Study Title: Transcriptomic deconvolution to dissect the immunological mechanism of B cell depletion therapy in ANCA associated vasculitis and impact on clinical outcomes.

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V1.0	09 November 2022	original protocol
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Summary

ANCA associated vasculitis is an autoimmune condition, characterised by inflammation of blood vessels, which can affect any organ in the body. It has been transformed from a largely fatal to a chronic relapsing condition following the introduction of immunosuppression, initially glucocorticoids and cyclophosphamide, and more recently, rituximab, a drug which targets a particular subset of immune cells, called B cells. B cells have many functions including the production of antibodies, one of which is ANCA. Rituximab is effective at inducing remission but must be given repeatedly to maintain remission and the effect is not sustained when treatment is discontinued in many patients. Unfortunately, repeated dosing of rituximab can be harmful by weakening the immune system, leading to an increased risk of infection and poorer vaccine responses. Vaccination is hugely topical at present in light of the Covid-19 pandemic, and many patients who have received courses of rituximab have mounted sub-optimal antibody responses, and remain vulnerable to the infection.

It is not clear why some patients have long lasting remissions and others relapse. The repopulation of B cells, or rising levels of ANCA cannot accurately predict future disease course. This is likely due to the interactions of B cells with other cells in the immune system. Furthermore, it is unclear why some patients appear more sensitive to rituximab developing low immunoglobulin levels and recurrent infections. Using new technologies and sophisticated computer methods, it is now possible to characterise in great detail the different types of immune cells and thereby better understand the impact of rituximab on the immune system.

As part of the RITAZAREM clinical trial, which compared repeated doses of rituximab to azathioprine as maintenance treatment in relapsing ANCA associated vasculitis, blood samples were collected at multiple time points. The analysis of these samples will allow us to model changes over time of both proportions of cells and individual cell characteristics to better understand the way B cell depletion with rituximab impacts other immune cell populations. This information, in conjunction with clinical data will allow us to further understand the effect of rituximab on the immune system and potentially explain the varied long-term clinical outcomes. This knowledge may in future allow more personalised treatment approaches.

Contents

1. Background and Rationale

a. ANCA associated vasculitis (AAV)

Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis (AAV) is an autoimmune condition characterised by inflammation of blood vessels leading to inflammation and necrosis of small blood vessels. All organs can be affected, but the most serious disease manifestations often include the kidneys and lungs. AAV is marked by the presence of antibodies against the neutrophil enzymes, proteinase 3 (PR3) and myeloperoxidase (MPO), produced by autoreactive B cells.

Prior to effective treatment, AAV had a mortality of 93% within 2 years, due to renal and respiratory failure. The introduction of glucocorticoids in 1948 and cyclophosphamide in the 1960s has transformed survival—with 5-year survival rates now approaching 80%. Despite refinement in the dosing regimens of these agents, and the introduction of novel biologic therapies, such as rituximab, the natural history of AAV is characterised by frequent relapses, necessitating periods of intensified immunosuppression, and its consequent toxicity, with progressive organ damage and disability, eventually affecting the majority of patients.

b. Role of B cells in AAV

B cells play a key role in the pathogenesis of AAV. Firstly, B cells are the precursors of ANCA secreting plasma cells, and evidence has accumulated over recent years demonstrating the potential pathogenic role of ANCA. CD20 positive B cell rich follicle like areas, containing autoreactive memory B cells with affinity for PR3 ANCA, are present in granulomata in GPA[1]. B cell activation also correlates with disease activity in GPA[2]. Not only are B cells the precursors of ANCA secreting plasma cells, but they also act as antigen presenting cells for autoreactive T cells, providing co-stimulatory support, and initiating T cell activation. In addition, B cells produce pro-inflammatory cytokines such as TNFα and IL-6 and initiate lymphangiogenesis and tertiary lymphoid formation[3].

