

# **FACTORS AFFECTING OUTCOME IN ANCA ASSOCIATED VASCULITIS**

A thesis submitted to Imperial College London in candidature for the  
degree of Doctor of Philosophy by

**Anisha Tanna**

**February 2021**

Renal and Vascular Inflammation Section

Department of Medicine

Imperial College London

Commonwealth Building

Hammersmith Hospital

Du Cane Road

London W12 0NN

## ABSTRACT

The anti-neutrophil cytoplasm antibody (ANCA) associated vasculitides (AAV) are associated with considerable morbidity and mortality. The main aim of this study was to assess factors affecting outcome for patients with AAV.

Factors affecting outcome for over 500 patients with AAV were assessed. For some patients, this assessment included histological factors. AAV patients with severe renal involvement were also studied. The clinical significance of the B cell survival factors BlyS and APRIL was assessed. In addition, the effect of a BlyS inhibitor was evaluated using the in vivo model of AAV, experimental autoimmune vasculitis (EAV).

Increased age and the requirement for renal replacement therapy (RRT) at presentation were associated with poorer survival. Decade of diagnosis was predictive of outcome which improved with increasing decade. Histological features were assessed for 104 patients and a validation study carried out of the International classification described by Berden *et al* in 2010. The histological classification was not predictive of renal outcome in our study. One hundred and eighty patients with AAV and an eGFR of  $<15 \text{ ml/min/1.73 m}^2$  were also studied. Fifty nine percent of patients who presented dialysis dependent gained independent renal function at 1 year. Patients with severe renal involvement who presented independent of dialysis had significantly improved outcome.

Serum BlyS levels were found to be raised in patients with active AAV and those in remission, treated with rituximab. APRIL levels were also raised in active patients.

The use of a BlyS inhibitor as an induction agent resulted in marked improvement in EAV but treatment of established disease was less effective. An understanding of factors predictive of prognosis in AAV is necessary to balance the risk of immunosuppression with that of the disease process itself and aid the individualisation of therapy for patients. Work described in this thesis has contributed to this understanding.

*For Dillan and Mila*

## STATEMENT OF CONTRIBUTION

I, Anisha Tanna, confirm that this thesis is composed of my own original work. Where work was performed in collaboration with other persons, or where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## COPYRIGHT DECLARATION

The copyright of this thesis rests with the author. Unless otherwise indicated, its contents are licensed under a Creative Commons Attribution-Non Commercial 4.0 International Licence (CC BY-NC).

Under this licence, you may copy and redistribute the material in any medium or format. You may also create and distribute modified versions of the work. This is on the condition that: you credit the author and do not use it, or any derivative works, for a commercial purpose.

When reusing or sharing this work, ensure you make the licence terms clear to others by naming the licence and linking to the licence text. Where a work has been adapted, you should indicate that the work has been changed and describe these changes.

Please seek permission from the copyright holder for uses of this work that are not included in this licence or permitted under UK Copyright Law.

## ACKNOWLEDGEMENTS

I am very grateful to the Wellcome Trust for awarding the Clinical Research Training Fellowship that enabled me to undertake this project.

I could not have carried out this work without the help and guidance of my friends and colleagues in the renal laboratory. Thank you to Gurjeet for showing me everything I know in the lab with the most incredible patience, to Steve for being my main data collection partner and helping me so much with the *in vivo* work, and to Maria for being the calmest advisor and finding a solution for any crisis. Thank you to Shenny and Fayaz for keeping me sane in the office and to Anjli for organising everything. Thank you so much to Nish for all the help with statistical analysis and to Professor Cook and Candice for the histological advice. Thank you to Kerry and Neil for helping me get everything I needed and thank you to Katrina Todd, John McDaid, Bynvant, Tabi, and everyone else in the lab who has helped me over the past decade. Thank you also to my amazing family for their continued patience. The greatest thanks also to the patients and colleagues of the Imperial College Vasculitis clinic without whom this work would not have been possible.

Finally, I would like to thank Professor Charles Pusey and Professor Fred Tam for being the most supportive, patient and inspiring supervisors anyone could ask for. I cannot thank you enough.

## Table of Contents

|                                                            |           |
|------------------------------------------------------------|-----------|
| TITLE.....                                                 | 1         |
| ABSTRACT.....                                              | 2         |
| STATEMENT OF CONTRIBUTION .....                            | 5         |
| COPYRIGHT DECLARATION .....                                | 5         |
| ACKNOWLEDGEMENTS .....                                     | 6         |
| TABLE OF CONTENTS .....                                    | 7         |
| LIST OF FIGURES .....                                      | 16        |
| LIST OF TABLES.....                                        | 21        |
| LIST OF ABBREVIATIONS.....                                 | 23        |
| PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK..... | 29        |
| PUBLICATIONS .....                                         | 29        |
| CONFERENCE ABSTRACTS: ORAL COMMUNICATIONS.....             | 33        |
| CONFERENCE ABSTRACTS: POSTER COMMUNICATIONS.....           | 36        |
| <b>CHAPTER ONE – INTRODUCTION AND BACKGROUND .....</b>     | <b>41</b> |
| 1.1 INTRODUCTION TO AAV .....                              | 42        |
| 1.1.1 CLASSIFICATION OF AAV.....                           | 42        |
| 1.1.2 EPIDEMIOLOGY AND AETIOLOGY .....                     | 44        |
| 1.1.3 CLINICAL MANIFESTATIONS.....                         | 45        |
| 1.1.4 ASSESSMENT OF DISEASE ACTIVITY AND DAMAGE .....      | 48        |
| 1.1.4.1 BIRMINGHAM VASCULITIS ACTIVITY SCORE (BVAS) .....  | 48        |

|                                                         |    |
|---------------------------------------------------------|----|
| 1.1.4.2: THE FIVE FACTOR SCORE .....                    | 50 |
| 1.1.4.3: THE VDI SCORE.....                             | 50 |
| 1.2 PATHOGENESIS OF AAV .....                           | 50 |
| 1.2.1 ANCA.....                                         | 50 |
| 1.2.2 NEUTROPHILS.....                                  | 53 |
| 1.2.3 COMPLEMENT .....                                  | 54 |
| 1.2.4 T CELLS .....                                     | 55 |
| 1.2.5 B CELLS .....                                     | 56 |
| 1.2.5.1: B CELL SURVIVAL FACTORS – BLYS AND APRIL ..... | 57 |
| 1.2.6 MONOCYTES AND MACROPHAGES:.....                   | 63 |
| 1.3 ANIMAL MODELS OF AAV .....                          | 64 |
| 1.3.1 MOUSE MODELS.....                                 | 64 |
| 1.3.2 RAT MODELS.....                                   | 65 |
| 1.4 TREATMENT OF AAV.....                               | 66 |
| 1.4.1 OLDER TREATMENTS FOR AAV .....                    | 66 |
| 1.4.2 ADJUNCTIVE TREATMENTS.....                        | 71 |
| 1.4.3 NEWER THERAPIES.....                              | 71 |
| 1.4.3.1 COMPLEMENT INHIBITION .....                     | 72 |
| 1.4.3.2 T CELL TARGETTED THERAPY .....                  | 72 |
| 1.4.3.3 B CELL TARGETTED THERAPIES.....                 | 73 |
| 1.4.3.3.1: BLYS INHBITORS .....                         | 74 |
| 1.5 FACTORS AFFECTING OUTCOME IN AAV.....               | 76 |

|                                                 |           |
|-------------------------------------------------|-----------|
| 1.5.1 ADVERSE EFFECTS OF THERAPY .....          | 76        |
| 1.5.2 PROGNOSTIC FACTORS .....                  | 77        |
| 1.6 EXPERIMENTAL HYPOTHESES AND STUDY AIMS..... | 79        |
| HYPOTHESES: .....                               | 79        |
| AIMS:.....                                      | 79        |
| <b>CHAPTER 2: MATERIALS AND METHODS.....</b>    | <b>81</b> |
| 2.1 HUMAN WORK .....                            | 82        |
| 2.1.1 PATIENT IDENTIFICATION.....               | 82        |
| 2.1.2 PATIENT RECRUITMENT .....                 | 82        |
| 2.1.3 SAMPLE COLLECTION.....                    | 82        |
| 2.1.4 BLYS ELISA.....                           | 82        |
| 2.1.5 APRIL ELISA .....                         | 83        |
| 2.1.6 IMMUNOHISTOCHEMISTRY .....                | 83        |
| 2.1.6.1: OBTAINING BIOPSY SLIDES .....          | 83        |
| 2.1.6.2: IMMUNOHISTOCHEMISTRY PROTOCOL .....    | 83        |
| 2.2 IN VIVO ANIMAL STUDIES.....                 | 87        |
| 2.2.1 ANIMAL STUDY APPROVAL.....                | 87        |
| 2.2.2 ANIMAL HUSBANDRY .....                    | 87        |
| 2.2.3 INDUCTION OF EAV .....                    | 87        |
| 2.2.4 SAMPLE COLLECTION.....                    | 88        |
| 2.2.4.1 URINE COLLECTION.....                   | 88        |
| 2.2.4.2 SERUM COLLECTION.....                   | 88        |

|                                                                                 |                                                           |           |
|---------------------------------------------------------------------------------|-----------------------------------------------------------|-----------|
| 2.2.4.3                                                                         | TERMINAL PROCESSING OF BLOOD AND TISSUES .....            | 88        |
| 2.2.5                                                                           | ANALYSIS OF RENAL AND PULMONARY INJURY IN EAV .....       | 89        |
| 2.2.5.1                                                                         | HAEMATURIA .....                                          | 89        |
| 2.2.5.2                                                                         | PROTEINURIA .....                                         | 90        |
| 2.2.5.2.1                                                                       | DIPSTICK PROTEINURIA .....                                | 90        |
| 2.2.5.2.2                                                                       | QUANTIFICATION OF PROTEINURIA .....                       | 90        |
| 2.2.5.3                                                                         | BIOCHEMICAL ANALYSIS OF SERUM AND URINE .....             | 90        |
| 2.2.5.4                                                                         | RENAL HISTOLOGY .....                                     | 91        |
| 2.2.5.4.1                                                                       | IMMUNOSTAINING FOR ED1 POSITIVE CELLS .....               | 91        |
| 2.2.5.5                                                                         | INDIRECT IMMUNOFLUORESCENCE FOR ANTI MPO ANTIBODIES ..... | 92        |
| 2.2.5.6                                                                         | ELISA FOR ANTI-MPO ANTIBODIES .....                       | 93        |
| 2.2.6                                                                           | STATISTICS .....                                          | 93        |
| 2.2.7                                                                           | GENERAL REAGENTS AND BUFFERS .....                        | 94        |
| 2.2.8                                                                           | BLYS INHIBITOR .....                                      | 96        |
| 2.3.9                                                                           | ISOTYPE CONTROL .....                                     | 96        |
| <br><b>CHAPTER 3: FACTORS AFFECTING LONG TERM SURVIVAL OF PATIENTS WITH AAV</b> |                                                           |           |
|                                                                                 | .....                                                     | <b>97</b> |
| 3.1                                                                             | INTRODUCTION .....                                        | 98        |
| 3.2                                                                             | METHODS .....                                             | 100       |
| 3.2.1                                                                           | WHOLE COHORT ANALYSIS .....                               | 100       |
| 3.2.2                                                                           | TREATMENT .....                                           | 101       |
| 3.2.3                                                                           | STATISTICS .....                                          | 102       |

|                                                                                                                        |            |
|------------------------------------------------------------------------------------------------------------------------|------------|
| 3.2.4 ETHICS.....                                                                                                      | 103        |
| 3.3 RESULTS.....                                                                                                       | 104        |
| 3.3.1 DEMOGRAPHICS.....                                                                                                | 104        |
| 3.3.2 CLINICAL FEATURES BY DECADE.....                                                                                 | 107        |
| 3.3.3 FACTORS AFFECTING RENAL FUNCTION AT PRESENTATION .....                                                           | 110        |
| 3.4 OUTCOMES.....                                                                                                      | 115        |
| 3.5 DISCUSSION .....                                                                                                   | 119        |
| 3.6 CONCLUSION.....                                                                                                    | 123        |
| <b>CHAPTER 4 - EVALUATION OF THE INTERNATIONAL HISTOLOGICAL<br/>CLASSIFICATION OF ANCA ASSOCIATED VASCULITIS .....</b> | <b>124</b> |
| 4.1: INTRODUCTION .....                                                                                                | 125        |
| 4.2 METHODS .....                                                                                                      | 128        |
| 4.2.1 DATA COLLECTION.....                                                                                             | 128        |
| 4.2.2 TREATMENT AND OUTCOMES .....                                                                                     | 130        |
| 4.2.3 STATISTICAL ANALYSIS .....                                                                                       | 130        |
| 4.3 RESULTS.....                                                                                                       | 131        |
| 4.3.1 DEMOGRAPHICS.....                                                                                                | 131        |
| 4.3.2 HISTOLOGICAL CLASSIFICATION.....                                                                                 | 133        |
| 4.3.3 PERCENTAGE NORMAL GLOMERULI .....                                                                                | 136        |
| 4.3.4 TUBULAR ATROPHY .....                                                                                            | 138        |
| 4.3.5 OTHER FACTORS AFFECTING OUTCOME IN THIS STUDY .....                                                              | 140        |
| 4.3.5.1 BASELINE EGFR .....                                                                                            | 140        |
| 4.3.5.3 AGE.....                                                                                                       | 141        |

|                                                                                                                                         |            |
|-----------------------------------------------------------------------------------------------------------------------------------------|------------|
| 4.3.5.4 ANCA TYPE .....                                                                                                                 | 141        |
| 4.3.5.5 TREATMENT.....                                                                                                                  | 142        |
| 4.3.6 MULTIVARIATE ANALYSIS.....                                                                                                        | 142        |
| 4.4 DISCUSSION .....                                                                                                                    | 1467       |
| <b>CHAPTER 5: OUTCOMES OF ANCA PATIENTS WITH SEVERE RENAL DYSFUNCTION<br/>AT PRESENTATION .....</b>                                     | <b>149</b> |
| 5.1 INTRODUCTION .....                                                                                                                  | 150        |
| 5.2 AIMS .....                                                                                                                          | 153        |
| 5.3 EXPERIMENTAL DESIGN .....                                                                                                           | 153        |
| 5.3.1 TREATMENT .....                                                                                                                   | 154        |
| 5.3.2 STATISTICAL ANALYSIS .....                                                                                                        | 154        |
| 5.4 RESULTS.....                                                                                                                        | 155        |
| 5.4.1 DEMOGRAPHICS.....                                                                                                                 | 155        |
| 5.4.2 OUTCOMES .....                                                                                                                    | 157        |
| 5.4.3 COMPARISON OF PATIENTS WHO WERE DIALYSIS DEPENDENT AT<br>PRESENTATION WITH PATIENTS WHO PRESENTED INDEPENDENT OF<br>DIALYSIS..... | 159        |
| 5.4.4 RENAL AND PATIENT SURVIVAL ACCORDING TO DECADE OF<br>PRESENTATION .....                                                           | 164        |
| 5.4.5 RENAL AND PATIENT SURVIVAL ACCORDING TO AGE AT PRESENTATION<br>.....                                                              | 166        |
| 5.4.6 RENAL AND PATIENT SURVIVAL ACCORDING TO ANCA STATUS .....                                                                         | 168        |
| 5.4.7 RENAL AND PATIENT SURVIVAL DIVIDED BY PERCENTAGE NORMAL<br>GLOMERULI ON BIOPSY. ....                                              | 170        |

|                                                                                                             |            |
|-------------------------------------------------------------------------------------------------------------|------------|
| 5.4.8 ANALYSIS OF PATIENTS WHO PRESENTED DIALYSIS DEPENDENT .....                                           | 172        |
| 5.4.9: HISTOLOGICAL ANALYSIS .....                                                                          | 174        |
| 5.4.10 CHARACTERISTICS OF PATIENTS WHO HAD PRESENTED DIALYSIS<br>DEPENDENT FROM 2000 .....                  | 181        |
| 5.5 CONCLUSIONS: .....                                                                                      | 183        |
| 5.6 DISCUSSION: .....                                                                                       | 184        |
| <b>CHAPTER 6: B CELL SURVIVAL FACTORS AND AAV .....</b>                                                     | <b>190</b> |
| 6.1 INTRODUCTION: .....                                                                                     | 191        |
| 6.2 ASSESSING THE CLINICAL SIGNIFICANCE OF SERUM BLYS AND APRIL.....                                        | 193        |
| 6.2.1 EXPERIMENTAL DESIGN: .....                                                                            | 193        |
| 6.2.2 RESULTS: MEASUREMENT OF SERUM BLYS LEVELS .....                                                       | 194        |
| 6.2.3 RESULTS: MEASUREMENT OF SERUM APRIL LEVELS .....                                                      | 197        |
| 6.2.4 CONCLUSIONS: .....                                                                                    | 202        |
| 6.3 ASSESSMENT OF BLYS AND ITS RECEPTORS IN RENAL BIOPSY SPECIMENS OF<br>PATIENTS WITH AAV .....            | 203        |
| 6.3.1 EXPERIMENTAL DESIGN .....                                                                             | 203        |
| 6.3.2 RESULTS: .....                                                                                        | 205        |
| 6.3.3 ANALYSIS .....                                                                                        | 214        |
| 6.4 DISCUSSION: .....                                                                                       | 217        |
| <b>CHAPTER 7 – ANTI-BLYS ANTIBODY AS A NOVEL TREATMENT FOR EXPERIMENTAL<br/>AUTOIMMUNE VASCULITIS. ....</b> | <b>220</b> |
| 7.1 INTRODUCTION AND AIMS: .....                                                                            | 221        |
| 7.2 METHODS .....                                                                                           | 222        |

|                                                                                                      |     |
|------------------------------------------------------------------------------------------------------|-----|
| 7.2.1 ETHICS.....                                                                                    | 223 |
| 7.2.2 ANIMALS .....                                                                                  | 223 |
| 7.2.3 BLYS INHIBITOR.....                                                                            | 223 |
| 7.2.4 IMMUNISATION.....                                                                              | 223 |
| 7.2.4.1 EXPERIMENT 1: .....                                                                          | 224 |
| 7.2.4.2 EXPERIMENT 2: .....                                                                          | 225 |
| 7.2.5 URINE COLLECTION AND ANALYSIS .....                                                            | 226 |
| 7.2.6 IMMUNOHISTOCHEMISTRY .....                                                                     | 226 |
| 7.2.7 IMMUNOFLUORESCENCE:.....                                                                       | 227 |
| 7.2.8 ANTI-MPO ANTIBODY DETECTION BY DIRECT ELISA .....                                              | 227 |
| 7.2.9 B-CELL ELISPOT .....                                                                           | 228 |
| 7.2.10 ASSESSMENT OF LUNG INJURY .....                                                               | 228 |
| 7.2.11 ANALYSIS .....                                                                                | 229 |
| 7.3 RESULTS.....                                                                                     | 229 |
| 7.3.1 EXPERIMENT 1: THE EFFECT OF AN ANTI-BLYS MONOCLONAL ANTIBODY<br>IN THE PREVENTION OF EAV. .... | 229 |
| 7.3.1.1 ASSESSMENT OF HAEMATURIA .....                                                               | 229 |
| 7.3.1.2 ASSESSMENT OF PROTEINURIA .....                                                              | 230 |
| 7.3.1.3 HISTOLOGICAL INJURY .....                                                                    | 232 |
| 7.3.1.4 IMMUNOFLUORESCENCE .....                                                                     | 233 |
| 7.3.1.5 PULMONARY HAEMORRHAGE .....                                                                  | 235 |
| 7.3.1.6 MEASUREMENT OF B CELL ELISPOT .....                                                          | 236 |
| 7.3.1.7 ASSESSMENT OF CIRCULATING ANCA TITRES BY DIRECT ELISA .....                                  | 236 |

|                                                                                                                        |            |
|------------------------------------------------------------------------------------------------------------------------|------------|
| 7.3.1.8 HAEMATOLOGICAL INDICES .....                                                                                   | 238        |
| 7.3.1.9 CONCLUSION AND BRIEF DISCUSSION: EXPERIMENT 1 .....                                                            | 239        |
| 7.3.2 EXPERIMENT 2: THE EFFECT OF AN ANTI-BLYS MONOCLONAL ANTIBODY<br>IN TREATING EAV AFTER ONSET OF THE DISEASE. .... | 239        |
| 7.3.2.1 SUMMARY OF TREATMENT GROUPS: .....                                                                             | 239        |
| 7.3.2.2: ASSESSMENT OF HAEMATURIA .....                                                                                | 239        |
| 7.3.2.3 ASSESSMENT OF PROTEINURIA .....                                                                                | 240        |
| 7.3.2.4: QUANTIFICATION OF PROTEINURIA .....                                                                           | 241        |
| 7.3.2.5 PULMONARY HAEMORRHAGE .....                                                                                    | 242        |
| 7.3.2.6 CIRCULATING ANTI-MPO ANTIBODIES.....                                                                           | 243        |
| 7.3.2.7 RENAL HISTOLOGY.....                                                                                           | 244        |
| 7.3.2.8 MACROPHAGE INFILTRATION .....                                                                                  | 245        |
| 7.3.2.9 CONCLUSION AND BRIEF DISCUSSION, EXPERIMENT 2: .....                                                           | 247        |
| 7.4 OVERALL CONCLUSION AND DISCUSSION: .....                                                                           | 248        |
| 7.5 ACKNOWLEDGMENTS: .....                                                                                             | 250        |
| <b>CHAPTER 8: DISCUSSION AND FUTURE WORK .....</b>                                                                     | <b>251</b> |
| 8.1 AIMS OF THE STUDY.....                                                                                             | 252        |
| 8.2 CONCLUDING REMARKS .....                                                                                           | 258        |
| <b>REFERENCES: .....</b>                                                                                               | <b>259</b> |

## LIST OF FIGURES

|                                                                                              |     |
|----------------------------------------------------------------------------------------------|-----|
| Figure 1.1 Examples of clinical manifestations of AAV. ....                                  | 47  |
| Figure 1.2 Example of a scoring sheet for assessing BVAS. ....                               | 49  |
| Figure 1.3 Pathogenesis of ANCA associated vasculitis .....                                  | 54  |
| Figure 1.4 The interaction of BlyS and APRIL with their receptors. ....                      | 59  |
| Figure 1.5: Stages of B cell development and B cell survival factors. ....                   | 61  |
| Figure 3.1 Clinical features by different decades. ....                                      | 109 |
| Figure 3.2: Factors affecting serum creatinine (Cr) at presentation .....                    | 111 |
| Figure 3. 3: Factors affecting patient survival .....                                        | 116 |
| Figure 3. 4: Factors affecting patient survival (2).....                                     | 117 |
| Figure 4.3.1 Schematic for the International Classification for AAV .....                    | 129 |
| Figure 4.3.2: Renal function at presentation, 1 and 5 years divided by histological class. . | 134 |
| Figure 4.3.3 Change in eGFR at 1 and 5 years divided by histological class.....              | 135 |
| Figure 4.3.4 Renal function according to histological class .....                            | 136 |
| Figure 4.3.5: Renal function according to percentage normal glomeruli at presentation ....   | 137 |
| Figure 4.3.6: Renal function by degree of tubular atrophy .....                              | 139 |
| Figure 4.3.7: Renal survival according to presenting eGFR .....                              | 140 |
| Figure 4.3.8 Renal survival according to ANCA type. ....                                     | 141 |
| Figure 5.1: Renal survival over 10 years .....                                               | 157 |

|                                                                                                                |     |
|----------------------------------------------------------------------------------------------------------------|-----|
| Figure 5.2: Patient survival over 10 years .....                                                               | 158 |
| Figure 5.3: Combined renal and patient survival .....                                                          | 158 |
| Figure 5.4 Renal survival of dialysis dependent vs dialysis independent patients.....                          | 162 |
| Figure 5.5: Patient survival dialysis dependent vs dialysis independent patients.....                          | 163 |
| Figure 5.6: Combined renal and patient survival in dialysis dependent vs dialysis<br>independent patients..... | 164 |
| Figure 5.7: Renal survival according to decade of presentation. ....                                           | 165 |
| Figure 5.8: Patient survival according to decade of presentation.....                                          | 165 |
| Figure 5.9: Combined renal and patient survival according to decade of presentation .....                      | 166 |
| Figure 5.10: Renal survival according to age. ....                                                             | 167 |
| Figure 5.11: Patient survival according to age.....                                                            | 168 |
| Figure 5.12: Renal survival according to ANCA type .....                                                       | 169 |
| Figure 5.13: Patient according to ANCA type. ....                                                              | 169 |
| Figure 5.14: Renal survival according to percentage normal glomeruli.....                                      | 170 |
| Figure 5.15: Patient survival according to percentage normal glomeruli.....                                    | 171 |
| Figure 5.16: Combined renal and patient survival according to percentage normal glomeruli.<br>.....            | 171 |
| Figure 5.17: Schematic of patients who presented post 2000. ....                                               | 176 |
| Figure 5.18: Schematic to demonstrate classification of biopsies into histological class....                   | 177 |
| Figure 5.19 Renal survival divided by histological class.....                                                  | 179 |
| Figure 5.20: Patient survival divided by histological class .....                                              | 178 |
| Figure 5.21: Combined renal and patient survival divided by histological class .....                           | 179 |
| Figure 5.22: Renal survival divided by percentage normal glomeruli. ....                                       | 180 |

|                                                                                                                                                        |     |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Figure 5.23: Patient survival divided by percentage normal glomeruli .....                                                                             | 180 |
| Figure 5.24 Combined renal and patient survival divided by percentage normal glomerul .                                                                | 181 |
| Figure 6.1 Graph to show serum BlyS concentrations in patients in active AAV vs those in remission vs healthy controls. ....                           | 195 |
| Figure 6.2 Correlation between serum BlyS and crp, serum creatinine, ANCA titre and BlyS level. ....                                                   | 196 |
| Figure 6.3 Graph to show all patients included in this study divided according to treatment with rituximab.....                                        | 197 |
| Figure 6.5: Graph to show comparison between serum APRIL levels in patients with active AAV compared with those in remission and healthy controls..... | 198 |
| Figure 6.6: Graph to show the effect of rituximab treatment on serum APRIL levels. ....                                                                | 201 |
| Figure 6.8 Graph to demonstrate correlation between BlyS and APRIL levels. ....                                                                        | 202 |
| Figure 6.9: Graph demonstrating sequential BlyS levels in ten AAV patients. ....                                                                       | 202 |
| Figure 6.10: Patient 1 immunohistochemistry.....                                                                                                       | 205 |
| Figure 6.11: Patient 2 immunohistochemistry.....                                                                                                       | 206 |
| Figure 6.12 Patient 3 immunohistochemistry.....                                                                                                        | 207 |
| Figure 6.13 Patient 4 immunohistochemistry.....                                                                                                        | 208 |
| Figure 6.14 Patient 5 immunohistochemistry.....                                                                                                        | 209 |
| Figure 6.15 Patient 6 immunohistochemistry.....                                                                                                        | 210 |
| Figure 6.16 Patient 7 immunohistochemistry.....                                                                                                        | 211 |
| Figure 6.17 Patient 8 immunohistochemistry.....                                                                                                        | 212 |
| Figure 6.18 Patient 9 immunohistochemistry.....                                                                                                        | 213 |
| Figure 6.19: Patient 10 immunohistochemistry.....                                                                                                      | 214 |

|                                                                                                                    |     |
|--------------------------------------------------------------------------------------------------------------------|-----|
| Figure 6.20: Heatmap to show percentage glomerular positivity of each stain versus classification of patient ..... | 216 |
| Figure 6.21: Heatmap to show percentage interstitial positivity of each stain vs classification of patient. ....   | 217 |
| Figure 7.1: Protocol for induction of EAV.....                                                                     | 224 |
| Figure 7.2: BlyS inhibitor experiment 1.....                                                                       | 225 |
| Figure 7.3: BlyS inhibitor experiment 2.....                                                                       | 226 |
| Figure 7.4 Dipstick haematuria by week in EAV prevention study .....                                               | 230 |
| Figure 7.5: Dipstick proteinuria by week in EAV prevention study .....                                             | 231 |
| Figure 7.6: Proteinuria per day measured using the sulphosalicylic acid method. ....                               | 231 |
| Figure 7.7: Histological injury .....                                                                              | 232 |
| Figure 7.8: Macrophage infiltration in EAV anti-BlyS study.....                                                    | 233 |
| Figure 7.9: Direct immunofluorescence for deposited IgG.....                                                       | 234 |
| Figure 7.10: Assessment of pulmonary haemorrhage.....                                                              | 236 |
| Figure 7.11: Assessment of MPO specific cells by B cell Elispot. ....                                              | 236 |
| Figure 7.12: Assessment of circulating ANCA titres by direct ELISA.....                                            | 238 |
| Figure 7.13: Assessment of haematological indices across treatment groups.....                                     | 238 |
| Figure 7.14: Assessment of haematuria, by week, following the induction of EAV. ....                               | 240 |
| Figure 7.15 Assessment of proteinuria, by week, following the induction of EAV. ....                               | 241 |
| Figure 7.16 Proteinuria by week as measured using the sulphosalicylic acid method.....                             | 243 |
| Figure 7.17 Assessment of pulmonary haemorrhage score using the visual assessment score, .....                     | 243 |
| Figure 7.18: Assessment of circulating ANCA titres by direct ELISA at weeks 2, 4 and 6..                           | 244 |

|                                            |     |
|--------------------------------------------|-----|
| Figure 7.19 Histological injury .....      | 245 |
| Figure 7.20: Macrophage infiltration. .... | 246 |

## LIST OF TABLES

|                                                                                                                                                                                               |     |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 1.1 Definitions for ANCA-associated vasculitis according to the Chapel Hill Consensus Criteria 2012.....                                                                                | 43  |
| Table 1.2 Definitions of disease severity according to EULAR (20).....                                                                                                                        | 48  |
| Table 2.1 Antibodies used for immunofluorescence and immunohistochemistry .....                                                                                                               | 86  |
| Table 2.2 Lung haemorrhage score .....                                                                                                                                                        | 89  |
| Table 2.3 Commonly used buffers.....                                                                                                                                                          | 95  |
| Table 3.1 Demographics and clinical features separated by decade of presentation.....                                                                                                         | 107 |
| Table 3.2 Factors associated with renal replacement therapy at presentation.....                                                                                                              | 114 |
| Table 3.3 Analysis of factors associated with mortality by Cox proportional hazards.....                                                                                                      | 119 |
| Table 4.3.1 Demographics of patients included in the study divided by histological class.                                                                                                     | 133 |
| Table 4.3.2 Biopsy features divided according to histological category .....                                                                                                                  | 133 |
| Table 4.3.3 Multivariable models of eGFR at 1 and 5 years including presenting eGFR, age, histological class and tubular atrophy.....                                                         | 144 |
| Table 4.3.4 Cox proportional hazards model for predicted renal survival.....                                                                                                                  | 144 |
| Characteristics of patients at baseline .....                                                                                                                                                 | 156 |
| Table 5.2 Demographics of patients divided by dialysis dependence or independence at presentation. ....                                                                                       | 160 |
| Table 5.3 Comparison of renal and patient survival in patients who presented dialysis dependent with those who were dialysis independent at presentation.....                                 | 161 |
| Table 5.4 Comparison of the 146 patients who presented dialysis dependent at presentation, divided by those who remained dialysis dependent at one year, and those who came off dialysis..... | 173 |

|                                                                                                                                                                   |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| Table 5.5 Demographics of patients presenting after 2000 .....                                                                                                    | 179 |
| Table 5.6 Comparison of patients who presented dialysis dependent from 2000 who came<br>off dialysis at one year with those who remained dialysis dependent. .... | 182 |
| Table 6.1 Patients included by Histological Classification .....                                                                                                  | 204 |
| Table 6.2 Percentage positive staining.....                                                                                                                       | 216 |

## LIST OF ABBREVIATIONS

|        |                                      |
|--------|--------------------------------------|
| AAV    | ANCA Associated Vasculitis           |
| ANOVA  | Analysis of variance                 |
| APRIL  | A Proliferation Inducing Ligand      |
| ATG    | Anti-thymocyte globulin              |
| BAFF R | Baff receptor                        |
| BCMA   | B-cell maturation antigen            |
| BLYS   | B Lymphocyte stimulator              |
| BMEM   | B memory                             |
| BR3    | BlyS receptor 3                      |
| BREG   | B regulatory                         |
| BSA    | Bovine serum albumin                 |
| BVAS   | Birmingham Vasculitis Activity Score |
| CD19   | Cluster of differentiation 19        |
| CD20   | Cluster of differentiation 20        |
| CD21   | Cluster of differentiation 21        |
| CD22   | Cluster of differentiation 22        |

|       |                                                               |
|-------|---------------------------------------------------------------|
| CD38  | Cluster of differentiation 38                                 |
| CD40  | Cluster of differentiation 40                                 |
| CD52  | Cluster of differentiation 52                                 |
| CFA   | Complete Freund's Adjuvant                                    |
| CLL   | Chronic lymphocytic leukaemia                                 |
| CNS   | Central Nervous System                                        |
| CXCL1 | Chemokine ligand 1                                            |
| CYP   | Cyclophosphamide                                              |
| DCVAS | Diagnostic and Classification Criteria in<br>Vasculitis Study |
| dsDNA | Double stranded deoxyribonucleic acid                         |
| EAE   | Experimental autoimmune encephalomyelitis                     |
| EAV   | Experimental Autoimmune Vasculitis                            |
| eGFR  | Estimated GFR                                                 |
| EGPA  | Eosinophilic Granulomatosis with Polyangiitis                 |
| ELISA | Enzyme-linked immunosorbent assay                             |
| EM    | Electron Microscopy                                           |
| ENT   | Ear, Nose & Throat                                            |

|              |                                    |
|--------------|------------------------------------|
| ESRD         | End Stage Renal Disease            |
| EULAR        | European League Against Rheumatism |
| EUVAS        | European Vasculitis Society        |
| FDA          | Food and Drug Administration       |
| FFS          | Five Factor Score                  |
| GI           | Gastrointestinal                   |
| GPA          | Granulomatosis with Polyangiitis   |
| GWAS         | Genome-wide association study      |
| H&E          | Haematoxylin and eosin stain       |
| HRP          | Horseradish peroxidase             |
| IF           | Immunofluorescence                 |
| IFN $\gamma$ | Interferon gamma                   |
| IgA          | Immunoglobulin A                   |
| IgD          | Immunoglobulin D                   |
| IGS          | Immunoglobulins                    |
| IL-1         | Interleukin 1                      |
| IL-10        | Interleukin 10                     |
| IL-17        | Interleukin 17                     |

|       |                                       |
|-------|---------------------------------------|
| IL-2  | Interleukin 2                         |
| IL-23 | Interleukin 23                        |
| IQR   | Interquartile range                   |
| LM    | Light Microscopy                      |
| LPS   | Lipopolysaccharide                    |
| MAB   | Monoclonal antibody                   |
| MAPK  | Mitogen Activated Protein Kinase      |
| MDRD  | Modification of Diet in Renal Disease |
| MHC   | Major Histocompatibility Complex      |
| MMF   | Mycophenolate Mofetil                 |
| MPA   | Microscopic polyangiitis              |
| MPO   | Myeloperoxidase                       |
| MS    | Multiple sclerosis                    |
| MTX   | Methotrexate                          |
| MWU   | Mann-Whitney U                        |
| NETS  | Neutrophil Extracellular Traps        |
| NFKB  | Nuclear Factor Kappa B                |
| NOD   | Non obese diabetic                    |

|         |                                            |
|---------|--------------------------------------------|
| NRES    | National Research Ethics Service           |
| OMERACT | Outcome Measures in Rheumatology           |
| PAS     | Periodic acid-Schiff stain                 |
| PBS     | Phosphate buffered saline                  |
| PCP     | Pneumocystis Pneumonia                     |
| PCR     | Polymerase chain reaction                  |
| PEX     | Plasma Exchange                            |
| PML     | Progressive multifocal leukoencephalopathy |
| PR3     | Proteinase 3                               |
| PRTN3   | Proteinase 3                               |
| RLV     | Renal Limited Vasculitis                   |
| ROS     | Reactive oxygen species                    |
| RPGN    | Rapidly Progressive Glomerulonephritis     |
| RRT     | Renal replacement therapy                  |
| RTX     | Rituximab                                  |
| SCID    | Severe combined immunodeficiency           |
| SD      | Standard deviation                         |
| slgG    | Serum Immunoglobulin G                     |

|              |                                                                              |
|--------------|------------------------------------------------------------------------------|
| sIgM         | Serum immunoglobulin M                                                       |
| SLE          | Systemic lupus erythematosus                                                 |
| SYK          | Spleen Tyrosine Kinase                                                       |
| TACI         | Transmembrane activator and calcium-modulating cyclophilin ligand interactor |
| TBS          | Tris Buffered Saline                                                         |
| TLR4         | Toll Like Receptor 4                                                         |
| TLR9         | Toll Like Receptor 9                                                         |
| TNF          | Tumour Necrosis Factor                                                       |
| TNF          | Tumour necrosis factor                                                       |
| TNF $\alpha$ | Tumour necrosis factor alpha                                                 |
| TPMT         | Thiopurine Methyltransferase                                                 |
| Tregs        | Regulatory T cells                                                           |
| VDI          | Vasculitis Damage Index                                                      |
| WKY          | Wistar Kyoto                                                                 |

## PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS WORK

### PUBLICATIONS

#### **Effect of Treatment on Damage and Hospitalization in Elderly Patients with Microscopic Polyangiitis and Granulomatosis with Polyangiitis.**

M Weiner, S Goh, A Mohammad, Z Hrušková, **A Tanna**, **P Sharp**, **A Kang**, A Bruchfeld, D Selga, Z Chocová, K Westman, P Eriksson, L Harper, C Pusey, V Tesaf, AD Salama, M Segelmark.

