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PHARMACOGENETICS OF RITUXIMAB IN ANCA-ASSOCIATED VASCULITIS

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ABSTRACT

Background: Rituximab is effective for induction and maintenance of remission in ANCA-associated vasculitis; however, response to treatment is highly variable and relapse is common after its discontinuation. Biomarkers predicting response to rituximab do not exist and are needed to improve patients' stratification.

Objective: This study assesses potential genetic determinants of response to rituximab in ANCA-associated vasculitis.

Methods: Genotyping of 18 candidate single nucleotide polymorphisms, chosen on the basis of a biological rationale or previous reports, was performed using TaqMan and Sequenom platforms. End-points were rituximab-failure rate at 6 months and time to rituximab-failure within a year of the first rituximab administration. Rituximab failure was defined as any form of active vasculitis requiring escalation of immunosuppressive treatment.

Results: Two hundred and thirteen patients were enrolled in the primary and 109 in the replication cohorts. One single nucleotide polymorphism in the *TNFSF13B* regulatory gene region (BAFF) was associated with rituximab failure risk at 6 months and time to rituximab-failure in the two cohorts (respectively after meta-analysis, OR 8.8, p=0.0065; HR 7.3, $p=8.5x10^{-06}$). Carriers of the risk genotype had a higher rate of detectable B-cells 6 months after rituximab (50% vs 14%, p=0.0146). This association was restricted to the PR3-ANCA patient subgroup.

Conclusion: We have identified a single nucleotide polymorphism of the regulatory region of the gene BAFF that may be able to predict response to rituximab in ANCA-associated vasculitis.

INTRODUCTION

Antineutrophil Cytoplasmic Associated (ANCA) vasculitis (AAV)

Antineutrophil Cytoplasmatic Associated (ANCA) vasculitis (AAV) is a group of rare disease including Granulomatosis with Polyangiitis (GPA, formerly Wegener Granulomatosis), Microscopic Polyangiitis (MPA) and Eosinophilic Granulomatosis and Polyangiitis (EGPA, formerly Churg Strauss Syndrome) (*1*). A trademark AAV feature is positivity in 90% of patients for anti-neutrophil cytoplasm auto-antibodies (ANCAs) directed against neutrophil proteinase 3 or myeloperoxidase (PR3 or MPO-ANCA) (*2*) with PR3-ANCA more frequent in GPA and MPO-ANCA more typical of MPA. Their common association with ANCA, similar histological features of inflammation and fibrinoid necrosis of small blood vessels, and response to therapy has led to the GPA and MPA subgroups being combined for clinical studies. Both GPA and MPA patients present with vasculitic manifestations, such as necrotising crescentic glomerulonephritis, alveolar haemorrhage, purpura and peripheral neuropathy. GPA is further characterised by granulomatous involvement of the ear, nose and throat (ENT) and lungs; ENT involvement may be the only manifestation in the localised subset of the disease (*1*). EGPA is defined by eosinophilia, necrotizing vasculitis and eosinophil-rich granulomatous inflammation, while ANCA are less frequent (*3, 4*). Although formally included within AAVs, EGPA is considered and studied more as a stand-alone entity compared to GPA and MPA and will not be object of this thesis.

Pathogenesis of AAVs

The pathogenesis of AAV is still debated. Infectious triggers facilitating neutrophil activation play a key role leading to the migration of cytoplasmic PR3-MPO antigens on the neutrophil surface where they become available target for ANCAs. Also B-cells play a central role, not only being the precursors of ANCA-producing plasma cells and plasmablasts, but also through their interaction with the T cell compartment ultimately leading to the development and activation of the T effector memory cells (T EM) *(Figure 1)*. Activated neutrophils coated by ANCAs and T EM are the final responsible of the inflammatory vasculitic process (*5*). Complement activation seems to play an important role locally for the development of the inflammatory process although C3 and C4 blood levels are usually normal (*6*).

The report of- albeit rare- familial cases of AAVs has drawn attention on the role of genetic factors in the pathogenesis of AAVs (*7, 8*). The identification of genetic associations for AAVs would allow not only better understanding of the disease pathogenesis and risk factors, but also clearer classification with a potential impact on clinical management of the disease.

Genetics and AAVs

Genetics is an important tool in the approach to the understanding of diseases in general and rare diseases in particular. It may provide important information regarding risk factors, susceptibilities, pathogenesis and classification. The typical approach is to analyse the presence of associations between genetic determinants and the risk of developing the diseases or other characteristics/outcomes. However, the deeper the developments of genetics are progressing the clearer it becomes that this research approach may help out in several other fields in particular providing information regarding the prognosis of the diseases and not only of their risk factors.

Genetics as a tool to identify predisposing conditions in AAVs

Several candidate gene approach studies have been performed over the years, however the understanding in the field of genetics and AAVs has been dramatically increased after the publication of two genome wide association studies (GWAS). The philosophy beyond the GWAS approach is the interrogation of a high number of single nucleotide polymorphisms (SNPs) that are covering a big proportion of the human genome. This approach may be very informative however, in order to avoid spurious associations, a very stringent p value is required forcing to a multicentre approach with large cohorts of patients. Two GWASs have been performed by now in AAVs, the first conducted by the European Vasculitis Genetic Consortium (EVGC) (2687 cases of GPA and MPA, 6858 controls) (*9*) and the second by the American Vasculitis Clinical Research Consortium (VCRC) (987 cases of GPA and 2731 controls) (*10*). The different genetics approaches have brought to the identification of several associations summarised in *Table 1* and *Table 2*.

HLA region

The role of the HLA region in autoimmunity is central. Several associations have been shown, for instance, in rheumatoid arthritis and type 1 diabetes (*11, 12*) with different SNPs. When acting as risk factors, *HLA* SNPs may promote selection of autoreactive T cells or prevent their negative selection (*13*).

The strongest evidence in AAVs is with the *HLA-DPB1.* This association has been initially described in a cohort of 150 German GPA patients ($p=1.51x10^{-10}$, OR 3.91) (*14*) and subsequently confirmed in an independent group of 108 German GPA patients (*15*); in a further analysis of the combined cohort the association remained significant only in the ANCA positive patients ($p=1.26x10^{-22}$). This finding has been then confirmed by the results of both GWASs: a strong association of a SNP in the *HLA-DPB1* area (OR 3.67, $p=1.5x10^{-71}$) outlined from the EVGC GWAS (*9*) as well as from the VCRC GWAS (OR 0.24, $p=1.91x10^{-50}$) (*10*). Interestingly, a sub analysis of the EVGC GWAS also showed that this association was stronger in the anti-PR3 subgroup ($6.2x10^{-89}$) independently from the clinical diagnosis while this was not confirmed in the ant-MPO subgroup. In the latter group a further re-analysis revealed an unexpected association with a SNP in the *HLA-DQ* region ($p=2.1x10^{-08}$), masked by the small numbers of such patients during the primary analysis. This finding was further confirmed in an independent cohort of Italian cases (*9*).

Other associations have been described as result of small or not replicated study. In a Dutch cohort of 241 patients with GPA a lower proportion of *HLA-DR13 (6)* and *HLA-DR1* and an increased proportion of *HLA-DR4* was found compared to controls (*16*); no replication of these data has been published so far. In two small cohorts of 32 Afro-American and 74 Caucasian patients, an association with *HLA-DRB1* was documented in anti-PR3 positive patients but not in the anti-MPO ones (*17*);

interestingly an association with a variation in the same HLA has been documented in a cohort of 152 Chinese patients with GPA and MPA (*18*). Relatively old and small studies documented an association of GPA with *HLA-B50, HLA-DR1, HLA-DR9, HLA-DQw7* and *HLA-DR3* (*19-21*).

In MPA, few data are available, the most interesting report is from a group of 50 anti-MPO positive Japanese patients where an association with the *HLA-DRB1*0901* (p=0.033) and *HLA-DQB1*0303* (p=0.022) was documented. The small numbers and the high linkage disequilibrium between the two alleles (D' 0.95, r^2=0.82) did not allow identifying the "independent variant" (*22*). Quite interesting is the confirmation of the association of the *HLA-DQB1* with anti-MPO positive patients emerged from the EVGC GWAS in a third cohort of different ethnicity. In 107 Chinese patients with MPA, *HLA-DRB1*1101* have been found more frequently represented (p=0.023) (*18*).

In summary, the associations for all AAVs with SNPs in the HLA region have confirmed an autoimmune component in their pathogenesis. Different loci in the HLA region seem to be involved in the different diseases suggesting a different genetic background. Moreover, the results of the EVGC GWAS seem to support a distinction between AAV according to the ANCA specificity rather than the clinical phenotype.

PRTN3 – SERPINA1 genes

The presence of autoantibodies against the neutrophil serum protease PR3 suggested the potential role of this protein in the development of GPA. PR3 may be stored in the neutrophils azurophilic granules as well as exposed on the neutrophil surface membrane (*23*). Interestingly, PR3 surface exposition is bimodal with the antigen present on the surface of a proportion of neutrophils (defined as mPR3+) but

not on all of them (mPR3-). The membrane bound form is the one able to interact directly with the ANCA (*24*) precipitating neutrophils activation and endothelial adhesion. The neutrophil priming is a key process in order to increase surface exposition of PR3 in the subgroup of mPR3+neutrophils (*25*) but, since the bimodal exposition of the PR3 antigen is unaffected by neutrophils status, the proportion of the ones mPR3+ and mPR3- remain stable over time. A phenotype with high proportion of mPR3+ neutrophils is more frequent in patients with AAVs compared to controls (85% vs 55%) (*26*). These observations, together with the demonstration of the high stability over time of the mPR3+/mPR3- neutrophil ratio in controls (r=0.937) and the high concordance of this proportion in monozygotic twins (r=0.99) but not in dizygotic twins (r=0.06) (*27*), have suggested a role of genetics in influencing the PR3 antigen membrane exposition.

A non replicated study screened the entire coding and promoter sequences of *PRTN3* in 79 GPA patients and 129 controls identifying a SNP of the promoter region (A-564G) affecting a transcription factor binding site as associated to the disease (OR 4.2, p<0.00001) (*28*). The association of the *PRTN3* gene SNP rs62132295 has been interrogated also in the EVGC GWAS where a subgroup analysis revealed an association in GPA patients (OR 0.78, $p=2.6x10^{-5}$). The strength of the signal increased in the anti-PR3 positive group independently from the diagnosis (OR 0.73, $p=2.6x10^{-7}$). No association emerged in the anti-MPO positive patients (*9*) supporting a role for *PRTN3* only in the pathogenesis of anti-PR3 positive AAVs.

PR3 is also central in mediating direct tissue damage after neutrophil activation has happened and the enzyme is released. Alpha-1 antitrypsin, encoded by the gene *SERPINA1* on the chromosome 14, represents the major inhibitor of PR3 activity.

Two alpha-1 antitrypsin alleles, Z and S, have been described as associated to low enzymatic activity; studies in small cohorts suggested an association of both of these alleles with GPA (*29-31*). The mechanism beyond this was, and still is, unclear: it has been proposed that an enhanced reactivity of the immune system against an insufficiently cleared PR3 antigen might play a central role (*32*); however other evidences pointed the attention to the insufficient inhibition of the PR3 proteasic activity in peripheral tissues as responsible of more severe damages rather than a role as proper risk factor for the development of the disease (*33*). The largest study performed in the pre-GWAS era screened 433 Caucasian patients with GPA and compared the frequency of the Z and S allele to that in 421 controls, showing in the 10 patients carrier of the SS, ZZ, SZ genotype an odds-ratio for the development of GPA of 14.58 (p=0.002); the small number of cases was responsible of a broad confidence interval suggesting caution in the interpretation of these findings (*34*). In this context, significant have been the results of the EVGC GWAS which showed an association of a SNP in the *SERPINA1* gene with AAVs (OR 0.59, $p=2.4x10^{-9}$). As per *PRTN3* findings, this association was stronger in the GPA group (OR 0.54, $p=4.4x10^{-10}$) and even more significant in the anti-PR3 positive group independently from the clinical diagnosis (OR 0.53, $p=5.6x10^{-12}$) (*9*). The tag-SNP identified in the GWAS is in linkage disequilibrium with the Z allele confirming a role for this gene as risk factor for the development of the disease.

