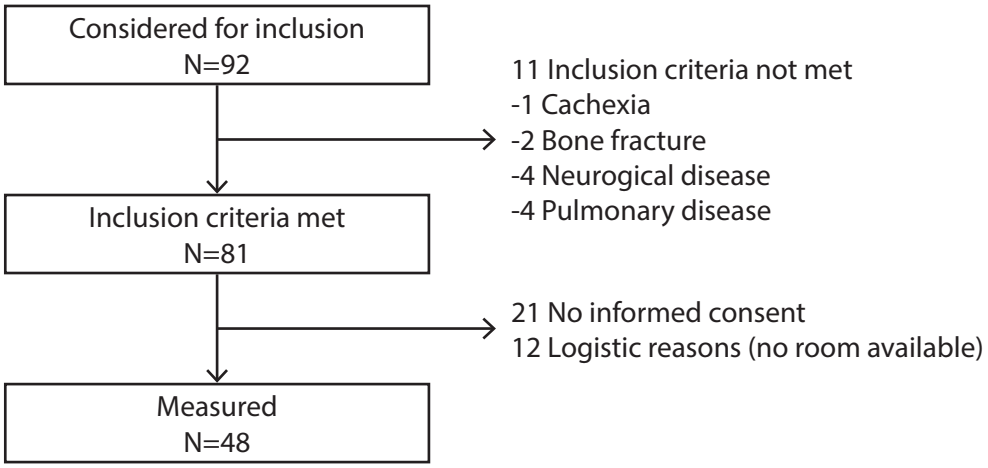


Table 1. Patient characteristics

	N (%) or Median (IQR)			
Variable	**All (n=48)**	**All (n=48)**	**Relapsing (n=35)**	**P-value**
Age (years)	62 (52-69)	64 (56-69)	61 (51-71)	0.86
Sex (n, % male)	25 (52%)	5 (39%)	20 (57%)	0.34
Diagnosis (N, % GPA)	41 (85%)	9 (69%)	32 (91%)	0.08
ANCA specificity (n,%)				0.30
Proteinase 3 (PR3)	35 (73%)	8 (62%)	27 (77%)	
Myeloperoxidase (MPO)	11 (23%)	5 (39%)	6 (17%)	
Negative	2 (4%)	0 (0%)	2 (6%)	
Treatment duration (weeks)	35 (11-104)	30 (9-62)	36 (14-119)	0.31
Months since diagnosis	83 (17-194)	7 (2-14)	141 (76-203)	<0.001***
Induction therapy (n, %)				0.001**
Cyclophosphamide	21 (44%)	11 (84%)	10 (29%)	
Rituximab	14 (29%)	0 (0%)	14 (40%)	
Other	13 (27%)	2 (16%)	11 (31%)	
Prednisolone	48 (100%)	13 (100%)	35 (100%)	
Currently on prednisolone	33 (69%)	6 (46%)	27 (77%)	0.08
Prednisolone dose (mg/d)	10.0 (0.0-28.8)	0.0 (0.0-50.0)	10.0 (3.8-25.0)	0.72
Cumulative prednisolone (g in last six months)	1.8 (0.1-3.3)	1.1 (0.0-3.8)	1.9 (0.6-2.9)	0.71
BVAS (diagnosis/relapse)	13 (11-17)	17 (13-21)	12 (8-16)	0.01*
CRP (mg/l) (at day of visit)	2.0 (0.7-5.2)	1.8 (0.8-2.8)	2.2 (0.6-5.5)	0.75

**P<0.05, **P<0.01, ***P<0.001.*

Muscle strength and quality of life
Out of all 48 patients, 46 had a successful measurement of knee extension. Overall intra-rater reliability was good to excellent. Intraclass correlations, overall and per assessor, are shown in **S1 Table**. Of 46 measured patients, 35 (76%) had a knee extension force less than 100% of their predicted value based on age, sex, height and weight. Elbow flexion force was below 100% of predicted for 30/45 measured patients (67%).

Fat free mass index (kg/m^2) showed a significant positive correlation with hip flexion (Rho= 0.56, P<0.001), knee extension (Rho= 0.51, P=0.001) and elbow flexion force in N (Rho=0.68, P<0.001). Urinary creatinine excretion (mmol/24h) also showed an association with hip flexion (Rho= 0.47, P=0.002), knee extension (Rho=0.47, P=0.002) and elbow flexion force (Rho=0.33, P=0.04).

Knee extension force (% of predicted) showed a significant positive correlation with the RAND-36 subscales physical functioning (Rho=0.31, P=0.04), physical role functioning (Rho=0.34, P=0.02), emotional role functioning (Rho=0.31, P=0.04) and general health (Rho=0.30, P=0.04). Elbow flexion force (% of predicted) did not show a significant correlation with any subscale of the RAND-36.

Physical activity and quality of life
Baecke score showed a positive trend with hip flexion force (Rho=0.29, P=0.05), but not with elbow flexion (Rho=0.22, P=0.15) or knee extension force (Rho=0.20, P=0.18). Accelerometer counts did not show a correlation with muscle strength (not shown).

Baecke total score showed positive associations with the RAND-36 subscales physical functioning (Rho=0.32, P=0.04), role limitations (physical problem) (Rho=0.33, P=0.03), role limitations (emotional problem) (Rho=0.46, P=0.001), mental health (Rho=0.41, P=0.005) and general health (Rho=0.42, P=0.003), as well as positive trends with vitality (Rho=0.28, P=0.06) and pain (Rho=0.26, P=0.08). Accelerometer counts did not show a significant correlation with any subscale of the RAND-36.

Associations of health-related quality of life

The age-adjusted PCS of the RAND-36 was significantly lower in AAV patients (mean 42, SD 10) compared to Dutch norm values (mean 50, SD 10, P<0.001). To a lesser degree, this was also true for the age-adjusted MCS (mean 46, SD 9, versus mean 50, SD 10; P=0.01). In univariable linear regression, knee extension force, Baecke total score and neurological vasculitis activity at most recent disease episode (peripheral neuropathy (n=4) or cranial nerve palsy (n=1)) were associated with the age-adjusted PCS. Prednisolone dose >5mg/d, ENT vasculitis activity and lower Baecke score were associated with lower age-adjusted MCS, see **Table 2**.

Table 2. Univariable linear regression for quality of life in ANCA associated vasculitis

Independent variables	Physical component summary			Mental component summary		
	B (95% CI)	P	Adj. R^2	B (95% CI)	P	Adj. R^2
Relapsing (versus new)	-6 (-13 to 0)	0.07	0.05	-1 (-7 to 6)	0.87	-0.02
Prednisolone dose >5 mg/d	-3 (-9 to 3)	0.35	0.00	-7 (-12 to -2)	0.01*	0.11
CRP ≥5mg/l	-4 (-11 to 3)	0.23	0.01	3 (-3 to 9)	0.25	0.01
ENT vasculitis activity	-1 (-7 to 6)	0.87	-0.02	-5 (-10 to 0)	0.04*	0.07
Neurological vasculitis activity	-12 (-22 to -1)	0.03*	0.08	-3 (-13 to 6)	0.49	-0.01
Knee extension (% of predicted)	0.1 (0.0 to 0.3)	0.04*	0.07	0.1 (0.0 to 0.2)	0.17	0.02
Elbow flexion (% of predicted)	0.1 (0.0 to 0.2)	0.06	0.06	0.0 (-0.1 to 0.1)	0.46	-0.01
Baecke total	3 (1 to 5)	0.01*	0.12	3 (1 to 4)	0.008**	0.13

*Univariable linear regression of factors potentially associated with age-adjusted physical and mental quality of life. B coefficient of independent variable in linear regression. Adj. R^2 adjusted R squared. *P<0.05. **P<0.01.*

In multivariable linear regression, age ≤69 years, previous relapse of vasculitis, elevated CRP and lower knee extension force were associated with lower age-adjusted physical quality of life, with a total adjusted R^2 of 0.55 for the regression model. Prednisolone dose >5mg/d, ENT involvement and lower Baecke total index were associated with lower age-adjusted mental quality of life, with a total adjusted R^2 of 0.36 for the regression model, see **Table 3**.