c. Role of T cells in AAV

To date, most attention has focused on B cell targeted approaches as a therapeutic strategy in AAV. The strongest evidence for T cell involvement in the pathogenesis of AAV comes from the GWAS studies showing the strong association of class II MHC gene polymorphisms with ANCA specificity and clinical syndrome. Recurrent exposure to (self) antigen, leads to persistent activation of T cells even after obtaining clinical remission in AAV[4-6]. Activated T cells, more specifically the pro-inflammatory Th17 subtype, are found in abundance at sites of inflammation and in the circulation[7-9]. Data suggest that even in clinical remission, T cells remain activated within lymph node tissue, where T follicular helper cells (TFH) help B cell maturation aiding autoantibody generation. T regulatory cells (Tregs) that keep effector T cells under check have been shown to be functionally deficient in patients with AAV and may explain the persistent T cell activation[10]. Additionally, peripheral TFH are also higher in frequency in AAV[11]. Thus, persistently activated T cells in combination with returning B cells after rituximab therapy may provide a perfect milieu for relapse and interventions which modulate T cells, dampening T cell activation may reduce this risk of relapse.

Using PD-1 as a marker of recent T cell activation multiple groups have shown the relevance of a circulating CD4+ T cell subset called peripheral T helper cells (cPTH), aiding B cell differentiation, in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, lupus nephritis and coeliac disease[12-14]. Our own group has shown that patients have a hyper expanded population of similar cells in the circulation, which are found at inflammatory sites and possess cytotoxic potential (unpublished data). These terminally differentiated T cells are likely to have resulted from persistent T cell stimulation in the context of dysfunctional T regulatory cells seen in AAV.

Furthermore, CD8+ T cell transcriptome profiling in patients with newly diagnosed AAV has identified two distinct groups predicting relapse risk highlighting the importance of T cells in this disease. Individuals with a CD8+ T cell exhaustion signature had fewer relapses and a more favourable outcome with sustained disease remission[15]. A preliminary study in GPA with the T cell co-

stimulator inhibitor, CTLa4Ig (abatacept) has shown promising results and a Phase III trials, ABROGATE, is ongoing[16].

d. Rituximab

Rituximab, an anti-CD20 B cell depleting agent is an established induction agent in AAV, and is superior to azathioprine for remission maintenance in both newly diagnosed and relapsing patients following cyclophosphamide or rituximab re-induction, respectively[17-20]. However, there is an absence of evidence to guide treatment decisions beyond a two-year maintenance course. Many clinicians stop rituximab therapy and monitor, but by 48 months 50% of patients will have relapsed[20]. Relapses necessitate further immunosuppression and lead to the accrual of organ damage thus increasing morbidity. Others continue repeated administration of rituximab, which although effective at reducing relapse rates is not without risk. In the RITAZAREM trial, 49% of patients experienced at least one infection and 29% developed hypogammaglobuliaemia (IgG < 5g/l) in the rituximab group. Furthermore, rituximab is clearly associated with suboptimal vaccine responses, which is particularly relevant in the Covid-19 pandemic.

Reconstitution of peripheral CD19+ B cells within 12 months and return of ANCA have been associated with disease relapse[21]. However, a randomised trial evaluating the usefulness of B cells and ANCA to inform treatment decisions (rituximab given for re-emergence of CD19+ B cells or ANCA reappearance/rise in titre versus fixed-interval rituximab administration), did not provide strong support for the biomarker-based regimen, highlighting the limitations of these commonly measured biomarkers[22]. It is becoming increasingly clear that measurement of CD19+ B cells is not sufficiently sensitive to predict future clinical course. However, there is the suggestion that CD27+ memory B cells are a better predictor of relapse risk following rituximab therapy in several conditions (nephrotic syndrome[23], neuromyelitis optica[24] and myasthenia gravis[25].

Pilot data from our clinic cohort suggests that individuals with delayed reconstitution of switched memory B cells (CD27+IgD-) are less likely to relapse. Immunophenotyping was performed on AAV patients (n=13) between 12 and 24 months following completion of a 2-year maintenance course of rituximab. Of those who had a CD19+ B cell count > 1% of total lymphocyte count (n=9), a lower naïve: memory B cell ratio (52 vs 115) and higher proportion of switched memory B cells (CD27+IgD-) (1.52% v 0.82% of CD19 + cells) was observed in patients who relapsed after rituximab compared to those who did not relapse. 7/8 (86%) of individuals who relapsed and 1/3 (33%) of those that did not had a CD19+ CD27+IgD- B cell proportion above a 1% threshold. In addition, there was a suggestion that the naïve: memory B cell ratio remained stable in individuals who maintained remission, but fell in those individuals who relapsed, prior to the event.