The Journal of Rheumatology. April 2020, 47(4) 580-588.

#### **High Incidence of Arterial and Venous Thrombosis in Antineutrophil Cytoplasm Antibody-associated Vasculitis.**

A Kang, M Antonelou, N Wong, **A Tanna**, N Arulkumaran, FWK Tam, CD Pusey.

The Journal of Rheumatology Nov 2018.

#### **Long-term follow-up of a combined rituximab and cyclophosphamide regimen in renal anti-neutrophil cytoplasm antibody-associated vasculitis.**

SP McAdoo, N Medjeral-Thomas, S Gopaluni, **A Tanna**, N Mansfield, J Galliford, M Griffith, J Levy, TD Cairns, D Jayne, AD Salama, CD Pusey.

Nephrol Dial Transplant. May 2018; 1;33(5):899

**Increased Prevalence of Thyroid Disease in Patients with Antineutrophil  
Cytoplasmic Antibody-associated Vasculitis.**

M Predecki, L Martin, **A Tanna**, M Antonelou, CD Pusey.

J Rheumatol. 2018 May; 45(5):686-689. doi: 10.3899/jrheum.170661. Epub 2018  
Mar 15.

**The Histopathological Classification of ANCA-associated Glomerulonephritis  
Comes of Age.**

**A Tanna**, CD Pusey

J Rheumatol. 2017 Mar;44 (3):265-267.

**Patients double-seropositive for ANCA and anti-GBM antibodies have varied  
renal survival, frequency of relapse, and outcomes compared to single-  
seropositive patients**

SP McAdoo, **A Tanna**, Z Hrušková, L Holm, M Weiner, N Arulkumaran, A Kang, V  
Satrapová, J Levy, S Ohlsson, V Tesar, M Segelmark, CD Pusey.

Kidney Int. 2017 May 12

**Long-term Outcomes of Rituximab Therapy in Ocular Granulomatosis with  
Polyangiitis: Impact on Localized and Non-localized Disease.**

L Joshi, **A Tanna**, SP McAdoo, N Medjeral-Thomas, SR Taylor, G Sandhu, RM  
Tarzi, CD Pusey, S Lightman.

Ophthalmology. 2015 Jun; 122(6):1262-8.

**Clinical trials: Rituximab for maintenance of remission in AAV.**

**A Tanna**, C Pusey.

Nat Rev Nephrol. 2015 Jan 20

**Outcome and treatment of elderly patients with ANCA-associated vasculitis.**

M Weiner, SM Goh, AJ Mohammad, Z Hruskova, **A Tanna**, A Bruchfeld, D Selga, Z Chocova, K Westman, P Eriksson, CD Pusey, V Tesar, AD Salama, M Segelmark.

Clin J Am Soc Nephrol. 2015 Jul 7;10(7):1128-35.

**Necrotizing and crescentic glomerulonephritis presenting with preserved renal function in patients with underlying multisystem autoimmune disease: a retrospective case series.**

SP McAdoo, **A Tanna**, O Randone, FW Tam, RM Tarzi, JB Levy, M Griffith, L Lightstone, HT Cook, T Cairns, CD Pusey.

Rheumatology (Oxford). 2014 Nov 26

**Long-term outcome of anti-neutrophil cytoplasm antibody-associated glomerulonephritis: evaluation of the international histological classification and other prognostic factors.**

**A Tanna**, L Guarino, FW Tam, B Rodriguez-Cubillo, JB Levy, TD Cairns, M Griffith, RM Tarzi, B Caplin, AD Salama, T Cook, CD Pusey.

Nephrol Dial Transplant. 2014 Jul 12

**Regulatory B cells are numerically but not functionally deficient in anti-neutrophil cytoplasm antibody-associated vasculitis.**

SK Todd, RJ Pepper, J Draibe, **A Tanna**, CD Pusey, C Muri, AD Salama.

Rheumatology (Oxford). 2014 Sep; 53(9):1693-703

**Spleen tyrosine kinase inhibition attenuates autoantibody production and reverses experimental autoimmune GN.**

SP McAdoo, J Reynolds, G Bhangal, J Smith, JP McDaid, **A Tanna**, WD Jackson, ES Masuda, HT Cook, CD Pusey, FW Tam.

J Am Soc Nephrol. 2014 Oct; 25 (10):2291-302

**B-cell-targeted therapy in adult glomerulonephritis.**

**A Tanna**, FW Tam, CD Pusey.

Expert Opin Biol Ther. 2013 Dec; 13(12):1691-706

## CONFERENCE ABSTRACTS: ORAL COMMUNICATIONS

### **ANCA associated vasculitis is associated with a high rate of arterial and venous thrombosis**

A Kang, M Antonelou, **A Tanna**, N Arulkumaran, F Tam, C Pusey

17<sup>th</sup> International Vasculitis and ANCA workshop, Tokyo, 2017

### **Long term follow up of a combined rituximab and low dose cyclophosphamide regimen for remission induction in renal ANCA associated vasculitis**

S McAdoo, S Gopaluni, N Medjeral-Thomas, **A Tanna**, M Griffith, J Levy, T Cook, T Cairns, A Salama, D Jayne

17<sup>th</sup> International Vasculitis and ANCA workshop, Tokyo, 2017

### **Organ damage and hospitalization in elderly patients with ant-neutrophil cytoplasm antibody (ANCA) associated vasculitis**

M Weiner, S Goh, A Mohammed, Z Hruskova, **A Tanna**, P Sharp, A Kang, A Bruchfeld, D Selga, Z Chocova

17<sup>th</sup> International Vasculitis and ANCA workshop, Tokyo, 2017

### **Outcomes in ANCA associated vasculitis – A 40 year retrospective cohort**

N Arulkumaran, S McAdoo, A Kang, M Antonelou, **A Tanna**, S West, M Montomoli, G Gaskin, A Salama, C Pusey

17<sup>th</sup> International Vasculitis and ANCA workshop, Tokyo, 2017

**Double Positivity for ANCA and Anti-GBM Antibodies: Clinical Characteristics and Long-Term Outcomes of a Multi-Centre Cohort**

S McAdoo, **A Tanna**, S Ohlsson, Z Hruskova, M Weiner, J Levy, V Tesar, M Segelmark, C Pusey.

American Society of Nephrology 2015

**Clinical importance of serum BlyS levels in AAV**

**A Tanna**, F Tam, C Pusey

17th International Vasculitis and ANCA workshop, London 2015

**The effect of anti-BLyS antibody in experimental autoimmune vasculitis**

**A Tanna**, S McAdoo, G Bhangal, J McDaid, F Tam, C Pusey

17th International Vasculitis and ANCA workshop, London 2015

**Factors predictive of prognosis in renal AAV. A study of 104 patients in a single UK centre**

**A Tanna**, L Guarino, F Tam, T Cairns, M Griffith, R Tarzi, J Levy, A Salama, G Gaskin, T Cook, C Pusey

16th International Vasculitis and ANCA workshop, Paris, 2013

**Long term outcomes in patients with both ANCA and GBM positivity**

**A Tanna**, S McAdoo, F Tam, T Cairns, M Griffith, J Levy, A Salama, T Cook, C Pusey

16th International Vasculitis and ANCA workshop, Paris, 2013

**B Regulatory cells are numerically but not functionally impaired in AAV**

S Todd, R Pepper, **A Tanna**, C Mauri, A Salama

16th International Vasculitis and ANCA workshop, Paris, 2013

**Outcome and prognostic factors in elderly patients with ANCA associated vasculitis**

M Weiner, S Goh, A Mohammad, Z Hruskova, **A Tanna**, A Bruchfield, D Selga, Q Ko, K Westman, P Eriksson, C Pusey, V Tesar, A Salama, M Segelmark

16th International Vasculitis and ANCA workshop, Paris, 2013

**Long term outcomes in biopsy proven acute interstitial nephritis treated with steroids**

M Predecki, **A Tanna**, A Salama, T Cairns, F Tam, D Taube, T Cook, C Pusey

UK Renal Association Annual Conference 2013

## CONFERENCE ABSTRACTS: POSTER COMMUNICATIONS

### **Rituximab Treatment for Eosinophilic Granulomatosis with Polyangiitis**

K Ward, A Douglas, **A Tanna**, S McAdoo, C Pusey, PW Ind

British Thoracic Society, London, 2019

### **Plasmapheresis, Rituximab and Low-Dose Cyclophosphamide for Remission Induction Therapy in Severe ANCA Associated Vasculitis**

K Gulati, S McAdoo, **A Tanna**, J Levy, M Griffith, T Cairns, C Pusey

19th International Vasculitis and ANCA Workshop, Philadelphia, 2019

### **Long - Term Outcomes of Patients with ANCA Associated Vasculitis Presenting with Severe Renal Dysfunction.**

S McAdoo, **A Tanna**, A Kang, S Azzam, K Gulati, F tam, M Griffith, T Cairns, J Levy, C Pusey

19<sup>th</sup> International Vasculitis and ANCA Workshop, Philadelphia, 2019

**Long term outcomes of patients with ANCA associated vasculitis presenting with severe renal dysfunction.**

**A Tanna**, S McAdoo, A Kang, S Azzam, K Gulati, F Tam, T Cairns, M Griffith, J Levy, C Roufousse, T Cook, C Pusey.

UK Kidney Week, 2019

**Long term outcomes of patients with ANCA associated vasculitis presenting with severe renal dysfunction.**

**A Tanna**, S McAdoo, A Kang, S Azzam, K Gulati, F Tam, T Cairns, M Griffith, J Levy, C Roufousse, T Cook, C Pusey.

19<sup>th</sup> International Vasculitis and ANCA workshop 2019

**Organ Damage and Hospitalization in Elderly Patients with Anti-Neutrophil Cytoplasmic Antibody (ANCA) Associated Vasculitis**

M Weiner, SM Goh, AJ Mogammad, Z Hruskova, **A Tanna**, P Sharp, A Kang, A Bruchfield, D Selga, Z Chocova, K Westman, P Eriksson, L Harper, CD Pusey, V Tesar, AD Salama, M Segelmark

18<sup>th</sup> International Vasculitis and ANCA Workshop, Tokyo, 2018

**Increased Prevalence of Thyroid Disease in Patients with ANCA Associated Vasculitis**

M Prendecki, L Martin, **A Tanna**, M Antonelou, C Pusey.

American Society of Nephrology 2016

**Long-Term Follow-Up of a Combined Rituximab and Low-Dose Cyclophosphamide Regimen for Remission Induction in Renal ANCA Associated Vasculitis**

S McAdoo, N Medjeral-Thomas, **A Tanna**, M Griffith, J Levy, T Cook, T Cairns, A Salama, C. Pusey

American Society of Nephrology 2016

**High Incidence of Arterial and Venous Thrombosis in Patients with ANCA Associated Vasculitis**

A Kang, M Antonelou, **A Tanna**, N Arulkumaran, F Tam, C Pusey

American Society of Nephrology 2016

**Serum B Lymphocyte Stimulator (BlyS) Levels as Markers of Disease Activity in Anti-Neutrophil Cytoplasm Antibody (ANCA) Associated Vasculitis (AAV)**

**A Tanna**, S McAdoo, F Tam, C Pusey

American Society of Nephrology 2015

### **Rituximab and Low-Dose Cyclophosphamide Therapy for Renal ANCA**

#### **Associated Vasculitis: Long-Term Follow-Up**

N Medjeral-Thomas, N Mansfield, S McAdoo, **A Tanna**, M Griffith, J Levy, T Cairns, T Cook, A Salama, R Tarzi, C Pusey.

American Society of Nephrology 2015

### **Treatment of Experimental Autoimmune Vasculitis with a SYK Inhibitor**

S McAdoo, **A Tanna**, J McDaid, G Bhargal, S Esteban, H Masuda, T Cook, F Tam, C Pusey

American Society of Nephrology 2014

### **Spleen Tyrosine Kinase (SYK) Expression Correlates with Disease Activity and Outcome in Glomerulonephritis**

S McAdoo, G Bhargal, **A Tanna**, L Lightstone, T Cook, C Pusey, F Tam.

American Society of Nephrology 2013

### **Crescentic and Focal Necrotizing Glomerulonephritis in Patients with Normal Serum Creatinine**

S McAdoo, **A Tanna**, O Randone, J Levy, M Griffith, F Tam, T Cook, T Cairns, C Pusey.

American Society of Nephrology 2013

**Focal Necrotizing and Crescentic Glomerulonephritis in Patients with Normal Serum Creatinine**

S McAdoo, **A Tanna**, O Randone, M Griffith, T Cook, T Cairns, C Pusey

UK Renal Association Annual Conference 2013

**Evaluation of the International histopathological classification of antineutrophil cytoplasm antibody associated vasculitis (AAV) in 104 patients at a single centre.**

**A Tanna**, L Guarino, F Tam, T Cairns, M Griffiths, R Tarzi, A Salama, T Cook, C Pusey

UK Renal Association Annual Conference 2013

**An Association between thyroid disease and ANCA associated vasculitis**

M Predecki, **A Tanna**, E Clutterbuck, C Pusey

16th International Vasculitis and ANCA workshop, Paris, 2013

**Focal necrotising and crescentic glomerulonephritis in patients with normal serum creatinine**

S McAdoo, **A Tanna**, O Rathbone, M Griffith, T Cook, T Cairns, C Pusey

16th International Vasculitis and ANCA workshop, Paris, 2013

**Long term outcomes in patients with both ANCA and GBM positivity**

**A Tanna**, S McAdoo, F Tam, T Cairns, M Griffith, J Levy, A Salama, T Cook, C Pusey

American Society of Nephrology 2012

## CHAPTER ONE –INTRODUCTION AND BACKGROUND

## 1.1 INTRODUCTION TO AAV

### 1.1.1 CLASSIFICATION OF AAV

The anti-neutrophil cytoplasm antibody (ANCA) associated vasculitides (AAV), are multisystem disorders characterised by necrotising inflammation of small blood vessels (1). Patients with AAV can be classified by their clinical phenotype as having granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA) or renal limited vasculitis (RLV). The definitions for these conditions, as described in the most recent Chapel Hill Consensus Criteria, are listed in Table 1.1.

| <b><u>Definitions for ANCA-associated vasculitis</u></b> |                                                                                                                                                                                                                                                                                                                                               |
|----------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| ANCA-associated vasculitis (AAV)                         | Necrotising vasculitis, with few or no immune deposits, predominantly affecting small vessels i.e. capillaries, venules, arterioles and small arteries), associated with MPO-ANCA or PR3 ANCA. Not all patients have ANCA. Add a prefix indicating ANCA reactivity, e.g. PR3-ANCA, MPO-ANCA, ANCA-negative                                    |
| Granulomatosis with polyangiitis (GPA)                   | Necrotising granulomatous inflammation usually involving the upper and lower respiratory tract, and necrotising vasculitis affecting predominantly small to medium vessels (e.g. capillaries, venules, arterioles, arteries and veins). Necrotising glomerulonephritis is common.                                                             |
| Eosinophilic granulomatosis with polyangiitis (EGPA)     | Eosinophil-rich and necrotising granulomatous inflammation often involving the respiratory tract, and necrotising vasculitis predominantly affecting small to medium vessels and associated with eosinophilia and asthma.                                                                                                                     |
| Microscopic polyangiitis (MPA).                          | Necrotising vasculitis with few or no immune deposits, predominantly affecting small vessels (i.e. capillaries, venules or arterioles). Necrotising arteritis involving small and medium arteries may be present. Necrotising glomerulonephritis is very common. Pulmonary capillaritis often occurs. There is no granulomatous inflammation. |
| Single organ AAV (including renal limited vasculitis)    | Vasculitis in arteries or veins of any size in a single organ that has no features that indicate that it is a limited expression of a systemic vasculitis. Vasculitis distribution may be unifocal or multifocal (diffuse) within an organ.                                                                                                   |

**Table 1.1 Definitions for ANCA-associated vasculitis according to the Chapel Hill**

**Consensus Criteria 2012 – adapted from Jennette et al (1, 2).**

ANCA = antineutrophil cytoplasmic antibody; MPO=myeloperoxidase; PR3=proteinase 3

These conditions are usually associated with a circulating ANCA, and proteinase 3 (PR3) and myeloperoxidase (MPO) have been identified as the main target antigens. The association of pauci-immune crescentic glomerulonephritis with circulating

autoantibodies against antigens in neutrophil cytoplasm (ANCA) was initially reported in 1985 (3). There is growing evidence that MPO + and PR3 + disease are two distinct disease entities.

The most recent Chapel Hill Consensus Criteria for the classification of vasculitis suggests the addition of a patient's PR3 or MPO status as a prefix to their clinical phenotype. Data are currently being collected for over 6000 patients as part of Diagnostic and Classification in Vasculitis study, DCVAS, with the aim of updating the classification system (4).

### 1.1.2 EPIDEMIOLOGY AND AETIOLOGY

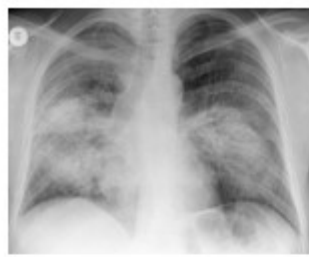
The incidence of AAV is considered to be 12-20 per million population per year in Europe and North America (5). There is no significant difference between the numbers of males and females affected, and there is rising incidence with age. Peak age of disease onset is between 60 and 70 years. There is marked geographical variation in disease patterns with a higher number of patients with GPA in northern Europe and Australia, and more patients in South East Asia with MPA (5). The incidence of GPA has been found to decrease with proximity to the equator (6). The epidemiology of AAV is difficult to ascertain, with less data being available from countries with limited access to ANCA testing and hence less diagnoses of AAV being made. In two recent studies carried out in multi-ethnic populations in France and USA, the incidence of GPA and MPA was twice as high in white populations than in other ethnic groups (7, 8). This was consistent with the findings of a recent UK study; however when adjusted for other factors, this was found to be primarily a consequence of the increased age of the white population (9).

The precise aetiology of AAV remains unclear and there is believed to be an environmental trigger in a genetically susceptible individual. The European Vasculitis Genetics Consortium reported the first genome-wide association study (GWAS) in AAV in 2012 (10). This study revealed both major histocompatibility complex (MHC) associations, for example the association of PR3 vasculitis with the HLA-DPB1\*04:01 allele (11), and non MHC associations. The GWAS identified GPA and MPA as being genetically distinct diseases (10). Sun exposure, vitamin D and exposure to silica (12) (13, 14) have been implicated as aetiological factors. In addition, several drugs have been associated with AAV, including penicillamine, minocycline and propylthiouracil, as well as levamisole contaminated cocaine (15). In addition, infections, including carriage of nasal Staph aureus, have been identified as risk factors for disease relapse (16). A double blinded trial of the use of trimethoprim in GPA patients resulted in a reduction in respiratory tract infections and relapses (17).

### 1.1.3 CLINICAL MANIFESTATIONS

Clinical manifestations are varied in terms of organ involvement and severity. These disorders are remitting, relapsing diseases with an untreated mortality of 80-90% (18). Diagnosis is often delayed and challenging as early symptoms are often non-specific, including generalised malaise, weight loss, fevers and polyarthropathy. GPA and MPA frequently present in a similar manner and are usually distinguished by the lack of granulomatous manifestations in MPA. Upper respiratory tract symptoms are more commonly seen in GPA than MPA and include ENT symptoms – nasal discharge, epistaxis, deafness, oral and nasal ulceration and cartilaginous involvement (collapse of the nasal bridge and subglottic stenosis). Forty- five to seventy percent of patients present with pulmonary manifestations. Up to eighty- five percent of patients with GPA

have pulmonary involvement at some stage. Pulmonary involvement ranges from nodules, pleural effusions and fibrotic changes, to pulmonary haemorrhage, which in itself is associated with poor outcomes. Renal manifestations, and in particular rapidly progressive glomerulonephritis (RPGN), are a major cause of both morbidity and mortality. RPGN results in end stage renal disease (ESRD) or death in 50% of AAV patients at 1 year (18). Renal manifestations include microscopic haematuria, proteinuria, hypertension, which occurs in 30% of patients, and a decline in estimated glomerular filtration rate (eGFR). Renal biopsy is the gold standard for diagnosis and there is classically crescentic glomerulonephritis on light microscopy (LM), with a paucity of immune deposits seen on immunofluorescence (IF) and electron microscopy (EM). Dermatological manifestations include rashes, purpura and oral ulceration. Neurological features include peripheral neuropathy and mononeuritis multiplex. Ocular manifestations are varied and include conjunctivitis, uveitis, episcleritis and orbital masses. Optic nerve vasculitis and renal artery thrombosis are rare but important complications (19). Vasculitic gastrointestinal manifestations can include haemorrhagic ulceration of the bowel and bloody diarrhoea, and can result in bowel perforation. Cardiac manifestations can be severe and include pericarditis and vasculitis of coronary vessels. The clinical manifestations of AAV are summarised in Figure 1.1. Disease severity has been defined according to European League Against Rheumatism (EULAR) guidelines, which are listed in Table 1.2.



(a)



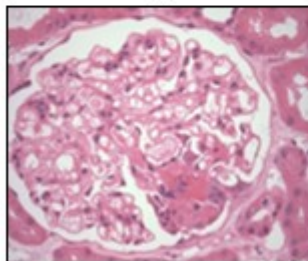
(b)



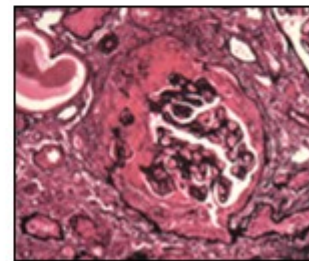
(c)



(d)



(e)



(f)

**Figure 1.1 Examples of clinical manifestations of AAV.**

(a) Xray demonstrating pulmonary haemorrhage and (b) CT scan demonstrating pulmonary haemorrhage Figure (c) Xray demonstrating pulmonary cavitation and (d) CT scan showing pulmonary cavitation. Figures (e) and (f) crescentic glomerulonephritis seen on renal biopsy.

|                       | Definition                                                                                                     |
|-----------------------|----------------------------------------------------------------------------------------------------------------|
| <b>Localised</b>      | Upper and/or lower respiratory tract disease without any other systemic involvement or constitutional symptoms |
| <b>Early Systemic</b> | Any symptoms without organ threatening or life- threatening disease                                            |
| <b>Generalised</b>    | Renal or other organ threatening disease, serum creatinine <500 µmol/l                                         |
| <b>Severe</b>         | Renal or other vital organ failure, serum creatinine > 500µmol/l                                               |
| <b>Refractory</b>     | Progressive disease unresponsive to glucocorticoids and cyclophosphamide                                       |

**Table 1.2: Definitions of disease severity according to EULAR (20)**

#### **1.1.4 ASSESSMENT OF DISEASE ACTIVITY AND DAMAGE**

Both the BVAS and VDI scores have been approved by EULAR and the Outcome Measures in Rheumatology (OMERACT) group for the recording of disease activity and damage in clinical trials (21).

##### *1.1.4.1 Birmingham vasculitis activity score (BVAS)*

Disease activity can be measured using the BVAS score. This is a validated tool to measure disease activity which has been used in international studies and trials. The physician scores the features deemed to be a result of active vasculitis, and a score between 0 and 68 is obtained. This score can be used to monitor disease progression

and response to treatment (22). A BVAS score of 0 is representative of remission and  $\geq 1$  active disease. An example of a BVAS scoring sheet is shown in Figure 1.2.

**Birmingham Vasculitis Activity Score (version 3)**

Patient ID: \_\_\_\_\_ Date of birth: \_\_\_\_\_ Total score: \_\_\_\_\_

Assessor: \_\_\_\_\_ Date of assessment: \_\_\_\_\_

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |                                                                 |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------|
| Tick an item <b>only</b> if attributable to active vasculitis. If there are no abnormalities in a section, please tick 'None' for that organ-system.                                                                                                                                                                                                                                                                                                                                                                                                                   |                                                                 | If <b>all</b> abnormalities are due to persistent disease (active vasculitis which is not new/worse in the prior 4 weeks), tick the <b>PERSISTENT</b> box at the bottom right corner                                                                                                                                                                                                                                                                                                                                                                       |                                                                 |
| <b>Is this the patient's first assessment?</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                                                                 | <b>Yes</b> <input type="radio"/>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | <b>No</b> <input type="radio"/>                                 |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | None <input type="radio"/> Active disease <input type="radio"/> |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | None <input type="radio"/> Active disease <input type="radio"/> |
| <b>1. General</b> <input type="radio"/><br>Myalgia <input type="radio"/><br>Arthralgia / arthritis <input type="radio"/><br>Fever $\geq 38^{\circ}\text{C}$ <input type="radio"/><br>Weight loss $\geq 2$ kg <input type="radio"/>                                                                                                                                                                                                                                                                                                                                     |                                                                 | <b>6. Cardiovascular</b> <input type="radio"/><br>Loss of pulses <input type="radio"/><br>Valvular heart disease <input type="radio"/><br>Pericarditis <input type="radio"/><br>Ischaemic cardiac pain <input type="radio"/><br>Cardiomyopathy <input type="radio"/><br>Congestive cardiac failure <input type="radio"/>                                                                                                                                                                                                                                   |                                                                 |
| <b>2. Cutaneous</b> <input type="radio"/><br>Infarct <input type="radio"/><br>Purpura <input type="radio"/><br>Ulcer <input type="radio"/><br>Gangrene <input type="radio"/><br>Other skin vasculitis <input type="radio"/>                                                                                                                                                                                                                                                                                                                                            |                                                                 | <b>7. Abdominal</b> <input type="radio"/><br>Peritonitis <input type="radio"/><br>Bloody diarrhoea <input type="radio"/><br>Ischaemic abdominal pain <input type="radio"/>                                                                                                                                                                                                                                                                                                                                                                                 |                                                                 |
| <b>3. Mucous membranes / eyes</b> <input type="radio"/><br>Mouth ulcers <input type="radio"/><br>Genital ulcers <input type="radio"/><br>Adnexal inflammation <input type="radio"/><br>Significant proptosis <input type="radio"/><br>Scleritis / Episcleritis <input type="radio"/><br>Conjunctivitis / Blepharitis / Keratitis <input type="radio"/><br>Blurred vision <input type="radio"/><br>Sudden visual loss <input type="radio"/><br>Uveitis <input type="radio"/><br>Retinal changes (vasculitis / thrombosis / exudate / haemorrhage) <input type="radio"/> |                                                                 | <b>8. Renal</b> <input type="radio"/><br>Hypertension <input type="radio"/><br>Proteinuria $>1+$ <input type="radio"/><br>Haematuria $\geq 10$ RBCs/hpf <input type="radio"/><br>Serum creatinine 125-249 $\mu\text{mol/L}^*$ <input type="radio"/><br>Serum creatinine 250-499 $\mu\text{mol/L}^*$ <input type="radio"/><br>Serum creatinine $\geq 500$ $\mu\text{mol/L}^*$ <input type="radio"/><br>Rise in serum creatinine $>30\%$ or fall in creatinine clearance $>25\%$ <input type="radio"/><br><b>*Can only be scored on the first assessment</b> |                                                                 |
| <b>4. ENT</b> <input type="radio"/><br>Bloody nasal discharge / crusts / ulcers / granulomata <input type="radio"/><br>Paranasal sinus involvement <input type="radio"/><br>Subglottic stenosis <input type="radio"/><br>Conductive hearing loss <input type="radio"/><br>Sensorineural hearing loss <input type="radio"/>                                                                                                                                                                                                                                             |                                                                 | <b>9. Nervous system</b> <input type="radio"/><br>Headache <input type="radio"/><br>Meningitis <input type="radio"/><br>Organic confusion <input type="radio"/><br>Seizures (not hypertensive) <input type="radio"/><br>Cerebrovascular accident <input type="radio"/><br>Spinal cord lesion <input type="radio"/><br>Cranial nerve palsy <input type="radio"/><br>Sensory peripheral neuropathy <input type="radio"/><br>Mononeuritis multiplex <input type="radio"/>                                                                                     |                                                                 |
| <b>5. Chest</b> <input type="radio"/><br>Wheeze <input type="radio"/><br>Nodules or cavities <input type="radio"/><br>Pleural effusion / pleurisy <input type="radio"/><br>Infiltrate <input type="radio"/><br>Endobronchial involvement <input type="radio"/><br>Massive haemoptysis / alveolar haemorrhage <input type="radio"/><br>Respiratory failure <input type="radio"/>                                                                                                                                                                                        |                                                                 | <b>10. Other</b> <input type="radio"/><br>a. <input type="radio"/><br>b. <input type="radio"/><br>c. <input type="radio"/><br>d. <input type="radio"/>                                                                                                                                                                                                                                                                                                                                                                                                     |                                                                 |
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |                                                                 | <b>PERSISTENT DISEASE ONLY:</b><br>(Tick here if <b>all</b> the abnormalities are due to persistent disease) <input type="checkbox"/>                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                 |

Figure 1.2: Example of a scoring sheet for assessing BVAS.