Table 3. Multivariable linear regression for quality of life in ANCA-associated vasculiits

Independent variables	**B (95% CI)**	**P-value**	**Beta**
	Age-adjusted Physical Component Summary		
Knee force (% of pred.)	0.19 (0.08 to 0.29)	<0.001***	0.41
Baecke total index	1.6 (-0.1 to 3.3)	0.06	0.21
Age >69 years	13 (7 to 19)	<0.001***	0.52
Relapsing (vs new)	-9 (-14 to -3)	0.003**	-0.36
Elevated CRP (≥5mg/l)	-8 (-13 to -2)	0.005**	-0.32
Neurological vasculitis	-8 (-16 to 1)	0.07	-0.21
Female sex	-3 (-8 to 1)	0.16	-0.15
Prednisolone >5mg/d	0 (-5 to 5)	0.99	0.00
	Age-adjusted Mental Component Summary		
Baecke total index	2.4 (0.7 to 4.1)	0.006**	0.36
Prednisolone (>5mg/d)	-7.1 (-11.9 to -2.3)	0.005**	-0.38
ENT involvement	-6.9 (-11.7 to -2.1)	0.006**	-0.38
Age >69 years	3.9 (-2.0 to 9.8)	0.19	0.17
Relapsing (vs new)	2.2 (-3.4 to 7.9)	0.43	0.11
Female sex	-0.3 (-4.9 to 4.2)	0.88	-0.02

*Multivariable final model of physical and mental quality of life. B coefficient of independent variable in linear regression. Beta standardised regression coefficient. *P<0.05. **P<0.01. ***P<0.001.*

Prednisolone use versus muscle strength and physical activity
Knee extension, hip flexion and elbow flexion forces were not correlated to current or cumulative prednisolone dose. Physical activity according to the accelerometer and Baecke total score showed significant negative correlations with cumulative prednisolone use, but not with current prednisolone dose, see **Table 4**. In paired measurements (n= 7), median elbow flexion, hip flexion and knee extension increased after discontinuation of GC therapy. However, this difference was only statistically significant for hip flexion force, see **S1 Figure.**

Table 4. Correlation of muscle strength and physical activity with prednisolone use

Variable	Rho current prednisolone	P-value	Rho cumulative prednisolone	P-value
Muscle strength				
Knee extension (N)	-0.14	0.37	-0.03	0.86
% of predicted	-0.14	0.34	-0.13	0.38
Hip flexion (N)	-0.11	0.45	0.05	0.74
Elbow flexion (N)	0.01	0.97	0.18	0.23
% of predicted	0.04	0.81	0.17	0.26
Physical activity				
Actiwatch (kcount/d)	-0.23	0.25	-0.39	0.04*
Baecke total index	-0.17	0.24	-0.33	0.03*

*Results of Spearman Rank correlation for measures of muscle strength and physical activity versus current prednisolone dose (mg/d) and cumulative use over the past six months (g). *P<0.05*

Disease duration versus muscle strength and physical activity
In relapsing patients, a longer time after diagnosis showed a trend with lower knee extension force (% of predicted) (Rho= -0.32, P=0.07) and lower Baecke index (Rho= -0.31, P=0.08), but not with elbow flexion force (Rho= -0.23, P=0.19). By contrast, in newly diagnosed patients, a longer time after diagnosis was associated with a higher Baecke index (Rho= 0.58, P=0.04). Time after diagnosis still showed no significant correlation with knee extension force (Rho= -0.08, P=0.79) or elbow flexion force (Rho= -0.17, P=0.60) in newly diagnosed patients.

Patients with knee extension force below 100% of predicted had significantly longer median follow-up (118 months, IQR 30-201) compared to patients with at least 100% of predicted knee extension force (22 months, IQR 7-80), P=0.006. This remained significant (P=0.03) after correction for age, sex, Baecke total score and cumulative prednisolone use. Patients with elbow flexion force below 100% of predicted did not have a significantly longer follow-up (median 123, IQR 32-202 months if below 100%; median 36, IQR 11-118 months if at or above 100% of predicted; P=0.13).

DISCUSSION

In this study, we found that the majority of AAV patients have a muscle strength below their predicted values based on age, sex, height and weight [18], and that physical QoL was significantly reduced in AAV patients compared to healthy norm values [27]. We also identified an association of self-reported physical activity measured by the Baecke questionnaire with mental QoL in AAV.

The reduced leg muscle strength in AAV patients, found in this and previous studies [12,29], might be the result of steroid myopathy. Indeed, studies in several other disease populations have previously shown associations between chronic glucocorticoid use and reduced muscle strength [30-32]. While leg muscle strength did not show a correlation with prednisolone use in the present cross-sectional study, this might be explained by large inter-individual variation in muscle strength and GC sensitivity. Inclusion of muscle imaging to assess for typical signs of muscle atrophy, such as ultrasonography or MRI, might be interesting for a future study.

Interestingly, relapsing patients with longer disease duration more frequently had a muscle strength below norm values. This suggests that accumulating damage from relapses of AAV and treatment of these relapses results in a reduction of muscle strength over time.

Physical activity, as measured using an accelerometer and the Baecke questionnaire, showed a negative association with cumulative prednisolone exposure, as well as a positive association with follow-up time in newly diagnosed patients. This indicates that prednisolone therapy negatively impacts exercise capacity and that physical activity increases when tapering prednisolone. Alternatively, the association might be confounded my more recent disease activity in patients with a higher cumulative prednisolone exposure in the past 6 months, as disease activity might also result in reduced physical activity. Contrary to our expectations, we did not find an association of muscle strength with cumulative prednisolone dose.

In agreement with earlier studies [4,5], especially the age-adjusted PCS was reduced in AAV patients compared to general population norms [27]. Leg muscle strength was independently associated with PCS. Therefore, muscle strength might be part of the explanation for reduced QoL in vasculitis patients. Leg muscle strength showed only a positive trend with physical activity. Also, leg muscle strength, in contrast to self-re-

ported physical activity, was associated with PCS in multivariable linear regression. This suggest that muscle strength directly affects physical QoL, not (only) through physical activity. Physical activity was a main factor associated with mental QoL. Based on these results, interventions focusing on improving muscle strength and exercise capacity might improve both physical and mental QoL in AAV patients.

This study has several limitations. First, due to logistic reasons, only some patients received accelerometer and bioelectric impedance analysis (BIA) measurements, limiting the sample size for these measurements. For this reason, these measurements were not included in linear regression analyses. Furthermore, due to the cross-sectional nature of the study, inferences about causality cannot be made and statistical power is limited by inter-individual variation in variables measured. Also, no matched control group was included, requiring comparison to literature values from an unmatched general population. Lastly, generic questionnaires were used for the study. While this enables comparison with reference values from literature, the questionnaires are less sensitive to change than disease-specific questionnaires would be.