The effect of rituximab on T cell populations in various autoimmune diseases has produced conflicting results. In a study of 51 patients with AAV, those in remssion who had received immunosuppression had proportionally fewer naïve CD4 T cells and more effector memory T cells compared to individuals with active disease. However, there was no differential effect on CD4 T cell populations, including regulatory T cells, with either rituximab or conventional immunosuppression. Rituximab reduced the proportion of CD8 effector memory T cells in AAV patients, whereas conventional immunosuppression had the opposite effect[26]. The effect of rituximab on T cell populations in AAV is yet to be fully elicudated.

e. Clinical challenge – balancing relapse and infection risk

The desire to sustain remission, whilst minimising drug exposure, is a clinical dilemma faced by clinicians managing autoimmune disease on a daily basis. It is not clear why rituximab induces long lasting remission in only about 50% of individuals. Return of B cells and rising ANCA levels are associated with relapse, but currently neither have sufficient sensitivity or specificity to be used as biomarkers to directly inform treatment decisions[21]. There is the suggestion that CD27+ memory B cells may more accurately predict disease course after rituximab in other conditions[23-25]. The longer-term response to rituximab and relapse risk is likely a combination of these B cell factors, as well as the complex, intimate relationship between B cells and T cells that has been shown to

underpin the pathophysiology of AAV. Yet, the effect of rituximab on T cell populations remains to be fully elucidated.

f. Transcriptomic deconvolution to dissect disease mechanism

In systemic autoimmune and inflammatory diseases, direct access to either affected tissue or secondary lymphoid organs is challenging, however collection of peripheral blood is straightforward. Multiple studies in both human and animal models from a range of autoinflammatory diseases[27-32] indicate that, while both functional properties and phenotypic markers vary between different anatomical locations, investigation of circulating cell populations provides a useful window through which disease-relevant mechanistic changes can be identified.

The identification and interpretation of changes in gene expression in peripheral blood populations has advanced our understanding of disease mechanism in numerous disease contexts, from cancer[34] to autoimmunity[15,33-35]. However, while access to peripheral blood is straightforward, analysis is not: major transcriptomic differences between haematopoietic lineages mean that whole blood signatures are driven by variable proportion of circulating cells, limiting our ability to identify mechanism from the resulting data. By contrast, single cell approaches allow granular dissection of gene expression changes but are limited by cost and a need to process samples rapidly to preserve viable cells. Consequently, they are challenging to apply in the context of prospective clinical trials where whole blood collection is more common.

Computational methods have been developed to allow inference of cell proportions from bulk RNA sequencing data and, in some cases, to also infer cell intrinsic transcriptomes (collectively termed 'deconvolution'). This creates the potential to unlock huge potential in data and samples collected during clinical trials, allowing analysis of longitudinal changes in both cell proportions and their associated gene expression levels. We have recently demonstrated the power of this approach, using a combination of deconvolution, network analyses and longitudinal mixed models to identify and interpret transcriptional changes accompanying progression of T1D[35].

2. Rationale

This project will use a transcriptomic approach to further characterise the effect of rituximab on B cell and T cell populations, using samples collected as part of the RITAZAREM trial – an international randomised controlled trial comparing fixed interval repeat dose rituximab to azathioprine as a remission maintenance strategy in AAV[36]. It is currently unclear why long-term responses following rituximab are so variable. Understanding the immunological mechanisms of action of rituximab, beyond B cell depletion, may explain disease course following rituximab administration. Using a cutting-edge machine learning based deconvolution method, cell intrinsic profiles will be analysed at multiple time points in each individual patient. Existing postulated predictors of sustained remission, including delayed reconstitution of memory B cells and a CD8 T cell exhaustion signature will be evaluated, in the context of clinical data collected as part of this clinical trial.