Fine mapping of the *PRTN3* and *SERPINA1* area will be required for a better understanding of the genetic variations responsible for the risk of developing GPA and for further improvement in the pathogenic understanding.

Other replicated associations – PTPN22, CTLA4

The interaction between ANCA and neutrophil is a central pathogenetic moment in AAVs. However, this represents only a component of a picture that indeed is more complex. The role of the B cell is central as attested by the effectiveness of the anti-CD20 agent rituximab (RTX) as therapeutic option (*5*). ANCA production is only one of the several B-cell functions with T cells support when acting as antigen presenting cells (*35*) and production of pro-inflammatory cytokine being other important activities. Also T cells are heavily involved as proved by their general hyperactivity in AAVs and by the prominent role of the $CD4^+$ T EM cells in causing endothelial damage (*36*). It is therefore not surprising that two B and T cell specific SNP have been identified as potential risk factors for AAVs.

The gene *PTPN22* encodes the protein Lymphoid tyrosine phosphatase (Lyp). Abnormal CD4 T_{reg} function, increased humoral activity (*37*) and enhanced neutrophil functions (*38*) have been described as features of the 620W *PTPN22* variant; its role in AAV seemed indeed reasonable driving three main genetic association studies by now. The first showed in a German cohort of 199 GPA patients and 399 controls an association (OR 1.75, p=0.002) even stronger in the subgroup ANCA positive (OR 2.01, p=0.0002) (*39*). The result has been subsequently replicated in two independent cohorts of 641 British (OR 1.40, $p=1.4x10^{-4}$) (*40*) and 344 Italian patients (*41*). Interestingly, the latter study showed a restriction of the association only to the 143 GPA patients (OR 1.91, p=0.005) but not in 102 MPA (p=0.1072) and 99 EGPA patients (p=0.1508). The GPA population was anti-PR3 positive in nearly 80% of the cases while the MPA subgroup was anti-MPO positive in around the 90%.

CTLA4 is an inhibitory glycoprotein expressed on activated T cells that competes with the co stimulatory molecule CD28 for the binding of CD80 or CD86 of the antigen-presenting cells (*42*). The monoclonal antibody abatacept, recently associated to effective disease control in GPA patients with non severe manifestations, contains the binding domain of CTLA4 reducing the interaction CD28-CD80/CD86 and therefore T-cell stimulation (*43*). A role for *CTLA4* or of its pathway may therefore be potentially of interest in AAVs (*44, 45*). The more robust finding by now regards the SNP rs3087243 interrogated in 2 big cohorts of British patients; the first study found an association in 222 AAV patients (p=0.0001) (*46*) confirmed in a cohort of 641 AAV patients ($p=6.4x10^{-3}$) (*40*).

Other associations

The second GWAS in AAV identified a potential association of GPA with the gene *SEMA6A* ($p=2.09\ x10^{8}$) encoding for the protein semaphorin 6A (*10*). The function of the proteins belonging to this family is unclear and it has been proposed that may be involved in regulating the immune system activity (*47*).

The association between the rs26595 SNP of the SEM6A6 gene and GPA was recently investigated in 879 GPA patients, 150 MPA patients and 191 EGPA patients, compared with 1,376 healthy control subjects (*48*). The authors did not observe any statistically significantly different allele frequencies between AAV patients and controls, not confirming the findings from the initial VCRC GWAS.

The pathogenetic role of this association, although above the GWAS threshold of significance, will need to be better clarified and explored in different cohorts of patients and through functional studies.

IL10 is a cytokine with mainly an anti-inflammatory activity. A study performed on 32 patients and replicated on another 125 proposed an association of its (-1082) SNP with GPA (*49, 50*); interestingly, the latter report showed a signal for the same SNP in 36 MPA patients ($p<0.00001$) (*50*). A subsequent study performed on 403 German GPA patients did not confirm this finding but showed an association in 75 patients with ANCA-negative EGPA for a specific *IL10* haplotype (X^2 19.14, $p=0.0003$) (*51*). Although it an association of *IL10* SNP with GPA now seems unlikely, further assessment will be necessary in MPA and ANCA-negative EGPA.

The high-affinity IL2 receptor, encoded by the gene *IL2RA*, is expressed not only on activated T cells but also on activated B cells, NK cells and monocytes (*52*). A normal IL2-IL2RA pathway is central for a physiological function of the immune system and for the development of a normal Treg repertoire (*53*). A weak association ($p=0.0122$) has been documented in 670 British patients with AAV for the SNP rs41295061 (*54*); replication in an independent cohort is required before considering this SNP as potential risk factor for AAVs.

CD226 is an adhesion molecule belonging to the immunoglobulin superfamily. In vitro stimulation of CD226 potentiates cytotoxicity NK mediated (*55*) and provides a positive costimulatory signal for T cell proliferation. An association between *CD226* and GPA was documented in two distinct cohorts of 520 and 122 German GPA patients (respectively OR 1.2; $p=0.020$, OR 1.37, $p=0.020$); however further replication failed in 105 British GPA patients (*56*). A further independent replication cohort made of 641 British AAV found no association ($p=0.21$) (*40*). Interestingly the two populations were of similar size and showed similar frequency for the SNP analysed (0.47 vs 0.50) suggesting as unlikely a different effect of the SNP in these populations.

Fc gamma (FCGRs) are a group of protein expressed on the surface of different cells with different affinity for the Fc portion of different immmunoglobulin subtypes (*57*) suggesting a reasonable potential role for FCGRs in AAVs. However these genes are encoded in a highly variable area (locus 1q23.3) difficult to study since characterised by several possible SNPs and copy number variations (CNV) (*57*). A Dutch study identified SNP of *FCGRIIA* and *FCGRIIIA* as potential risk factor for GPA in a cohort of 91 patients (*58*) but the finding was not confirmed, at least for the *FCGRIIA* SNP, in a cohort of 107 British patients (*59*). CNV of *FCGRIIIB* have been investigated in two cohorts of patients with contrasting results: an association was documented using qPCR in 80 UK patients with GPA and replicated in 77 French and 76 MPA patients (*60*). However this was not confirmed in 567 AAV patients when a different approach was used (paralogue ratio test) (*61*). The availability of new genotyping techniques will allow shedding light on the potential association of this complex area with AAVs.

Interferon regulatory factor 5 (IRF5) is a transcriptional factor able to induce transcription of IFN-alfa mRNA (*62*). A large association study on 644 GPA failed to identify a correlation between *IRF5* SNPs and disease, however a potential protective haplotype was identified (p=0.0012) (*63*). Interestingly, one of these SNPs (rs10954213) has recently been identified as in association with anti-MPO positive AAV in 177 Japanese patients (OR 1.27, p=0.023) (*64*).

A key role in the development of AAVs is played by infection. Any chronic infection, in particular nasal carriage of *Staphylococcus aureus,* is considered risk factor for driving disease activity (*65*). Toll like receptors (TLRs) are a group of protein able to recognise microbiological structures and activate immune response. SNPs of *TLR9*

has been showed as associated to GPA, MPA and PR3-associated AAV in a cohort of 863 AAV patients, however the findings were not replicated in a small cohort. Interestingly, patients anti-MPO positive were associated to the contrary allele compared to the anti-PR3 ones leading to a significant difference between the two groups for the SNP rs352140 (p=0.000016) (*66*). Defensins are cationic proteins characterised by intrinsic antimicrobial activity (*67*), a weak association between number of CNVs of the defensin gene *DEFB4* with GPA has been documented in a small cohort of Chinese patients (*68*).

Few further associations have been documented by now. In a cohort of 460 GPA patients an association with a leptin SNP was found and confirmed in a replication cohort of 226 patients (OR 0.72 p=0.0013); interestingly the genotyping for the same SNP in 196 EGPA patients confirmed the association but with contrasting allele distribution (OR 1.41, p=0.0067) (*69*). A small Japanese study identified a weak association between a leukocyte immunoglobulin like receptor (*LILRA2)* and 50 MPA patients (p=0.049) (*70*) and an old study explored association of complement genes and risk of AAV in 67 patients identifying a potential role for the gene *C3F* and *C4A3* (*71*).

Treatment of AAV

The treatment of AAVs has developed into an induction phase to stop vasculitis activity and prevent or reverse vital organ dysfunction, and a longer maintenance stage. Current induction regimens are effective in 70-90% of the patients but complicated by high adverse event rates. Subsequent maintenance therapy is better tolerated but relapse occurs in 70% despite initial disease control (*72*). The natural history of AAV has evolved from one of high mortality, end-stage renal failure and high organ damages risk to a chronic relapsing disease (*73*) where a fine balance exists between the benefits and risks of therapy.

Glucocorticoids (GCS) and cyclophosphamide (CYC) are the mainstays of the induction phase for AAV patients with vital organ, especially renal, involvement (*74*); plasma exchange is recommended for presentations with severe renal impairment or alveolar haemorrhage (*75*). Methotrexate and mycophenolate mofetil have been evaluated as alternatives to CYC, in particular for non-organ threatening disease (*72*); although effective these regimens are associated with a higher relapse risk (*76*). Bladder, haematological and gonadal toxicity (*77, 78*) as well as an increased cancer risk have limited the duration of CYC and inspired lower CYC dosing as used in intravenous protocols (*79*). Azathioprine and methotrexate have been equally effective for preventing relapse, with weaker evidence for leflunomide or mycophenolate mofetil in this role (*80, 81*). Other drugs that have been studied for AAV but are not in routine use include TNF-alpha blockade (*82*), gusperimus (deoxyspergualin) (*83*) and intravenous immunoglobulin (*84*). Alemtuzumab is a humanised anti-CD52 monoclonal antibody that depletes lymphocytes, including B-cells, and monocytes. Compassionate use studies have indicated potential in AAV

but also immune suppressive complications especially in the elderly and those with renal impairment (*85*).

Rituximab

Rituximab (RTX) is an anti-CD20 chimeric monoclonal antibody (*86*) that depletes circulating and tissue resident B-cells by direct induction of apoptosis, complement dependent cytotoxicity (CDCC) and antibody dependent cytotoxicity (ADCC) (*86*). CDCC and ADCC are mediated respectively by the complement protein C1q and the FCGRs. There has been extensive experience of RTX following its approval in 1997 for B-cell lymphoma and in 2005 for rheumatoid arthritis (*87, 88*). A rationale for its use in AAV has been based on the pathogenicity of ANCA (*89*), the presence of B-cells in vasculitic lesions and the association of B-cell activation with clinical disease activity (*Figure 1*) (*90*). Moreover the efficacy of CYC in vasculitis has been linked with its anti-B-cell effects (*89*), and B-cell autoreactivity is linked with the T cell dysregulation seen in AAV (*35*).

Rituximab as induction treatment in GPA and MPA

The first report of successful treatment of GPA with RTX was in 2001 (*91*), in a patient with PR3-ANCA positivity and refractory disease. Twenty-five studies have examined RTX and GCS as induction treatment in GPA and MPA of more than five patients, seven prospective (*92-98*) and eighteen retrospective (*99-115*) (*Table 3*).

Twelve included patients with refractory-relapsing disease (*95-97, 99-105, 111, 116*), one newly diagnosed patients (*93*) and twelve relapsing and new-onset AAV (*92, 98, 106-110, 112-115, 117*).

Relapsing patients treated with RTX had had previous CYC exposure and most were receiving another immunosuppressive at the time of RTX (*94-97, 99-103, 107, 109, 110, 113-115, 117*). There was variable use of CYC with RTX and continuation or withdrawal of other immunosuppressants. Although not formally compared, no

obvious benefit of concomitant CYC on response rates, or continued immunosuppression on relapse rates, was seen. RTX has been dosed at 375 mg/m^2 a week for four consecutive weeks (*92, 93, 95-97, 100, 103-106, 109, 111, 113-115*), two doses of 1000 mg with a two week interval (*94, 98, 99, 101, 102, 106, 108-110, 112, 114*) and in some reports with alternative approaches including low dosing regimens (*114, 117*); interestingly no differences in the duration of B-cell depletion or disease-free interval were observed.