Our findings that most AAV patients have a lower muscle strength than expected based on healthy population norms and that muscle strength and self-reported physical activity are positively associated with QoL suggest that AAV patients might benefit from interventions aimed at improving muscle strength and physical activity. Studies in other disease populations have demonstrated clinically relevant improvements with simple interventions. For example, improvement of fatigue and physical function was achieved in Rheumatoid Arthritis patients by giving them a pedometer and a step-monitoring diary [33]. As fatigue negatively influences QoL in AAV [34], and self-reported physical activity was positively related to QoL in the present study, physical activity interventions might also reduce fatigue and improve QoL in AAV patients.

In conclusion, the majority of AAV patients have reduced leg muscle strength and physical QoL compared to norm values. Knee extension strength is independently associated with physical QoL, while self-reported physical activity is independently associated with mental QoL. Therefore, interventions promoting leg muscle force and physical activity might improve both aspects of QoL and should be evaluated in clinical trials.

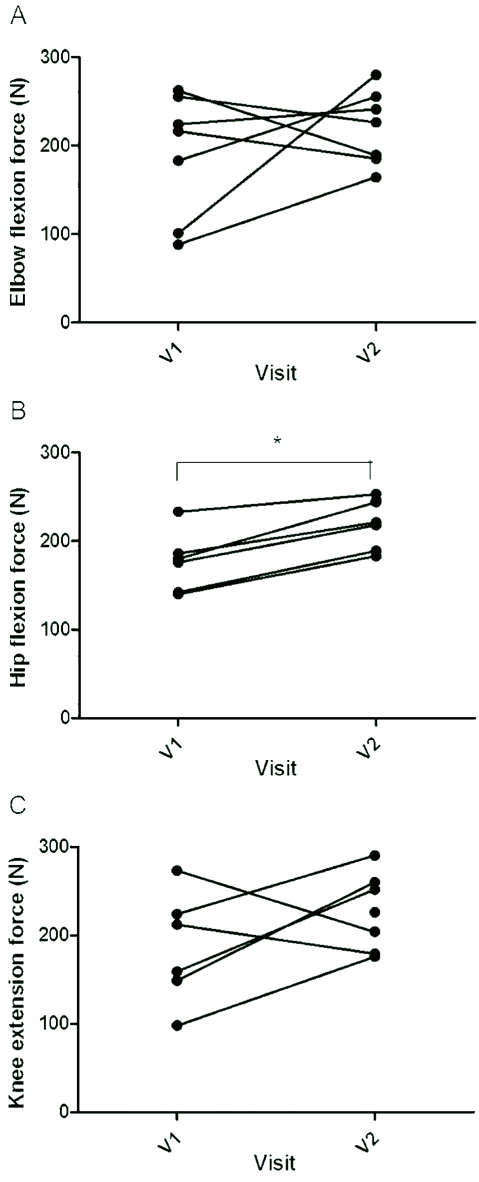
SUPPORTING INFORMATION

S1 Table. Intra-rater reliability of handheld dynamometry muscle groups, overall and per assessor.

Muscle group	Overall (n=48)	Assessor A (n=23)	Assessor B (n=5)	Assessor C (n=6)	Assessor D (n=14)
Elbow flexion (n=45)	0.91 (0.85 to 0.95)	0.91 (0.82 to 0.97)	0.70 (0.27 to 0.96)	0.93 (0.78 to 0.99)	0.73 (0.48 to 0.90)
Hip flexion (n=46)	0.86 (0.78 to 0.92)	0.84 (0.70 to 0.93)	0.81 (0.49 to 0.98)	0.96 (0.86 to 0.99)	0.76 (0.54 to 0.92)
Knee extension (n=46)	0.83 (0.74 to 0.90)	0.86 (0.73 to 0.95)	0.81 (0.45 to 0.98)	0.83 (0.51 o 0.98)	0.66 (0.40 to 0.86)

Results shown are intraclass correlation coefficients (ICC) with 95% conficence intervals.

S1 Figure. Paired comparisons of muscle strength during and after high-dose prednisolone.



*Muscle strength of individual patients at visit V1 (prednisolone dose ≥ 30mg/d) and visit V2 (≤ 2.5 mg/d) for elbow flexion (A), hip flexion (B) and knee extension (C). *P<0.05.*

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* Formerly, at the time of study conduction.

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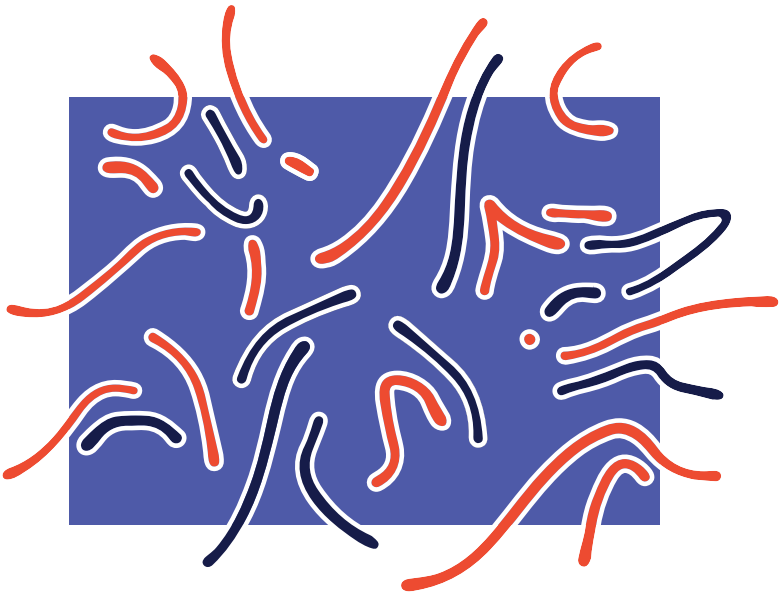
08 Chapter

Summary and future perspectives



SUMMARY

In **Chapter 1**, characteristics of the different diagnostic classes of ANCA-associated vasculitis (AAV) (i.e., granulomatosis with polyangiitis [GPA], microscopic polyangiitis [MPA], eosinophilic granulomatosis with polyangiitis [EGPA]) are summarized. We conclude that evidence for classification based on ANCA specificity (PR3-ANCA versus MPO-ANCA) rather than clinical diagnosis is increasing [1,2]. We also note that the relation between ANCA specificity and clinical characteristics differs between geographical regions [3].

Subsequently, we provide an overview of important developments in AAV treatment. An example is the introduction of azathioprine and low-dose glucocorticoids as maintenance therapy following remission induction with cyclophosphamide and high-dose glucocorticoids [4]. Another example is the introduction of rituximab as an alternative to cyclophosphamide and azathioprine for induction and maintenance of remission, respectively [5,6]. We also note the recent shift of research focus towards disease- and patient-tailored medicine [1].

We conclude the first chapter with the challenges we still face in AAV treatment, including a high risk of relapse [7], disease- and treatment-related mortality [8,9], accumulation of damage from disease and treatment [10,11], and a reduced quality of life (QoL) despite control of disease activity [12,13]. All of these are items that may benefit from individualized therapy based on disease and patient characteristics. Currently, few studies are available that study this topic in AAV.

Part 1: Pharmacogenetics
In the chapters of part 1 we explore the current knowledge and results of our own studies with respect to genetic factors that are potentially associated with treatment outcome of AAV.

In **Chapter 2**, we summarize the current literature on gene variants in relation to efficacy and/or toxicity of current treatment in AAV. In addition to the results from chapters 3 and 4 from this thesis, we find that the Cytochrome P (CYP) 450 related CYP2C9 polymorphism has been associated with clinical response to cyclophosphamide [14]. Additionally, genetic polymorphisms related to B cell activator of the tumor necrosis factor family (BAFF) [15], the interleukin (IL)2-IL21 genetic region [15], Fcγ receptor IIa [16], and IL6 were associated with response to rituximab [17]. These studies add genetic markers for potential application of personalized therapy in AAV. Their value for clinical practice will need to be evaluated in future studies.