There is an unmet need to understand the effect of rituximab on multiple interacting components of the immune system, and subsequent disease course. Reducing relapse rates is one of the key objectives in AAV treatment strategies, as relapses have a huge impact on patient morbidity, with the accrual of damage and adverse effects on quality of life. Ultimately, this could enable therapy to be individually tailored, which will have direct individual patient benefit as well as cost saving benefits for the NHS. Although AAV is a rare disease (annual incidence in England is ~20/million and prevalence 250/million), rituximab is also used for other autoimmune conditions including rheumatoid arthritis and systemic lupus erythematosus. A better understanding of the mechanism of action could inform treatment strategies in these more common conditions too.

3. Research question / aims and objectives

a. Hypothesis:

A greater understanding of the mechanism of B cell depletion in AAV will give insight into the subsequent disease course and will enable individualisation of therapeutic strategies.

b. Objective:

To better understand the processes that lead to either the establishment of long-term remission or disease recurrence on stopping rituximab in AAV.

c. Specific Aims:

1. To quantify longitudinal changes in deconvoluted immune cell proportions following B cell depletion therapy and relate these to clinical outcomes.
2. To identify longitudinal changes in deconvoluted cell intrinsic immune cell transcriptomes following B cell depletion and relate these to clinical outcomes.
3. To compare longitudinal trajectories of deconvoluted immune cell proportions and transcriptomes between rituximab and azathioprine treated individuals.
4. To directly query the longitudinal trajectory of a previously identified T cell exhaustion signature, known to be associated with clinical outcome at baseline in AAV.
5. To identify longitudinal changes in deconvoluted cell proportions or cell intrinsic transcriptomes that occur after withdrawal of immunosuppression, comparing changes in those who relapse against those who do not.
6. To identify a transcriptomic signature which could be used, once appropriately validated to guide relapse prevention strategies with rituximab leading to more personalised therapy more efficient use of rituximab and better patient outcomes

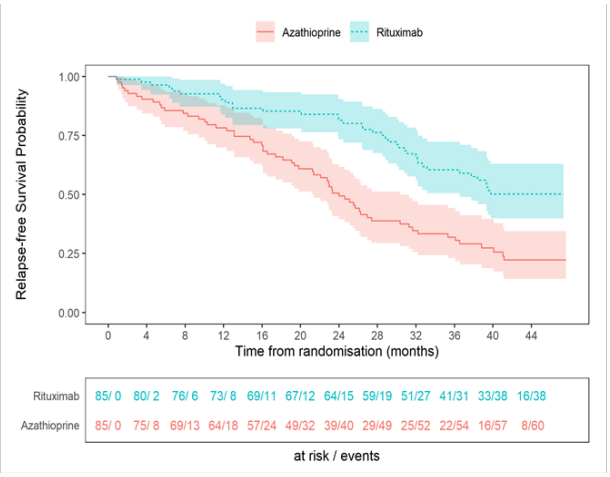
4. Project Plan

This project will utilise the clinical data and stored biological specimens collected as part of the RITAZAREM trial.

a. RITAZAREM Trial

The RITAZAREM trial was an international, randomized controlled trial comparing repeat dose rituximab to azathioprine as a maintenance strategy in AAV funded by Versus Arthritis. Patients with AAV were recruited at the time of disease relapse and received induction therapy with rituximab (4 x 375mg/m^2) and glucocorticoids (either high dose starting at 1mg/kg/day, maximum 60mg, or lower dose starting at 0.5mg/kg/day, maximum 30mg, with both regimens tapering to 10mg daily by month 4). Those achieving remission by four months were randomized to receive either rituximab (1000 mg every 4 months for 5 doses) or azathioprine (2 mg/kg/day, tapered after 24 months) as maintenance therapy. Glucocorticoid doses were protocoled in both groups reducing to 5mg daily at 6 months, 2.5mg daily at 16 months and were completely withdrawn at 20 months. Patients were followed either until 48 months, or until the final patient reached 36 months. The primary outcome was time to disease relapse.