From Mukhytar et al, 2008 (22)

#### *1.1.4.2: The five factor score*

The five factor score (FFS) was initially devised in 1996 and included the five factors, serum creatinine, proteinuria, cardiomyopathy, gastrointestinal (GI) involvement and central nervous system (CNS) involvement. It was validated for MPA and EGPA but not GPA (23). This score was revised in 2009 but has not been extensively validated (24).

#### *1.1.4.3: The VDI score*

The Vasculitis Damage Index (VDI) is a score used to assess chronic damage caused by the disease itself and treatment. There are ten systems included in this score, skin, mucous membranes, ocular, ENT, cardiovascular, pulmonary, gastrointestinal, renal, neuropsychiatric and musculoskeletal. A further eleventh category is included for involvement of additional systems.

## **1.2 PATHOGENESIS OF AAV**

The pathogenesis of AAV has yet to be fully elucidated and both cellular and humoral immune responses have been implicated.

### **1.2.1 ANCA**

There is evidence both from human studies and in vitro work, that ANCA play an important role in pathogenesis of disease. In vitro work with MPO and PR3 ANCA has demonstrated their ability to activate cytokine-primed leucocytes and cause damage to endothelial cells in culture (25, 26). A study in which rolling neutrophils were treated with ANCA-IgG in flow conditions, using chambers lined with activated platelets or endothelial cells, has been shown to cause adhesion and transmigration of neutrophils (27). This study therefore suggested a role for ANCA in the causation of vascular

inflammation and damage. A number of further experimental studies have implicated ANCA in the pathogenesis of tissue damage, and suggested possible mechanisms for this pathogenicity. One such mechanism includes the phosphorylation of spleen tyrosine kinase (SYK)(28). Upregulation of SYK expression has been demonstrated in both inflamed pulmonary and renal tissue. The SYK inhibitor Fostamycin has been found to reduced ANCA induced damage in rat leucocytes in vitro and has also been proved effective in vivo (29).

A clear role for ANCA in pathogenesis has also been demonstrated using animal models. Xiao *et al*, demonstrated a role for ANCA in pathogenicity, using a passive transfer mouse model of AAV. This model involved the immunisation of MPO knock-out mice with purified murine MPO, and the transfer of splenocytes or purified antibodies from these animals, into RAG deficient or wild type mice. Transfer of the purified antibodies resulted in development of a pauci-immune glomerulonephritis and, in some cases, pulmonary haemorrhage (30). Further evidence for the pathogenic role of ANCA has been obtained using the autoimmune rat model of AAV, experimental autoimmune vasculitis (EAV) developed by Little *et al* (31). This model involves the immunisation of Wistar Kyoto (WKY) rats, a strain with known susceptibility to glomerulonephritis, with human MPO in Complete Freund's Adjuvant (CFA). Anti-MPO antibodies are produced and animals develop crescentic glomerulonephritis, some with pulmonary capillaritis. Anti-human MPO antibodies have been shown to react with rat MPO which exhibits significant homology to human MPO. Using intravital microscopy, this model has demonstrated increased leucocyte endothelial interaction in response to CXCL1 in the presence of ANCA-IgG. This finding is also present in healthy WKY rats when infused with anti-MPO antibodies isolated from rats with disease (32).

In terms of evidence for the pathogenesis of ANCA in humans, Bansal *et al* reported a case of transplacental transfer of MPO-ANCA from a mother to her neonate at 33 weeks gestation. The neonate was found to have raised MPO titres and developed pulmonary haemorrhage and renal involvement (33). A further case of maternal transfer of MPO was described by Silva *et al* in 2009. In this case, a Mother with MPA had high titres of MPO but remained in clinical remission. Her baby was found to have high MPO levels but was clinically well with no clinical manifestations of vasculitis (34). This finding suggests that additional factors are needed in conjunction with the antibody to cause clinical manifestation in humans. From clinical studies, there is evidence that ANCA target specificity correlates with disease phenotype (35). Some studies have also demonstrated that ANCA titres correlate with disease severity and have reported the association between increasing titres and relapse (36-39). In patients, the successful use of B cell depletional therapies and improved outcomes following antibody removal by plasma exchange, gives further support to the role of ANCA in pathogenesis of disease (40, 41) (42).

As mentioned previously, Lyons *et al* described a genome wide association study (GWAS) which identified that there were stronger genetic associations for patients with AAV with ANCA specificity (PR3 or MPO), than with the clinical syndromes GPA or MPA (10). Anti-PR3 vasculitis has been demonstrated to be strongly associated with the gene coding proteinase 3 (PRTN3, the autoantigen), alpha 1 antitrypsin (SERPINA1; the circulating inhibitor of the autoantigen), and HLA DP. This gives further evidence for the pathogenicity of ANCA itself. Despite this evidence however, ANCA are not always pathogenic or representative of disease activity. Clinically, there are patients in whom ANCA titres remain elevated during clinical remission and patients who experience relapses in the absence of ANCA. Anti-MPO antibodies

have been identified in healthy individuals but in lower quantity and of lower avidity than in patients (43). Research continues into the features which make certain ANCAs pathogenic and includes investigation of different subclasses of ANCA and disease causing epitopes. One hypothesis is that ANCA become pathogenic by epitope spreading which involves an increase in the number of epitopes exposed to antibody (44) .

### 1.2.2 NEUTROPHILS

The role of the neutrophil as a major component in the pathogenesis of AAV was described by Falk *et al* in 1990 (45). In inflammatory and infective conditions, neutrophils become primed in the presence of pro-inflammatory cytokines such as tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interleukin 1 (IL-1). During this priming, intracellular granules containing MPO and PR3 move to the surface of the cells where they are accessible for binding with ANCA. Binding of ANCA results in activation of the neutrophil, with degranulation, release of adhesion molecules and binding to the endothelium. There is also release of reactive oxygen species and proteases which contribute to the endothelial damage (46). Activated neutrophils may also undergo a process called NETosis, a form of cell death. This involves the release of neutrophil extracellular traps (NETS), chromatin webs containing PR3, MPO and complement. These NETS can directly damage the endothelial surface and also play a role in antigen presentation to dendritic cells and the activation of complement (47). Autoreactive T cells and monocytes are also recruited to the site of injury and exacerbate this further (Figure 1.3).

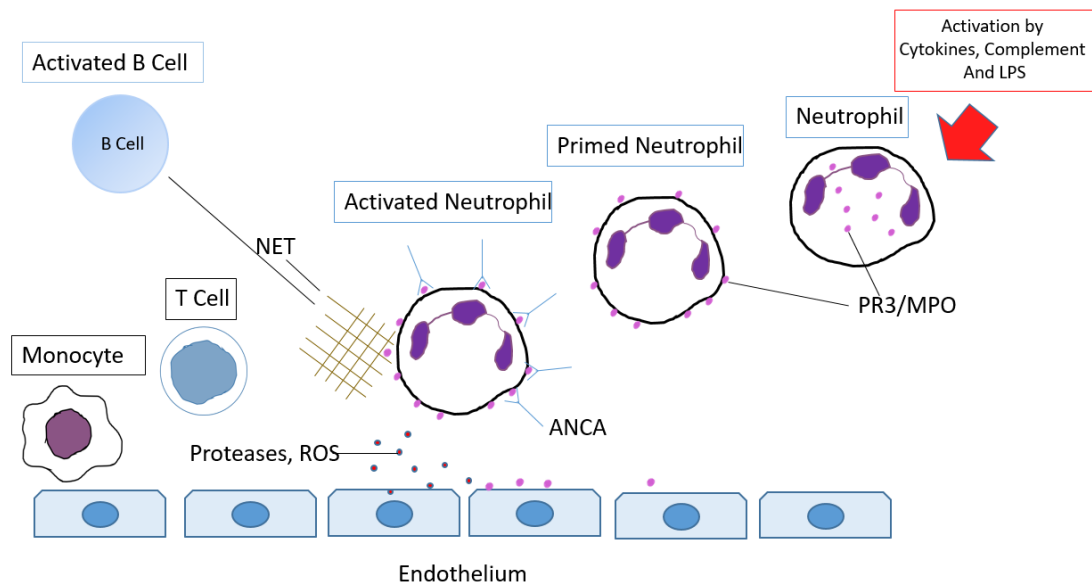


**Figure 1.3 Pathogenesis of ANCA associated vasculitis**

Adapted from Geetha et al – vasculitis core curriculum 2020 (48) . Stimulation of neutrophils by cytokines, complement and LPS results in neutrophil priming and the expression of PR3/MPO on the cell surface. ANCA binding results in neutrophil activation, with consequent degranulation and adherence to the vascular endothelium. This, in addition to the release of proteases, reactive oxygen species (ROS) and neutrophil extracellular traps (NETS), results in endothelial damage. There is activation of B cells by NETS and recruitment of autoreactive T cells and monocytes to the site of endothelial injury in response to chemokines and the tissue deposition of PR3 and MPO. This creates further tissue damage.

The binding of ANCA to endothelial surfaces also activates intracellular pathways which cause conformational change and altered adhesion molecule expression. These changes promote adhesion of neutrophils to the endothelial surface, and transmigration (49).

### 1.2.3 COMPLEMENT

Animal studies and in particular the MPO passive transfer mouse model, have identified a role for complement in the pathogenesis of AAV. Anti-MPO antibodies transferred to mice deficient in C5 or the C5a receptor did not cause disease. C4

deficiency did not however protect from disease. This demonstrates the alternative complement pathway as being contributory to disease (50). The C5a receptor, in particular, has been implicated.

In AAV patients, a phase II trial found the C5a receptor antagonist avacopan to be successful in the induction of remission of AAV when used in place of high dose steroids, alongside cyclophosphamide (CYP) or rituximab (RTX) (51). The results of Avacopan Development in Vasculitis to Obtain Corticosteroid Elimination And Therapeutic Efficacy study (ADVOCATE) are awaited. This study is a large randomised multicentre controlled trial aiming to include 300 patients, evaluating the safety and efficacy of avacopan vs prednisolone for the induction of remission in AAV, when used in combination with other immunosuppressive therapy (Clinical trials NCT02994927) (52).

#### 1.2.4 T CELLS

Abnormalities in T cells have been shown to contribute to pathogenesis in AAV. The main mechanism by which this occurs is thought to be through their disordered regulatory capacity. A study in patients with GPA has identified functionally impaired regulatory T cells (Tregs) (53). This finding was not replicated in MPO ANCA positive patients (54). In 2013, Bunch *et al* described a suppressor resistant CD4<sup>+</sup> effector T cell population responsible for the production of pro-inflammatory cytokines (55). Serum levels of IL-23 and IL-17 have been shown to be elevated in patients with acute AAV (56). IL-23 induces differentiation of these T cells into the TH17 subset leading

to production of IL-17. The correlation with serum ANCA titres suggests a role for IL-23 in the production of ANCA (48).

### 1.2.5 B CELLS

The role of the B cell in the pathogenesis of AAV is now well established and use of the anti- CD20 B cell depletion agent rituximab is widespread, in both the induction and maintenance phases of treatment (40, 41).

There are a number of mechanisms by which B lymphocytes potentially contribute to the pathogenesis of AAV. These include the production of ANCA, antigen presentation to T cells, and the production of pro –inflammatory cytokines (57). In the murine disease model described previously, in which anti-MPO antibodies were transferred and caused disease, a direct role for B cells was also suggested as splenic B cells were also able to transfer disease (30). Clinically, B cell clusters have been identified in the kidneys of AAV patients and the nasal mucosa of GPA patients (66).

Subsets of B cells capable of inhibiting inflammatory responses and inducing immune tolerance have been identified in both humans and rodents, and termed regulatory B cells (Bregs) (58). The balance between these regulatory B cells and effector B cells is a factor which contributes to the disease process in AAV. One such population of B cells, found to act in a regulatory manner, are of the phenotype CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup> (59). These cells have been shown to suppress T cell proliferation and production of pro-inflammatory cytokines *in vitro*, by both IL-10 related mechanisms, and through direct T cell contact (60). Todd *et al* investigated the balance between the B cell subsets Breg (CD24<sup>hi</sup>CD38<sup>hi</sup>), B memory (Bmem, CD24<sup>hi</sup>CD38<sup>lo</sup>) and naive B cells (Bnaive, CD24<sup>int</sup>CD38<sup>int</sup>) in AAV patients with active disease and in remission (59). In

this study, the frequency of Breg (CD24<sup>hi</sup>CD38<sup>hi</sup>) was significantly reduced in patients with PR3 and MPO positive vasculitis in remission, compared with healthy controls. Their frequency was also reduced in acute disease in PR3 positive patients. In contrast, the number of memory cells (CD24<sup>hi</sup>CD38<sup>lo</sup>) was reduced during active disease and maintained in remission. These findings suggest that, in remission, there is a numerical but not functional deficiency in the Breg population, and that this may contribute to the propensity to relapse, particularly in PR3 AAV patients (59). The Bmem population was found to be relatively preserved in remission, and reduced in active disease. The number of memory and naïve B cells were found to inversely correlate with IL10 production whilst there was a positive correlation with Bregs. This imbalance between the memory and naïve B cells with the Breg population is likely to contribute to the risk of relapsing disease. CD5+ B cells are a further B cell population which have been identified as having regulatory properties. It has been reported that, following B cell depletion, patients who re-populate their B cells with a lower proportion of CD5+ cells experience a shorter time to relapse (61). Therefore, understanding the precise role of different B cell subsets during AAV disease evolution, and following RTX or CYP therapy, is critical to understanding how best to use B cell depletion or inhibitory therapy, and to develop novel strategies of enhancing Breg production and function whilst diminishing the pro-inflammatory effects of effector B cells.

#### *1.2.5.1: B Cell survival factors – BlyS and APRIL*

In addition to the presence of different B cell subsets in patients with AAV, studies have also reported a difference in B cell survival factors. B-Lymphocyte stimulator (BlyS) and A proliferation inducing ligand (APRIL) are two such survival factors, which form part of the tumour necrosis factor (TNF) ligand superfamily. These cytokines

are produced by a variety of cell types including myeloid cells, activated lymphocytes, epithelial cells, stromal cells and some neoplastic cells. Production is stimulated by type I interferons, IL-10 and interferon gamma (IFN gamma). Activation of TLR4 and TLR9 also stimulates their production (62). In addition to these molecules, ANCA has been shown to stimulate the production of BlyS (63). BlyS is produced as a 285 amino acid membrane bound protein which becomes active as its soluble form, on cleavage by furin proteases. A splice variant of BlyS has also been identified and named delta BlyS, and has been shown to downregulate the secretion of functional BlyS (64). APRIL shares some homology with BlyS and is also activated following cleavage to its soluble form. BlyS and APRIL bind to three receptors found on B cells of varying developmental stages. These receptors are BR3 (BAFF R), transmembrane activator and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA). BlyS binds to all three receptors but with the highest affinity to BR3, and APRIL binds only to TACI and BCMA. BCMA is primarily expressed on mature plasma cells.

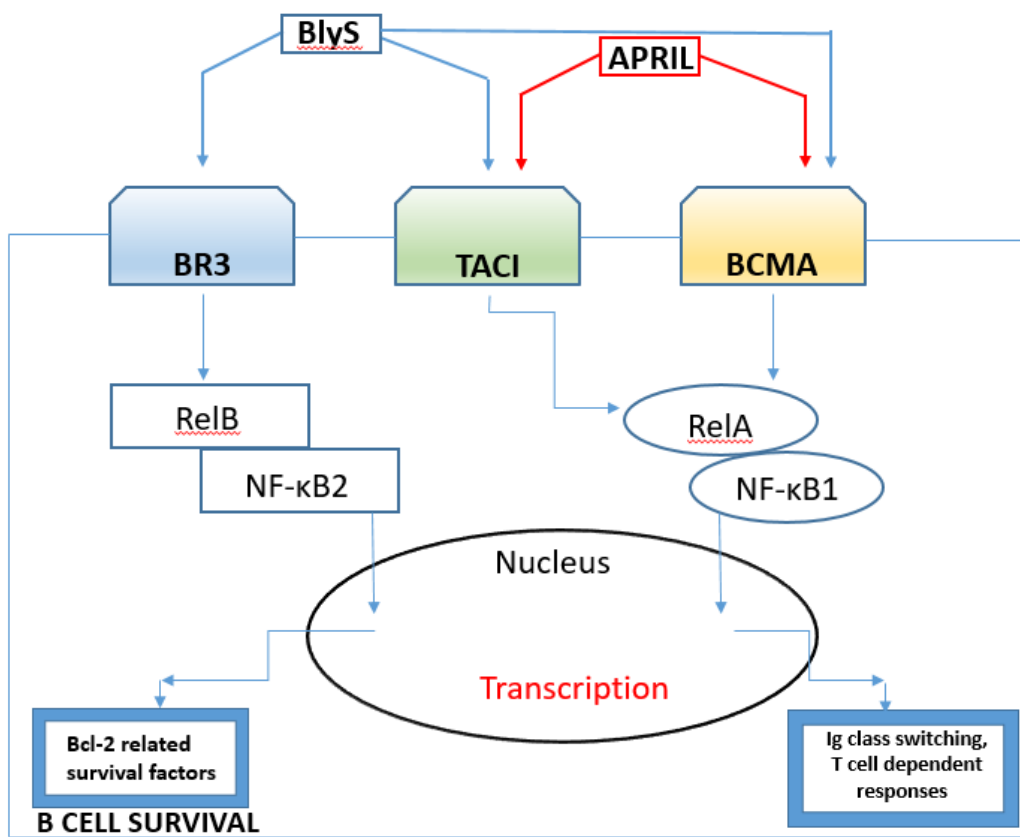


**Figure 1.4 – The interaction of BlyS and APRIL with their receptors.**

Adapted from Cancro et al: The Journal of Clinical Investigation 2009(65). BlyS binds to all three of the receptors, BR3, TACI and BCMA whereas APRIL binds to TACI and BCMA but not BR3. Receptor binding results in the transcription of anti-apoptotic transcription factors and Ig Class switching via the NF-κB pathway.

Binding to these receptors stimulates production of anti-apoptotic proteins via the NFκB and MAPK pathway, and hence the promotion of B cell survival at various stages of B cell development. This is depicted in Figure 1.4.

BlyS binding to BAFFR is essential for the survival of early B cells and in particular their development from immature T1 B cells to immature T2 B cells in the spleen. BlyS deficient mice have been shown to have minimal B cells beyond the T1 stage.

Patients with BAFFR deficiency have been demonstrated to present with adult onset antibody deficiency (66). BlyS is also required at a number of other points during B cell maturation. These include germinal centre reactions during which centroblasts develop into centrocytes, and in the production of high affinity plasmablasts. The binding of BlyS and APRIL to BCMA is essential for the differentiation of plasma cells in the bone marrow, and animals deficient in BCMA have been shown to maintain normal immune responses but experience impaired survival of plasma cells (67). The precise functions and interactions of the three receptors have yet to be fully elucidated. It has been suggested that TACI is a negative regulator of B cells, as TACI deficient mice have been shown to have increased numbers of B cells and immunoglobulins (68). Figure 1.5 demonstrates the stages of B cell development. BAFFR is expressed on the mature B cells and memory B cells, TACI on both plasma cells and memory B cells, and BCMA on plasma cells.

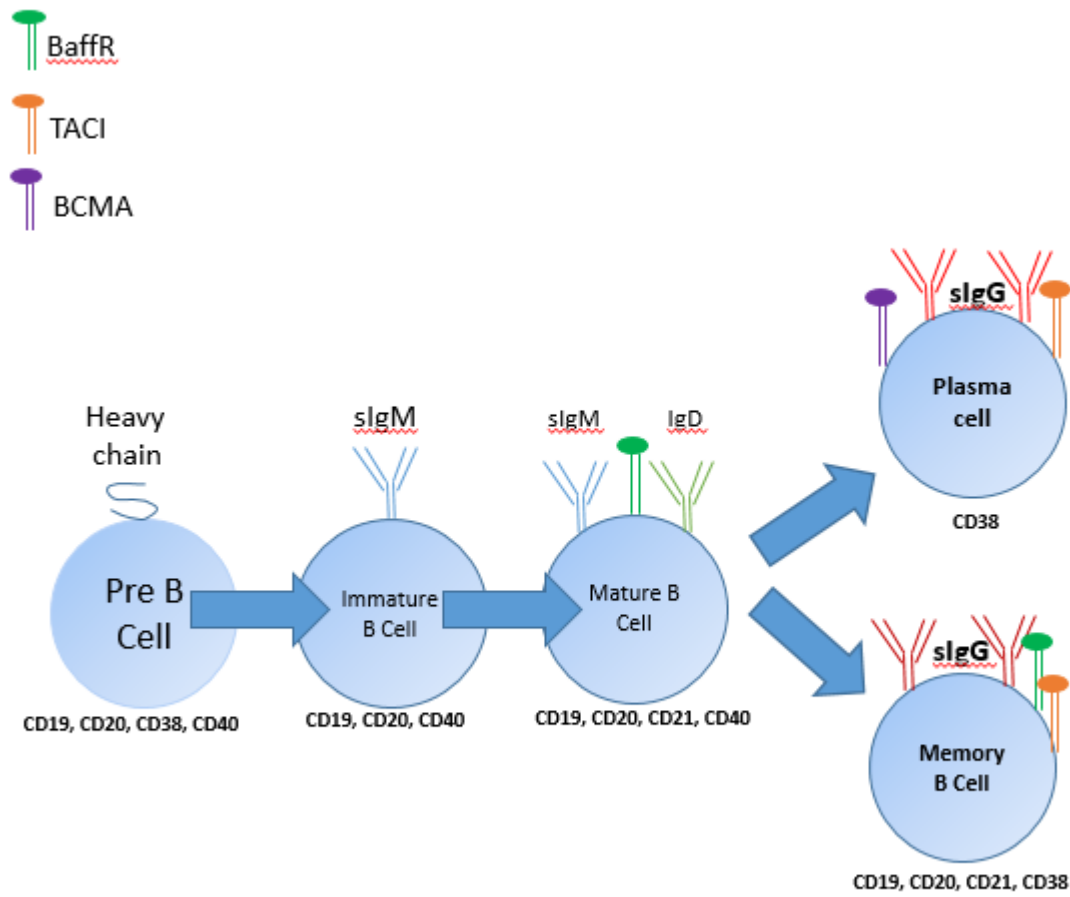


**Figure 1.5: Stages of B cell development and B cell survival factors.**

BAFFR is expressed on mature B cells and memory B cells, TACI on both plasma cells and memory B cells, and BCMA on plasma cells.

In addition to ensuring B cell differentiation and survival, the balance of B cell survival factors is important in preventing autoimmunity. The T1/T2 transition point for B cells, which is known to be BlyS dependent, also functions as an important checkpoint for deletion of autoreactive B cells. Increased concentrations of BlyS have been shown to enable these autoreactive B cells to escape deletion (69). Mackay *et al* developed a transgenic mouse with over expression of BlyS (70). This mouse demonstrated signs of autoimmunity representative of human autoimmune diseases and, in particular,

systemic lupus erythematosus (SLE). An increase in the proportion of mature B and effector T cells was observed as well as the production of autoantibodies such as anti-double stranded DNA (dsDNA). This led to further studies into the role of BlyS in autoimmune disease and its therapeutic potential (70).

Serum BlyS levels have been shown to be elevated in many autoimmune diseases including SLE, Sjogren's disease and rheumatoid arthritis (RA) (71). Studies have revealed an increase in serum levels of BlyS in patients with GPA compared with healthy controls (72, 73). Patient numbers in these studies were however relatively small and there is discrepancy as to the correlation with ANCA titres. A study investigating the association between BlyS levels and disease activity in 37 patients with MPO positive vasculitis, revealed that patients with active MPO-ANCA vasculitis had significantly higher levels of BlyS when compared with controls. An inverse correlation was made with ANCA titres but there was no correlation with serum APRIL levels (74). Holden *et al.* reported that ANCA stimulated neutrophils release BlyS and also confirmed that serum BlyS levels increase in patients with active disease. Serum BlyS levels also increased 3 months following therapy with Rituximab (63).

These studies in mice and humans implicate BlyS in autoimmunity. BlyS has been shown to effect both the extra-follicular and germinal centre pathways of B cell development. Ectopic germinal centres (tertiary lymphoid organs) have been identified in non-lymphoid tissues in a number of autoimmune conditions (75). Both BlyS and APRIL and their receptors have been identified by immunohistochemistry in the nasal mucosa of patients with GPA (76). The influence of these survival factors may be implicated in the autoimmune tendencies of B cells found in these tissues.

As mentioned above, ANCA itself can stimulate the production of B cell survival factors. The pathogenicity of neutrophils in AAV is well established and neutrophils, particularly when stimulated with ANCA, have been shown to express BlyS (77). It has been proposed that ANCA stimulated neutrophils may release B cell survival factors and that this could explain the elevated levels of Blys seen in AAV patients compared with controls (62). BlyS and APRIL have also been detected in granulomas, along with activated B cells, in patients with AAV (76). These findings therefore implicate BlyS in the pathogenesis of AAV. There is a potential role for its use in monitoring of the disease and as a marker of impending relapse. The effect of therapy and, in particular, B cell depletional agents, needs to be fully investigated as a rise in BlyS levels as a result of such therapy could increase the risk of relapse. It is thought that B cell depletion results in a higher level of B cell survival factors and an environment which favours the survival of autoimmune cells. Together these findings suggest a therapeutic role for BlyS inhibition in AAV.

#### 1.2.6 MONOCYTES AND MACROPHAGES:

Monocytes have also been implicated in the pathogenesis of AAV. Stimulation of monocytes with ANCA in vitro has been shown to result in the production of cytokines and generation of reactive oxygen species (ROS) (78, 79). Monocytes from patients with active AAV have been shown to contain higher levels of the LPS receptor CD14, which is suggestive of cell activation, than patients in remission and healthy controls (80). Increased levels of cell surface markers required for leucocyte-endothelial interaction, have also been found on circulating monocytes from patients with active

AAV (81). Monocytes and macrophages have been identified as being the predominant cell in renal biopsies from patients with AAV (82, 83).

### 1.3 ANIMAL MODELS OF AAV

There have been multiple attempts to establish reliable, reproducible animal models of AAV in order to further understanding of pathophysiology and to evaluate therapy. Both rat and mouse models have been used with varying degrees of success. Due to significant differences between human and rodent PR3, the majority of animal models use MPO (84).

#### 1.3.1 MOUSE MODELS

In 2002, Xiao *et al.* developed a passive transfer mouse model of AAV (30). This model involved the immunisation of MPO knock-out mice with purified murine MPO, and the transfer of splenocytes or purified antibodies from these animals into RAG deficient or wild type mice. Transfer of the purified antibodies resulted in a pauci-immune glomerulonephritis at between 6 and 13 days and, in some cases, pulmonary haemorrhage. This model demonstrated the pathogenicity of the neutrophil in AAV, in addition to the involvement of B cells (22). The addition of lipopolysaccharide (LPS) has been shown to augment the response to MPO in a manner analogous to infective triggers of autoimmunity in humans (85). This model has also demonstrated varying degrees of susceptibility to disease in mice of different genetic backgrounds, and exacerbation of disease in mice deficient in the inhibitory FcγII receptor (86). The alternative complement pathway has been implicated using this model, an unexpected finding given the paucity of complement consumption in human disease (87). Transgenic mice with human C5a receptors demonstrated the efficacy of anti-C5a agents in reducing renal damage (85). These findings have now been translated to

human clinical trials and demonstrate the importance of this murine model. Such a passive transfer model is not however truly representative of autoimmunity. In an attempt to induce autoantibodies, C57Bl/6 mice were immunised with both human and murine MPO with adjuvant by Ruth *et al.* in 2006 (88). This model required a boost of subnephritogenic nephrotoxic serum in order to localise inflammation to the glomerulus and was found to be T cell dependent (in contrast to previous studies), and dependent on IL-17 (89). Attempts to produce successful rodent models of anti-PR3 vasculitis continue. In contrast to MPO, human and mouse PR3 have limited homology of 68% (90). In addition, murine PR3 does not contain the antigenic determinants of human PR3. These findings make it more challenging to produce a rodent model of anti-PR3 vasculitis. Little *et al.* appear to have developed the most successful current model, using humanised NOD SCID IL-2 receptor knockout mice (91). These mice developed mild renal and pulmonary damage following transfer of anti-PR3 antibodies from patients with severe AAV.

### 1.3.2 RAT MODELS

The Wistar Kyoto (WKY) strain of rat has demonstrated extreme susceptibility to glomerulonephritis (92). The experimental autoimmune vasculitis (EAV) model developed in our laboratory by Little *et al.* involves the immunisation of female WKY rats with human MPO in complete Freund's adjuvant (CFA) (31). Anti-MPO antibodies are produced and animals develop crescentic glomerulonephritis, some with pulmonary capillaritis. Anti-human MPO antibodies have been shown to react with rat MPO which exhibits significant homology to human MPO. This model has demonstrated increased leukocyte endothelial interaction in the presence of ANCA-IgG; however ANCA itself has not been demonstrated to transfer disease [50].

Interesting observations have also been made regarding the genetic basis of disease susceptibility as Lewis rats, which share a similar MHC to WKY rats, have been shown to be protected from disease despite producing anti- MPO antibodies. Differences in numbers of activatory Fc receptors, in particular FCGR3, have been implicated in this finding (93). FCGR3 genes have been identified as risk factors for the development of immune mediated glomerulonephritis.

## 1.4 TREATMENT OF AAV

### 1.4.1 OLDER TREATMENTS FOR AAV

Treatment of AAV is divided into two phases. The first of these is the induction of remission and continues for between 3 and 6 months. The next phase is maintenance of remission and this phase of treatment is usually continued for at least 2 years.

Induction treatment with CYP and glucocorticoids has revolutionised the management of AAV. Almost 90% of patients achieve remission with this regimen, however relapses still occur in up to 50% of patients within 5 years (94). There is significant toxicity associated with this regimen particularly in elderly patients (95). Little *et al* reviewed the data of 524 participants in EUVAS trials with a new presentation of AAV, and found that the highest risk of death in these patients in the first year post diagnosis was as a result of treatment toxicity (96). These adverse effects included not only a higher risk of infection, but the longer- term effects of malignancy and infertility.

Cyclophosphamide can be administered orally or intravenously. CYCLOPS was a randomised controlled trial carried out by EUVAS, which compared these two routes of administration in 149 newly diagnosed AAV patients. Patients were randomised to receive either oral CYP at a dose of 2 mg/kg per day, or intravenous CYP at 15 mg/kg

every 2-3 weeks for 3-6 months. Both regimens were found to be equivalent in the induction of remission, but the cumulative dose of CYP was higher when administered orally, with higher rates of leukopenia. However, higher rates of relapse were seen in patients treated intravenously. Long term follow up of these patients revealed no difference in terms of renal or patient survival in this cohort (97).

As knowledge of the pathogenesis of disease has increased, more directed therapies have been introduced, including B cell depletion with rituximab. In 2011, RTX was approved by the FDA as induction therapy for GPA, following the results of two large multicentre trials which demonstrated its non-inferiority, when compared with CYP, in the induction of remission in AAV (40, 41). The Rituximab versus Cyclophosphamide in ANCA-associated Vasculitis (RITUXVAS) trial included 44 patients with AAV and renal impairment, randomised to receive CYP or rituximab as induction therapy (41). Patients in the RTX arm received 4 doses of RTX, weekly at 375 mg/m<sup>2</sup>, and in addition received two doses of IV CYP and the standard glucocorticoid regimen. Patients in the CYP arm received the standard regime of IV CYP for 3-6 months, and glucocorticoids. All patients who could tolerate it received azathioprine maintenance therapy. There was no significant difference between the groups with regards to the primary end points of remission and adverse effects at 1 year. The rituximab in ANCA associated vasculitis (RAVE) trial included 197 AAV patients who were treated with either rituximab or CYP at induction. Patients with severe renal disease or pulmonary haemorrhage were excluded. There was no CYP given in the RTX arm. RTX was shown to be non-inferior to CYP for the induction of remission at six months, by which time corticosteroids were weaned to 0. In subgroup analysis, RTX was found to be superior to CYP for induction of remission in patients who were PR3 positive and in relapsing patients.

Both RTX and CYP induction regimes are associated with significant toxicity. Some centres, including ours, combine the two agents in an attempt to reduce the overall CYP dose and minimise the dose of glucocorticoids. Mcadoo *et al* recently reported on the outcomes of the long term follow up of a combined rituximab and low dose iv cyclophosphamide regimen in renal AAV (98). Sixty- six patients treated with this regimen were compared with a cohort of propensity matched cases from EUVAS studies, who had received either rituximab or CYP induction treatment. Ninety four percent of patients had achieved remission at six months, and patient and renal survival were reported to be 84 and 95% respectively. These outcomes were better than those reported for the EUVAS cohort. The implication of these results was that the combination regime is potentially superior to current standards of care but that further studies are necessary. A clinical trial of combination treatment with CYP and RTX – Exploring Durable Remission with Rituximab in Antineutrophil Cytoplasm Antibody (ANCA) Associated Vasculitis, (the ENDURRANCE study; NCT03942887) is currently recruiting to assess primary immunological responses and clinical outcomes in patients treated with RTX, low dose CYP and low dose glucocorticoids.