The remission rates (partial and complete remission) after RTX have varied from 62 to 100%, with differences in remission definitions influencing the results; in particular the lower response rates were observed in the two randomised trials (*92, 93*) which employed more stringent remission definitions and were not subject to publication bias. Most patients achieve B-cell depletion, with rates of 94% (*92*) and 82% (*93*) seen in two randomised trials; ANCA levels fall predictably after six weeks and parallel the achievement of remission (*94, 95, 97-102, 104, 105*). Relapses following RTX respond successfully to further anti CD20 treatment (*94-105, 108, 109, 117*). All the studies contained a majority of PR3-ANCA positive GPA patients, for this reason some caution is required in extrapolating the results to MPO-ANCA positive or MPA patients.

Both randomised trials (*92, 93*) found similar efficacy between RTX- and CYC-based regimens for patients with new diagnosis of vasculitis; moreover the RAVE trial (*92*) found a higher remission rate in the RTX subgroup with relapsing, as opposed to newly diagnosed, disease. There were no differences in the time to remission for nephritis or pulmonary manifestations between RTX and CYC groups. Despite the reduction or removal of CYC in the RTX treatment groups, there were no differences in severe adverse events rates (*92, 93*). The reasons for this are unclear but a major

contribution of GCS to adverse events in these short-term studies appears likely. Attention is now turning to whether RTX permits early GCS reduction, and this has been tested in a cohort study of 23 patients with ANCA associated renal vasculitis when all remitted on a regimen of RTX, reduced dose CYC, and prednisolone starting at 20 mg/day (*98*). Thus therapy in the future may be more finely adjusted, or stratified, according to severity and comorbidity (*118*).

Rituximab in vasculitic and granulomatous manifestations

The manifestations of GPA can be divided into those with predominant vasculitis: glomerulonephritis, alveolar haemorrhage, arthritis, polyneuropathy; and those with granulomata: orbital and pulmonary masses, granulomatous sinusitis, subglottic stenosis, tracheobronchial and meningeal involvement. Granulomatous lesions are more difficult to treat: in localised (ENT and lung) GPA relapse rates exceed 50% at five year, and two thirds have damage due to vasculitis (*119*). Furthermore, orbital involvement leads to frequent visual impairment and blindness in 20% (*120*).

CD20+ B-cells contribute to granulomatous inflammation and have been identified locally in tissue biopsies (*121, 122*); moreover, in patients who achieved remission after RTX, tissue B-cells were depleted along with circulating cells (*121*). However, the rates of response of granulomatous manifestations to RTX have been variable among different reports and the effectiveness of RTX in this subgroup of patients is still unclear (*105, 110, 121, 123-128*). In a cohort of 59 patients, the response of granulomatous manifestations was low with a higher rate of refractory-unchanged disease compared to vasculitic manifestations (respectively 41.8% and 9.4%); moreover some localisations (e.g. pulmonary masses) had a better outcome compared to orbital and meningeal involvement which had the highest rate of

refractivity (*129*). In another study 34 patients with mainly ENT granulomatous disease showed an 88% response rate after one RTX course and the failing patients improved after a second RTX dose; moreover repeated RTX administration were effective in preventing relapse (*130*). The differences in response rates may have been influenced by patient selection and the level of non-healing tissue damage (*129*). Ongoing infection, such as with *Staphylococcus aureus*, provides a local stimulus to vasculitis and hinders efforts to attain disease control (*65*); routine ENT assessment and nasal swabs culture should be performed and an aggressive treatment of local colonisations and infections is suggested. The microenvironment within granulomatous lesions may protect B cells from RTX-associated depletion: both tissue B-cells activating factor levels (BAFF) and adhesion molecules have prevented depletion and persisting tissue B-cells have been demonstrated in patients despite depletion of the circulating ones (*131*); this may explain why higher RTX dosing may be necessary to achieve remission in some GPA patients (*130, 132*).

Rituximab as maintenance treatment

With conventional therapy 50% of AAV patients will relapse by 5 years and 15-20% will experience a refractory disease course (*72, 79, 133*) resulting in high GCS exposure and increased vasculitis-induced damage (*134*). Following RTX induction for relapsing or refractory disease, 75% will relapse again with a median time to relapse of 12 months; the relapse is usually preceded by B-cell return and a positive ANCA assay although both B-cells and ANCAs lack sensitivity as relapse predictors (*99*). A memory, CD27+ B-cell phenotype in the recovering population has been associated with relapse in rheumatoid arthritis and SLE, while falls in regulatory

CD5+ B-cells have been associated with relapse in AAV (*135, 136*). Although RTX treatment of relapse is effective, relapse avoidance is preferable in order to prevent disease related damage and exposure to GCS. RTX has then been given pre-emptively at different doses with intervals ranging between 3 and 12 months: high rates of sustained remission have been obtained during the treatment period suggesting a role of RTX also as maintenance treatment (*Table 4*) (*113, 114, 116, 137-143*).

Although several retrospective reports were already pointing toward this direction, the publication of the MAINRITSAN trial (*141*) definitely confirmed a role for pre-emptive RTX as maintenance approach in AAV. Moreover, this randomised trial showed the superiority of this approach compared to the conventional maintenance agent (azathioprine) in a cohort of 115 patients that received CYC as induction.

Although informative, this study is not clarifying all the aspects of the problem. First of all, the study population was mainly represented by patients with a first diagnosis of AAV, namely the group more likely to show a good response to first line approaches and therefore the one that in the common clinical practice scenario is less likely to be treated with RTX in first place. Second, the patients received induction with CYC and RTX was used only with maintenance purposes, moreover RTX dosing was on the low side among the ones employed in AAV so far. Further information on this topic will be provided by the on-going trial RITAZAREM trial (Clinicaltrials.gov NCT01697267) that is exploring a RTX-based maintenance approach with higher dosing (1 g every 4 months for 5 administrations) compared to azathioprine after a RTX based induction of the remission in patients with relapsing refractory disease.

Another open topic is the identification of the ideal RTX-based maintenance treatment: according to some Authors, a fully fixed term pre-emptive approach may expose patients to an excessively high cumulative dose of the drug and a role for CD19+ B-cells and ANCA as biomarkers for re-treatment has been proposed; however this should be seen in the context of conflicting results of studies aimed at the exploration for B-cells and ANCA as relapse predictors. The MAINRITSAN2 trial (NCT01731561) is comparing effectiveness of two RTX maintenance regimens, one based on a pure pre-emptive philosophy and the second guided by B-cells and ANCA reconstitution; this will help clarify whether or not these may be useful as biomarkers in this setting and whether or not re-treatment based on their levels would allow benefits in terms of RTX exposure, costs, side effects and disease control.

In conclusion, although it seems now clear that a RTX-based maintenance course is feasible in terms of efficacy, the optimal duration as well as dosing, schedule of administration and the role of biomarkers is still uncertain. Despite that, quite interestingly, the long-term follow-up of retrospective relapsing-refractory cohorts is showing that the relapse risk after a maintenance course with RTX is still very high although the kinetics of the flares is slightly different and delayed compared to patients treated with a single infusion (*Figure 2*) (*116*). This confirms how in AAV the identification of the optimal RTX-based maintenance regimen with the best profile in terms of cost-effectiveness-toxicity is still an open problem. Moreover, the relapse risk after this approach remains significant and therefore the identification of therapeutic alternatives is required.

Rituximab safety

Mild to moderate infusion reactions, including bronchospasm, occur in 20% of patients during or after RTX and prophylaxis with 100mg IV methyl prednisolone and an anti-histamine drugs is recommended (*144*). Severe reactions are rare (*145*). It is unclear the extent to which RTX increases infection risk: there was no reduction in infections with RTX in the randomised trials (*92, 93*), and although infections were seen in non-randomised surveys, the impact of RTX could not be determined. Even in the context of repeat administrations, the rate of adverse events was overlapping with the standard maintenance treatment in a prospective trial mainly including patients with new diagnosis of the disease (*141*). Progressive multifocal leucoencephalopathy is a very rare complication that may be more common in patients who have received several immunosuppressives treatment (*146*). The serology for hepatitis C (HCV) and B viruses (HBV) should be checked before starting treatment, HCV positive patients have in fact an increase risk of hepatic flares after RTX (*147*); HBV reactivation has been described after RTX and pre-emptive lamivudine may be considered (*148*). Prophylactic treatment of *pneumocystis jiruvecii pneumonia* is recommended (*149*). Great concern is related to the risk of developing hypogammaglobulinemia secondary to RTX administration: a reduction in immunoglobulin levels has indeed been noted in long term observational studies in 33-71% of patients, a small number of patients have required intravenous immunoglobulin replacement (*116, 137, 138, 150, 151*). We have shown how immunoglobulin tend to be decreased compare to the baseline values after a maintenance treatment with RTX but their levels stabilise once the drug is suspended (*Figure 3)*. Interestingly, the risk of hypogammaglobulinemia seems to correlate with the burden of previous immunosuppression and in particular to the

cumulative dose of CYC administered as well as the IgG levels at the moment of RTX administration; no role for RTX cumulative dose has been described although a maintenance regimen of 1 g administered biannualy may represent a risk factor (*152, 153*). Moreover, no clear association between hypogammaglobulinemia and risk of infection has been yet described with some patients developing recurrent infections with only a mild reduction of the IgG while some other experiencing no infective complications despite a severe reduction (*142*). Interestingly, hypogammaglobulinemia may be reversible after RTX cessation and the IgG replacement seems to be an effective therapeutic option in case of recurrent infection (*142, 154, 155*). In conclusion, RTX administration should be associated with a thorough screening aimed at the identification of chronic infections as well as a close monitoring of the IgG levels especially in patients with relapsing-refractory disease who have a significant burden of immunosuppression in their past medical history.

Rituximab dosing

The first licensed use for RTX has been in haematological malignancies. Once a role for this drug in autoimmune-immunological diseases has been proposed, the same dosing regimen has been employed ("the haematological approach", 375 mg/m^2 weekly for four infusion). Some reports started then to claim that this approach may be as effective as a dosing of 1 g every 2 weeks for 2 administrations (*99*) with the benefits of reducing costs due to fewer hospital admissions and a lower amount of drug required. This second scheme became more popular in the Rheumatological-Immunological world as "the rheumatological approach". Now RTX is widely used in several immunological diseases and an increasing number of reports are suggesting

that even lower dosing regimens (for example 375 mg/m^2 in single administration, 500 mg in single administration), associated or not with other immunosuppressive drugs, may be potentially effective providing B-cell depletion is reached (*117, 156, 157*).

It is however important to consider that several mechanisms of action have been postulated for RTX and these may differ across different settings justifying the fact that indeed different dosing approaches might be effective across different diseases (*158*). In AAV B-cells seem to act at several levels in the pathogenic cascade and RTX may act allowing not only reduction of the ANCA levels but also reducing the stimulation that B-cells may provide to the T lymphocytic compartment through their interaction toward the immunological synapsis (*Figure 1)* (*159*). Interestingly, CD20 positive B-cells have been observed in the tissues of relapsing AAV patients despite peripheral depletion (*132*) suggesting that these cells may play a role in the relapse risk also in patients with undetectable circulating CD20+ B-cells and in this context a higher cumulative dose of the drug may be a factor in order to reach satisfactory tissues concentration.

In conclusion, the ideal dosing regimen of RTX in several diseases including AAV is still to be determined and studies aimed at defining the risk stratification are therefore required in order to optimise the therapeutic approach.