188 patients were enrolled and 170 randomized at 4 months (85 to rituximab; 85 to azathioprine). Rituximab was superior to azathioprine in preventing relapse with an overall hazard ratio estimate of 0.41 (95% CI 0.27-0.61, p <0.001) (Figure 1A). 152 relapses in 98 patients occurred during the trial (3 relapses in the induction phase, 61 relapses in 45 subjects in the treatment period and 88 relapses in 53 subjects in the follow up phase) (Figure 1B).



	Azathioprine (N=85)	Rituximab (N=85)
Patients relapsing during treatment (up to month 24)	32	13
Patients relapsing once maintenance treatment discontinued (post month 24)	28	25
TOTAL	**60**	**38**

Figure 1: A - Probability of relapse-free survival: rituximab compared to azathioprine. B – Number of patients experiencing a disease relapse during and beyond the maintenance treatment period according to maintenance therapy.

19/85 (22%) patients in the rituximab group and 31/85 (36%) patients in the azathioprine group experienced at least one severe adverse event. Fifteen patients experienced 19 severe infections in the rituximab group, and 19 patients had 33 severe infections in the azathioprine group. One-hundred and ninety-seven and 207 non-severe infections occurred in 54 and 62 patients in the rituximab and azathioprine groups respectively. Thirty-six (42%) patients in the rituximab group had an IgG level < 5g/l at some point during the trial and 8 (9%) patients had an IgG level < 3g/l compared to 26 (31%) and 6 (7%) in the azathioprine group. Lower baseline IgG (Odds Ratio (OR) 0.52 baseline IgG; 95% CI 0.40-0.65, p<0.001) and receipt of higher-dose glucocorticoids during the induction phase (OR 8.6; 95% CI 3.02-27.58, p<0.001) were associated with the development of hypogammaglobulinaemia by multivariate analysis.

b. RITAZAREM Samples

Whole blood RNA (PAXgene stabilised) and serum were collected at baseline, months 1.5, 3, 4 (remission), 8, 12, 16, 20, 24 (end of maintenance treatment), 30, 36, 42, 48 and at time of relapse). Longitudinal samples including those collected at baseline and months 4 (remission), month 20 (last rituximab dose in maintenance course) and month 30 (off immunosuppression) will be used in this project. Clinical follow-up (including relapses, occurrence of infections and glucocorticoid dosage) was collected up to month 48 allowing comparison of transcriptional changes on stopping immunosuppression with subsequent clinical course.

5. Methods

We will undertake bulk RNA sequencing of peripheral blood samples taken longitudinally from patients recruited into the RITAZAREM trial. We will then perform a detailed deconvolution analysis and will model longitudinal trajectories of both deconvoluted cell proportions and cell intrinsic transcriptomic signatures to better understand the mechanism of B cell depletion therapy on other leucocyte populations. The results will be integrated with the clinical trial data set to associate signatures with the longitudinal changes disease activity.

a. Sequencing Pipeline

- extraction of RNA from each sample in collaboration with the Cambridge Stratified Medicine Core Laboratory using established protocols incorporating a PAXgene extraction kits.
- RNA amplification, with a ribosomal cDNA reduction step and confirmation of RNA quality
- RNA sequencing will be performed using an Illumina HiSeq4000 platform to generate a target of 20M 75bp paired-end reads per sample for bulk mRNAseq. Library preparations and sequencing will be performed in batches of 96 samples.

b. Data QC

In the pipeline RNAseq data is internally QC'd and processed by those directly involved in downstream analysis, using a combination of Illumina Basespace and FastQC metrics for processing of sequencer-derived Fastq files with further QC metrics derived using the BioConductor QoRTs package in R. Standard QC processing for our RNAseq pipeline also includes filtration on the basis of Phred score (>30), reads >20bp, paired reads only, illumina adaptor matching/trimming and in silico ribosomal read reduction (note that chemical reduction is also performed during library prep stage).

c. Bioinformatics analysis

The study will use multiple deconvolution methods run in parallel to:

- extract time and treatment dependent changes in cell frequencies
- undertake unsupervised identification and interpretation of transcriptional changes, relating each to clinical outcomes from the index trial
- extract cell intrinsic transcriptomes through deconvolution methods
- validate the quantification of cell-intrinsic transcriptomes through parallel comparison to those derived from physically sorted cell subsets