Plasma exchange (PEX) has traditionally been used alongside induction therapy in patients with organ threatening involvement including dialysis dependence, or a serum creatinine of over 500  $\mu\text{mol/l}$ , and/or pulmonary haemorrhage. This practice was largely based on data from the MEPEX study, a randomised trial of PEX vs high dose methylprednisolone in severe renal AAV (42). This trial included patients with severe renal involvement with either a creatinine of over 500  $\mu\text{mol/l}$  or dialysis dependence. At 12 months, there was a significant reduction in progression to ESRD in patients treated with PEX when compared with the methylprednisolone group (39). The reduction in dialysis requirement was significant at around 50%. There was however

a high overall mortality in MEPEX and a concern was raised that this marked improvement in outcome with PEX at one year would not be longstanding. Walsh *et al* carried out a study assessing the long-term outcomes of patients from the MEPEX trial. One hundred and thirty-seven patients who presented with a serum creatinine of 500µmol/l or dialysis dependence were included in the study and were treated with either IV methylprednisolone or PEX. Patients were followed up for a median of 3.95 years, and outcome determined using a composite endpoint of death or end stage renal disease (ESRD) (99). This study highlighted the poor prognosis for patients presenting with severe AAV, with 50% of patients having died at the end of follow up and two thirds of patients reaching an endpoint of either death or ESRD. There was no significant difference in outcome found between PEX and methylprednisolone (85). Following these results, there was a need for a larger study to evaluate the benefit of PEX in patients with severe AAV, and this was the aim of Plasma Exchange and Glucocorticoids for Treatment of Anti-Neutrophil Cytoplasm Antibody (ANCA) - Associated Vasculitis study (PEXIVAS). The PEXIVAS study was a two by two factorial randomised controlled trial to evaluate the benefit of PEX and two different oral glucocorticoid regimens in the treatment of severe AAV (86). This study included 500 patients with severe AAV recruited from worldwide centres. All patients received induction with either CYP or rituximab and were then randomised to receiving PEX or no PEX and high dose vs low dose corticosteroids. The primary outcome was the time to the combined endpoint of patient death and ESRD, and the median follow up was 2.9 years. Treatment with plasma exchange did not result in any improvement in long-term outcome in this study although the effect of dialysis dependent cases was not reported. The use of low dose corticosteroids was not inferior to the higher dose regimen but patients treated with the lower dose experienced significantly less

infective complications than those treated with the higher dose. A study in our centre reviewing the outcomes of patients with RPGN, is discussed in chapter 5.

Mycophenolate Mofetil (MMF) has also been trialled for use in induction of remission. MYCYC was a randomised controlled trial comparing oral MMF with intravenous CYP (100). The primary endpoint for this study was remission at 6 month and was achieved in 67% of patients treated with MMF, compared with 61% in the CYP group. Relapses were however, more common in the MMF treated group, especially in the PR3 ANCA positive patients. There was a suggestion from this study that MMF could potentially be used, with glucocorticoids, in the induction of remission of AAV for patients without organ threatening involvement (100). Methotrexate has also been used as an induction agent but is less effective than CYP, and used primarily for patients with localised mild disease in the absence of organ threatening involvement.

The aim of maintenance therapy for AAV is the maintenance of remission and the prevention of relapse. Relapses are most common in the first 12-18 months after stopping immunosuppression, and have been reported to occur in 30-50% of patients by 5 years when using traditional induction and maintenance therapy (48). Azathioprine is the recommended agent for maintaining remission, when tolerated. The CYCAZAREM trial compared relapse rates in patients who continued oral CYP with those who were commenced on azathioprine at six months. There was no difference in relapse rates at 18 months (101). For patients who are intolerant of azathioprine, Mycophenolate Mofetil (MMF) is often used as a substitute. The IMPROVE study showed higher levels of relapse in patients treated with MMF when compared to azathioprine (102).

A randomised controlled trial, Maintenance of Remission using Rituximab (MAINRITSAN), found a reduction in relapses with pre-emptive treatment with RTX when compared with azathioprine (103). MAINRITSAN compared the maintenance of remission by azathioprine with 5 doses of RTX administered over 18 months. Only 5% of patients in the RTX arm experienced a relapse, compared with 29% in the azathioprine arm and this benefit was maintained at 60 months. A second study, MAINRITSAN 2 compared RTX dosing at fixed time intervals, with dosing tailored to B cell repopulation or rise in ANCA, and found that at 28 months there was no difference in the two groups with regards to relapse or adverse events, but the tailored approach required a lower cumulative dose of RTX (104).

#### 1.4.2 ADJUNCTIVE TREATMENTS

In order to minimise treatment related toxicity whilst immunosuppressed, European League Against Rheumatism (EULAR) guidelines recommend patients are given *Pneumocystis jirovecii* prophylaxis, usually in the form of co-trimoxazole, antifungal agents such as nystatin, bone protection and gastric protection, and undergo regular bone densitometry.

#### 1.4.3 NEWER THERAPIES

As knowledge of the pathogenic mechanisms of AAV increases, a number of new, more targeted treatments are under development. In this section, I will discuss some of the treatments which have been trialled in patients with AAV.

#### 1.4.3.1 COMPLEMENT INHIBITION

A number of targets in the complement pathway have been identified following increased understanding of the role of complement in the pathogenesis of AAV (105). Complement deposition has been demonstrated at sites of inflammation in patients with AAV, and complement components detected in blood and urine. Levels of complement have been found to correlate with severity of disease (106) (107). Avacopan is a selective C5a receptor inhibitor and was compared when given alone and in combination with low dose steroid, to standard dose prednisolone, for maintenance of remission following induction with either CYP or RTX in a phase 2 trial (108). The outcome of this trial was that the avacopan groups were non inferior to the standard glucocorticoid group at maintaining remission. It was a small trial of only 67 patients, and following on from this, a phase 3 trial of avacopan (ADVOCATE; NCT02994927) completed recruitment of 300 patients in 2018. The outcomes of this trial are awaited.

#### 1.4.3.2 T CELL TARGETTED THERAPY

The pathogenicity of T cells has been described previously. T cell targeted therapy has been trialled mainly in small groups of AAV patients with refractory disease. The anti-CD52 monoclonal antibody alemtuzumab depletes all lymphocytes and has been shown to have longstanding effects on T cell depletion, with CD4+ cells reportedly taking 5 years to recover (109). Repopulation of T cells following alemtuzumab has been reported in studies of patients with multiple sclerosis to be skewed towards a regulatory phenotype (110). Walsh *et al* described the long term follow up of 71 alemtuzumab treated refractory AAV patients (111). Eighty percent of patients in this study achieved remission, but relapse and adverse event rates were high (111). High and low dose alemtuzumab were compared in a phase 2 randomised controlled trial,

Alemtuzumab for ANCA Associated Refractory Vasculitis (ALEVIATE), in patients with AAV or Behcet's disease (ClinicalTrials.gov: NCT01405807).

Initial findings from this study suggested that 65% of alemtuzumab treated patients had achieved remission at six months and that this was sustained at one year for 35%, although at this time more than 25% had experienced adverse events. It seems therefore, that there is potential for the use of alemtuzumab in refractory disease; however its adverse event profile would currently limit its use. An open label study also reported on the use of the anti-T cell therapy anti-thymocyte globulin (ATG) in 15 patients with refractory disease, of whom 13 achieved remission (112).

Abatacept is a fusion protein of the Fc region of IgG1 and CTLA4. This protein can inhibit co-stimulatory signals which are necessary for T cell activation by antigen presentation (113). A study was carried out using abatacept in addition to methotrexate, MMF or azathioprine, in 20 relapsing, non-severe GPA patients. Remission rate were reported as 80% in this study (114).

#### *1.4.3.3 B CELL TARGETTED THERAPIES*

Alternative anti-CD20 agents to RTX are at varying stages of development, and have been used particularly in patients unable to tolerate RTX. These agents differ from RTX in terms of their binding to the CD20 molecule. Ofatumumab is an example of one of these agents and is a fully humanised monoclonal antibody to CD20, approved by the FDA for the treatment of resistant CLL (115). The use of ofatumumab has been described in a case series from our centre, and shown to be of therapeutic benefit (116). There is ongoing work with other secondary anti- CD20 antibodies including obinutuzumab, ocrelizumab and veltuzumab.

CD22 is a 135 kDa transmembrane sialoglycoprotein which is expressed mainly on the surface of the mature B cell, and is involved in immune regulation (117). Epratuzumab is a humanised monoclonal antibody to CD22. Two randomised clinical trials of epratuzumab in SLE did not meet their primary endpoints; however work is ongoing in AAV (118).

The proteasome inhibitor bortezomib can cause apoptosis of antibody producing plasma cells. This agent has been used successfully in an AAV patient with PR3-AAV resistant to treatment (119). Bortezomib is associated with adverse effects including severe peripheral neuropathy, which limits its use. Newer protease inhibitors are currently under development and could be valuable future agents in the treatment of AAV (120).

#### 1.4.3.3.1: BLYS INHIBITORS

There have been multiple successful in vivo studies of BlyS inhibitors in mice, mainly with lupus-like diseases, but also with a variety of conditions including experimental autoimmune encephalitis (EAE) (121). Belimumab is a fully human monoclonal antibody which binds to BlyS. Atacicept is a fusion protein between TACI and the Fc portion of IgG. There have been clinical trials of the use of belimumab and atacicept in multiple autoimmune diseases in humans with varying results. Results in both multiple sclerosis (MS) and RA have been disappointing. A clinical trial of the use of atacicept in MS was terminated prematurely due to an increase in disease severity (122). This was in spite of improvement of EAE in the murine model and the finding of increased production of BlyS by astrocytes of patients with disease (121, 122). There has been more success with the use of anti-BlyS agents in the treatment of SLE. The BlyS inhibitor belimumab (Benlysta) was the first new drug to be licensed for the

treatment of lupus for over 40 years, following two successful phase III trials (123, 124). Pooled data from these trials demonstrated the ability of belimumab to reduce autoantibodies, normalise complement levels and alter B cell subsets in patients suffering from the disease [40]. Stohl *et al.* reported a significant reduction in naive and activated B cell populations with no change in plasma cell populations (125).

BREVAS (Belimumab in Remission of Vasculitis), a phase III multicentre randomised controlled trial comparing azathioprine + placebo and azathioprine + belimumab in the maintenance of remission in AAV, was terminated early due to under recruitment and no initial difference between the groups in the primary endpoint of relapse rate (126). However, patients receiving RTX for induction did not relapse. The combination of RTX and belimumab is being investigated in the COMBIVAS (rituximab and belimumab combination therapy in PR3 AAV trial (Combivas: NCT03967925). This trial is currently recruiting and will include patients treated with rituximab and steroids as induction therapy, and then randomised to receive either belimumab or placebo for one year (62) .

## 1.5 FACTORS AFFECTING OUTCOME IN AAV

The outcome for a patient with AAV depends on a number of factors, including both the success of treatment and also the impact of adverse effects of therapy.

### 1.5.1 ADVERSE EFFECTS OF THERAPY

As described by *Little et al* in 2010, in a cohort of over 500 patients with AAV, the majority of deaths in the first year following diagnosis were due to the adverse effects of therapy (96). The main side effect of immunosuppressive therapy with CYP is infection. Leucopenia is common and monitoring of white cell count at intervals during a course of CYP therapy is essential. As discussed previously, prophylaxis against *Pneumocystis Jiroveci* is routinely given. Patients are closely monitored for signs of infection. Immunoglobulin levels are monitored and immunoglobulin replacement in the form of IVIG administered when necessary.

Severe infection following RTX administration is also seen. In the trials RAVE and RITUXVAS, rates of infection in the RTX arm were not reduced when compared with CYP although the use of glucocorticoids may have contributed to this. Reactivation of hepatitis B and C is possible post RTX, and all AAV patients embarking on this treatment should have screening for hepatitis. Progressive multifocal leukoencephalopathy is a rare but crippling neurological condition linked with JC viral infection post RTX treatment (127). The multiple side effects of corticosteroids, including impairment of glucose tolerance, are well known and newer treatment regimens aim to reduce cumulative steroid exposure. In the longer term, CYP administration is also associated with a high risk of malignancy, primarily of urothelial origin and skin. All patients who have had exposure to CYP should undergo regular screening for malignancy. With reduction in cumulative doses of CYP more recently,

rates of malignancy appear to be decreasing (23) . There is also a marked impact of CYP treatment on fertility. Female patients are administered ovarian protection if indicated and male patients, who are at risk of permanent azoospermia, are often referred for semen cryopreservation prior to therapy. The risk of cardiovascular events following treatment of AAV is also high, with long term data from European vasculitis patients suggesting that 14% of patients with AAV develop cardiac complications within 5 years of diagnosis (48).

### 1.5.2 PROGNOSTIC FACTORS

Given the spectrum of adverse effects of therapy, one of the main challenges in the management of patients with AAV remains to balance the risks of treatment with the risks of damage from the disease process itself. Factors affecting prognosis are sought with the aim of providing patients with an indication of their prognosis in terms of renal and patient survival and risk of relapse, and to balance this with the risks of therapy. A number of factors have been shown to be predictive of prognosis in AAV. Age at presentation is one such factor, with older patients experiencing a worse prognosis in terms of both renal and patient survival. Presenting creatinine has also been shown to be associated with prognosis, with worse overall outcomes in patients presenting with higher serum creatinine levels and/or dialysis dependent (18).

A number of histological factors have also been shown to be predictive of outcome. A greater percentage of normal glomeruli on renal biopsy has been associated with better renal outcomes in a number of studies. A higher proportion of crescents has also been shown to correlate with response to therapy. A greater proportion of sclerotic glomeruli has been associated with poor renal outcomes and reduced response to therapy. Based on these findings, an international working group of renal

pathologists proposed a histological classification for AAV. This classification system was described by Berden in 2010 (128). Biopsies of patients with AAV were divided into four subgroups, focal, crescentic, mixed and sclerotic, based on light microscopy features. Biopsies with at least 10 glomeruli were included for analysis. The focal group was defined as those biopsies with > 50% normal glomeruli. Biopsies in the crescentic group contained >50% crescents and those in the sclerotic category, >50% sclerotic glomeruli. The mixed category included biopsies with <50% normal, <50% crescentic and <50% sclerotic glomeruli.

The prognostic value of this histological classification has been evaluated in multiple studies. The first of these studies was a validation study in 100 patients recruited to EUVAS trials (128). This study found the classification system to be predictive of prognosis at 1 and 5 years, with the best outcome in the focal category and the worst in the sclerotic (102). Multiple further validation studies have been carried out and have largely agreed on the best prognosis being in the focal and the worst in the sclerotic categories. These studies are discussed further in Chapter 4, where a validation study carried out in patients at our centre is described. There are thus a number of factors predictive of prognosis in AAV, both histological and otherwise. With the advent of more targeted therapies, and the use of biomarkers for disease, the hope is of a patient tailored management strategy for each patient presenting with AAV and a consequent improvement in outcome.

## 1.6 EXPERIMENTAL HYPOTHESES AND STUDY AIMS

The overall aim of this work was to assess factors predictive of prognosis in our cohort of patients with AAV at Hammersmith hospital, and the role of B cell survival factors.

### HYPOTHESES:

- A number of factors are predictive of prognosis in AAV patients, including degree of renal dysfunction and histological classification.
- B cell survival molecules are important in the pathogenesis of AAV.

### AIMS:

The aims of the study are listed below:

- 1) To assess factors affecting outcome in our entire cohort of over 500 patients at a single centre with AAV. This work forms Chapter 3 of this thesis.
- 2) To address clinical and histological factors affecting outcome in a smaller subgroup of patients and carry out a validation study of the international histological classification described by Berden. This work is described in Chapter 4.
- 3) To assess factors affecting outcome in patients presenting with severe renal disease.  
  
This work is described in Chapter 5.
- 4) To assess the clinical significance of the B cell survival factors BlyS and APRIL in AAV  
  
This work is shown in Chapter 6.

- 5) To assess the use of a BlyS inhibitor in vivo using the rat model of AAV, EAV.

This work is described in Chapter 7.

## CHAPTER 2: MATERIALS AND METHODS

## 2.1 HUMAN WORK

### 2.1.1 PATIENT IDENTIFICATION

Ethical approval for this study was granted by the NRES committee London-West London (formerly the Hammersmith and Queen Charlottes Research Ethics Committee).

Patients with AAV, as diagnosed by the Chapel Hill Consensus Criteria were identified using biopsy databases and clinic lists. Patient records, pathology results and electronic data were used to collect data and create a database of all patients.

### 2.1.2 PATIENT RECRUITMENT

Patients with AAV were recruited from the Imperial College Vasculitis clinic. These patients provided written consent for the use of their samples, and were free to withdraw their participation at any time.

### 2.1.3 SAMPLE COLLECTION

Patient blood samples were collected alongside routine bloods either on the wards, or in the outpatient clinic. Urine samples were also collected. Blood samples were centrifuged for 15 minutes at 1000 x g. Serum was extracted and either used immediately for ELISA, or aliquoted and stored at <20 degrees.

### 2.1.4 BLYS ELISA

The BlyS sandwich ELISA was carried out in accordance with the protocol for the Quantikine ELISA kit: Human BAFF/BlyS/TNFSF13B (R&D Systems).

Plates were read using a microplate reader set to 450 nm.

### 2.1.5 APRIL ELISA

The APRIL sandwich ELISA was carried out in accordance with the protocol for the human APRIL platinum ELISA kit (Ebiosciences).

Plates were read using a microplate reader set to 450 nm.

### 2.1.6 IMMUNOHISTOCHEMISTRY

#### *2.1.6.1: OBTAINING BIOPSY SLIDES*

Human renal tissue samples were obtained from patients under local Research Ethics Committee approval (04/Q0406/25 Hammersmith and Queen Charlotte's & Chelsea Hospitals Research Ethics Committee). These samples were paraffin sections which were surplus to clinical need and were obtained from the histology archives at Hammersmith Hospital.

#### *2.1.6.2: IMMUNOHISTOCHEMISTRY PROTOCOL*

Sections were placed in two sequential baths of xylene for 1-5 minutes each, for dewaxing. They were then placed in graded ethanol concentrations and then plain water. Antigen retrieval was performed using 0.1M sodium citrate buffer, to pH 6.0 for 15 minutes at 95 degrees. Sections were then blocked with 0.3 % hydrogen peroxide for 10 minutes. Following rinsing with PBS, tissue were encircled using a wax pen (Dako), and normal rabbit serum applied for 30 minutes in order to block non-specific binding of subsequent antibodies.

Primary antibodies at the appropriate concentration were then applied in sufficient volume to cover the section (see table 2.1). Slides were placed in a humidified staining chamber for 1 hour, for incubation. Following washing in PBS, secondary antibody, as detailed in Table 2.1 was applied to each section at a concentration of 1/100 and allowed to incubate for 45 minutes. The sections were washed in PBS and incubated

with an Extravidin- HRP conjugate (Sigma) at a dilution of 1:100 for 30 minutes, then washed again three times with PBS. A 3,3'-diaminobenzidine (DAB) chromagen solution was prepared by mixing DAB+ chromagen with DAB+ substrate buffer in a ratio of 1:50 (Dako). This was applied to tissue sections for 30 seconds to 5 minutes depending on the primary antibody. The reaction was terminated by placing slides in PBS. Slides were then rinsed in water and counterstained by immersion for 30 seconds in filtered Harris haematoxylin (Cellpath, Powys, UK). After rinsing, slides were immersed very briefly (<1 second) in acid-alcohol solution (1% HCL in 70% ethanol), to remove excess stain. The sections were then washed in water and dehydrated by passing through ascending alcohol concentrations and then xylene. Slides were then mounted with DPX (Distrene, Plasticiser, Xylene; VWR International, Lutterworth, UK) and glass coverslips and allowed to dry before examination.

| <b>Target Protein<br/>(Species)</b> | <b>Blocking<br/>solution</b>     | <b>Primary<br/>antibody</b>                                   | <b>Primary<br/>antibody<br/>dilution and<br/>incubation</b> | <b>Secondary<br/>antibody</b>                                                                                                                     |
|-------------------------------------|----------------------------------|---------------------------------------------------------------|-------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| BAFF-R<br><br>(human)               | 20% goat<br>serum<br>(DAKO)      | BAFF-R<br><br>ABCAM<br><br>Ab16323<br><br>Mouse<br>monoclonal | 1:100<br><br>1 hour, room<br>temperature                    | Biotinylated goat<br>anti-mouse<br>immunoglobulin<br>(Sigma;dilution1/100)<br><br>Followed by<br>extravidin HRP<br>(Sigma;dilution<br>1:100)      |
| TACI<br><br>(human)                 | 20% goat<br>serum<br>(DAKO)      | TACI<br><br>ABCAM<br><br>Ab5994<br><br>Rabbit<br>polyclonal   | 1:50<br><br>1 hour, room<br>temperature                     | Biotinylated goat<br>anti-rabbit<br><br>immunoglobulin<br>(Sigma;dilution1/100)<br><br>Followed by<br>extravidin HRP<br>(Sigma;dilution<br>1:100) |
| BlyS/BAFF<br><br>(human)            | 20%<br>normal<br>rabbit<br>serum | BAFF<br><br>ABCAM<br><br>AB16081<br><br>Rat<br>monoclonal     | 1/200<br><br>1 hour, room<br>temperature                    | Biotinylated rabbit<br>anti-rat<br>immunoglobulin<br>(Sigma;dilution1/100)<br><br>Followed by<br>extravidin HRP<br>(Sigma;dilution<br>1:100)      |

|                     |                                  |                                                             |                                          |                                                                                                                                               |
|---------------------|----------------------------------|-------------------------------------------------------------|------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|
| CD20<br><br>(human) | 20% goat<br>serum<br>(DAKO)      | CD 20<br><br>ABCAM<br><br>Ab9475<br><br>Mouse<br>Monoclonal | 1:100<br><br>1 hour, room<br>temperature | Biotinylated goat<br>anti-mouse<br>immunoglobulin<br>(Sigma;dilution1/100)<br>Followed by<br>extravidin AP<br>(Sigma;dilution<br>1:100)       |
| BCMA<br><br>(human) | 20% goat<br>serum<br>(DAKO)      | BCMA<br><br>ABCAM<br><br>AB5972<br><br>Rabbit<br>polyclonal | 1:50<br><br>1 hour, room<br>temperature  | Biotinylated goat<br>anti-rabbit<br>immunoglobulin<br>(Sigma;dilution1/100)<br>Followed by<br>extravidin AP<br>(Sigma;dilution<br>1:100)      |
| CD 68<br><br>(rat)  | 20%<br>Rabbit<br>serum<br>(DAKO) | Clone ED1<br><br>Serotec<br><br>Mouse<br>monoclonal         |                                          | Biotinylated rabbit<br>anti-mouse<br>immunoglobulin<br>(Sigma;dilution<br>1:100); Followed by<br>Extravidin HRP<br>(Sigma; dilution<br>1:100) |

**Table 2.1 Antibodies used for immunofluorescence and immunohistochemistry**

## 2.2 IN VIVO ANIMAL STUDIES

These studies were carried out in collaboration with Dr. Stephen McAdoo.

### 2.2.1 ANIMAL STUDY APPROVAL

All animal procedures were licensed by the Home office Science Unit (personal license and project license 70/7104 (Professor Charles Pusey), and conducted in accordance with the UK Animals Scientific Procedures act 1986.

### 2.2.2 ANIMAL HUSBANDRY

Female WKY rats were purchased from Charles River (Margate, UK) and maintained in a pathogen-free animal facility at the Central Biomedical Services Unit, Imperial College London,

Hammersmith Hospital campus. They were cared for in accordance with institutional guidelines in individually ventilated cages with free access to water and a standard laboratory diet. Cage occupancy was dependent on body weight and varied between two to five rats per cage.

### 2.2.3 INDUCTION OF EAV

The rats were immunized with human myeloperoxidase at 6-8 weeks of age, at a weight of 100-120 grams. Human MPO (MPO; Calbiochem, Merck Millipore, Darmstadt, Germany) was reconstituted in sterile water for injection and a dose of 1600 µg was administered following emulsification with an equal volume of Complete Freund's Adjuvant (CFA). Killed *Mycobacterium butyricum* was added, to a final concentration of 4 mg/ml. A maximum injection of 200 µl was administered as 2 x 100µl injections to each thigh. Immunised animals received 500 ng of pertussis toxin

(Invitrogen, Live Technologies) in PBS on days 0 and 2. This was administered intraperitoneally.

## 2.2.4 SAMPLE COLLECTION

### 2.2.4.1 URINE COLLECTION

In order to collect urine samples, rats were placed overnight in individual metabolic cages. They had free access to food and water and the total volume of urine they produced was collected and measured. Aliquots of urine were centrifuged at 1500 rpm for 5 minutes in order to sediment any visible debris and stored at -80°C until needed for use. Urine samples were collected weekly.

### 2.2.4.2 SERUM COLLECTION

For collection of serum, vasodilatation was promoted by placing rats in a warming chamber at 30°C and then anaesthetized using inhaled isoflurane, on a warming mat. A maximum of 0.5 ml whole blood was collected by superficial tail vessel puncture using a 23 gauge needle. Samples were centrifuged at 1500 rpm for 5 minutes, serum collected and stored at -80°C until use.

### 2.2.4.3 TERMINAL PROCESSING OF BLOOD AND TISSUES

At the end of the study period, blood and tissues were collected. Rats were anaesthetized using isoflurane and blood was obtained either by cardiac puncture using a 10ml pre-heparinised syringe and 23 gauge needle, or by transection of the great vessels and collection of blood into plain and heparinized collection tubes. Once the animals had been sacrificed, their tissues were dissected out and stored. For each animal, a sample of kidney (coronal mid-pole section), lung, spleen and liver tissue was placed in a histology cassette and transferred to 10% neutral buffered formalin for fixation. The remaining kidney pole was snap frozen in liquid nitrogen having been

placed in optimal cutting temperature (OCT) compound embedding matrix (ThermoFisher Scientific) on a cork disc and immersed in isopentane. Lung surfaces were visually inspected and a lung haemorrhage score was given based on the number of visible petechiae. This score was devised by Dr. Stephen McAdoo (Table 2.2).

| <b>Visual appearance of lung</b>         | <b>Pulmonary haemorrhage Score</b> |
|------------------------------------------|------------------------------------|
| Normal macroscopic appearance            | 0                                  |
| Less than 10 visible petechiae           | 1                                  |
| 10-20 visible petechiae                  | 2                                  |
| >20 visible petechiae                    | 3                                  |
| Large areas of infarction or haemorrhage | 4                                  |

**Table 2.2: Pulmonary haemorrhage score (29)**

## 2.2.5 ANALYSIS OF RENAL AND PULMONARY INJURY IN EAV

### 2.2.5.1 HAEMATURIA

Haematuria was quantified by dipstick analysis (Multistix 8 SG, Siemens Healthcare Diagnostics, Tarrytown, New York), and expressed as 0 (negative), 0.5 (trace), 1+, 2+ or 3+ for each animal.

#### 2.2.5.2 *PROTEINURIA*

##### 2.2.5.2.1 *DIPSTICK PROTEINURIA*

Dipstick proteinuria was also quantified by dipstick analysis (Multistix 8 SG, Siemens Healthcare Diagnostics, Tarrytown, New York), and expressed as 0 (negative), 0.5 (trace), 1+, 2+ or 3+ for each animal.

##### 2.2.5.2.2 *QUANTIFICATION OF PROTEINURIA*

Proteinuria was quantified using the sulphosalicylic acid method. Urine samples, diluted between 1:3 and 1:100 in water, were added to a 96-well microtitre plate in triplicate. Ten µl of 25% sulphosalicylic acid was added to two of three replicates, and 10µl of water to the third replicate (providing a 'blank' reading for that sample). Known quantities of bovine serum albumin (BSA; Sigma) in water were used to define a standard curve, and absorbance read at 450nm on a microplate reader and dedicated software (Biotek EL800; Gen5 Analysis Software; both Biotek Instruments Ltd, Potton, UK). The protein concentration in each sample was calculated from a regression equation described by the standard curve, and the 24 hour protein excretion rate was calculated by multiplying by the total urine volume. Urinary protein creatinine ratio was calculated by dividing the urinary protein concentration by the urinary creatinine concentration.

##### 2.2.5.3 *BIOCHEMICAL ANALYSIS OF SERUM AND URINE*

Concentrations of urea and creatinine in serum and urine samples were kindly measured by Dr. Olatunji Rowland and Dr. John Morris in the Department of Clinical Biochemistry, Hammersmith Hospital UK, using an AU700 analyser (Olympus, Southend, UK). Urinary clearance of each marker was calculated using the following

equation: urinary clearance per unit time = (urine concentration x urine volume per unit time)/serum concentration.

#### 2.2.5.4 RENAL HISTOLOGY

Kidney tissue collected at the time of sacrifice was fixed in 10% neutral buffered formalin overnight, then transferred to 70% ethanol and processed to paraffin blocks. Sections of 4µm were cut on a rotary microtome and stained with haematoxylin and eosin (H&E), periodic acid Schiff (PAS), and Jones methanamine silver stain, for assessment of renal injury. This sectioning and staining was performed by Ms. Lorraine Lawrence, Department of Leucocyte Biology, Imperial College London (except silver stain, performed by Dr. Donna Horncastle, Department of Histopathology, Hammersmith Hospital). In order to quantify renal injury, 50 consecutive glomeruli were assessed by light microscopy in a blinded fashion, and graded as normal or abnormal. Results are expressed as the percentage of abnormal glomeruli per animal.

##### 2.2.5.4.1 IMMUNOSTAINING FOR ED1 POSITIVE CELLS

Immunohistochemistry for ED1 positive cells was performed on formalin-fixed paraffin embedded kidney sections. Four µm formalin-fixed paraffin embedded sections were rehydrated. Antigen retrieval was carried out by heating the slides in a microwave in tri-sodium citrate buffer (pH6) for 15 minutes. Following washing, slides were incubated in 0.03% hydrogen peroxide for 10 minutes. The slides were then incubated with a mouse mAb for ED1 (Serotec, Oxford) at a dilution of 1:500 in PBS at room temperature for 1 hour. Slides were then incubated with an HRP labelled anti-mouse polymer (DAKO) for 30 minutes at room temperature and developed in 3'3

diaminobenzidine (DAKO) for 10 minutes. They were then counterstained with haematoxylin and dehydrated. After drying, the slides were mounted in De Pex medium (BHD laboratory supplies) and analysed for the number of positively stained cells in 10-25 sequential glomeruli using automated image analysis software (ImagePro Plus, Media Cybernetics, Rockville, Maryland).

#### *2.2.5.5 INDIRECT IMMUNOFLUORESCENCE FOR ANTI MPO ANTIBODIES*

Whole rat peripheral blood was added to rat red cell lysis buffer (Table 2.3) which had been filtered and centrifuged at 2400 rpm for 5 minutes to remove particles and incubated for 10 minutes on ice. The ratio of rat peripheral blood to lysis buffer was 1:2 (blood:buffer). The samples were mixed intermittently then centrifuged at 1500 rpm for 5 minutes. Cells were resuspended in PBS to a concentration of 50 000 cells/ml, and 100µl of cell suspension applied to coated slides (Leica) using a cytopspin centrifuge (450rpm for six minutes; Cytospin 2, Shandon, ThermoFisher Scientific). After drying, slides were fixed in 100% acetone, dried, and then stored in sealed boxes with silica crystals at -80°C until use. Prior to use, slides were thawed and rinsed in PBS, then blocked in 20% rabbit serum (Dako) for 30 minutes. Rabbit serum was tapped off, and serum from EAV and control rats (1:20 dilution in PBS) applied for 30 minutes in a covered humidified chamber. Slides were washed three times in PBS, then incubated with a secondary rabbit anti-rat IgG FITC (1:100) for 30 minutes, washed again, mounted in PBS/Glycerol (Citifluor) and inspected immediately by fluorescence microscopy.

#### 2.2.5.6 ELISA FOR ANTI-MPO ANTIBODIES

Anti-MPO antibody levels in animal sera was measured using direct ELISA. Wells were coated with commercially available purified human MPO (Calbiochem) at a concentration of 1.33µg/ml in carbonate buffer and stored at 4°C overnight. The following day, plates were washed three times in 0.1% PBS/T and 100 µl of serum samples (diluted to 1:100 in PBS/T plus 1% BSA) were applied in duplicate, with a negative control “blank”. Negative controls did not contain serum. Plates were incubated at 37°C for 1 hour and then washed 3 times in PBS/T. 100 µl of rabbit anti-rat IgG with alkaline phosphatase conjugate (Sigma) in 0.1% PBS/T with BSA (dilution: 1:1000) was then added to each well. After 1 hour of incubation at 37°C and 3x wash with PBS/T, 100 µl of SIGMA FAST p-nitrophenyl phosphate were applied to each well. The colour change was monitored and quantified on a microplate plate reader at 405nm. A co-efficient of variance of 10% between sample replicates was accepted. Historical pooled sera were used to develop a standard curve from which subsequent samples could be assigned an interpolated value in arbitrary units.