Treatment response stratification

As already discussed, RTX is an anti-CD20 monoclonal antibody effective at inducing and maintaining remission in AAVs (*92, 93, 141*). The ideal induction regimen (*116, 117*) as well as the optimal maintenance strategies are still debated with wide inter-patient variability in the duration of B-cell depletion and the time to relapse after RTX. Although effective while administered with maintenance purposes (*141*), results of long-term follow-up of retrospective maintenance studies suggest that relapses remain frequent once the treatment is withdrawn (*142*). This should be seen in the context of costs and adverse events, especially in patients with a long immunosuppression history, not negligible. The risk stratification is therefore becoming a high priority issue in this setting. (*142, 152*).

The most obvious candidate biomarkers in the setting of RTX treated AAV remain the circulating levels of CD20+ B-cells and the ANCA titre. However, although relapsing patients have an increased risk of being ANCA positive and CD20+ B-cells reconstituted at the moment of the flare (OR 4.6 95%CI 1.17-16.72, p=0.01) (*Alberici F. et al, Unpublished Data)*, sensitivity and specificity of B-cells and ANCA in predicting flares is very low.

Several other biomarkers have been associated with response to RTX across several diseases; these include granulocyte granularity index (*160*), the phenotype of the returning B-cells (*135*), the degree of intracellular internalisation of the drug (*161*), the levels of circulating CD4+ T-cells in patients with rheumatoid arthritis (*162*), B-regulatory cell levels (*163*) and the granulocyte gene signature (*164*).

However, all require validation before they can be used in clinical practice and some of them would be based on expensive technologies. A role for genetics as a tool to

define prognosis and response to treatment has been proposed; moreover SNPs would be ideal biomarkers since they can be tested in an economical and repeatable way.

Genetics as a prognostic tool

Historically, genetic approaches have been employed for the identification of associations between disease risk and genetics factor. However, of significant importance would also be the identification of factors able to predict the disease course as well as the response to treatment.

New fields of genetics are now committed in identifying determinants associated with disease outcome (*165*), this may end up in allowing the identification *a priori* of groups of patients with poorer prognosis.

Of similar importance is the identification of factors able to predict how a patient is going to respond to a treatment. This approach, named *pharmacogenetics*, is becoming critical for the therapeutic approach in several diseases; in particular it may allow the identification of patients less likely to respond to a medication or with an unacceptable high risk of side effects.

A role for SNPs in predicting response to RTX has been proposed in rheumatoid arthritis, systemic lupus erythematosus and haematological malignancies (*166-171*); however, none of the ones tested so far have shown a reliable association with response to treatment, mainly due to lack of replication, small sample size or contradictory results across different cohorts. Moreover, no such study had been performed in AAV at the moment we planned this project.

AIM OF THE STUDY

Herein, we tested a panel of candidate SNPs to investigate their potential association with response to RTX in two large cohorts of patients with AAV.

METHODS

Patients

Eligibility criteria were a clinical diagnosis of granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA) defined according to the EMEA algorithm (*172*) and the administration of RTX for either relapsing or refractory disease or remission induction in few patients (see also section Eligibility Criteria, *Appendix)*. Patients with a clinical diagnosis of eosinophilic granulomatosis with polyangiitis (formerly known as Churg-Strauss syndrome) were not included in this study. The primary cohort included patients enrolled at European centres with expertise in vasculitis and members of the European Vasculitis Genetics Consortium (EVGC) and/or of the European Vasculitis Society (EUVAS) *(Table 5)*; the replication cohort was enrolled at the Vasculitis and Lupus Clinic, Addenbrooke's Hospital (Cambridge, UK). All patients gave written informed consent before participation. Disease severity was assessed using the Disease Extent Index (DEI) (*173*); B-cell return was defined as a B-cell count ≥ 0.01 x 10^9/l.

End points

The end-points of the study were to identify associations between the tested SNPs and rate of RTX-failure at 6 months and time to RTX-failure (relapse) within 12 months of the first RTX administration. We defined RTX-failure as active vasculitis requiring escalation of immunosuppressive treatment. SNPs showing an association with one of the two end-points in the primary cohort were analysed in the replication cohort for confirmation and then meta-analysis of the results was performed. Subgroup analyses were performed after merging the two cohorts.

Genotyping

Eighteen candidate SNPs were chosen according to a biological rationale or previous reports *(Table 6)* (*166-171*). DNA was extracted from peripheral blood using the Qiagen DNA extraction kit; genotyping was performed using TaqMan and Sequenom platforms with the exception of the *FCGR2B* SNP rs1050501 that was genotyped via a "modified" TaqMan approach as previously described (*174*).

Statistics

Statistical analysis was performed using the software R *(http://www.r-project.org)* and the packages coin (*175*), survival (*176*), SNPassoc (*177*) and hapassoc (*178*).

For exploratory purposes the primary cohort has been analysed using the Cochrane-Armitage test with log-additive model. As a result of the exploratory analysis (recessive mechanism for the SNP rs3759467), the replication cohort has been analysed immediately using a recessive model of the same test. We have then re-analysed the primary cohort with the same model and a meta-analysis has been performed.

The time to RTX-failure has been assessed by Kaplan-Meier survival analysis and a log-rank test has been used to compare populations. Cox-proportional Hazards regression model has been used for the re-analyses of the time to RTX-failure in the primary and replication cohort using a recessive model and the results have been used for the meta-analyses for this end-point.

Delta of reduction of IgG and IgM levels has been assessed as the ratio between the baseline value and the value at the time point of interest.

Results are expressed as value and percentage for categorical variables and median and interquartile range (IQR) or mean and standard error of mean for continuous variables when appropriate.

In view of the observation of an association in the 5' regulatory region of the gene *TNFSF13B,* we decided to study the haplotypes of this region since 4 of the SNPs included in the study were in strong linkage disequilibrium and organised in well renowned haplotype blocks. We used the R package hapassoc (*178*) for the identification of the haplotypes and the Cochrane-Armitage test to explore association between haplotypes and the risk of treatment failure 6 months after treatment with RTX.

Meta-analyses were performed via a fixed effects-weighted method using the Linux version of the software metal (*http://csg.sph.umich.edu/abecasis/Metal/download/*). Bonferroni corrections for multiple testing were performed, with corrected p values <0.05 considered significant.

RESULTS

Two hundred and thirteen patients were enrolled in the primary and 109 in the replication cohort, patient characteristics reported in *Table 7.* Across the entire primary cohort, a mean of 0.88 ± 0.19 SNPs per sample could not be called and no SNP had a p-value for departure from Hardy-Weinberg Equilibrium (HWE) <0.05 *(Table 6)*.

In the primary cohort the *TNFSF13B* SNP rs3759467 was associated with time to RTX-failure ($p=2.86 \times 10^{-04}$, $p_{corr}=0.01$) *(Figure 4 panel A and Table 8)*. We genotyped this SNP in the replication cohort (test for deviation from HWE p=0.7627; rate of missing calls 3%) where the association with time to RTX-failure was confirmed (p=0.002) *(Figure 4 panel B).*

Since the results suggested a recessive effect for the SNP rs3759467, we used a Cochrane Armitage test for recessive models for the analysis of the risk of RTX-failure at 6 months in the replication cohort (p=0.008996) and proceeded to re-analyse the primary cohort with the same model (p=0.06489). We also re-assessed the time to RTX-failure using a Cox-proportional hazards regression model for both cohorts using a recessive model ($p=7x10^{-04}$ and p=0.0024). Meta-analyses of the two cohorts confirmed an association between the *TNFSF13B* SNP rs3759467 and risk of RTX-failure at 6 months and time to RTX-failure (p=0.006184, $p=8.5 \times 10^{-06}$) *(Table 9)*.

We then compared the main clinical characteristics of the carriers of the CC genotype to the carriers of the TC and TT genotypes at different time points (*Table 10*) and found a higher rate of detectable peripheral B-cells 6 months after RTX in

carriers of the CC genotype (50% vs 14%, p=0.0146) as well as a smaller reduction in IgM levels (1.5 [1.4-10.92] and 1 [1-1.33], p=0.01539).

The haplotype analyses of the 5' regulatory region of the *TNFSF13B* gene based on the genotyped SNPs confirmed an association of the risk of RTX-failure at 6 months for the haplotype including the risk allele of the SNP rs3759467 *(Table 11)*.

In view of the small numbers of MPO-ANCA positive patients and the fact that all the minor homozygous carriers for the rs3759467 SNP were PR3-ANCA positive, we re-analysed the primary cohort according to ANCA specificity (*Table 12)*.

The association with the BAFF SNP rs3759467 was limited to the PR3-ANCA subgroup (RTX-failure risk at 6 months and time to RTX-failure after meta-analyses of primary and replication cohorts, respectively p=0.0141 and $p=8.7x10^{-06}$) (*Table 12 and Table 13).* However, we cannot exclude the possibility that the lack association in the MPO-ANCA subgroup reflects lack of power due to the small sample size (n=48 across the two cohorts).

In the MPO-ANCA subgroup a different association emerged in the primary cohort with the SNP rs6822844 in the *IL2-IL21* area (RTX-failure risk at 6 months and time to RTX-failure respectively $p=4.2x10^{-04}$ – $p_{cor}=0.03$ and $p=1.9x10^{-04}$ – $p_{cor}=0.0068$) *(Table 12)*. However this was not replicated in the replication cohort (RTX-failure risk at 6 months and time to RTX-failure respectively p=0.153 and p=0.172) in the context of a small sample size (19 patients), although there was a trend to an increased risk of treatment failure at 6 months for the carriers of the allele T (RTX-failure risk at 6 months of the 33% in the GT genotype vs 6% in the GG genotype) *(Table 14).*

DISCUSSION

The identification of factors able to predict response to treatments is a key step toward stratified medicine; SNPs have been investigated for this purpose since they are at low-cost and unaffected by disease status or treatment. Some potential candidates (*166-171*) have been proposed in patients treated with RTX for diseases other than AAV; however no reliable marker has been identified as yet, with small sample size, lack of replication or, for some of the replicated findings, contradictory results across different cohorts, being the main limitations of these studies.

We have identified a SNP (rs3759467) in the 5' regulatory region of the gene *TNFSF13B* (BAFF) able to predict response to RTX in two cohorts of AAV patients, moreover a relative frequent haplotype (18%) including the risk allele showed association with the two outcomes. Interestingly, the carriers of the unfavourable genotype showed, as well as a poor response to RTX, a higher proportion of detectable B-cells 6 months after infusion. BAFF increase after treatment with RTX has been described (*179-181*) and a central role for this cytokine in AAV pathogenesis has also been proposed (*180*); however, little is known regarding factors that may modulate its levels in the context of B-cell depletion. Interestingly BAFF has also been shown to protect tissue resident B-cells from rituximab-induced cell-lysis (*131*). The 5' regulatory region of the BAFF gene includes several SNPs that may have a modulatory effect. The rs9514828 SNP has been associated with BAFF levels (*182*) in Sjögren's syndrome and, more recently, the -TTTT- haplotype of this region showed a correlation with higher BAFF levels (*183*). Interestingly, the

same haplotype has been associated with a better response to RTX in two small cohorts of patients with seropositive rheumatoid arthritis that failed infliximab (*166*).

In our study, while no association was documented for the SNP rs9514828, a trend toward better response in carriers of the -TTTT- haplotype was observed that merits investigation in a larger cohort. The role of the SNP rs3759467 as well as that of the haplotype -TCAC- which includes the unfavourable allele, will need to be clarified. This SNP is part of a TAAT binding site with the minor allele affecting the ability to bind transcriptional factors such as HOXA3 and SOX1-11-12-15; the lack of a heterozygous effect may suggest that the losing of both binding sites is required to obtain the phenotype causing poor response. It seems likely that this SNP modulates B-cell survival and/or activity; this might be a consequence of higher baseline BAFF levels or greater BAFF increase after B-cell depletion.