Deconvolution of whole blood transcriptomes will be facilitated by the availability of cell intrinsic transcriptomes from purified immune cell populations in a subset of cases (collected in parallel

studies from AAV patients). For this analysis cell intrinsic transcriptomes using comparable methodology are available for up to 17 immune cell types[37] and from single cell datasets with prior immune cell sorting of immune cell subsets (to enrich for less prevalent populations). Both datasets are already available from the applicants' laboratories as reference material for algorithms requiring them. This will allow direct validation testing of the deconvolution methods that will be applied to the larger cohort.

Multiple deconvolution methods will be applied including those relying on bulk immune cell sequence data and relying on single cell sequence data. These will include, but will not be restricted to, ABIS[37], CDSeq[38] and MuSiC[39] facilitating deconvolution using different reference sets (single cell vs bulk sequencing) and comparisons between different methods. A key strength of the proposed study is the availability of a parallel dataset (in the co-applicants and collaborators laboratories) for which RNA sequencing data from both whole blood and concurrently sampled, purified immune cell subsets are already available. This will allow direct comparison of deconvoluted with directly measured cell proportions and cell-intrinsic gene expression profiles (Figure 2). This will allow robust validation of the approach and rapid independent validation of findings.

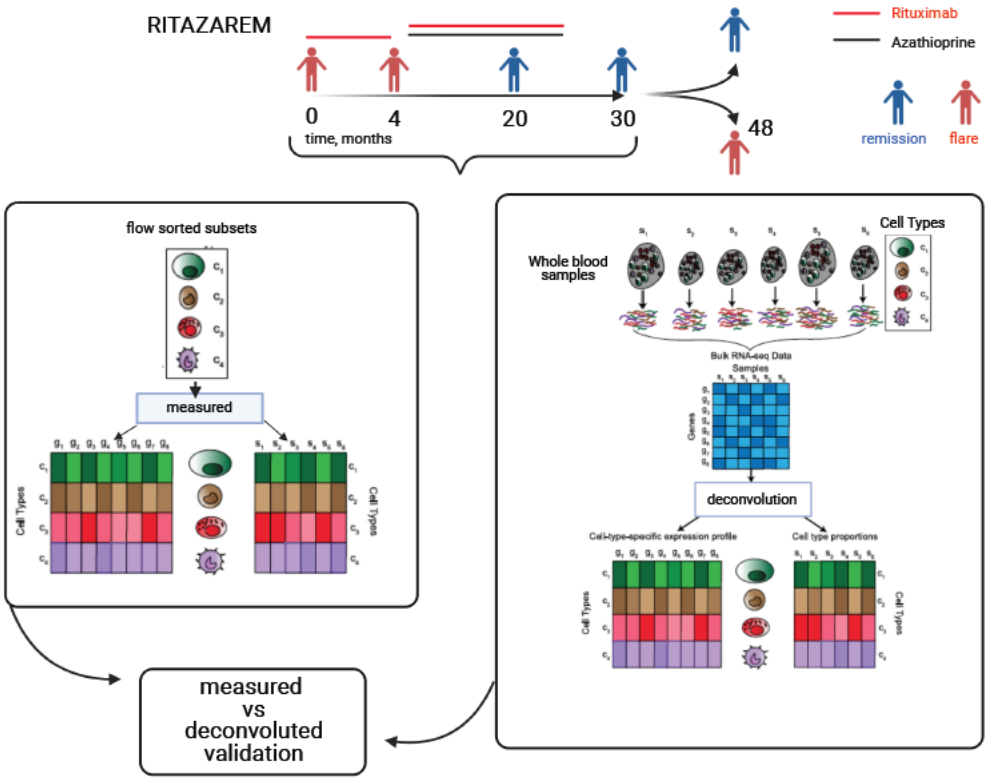


Figure 2. Schematic illustration of proposed analysis

Parallel analysis of flow sorted immune cell subsets (n=17 populations) for which both RNAseq and flow cytometric data is available will allow direct validation of deconvoluted cell-intrinsic transcriptomes (modified from Kang et al. PLoS Comput Biol 15(12): e1007510).