#### 2.2.6 STATISTICS

Graphs were constructed and statistical analysis conducted using Prism 5.0 (GraphPad Software Inc., San Diego, California). Whenever a statistical test was used, this is indicated in the text. The means of two or more normally distributed variables were compared with a t test or analysis of variance (ANOVA) respectively, with Bonferroni post-hoc test to compare individual groups. For non-parametric datasets, or where <8 samples were available, Mann-Whitney U, Kruskal-Wallis or Friedman tests were used to assess the difference between 2, >2 and >2 (repeated measures)

groups respectively, with Dunn's post-hoc test to compare individual groups. All tests were two-tailed. Multivariate statistical analysis was carried out by Dr. Nish Arulkumaran and Dr. Ben Caplin, who used SPSS software, version 24 (IBM corp).

### 2.2.7 GENERAL REAGENTS AND BUFFERS

Unless otherwise stated, laboratory chemicals were purchased from Sigma-Aldrich (Poole, UK), plastic consumables from Becton Dickinson (BD) Biosciences (Oxford, UK), tissue culture plastic-ware from Corning (Corning, New York) and tissue culture media from Gibco, Life Technologies (Paisley, UK). Microcentrifuge tubes were supplied by Eppendorf (Hamburg, Germany). Specific materials and their source are described in the relevant sections below. Commonly used solutions are described below (Table 2.3).

**Phosphate buffered saline (PBS):**

NaCl            8g/l  
KCL            0.2g/l  
Na<sub>2</sub>PO<sub>4</sub>2H<sub>2</sub>O      1.44 g/l  
KH<sub>2</sub>PO<sub>4</sub>        0.2g/l  
Adjusted to pH: 7.2

**Tri buffered saline (TBS):**

Tris HCL      7.88 g/l  
NaCl           8.76g/l  
Adjusted to pH: 7.4

**Carbonate Buffer:**

Na<sub>2</sub>CO<sub>3</sub>      1.59 g/l  
NaHCO<sub>3</sub> 2.93 g/l  
Adjusted to pH: 9.6

**ELISA wash buffer**

0.05% Tween 20 in PBS

**ELISA Blocking buffer**

1% BSA in PBS

**ELISA Reagent Diluent**

0.1-1% BSA

**Substrate solution**

1:1 mixture of Colour Reagent A (H<sub>2</sub>O<sub>2</sub>) and Colour Reagent B (Tetramethylbenzidine) (R&D systems).

**Stop solution**

2NH<sub>2</sub>SO<sub>4</sub>

**Rat Cell Lysis Buffer**

NH<sub>4</sub>CL        8.34 mg/l  
  
EDTA        3.7 mg/l  
  
NaHCO<sub>3</sub> 1g/l

**Table 2.3 Commonly used buffers**

#### 2.2.8 BLYS INHIBITOR

The BlyS inhibitor used for in-vivo studies was provided by Human Genome Sciences and was a murinised chimeric compound, 10F4, a monoclonal antibody to BlyS.

#### 2.2.9 ISOTYPE CONTROL

The isotype control used for in vivo studies was ANO2-mIgG2A/kappa, obtained from Human Genome Sciences.

## CHAPTER 3: FACTORS AFFECTING LONG TERM SURVIVAL OF PATIENTS WITH AAV

### 3.1 INTRODUCTION

As discussed in the previous chapter, the ANCA associated vasculitides (AAV) are multisystem disorders characterised by a wide range of clinical manifestations and varying degrees of organ damage. Without treatment, the mortality of these diseases is reported to be 90% (129). Patients experience significant morbidity, both as a consequence of the disease process itself, and as a result of adverse effects of therapy. A study by Little *et al*, which included over 500 AAV patients recruited in European Vasculitis Study Group (EUVAS) trials, attributed over a third of deaths in the first year and 60% of deaths at 5 years, to treatment related toxicity (96). The remitting relapsing nature of these diseases also contributes to morbidity, with the risk of relapse reported to be as high as 40% at 5 years (96). This increases damage, not only from the disease process itself, but from further exposure to treatment. There is therefore a significant impact on quality of life of patients suffering with AAV. There is also a marked economic burden posed by these diseases. Recent studies have reported an increased prevalence of AAV at between 300 and 421 cases per million population (130), likely due to improved diagnosis and survival (23). It is important to identify factors affecting the long- term outcome of these patients and these factors will be discussed in this chapter.

Outcomes for patients with AAV have been reported to have significantly improved over the decades (131-134). The reasons for this improvement are multifactorial. One of the major advances in the management of AAV has been the use of cyclophosphamide and steroids for the induction of remission. This regimen was

introduced in the 1970s and resulted in over 90% of patients treated in this manner achieving remission. The ability to test ANCA serology in the 1990s enabled earlier diagnosis and contributed to improved prognosis and clinical outcome. Advances in therapy and general supportive management, have also improved prognosis. Most studies describing the demographics and outcomes of AAV patients presenting between 1976 and 2015 have reported an improvement in patient survival over time, even when correcting for baseline factors including age at presentation and presenting renal function (131-134). With improvement in survival at early time points in the disease, it has become even more pertinent to assess the long-term outcomes of these patients. A number of therapeutic trials have been carried out, particularly by EUVAS, with the intention of evaluating long term outcomes. Patients included in these studies had varying degrees of clinical involvement and were from multiple centres, treated with a number of different trial medications. Flossman *et al* collected outcome data for 535 patients who were participants in four EUVAS trials between 1995 and 2002. These patients were followed up for an average of 5.2 years and were demonstrated to have a mortality ratio of 2.6 times that of an age and sex matched general population (135). Factors associated with a poorer outcome in this study were severe renal dysfunction, in the form of an eGFR <15 ml/min, anaemia, a raised white count, high starting BVAS score and advancing age (135). Very elderly patients were excluded from this study, in addition to those with pulmonary haemorrhage.

The Hammersmith Hospital is a tertiary referral centre for patients with AAV. These patients have been managed in a consistent manner over the years. This chapter will describe a retrospective, observational review of patients with AAV presenting to Hammersmith Hospital over a period of four decades. The primary objectives of this

study was to ascertain demographics of these patients and differences in their presentation and outcome as the decades progressed. Secondary objectives included assessment of other factors contributing to long term outcome.

## 3.2 METHODS

### 3.2.1 WHOLE COHORT ANALYSIS

Patients with a diagnosis of systemic vasculitis consistent with the Chapel Hill Consensus Conference definitions (136), and positive ANCA serology where available, between the years 1976 and 2015, were identified and included in the study. Patients were sub-classified according to their clinical syndromes as having granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA), or renal limited vasculitis (RLV).

Clinical notes were reviewed and demographic, clinical and laboratory data for each patient were recorded on an electronic database using patient case notes, electronic records and histopathology.

Retrospective BVAS scores were not carried out, but the following systemic features were recorded:

- Renal dysfunction
- Ear, nose and throat involvement
- Skin lesions
- Central nervous system complications
- Peripheral nervous system complications

- Ocular lesions
- Musculoskeletal involvement
- Gastrointestinal complications
- Cardiac involvement

Renal replacement therapy (RRT) at presentation was defined as dialysis requirement during the initial hospital admission. End stage renal disease (ESRD) was defined as a sustained requirement for RRT for greater than three months, which did not recover during the period of follow up of the patient. Patients were followed from presentation until their last clinical encounter prior to December 31<sup>st</sup> 2016.

Hammersmith Hospital is a tertiary referral centre for AAV and as such, some patients from outside the area are transferred for initial diagnosis and management and then followed up at local centres. Follow up information for these patients was obtained where possible from referring centres or their general practitioner (GP).

### 3.2.2 TREATMENT

All patients were treated according to our local protocols at the time they presented. For patients who presented prior to 2006, these protocols included induction with either oral or intravenous cyclophosphamide (CyP) in addition to oral prednisolone, followed by maintenance therapy with azathioprine and prednisolone from 3 months. Ninety-five percent of patients received CyP induction. The patients who did not receive CyP could not due to contraindications. Those who were intolerant of azathioprine were treated with Mycophenolate Mofetil (MMF). In 2006, a new protocol was introduced incorporating the addition of Rituximab (RTX) as induction therapy in an attempt to reduce cyclophosphamide and steroid exposure. This approach was

similar to that used in the Rituxvas trial using a combination of RTX and low dose IV CYP (*Jones et al, Rituximab versus cyclophosphamide in ANCA –associated renal vasculitis. N Engl J Med.2010; 363(3): 211-20*). This protocol was the basis of our published CYCLOWVAS regimen (98). Patients who had evidence of organ threatening disease (cerebral involvement, pulmonary haemorrhage, serum creatinine over 500 µmol/l or dialysis dependence) were also treated with plasma exchange at induction. Rituximab was initially added for those patients who did not meet requirements for plasma exchange, but in 2010 was also added for those patients with organ threatening involvement.

Minor relapses on baseline immunosuppression were treated with a temporary increase in oral treatment, including steroids or azathioprine/MMF. More severe relapses were treated with the addition of one or more agents including intravenous steroids, cyclophosphamide or rituximab. For all patients, protocols included adjunctive treatment with oral nystatin, proton pump inhibitors, and co-trimoxazole whilst on cyclophosphamide, rituximab or high dose steroids. Bone prophylaxis included vitamin D supplementation and bisphosphonates were given to high risk patients. Bone densitometry scans were carried out two yearly.

### 3.2.3 STATISTICS

The statistical analysis for this study was carried out with Dr. Nish Arulkumaran.

Continuous (non-parametric) variables are presented as median (interquartile range). For comparison of continuous variables, the Mann Whitney U test was used for comparison of 2 groups and the Kruskal-Wallis test with post-hoc Dunn's test for more than 2 groups. Categorical data were compared using the chi-square test. Differences

in column proportions of decade of presentation were assessed using Bonferroni-adjusted p-values. Kaplan- Meier survival plots were used to display cumulative survival, and the log rank test was used to ascertain unadjusted survival differences between groups. Logistic regression was used to assess factors associated with RRT at presentation and data presented as adjusted odds ratio (adjOR) with 95% confidence intervals. Univariate and multivariate analyses using a Cox survival model were constructed to determine risk factors for mortality and data presented as adjusted hazards ratio (adjHR) with 95% confidence intervals. Candidate factors included in the univariate analysis to identify risk factors associated with mortality included gender, race, diagnosis, ANCA type, age, decade of presentation, RRT at presentation, pulmonary haemorrhage at presentation, and number of systemic features. Graphs were constructed and statistical analysis performed using Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and SPSS version 24.0 (IBM Corp). We recognised the limitation of using the “ANCA not tested” as a reference group given that this group likely represents those diagnosed earlier in the study period but did not want to exclude these patients from the study.

### 3.2.4 ETHICS

The early data collection for this study was carried out as part of a service evaluation/quality improvement project and as such was approved internally by the renal and transplant directorate. The collection of samples from patients and the clinical data for these patients was carried out under the study SALA1004. This retrospective study was approved internally by the Imperial glomerulonephritis research group. Human AAV biopsy and surgical tissue samples surplus to clinical need were used according to the Imperial College Healthcare tissue bank.

## 3.3 RESULTS

### 3.3.1 DEMOGRAPHICS

Data were collected for 563 patients who presented to our centre with AAV between the years 1976 and 2015. These patients were identified from clinical and biopsy databases. Their data was collected retrospectively from paper and electronic records stored at Hammersmith Hospital. We acknowledge that there were some missing data from the earlier decades.

Table 3.1 shows the demographics of these patients. The median age at presentation was 55.3 years and 52% of patients were male. The majority of patients were white/Caucasian (n=384; 76%) according to self-reported ethnicity. Thirteen percent of patients were of South Asian origin, 3% black, and the remainder reported themselves to be in the category “other”. This group included patients of Middle Eastern origin and of “mixed race”.

Patients were diagnosed as GPA, MPA, EGPA and RLV. The most common clinical diagnosis was GPA (n=287; 50.9%), followed by MPA (n=207; 36.8%), EGPA (n=43; 7.3%) and then RLV (n=26; 4.6%). All the earlier patients included in our study with MPA had a diagnosis of microscopic polyarteritis as described by Professor Caroline Savage (137), which was renamed microscopic polyangiitis at the first Chapel Hill Consensus Conference 2012 (1). Patients with a constellation of clinical features which would now be diagnosed as PAN were not included in this study.

The median number of clinical features on presentation was 3. Thirty percent of patients were dialysis dependent at presentation and 19% of patients experienced pulmonary haemorrhage as one of their presenting features.

The median serum creatinine at presentation was 192  $\mu\text{mol/l}$ . Testing for ANCA was carried out in 66% of patients. Seventy-six patients (13.5%) tested negative for ANCA, 116 (20.6%) were positive for MPO- ANCA, and 177 (31.4%) were positive for PR3- ANCA. Eight percent of patients were lost to follow up. The median follow- up time was 9.34 years.

|         |  | 1976-1985 | 1986-1995 | 1996-2005 | 2006-2015 | Total | p-value |
|---------|--|-----------|-----------|-----------|-----------|-------|---------|
| Numbers |  | 117       | 139       | 118       | 189       | 563   | -       |

|                                |                          |                    |                    |                    |                  |                   |        |
|--------------------------------|--------------------------|--------------------|--------------------|--------------------|------------------|-------------------|--------|
| <b>Follow up<br/>(years)</b>   | <b>Median<br/>(IQR)</b>  | 16.5<br>(2.7-33.7) | 13.9<br>(4.2-23.3) | 12.8<br>(7.5-17.1) | 6.5<br>(4.2-8.8) | 9.3<br>(4.4-18.1) | -      |
| <b>Gender</b>                  | <b>Male</b>              | 70 (59.8)          | 71 (51.1)          | 66 (55.9)          | 88 (46.6)        | 295 (52.4)        | 0.119  |
|                                | <b>Female</b>            | 47 (40.2)          | 68 (48.9)          | 52 (44.1)          | 101 (53.4)       | 268 (47.6)        |        |
| <b>Ethnicity</b>               | <b>Black</b>             | 1 (0.9)            | 1 (0.8)            | 2 (2.4)            | 11 (6.3)         | 15 (3.0)          | <0.001 |
|                                | <b>Asian</b>             | 10 (8.7)           | 9 (6.9)            | 15 (17.6)          | 32 (18.3)        | 66 (13.0)         |        |
|                                | <b>Other</b>             | 1 (0.9)            | 1 (0.8)            | 3 (3.5)            | 36 (20.6)        | 41 (8.1)          |        |
|                                | <b>White</b>             | 103 (89.6)         | 120 (91.6)         | 65 (76.5)          | 96 (54.9)        | 384 (75.9)        |        |
| <b>Diagnosis</b>               | <b>MPA</b>               | 42 (35.9)          | 46 (33.1)          | 52 (44.1)          | 67 (35.4)        | 207 (36.8)        | <0.001 |
|                                | <b>GPA</b>               | 47 (40.2)          | 70 (50.4)          | 62 (52.5)          | 108 (57.1)       | 287 (50.9)        |        |
|                                | <b>EGPA</b>              | 21 (17.9)          | 7 (5.0)            | 4 (3.4)            | 11 (5.8)         | 43 (7.6)          |        |
|                                | <b>RLV</b>               | 7 (6.0)            | 16 (62.5)          | 0 (0.0)            | 3 (1.6)          | 26(4.6)           |        |
| <b>Age (years)</b>             | <b>&lt;50</b>            | 51 (43.6)          | 46 (33.1)          | 40 (33.9))         | 77 (40.7)        | 214 (38.0)        | 0.023  |
|                                | <b>50-59</b>             | 32 (27.4)          | 26 (18.7)          | 27 (22.9)          | 45 (23.8)        | 130 (23.1)        |        |
|                                | <b>60-69</b>             | 28 (23.9)          | 38 (27.3)          | 29 (24.6)          | 35 (18.5)        | 130 (23.1)        |        |
|                                | <b>&gt;70</b>            | 6 (5.1)            | 29 (20.9)          | 22 (18.6)          | 32 (16.9)        | 89 (15.8)         |        |
| <b>Age (years)</b>             | <b>Median<br/>(IQR)</b>  | 53 (38.3-61.1)     | 59.4 (39.7-68.0)   | 55.4 (44.6-67.8)   | 54.5 (40.6-65.1) | 55.3 (40.6-66.2)  | 0.071  |
| <b>RRT at<br/>presentation</b> |                          | 38 (33.3)          | 54 (39.7)          | 34 (29.1)          | 39 (20.9%)       | 165 (29.8)        | <0.001 |
| <b>Creatinine<br/>(μmol/l)</b> | <b>Median<br/>n(IQR)</b> | 364 (55-1842)      | 382 (49-1840)      | 135 (51-1700)      | 116 (46-1543)    | 192 (46-1842)     | <0.001 |

|                                   |                               |             |           |           |           |            |        |
|-----------------------------------|-------------------------------|-------------|-----------|-----------|-----------|------------|--------|
| <b>Number of Systems involved</b> | <b>Median n<br/>n(IQR)</b>    | 4 (3-6)     | 3 (2-4)   | 2 (1-3)   | 2 (1-3)   | 3 (2-4)    | <0.001 |
| <b>Pulmonary haemorrhage</b>      | <b>N (%)</b>                  | 24 (20.5)   | 35 (25.2) | 19 (16.1) | 29 (15.3) | 107 (19.0) | 0.116  |
| <b>ANCA Type</b>                  | <b>Neg N(%)</b>               | 0 (0.0)     | 10 (7.2)  | 30 (25.4) | 36 (19.0) | 76 (13.5)  | <0.001 |
|                                   | <b>MPO N(%)</b>               | 0 (0.0)     | 21 (15.1) | 35 (29.7) | 60 (31.7) | 116 (20.6) |        |
|                                   | <b>PR3 N (%)</b>              | 0 (0.0)     | 31 (22.3) | 53 (44.9) | 93 (49.2) | 177 (31.4) |        |
|                                   | <b>Not measured<br/>N (%)</b> | 117 (100.0) | 77 (55.4) | 0 (0.0)   | 0 (0.0)   | 194 (34.5) |        |
| <b>Patient Survival</b>           | <b>1 year (%)</b>             | 82%         | 84%       | 86%       | 91%       | 87%        | <0.001 |
|                                   | <b>5 year (%)</b>             | 56%         | 67%       | 84%       | 88%       | 76%        |        |

**Table 3.1: Demographics and clinical features separated by decade of presentation**

MPA: microscopic polyangiitis; GPA: granulomatosis with polyangiitis; EGPA: eosinophilic granulomatosis with polyangiitis; RLV: renal-limited vasculitis; MPO: myeloperoxidase; PR3: proteinase 3.

### 3.3.2 CLINICAL FEATURES BY DECADE

The study period was divided into four decades as shown in Table 3.1. Clinical features and demographics were compared across these decades. It is shown that the largest proportion of patients presented between 2006 and 2015 (n=189 (33.6%)), with a

rather lower proportion presenting across the other decades. There were 118 patients (21%) presenting between 1996 and 2005, 139 patients between 1986 and 1995, and 117 patients (20.8%) between 1976 and 1985. In terms of age at presentation, this was similar across the decades ( $p=0.071$ ); however there were fewer patients presenting over the age of 70 years in the earliest decade when compared to the later years (Table 3.1,  $p=0.023$ ). As the decades progressed there was a difference between the proportions of patients with the different clinical diagnoses. The proportion of patients with GPA increased from 40.2% in the first decade (1976 to 1985), to 57.1% between 2006 and 2015. The proportion of patients presenting with EGPA fell after the first decade ( $p<0.001$ ) whilst the proportion of patients with MPA and RLV remained consistent over the decades (Table 3.1).

There was no ANCA serological testing between 1976 and 1985. Consequently, the proportion of patients with results for ANCA testing increased to 55% between 1986 and 1995, and from 1995 all patients were tested for ANCA ( $p<0.001$ ). As shown in Table 3.1, in the earlier decades, the number of systems involved at time of diagnosis was significantly higher than during later decades. Between 1976 and 1985, the median number of systems involved was 4 and this fell to 2 systems between 2006 and 2015 ( $p<0.001$ ). There was no difference in the proportion of patients with pulmonary haemorrhage ( $p=0.116$ ) over the decades.

Figure 3.1 illustrates changing clinical features over the decades. Figure 3.1 (a) illustrates a significantly reduced creatinine at presentation in the 2006-2015 decade compared with 1976-1985. There is a corresponding reduction in the percentage of patients requiring RRT at presentation, Figure 3.1(b). There is also a reduction in the number of clinical features a patient presents with, Figure 3.1(c), and significantly

improved patient survival at 5 years in the last compared with the first decade studied, Figure 3.1 (d).

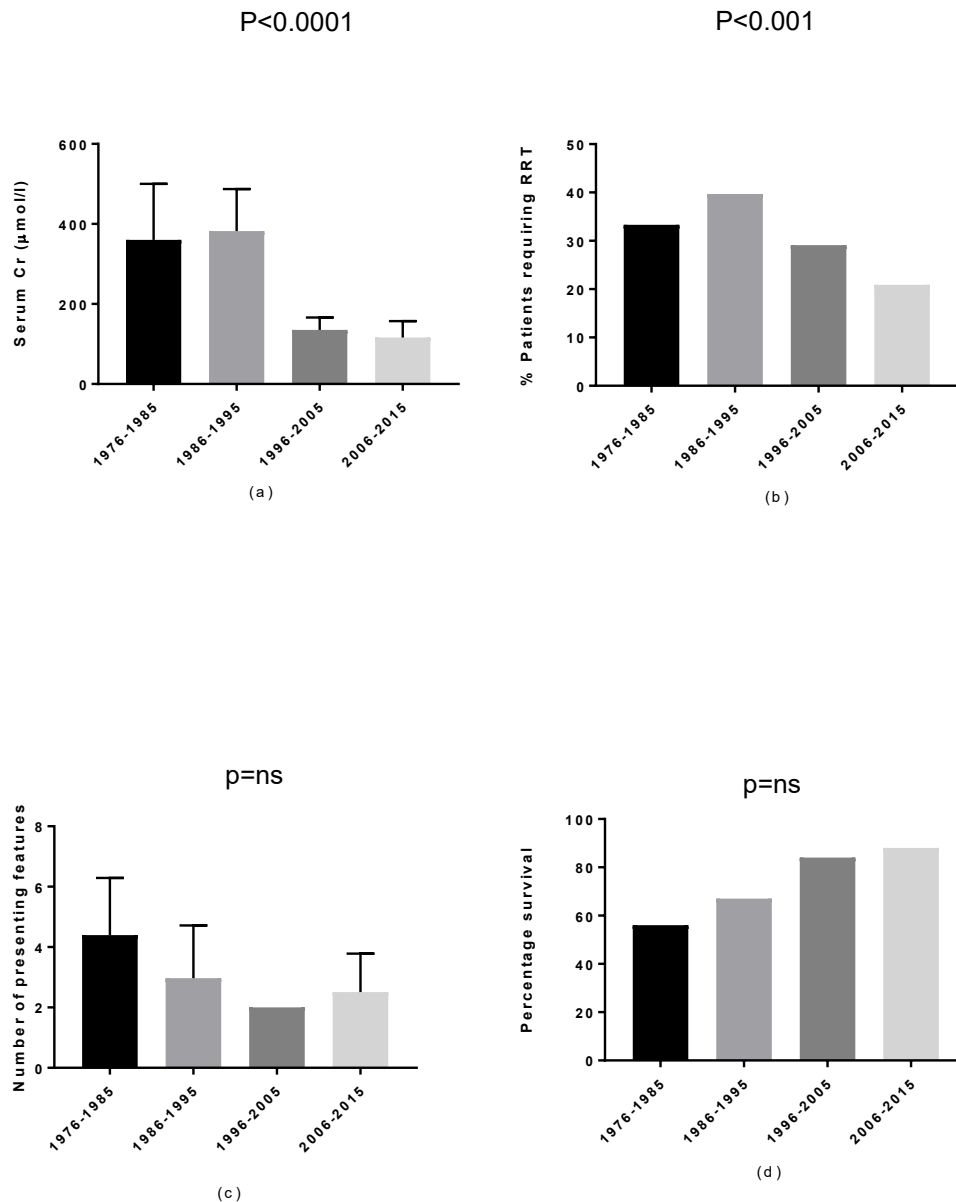


**Figure 3.1 Clinical features by different decades.**

(a) Presenting serum creatinine by decade;  $p < 0.0001$  (Kruskal Wallis); (b) Percentage of patients requiring RRT according to decade  $p < 0.001$  (Kruskal Wallis); (c) Number of presenting features by decade  $p = \text{ns}$  (Kruskal Wallis); (d) Percentage patient survival at 5 years according to decade  $p = \text{ns}$  (Kruskal Wallis).

### 3.3.3 FACTORS AFFECTING RENAL FUNCTION AT PRESENTATION

Patients presenting prior to 1995 had a significantly higher serum creatinine than those presenting after 1995 (Figure 3.1a). The median serum creatinine at presentation was 364  $\mu\text{mol/l}$  between 1986 and 1995, compared to 135  $\mu\text{mol/l}$  between 1996 and 2005 ( $p<0.001$ ). There was a corresponding decrease in the proportion of patients requiring RRT at presentation, as demonstrated in Figure 3.1b. Figure 3.2 demonstrates other factors affecting presenting creatinine. Patients presenting with pulmonary haemorrhage were demonstrated to have a higher presenting serum creatinine than those without (345 $\mu\text{mol/l}$  vs. 130 $\mu\text{mol/l}$ ;  $p<0.001$ ) (Figure 3.2a). As expected, the median serum creatinine among patients who required RRT at presentation was significantly higher than for those who were RRT-independent (580  $\mu\text{mol/l}$  vs. 103  $\mu\text{mol/l}$ ;  $p<0.001$ ) (Figure 3.2b). Serum creatinine at presentation was also shown to be increased with increasing age ( $p<0.001$ ) (Figure 3.2c). Patients with RLV had the highest presenting serum creatinine (596  $\mu\text{mol/l}$ ), followed by patients with MPA (267  $\mu\text{mol/l}$ ), GPA (110  $\mu\text{mol/l}$ ), and EGPA (100 $\mu\text{mol/l}$ ) ( $p<0.001$ ) (Figure 3.2e). Patients who had not undertaken ANCA testing presented with the highest serum creatinine (283  $\mu\text{mol/l}$ ), followed by MPO positive patients (253  $\mu\text{mol/l}$ ), PR3 positive patients (161  $\mu\text{mol/l}$ ), and ANCA negative patients (81  $\mu\text{mol/l}$ ) ( $p<0.001$ ) (Figure 3.2f). There

was no statistically significant difference between the starting creatinine of patients according to the number of presenting clinical features (Figure 3.1c).

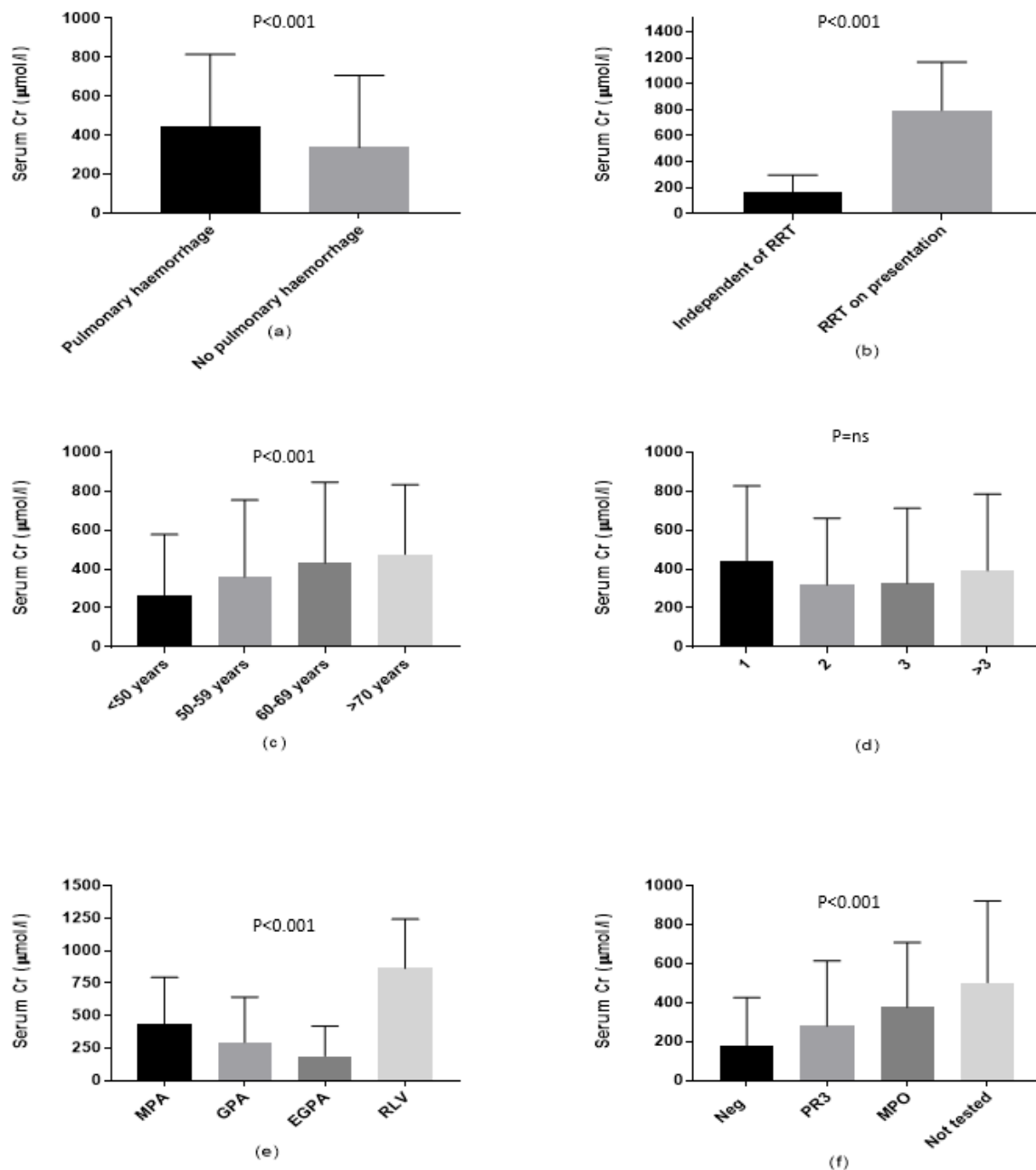


**Figure 3.2: Factors affecting serum creatinine (Cr) at presentation**

(a) Differences in serum Cr by pulmonary haemorrhage; (b) Starting serum Cr in patients presenting dialysis dependent vs those independent of dialysis; (c) Starting serum Cr compared across age groups; (d) Association of serum Cr with number of systems affected at presentation; (e) Serum Cr according to clinical diagnosis; (f) Serum Cr by ANCA type. Data are presented as bar charts with medians and interquartile range. Differences between categorical data are compared by chi-squared

test. Differences in column proportions of categorical variables were assessed using adjusted standardised residuals and Bonferroni-adjusted p-values.