Interestingly, our findings were restricted to the subgroup of patients with positive PR3-ANCA; the small size of the MPO-ANCA subgroup as well as the lack of minor homozygous for the SNP rs3759467 in this population, does not allow us to rule out that this SNP may act as predictor of response also in this group. However, the re-analyses of the MPO-ANCA positive patients, identified a different association with the SNP rs6822844 of the *IL2-IL21* area in the primary cohort, which did not replicate in the replication cohort, a finding that may be due to the small sample size. The association of this SNP with response to RTX has been previously described in an un-replicated cohort of SLE patients (*184*) confirming that it has a likely role in the modulation of the response at least in some settings.

Limitations to our study need to be acknowledged: although larger than previous reports and the first in AAVs, our sample size was still small for a genetic study; the retrospective nature of the data collection may be another weakness of our survey.

However, at the time of this writing, it would not have been possible to enrol a comparable prospective cohort of AAV patients treated with RTX.

Pharmacogenetics is becoming a key discipline in patients' risk stratification. In settings where the understanding of the impact of SNPs on influencing drug and metabolite levels is more advanced (such as azathioprine and SNPs of the thiopurine S-methyltransferase) this approach is already routinely implemented in the clinical practice in order to identify subsets that may require lower drug doses or closer monitoring (*185*). At the moment our study was designed and performed, no paper had been published regarding pharmacogenetics approaches in AAV treated with RTX. At the same time of the publication of our report (*186*), a similar project has been published by the American Vasculitis Consortium (*187*). Although of interest, several differences must be acknowledged between the two research projects. Cartin-Ceba et Al. explored the role of fewer SNPs in predicting response to RTX (3 SNPs) and CYC (2 SNPs); the analyses was performed on a cohort of a prospective trial and the numbers were relatively small (96 patients treated with RTX, 93 with CYC); of note there was no benefit of a replication cohort. The 3 SNPs tested on the group treated with RTX were belonging to the FCGR region; historically these are the SNPs more often investigated in the context of pharmacogenetic studies of patients treated with RTX. The receptors encoded by these genes are in fact involved in the binding of the monoclonal antibodies on the effector cells and a possible role for the variants of the receptors characterised by higher affinity for the antibodies binding has been suggested. However this has failed to be proven consistently in diseases different from AAVs and no signal emerged in our study as well as the one from Cartin-Ceba et al. Interestingly an association between the

tested SNP of the FCGRIIA and probability of achieving complete remission as well as time to complete remission was identified in the overall population (combination of RTX and CYC treated patients) of the latter report. However it must be underlined as small numbers, lack of multiple test correction and of replication in an independent cohort pose challenges to the interpretation of these findings. Moreover it appears unclear why this SNP, not involved in the CYC metabolism, should have a prognostic role in the whole population of the study in absence of a signal detected in the patients treated with RTX.

The analyses of strengths and weaknesses of these two studies are good examples of the difficulties in performing research in the field of rare diseases: in order to increase the possibilities of detecting associations is key the identification of a research question able to be fulfilled by the statistical power that the sample size may allow. Moreover, replication and multiple test corrections remain a key step of the process in order to avoid spurious associations with weak biological rational and eventually of doubtful use.

Our involvement as group in the field of pharmacogenetics is an ongoing commitment. As part of an initiative of the European Vasculitis Society the CYCLOGENE project is now testing SNPs of the genes encoding for proteins involved in the metabolism of the CYC to identify possible associations of response to treatment as well as risk of side effects. The primary cohort has been so far entirely enrolled across several Italian centers and includes 239 patients affected by AAV and treated with CYC. Thirteen SNPs were carefully shortlisted among several candidates since potentially able to increase the chances of identifying association according to their role in the metabolic pathways, to their minor allele frequency and

to results of previous reports. We have so far identified a SNP (rs4244285) associated to time of remission (recessive model, $p=5.8 \times 10^{-04}$) and one (rs1799853) associated to the risk of developing infertility (log additive model, p=0.008); these associations were independent of the cumulative dose of CYC administered. These preliminary findings, if confirmed in an independent cohort, might contribute to a more personalized approach for AAV patients due to be treated with CYC.

The association studies based on candidate SNPs, although allowing sufficient statistical power for settings characterized by relatively small population and therefore ideal for rare diseases, have intrinsic limitations mainly in terms of the identification of potentially unexpected associations. The exploratory purposes of the research in genetics remain a key step able to allow a deeper understanding of the pathogenesis of the diseases as well as the identification of potential new therapeutic targets. More inclusive approaches in the field of the research in genetics such as genome wide associations studies (GWAS), whole genome sequencing and exome sequencing are now available and allow the exploration of millions of potential variants at the same time. These are very powerful approaches, however the risk of spurious associations is very high; very stringent p values are therefore needed in order to consider an association as of potential interest (in case of GWASs, $p < 5 \times 10^{-08}$). Limitations and the high risk of failure of such approaches in the field of the rare disease is quite understandable being the statistical power directly correlated with the samples size; careful study design is therefore needed in order to increase the probabilities of detecting meaningful association. Strategies with this purpose therefore need to be put in place, for example instead of running a

classical case-control approach, it may be planned a comparison between “extreme phenotypes” (for example patients with opposed profile of response to a drug); another option might be to enroll very homogenous groups of patients and compare them to controls (for examples, all patients with certain localization of disease treated in a very consistent way and with a very good response to treatment). Despite all these efforts at the stage of study design, sample size remains a key determinant and therefore recruitment among several centers in the context of international societies and consortia as well as the planning of such projects as spin-offs of clinical trials or other clinical studies is a key step. Moreover, the costs of these studies remain significant and all the logistic aspects need to be carefully planned including a very well organised pipeline in terms of patients’ identification, sample collection and process as well as cutting edge technologies from the point of view of laboratory equipment and bioinformatics structure.

CONCLUSIONS

We have identified a *TNFSF13B* (BAFF) SNP (rs3759467) associated with response to RTX in two independent cohorts of patients with AAV. The SNP localisation and the presence of signs of increased activity of the B-cells compartment in the carriers of the unfavourable genotype, suggest a potential role for an augmented BAFF activity in this group. Study aimed at the assessment of BAFF levels in populations stratified according to this SNP genotype before and after B cell-depleting events, might be able to confirm this hypothesis. If the role of this SNP is confirmed, it might be employed as biomarker to identify a subgroup of patients more likely to fail RTX and therefore requiring alternative approaches including combination of RTX with other biologics such as a BAFF inhibitor.

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TABLES AND FIGURES

Table 1. Main genetic associations with ANCA-associated vasculitis excluding those deriving from genome-wide association studies (GWASs)

GENE	VARIATION	POPULATION	CASES	CONTROLS	OR	P-VALUE	Author, year (reference)
CD226	*rs763361*	German	642 GPA	1226	1.02	0.016	Wieczorek, 2009 (56)
CTLA4	*rs3087243*	British	641 (GPA, MPA and EGPA)	9.115	1.19	$6.4x10^{-3}$	Carr, 2009 (40)
GHSR	*Haplotype*	German	460 GPA	878	1.30	0.026	Wieczorek, 2010 (69)
*HLA-DQB1*0303*		Japanese	50 MPA	77	2.35	0.017	Tsuchiya, 2006 (22)
*HLA-DRB1*0901-DQB1*0303*		Japanese	50 MPA	77	2.44	0.0037	Tsuchiya, 2007 (22)
IL2RA	*rs41295061*	British	675 (GPA, MPA and EGPA)	8.936	0.05	0.012	Carr, 2009 (54)
IRF5	*Haplotype*	German	664 GPA	952	0.05	0.0012	Wieczorek, 2010 (63)
KIR2	*DS3*	Japanese	43 MPA	239	0.24	0.038	Miyashita, 2006 (80)
LEPR	*Lys656Asn*	German	460 GPA	878	0,05	0.0013	Wieczorek, 2010 (69)
LILRA2	*rs2241524 AA genotype*	Japanese	50 MPA	284	2.52	0.049	Mamegano, 2008 (70)
PTPN22	*rs2476601*	German	199 GPA	399	1.08	0.002	Jagiello, 2005 (39)
PTPN22	*rs2476601*	Italian	143 GPA, 102 MPA and 99 EGPA	945	1.91 GPA; 2.31 ANCA+ GPA	0.005 GPA; 0.00012 ANCA+GPA	Martorana, 2012 (41)

PTPN22	*rs2476601*	British	641 (GPA, MPA and EGPA)	9.115	1.04	0.000140	Carr, 2009 (40)
TLR9	*3-SNP haplotype*	German, Dutch, British	646 GPA, 164 EGPA and 53 MPA German; 273 GPA, 53 EGPA and 100 MPA Dutch and British AAV cases	1898	0.60	0.000044	Husmann, 2013 (66)
FCGR3B CNVs	*High CNVs*	British	556 (GPA, MPA and EGPA)	286	/	1x10-8	Willcocks, 2008 (81)
FCGR3B CNVs	*Low CNVs*	British	76 MPA	190	/	0.0003	Fanciulli, 2007 (60)

All genetic association studies on MPA were reported, while for GPA we only included studies with a sample size of >400 patients

Table 2. Main genetic associations with ANCA-associated vasculitis deriving from the two genome-wide association studies (GWASs).

GENE	VARIATION	POPULATION	CASES	CONTROLS	OR	P-VALUE
GWAS performed by the European Vasculitis Genetics Consortium (Lyons et al. 2012) (9)						
HLA−DP	rs3117242	European	2267 GPA and MPA	6858	3.67	1.5×10^{-71}
HLA−DQ	rs5000634	European	2267 GPA and MPA	6858	0.80	2.9×10^{-9}
COL11A2	rs3130233	European	2267 GPA and MPA	6858	1.51	7.8×10^{-15}
COL11A2	rs3117016	European	2267 GPA and MPA	6858	1.83	6.4×10^{-24}
SERPINA1	rs7151526	European	2267 GPA and MPA	6858	0.59	2.4×10^{-9}
HLA−DP	rs3117242	European	1683 GPA	6858	5.39	3.1×10^{-85}
HLA−DQ	rs5000634	European	1683 GPA	6858	0.83	2.2×10^{-6}
ARHGAP18	rs1705767	European	1683 GPA	6858	0.78	3.3×10^{-7}
SERPINA1	rs7151526	European	1683 GPA	6858	0.54	4.4×10^{-10}
PRTN3	rs62132295	European	1683 GPA	6858	0.78	2.6×10^{-5}
MOSPD2	rs6628825	European	1683 GPA	6858	0.80	2.6×10^{-6}
HLA−DP	rs3117242	European	489 MPA	6858	1.60	1.3×10^{-3}
HLA−DQ	rs5000634	European	489 MPA	6858	0.67	1.4×10^{-5}
ARHGAP18	rs1705767	European	489 MPA	6858	0.84	1.8×10^{-2}
SERPINA1	rs7151526	European	489 MPA	6858	0.76	1.7×10^{-1}
PRTN3	rs62132295	European	489 MPA	6858	0.99	9.3×10^{-1}
MOSPD2	rs6628825	European	489 MPA	6858	0.79	2.2×10^{-1}
HLA−DP	rs3117242	European	1521 PR3+	6858	7.03	6.2×10^{-89}
HLA−DQ	rs5000634	European	1521 PR3+	6858	0.86	3.3×10^{-5}
ARHGAP18	rs1705767	European	1521 PR3+	6858	0.73	5.2×10^{-8}
SERPINA1	rs7151526	European	1521 PR3+	6858	0.53	5.6×10^{-12}
PRTN3	rs62132295	European	1521 PR3+	6858	0.73	2.6×10^{-7}
MOSPD2	rs6628825	European	1521 PR3+	6858	0.77	6.1×10^{-7}
HLA−DP	rs3117242	European	556 MPO+	6858	1.55	3.2×10^{-2}
HLA−DQ	rs5000634	European	556 MPO+	6858	0.65	2.1×10^{-8}
ARHGAP18	rs1705767	European	556 MPO+	6858	0.87	1.0×10^{-2}
SERPINA1	rs7151526	European	556 MPO+	6858	0.84	2.8×10^{-1}
PRTN3	rs62132295	European	556 MPO+	6858	1.10	2.2×10^{-1}
MOSPD2	rs6628825	European	556 MPO+	6858	0.86	6.3×10^{-1}