The gene encoding the enzyme Thiopurine methyltransferase (TPMT) is well-known and used for pharmacogenetics. Genotyping before starting thiopurine therapy and adjustment of initial dose in patients carrying genetic variants is recommended to prevent bone marrow toxicity [18,19]. In **Chapter 3**, we find that AAV patients carrying a TPMT variant do not have an increased risk of adverse effects during azathioprine maintenance therapy, at least if they receive strict surveillance of hematological parameters and clinical follow-up. On the other hand, lower leukocyte counts after cyclophosphamide therapy, possibly reflecting a small bone marrow reserve or 'fitness', are strongly associat-

ed with bone marrow toxicity during azathioprine treatment and relapse, indicating that response to cyclophosphamide might be a stronger predictor of these outcomes than TPMT variants, even after switch to azathioprine [20]. Of note, none of the patients in the study is homozygous for TPMT variants, while this is associated with the highest risk of severe thiopurine-induced bone marrow suppression [21]. Also, frequent laboratory evaluation is performed in the UMCG after switch to azathioprine, allowing for early dose reduction in TPMT variant carriers. The results of this chapter indicate that TPMT pretesting might be less useful for AAV patients compared to other populations.

Several haplotypes of the glucocorticoid receptor (GR) have been identified that affect glucocorticoid (GC) sensitivity in the general population [22].In **Chapter 4**, we investigate whether these haplotypes affect disease- and treatment related outcomes in a cohort of AAV patients treated with GCs combined with other immunosuppressive drugs. We find that GR haplotype 1 (minor variant of BclI), associated with increased GC sensitivity, results in an increased risk of developing dyslipidemia. GR haplotype 4 (minor ER22/23EK+9β+TthIII1), associated with glucocorticoid resistance, results in increased risks of end-stage renal disease and death, suggesting a more severe inflammatory disease phenotype and/or reduced treatment response. A genetic variant of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), associated with reduced local GC activation [23], results in an increased risk of relapse, but only in non-carriers of GR haplotype 1. We conclude that inter-individual differences in glucocorticoid sensitivity affect clinically relevant outcomes in AAV. As no such data are currently available, future studies will need to assess whether adjustment of treatment based on 11β-HSD1 genotype or GR haplotype will result in improvement of inflammatory and/or metabolic outcomes.

Part 2: characterizing treatment outcomes
In the second part, several treatment outcomes are characterized in more detail, in order to formulate points of consideration for clinical practice.

Clinical characteristics of AAV differ between geographical regions and ethnic groups [3,24,25]. Also, MPO-ANCA is associated with a different disease phenotype, different clinical outcomes and different genetic associations than PR3-ANCA [1,2,26]. In **Chapter 5**, we compare Dutch, Brazilian and Chinese cohorts of AAV patients and test whether geographical differences in organ manifestations and clinical outcomes are independent of ANCA-specificity. We find that differences in eye/mucosa and ear-nose-throat involvement between China and other countries can be explained by the lower frequency of PR3-ANCA positive patients in this country. Other differences in organ manifestations between countries cannot be explained by differences in ANCA-specificity. This is in agreement with earlier studies comparing organ manifestations of GPA and MPA patients between Japan and the United Kingdom [24,25]. Despite an expected lower risk of relapse based on a lower frequency of PR3-ANCA positive patients and ear-nose-throat involvement, as well as a higher frequency of end-stage renal disease, relapse risk of Chinese patients is similar to Brazilian and Dutch patients. Also, Chinese patients have a lower overall survival compared to both other countries, even after adjustment for other factors associated with mortality such as age, end-stage renal disease, pulmonary involvement and induction treatment. These findings indicate additional, yet unidentified, risk factors for relapse and mortality in this Chinese AAV population.

Toxicity and severe adverse effects are well-known and important aspects related to induction and maintenance therapy in AAV. A somewhat neglected and probably underestimated adverse effect of azathioprine is the azathioprine hypersensitivity syndrome (AHSS), characterized by fever and several other possible skin and systemic symptoms [27]. In **Chapter 6**, we describe the AHSS within our observational cohort of AAV patients. We find an estimated incidence of 9% (95% CI 6 to 13%) in AAV, which is more frequent than the 2% previously reported for IBD patients using 6-mercaptopurine (6-MP) [28]. AHSS is associated with lower TPMT activity in our cohort, although most patients (80%) still have a TPMT activity in the normal range. We find similar (frequencies of) symptoms to those described previously [27]. Eosinophilia that is characteristic of other drug hypersensitivity reactions is absent. Instead, the laboratory abnormalities of elevated CRP, leukocytosis and neutrophilia resemble infection and a relapse of vasculitis. In multivariable Cox regression, after adjustment for PR3-ANCA (lower frequency in hypersensitive patients of our cohort) and TPMT activity (lower in hypersensitive patients of our cohort), AHSS is a risk factor for relapse. A likely explanation for this is that immunosuppressive therapy in patients with AHSS is often temporarily discontinued because it is confused with an infection. We conclude that AHSS is more common than previously reported, that lower TPMT activity might facilitate its initial development, and that AHSS negatively impacts the efficacy of remission maintenance therapy.

Physical quality of life (QoL) is reduced in AAV patients compared to the general population, even in remission [13]. In **Chapter 7**, we sought to investigate whether reduced physical QoL in AAV might in part be explained by steroid myopathy and reduced physical performance. The main predictors of lower age-adjusted physical QoL in our study are lower knee extension force, younger age and vasculitis relapses. We find that the majority of AAV patients have lower muscle strength than expected based on age, sex, height and weight. While cross-sectional measurements do not show an association between muscle strength and prednisolone use, muscle strength improves within patients after prednisolone tapering. Lastly, knee extension force of relapsing patients decreases over time. We conclude that reduced muscle strength results from prednisolone use as well as damage from vasculitis activity, and contributes to reduced physical QoL in AAV. Alternatively, reduced physical QoL results in decreased exercise capacity and less physical activity, in turn resulting in muscle wasting and reduced muscle strength.

DISCUSSION AND FUTURE PERSPECTIVES

Pharmacogenetics: possible role in AAV

Several genetic factors have been identified by others (**Chapter 2**) and our group (**Chapters 3 and 4**) that have been associated with response to drugs used for AAV treatment [14-17,20,29]. In theory, adjusting therapy based on these genetic factors could improve treatment outcomes for individual patients by reducing treatment toxicity while maximizing efficacy.

The cytochrome P (CYP)2C9 variant was associated with a higher risk of leukopenia and showed a trend towards increased treatment response in cyclophosphamide-treated

AAV patients. Also, gene variants of B cell activator of the tumor necrosis factor family (BAFF), Fcγ receptor (FcγR) IIA, the interleukin (IL)2-IL21 region and IL-6 have been associated with response to rituximab therapy, although these effects are most likely independent of induction therapy used. Treatment outcomes might improve by adjusting cyclophosphamide dose based on CYP2C9 genotype. Also, it may be interesting to investigate additional targeted therapy, such as belimumab (targeting BAFF signaling), and tocilizumab (targeting the IL-6 receptor), in patients carrying genetic variants related to BAFF and IL-6.