Multivariate analysis was also carried out to assess factors affecting RRT at presentation. This analysis is shown in Table 3.2. As demonstrated, patients presenting between 1976 and 1985 (adjOR 2.94 (95% CI 1.52 to 5.66);  $p < 0.001$ ) and between 1986 and 1995, (adjOR 2.59 (95% CI 1.44 to 4.64);  $p < 0.001$ ) had greater odds of RRT requirement at presentation than those presenting between 2006 and 2014. Patients with a diagnosis of RLV were found to have significantly greater odds of RRT requirement compared to those with MPA (adjOR 0.13 (95% CI 0.04 to 0.49);  $p = 0.002$ ), GPA (adjOR 0.08 (95% CI 0.02 to 0.29);  $p < 0.001$ ), or EGPA (adjOR 0.03 (95% CI 0.01 to 0.013);  $p < 0.001$ ). Increased age was also associated with a higher likelihood of RRT requirement at presentation, with patients over 70 years being more likely to require RRT than those under 50 years (adjOR 0.32 (95% CI 0.17 to 0.60);  $p < 0.001$ ) and patients between 50 and 59 years (adjOR 0.49 (95% CI 0.25 to 0.95);  $p = 0.036$ ). Patients who did not have ANCA testing had a greater odds of requiring RRT compared to patients who were MPO positive (adjOR 0.17 (95% CI 0.05 to 0.54);  $p < 0.001$ ) or patients who were ANCA negative (adjOR 0.40 (95% CI 0.17 to 0.95);  $p = 0.040$ ). We acknowledge the limitation that the ANCA not tested group could be seen as a surrogate for patients who were diagnosed earlier in the study and that this could be a confounding factor. Patients for whom pulmonary haemorrhage was a presenting feature were also shown to be at higher risk of requiring RRT than those for whom this was not a disease manifestation (adjOR 2.17 (95% CI 1.33 to 3.57);  $p = 0.002$ ) (Table 3)

|                  | <b>Odds ratio</b>            | <b>P-value</b> |
|------------------|------------------------------|----------------|
|                  | <b>(Confidence interval)</b> |                |
| <b>Race</b>      |                              | 0.928          |
| <b>White</b>     | Reference                    |                |
| <b>Black</b>     | 1.139 (0.33-3.92)            | 0.836          |
| <b>Asian</b>     | 1.039 (0.54-2.01)            | 0.909          |
| <b>Other</b>     | 0.767 (0.32-1.86)            | 0.557          |
| <b>Diagnosis</b> |                              | <0.001         |
| <b>RLV</b>       | Reference                    |                |
| <b>MPA</b>       | 0.133 (0.04-0.49)            | 0.002          |
| <b>GPA</b>       | 0.079 (0.02-0.29)            | <0.001         |
| <b>EGPA</b>      | 0.026 (0.01-0.13)            | <0.001         |
| <b>Decade</b>    |                              | <0.001         |
| <b>2006-2015</b> | Reference                    |                |
| <b>1976-1985</b> | 2.935 (1.52-5.66)            | 0.001          |
| <b>1986-1995</b> | 2.585 (1.44-4.64)            | 0.001          |

|                                 |                   |        |
|---------------------------------|-------------------|--------|
| <b>1996-2005</b>                | 0.857 (0.44-1.67) | 0.650  |
| <b>Age range</b>                |                   | 0.005  |
| <b>&gt;70</b>                   | Reference         |        |
| <b>&lt;50</b>                   | 0.318 (0.17-0.60) | <0.001 |
| <b>50-59</b>                    | 0.485 (0.25-0.95) | 0.036  |
| <b>60-69</b>                    | 0.551 (0.29-1.05) | 0.070  |
| <b>ANCA type</b>                |                   | 0.02   |
| <b>Not tested</b>               | Reference         |        |
| <b>MPO</b>                      | 0.168 (0.05-0.54) | <0.001 |
| <b>PR3</b>                      | 0.597 (0.23-1.53) | 0.28   |
| <b>Negative</b>                 | 0.397 (0.17-0.95) | 0.04   |
| <b>Total number of features</b> | 1.008 (0.88-1.16) | 0.915  |
| <b>Lung haemorrhage</b>         | 0.455 (0.28-0.75) | 0.002  |

---

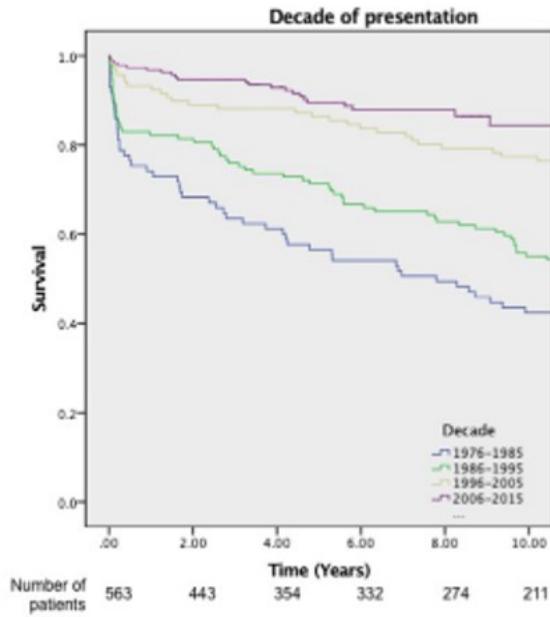
**Table 3.2: Factors associated with renal replacement therapy at presentation**

MPA: microscopic polyangiitis; GPA: granulomatosis with polyangiitis; EGPA: eosinophilic granulomatosis and polyangiitis; RLV: renal-limited vasculitis; MPO: myeloperoxidase; PR3: proteinase 3.

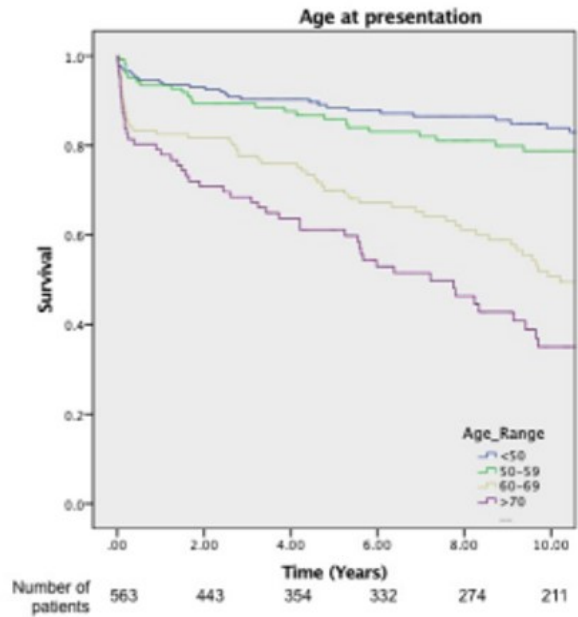
### 3.4 OUTCOMES

The main outcome assessed in this study was patient survival. Table 3.4 is an analysis of factors associated with mortality by Cox proportional hazards. The patient survival for the entire cohort was 87% at 1 year and 76% at 5 years. In the period 1976 to 1985, 1 year survival was 73%, and 5 year survival 56%. In the decade 2006 to 2015 survival was up to 91% at 1 year and 88% at 5 years (Table 3.2). On univariate analysis, factors at presentation found to be predictors of mortality included advancing age ( $p<0.001$ ), ethnicity ( $p<0.001$ ), decade of presentation ( $p<0.001$ ), ANCA type ( $p<0.001$ ), diagnosis ( $p<0.001$ ), pulmonary haemorrhage ( $p=0.009$ ), RRT ( $p<0.001$ ), and number of systemic features ( $p=0.008$ ).

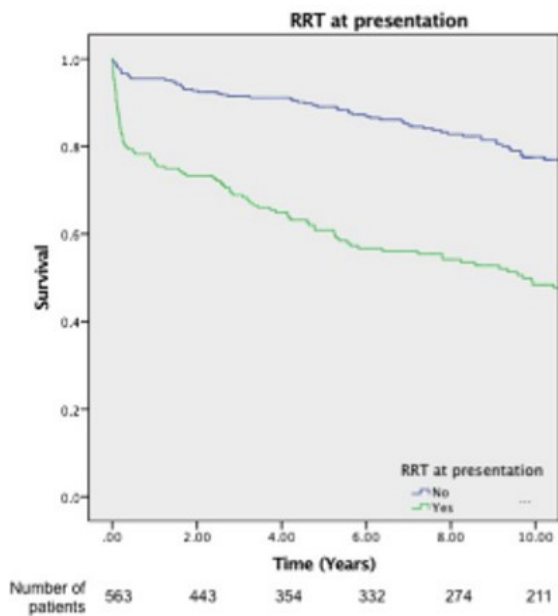
Figures 3.3 and 3.4 show unadjusted long- term patient survival according to the variables described, and illustrated by Kaplan-Meier plots. Figure 3.3 (a) demonstrates an improvement in survival with increasing decade of presentation ( $p<0.001$ ). Figure 3.3(b) shows a reduction in survival with increasing age at presentation. Figure 3.3 (c) illustrates better survival in patients presenting with independent renal function than those presenting on RRT ( $p<0.001$ ). Figure 3.3 (d) shows poorer survival in patients for whom pulmonary haemorrhage was a presenting feature ( $p<0.001$ ). Figure 3.4(a) compared survival for the different clinical syndromes and shows the best survival in patients with GPA and EGPA, and the poorest for patients with RLV ( $p<0.001$ ). Figure 3.4 (b) confirms the previous findings that patients without a test for ANCA had significantly poorer survival than those who had been tested, and either found to be negative or positive for MPO or PR3 ( $p<0.001$ ).



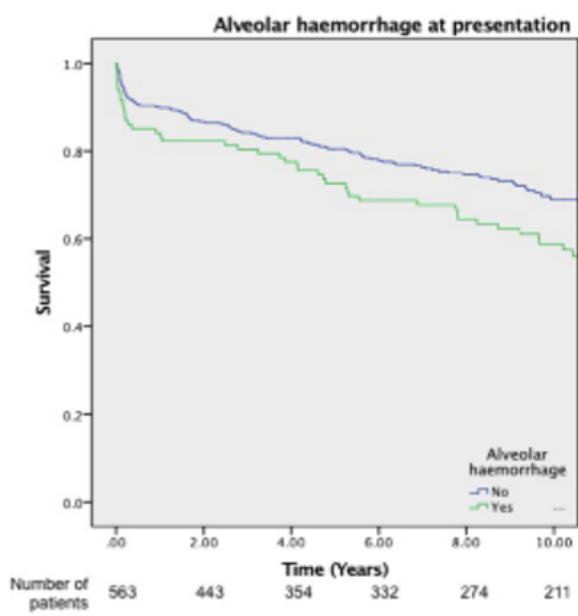
(a)



(b)



(c)



(d)

**Figure 3. 3: Factors affecting patient survival**

Unadjusted long-term patient survival depicted by Kaplan-Meier plots. Follow up time (x-axis) is illustrated in years against the proportion surviving (y-axis). Differences in survival between groups are assessed by log rank test. (a) Patient survival by decade of presentation ( $p < 0.001$ ); (b) Patient survival by age at presentation ( $p < 0.001$ ); (c) Patient survival by RRT at presentation ( $P < 0.001$ ); (d) Patient survival by pulmonary haemorrhage ( $p < 0.001$ ).

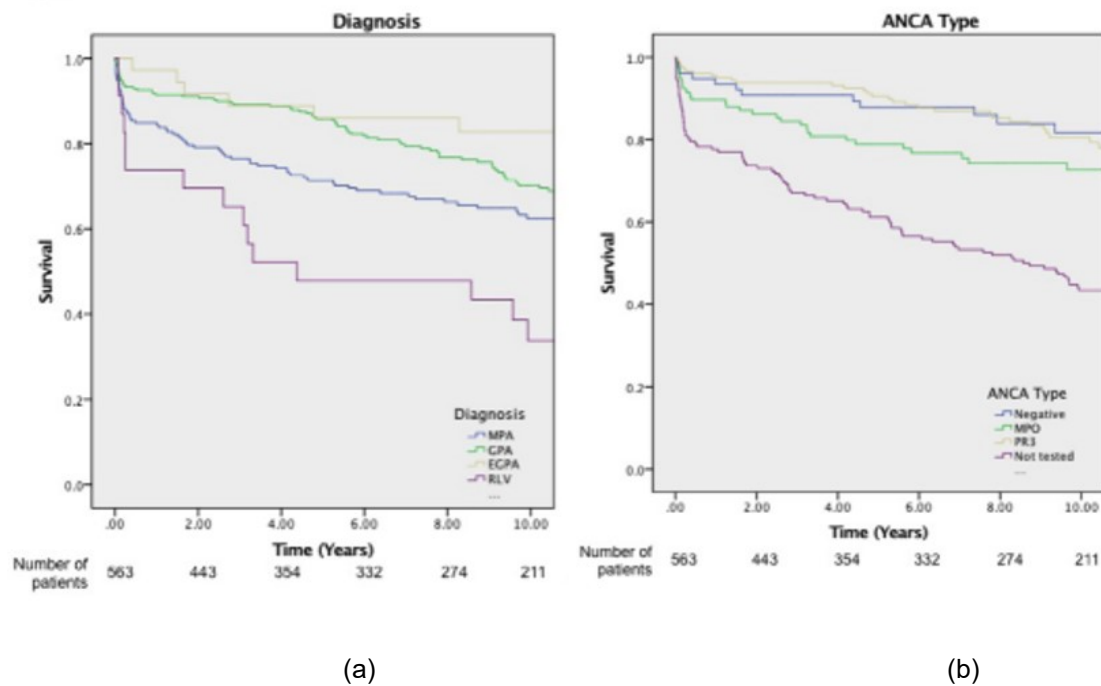


**Figure 3. 4: Factors affecting patient survival (2)**

Unadjusted long-term patient survival depicted by Kaplan-Meier plots. Follow up time (x-axis) is illustrated in years against the proportion surviving (y-axis). Differences in survival between groups are assessed by log rank test. (a) Patient survival by diagnosis ( $p < 0.001$ ); (b) Patient survival by ANCA type ( $p < 0.001$ ).

Upon multivariate analysis, age (adjHR 1.04 (95% CI 1.02 to 1.05),  $p < 0.001$ ), renal replacement therapy (adjHR 2.04 (95% CI 1.55 to 2.69),  $p < 0.001$ ), ANCA type ( $p < 0.001$ ) and decade of presentation ( $p < 0.001$ ) were independently associated with long-term patient survival (Table 3.3). When compared with patients presenting in the latest decade, between 2006 and 2015, patients with AAV who presented between 1976 and 1985 (adjHR 5.77 (95% CI 3.36 to 9.91);  $p < 0.001$ ) and 1986 to 2005 (adjHR 3.24 (95% CI 1.98 to 5.31);  $p < 0.001$ ) had a significantly worse prognosis in terms of mortality. Patients for whom ANCA testing was not available had a higher risk of mortality compared to those who had tested positive for PR3 (adjHR 0.35 (95% CI 0.26 to 0.50),  $p < 0.001$ ), MPO (adjHR 0.33 (95% CI 0.18 to 0.59),  $p < 0.002$ ), or tested but found to be ANCA negative (adjHR 0.36 (95% CI 0.26 to 0.50),  $p < 0.001$ ).

Pulmonary haemorrhage (adjHR 0.86 (0.61 to 1.20)), p=0.371) was no longer associated with mortality on multivariate testing.

|                               | Unadjusted                             |         | Adjusted                                        |         |
|-------------------------------|----------------------------------------|---------|-------------------------------------------------|---------|
|                               | Hazards ratio<br>(Confidence interval) | p-value | Adjusted hazards ratio<br>(Confidence interval) | p-value |
| <b>Age (years)</b>            | 1.04 (1.03-1.05)                       | <0.001  | 1.04 (1.02-1.05)                                | <0.001  |
| <b>Diagnosis</b>              |                                        | <0.001  |                                                 | 0.154   |
| <b>RLV</b>                    | Reference                              |         | Reference                                       |         |
| <b>MPO</b>                    | 0.58 (0.35-0.95)                       | 0.030   | 0.84 (0.48-1.43)                                | 0.287   |
| <b>GPA</b>                    | 0.41 (0.25-0.67)                       | <0.001  | 0.70 (0.40-1.24)                                | 0.225   |
| <b>EGPA</b>                   | 0.34 (0.18-0.65)                       | 0.001   | 0.45 (0.21-0.94)                                | 0.713   |
| <b>RRT at presentation</b>    | 2.78 (2.14-3.61)                       | <0.001  | 2.04 (1.55-2.69)                                | 0.003   |
| <b>Alveolar haemorrhage</b>   | 1.46 (1.10-1.95)                       | 0.009   | 0.86 (0.61-1.20)                                | 0.371   |
| <b>Total Features</b>         | 1.10 (1.03-1.18)                       | 0.008   | 1.02 (0.94-1.11)                                | 0.642   |
| <b>ANCA type</b>              |                                        | <0.001  | -                                               | <0.001  |
| <b>Not tested</b>             | Reference                              | -       | Reference                                       | -       |
| <b>MPO</b>                    | 0.58 (0.35-0.95)                       | <0.001  | 0.33 (0.18-0.59)                                | <0.001  |
| <b>PR3</b>                    | 0.41 (0.25-0.67)                       | <0.001  | 0.35 (0.24-0.52)                                | <0.001  |
| <b>Negative</b>               | 0.34 (0.18-0.65)                       | <0.001  | 0.36 (0.26-0.50)                                | <0.001  |
| <b>Gender (Male)</b>          | 0.92 (0.71-1.19)                       | 0.543   | 0.911 (0.69- 1.21)                              | 0.517   |
| <b>Ethnicity</b>              |                                        | <0.001  | -                                               | 0.491   |
| <b>White</b>                  | Reference                              |         |                                                 |         |
| <b>Black</b>                  | 0.40 (0.13-1.25)                       | 0.114   | 1.56 (0.71-3.41)                                | 0.270   |
| <b>Asian</b>                  | 0.30 (0.17-0.55)                       | <0.001  | 0.90 (0.57-1.43)                                | 0.653   |
| <b>Other</b>                  | 0.50 (0.23-1.51)                       | 0.070   | 1.88 (1.01-2.30)                                | 0.027   |
| <b>Decade of presentation</b> |                                        | <0.001  |                                                 | <0.001  |
| <b>2006-2015</b>              | Reference                              |         | Reference                                       |         |
| <b>1976-1985</b>              | 5.28 (3.44-8.60)                       | <0.001  | 5.77 (3.36-9.91)                                | <0.001  |
| <b>1986-1995</b>              | 3.49 (2.16-5.64)                       | <0.001  | 3.24 (1.98-5.31)                                | <0.001  |
| <b>1996-2005</b>              | 1.68 (0.99-2.87)                       | 0.057   | 1.67 (0.96-2.89)                                | 0.068   |
|                               |                                        | <0.001  | -                                               | -       |

|                            |                  |        |   |   |
|----------------------------|------------------|--------|---|---|
| <b>Creatinine (μmol/L)</b> |                  |        |   |   |
| <b>&lt;120</b>             | Reference        |        | - | - |
| <b>&gt;500 or RRT</b>      | 2.92 (2.07-4.12) | <0.001 | - | - |
| <b>250- 500</b>            | 2.09 (1.27-3.42) | <0.001 | - | - |
| <b>120-249</b>             | 1.33 (0.87-2.03) | 0.190  | - | - |
| <b>Age range (years)</b>   |                  | <0.001 | - | - |
| <b>&gt;70</b>              | Reference        |        | - | - |
| <b>&lt;50</b>              | 0.22 (0.15-0.32) | <0.001 | - | - |
| <b>50-59</b>               | 0.32 (0.22-0.48) | <0.001 | - | - |
| <b>60-69</b>               | 0.68 (0.48-0.96) | 0.029  | - | - |

**Table 3.3: Analysis of factors associated with mortality by Cox proportional hazards**

MPA: microscopic polyangiitis; GPA: granulomatosis with polyangiitis; EGPA: eosinophilic granulomatosis with polyangiitis; RLV: renal-limited vasculitis; MPO: myeloperoxidase; PR3: proteinase 3; Adjusted HR (CI): Hazards ratio (confidence intervals).

## 3.5 DISCUSSION

Patient cohorts of this size at a single centre, treated in a uniform fashion, are not common. We have been fortunate in obtaining data from our centre for over 500 patients with AAV spanning four decades. In terms of demographics, the patients in this cohort presented at a median age of 55 years, in keeping with other studies carried out during this time. There were similar proportions of male and female patients and the majority had a self-reported ethnicity of white/Caucasian, also in keeping with the literature. Thirteen percent of our patients were of Indo-Asian origin consistent with the population served by our centre and the proportion of patients with EGPA increased over the decades. Thirty seven percent of patients had a diagnosis of MPA, and the remainder, either EGPA or RLV. Once ANCA testing was established, and by the third decade of analysis, all patients were tested for ANCA. Thirteen percent of those tested were found to be ANCA negative, 21% MPO-ANCA positive and 32% PR3-ANCA positive. The median serum creatinine at presentation was 157 for the whole group and 30% of patients were dialysis dependent at presentation. Pulmonary

haemorrhage was a feature in 19% of patients. Both dialysis dependence and pulmonary haemorrhage are associated with poor prognosis in AAV and at our centre are treated with plasma exchange.

Patients with AAV have an increased standardized mortality ratio of death compared to the general population (129, 134, 138). Age at presentation is a factor which consistently predicts survival (131-134). Patient survival is significantly reduced with increasing age at presentation. In our study, although the median age was similar between decades, there was a lower proportion of patients over the age of 70 in the earliest decade. By 2006-2015, 17% of patients were over the age of 70 at presentation. This was as low as 6% in the 1976-1985 decade. It is possible that at this time, prior to ANCA testing, these older patients, with other co-morbidities, may not have received a diagnosis of AAV. It is also possible that during this time period, there was less referral of older patients to specialist services.

We demonstrate improved patient survival over the four decades, following correction for baseline differences in age, RRT requirement, pulmonary haemorrhage, number of clinical features, ANCA type and testing, and diagnosis. This is in keeping with a number of other studies reporting outcomes of patients with AAV between 1975 and 2010 (131-134, 138). There have been some studies, however, where patient survival has not improved over time (133, 139). It has been proposed that this could be in part explained by the increased age of presentation of patients in the later decades and their associated co-morbidities (139).

In our cohort, improvement in outcome over the decades could be attributed, in part, to the ability to test for serum ANCA and make an earlier diagnosis. There was a “stepwise” reduction in the presenting creatinine over the four decades. This appears

to have coincided with the introduction of ANCA testing. In the earlier decades, prior to testing, studies have reported longer intervals between the onset of symptoms and diagnosis of AAV (131, 138, 139). It is likely that patients experienced greater degrees of renal decline due to these delays. Following the introduction of ANCA testing, patients in our cohort were also found to have fewer presenting features, likely as their time to diagnosis was reduced. The fall in presenting creatinine over the decades and reduction in the proportion of patients requiring RRT can therefore be, at least in part, attributed to the introduction of ANCA testing. Patients who have not been tested for ANCA have a significantly greater mortality than other patients. ANCA testing is thus likely to have been a significant factor to improved patient survival over time. However, it needs to be considered that patients for whom ANCA testing was not available largely presented from the earlier decades. Immunotherapy, with its known associated toxicity, has evolved and become more targeted over the years. There is also increased knowledge of and monitoring of side effects. This, coupled with advances in general medical care may also have contributed to reduction in mortality in our cohort over time.

Renal dysfunction and increased renal damage on histological analysis are consistently associated with increased mortality (128, 131-134, 138, 140). We found a stepwise increment in hazard ratio of death with increasing creatinine levels. Consistent with other studies, patients requiring RRT at presentation had an increased risk of long term mortality. Predictors of RRT at presentation included advancing age, diagnosis of RLV, presentation in earlier decades, lack of ANCA testing and pulmonary haemorrhage. Older patients were also more likely to require RRT at presentation, which may relate to underlying co-morbid disease. Increased disease severity may explain why patients with pulmonary haemorrhage had a higher serum creatinine at

presentation and were more likely to require RRT than those without. The association between lack of ANCA testing and need for RRT on presentation is likely to reflect a delay in diagnosis. Consistent with other studies, patients with RLV presented with a higher serum creatinine and were more likely to require RRT than patients with GPA, MPA or EGPA (141). This could represent later diagnosis due to the lack of multisystem symptomatic disease. For our entire cohort of AAV patients, we found that the greater the number of organ systems involved, the lower the creatinine. Consequently, patients with RLV had a higher mortality than other diagnoses on unadjusted analysis. However, when adjusted for renal function and age, we found no differences in survival between RLV and other AAV clinical syndromes, as demonstrated in other studies (134, 141). Over half of the patients who presented dialysis dependent became dialysis independent following treatment, at one year. Renal survival by decade was not analysed due to confounding survival bias (with a larger proportion of deaths in earlier years).

The main limitations of this study were its retrospective nature, size and timescale. Patient data has been recorded on different systems over the years and this has limited data collection. A number of patients were also lost to follow up or died outside of this centre. The main aim of this study was to determine features on presentation which could be predictive of long-term patient survival. We have not included data on renal histopathology or renal outcomes, this is commented on in Chapters 4 and 5. We have described our published treatment regimens, but were unable to obtain precise details of cumulative doses of medications or adverse effects.

The main strength of this study is the inclusion of a large cohort of patients followed up for a long period of time at a single centre with uniformity of management.

Diagnostic criteria were consistent, and treatment was by agreed protocols. This has allowed robust statistical analysis of factors associated with long- term mortality.

### 3.6 CONCLUSION

We have demonstrated that in addition to age and requirement for renal replacement therapy at presentation, the decade of diagnosis is a significant factor determining patient survival in AAV. Early diagnosis facilitated by ANCA testing, advances in immunotherapy and better supportive care, are likely to have contributed to this.

## CHAPTER 4 - EVALUATION OF THE INTERNATIONAL HISTOLOGICAL CLASSIFICATION OF ANCA ASSOCIATED VASCULITIS

## 4.1: INTRODUCTION

As discussed previously, one of the major challenges in the management of AAV, is the identification of factors predictive of prognosis, which can then be useful in determining a patient specific plan for immunosuppression. In the previous chapter, factors affecting outcome have been described for the entire cohort of patients with AAV treated at our centre. In this chapter the results of a study primarily assessing histological factors affecting outcome will be described. This work has been published in the journal Nephrology Dialysis Transplantation (NDT), in 2015 (140).

The renal biopsy remains the “gold standard” for diagnosis of renal AAV. A number of histopathological features have been reported to correlate with renal prognosis in AAV. For example, studies have shown a marked correlation between the proportion of normal glomeruli on biopsy, and renal outcome (18, 142-146). A higher proportion of crescents has also been associated with improved response to therapy (143). Sclerotic glomeruli are characteristically associated with poor renal prognosis and reduced response to treatment (146). A higher percentage of tubular atrophy on renal biopsy has also been correlated with poorer outcome in AAV (147).

A number of renal diseases including lupus and IgA nephropathy, have an established histological classification system (148, 149). The histological classification guides immunosuppressive therapy, and also enables appropriate stratification and uniformity of reporting of results of clinical trials in these diseases. The first histological classification of GN in AAV was proposed by an international working group of renal pathologists in 2010, and was described by Berden *et al* (128).

An international working group of renal pathologists divided biopsies into four subgroups, focal, crescentic, mixed and sclerotic, based on histological features on light microscopy. All biopsies were characterised as pauci-immune and included at least 10 glomeruli for analysis. The focal group was defined as those biopsies with  $\geq 50\%$  normal glomeruli. Biopsies in the crescentic group contained  $\geq 50\%$  crescents and those in the sclerotic group  $\geq 50\%$  globally sclerotic glomeruli. The mixed category included biopsies with  $<50\%$  normal,  $<50\%$  crescentic and  $<50\%$  sclerotic glomeruli.

A number of validation studies have been carried out to evaluate the prognostic value of this classification system. The first of these assessed its use in 100 patients recruited in EUVAS trials from 32 centres across 9 European countries (128). This study reported favourably on the prognostic value of the classification system at 1 and 5 years, with the best outcome in the focal category and the least favourable in the sclerotic. The crescentic category was associated with better survival than the mixed. Following this, a number of validation studies have been carried out with varying results (140, 150-154) (155, 156). One of the largest of these studies was a Norwegian study carried out by Bjornekleit *et al* (154). This study included 250 patients with AAV and concluded that the international classification was a useful predictor of outcome with patients in the focal category experiencing the best and the sclerotic category the worst outcomes. There was no significant difference in outcome found between the mixed and crescentic categories. The prognostic accuracy of the classification system was also assessed using receiver-operator curve characteristics, and determined to be of moderate quality (154).

The different validation studies, some of which were carried out after our study, have largely confirmed the value of the international classification system however there

has been some variability, in particular with regards to the mixed and crescentic classes. Chen *et al*, carried out a meta-analysis in addition to their validation study of the classification system (157). This meta-analysis included 1601 patients from 16 worldwide studies and confirmed the best outcome in the focal class, intermediate outcomes in the mixed and crescentic classes, and the poorest outcomes in the sclerotic classes. Results were similar across the different populations with the implication that the classification can be used across different ethnic groups. As acknowledged by the authors of this study, it was limited by its retrospective nature and also due to variations with regards to information available for treatment, BVAS scores and eGFR assessment. Despite these limitations however, this meta-analysis has been influential in the validation of this classification system.

The majority of studies mentioned have also suggested the inclusion of additional parameters such as percentage normal glomeruli and degree of tubular atrophy as formal components. The aim of this chapter was thus to evaluate the International classification system for AAV in the patients treated uniformly at a single centre, and in doing so identify other possible histopathological features of potential prognostic value.

## 4.2 METHODS

### 4.2.1 DATA COLLECTION

The renal biopsies of 104 patients presenting between 1997 and 2011 with a diagnosis of AAV as defined by the Chapel Hill Consensus Criteria (158), were reviewed. Patients were excluded if there were fewer than 10 glomeruli present on renal biopsy, if there was no follow up data available within one year, or if there was evidence of concomitant renal disease including anti-GBM disease, diabetic nephropathy or IgA nephropathy.

Patient records and electronic data were retrospectively reviewed. Demographic data were recorded, in addition to ANCA titres, presenting renal function and renal and patient outcomes. Biopsies were reviewed by a renal histopathologist, blinded to outcomes. They were classified according to the International Classification (Berdn *et al*, figure 4.3.1). Information was also collected on tubular atrophy. Patients were divided into three groups according to degree of tubular atrophy <20%, 20-50% and >50%. The four variable Modification of Diet in Renal Disease study questionnaire (MDRD4) was used to calculate eGFR. Patients were described as dialysis dependent at presentation if they required dialysis within 48 hours of their admission to hospital. The end point of renal death was used for those who had developed end stage renal disease.

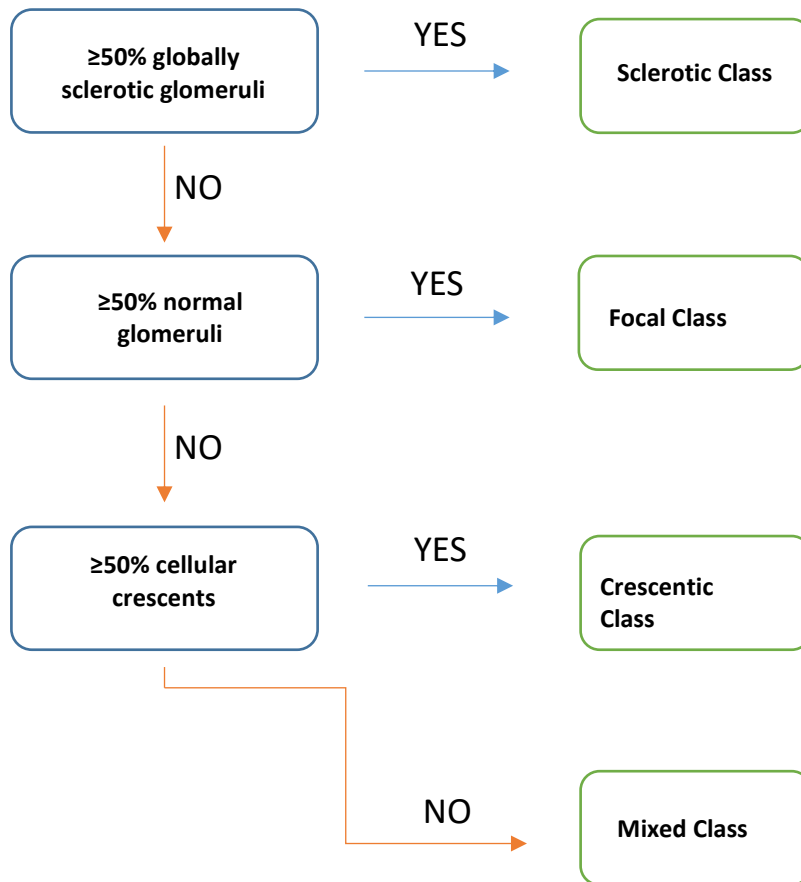


**Figure 4.3.1 Schematic for the International Classification for AAV**

Adapted from Berden *et al*, 2010 (128). Patients' biopsies were classified according to the chart above.

#### 4.2.2 TREATMENT AND OUTCOMES

For patients presenting between 1997 and 2006, treatment was according to established vasculitis protocols for our centre (159). Ninety-five percent of patients received cyclophosphamide as induction therapy, either intravenous or oral, with steroids as induction treatment, followed by azathioprine and oral steroids from 3 months, with mycophenolate mofetil (MMF) for those who were intolerant of azathioprine. Patients with organ threatening disease, including dialysis requirement, pulmonary haemorrhage or cerebral vasculitis were treated in addition, with plasma exchange on induction. Twenty eight out of the 104 patients included, underwent plasma exchange.

In 2006, the protocol included the addition of rituximab as induction therapy, with the aim of reducing cumulative exposure to cyclophosphamide and steroids (160). Forty one of the patients included in this study were treated with rituximab, usually together with low dose IV cyclophosphamide. Clinical endpoints recorded included eGFR at 1 and 5 years and development of ESRD.

#### 4.2.3 STATISTICAL ANALYSIS

(As described in paper Tanna *et al*, multivariate analysis was carried out by Dr. Benjamin Caplin).

Variables are presented as mean (SD) or median [interquartile range (IQR)] according to their distribution. One way ANOVA, X2 test and Fisher's exact test were used to compare continuous variables between groups and categorical data as appropriate. The Kaplan –Meier survival analysis was carried out to calculate the probability of renal survival. Log –rank tests were used to assess differences across categories of

variables studied. All p-values are two sided, with significance defined as  $p < 0.05$ . Analyses were carried out using Graphpad PRISM (version 4) and SPSS (version 19.0). Multivariable linear regression was used to examine the association between predesignated potential predictors of renal function at follow-up (baseline eGFR, age, ANCA class, degree of tubular atrophy and histological class as a categorical variable), and eGFR at 1 and 5 years. Variables were retained in the model where there was an improvement in model fit. This was judged to have occurred where there was a significant reduction in the log likelihood (using a likelihood ratio test). The association between histological classification (as a categorical variable) and renal survival (i.e. death censored renal replacement therapy) was estimated using a Cox proportional hazards model before and after adjustment for baseline eGFR. Departure from the proportional hazards assumption was tested by examining Schoenfeld residuals both globally and for each covariate.