GWAS performed by the Vasculitis Clinical Research Consortium (Xie et al. 2013) (10)						
HLA-DPB1	rs9277554	European Descent	750 GPA	1820	0.24	1.92×10^{-50}
HLA-DPA1	rs9277341	European Descent	750 GPA	1820	0.33	2.18×10^{-39}
WSCD1	rs7503953	European Descent	750 GPA	1820	1.50	1.93×10^{-7}
COBL	rs1949829	European Descent	750 GPA	1820	1.78	4.19×10^{-7}
CCDC86	rs595018	European Descent	750 GPA	1820	1.46	1.60×10^{-7}
DCTD	rs4862110	European Descent	750 GPA	1820	1.44	2.14×10^{-6}
SEMA6A	rs26595	European Descent	750 GPA	1820	0.74	2.09×10^{-8}
HLA-DPB1	rs9277554	European Descent	578 c-ANCA+	1820	0.16	4.7×10^{-57}
HLA-DPA1	rs9277341	European Descent	578 c-ANCA+	1820	0.27	2.30×10^{-42}

Table 3. Clinical trials of more than five patients exploring the effectiveness of rituximab as induction treatment in GPA/MPA

Study	Type of Study	Number of Patients (GPA%)	Type of disease	Other add - on immunosuppressive with RTX	Disease Score	Follow-up (months)	Remission Rate	Remission rate in relapsing patients re-treated with RTX (%)
Stone J.H. et Al 2010[92]	Prospective Randomised	99 (75%)	New Onset (48%) Relapse (52%)	None	Mean BVAS 8.5 (SD ± 3.2)	Trial follow-up 6	65%	NA
Jones R.B. et Al 2010[93]	Prospective Randomised	33 (55%)	New Onset (100%)	CYC 100% *	Median BVAS 19 (IQR 14-24)	Trial Follow-up 12	76%	NA
Mansfield N. et Al 2011[98]	Prospective	23 (57%)	New Onset (96%) Relapse (4%)	CYC 100% AZA 100%	Median BVAS 21 (range 12-27)	Median (range) 39 (8-51)	100%	100%
Smith K. G. et Al 2006[94]	Prospective	11 (45%)	Relapsing Refractory	CYC 100% *	BVAS > 8	Median (range) 24 (12-31)	82%	100%
Stasi R. et Al 2006[95]	Prospective	10 (80%)	Relapsing Refractory	None	Median BVAS 5.5 (range 3-11)	Median (range) 33.5 (26-45)	100%	100%
Keogh K. A. et Al 2006[96]	Prospective	10 (NA)	Relapsing Refractory	None	Median BVAS 6 (range 5-10)	Trial follow-up 12	100%	100%
Keogh K. A. et Al 2005[97]	Prospective	11 (91%)	Refractory	None	Median BVAS 5 (range 3-11)	Median (range) 14 (10-42)	100%	100%
McGregor JG et Al. 2015[109]	Retrospective	120 (47%)	New Onset (14%) Relapse (86%)	CYC 33%	NA	NA	86%	84%
Charles P. et Al. 2014[114]	Retrospective	80 (88%)	Relapse New Onset	Non specified 25%	Median BVAS 7 (IQR 5-12)	Median (IQR) 18 (12-37)	91%	NA
Calich AL et Al. 2014[113]	Retrospective	66 (100%)	New Onset (5%) Relapsing (95%)	Various 24% [£]	Mean BVAS 9.5 (SD ± 5.2)	Mean (SD) 34.2 (26.2)	79%	NA
Jones R.B. et Al 2009[99]	Retrospective	65 (71%)	Relapsing Refractory	CYC 43% *	Median DEI 4 (range 2-11)	Median (range) 20 (3-55)	98% #	84-95%

Joshi L. et Al. 2015[110]	Retrospective	37 (100%)$	New Onset (14%) Relapse	CYC 35%	NA	Median (range) 36.5 (6-56)	86%	NA
Geetha D. et Al. 2015[115]	Retrospective	37 (38%)	New Onset (78%) Relapsing	CYC 68%	All Active Renal Vasculitis and GFR ≤ 20 ml/min	Median (range) 32.4 (6.7-55.2)	97%	NA
Md Yusof MY et Al. 2015[108]	Retrospective	35 (NA)	New Onset (11%) Relapsing	None	Mean BVAS 10.5 (SD ± 6)	Patient-year 162	94%	83-100%
Timlin H et Al. 2015[115]	Retrospective	31 (61%)	New Onset (58%) Relapsing	CYC 13%	Mean BVAS 4.4 (SD ± 1.5)	Mean (SD) 35.4 (32.4)	97%	NA
Pullerits R. et Al 2012[100]	Retrospective	29 (97%)	Relapsing Refractory	None	Median BVAS 6 (IQR 3-8)	Median (IQR) 21 (14-31)	62%	100%
Turner-Stokes T et Al 2014[117]	Retrospective	19 (NA)	Relapsing New Onset	MMF 32% CYC 11%	Median BVAS 7 (range 2-18)	Median (range) 11.5 (1.5-36.9)	100%	100%
Chocova Z. et Al 2015[112]	Retrospective	18 (NA)	New Onset (11%) Relapsing	NA	NA	Median (range) 26 (3-82)	72%	NA
Wendt M. et Al 2012[101]	Retrospective	16 (88%)	Relapsing Refractory	CYC 19% Other (69%)	Median BVAS 9.5 (range 2-27)	Median (range) 20 (3-48)	94%	100%
Lovric S. et Al 2009[105]	Retrospective	15 (87%)	Relapsing Refractory	None	Median BVAS 12 (range 6-21)	Median (range) 15 (3-39)	93%	100%
Shah S. et Al 2015[106]	Retrospective	14 (43%)	New Onset Relapsing	None	All Active Renal Vasculitis and GFR ≤ 20 ml/min	Median (range) 18 (4.5-67.1)	100%	NA
Rees F. et Al 2011[102]	Retrospective	12 (92%)	Relapsing Refractory	CYC 67% *	Median BVAS 13.5 (range 7-26)	Median (IQR) 32 (19-47)	100%	100%
Roccatello D. et Al 2011[104]	Retrospective	11 (82%)	Relapsing Refractory	None	Median BVAS 18 (IQR 15-20)	Median (IQR) 40 (36-52)	100%	100%

Nagafuchi H. et Al. 2015[111]	Retrospective	7 (NA)	Relapsing Refractory	NA	Mean BVAS 16.7 (range 2-34)	Mean (range) 62.9 (4.8-81)	86%	NA
Henes J.C. et Al 2007[103]	Retrospective	6 (100%)	Relapsing Refractory	LEF 83%	Median BVAS 5 (range 3-8)	Mean (range) 16 (12-21)	100%	100%

Remission Rate includes complete and partial response. If not specified, the remission rate was assessed 6 months after the RTX administration.

Study assessing the response to therapy before the 6th month after RTX treatment.

* CYC administered in a low-dose fashion (low dose defined in case of cumulative dose lower than the 50% of what suggested by EUVAS guideline).

$ Only cases with ocular involvement

£: cyclophosphamide (6 patients), azathioprine (4), methotrexate (3), mycophenolate mofetil (3)

IQR: interquartile range.

LEF: leflunomide.

BVAS:Birmingham Vasculitis Activity Score.

NA: Not Available.

Table 4.

Clinical trials exploring the effectiveness of the pre-emptive rituximab administration as maintenance treatment in GPA and MPA.

Study	Number of patients	Induction treatment	Reason for re-treatment	RTX maintenance schedule	Number of infusions	Response to treatment	Patients on steroids before treatment	Patients on steroids after treatment	Patients on immunosuppressive before treatment	Patients on immunosuppressive after treatment
Guillevin L. et Al 2014[141]	57 £	CYC	Pre-emptive	500 mg x2/month, then 500 mg every 6 months (3 administrations)	5	95%	100%	100%	100%	NA
Pendergraft WF et Al 2014[143]	172	RTX	Pre-emptive	1 g every 3-4 months, according to B-cell repopulation time	Continuous administration (median FUP 2.1 years)	80% (95% considering only major relapse)		Effective reduction (numbers not provided)		Effective reduction (numbers not provided)
Alberici F. et Al 2014[159]	69	RTX	Pre-emptive	1 g twice a year	Median 5	87%	96%	48%	20%	9%
Calich AL. et Al 2014[113]	60	RTX	Pre-emptive	375 mg/m^2 or 500 mg every 6 months	3	90%	100%	NA	24%	NA
Smith R. et Al 2012[116]	45	RTX	Pre-emptive	1g twice a year	Median 6 (range 2-11)	88%	100%	62%	89%	3%
Roubaud-Baudron C. et Al 2012[137]	28	Variable⊥	Miscellaneous♯	375 mg/m^2 or 1 g twice a year (60%) Other (40%)	Median 4 (range 2-10)	93%	82%	57%	57%	29%
Cartin-Ceba R. et Al 2012[138]	53	RTX	Miscellaneous§	375 mg/m^2 in 4 weeks 1 g twice in a month	Median 4 (IQR 3-5)	100%	NA	NA	NA	NA

De Menthon M. et Al 2011[139]	8	RTX	Pre-emptive	375 mg/m2 at month 4, 8, 12	5 patients completed the planned 7 infusions	63%¶	88%	88%	100%	100%
Rhee E.P. et Al 2010[140]	39	RTX	Pre-emptive	1 g every 4 months	On average 6.5 infusions per patient	92%	92%	55-59%	87%	30-41%

£ Randomized controlled trial. 57 patients were treated with rituximab as maintenance therapy.
⊥ Induction treatment include RTX alone, RTX in association with another immunosuppressive, CYC, IVIG or MTX.

♯ RTX re-treatment was given pre-emptively, for intolerance of more conventional maintenance treatment, for grumbling disease despite treatment with other drugs and for kidney failure.

§ RTX re-treatment was given for relapses, for rise of PR3 ANCA levels after B-cells return, for B-cells return in patients previously ANCA negative. Only 64% of the RTX courses were given pre-emptive.

¶ The decision whether or not completing the treatment was based upon the response 2 months after the first RTX infusion of the induction phase.

IQR: interquartile range

NA: not available

Table 5.
Primary cohort recruitment according to country.

Country	Number of patients
Germany	53
Italy	49
Sweden	46
Denmark	41
Czech Republic	13
Spain	11

Table 6.
Single nucleotide polymorphisms (SNPs) tested, Minor allele frequency observed (MAF) and test for deviation from Hardy-Weinberg Equilibrium (HWE) in primary cohort of 213 patients with AAVs treated with rituximab.

SNP	Alleles	Gene	MAF	HWE - p
rs396991	G/T	*FCGR3A*	0.439	0.407
rs1050501	T/C	*FCGR2b*	0.11	0.716
rs1224141	G/T	*TNFSF13B*	0.235	0.839
rs16972216	A/G	*TNFSF13B*	0.173	1.000
rs1224147	T/C	*TNFSF13B*	0.221	0.680
rs10508198	C/G	*TNFSF13B*	0.339	0.058
rs12583006	A/T	*TNFSF13B*	0.231	0.554
rs8181791	A/G	*TNFSF13B*	0.307	0.327
rs172378	A/G	*C1QA*	0.38	0.303
rs9514828	C/T	*TNFSF13B*	0.442	0.888
rs1801274	C/T	*FCGR2A*	0.495	0.407
rs1800795	C/G	*IL6*	0.359	0.881
rs6822844	G/T	*IL2-IL21*	0.127	0.542
rs3759467	A/G	*TNFSF13B*	0.172	0.057
rs28362491	DEL/ATTG	*NFKB1*	0.383	0.102
rs1800471	C/G	*TGFB1*	0.09	1.000
rs1041569	A/T	*TNFSF13B*	0.166	1.000
rs9514827	T/C	*TNFSF13B*	0.318	0.626

MAF: Minor Allele Frequency; HWE: Hardy Weinberg Equilibrium.