An example of clinical application of pharmacogenetics is adjustment of thiopurine dose based on TPMT genotype, which reduces thiopurine toxicity in IBD patients carrying a TPMT variant while maintaining efficacy [30]. TPMT gene variants have less predictive value for outcome of azathioprine maintenance therapy in AAV [20,31], although we do see a trend of higher relapse-free survival in patients with low TPMT activity, as well as an increased bone marrow susceptibility to azathioprine in carriers of a genetic TPMT variant. TPMT genotyping may still be relevant to detect the occasional patient that is homozygous for TPMT variants, since these patients have an increased risk of severe bone marrow toxicity [32], and require a 10-fold dose reduction or alternative therapy [30].

Carriers of genetic variants that reduce GC sensitivity are associated with adverse disease outcomes such as relapse and end-stage renal disease. This suggests that AAV patients with these genetic variants might benefit from more intensive treatment. On the other hand, a genetic variant that increases glucocorticoid sensitivity is associated with lower relapse risk and an increased risk of adverse metabolic outcomes. AAV patients carrying this variant might benefit from reduced GC exposure. Interestingly, none of the genetic factors was associated with speed of GC tapering. While this may be the result of the protocol-based tapering schedule, another possibility is that genetic variation in 11β-HSD1 and the GR mainly affect the effects of cortisol, in patients not receiving prednisolone treatment. Indeed, prednisolone is given in supra-physiological dosages, suppressing the endogenous production of cortisol, thereby removing (inter-individual variation in) hypothalamic-pituitary-adrenal axis regulation of GC production. The most appropriate way to adjust treatment in order to improve the measured outcomes will need to be investigated in randomized controlled trials.

Before gene variants can be applied for clinical practice, several hurdles need to be overcome. Firstly, most of the findings were only shown in a single cohort; they require confirmation in independent replication cohorts. Because of the low prevalence of AAV [33], replication of findings in a large enough cohort will require a multicenter approach. Secondly, after confirmation of results, a multicenter randomized controlled trial (RCT) would need to be performed to find the appropriate dose reduction and to evaluate the effect of this adjustment on treatment efficacy and toxicity. Because most gene variants are only present in a minority of patients, the number of patients included will need to be much larger than the number of patients receiving dose adjustment.

Characterizing treatment outcomes

Recent studies indicate that ANCA specificity is associated with distinct clinical manifestations and outcomes [26]. In **Chapter 5**,we show that a lower frequency of mucosa/eye

and ENT involvement in China can be explained by the lower frequency of PR3-ANCA positive patients in this country. ANCA-specificity does not fully predict clinical manifestations and outcomes, however. Several differences in organ manifestations between Brazil, China and the Netherlands could not be explained by ANCA-specificity. Also, Chinese patients had a higher risk of relapse and mortality than expected based on known risk factors for these outcomes. Therefore, we expect additional environmental and/or genetic factors to affect disease characteristics. Most of the RCTs performed so far have mostly included Caucasian patients [4,5,34]. Therefore, inter-ethnic differences in clinical response to drugs cannot be excluded, which may be mediated by genetic factors. Research into personalized treatment based on genetic factors in addition to ANCA specificity may be worthwhile.

In **Chapter 6**, the first cohort study on the azathioprine hypersensitivity syndrome in AAV, we found that it is more common in AAV than previously reported for IBD [28]. One explanation for this finding could be that patients in the previous cohort study received 6-MP, which lacks the imidazole group of azathioprine as an additional epitope [28]. Indeed, successful switch of azathioprine to 6-MP and vice versa have been reported for patients with hypersensitivity to either drug [27,28,35,36]. This indicates that the imidazole group of azathioprine as well as epitopes from other metabolites can trigger a hypersensitivity response. Also, other studies in patients using azathioprine rather than 6-MP, including a clinical trial conducted in AAV patients, found frequencies of azathioprine hypersensitivity closer to the one described in **Chapter 6** [4,37,38]. Another explanation could be the existence of a common susceptibility factor to both AAV and azathioprine hypersensitivity, for example a human leukocyte antigen (HLA) genetic association. This hypothesis requires further study for verification.

The mechanism of azathioprine hypersensitivity has not been fully elucidated. Most likely, based on the timing and clinical symptoms, the mechanism is either a type IV hypersensitivity reaction [39], or a pharmacological interaction of azathioprine with an immune receptor such as HLA or a T-cell receptor (TCR) [40].

Azathioprine hypersensitivity has previously been considered a dose independent reaction, supported by the low dose required upon rechallenge to elicit the same symptoms [27]. On the other hand, the association we found with a lower TPMT activity indicates that a higher exposure to some metabolites of azathioprine facilitates development of azathioprine hypersensitivity. Also, several cases of successful desensitization with very low doses of azathioprine have been reported [28].

Based on the results of **Chapter 7**, relapses of vasculitis have a cumulative negative effect on muscle strength. Whether this is the result of vasculitis activity and/or treatment toxicity could not be determined in this study. Nevertheless, muscle strength, exercise capacity and QoL are all reduced in vasculitis patients even after remission has been achieved [13,41,42], warranting intervention studies aimed at improving these outcomes. The difficulty in designing an effective physical activity intervention for AAV patients is that the cause of reduced exercise capacity likely differs between individual patients. While one patient may suffer from dyspnea due to subglottic stenosis, nasal obstruction or pulmonary damage, another patient may have difficulty walking due to pe

ripheral nerve damage. These differences make it difficult, if not impossible, to develop one intervention that is effective for all AAV patients. Preferably, a personalized training program should be designed based on the type of disease and treatment damage present. Alternatively, muscle strength, exercise capacity and QoL can improve by reducing exposure to glucocorticoids. The currently investigated drug CCX168 (avacopan) might help achieve a reduction of glucocorticoid exposure, possibly resulting in less muscle wasting [43].

The questionnaire most frequently used to measure QoL in AAV patients, the SF-36 [13,41,42], is a generic questionnaire, lacking items specific for the functioning of AAV patients. Recently, the AAV-PRO questionnaire has been developed, which includes categories such as disease symptoms , treatment side effects and physical functioning [44]. It may be interesting to investigate whether this measure will prove useful to monitor interventions aimed at improving physical functioning of AAV patients.

CONCLUSION

Treatment of AAV has greatly improved over the years. Some interesting targeted therapies are on their way, but the balance between disease inflammation and treatment toxicity still needs improvement. The use of gene variants to optimize outcomes of currently used drugs is an interesting approach, but several hurdles need to be overcome before it can be implemented. Further improvement could be achieved by designing separate treatments based on ANCA type, gene variants related to drug efficacy or toxicity, and possibly other genetic factors.

Early recognition and further understanding of azathioprine hypersensitivity will hopefully prevent unnecessary hospitalizations and treatments, as well as improve efficacy of remission maintenance therapy. Lastly, specific interventions based on the underlying type of damage need to be developed to improve physical functioning of AAV patients during and after successful treatment of disease activity.

In summary, with this thesis we have made some small steps towards personalized medicine in AAV, which will hopefully be expanded upon in the coming years.

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NEDERLANDSE SAMENVATTING

Vasculitis en het Vasculitis Expertise Centrum Groningen
Vasculitis betekent ontsteking van de bloedvatwand. Dit verschijnsel kan optreden bij ontstekingsziekten zoals lupus en reumatoïde artritis, na gebruik van bepaalde medicatie, of bij infecties met bacteriën (bijvoorbeeld streptokokken en stafylokokken) of virussen (bijvoorbeeld hepatitis B en C). Wanneer er echter geen duidelijke oorzaak of onderliggende ziekte aanwezig is, wordt gesproken van 'primaire' of 'idiopathische' vasculitis.