## 4.3 RESULTS

### 4.3.1 DEMOGRAPHICS

One hundred and four patients were included in this study. The median age of patients was 62 years (17.3-87.2) and 56% of patients were male. Seventy-six percent of patients reported their ethnicity as Caucasian, 14% of patients Indoasian, and 6% of patients Afrocaribbean. Equal numbers of patients were anti-PR3 and anti-MPO positive and the mean eGFR was 30.3 ml/min/1.73 m<sup>2</sup>. Eight patients were dialysis dependent at time of biopsy. There was a higher proportion of deaths at last follow up in the mixed (35.2%) and sclerotic (42.9%) categories, than the crescentic (17.1%) and focal (17.4%) categories. The demographics of these patients are described in Table 4.3.1.

|                                                | <b>Focal</b>        | <b>Crescentic</b>   | <b>Mixed</b>        | <b>Sclerotic</b>    | <b>Total</b>         |
|------------------------------------------------|---------------------|---------------------|---------------------|---------------------|----------------------|
| <b>N</b>                                       | 23                  | 26                  | 48                  | 7                   | 104                  |
| <b>Median age (in years)</b><br><b>(range)</b> | 61.2<br>(28.9-80.4) | 54.9<br>(17.3-74.3) | 66.3<br>(23.7-87.2) | 66.6<br>(51.1-76.9) | 62.2<br>(17.3-87.2)  |
| <b>Male sex n (%)</b>                          | 10(43)              | 15(58)              | 29(60)              | 4 (57)              | 58 (56)              |
| <b>Ethnicity</b>                               |                     |                     |                     |                     |                      |
| Caucasian n (%)                                | 19(83)              | 16 (61)             | 37(77)              | 4.(57)              | 76(73)               |
| Indoasian n (%)                                | 2(9)                | 6 (23)              | 5(10)               | 1 (14)              | 14 (13)              |
| Afrocaribbean n (%)                            | 1(4)                | 2 (8)               | 2(4)                | 1 (14)              | 6 (6)                |
| Other n (%)                                    | 1(4)                | 2 (8)               | 4(8)                | 1(14)               | 8 (8)                |
| <b>ANCA Status</b>                             |                     |                     |                     |                     |                      |
| PR3 (%)                                        | 7(30)               | 17(65)              | 24 (50)             | 1(14)               | 49 (47)              |
| MPO (%)                                        | 14(60)              | 8 (31)              | 22 (46)             | 5 (71)              | 49 (47)              |
| ANCA negative                                  | 2(9)                | 1(4)                | 2 (4)               | 1 (14)              | 6(6)                 |
| <b>eGFR (ml/min/1.73 m<sup>2</sup>)</b>        | 51.5                | 19.5                | 28.3                | 13.5                | 30.3                 |
| <b>Histology</b>                               |                     |                     |                     |                     |                      |
| Mean total glomeruli                           | 19                  | 19                  | 19                  | 24.4                | 18.5                 |
| Normal (%)                                     | 65.5                | 26.9                | 17.0                | 2.3                 | 26.8                 |
| Crescents (%)                                  | 9.6                 | 67.7                | 21.6                | 15.9                | 29.9                 |
| Sclerotic (%)                                  | 15.3                | 5.0                 | 19.2                | 59.1                | 15.7                 |
| Tubular atrophy (%)                            | 18.4                | 8.8                 | 23.9                | 46.4                | 19.5                 |
| <b>Median time of follow in months (range)</b> | 38.0<br>(0.4-182.4) | 62.9<br>(0.3-146.3) | 39.4<br>(0.8-166.7) | 64.5<br>(0.1-149.6) | 49.9<br>(0.07-182.4) |
| <b>Patient death (%)</b>                       | 17.4                | 17.1                | 35.4                | 42.9                | 26.9                 |

**Table 4.3.1: Demographics of patients included in the study divided by histological class.**

### 4.3.2 HISTOLOGICAL CLASSIFICATION

When divided according to the International Classification (15), 23 patients were classified as focal, 26 crescentic, 48 mixed and 7 sclerotic. The demographics of these patients are described in Table 4.3.1. Table 4.3.2 demonstrates the features of the biopsies divided by histological category.

|                               | Focal | Crescentic | Mixed | Sclerotic | Total |
|-------------------------------|-------|------------|-------|-----------|-------|
| Total Glomeruli<br>(mean no.) | 19.0  | 19.0       | 19.0  | 24.4      | 18.5  |
| Normal Glomeruli<br>(%)       | 65.5  | 26.9       | 17.0  | 2.3       | 26.8  |
| Crescentic Glomeruli<br>(%)   | 9.6   | 67.7       | 21.6  | 15.9      | 29.9  |
| Sclerotic Glomeruli<br>(%)    | 15.3  | 5.0        | 19.2  | 59.1      | 15.7  |
| Tubular atrophy<br>(%)        | 18.4  | 8.8        | 23.9  | 46.4      | 19.5  |

**Table 4.3.2 Biopsy features divided according to histological category.**

Figure 4.3.2 demonstrates renal function at presentation, 1 and 5 years according to histological category. There is a significant difference in presenting eGFR across the groups. This is significantly higher in the focal category and lowest in the sclerotic ( $p=0.0001$ ). The difference was maintained at 1 year ( $p<0.0001$ ) but at 5 years, it did not reach statistical significance. Figure 4.3.3 illustrates the change in eGFR at 1 and 5 years. There was a significant difference in the categories when assessing change in eGFR at 1 year ( $p=0.0042$ ) and 5 years ( $p=0.0224$ ). Eight patients presented dialysis dependent. This included 5 patients in the crescentic category (of whom 3 recovered renal function), and 2 patients in the sclerotic category, one of whom recovered renal function. One patient in the mixed category presented dialysis dependent and did not regain independent renal function.

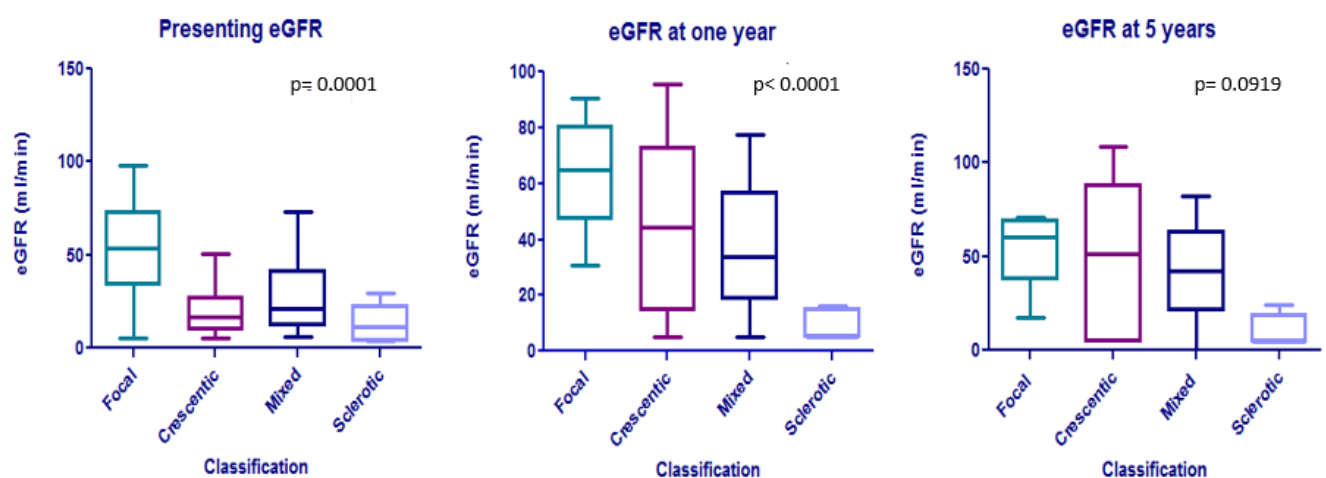


**Figure 4.3.2: Renal function at presentation, 1 and 5 years divided by histological class.**

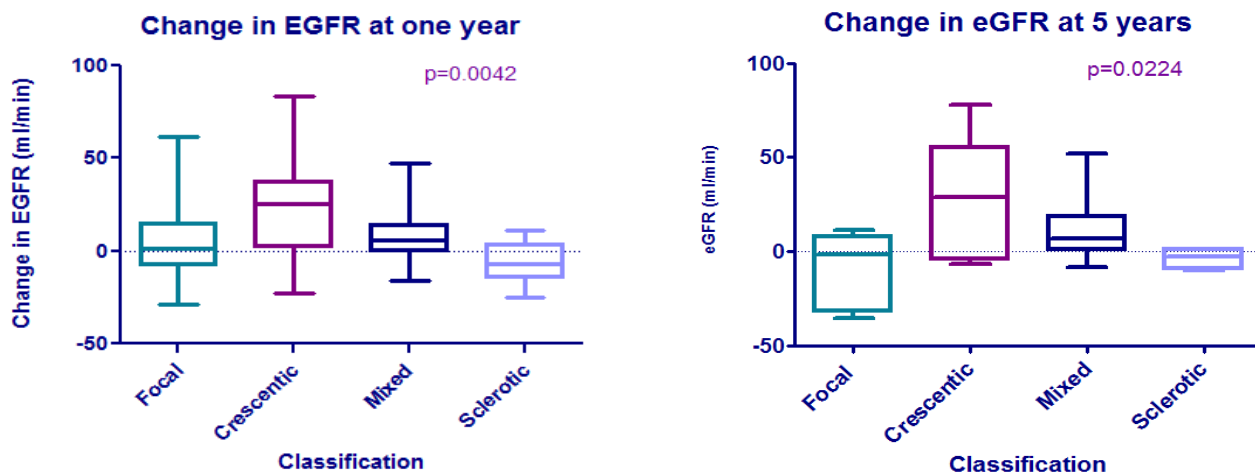


**Figure 4.3.3 Change in eGFR at 1 and 5 years divided by histological class**

Figures 4.34 (a) and (b), show a comparison of renal survival according to histological class and also assesses survival using a composite endpoint of renal and patient survival. In terms of renal survival, this was best in the focal category, followed sequentially by the mixed, crescentic and sclerotic categories (log-rank  $p=0.01$ ). At 1 year there was 100% renal survival in the focal class, 85% in the mixed, 74% in the crescentic and 50% in the sclerotic category. At 5 years, renal survival remained 100% in the focal category, mixed and crescentic categories had similar survival at 77% and 74 % respectively, and survival was only 25% in the sclerotic category. Longer term, renal survival was better in the crescentic than the mixed category.

We subdivided the mixed category further by percentage crescents (< or >20% crescents), percentage normal glomeruli (< or > 20% normal glomeruli), and percentage sclerotic glomeruli (< or >20% sclerotic glomeruli). Using these subdivisions, outcome was best in patients with a higher percentage of crescents and normal glomeruli, and worse with a higher percentage of sclerotic glomeruli.

When using a composite endpoint of patient and renal survival, findings were in keeping with those for renal survival. Survival was best at one year in patients in the focal category but this was similar to survival in the crescentic category by five years. Survival was significantly worse in the mixed and sclerotic categories.

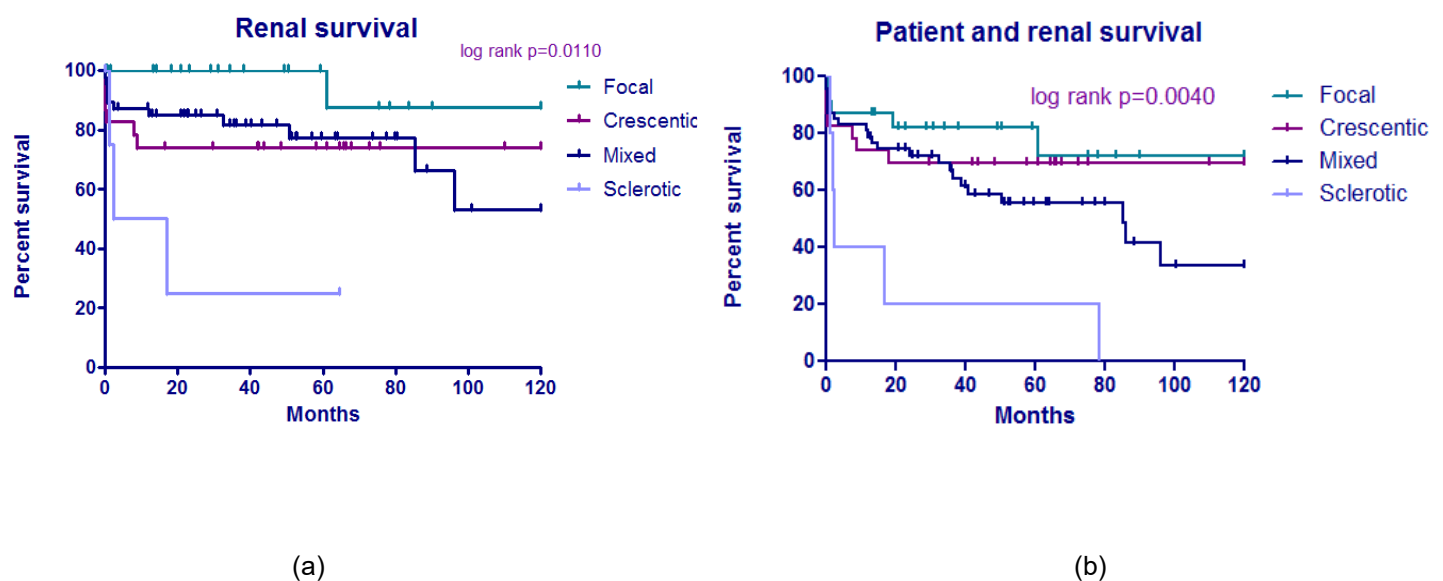


**Figure 4.3.4 Renal function according to histological class**

(a) Kaplan-Meier curve demonstrating renal survival according to histological category ( $p=0.01$ ) and (b) Kaplan-Meier curve assessing a combined endpoint of patient and renal survival divided by histological category ( $p=0.004$ ).

### 4.3.3 PERCENTAGE NORMAL GLOMERULI

Figure 4.3.5(a) demonstrates eGFR at presentation, 1 and 5 years divided by percentage normal glomeruli at presentation. There was a significant difference across the groups at presentation at 1 year, with a larger proportion of normal glomeruli correlating with an improved eGFR. This difference was not maintained at 5 years likely due to numbers being reduced at this time point.

Figure 4.3.5 (b) shows significantly improved renal survival with an increased percentage of normal glomeruli (log rank  $p=0.04$ ).

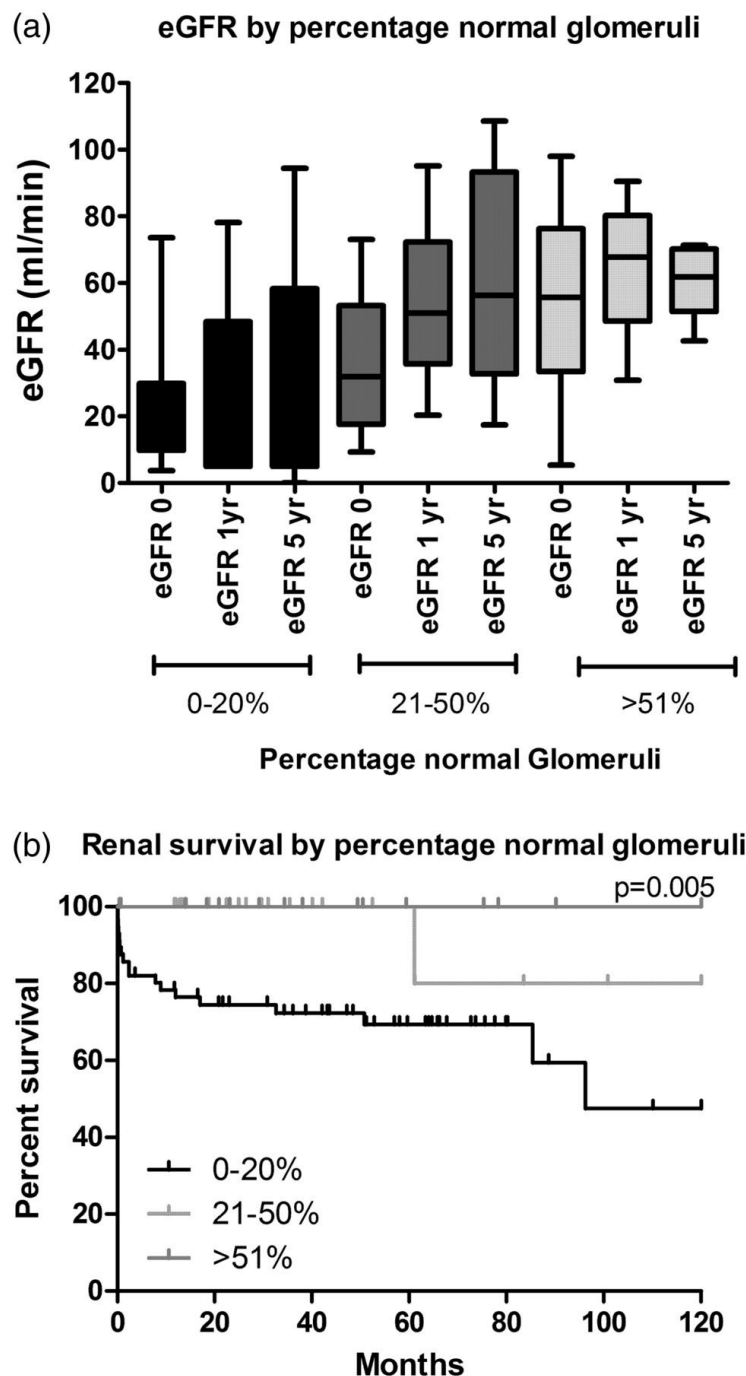


**Figure 4.3.5: Renal function according to percentage normal glomeruli at presentation**

(a) eGFR by percentage normal glomeruli at presentation, 1 year and 5 years. (b) Kaplan-Meier curve demonstrating renal survival by percentage normal glomeruli (censored for patient death). (Figure used with permission from Nephrol Dial Transplant (2015) 30: 1185-1192 doi: 10.1093/ndt/gfu237 figure 3, page 1189).

#### 4.3.4 TUBULAR ATROPHY

Biopsies were divided into three groups according to percentage tubular atrophy, (i) TA<20%, (ii) 21-30% and (iii) >50%. Figure 4.3.6 (a) demonstrates the effect of tubular atrophy on renal outcome at 1 and 5 years. There was a statistically significant difference in eGFR at both 1 and 5 years, with increasing degree of tubular atrophy correlating with a reduction in eGFR. Figure 4.3.6 (b) demonstrates renal survival according to percentage tubular atrophy and shows clearly that there is improved renal survival with reduction in percentage tubular atrophy (log-rank 0.04).

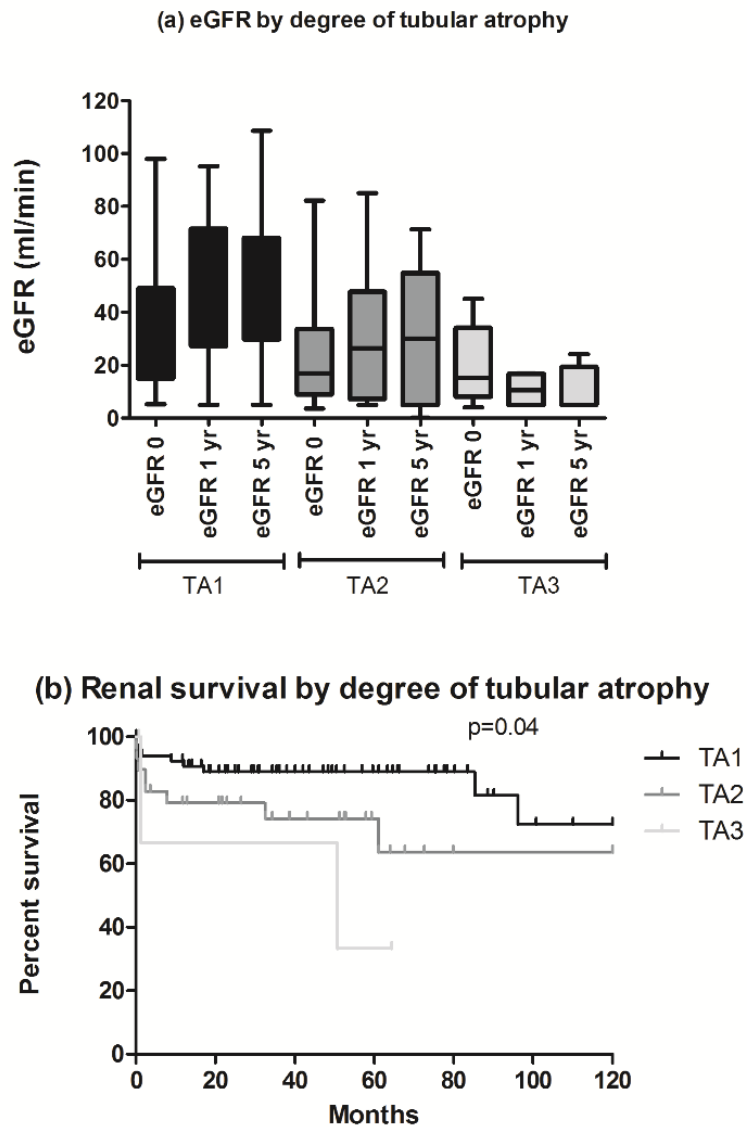


**Figure 4.3.6: Renal function by degree of tubular atrophy**

(a) eGFR by degree of TA at presentation, 1 year and 5 years. TA1- 0-20%, TA2- 21-50%, TA3: >51%(b) Kaplan-Meier curve demonstrating renal survival by degree of TA. Figure used with permission from Nephrol Dial Transplant (2015) 30: 1185-1192

### 4.3.5 OTHER FACTORS AFFECTING OUTCOME IN THIS STUDY

#### 4.3.5.1 BASELINE EGFR

Patients were divided according to their starting eGFR into those with eGFR <15 ml/min, eGFR: 16-60 ml/min and eGFR>61 ml/min. There was significantly improved renal survival with a higher eGFR at presentation (Figure 4.3.7 (a) log rank  $p<0.0001$ ). There was also a significantly improved survival using a combined endpoint of renal and patient survival (figure 4.3.7 (b) log rank  $p=0.0014$ ).

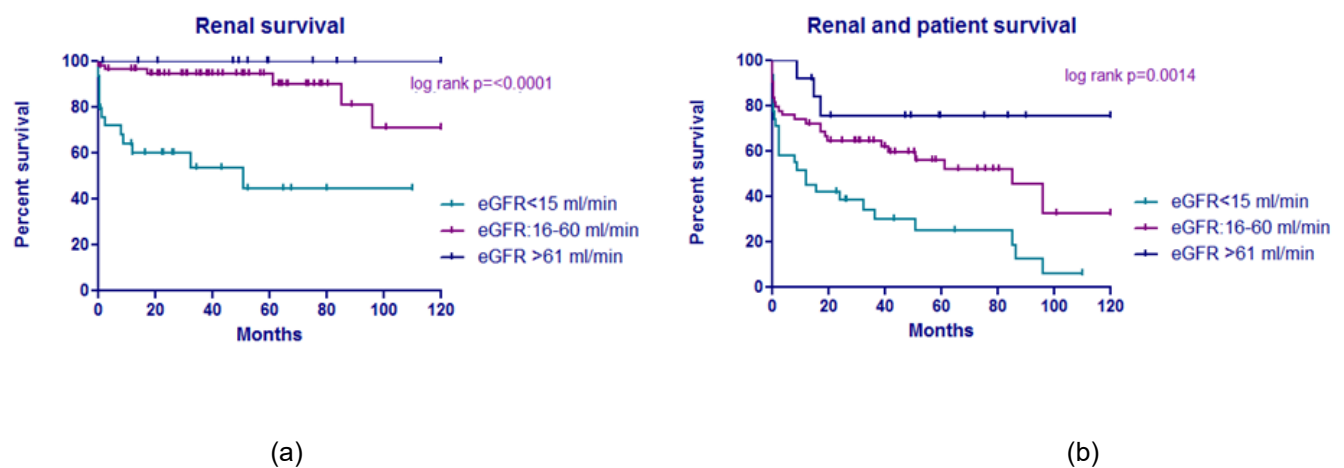


**Figure 4.3.7: Renal survival according to presenting eGFR**

(a) Kaplan-Meier survival curve to demonstrate renal survival according to presenting eGFR (log rank  $p<0.0001$ ). (b) Kaplan-Meier survival curve demonstrating a combined endpoint of renal and patient survival according to starting eGFR ( $p=0.0014$ ).

#### 4.3.5.3 Age

The effect of age at presentation was assessed and in keeping with our previous work, renal survival was significantly worse with increasing age (log-rank  $p=0.04$ )

#### 4.3.5.4 ANCA type

When assessing the impact of ANCA type on renal survival, there was significantly improved renal survival in patients who were P-ANCA positive compared with those with C-ANCA positivity (log-rank  $P=0.03$ ) (Figure 4.3.8). There was no difference in survival using a combined endpoint of renal and patient survival.

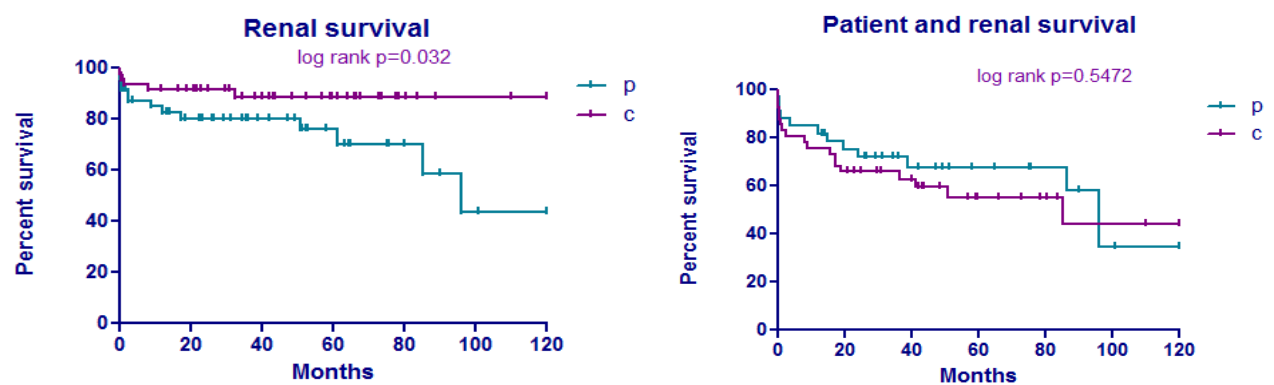


**Figure 4.3.8 Renal Survival according to ANCA type.**

(a) Kaplan-Meier survival curve to demonstrate renal survival according to ANCA type (log rank  $p=0.032$ ). (b) Kaplan-Meier survival curve demonstrating a combined endpoint of renal and patient survival according to ANCA type ( $p=ns$ ).

#### 4.3.5.5 TREATMENT

There was no difference in outcome when dividing patients into those treated with rituximab upon induction after 2006 and those treated before this.

#### 4.3.6 MULTIVARIATE ANALYSIS

(This analysis was carried out by Dr. Benjamin Caplin)

Table 4.3.3 demonstrates the results of multivariable models of eGFR at 1 and 5 years. After adjusting for the variables age, baseline eGFR and tubular atrophy, there was no longer a significant association between histological class and renal outcome. Inclusion of histological class as a variable, did not improve the model fit for either the 1 or 5 year model in a sensitivity analysis.

Despite being predictive of renal survival using Kaplan-Meier survival analysis, this association was no longer apparent following adjustment for baseline eGFR using a Cox proportional hazards model, shown in Table 4.3.4.

Multivariable models of eGFR at 1 and 5 years

| Model              | Variable         | 1 year    |                 |                                           | 5 years   |             |                                           |
|--------------------|------------------|-----------|-----------------|-------------------------------------------|-----------|-------------|-------------------------------------------|
|                    |                  | $\beta$   | 95% CI          | P-value for LR test versus previous model | $\beta$   | 95% CI      | P-value for LR test versus previous model |
| 1                  | Baseline eGFR    | 0.73      | 0.53–0.92       |                                           | 0.87      | 0.61–1.14   |                                           |
| 2                  | Baseline eGFR    | 0.67      | 0.49–0.85       | <0.005                                    | 0.83      | 0.60–1.06   | <0.005                                    |
|                    | Age              | –0.58     | –0.83–0.32      |                                           | 0.73      | –1.06–0.42  |                                           |
| 3                  | Baseline eGFR    | 0.62      | –16.55 to –2.85 | 0.005                                     | 0.78      | 0.55–1.01   | 0.0345                                    |
|                    | Age              | –0.52     | –0.77–0.26      |                                           | –0.67     | –0.98–0.35  |                                           |
|                    | TA (per tertile) | –9.70     | –16.55–2.85     |                                           | –9.11     | –17.83–0.38 |                                           |
| 4                  | Baseline eGFR    | 0.64      | 0.42–0.85       | 0.43                                      | 0.82      | 0.51–1.13   | 0.37                                      |
|                    | Age              | –0.49     | –0.77 to –0.26  |                                           | –0.56     | 0.93–0.20   |                                           |
|                    | TA (per tertile) | –8.02     | –15.58 to –4.52 |                                           | –5.40     | –15.19–4.38 |                                           |
| Histological class |                  |           |                 |                                           |           |             |                                           |
|                    | Sclerotic        | Reference |                 |                                           | Reference |             |                                           |
|                    | Crescentic       | 13.38     | 5.01–31.77      |                                           | 19.01     | –3.64–41.68 |                                           |
|                    | Mixed            | 12.14     | –4.42–28.69     |                                           | 11.64     | –8.09–31.34 |                                           |
|                    | Focal            | 9.47      | –10.24–29.18    |                                           | 11.79     | 13.15–36.73 |                                           |

eGFR, estimated glomerular filtration rate; CI, confidence interval.

**Table 4.3.3: Multivariable models of eGFR at 1 and 5 years including presenting eGFR, age, histological class and tubular atrophy.**

Figure used with permission from Nephrol Dial Transplant (2015) 30: 1185-1192

Cox proportional hazards model for predicted renal survival

|                       | HR   | SE   | Z     | P > Z | 95% CI    |
|-----------------------|------|------|-------|-------|-----------|
| Sclerotic (reference) |      |      |       |       |           |
| Crescentic            | 0.55 | 0.40 | -0.82 | 0.412 | 0.13-2.29 |
| Mixed                 | 0.65 | 0.44 | -0.64 | 0.523 | 0.17-2.45 |
| Focal                 | 0.66 | 0.83 | -0.33 | 0.742 | 0.06-7.59 |
| eGFR at presentation  | 0.92 | 0.02 | -3.20 | 0.001 | 0.87-0.97 |

HR, hazard ratio; SE, standard error; CI, confidence interval.

**Table 4.3.4: Cox proportional hazards model for predicted renal survival**

Figure used with permission from Nephrol Dial Transplant (2015) 30: 1185-1192

| <b>Variable</b>           | <b>Coef</b> | <b>SE</b> | <b>T</b> | <b>P&gt;[t]</b> | <b>95% CI</b> |
|---------------------------|-------------|-----------|----------|-----------------|---------------|
| Mdrd px                   | 0.85        | 0.16      | 5.45     | 0.00            | 0.54-1.16     |
| <u>Histological Class</u> |             |           |          |                 |               |
| Sclerotic                 | Reference   |           |          |                 |               |
| Crescentic                | 24.60       | 10.15     | 2.42     | 0.02            | 4.31-44.89    |
| Mixed                     | 15.13       | 9.37      | 1.62     | 0.11            | -3.59-33.86   |
| Focal                     | 15.73       | 11.97     | 1.31     | 0.19            | -8.20-39.66   |
| Age                       | -0.57       | 0.18      | -3.10    | 0.00            | -0.93—0.20    |
| Cons                      | 32.63       | 15.25     | 2.14     | 0.036           | 2.15-63.11    |

**Table 4.3.6: Multivariate model to predict eGFR at 5 years using eGFR at presentation, histological class and age, without the inclusion of tubular atrophy. (Prob>F = 0.12).**

## 4.4 DISCUSSION

The aim of this large single centre study was to assess the long term prognostic value of the international histological classification for AAV (128), along with other prognostic factors, in our cohort of patients. By univariate analysis, histological class was found to have a significant impact on renal outcome. The sclerotic category of patients had the worst prognosis at all time points, and the focal the best. These findings were in accordance with other validation studies (128, 151). There was no difference in prognosis between the mixed and crescentic classes in our study. Upon dividing the mixed category by percentage normal glomeruli, there were no differences in outcome seen. The percentage of crescents present in biopsies of those categorised as “mixed” did however have affect outcome. There was better renal function at 1 year in patients with >20% crescents possibly reflecting better responsiveness to treatment. There was also worse renal function at 5 years in patients in the mixed category with >20% sclerotic glomeruli.

Validation cohorts have varied with regards to which of the crescentic and mixed classes have a better overall prognosis. The EUVAS cohort, for example, demonstrated better outcomes in the crescentic category (128) whilst a Chinese cohort (151) experienced better outcome in the mixed category. It is difficult to compare different cohorts as there are known differences in disease manifestations in AAV patients of varying ethnic backgrounds. European and Chinese AAV patients have different human leucocyte antigen associations (152). Patients with MPO-ANCA have also been shown to have less favourable renal outcomes than those with PR3-ANCA. In the Chinese cohort, 89% of patients were MPO ANCA positive. Treatment variation may also have impacted these studies.