The test for deviation from HWE has been calculated using the R package SNPasso *(177)*, threshold for significance has been established to $p < 0.05$.

Table 7. Patient characteristics

Characteristic			Primary cohort (n=213)	Replication cohort (n=109)
Age (years)			53.3 (40.2 - 64.7)	57.1 (41.9-65)
Gender (Male)			111 (52%)	48 (44%)
Diagnosis	GPA		185 (87%)	94 (86%)
	MPA		29 (13%)	15 (14%)
ANCA specificity	PR3		148 (70%)	78 (72%)
	MPO		29 (13%)	19 (17%)
	Negative		36 (17%)	12 (11%)
Prior disease duration (months)			56.3 (12.8 - 113.8)	48.2 (16.5 - 138)
Indication for RTX	Relapse		108 (51%)	53 (49%)
	Refractory disease		76 (36%)	15 (14%)
	Other¶		26 (13%)	41 (38%)
RTX dose for induction regimen	375 mg/m^2 weekly for 4 weeks		68 (32%)	28 (26%)
	1 g two weeks apart		143 (67%)	79 (72%)
	Otherß		2 (1%)	2 (2%)
Number of previous immunosuppressive				
Agents			2 (1-3)	3 (2-3)
	Cyclophosphamide		175 (82%)	94 (86%)
		Cumulative dose (g)	10 (4 - 28)	10 (4 - 27)

	Methotrexate	92 (43%)	28 (26%)
	Azathioprine	91 (42%)	76 (70%)
	MMF	49 (23%)	60 (55%)
Immunosuppression at study entry *		1 (0 - 1)	1 (0 - 1)
	Cyclophosphamide	35 (16%)	15 (14%)
	Methotrexate	30 (14%)	8 (7%)
	MMF	18 (9%)	17 (16%)
	Azathioprine	14 (8%)	11 (10%)
	IV methylprednisolone	8 (4%)	17 (16%)
	Oral prednisolone dose	25 mg (12.5 - 50)	12.5 mg (10 - 20)
Organ involvement at study entry	ENT	132 (62%)	60 (46%)
	Lungs	88 (41%)	21 (19%)
	Joints	83 (39%)	26 (24%)
	Kidneys	82 (39%)	24 (22%)
	Eye	59 (28%)	12 (11%)
	Peripheral nervous system	25 (12%)	9 (8%)
	Central nervous system	11 (5%)	3 (3%)
	Gastrointestinal	4 (2%)	0 (0%)
	Cardiac	4 (2%)	0 (0%)

DEI			5 (3.75 - 7)	3 (2 - 5)
ANCA status at study entry	Positive by ELISA		174 (82%)	73 (67%)
		PR3	148 (70%)	59 (54%)
		MPO	26 (12%)	14 (13%)
		Negative	39 (18%)	36 (33%)

* Excluding oral steroids, but including oral immunosuppressive agents continued for at least 3 months after the administration of the first RTX dose.

Results are expressed as *n/N (%)* or *median (IQR)* when appropriate.
¶ Other. Includes: First presentation of disease, contraindication to standard treatment, grumbling disease, patients unable to taper the prednisolone dose, need for steroid free regimen.
ß Other. Includes: 500 mg 2 weeks apart (2 cases), administration of a single dose of 1 g of RTX (1 case), administration of 3 doses of RTX at the dose of 375 mg/m2

Table 8.
Association of 18 candidate SNPs with the two main outcomes explored in the study: RTX-failure risk at 6 months and time to RTX-failure.

SNP	Alleles	Gene	RTX-failure risk at 6 months		Time to RTX-failure	
			p	p cor	p	p cor
rs396991	G/T	*FCGR3A*	0.275	1	0.495	1
rs1050501	T/C	*FCGR2b*	0.399	1	0.089	1
rs1224141	G/T	*TNFSF13B*	0.006*	0.216	0.268	1
rs16972216	A/G	*TNFSF13B*	0.328	1	0.521	1
rs1224147	T/C	*TNFSF13B*	0.01*	0.36	0.366	1
rs10508198	C/G	*TNFSF13B*	0.295	1	0.083	1
rs12583006	A/T	*TNFSF13B*	0.625	1	0.129	1
rs8181791	A/G	*TNFSF13B*	0.489	1	0.968	1
rs172378	A/G	*C1QA*	0.987	1	0.441	1
rs9514828	C/T	*TNFSF13B*	0.854	1	0.943	1
rs1801274	C/T	*FCGR2A*	0.949	1	0.605	1
rs1800795	C/G	*IL6*	0.574	1	0.735	1
rs6822844	G/T	*IL2-IL21*	0.184	1	0.853	1
rs3759467	A/G	*TNFSF13B*	0.911	1	2.6×10^{-04} *	0.009 *
rs28362491	- /ATTG	*NFKB1*	0.012*	0.432	0.245	1
rs1800471	C/G	*TGFB1*	0.727	1	0.978	1
rs1041569	A/T	*TNFSF13B*	0.461	1	0.88	1
rs9514827	T/C	*TNFSF13B*	0.577	1	0.825	1

p cor = p corrected for multiple testing according to Bonferroni.
* = statistically significant.

Table 9.
Association results for the SNP rs3759467 after fixed-effects weighted meta-analysis for the two main outcomes assessed in our study assuming a recessive model.

Outcome assessed	Primary cohort		Replication cohort		Meta-analysis	
	OR-HR	p	OR-HR	p	OR-HR	p
RTX-failure risk at 6 months	9.1	0.06489	8.6	0.008996	8.8	0.0065
Time to RTX-failure	12.4	7×10^{-04}	5.39	0.0024	7.3	8.5×10^{-06}

OR: Odds ratio; HR: Hazard ratio;
RTX-failure risk at 6 months has been explored using a recessive model of the Cochrane-Armitage test.
Time to RTX-failure has been explored using a recessive model of Cox-proportion Hazards regression model.
OR and HR have been reported when appropriate.

Table 10.
Main clinical characteristics of patients at time of study entry and 6 months after rituximab according to the different genotype for the SNP rs3759467 of the gene *TNFSF13B* in the overall study population (primary + replication cohorts merged).

Characteristic	Genotype TT+TC	Genotype CC	p value
Diagnosis (GPA)	260/301 (86%)	7/7 (100%)	0.2951
ANCA specificity (PR3)	209/294 (71%)	7/7 (100%)	0.2453
DEI	4 (2-6)	4 (3.5-4.5)	0.6925
Age	54.2 (40.8 - 65)	54.8 (37.5 - 58.5)	0.5965
Rituximab indication (active flare)^	233/299 (78%)	6/7 (86%)	0.6229
B cell return at 6 months	22/161 (14%)	3/6 (50%)	0.0146 *
ANCA positivity at 6 months	137/244 (56%)	6/7 (86%)	0.12
IgG delta at 6 months #	1.06 (0.96 - 1.34)	0.97 (0.76-0.97)	0.09389
IgM delta at 6 months #	1.5 (1.04 - 10.92)	1 (1 - 1.33)	0.01539 *
DEI at RTX-failure	4 (2-5)	4 (2.5 - 4.75)	0.703

Results are expressed as *n/N (%)* or *median (IQR)* when appropriate.
B cell return has been defined as B cell count ≥ 0.01 x 10^9 /l.
^ Active flare defined as rituximab given either for relapsing or refractory disease.
# Delta has been calculated as the ratio between the value at the time of rituximab administration and the value at the time point of interest.
GPA: Granulomatosis with Polyangiitis; DEI: Disease Extent Index Score.
Associations have been tested using the Mann-Whitney Rank Sum Test.
* Statistically significant.

Table 11.
Haplotypes of the 5' regulatory region of the gene *TNFSF13B* and their association with risk of TF 6 months after treatment with RTX according to a log-additive and recessive models.

Haplotype	Frequency	RTX-failure risk at 6 months			
		Log -additive model		Recessive model	
		p	p cor	p	p cor
TTAC	34%	0.635	1	0.58	1
CTAT	31%	0.73	1	0.699	1
TCAC	18%	0.028*	0.28	2.6×10^{-05}*	2.6×10^{-04}*
TTTT	13%	0.028*	0.28	0.386	1
Pooled	4%	0.475	1	0.741	1

* = statistically significant.

Table 12.
Association of 18 candidate SNPs with the two outcomes explored in our study (RTX-failure risk at 6 months and time to RTX-failure) according to the historical ANCA specificity.

SNP	Alleles	Gene	PR3 – AAVs (n = 148)				MPO – AAVs (n = 29)			
			RTX-failure 6/12		Time to RTX-failure		RTX-failure6/12		Time to RTX-failure	
			p	p corr	p	p corr	p	p corr	p	p corr
rs396991	G/T	*FCGR3A*	0.150	1	0.815	1	0.388	1	0.25	1
rs1050501	T/C	*FCGR2b*	0.073	1	0.401	1	0.433	1	0.808	1
rs1224141	G/T	*TNFSF13B*	0.007	0.53	0.208	1	0.380	1	0.672	1
rs16972216	A/G	*TNFSF13B*	0.164	1	0.806	1	0.667	1	0.705	1
rs1224147	T/C	*TNFSF13B*	0.012	0.84	0.253	1	0.360	1	0.675	1
rs10508198	C/G	*TNFSF13B*	0.775	1	0.206	1	0.546	1	0.824	1
rs12583006	A/T	*TNFSF13B*	0.980	1	0.162	1	0.966	1	0.702	1
rs8181791	A/G	*TNFSF13B*	0.645	1	0.829	1	0.625	1	0.603	1
rs172378	A/G	*C1QA*	0.733	1	0.481	1	0.551	1	0.671	1
rs9514828	C/T	*TNFSF13B*	0.390	1	0.533	1	0.434	1	0.782	1
rs1801274	C/T	*FCGR2A*	1	1	0.401	1	0.194	1	0.562	1
rs1800795	C/G	*IL6*	0.574	1	0.392	1	0.135	1	0.287	1
rs6822844	G/T	*IL2-IL21*	0.987	1	0.874	1	4.2×10^{-04}*	0.03 *	1.9×10^{-04}*	0.0068 *
rs3759467	A/G	*TNFSF13B*	0.667	1	4.8×10^{-04}*	0.017 *	0.452	1	0.825	1

rs28362491	-/ATTG	*NFKB1*	0.044	1	0.263	1	0.212	1	0.268	1
rs1800471	C/G	*TGFB1*	0.609	1	0.148	1	0.626	1	0.363	1
rs1041569	A/T	*TNFSF13B*	0.306	1	0.648	1	0.899	1	0.668	1
rs9514827	T/C	*TNFSF13B*	0.111	1	0.598	1	0.706	1	0.534	1

RTX-failure rates at 6 months were compared using a Cochrane-Armitage test with log-additive model and time to RTX-failure using log–rank test.
n = number.
* Statistically significant.

Table 13.
Association results for the SNP rs3759467 in the subgroup of the patients with PR3-ANCA after fixed-effects weighted meta-analysis for the two outcomes assessed in our study assuming a recessive model for the SNP.

Outcome assessed	Primary cohort (n=148)		Replication cohort (n=78)		Meta-analysis	
	OR/HR	P	OR/HR	p	OR/HR	p
RTX-failure risk at 6 months	8.2	0.0853	9.2	0.0089	8.8	0.007
Time to RTX-failure	11.6	0.0012	6.2	0.002	8.2	8.7×10^{-06}

RTX-failure risk at 6 months has been explored using a recessive model of the Cochrane-Armitage test.
n = number.
Time to RTX-failure has been explored using a recessive model of Cox-proportion Hazards regression model.

Table 14.
Genotype distribution for the SNP rs6822844 of the gene *IL2-IL21* in the subgroup of patients MPO-ANCA positive of the replication cohort. The difference is not statistically significant (p=0.1722, Cochrane Armitage test, log-additive model) although there was a trend towards an increased risk of treatment failure in the carriers of the T allele in small sample size (19 patients).