Vasculitis kan optreden in alle bloedvaten, van de grootste (aorta) tot aan de kleinste haarvaten. Dit levert een veelheid aan verschillende verschijnselen en ziektebeelden op. Vaak is bij een bepaalde vorm van vasculitis een specifiek type of kaliber van bloedvat het meest aangezien. Daarom wordt vasculitis vaak op de grootte van de betrokken bloedvaten ingedeeld.

Antineutrofiel cytoplasma antistoffen- (ANCA-) geassocieerde vasculitis is de naam voor een groep aandoeningen die met name ontstekingen van de kleinere bloedvaten geven. Kenmerkend is de aanwezigheid van bepaalde antistoffen in het bloed, de ANCA's, gericht tegen eiwitten in de witte bloedcel. Mede vanwege deze antistoffen wordt ANCA-geassocieerde vasculitis een auto-immuunziekte genoemd. De ANCA's zijn in 1985 ontdekt in Groningen door onder anderen dr. F. van der Woude (1953-2006). Jaren later is het Universitair Medisch Centrum Groningen nog steeds een belangrijk expertisecentrum op het gebied van vasculitis.

Er zijn verschillende vormen van ANCA-geassocieerde vasculitis. De meest voorkomende in Nederland is granulomatose met polyangiitis (GPA), voorheen de ziekte van Wegener genoemd. Andere vormen zijn microscopische polyangiitis (MPA) en eosinofiele granulomatose met polyangiitis (EGPA), voorheen de ziekte van Churg-Strauss.

Symptomen
In principe kan elk orgaan bij ANCA-geassocieerde vasculitis aangedaan worden, maar bepaalde symptomen komen veel vaker voor dan andere. Welke organen het meest betrokken zijn verschilt per vorm van vasculitis. GPA geeft vooral klachten van het keel-neus-oor gebied (zoals neusbloedingen) en de longen, maar ook ontstekingen van de nieren komen vaak voor. MPA geeft vooral longbloedingen en ontsteking van de nieren. EGPA geeft vooral neuspoliepen en astma, met soms ontsteking van de hartspier of de darmen.

In plaats van in diagnosen (GPA, MPA, EGPA) kunnen patiënten ook ingedeeld worden op ANCA type. Er zijn twee belangrijke soorten ANCA die gemeten kunnen worden in het bloed, de PR3-ANCA (antistoffen gericht tegen proteinase-3 (PR3))en de MPO-ANCA (antistoffen gericht tegen myeloperoxidase (MPO)). Over het algemeen hebben patiënten met PR3-ANCA meer klachten passend bij GPA en hebben ze grotere kans op opvlammingen van vasculitis. Patiënten met MPO-ANCA hebben meer klachten passend bij MPA en hebben volgens sommige onderzoeken een grotere kans op nierfalen en overlijden. Bij EGPA patiënten wordt in de helft van de gevallen geen ANCA gevonden. EGPA patiënten die wel ANCA in het bloed hebben, hebben vaker longbloedingen en nierontstekingen.

Vóórkomen en risicofactoren
ANCA-geassocieerde vasculitis is zeldzaam. Naar verwachting krijgen jaarlijks 13-20 per miljoen mensen de diagnose, in Nederland komt dit neer op tussen de 225 en 345 mensen per jaar. De ziekte doet iets meer mannen dan vrouwen aan. Het kan op elke leeftijd ontstaan, maar treedt vooral op bij oudere patiënten (ouder dan 60 jaar).

De ziekte en het meest voorkomende ANCA type is niet in elk land of regio hetzelfde. In Noord-Europa, het Midden Oosten en India komt PR3-ANCA het meeste voor. In Japan en China heeft juist de meerderheid van de patiënten MPO-ANCA. Zowel erfelijke aanleg als omgevingsfactoren spelen waarschijnlijk een rol bij de verschillen. Voorbeelden van omgevingsfactoren zijn Staphylococcus aureus (deze bacterie wordt vaak in de neus van GPA patiënten gevonden) en de breedtegraad/blootstelling aan UV licht (verder van de evenaar en in landen met minder zon komt meer ANCA-geassocieerde vasculitis voor). Bij mensen van Aziatische afkomst komt MPO-ANCA het meest voor, terwijl bij Kaukasiërs juist PR3-ANCA meer voorkomt. Bij mensen van Afrikaanse komaf komt ANCA-geassocieerde vasculitis nauwelijks voor. In een grootschalig DNA onderzoek zijn een aantal genen gevonden die meer bij ANCA-geassocieerde vasculitis patiënten aanwezig zijn dan bij gezonde mensen. Deze genen verklaren echter maar een klein deel van het voorkomen, dus ANCA-geassocieerde vasculitis is geen duidelijk erfelijke ziekte.

Behandeling
Onbehandeld is ANCA-geassocieerde vasculitis een in veel gevallen dodelijke ziekte. Toen er nog geen behandeling was, overleed 80-90% van de patiënten binnen een jaar. Hier kwam in de jaren ’60 en ’70 van de vorige eeuw verandering in, toen een gecombineerde behandeling met cyclofosfamide (in hogere doseringen ook gebruikt voor chemotherapie) en prednisolon werd geïntroduceerd. Met deze behandeling kan de ziekte bij de meeste patiënten tot rust worden gebracht en is de kans op overlijden veel kleiner geworden. Tegenwoordig is meer dan 80-90% van de patiënten na 5 jaar nog in leven.

Helaas geeft de behandeling ook veel bijwerkingen. Voorbeelden van bijwerkingen bij cyclofosfamide zijn beschadiging van het blaasslijmvlies, tekort aan witte bloedcellen (waardoor er een verhoogd risico bestaat op infecties) en later mogelijk ook verhoogde kans op blaaskanker en mogelijk enkele andere vormen van kanker. Bijwerkingen van prednisolon zijn onder andere forse gewichtsstijging, suikerziekte, botontkalking en verlies van spiermassa. Schade door de behandeling stapelt zich op door de jaren heen, zeker als de ziekte een of meerdere keren weer opvlamt en opnieuw moet worden behandeld. De schade door cyclofosfamide is teruggebracht door na het tot rust brengen van de ziekte te wisselen naar een minder schadelijke onderhoudsbehandeling, meestal met azathioprine. Tevens blijkt rituximab voor een deel van de patiënten een potentieel minder schadelijk alternatief dan cyclofosfamide bij de behandeling van actieve ziekte. Dit middel blijkt net zo goed te werken als cyclofosfamide. Met uitzondering van de vruchtbaarheid geeft rituximab helaas niet minder bijwerkingen op korte en middellange termijn, waardoor het als een gelijkwaardig alternatief wordt gezien. Recente onderzoeken suggereren dat rituximab als onderhoudsbehandeling beter werkt dan azathioprine. Helaas zijn op dit moment de lange termijn bijwerkingen van rituximab nog niet goed bekend, maar de verwachting is dat naast het ontbreken van aantasting van de vruchtbaarheid ook het risico op kanker niet door rituximab wordt verhoogd.

Nieuwe medicijnstudies bij ANCA-geassocieerde vasculitis richten zich op het vinden van een gerichtere behandeling. In plaats van middelen die breed werken en daardoor veel bijwerkingen hebben, zoals cyclofosfamide, azathioprine en prednisolon, wordt met nieuwe medicijnen geprobeerd meer specifiek de ontsteking die bij vasculitis optreedt te remmen. Een voorbeeld van zo'n mogelijk nieuw medicijn is avacopan, dat zich richt op een deel van de aangeboren afweer (complementsysteem) dat extra actief is bij ANCA-geassocieerde vasculitis. In onderzoeken wordt gekeken of dit middel in de toekomst prednisolon (deels) kan vervangen.