Analyses will be performed in R using packages from Biconductor/CRAN with additional bespoke elements conducted in Unix/python script. Methods include clustering (k-means, self-organising maps, hierarchical, principal components), pattern recognition (non-negative matrix factorization), multi-dimensional scaling tools, network analyses (WGCNA, differential co-expression) and surrogate eigenvector identification using random forests or adaptive elastic networks. There is an increasing range of databanks available for comparative purposes and MSigDB, ImmuneSigDB, BluePrint, ENCODE, KEGG, BIOCARTA, NIH LINCS, Connectivity Map and Human Gene Atlas and EnrichR collections are routinely incorporated into analyses together with an established local databank of purified immune cell transcriptomes from patients with a broad range of autoinflammatory disease (some publicly available, others still under collection). Comparison with this extensive collection will allow direct comparison of identified signatures with those seen in comparable cell types of other conditions and other healthy control datasets.

The final phase of analysis will be to validate data-driven hypotheses to ensure that transcriptomic signatures are not over-fitted to the discovery dataset they will be sought and their clinical associations validated in independent datasets. Serological biomarkers reflecting any transcriptomic signatures identified will be sought in paired serum samples collected in the RITAZAREM trial.

6. Data Management Plan

Established pipelines for the storage, processing and linkage of complex high-throughput genomic datasets with extensive clinical metadata are already in place. All data will be centrally linked using pseudonymised identifiers alongside a controlled vocabulary for entry and recall of metadata.

The data management plan comprises three inter-related systems hosted by the Cambridge Service for Data-Driven discovery (CSD3):

- The **Research Data Store (RDS)** provides large scale, enterprise grade storage that is optimised for performance on large, active datasets that are accessed frequently
- The **Research File Share (RFS)** provides large scale, enterprise grade storage for data sets that delivers a highly resilient storage solution that provides snapshots to allow fast recovery of lost files. Both systems are run in failover mode, which means the service can be switched to the secondary storage system if there is ever a major failure of the primary system
- **Research Cold Store (RCS)** is a cost effective way to manage the data lifecycle and can be used to create space on more expensive storage platforms, making it available for new active data.

CSD3 has a robust security setup. It is founded on mandatory multi factor authentication including SSH key/password control combined with a 2-factor time-based one-time password. Any user in addition has to have university approval to join CSD3 and project approval (from the data manager) to gain access to the relevant shared directories on one or more of the shared areas of CSD3 (rds/rfs/rcs).

7. Regulatory and ethical considerations

a. Risk Assessment

For this study, only pseudonymized data and samples already collected will be used and all patients have an unique identifier. Researchers working with the samples will be unaware of the identity of the donor. Patients have already consented to the use of clinical data and samples collected as part of the original AAV trials in future ethically approved studies, and this research does not place any additional burden on patients.

The subjects whose samples and data are being used are unlikely to benefit directly from the proposed research. However, since the majority of patients with AAV experience at least one disease relapse and will require further treatment, advances in disease understanding and therapeutic approaches may be of benefit in future.

b. Informed consent

No informed consent is necessary for this study since participants provided written informed consent for clinical data and biological samples collected during the original AAV studies to be used in future ethically approved studies.

8. Publications

Results will be disseminated at scientific meetings and in the academic literature, and via Vasculitis UK newsletters and media sources to reach both clinicians and patients.

9. Intellectual Property and Commercialisation

Any arising intellectual property will be pursued through Cambridge Enterprise with all due consideration to the funding sources in accordance with University of Cambridge policy.

10. Project research timetable

The **Table 1** provides an overview of the work plan and estimated duration showing the overall study analysis period including the sample analysis and write up periods.

Task	Mai-23	Jun-23	Jul-23	Aug-23	Sep-23	Okt-23	Nov-23	Dez-23	Jan-24	Feb-24	Mär-24	Apr-24
RNA extraction and QC												
Sequencing and library prep												
Comparison of purified immune subsets and whole blood												
Deconvolution analysis												
Preparation of manuscript												

Table 1. Project timetable

11. Acknowlegements

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