Genotype rs6822844	Response at 6 months		
	Positive response	TF	TF percentage
GG	15	1	6%
GT	2	1	33%
TT	0	0	0%

Figure 1.

Role of B cells in the pathogenesis of AAVs. The interaction between CD20+ B-cells and T lymphocytes leads to (i) the development of ANCA-producing plasmablasts (CD20+) and plasma cells (CD20−), (ii) the maturation of T effector memory cells (T EM) and (iii) the production of pro-inflammatory cytokines. The priming of neutrophils is facilitated by the circulating cytokines and by microorganisms including Staphylococcus aureus. The primed neutrophils are activated by the binding of ANCA on the surface and together with T EM cells are responsible for vessel inflammation and tissue damage. The activated neutrophils may produce BAFF contributing to further B lymphocyte activation. In the peripheral tissues, a further interaction between B and T cells occurs inside the tertiary lymphoid tissues sites where B cells are more protected compared with the circulation due to stromal cell adhesion molecules and BAFF production *(5)*.

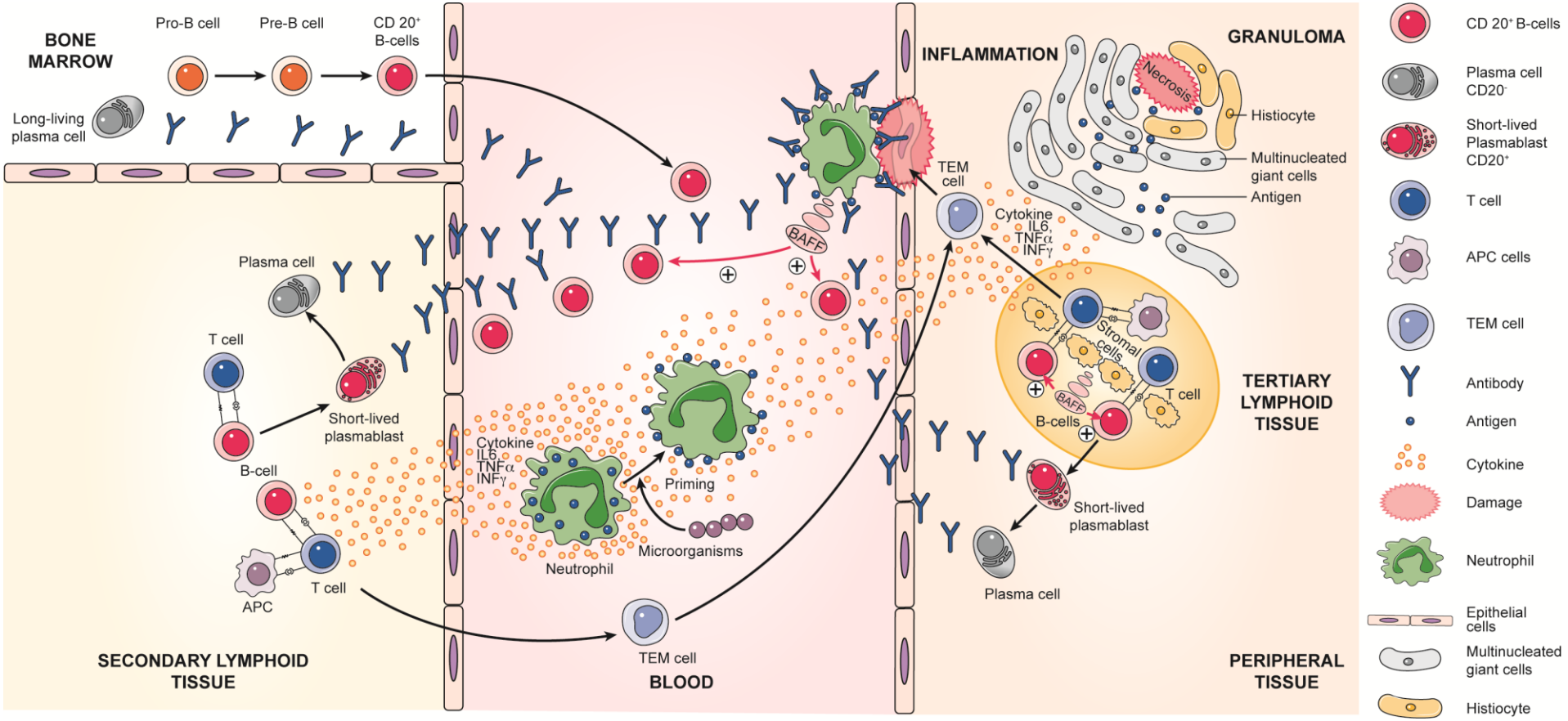


Figure 2.

Relapse free survival in two cohorts of relapsing AAV patients treated with rituximab at the moment of the relapse according to whether or not pre-emptive re-treatment with rituximab was performed *(142)*.

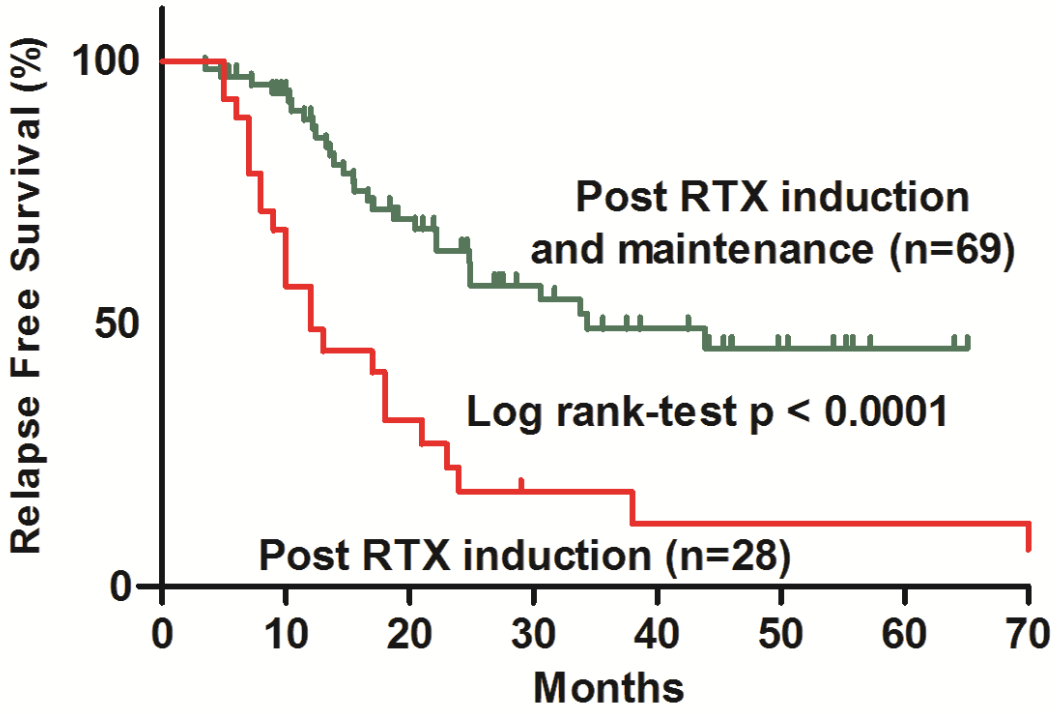


Figure 3. Change in IgG levels in a cohort of patients treated with rituximab (RTX) as maintenance treatment.

Data includes 69 AAV patients that received RTX induction and maintenance treatment (paired t test).

The lines inside the boxes represent the median level, the edge of the boxes the 25^{th}-75^{th} percentile, the whiskers the 5^{th}-95^{th} percentiles and the dots the outliers. The grey area represents the reference range *(142).*

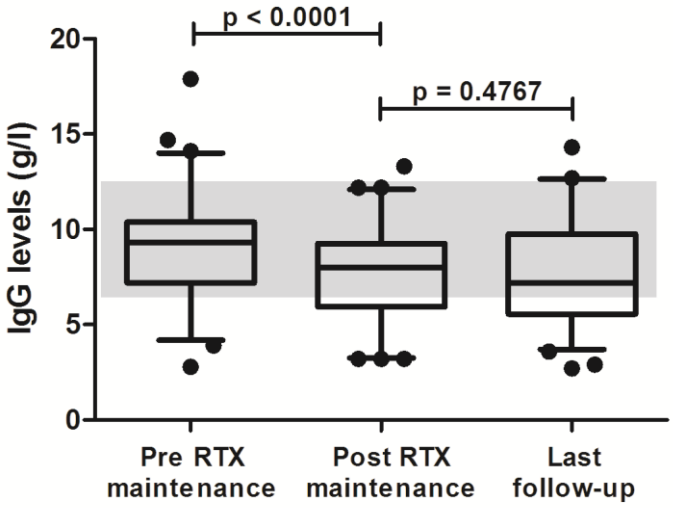
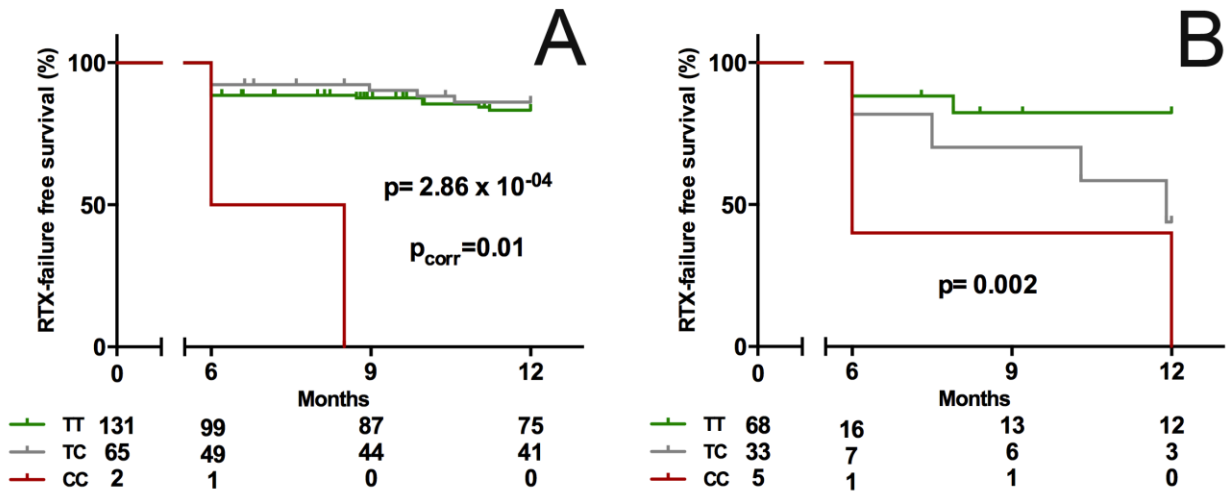


Figure 4.
RTX-failure free survival in an exploratory cohort of 213 patients (**panel A)** and in a replication cohort of 109 patients (**panel B**) stratified according to the genotypes of the SNP rs3759467 of the gene *TNFSF13B*.



APPENDIX

Eligibility Criteria

Patients affected by granulomatosis with polyangiitis (Wegener's, GPA) or microscopic polyangiitis (MPA) for whom there was indication to the beginning of treatment with RTX as follows:

- Relapsing disease: disease flare defined as DEI score >2, physician assessment of relapsing disease and need for immunosuppression escalation.
- Refractory disease: persistent activity of disease despite treatment with IV and/or oral steroids and other immunosuppressive agents and physician assessment of refractory disease for at least 3 months.
- Other indications:
    - First presentation of the disease.
    - Contraindication to standard treatment (e.g. recurrent infection, fertility preservation).
    - Grumbling disease: low-grade disease activity according to physician assessment that is not formally fulfilling the inclusion criteria in terms of disease activity scoring.
    - Patients unable to taper the prednisolone below the 15 mg/day on their previous therapeutic regimen.
    - Need for steroid free regimen.

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