Uitdagingen bij de behandeling van ANCA-geassocieerde vasculitis
Zoals genoemd zijn de huidige inductie- en onderhoudsbehandeling effectief in het tot rust brengen van vasculitis en het tegengaan van overlijden. Er zijn nog wel een aantal uitdagingen.

Zo krijgt zeker 35% van de patiënten een opvlamming van de ziekte binnen 5 jaar na diagnose. Deze opvlamming moet op tijd herkend en behandeld worden. Daarom blijft controle nodig, zelfs nadat de ziekte tot rust is gekomen.

Hoewel de overgrote meerderheid van de patiënten niet meer overlijdt aan vasculitis, is de sterfte nog steeds hoger dan bij gezonde leeftijdsgenoten. In het verleden overleden patiënten vooral aan de vasculitis zelf die onvoldoende onder controle was te krijgen. Nu zijn bijwerkingen van de behandeling de belangrijkste doodsoorzaak. Overlijden in het eerste jaar komt vooral door infecties die optreden als complicatie bij de afweer-onderdrukkende medicijnen. In latere jaren lijkt ook het risico op hart- en vaatziekten en bepaalde vormen van kanker (o.a. blaaskanker, huidkanker), waarschijnlijk mede ten gevolge van de behandeling met prednisolon en cyclofosfamide, iets verhoogd.

Door opvlammingen van de ziekte en bijwerkingen van de behandeling lopen veel vasculitis patiënten schade op. Uiteindelijk heeft na 5 jaar 90% van de patiënten een vorm van schade opgelopen. Schade bij MPA patiënten is veelal nierschade. GPA patiënten hebben relatief vaak gehoorschade, chronische klachten van de neus/neusbijholten en schade aan zenuwen. Circa twee derde van de patiënten krijgt schade of zwakte die mede te wijten kan zijn aan de behandeling, zoals hoge bloeddruk, botontkalking of suikerziekte.

Het is niet verbazingwekkend dat veel ANCA-geassocieerde vasculitis patiënten door de opgelopen schade beperkingen ondervinden in het dagelijks leven, zelfs nadat de ontstekingen tot rust zijn gekomen. Vooral patiënten die ouder zijn en/of schade aan zenuwen hebben geven meer beperkingen aan. Daarnaast is een veelgehoorde klacht van patiënten vermoeidheid, waardoor ze niet hun oude niveau van functioneren kunnen halen.

Dit proefschrift
Dit proefschrift richt zich op de ontwikkeling van een behandeling op maat bij ANCA-geassocieerde vasculitis. We streven ernaar om met zo min mogelijk bijwerkingen zo goed mogelijk de vasculitis te behandelen.

Na een inleiding in **hoofdstuk 1** worden de verschillende onderzoeken besproken. Deze onderzoeken zijn in twee overkoepelende thema's verdeeld.

In het **eerste thema** kijken we naar genen die de gevoeligheid voor de medicijnen gebruikt bij vasculitis behandeling zouden kunnen beïnvloeden. Het uiteindelijke doel is om op basis van analyse van deze genen de behandeling met ontstekingsremmende medicijnen beter op de individuele patiënt af te stemmen. Nu krijgt iedere patiënt min of meer hetzelfde behandelschema. Mogelijk is op grond van analyse van de set genen een meer passende behandeling per patiënt te geven.

In **hoofdstuk 2** kijken we naar een gen dat de gevoeligheid voor de effecten van azathioprine beïnvloedt. Dragers van een afwijkende versie van dit gen kunnen de actieve vorm van azathioprine minder snel afbreken. Bij de ziekte van Crohn en colitis ulcerosa krijgen patiënten die drager zijn van het afwijkende gen een lagere dosis azathioprine, omdat ze anders meer bijwerkingen hebben van azathioprine. De behandeling bij ANCA-geassocieerde vasculitis patiënten is echter anders. Patiënten krijgen namelijk andere sterke ontstekingsremmers zoals cyclofosfamide en prednisolon, waardoor de invloed van azathioprine op bijwerkingen kleiner is. Daarom vroegen wij ons af of drager zijn van een afwijkend gen bij ANCA-geassocieerde vasculitis ook meer bijwerkingen geeft. Dit blijkt niet zo te zijn, mede door de strenge controle van het bloedbeeld, vooral de witte bloedlichaampjes, die we standaard hanteren. We concluderen dat de startdosis van azathioprine niet aangepast hoeft te worden bij ANCA-geassocieerde vasculitis patiënten met één afwijkende kopie van het gen, als maar regelmatig de witte bloedlichaampjes gecontroleerd worden en zo nodig de azathioprine dosering wordt verlaagd. Op basis van eerder onderzoek is het wel belangrijk om patiënten met twee afwijkende kopieën van het gen op te sporen. Patiënten met deze zeldzame combinatie van genen hebben namelijk een zeer sterk verhoogde kans op ernstige problemen met de witte bloedli-

chaampjes. Bij deze patiënten is het beter een ander middel te geven of anders een sterk verlaagde dosering van azathioprine.

In **hoofdstuk 3** kijken we naar verschillende genen die de prednisolon gevoeligheid beïnvloeden. We laten zien dat sommige van deze genen invloed hebben op de ernst van de ontsteking, de kans op opvlammingen van en overlijden aan vasculitis en de kans op bijwerkingen van prednisolon (specifiek een verhoogd cholesterol). We concluderen dat genetisch bepaalde prednisolon gevoeligheid invloed heeft op het beloop van ziekte en behandeling. In theorie zou aanpassen van de behandeling op prednisolon gevoeligheid kunnen zorgen voor minder nierfalen en overlijden door vasculitis, minder opvlammingen van vasculitis en minder bijwerkingen van prednisolon. De bevindingen moeten wel eerst bevestigd worden in onafhankelijk onderzoek. Daarna moet onderzocht worden of aanpassen van de behandeling op grond van de vooraf gedane gen bepalingen inderdaad het gewenste effect heeft.
In **hoofdstuk 4** zoeken we in de literatuur naar andere genen die mogelijk gebruikt kunnen worden voor een behandeling op maat bij ANCA-geassocieerde vasculitis. We vinden verschillende genen die werking en bijwerkingen van cyclofosfamide en rituximab beïnvloeden. Mogelijk kan aanpassing van de behandeling op basis van bepaling van deze genen dus zorgen voor een effectievere behandeling met minder bijwerkingen. We merken wel op dat de meeste effecten in slechts één onderzoek zijn gevonden en vrij beperkt lijken te zijn. Bij voorkeur dienen ze eerst bevestigd te worden in een onafhankelijk onderzoek. Na bevestiging kan gekeken worden of aanpassing van de behandeling op deze genen inderdaad zorgt voor een betere behandeling. Dit zijn langdurige onderzoeken waarbij veel patiënten moeten worden ingesloten, zodat dit in onze ogen alleen uitvoerbaar is door samenwerking tussen meerdere ziekenhuizen en misschien zelfs landen.

In het **tweede thema** beschrijven we verschillen tussen vasculitis patiënten uit verschillende landen, het vóórkomen en de kenmerken van azathioprine overgevoeligheid en de relatie tussen spierkrachtverlies bij vasculitis en het functioneren in het dagelijks leven. Overkoepelend beschrijven deze onderzoeken extra aandachtspunten bij negatieve uitkomsten van de behandeling van ANCA-geassocieerde vasculitis.

In **Hoofdstuk 5** vergelijken we ANCA-geassocieerde vasculitis patiënten uit Nederland met patiënten uit Brazilië en China, omdat we benieuwd waren of verschillen in symptomen en beloop komen door de verschillen in het voorkomen van de verschillende typen ANCA die tussen geografische regio's worden gezien. We zien dat verschillende organen niet in elke regio even vaak betrokken zijn. Deze verschillen zijn inderdaad deels, maar niet volledig, te verklaren door meer MPO-ANCA in China en meer PR3-ANCA in Brazilië en Nederland. We zien verder dat Chinese patiënten even vaak opvlammingen hebben als Nederlandse en Braziliaanse patiënten, terwijl ze op basis van bekende risicofactoren minder opvlammingen zouden moeten hebben. We zien ook dat Chinese patiënten vaker overlijden dan Nederlandse en Braziliaanse patiënten. Een goede verklaring hiervoor hebben we in dit onderzoek niet kunnen vinden. Mogelijk gaat het om erfelijke of omgevingsfactoren die in China anders zijn. Een alternatieve verklaring is dat de behandeling tussen de landen verschilt of dat de diagnose in China later wordt gesteld met uitgebreidere ziekte en meer irreversibele schade (nier) tot gevolg en daar-

mee slechtere uitkomst. Helaas zijn de gegevens niet in zodanig detail aanwezig dat dit aangetoond of uitgesloten kan worden.

In **Hoofdstuk 6** beschrijven we een overgevoeligheidsreactie op azathioprine met koorts en klachten als gewrichtspijn, misselijkheid en huiduitslag. Naar ons idee wordt het aantal patiënten met deze reactie onderschat. De reactie wordt vaak aangezien voor een infectie of een opvlamming van vasculitis. Hierdoor worden patiënten onterecht opgenomen in het ziekenhuis of krijgen ze onnodig antibiotica of verhoging van de prednisolon of andere ontstekingsremmers. We zien dat deze reactie op azathioprine bij ongeveer 9% van de patiënten met vasculitis optreedt. Dit is beduidend meer dan de 2% die op basis van een eerder onderzoek bij patiënten met een andere ziekte wordt genoemd. We zien dat de symptomen van de reactie erg lijken op die van een infectie of opvlamming van vasculitis. In het bloed zien we tekenen van ontsteking die ook aanwezig zijn bij een infectie of een opvlamming van vasculitis. De kenmerkende afwijkingen die bij allergieën worden gezien ontbreken vrijwel volledig. De klachten van de reactie verdwijnen binnen enkele dagen na het stoppen van azathioprine en komen binnen een paar uur terug bij hervatten van het middel. We concluderen dat artsen rekening moeten houden met een overgevoeligheidsreactie op azathioprine als patiënten binnen een maand na starten van dit middel koorts en andere bijpassende klachten ontwikkelen. Omdat de klachten snel verdwijnen na het stoppen van azathioprine, kan tijdige herkenning onnodige opnames en behandelingen van patiënten besparen.

In **Hoofdstuk 7** onderzoeken we of de beperkingen die vasculitis patiënten ervaren ondanks rustige ziekte deels te verklaren zijn door spierkrachtverlies bij prednisolon en ontsteking. We zien inderdaad dat spierkracht, vooral in de benen, verminderd is bij vasculitis, dat deze afneemt tijdens behandeling met prednisolon en na opvlammingen van vasculitis, en samengaat met door de patiënt aangegeven beperkingen in het dagelijks leven. Ook zien we dat patiënten die per week meer bewegen minder beperkingen ervaren. Helaas is door de opzet van het onderzoek niet duidelijk of minder bewegen leidt tot meer beperkingen of juist dat patiënten met meer beperkingen minder gaan bewegen. We concluderen dat het interessant is om te onderzoeken of trainen van spierkracht en inspanningsvermogen tijdens de behandeling met prednisolon zorgt voor minder beperkingen in het dagelijks leven van ANCA-geassocieerde vasculitis patiënten.

Conclusie en toekomstperspectief

In de afgelopen halve eeuw is ANCA-geassocieerde vasculitis veranderd van een vrijwel zonder uitzondering op korte termijn dodelijke ziekte naar een chronische ziekte met een veel betere levensverwachting. Dit heeft geleid tot nieuwe uitdagingen in de behandeling van deze ziekte. Zo krijgen veel patiënten nog opvlammingen van de ziekte, wat weer leidt tot schade door actieve vasculitis en door bijwerkingen van medicatie. Door deze schade ervaren patiënten beperkingen in het dagelijks leven, zelfs na succesvolle behandeling van ziekte. Een behandeling op maat zou de balans tussen het effect op de ziekte en de bijwerkingen van de behandeling kunnen verbeteren. In dit proefschrift hebben we genen gevonden die de gevoeligheid voor medicijnen beïnvloeden en laten we zien dat sommige hiervan inderdaad effect en bijwerkingen van de medicatie beïnvloeden. Als dit bevestigd wordt in onafhankelijk onderzoek en als een interventie studie laat zien dat aanpassen van de behandeling op basis van deze genen helpt, kan in de toekomst

een behandeling op maat gegeven worden die met zo min mogelijk bijwerkingen zo goed mogelijk de vasculitis behandelt.

We hebben laten zien dat indeling op basis van ANCA type belangrijk is, maar ook dat er verschillen tussen geografische gebieden zijn die niet door het soort ANCA alleen worden verklaard. Patiënten die azathioprine onderhoud krijgen hebben een grotere kans op overgevoeligheid voor dit middel dan eerder gedacht. Aangezien de klachten erg lijken op een infectie en opvlamming is het belangrijk beducht te zijn op deze bijwerking, die eenvoudig behandeld kan worden door stoppen en vervangen van azathioprine. Tenslotte is, gezien onze gegevens en ook enkele studies van anderen, onderzoek belangrijk naar een revalidatietraject of trainingsprogramma ter verbetering van het dagelijks functioneren. Omdat elke vasculitis patiënt anders is, onder andere door verschillen in betrokken organen, moet hiervoor naar de individuele patiënt gekeken worden en kan niet worden volstaan met één trainingsprogramma voor alle vasculitis patiënten.

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Arno

ABOUT THE AUTHOR

Arno Christiaan Hessels was born at 28 July 1992 in Zwolle, the Netherlands. He attended the Gymnasium Celeanum in Zwolle, from which he graduated cum laude in 2010. Later that year, he started studying Medicine at the University of Groningen. During his Bachelor he attended research-related courses and worked on research projects as part of the Junior Scientific Masterclass (JSM). In 2013, he worked on a JSM Pilot Project on thiopurine methyltransferase genotype and activity in relation to efficacy and toxicity of azathioprine maintenance therapy in ANCA-associated vasculitis. This project was supervised by dr. Bram Rutgers, dr. Jan Stephan F. Sanders and prof. dr. Coen A. Stegeman. After attaining his Bachelor degree in 2013 cum laude and with honours for science, he continued working on this research project under the same supervisors during his Scientific Internship. In 2014, he successfully applied for a position in the MD/PhD programme. During the remainder of his Master, he alternated Internships in the University Medical Center Groningen and the Isala Clinics in Zwolle with PhD research.

His research focuses on identifying genetic predictors of treatment efficacy and toxicity in ANCA associated vasculitis, as well as characterising treatment outcomes. During his PhD research, he streamlined a database for a large observational cohort of ANCA-associated vasculitis patients, coordinated two prospective observational studies, supervised multiple students for JSM Pilot Projects and Master Projects and presented data for ANCA-associated vasculitis patients and at international scientific conferences including Vasculitis Workshop 2015 and the EULAR 2018.

From September 2018 through March 2019, he worked as a Junior Doctor at the department of Rheumatology and Clinical Immunology of the University Medical Center Groningen. In April 2019, he will start his residency programme of Internal Medicine at the MST in Enschede.