In this study, when histological classification was included in multivariate models there was no improvement in model fit or association with renal outcome, following adjustment for other established prognostic factors such as age, baseline eGFR and tubular atrophy. This was despite clear differences in outcome with histological group on univariate testing. This finding suggests that for our cohort of patients, in contrast to the EUVAS validation study, the histological classification is not an independent prognostic factor when taking other variables into account. Although the sclerotic class was strongly predictive of poor renal outcome in univariate analysis, this category was also highly associated with lower baseline eGFR and increased tubular atrophy. In the multivariate analysis, these variables were independently associated with renal outcome, rather than histological class.

In terms of other prognostic factors, we found age, presenting eGFR and percentage normal glomeruli on biopsy to be important in our study. Patients who presented dialysis dependent, or with an eGFR of less than 15 ml/min experienced a markedly reduced renal survival over the whole study period. Elderly patients also experienced poorer outcomes. A greater proportion of normal glomeruli conferred an improved renal prognosis. These findings were in keeping with other studies of these patients. (129, 161, 162).

We also found a significant association between the degree of tubular atrophy and renal outcome, with a greater degree of tubular atrophy being associated with poorer renal outcome. This was in accordance with Berden *et al*, who also found tubular atrophy to be a significant predictor of renal outcome at 1 year in rituximab treated patients (147).

Upon dividing patients by ANCA type, we also demonstrated better renal survival in patients who were PR3 positive than those who were MPO positive. This finding is in keeping with previous studies(163). PR3- ANCA and MPO-ANCA positive AAV are increasingly considered as genetically distinct disease subsets (10).

The main advantage of our study was that of being from a single centre with consecutive unselected patients with consistent sample preparation and processing. Treatment was also uniformly given as per protocol. The study was limited by its retrospective nature and also time points for analysis of eGFR. As there were only two points for analysis of eGFR at follow up, 1 and 5 years, patients without follow up at these particular time-points were excluded and could have provided additional information. Although a mixed model approach would account for missing data and potentially increase the power of such an analysis, we attempted to replicate the analysis used by Berden *et al* in their validation study, and so used a simple linear regression approach. Further validation in varied populations would be of value, particularly including larger numbers of patients in the various categories.

We conclude that, in our cohort of patients, the international histological classification is predictive of renal outcome in AAV, but did not appear to be additionally informative over other established prognostic factors in multivariate analysis. It may be of value to combine the current histological classification with other established parameters including tubular atrophy and percentage normal glomeruli.

## CHAPTER 5: OUTCOMES OF ANCA PATIENTS WITH SEVERE RENAL DYSFUNCTION AT PRESENTATION

## 5.1 Introduction

Renal dysfunction is a common manifestation of AAV, affecting over 50% of patients at presentation and up to 80% of patients during the course of their illness (18). On renal biopsy, the classical finding is of a focal necrotising pauci-immune glomerulonephritis (164). Untreated, this glomerular inflammation can result in rapidly progressive glomerulonephritis (RPGN), leading to end stage renal failure within weeks or months (18).

Patients presenting dialysis dependent or with severe renal dysfunction have been shown to have a poor prognosis both in terms of renal and patient survival (165, 166). Treatment with immunosuppression is associated with significant toxicity. Up to one third of deaths in patients with AAV at 1 year following diagnosis have been attributed to treatment related effects (96). These adverse effects have also been demonstrated as being more severe and frequent in AAV patients with significant renal impairment (167, 168).

There is therefore the need to balance the risks of treatment with the effects of the disease itself, in order to minimise harm to these patients. It is important to assess factors affecting outcome, with the aim of being able to tailor therapy and also to give patients as accurate a prognosis as possible at presentation.

Few studies have specifically addressed the outcome of patients presenting with severe renal injury, traditionally defined as dialysis dependence or a serum creatinine (Scr) of over 500  $\mu\text{mol/l}$ . Current outcome data for patients with severe renal failure is

largely based on small studies (169). These patients are often excluded from randomised controlled trials.

The MEPEX (methylprednisolone versus plasma exchange) trial was a multicentre randomised controlled trial which included patients with severe renal disease defined as above, as a serum creatinine  $> 500 \mu\text{mol/l}$  or dialysis dependence. Plasma exchange (PEX) has been used in the treatment of severe AAV since the 1990s (170) (171). As part of the MEPEX trial patients were treated with oral cyclophosphamide (CYP) and either PEX or pulsed methylprednisolone. Serious adverse effects developed in almost 50% of patients in both patient groups. This trial concluded that renal recovery was improved at 3 and 12 months in patients treated with PEX, and formed the basis of the “MEPEX” protocol for AAV patients with severe renal involvement, at our centre.

Recent research has challenged the use of PEX in this patient group. The long term follow up of MEPEX concluded that PEX conferred no survival benefit beyond 12 months (99). PEXIVAS was recently published. This was a trial assessing the use of PEX for patients with “severe” AAV. The median starting creatinine for patients in this study was  $327 \mu\text{mol/l}$  and inclusion criteria included an eGFR of  $<50 \text{ ml/min}$ . Under 1/3 of patients had a starting creatinine of over  $500 \mu\text{mol/l}$ . The overall conclusion of this trial was that there was no benefit in terms of renal outcome, from treatment with PEX. Subgroup analysis of patients presenting with a creatinine of over 500 however concluded that “PEX as an adjunctive therapy may reduce the need for dialysis at 3 and 12 months”.

The RITUXVAS (rituximab versus cyclophosphamide in ANCA-associated vasculitis) study also included patients with severe renal disease (41). Rituximab, together with

iv CYP was shown to be effective in the induction of remission for these patients. Rituximab was introduced as part of the treatment regime for patients at our centre from 2011 together with iv CYP and low dose prednisolone. Pepper *et al* showed iv CYP to be as effective as oral CYP in the induction of remission of AAV in those presenting with dialysis dependence, and enabled a reduction in cumulative CYP dose (172).

Although a number of studies have been carried out in AAV patients attempting to determine factors predictive of prognosis, patients with severe renal impairment have again, largely been excluded. From previous studies, the main factors affecting overall outcome have been age at presentation and starting creatinine (173).

As described in chapter 4, histological class as determined by the Berden classification, has been used as a prognostic factor with the best prognosis demonstrated in patients with a biopsy classified as focal, and the worse in those with sclerotic biopsies (128). In patients with an eGFR of  $<15$  ml/min/1.73m<sup>2</sup> however, this classification has not been demonstrated to be predictive of outcome (166).

Brix *et al* described a renal risk score to assess prognosis of AAV (174). Their initial study included 205 patients with a new diagnosis of AAV and confirmed the findings that percentage normal glomeruli on biopsy and starting eGFR were the factors most predictive of renal outcome (174). In this study the median eGFR was 28 ml/min/1.73m<sup>2</sup> (20-45.8) and only 19% of patients presented dialysis dependent. This therefore cannot be applied fully to our patient cohort.

Studies to date have combined patients who are dialysis dependent with those who have a Scr of over 500µmol/l but remain dialysis independent. We decided to divide these patients by dialysis dependence and compare their outcomes.

## 5.2 Aims

The overall aim of this chapter is to describe the long term patient and renal outcomes in a large cohort of patients, treated consistently with a regimen including PEX, at a single centre over a 40 year period. We aimed to identify factors predicting both renal and patient outcome in this cohort. We also aimed to determine if there was a difference in prognosis and outcome for patients who presented with a creatinine of over 500  $\mu\text{mol/l}$  but who were independent of dialysis, with those who were dialysis dependent.

## 5.3 Experimental Design

We carried out a retrospective analysis of patients with newly presenting AAV and severe renal dysfunction, treated at Imperial College Healthcare Trust, between 1977 and 2017. The diagnosis of AAV was based on clinical features, ANCA serology, where available, and biopsy findings. Patients with relapsing disease and those with a concomitant anti-GBM antibody, were excluded.

Severe renal dysfunction was defined as patients who were either dialysis dependent upon presentation, or those who were dialysis independent but had an eGFR of  $<15 \text{ ml/min/1.73m}^2$ .

Dialysis dependent renal failure was defined as the need for dialysis within 72 hours of admission to hospital. An outcome of renal death (ESRD) was defined as persistent (more than 3 months) need for renal replacement therapy, or long-term reduction in eGFR to  $15 \text{ ml/min}$  by the CKD EPI equation.

Clinical, laboratory and histopathological data were collated from anonymised patient records. Patient and renal outcomes were assessed and factors affecting outcome analysed. As this was a retrospective review, this study was carried out as a service evaluation study and hence did not require approval from a research ethics committee.

### 5.3.1 Treatment

Patients were treated consistently as per unit protocol. This protocol included a combination of CYP (oral in the earlier years and then intravenous), a rapidly tapering course of oral steroids and plasma exchange for induction of remission. Rituximab was also added in 2011.

Following induction, patients were commenced on azathioprine maintenance therapy at a dose of 2 mg/kg (maximum 150 mg) or 1 mg/kg if Thiopurine Methyltransferase (TPMT) activity was low. Mycophenolate Mofetil (MMF) was prescribed as maintenance therapy for those who were azathioprine intolerant. Maintenance therapy was continued for at least two years.

Patients also received prophylaxis for *Pneumocystis jirovecii* pneumonia, gastric protection, bone protection and TB prophylaxis when in an “at risk” group.

### 5.3.2 Statistical Analysis

Statistical analysis comparing the survival of groups of patients was carried out using the Log-rank test and Kaplan–Meier survival curves created using Graph Pad Prism software (Graph Pad Software, San Diego, California, US). Multivariate statistical analysis was carried out using SPSS software, version 24 (IBM corp).

## 5.4 Results

### 5.4.1 Demographics

We included 180 AAV patients who presented to our centre with a serum creatinine of over 500µmol/l, an eGFR of <15ml/min/1.73m<sup>2</sup>, or dialysis dependence, between 1977 and 2017 (Table 1).

One hundred and eight (108) patients included in our study were male (60%). The mean age of patients was 59.5 years. Fifty two percent of patients were MPO positive and forty percent PR3 with the remainder ANCA neg or not tested for ANCA. The mean follow up time was 88 months (range 0.13-514). The mean presenting creatinine was 738.1 µmol/l. One hundred and forty six patients presented dialysis dependent and 36 patients independent of dialysis.

| Characteristic:                                        | N              |
|--------------------------------------------------------|----------------|
| Number of patients                                     | 180            |
| No of males – no. (%)                                  | 108 (60)       |
| Mean age - yrs.                                        | 59.5           |
| MPO – no. (%)                                          | 93 (52)        |
| PR3 – no. (%)                                          | 72 (40)        |
| ANCA negative or untested for ANCA– no (%)             | 15 (8)         |
| Mean follow up - months (range)                        | 88 (0.13-514)  |
| Mean presenting creatinine – $\mu\text{mol/L}$ (range) | 738 (200-1840) |
| Number presenting dialysis dependent – no. (%)         | 146 (81)       |
| Number presenting dialysis independent – no. (%)       | 34 (19)        |

**Table 5.1: Characteristics of patients at baseline**

### 5.4.2 Outcomes

One year patient survival was 74% and of the people who were alive at one year, renal survival was 64%. Five year patient survival was 55.8 % and of these patients, 51% had independent renal function.

Kaplan-Meier plots were constructed to describe renal and patient survival of the whole cohort over a ten year period, and a composite end point of renal and patient death was also used.

Figure 5.1 demonstrates renal survival of the entire cohort over a 10 year period. Figure 5.2 is a Kaplan-Meier survival curve of patient survival over 10 years. A combined endpoint of renal or patient death was used in Figure 5.3.

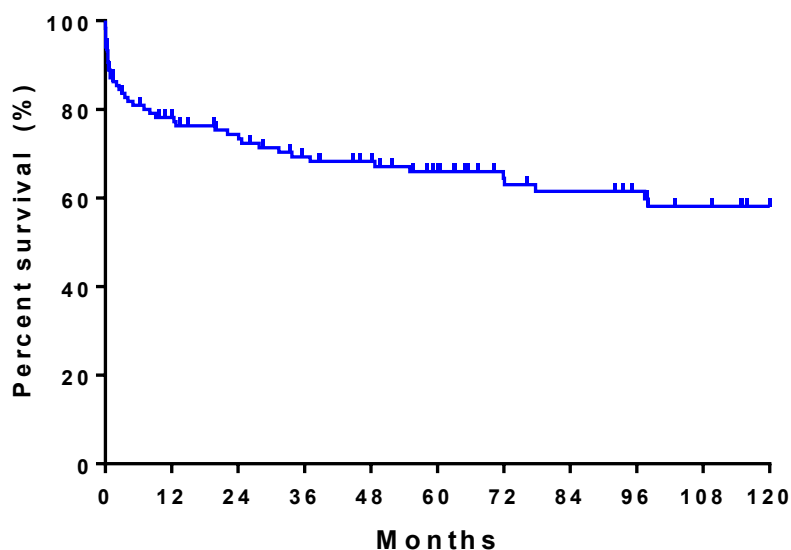


**Figure 5.1: Renal survival over 10 years**

Unadjusted Kaplan-Meier survival plot describing renal survival over 10 years. The primary outcome demonstrated by this chart is renal survival.

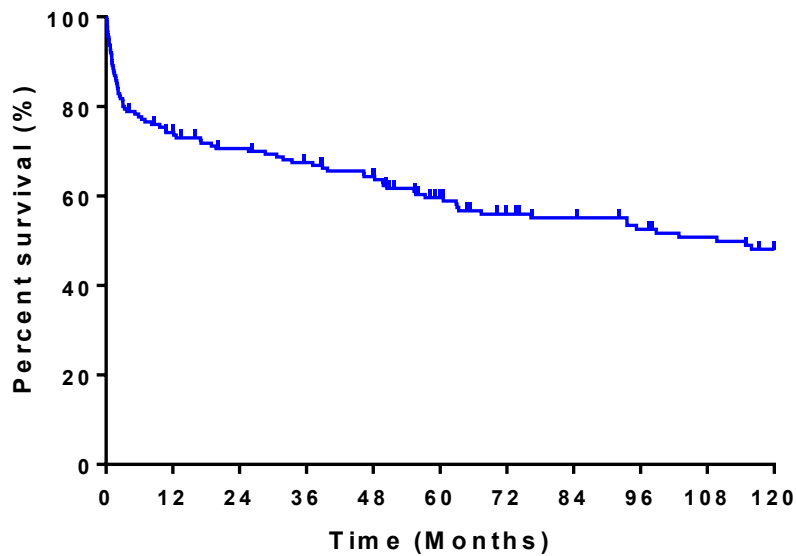


**Figure 5.2: Patient survival over 10 years**

Unadjusted Kaplan-Meier survival plot describing patient survival over 10 years.

The primary outcome demonstrated by this chart is patient survival, censored for patient death.

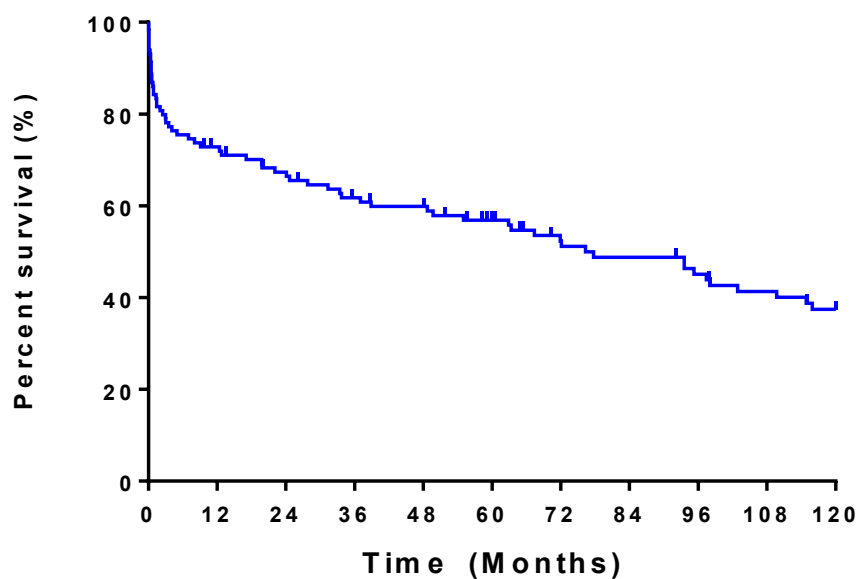


**Figure 5.3: Combined renal and patient survival**

Unadjusted Kaplan-Meier survival plot describing a composite end point of renal or patient survival of the study cohort during follow up over 10 years.

#### 5.4.3 Comparison of patients who were dialysis dependent at presentation with patients who presented independent of dialysis.

Patients were divided into those who were dialysis dependent at presentation, and those who were dialysis independent but presented with a creatinine of over 500 $\mu$ mol/l and/or an eGFR < 15ml/min/1.73m<sup>2</sup>. The demographics of these patients are listed in Table 2. Of the 180 patients included in the study, 146 patients (80%) presented dialysis dependent. Sixty percent of these patients were male. The mean presenting creatinine of these patients was 792  $\mu$ mol/l compared with 511.7 $\mu$ mol/l for those who presented dialysis independent.

As demonstrated in Table 5.2, 1 year patient survival was 79% in patients who were dialysis independent at presentation, compared with 69% for those who were dialysis dependent. There was also significantly improved renal survival of 96% at 1 year in patients who were dialysis independent, compared with 59% of patients in the group who presented dialysis dependent. Allowing for the 12 patients who did not reach five year follow up, patient survival at 5 years was 52% for those who presented dialysis dependent, compared with 74% for the dialysis independent group. Renal survival was significantly different at 5 years. It was 80% for the dialysis independent cohort and only 42% in the dialysis dependent patients. In previous studies patients presenting with a creatinine of over 500 were analysed together. Although the precise explanation for the improved survival in the dialysis independent cohort cannot be deduced from this study, the implication is that these patient groups could be separated in future trials or studies.

|                                             | <b>All patients</b> | <b>Dialysis dependent</b> | <b>Dialysis independent</b> |
|---------------------------------------------|---------------------|---------------------------|-----------------------------|
| Number                                      | 180                 | 146 (81)                  | 34 (19)                     |
| Male                                        | 108 (60)            | 87(60)                    | 21 (62)                     |
| Age (yrs.)                                  | 59.5                | 59.9                      | 57.9                        |
| PR3 ANCA                                    | 72 (40)             | 60 (41)                   | 12 (35)                     |
| MPO ANCA                                    | 93 (52)             | 73 (50)                   | 20 (59)                     |
| Mean Presenting<br>creatinine( $\mu$ mol/L) | 738.1               | 792                       | 511.7                       |

**Table 5.2: Demographics of patients divided by dialysis dependence or independence at presentation.**

|                                                    | <b>Patients<br/>(N)</b> | <b>1 yr. patient<br/>survival<br/><br/>N (%)</b> | <b>1 yr. renal<br/>survival<br/><br/>N (%)</b> | <b>5 year patient<br/>survival<br/><br/>N (%)</b> | <b>5 year renal<br/>survival<br/><br/>N (%)</b> |
|----------------------------------------------------|-------------------------|--------------------------------------------------|------------------------------------------------|---------------------------------------------------|-------------------------------------------------|
| All patients                                       | 180                     | 134 (74)                                         | 84 (63)                                        | 92 (56)                                           | 47 (51)                                         |
| Dialysis dependent at<br>presentation<br><br>(%)   | 146<br><br>(81)         | 101<br><br>(69)                                  | 60<br><br>(59)                                 | 72<br><br>(52)*                                   | 30<br><br>(42)                                  |
| Dialysis independent<br>at presentation<br><br>(%) | 34<br><br>(19)          | 27<br><br>(79)                                   | 26<br><br>(96)                                 | 20<br><br>(74)**                                  | 16<br><br>(80%)                                 |

**Table 5.3: Comparison of renal and patient survival in patients who presented dialysis dependent with those who were dialysis independent at presentation.**

\* 8 patients did not reach 5 year follow up. \*\* 7 patients did not reach 5 year follow up

Kaplan- Meier Curves were used to compare both patient and renal survival in the two groups, dialysis dependent at presentation, vs dialysis independent (Figures 5.4 and 5.5). As demonstrated in Figure 5.4, there was a significant improvement in renal

survival over 10 years for patients who were independent of dialysis at presentation. Figure 5.5 demonstrates a statistically significant difference between the two groups in terms of patient survival, which is improved over 10 years for patients who presented dialysis independent. Figure 5.6 demonstrates survival when using a combined endpoint of renal or patient death.

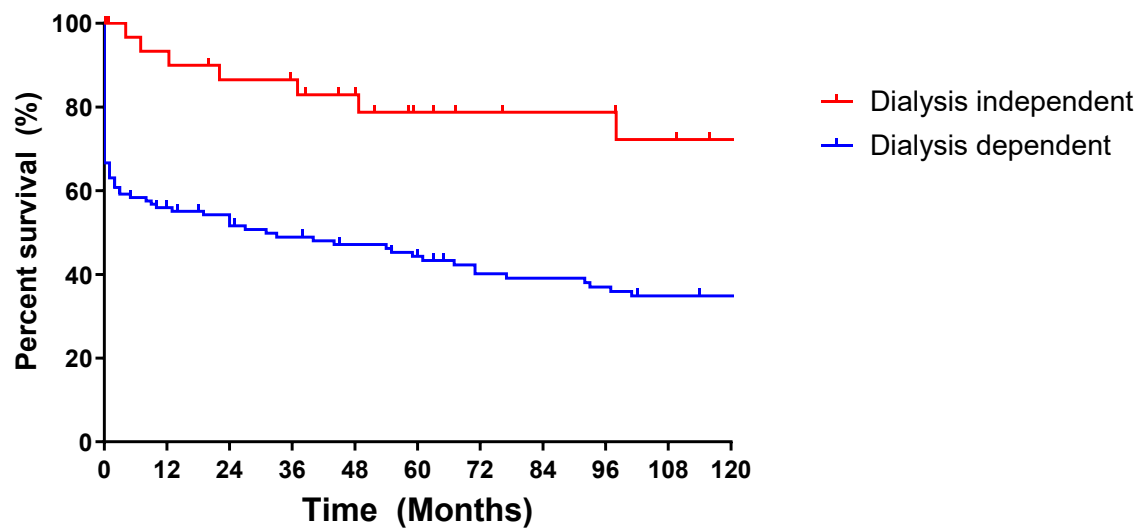


**Figure 5.4: Renal survival in dialysis dependent vs dialysis independent patients at presentation**

Unadjusted Kaplan-Meier survival plot describing the renal survival of patients who were dialysis dependent at presentation vs those who were dialysis independent. There was significantly improved renal survival over 5 years in patients who presented dialysis independent.

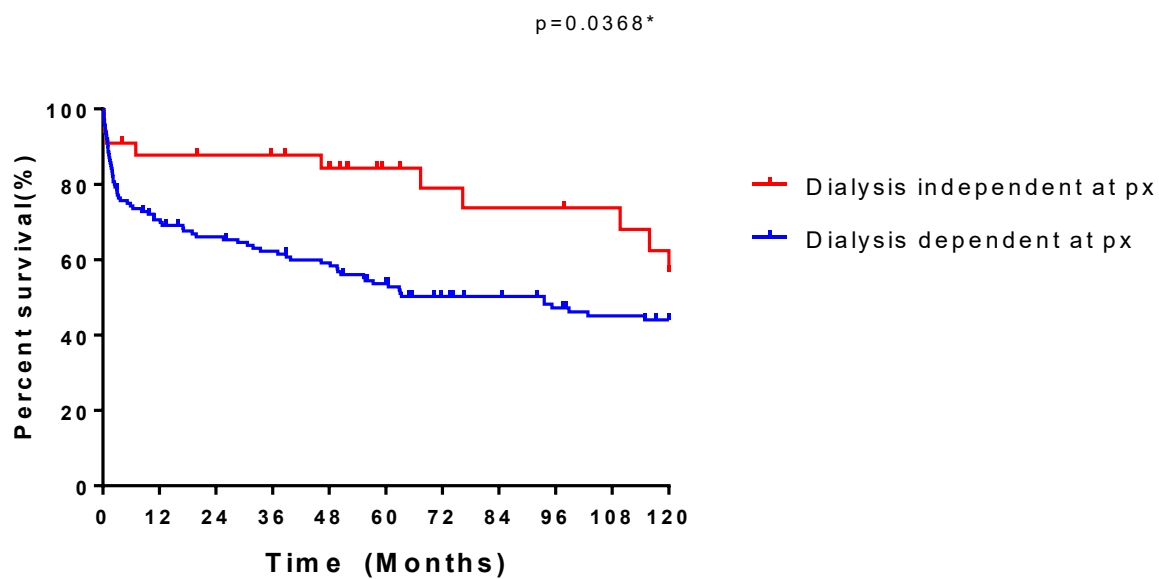


**Figure 5.5: Patient survival dialysis dependent vs dialysis independent patients at presentation**

Unadjusted Kaplan- Meier survival plot describing the patient survival of patients who presented dialysis dependent vs those who presented dialysis independent over a 10 year period. There was a significantly improved patient survival in patients who were dialysis independent at presentation (p=0.0368)

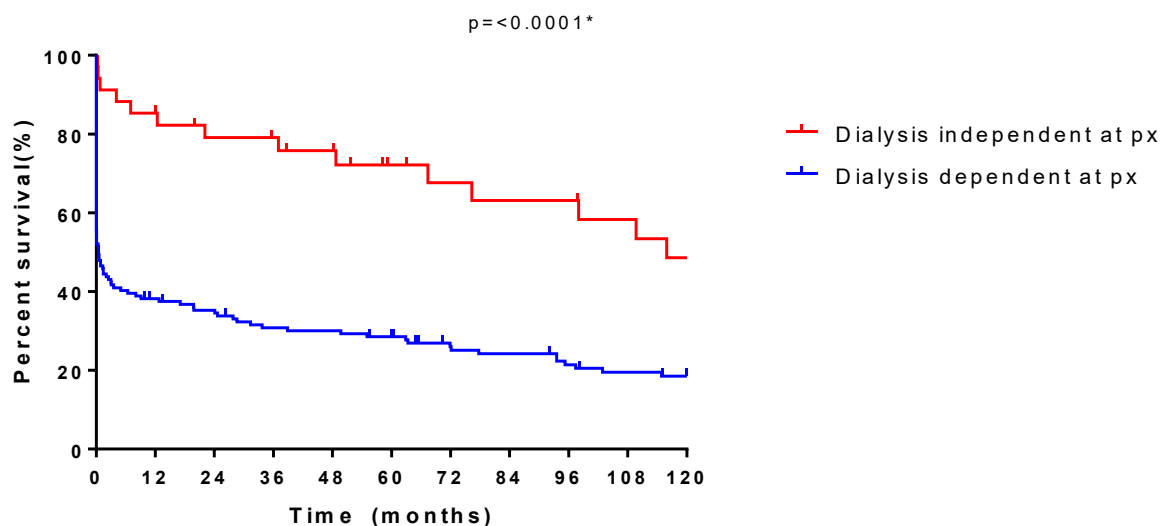


**Figure 5.6: Combined renal and patient survival in dialysis dependent vs dialysis independent patients at presentation.**

Unadjusted Kaplan – Meier survival plot describing the composite endpoint of renal and patient death and comparing patients who were dialysis dependent at presentation with those who were dialysis independent, over a ten year period. There a highly significant difference between the two groups with better survival in those who presented independent of dialysis ( $p < 0.0001^*$ ).

#### 5.4.4 Renal and patient survival according to decade of presentation

Our patient cohort included patients who presented over a period of 40 years. Patients were divided into five decades of presentation, from 1970 to 2020. Kaplan- Meier curves were used to compare renal and patient survival across these decades. As demonstrated in Figure 5.7, there was no significant difference in terms of renal survival when patients were compared by decade of presentation. There was however, a significant difference in patient survival between decades 1 and 5, as shown in Figure 5.8. Patients who presented between 2010 and 2020 had a significantly better survival over ten years, than those who presented between 1970 and 1980. There was also a trend towards better survival in patients presenting in the fifth decade than the first, using a composite endpoint of renal or patient death (Figure 5.9).

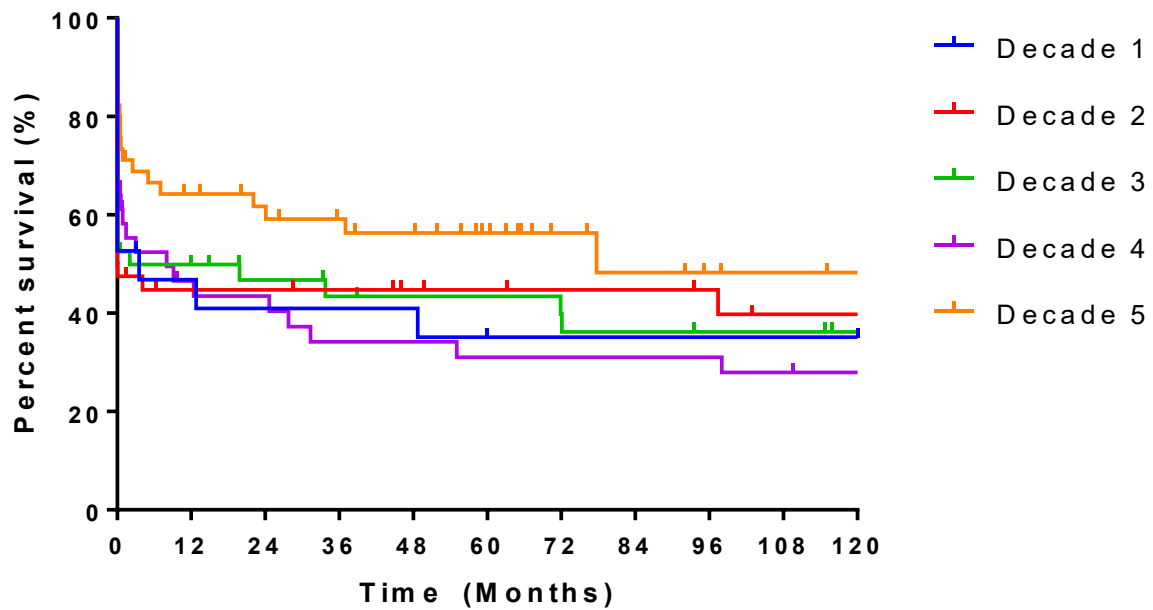


**Figure 5.7: Renal survival according to decade of presentation.**

Unadjusted Kaplan Meier graph describing renal survival in the patient cohort divided by decade of presentation. Decade 1: 1970-1980; Decade 2: 1980-1990; Decade 3: 1990-2000; Decade 4: 2000-2010; Decade 5: 2010-2020.

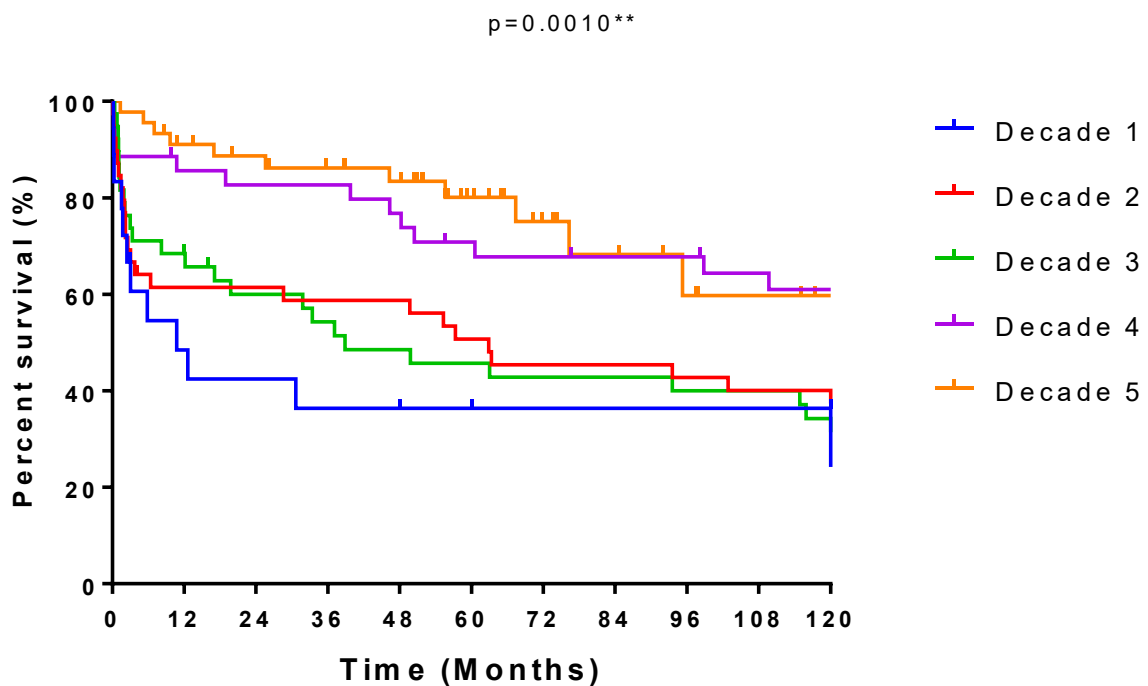


**Figure 5.8: Patient survival according to decade of presentation**

Unadjusted Kaplan Meier Curve illustrating patient survival in the patient cohort divided by decade of presentation. Decade 1: 1970-1980; Decade 2: 1980-1990; Decade 3: 1990-2000; Decade 4: 2000-2010; Decade 5: 2010-2020 ( $p=0.0010$ ).

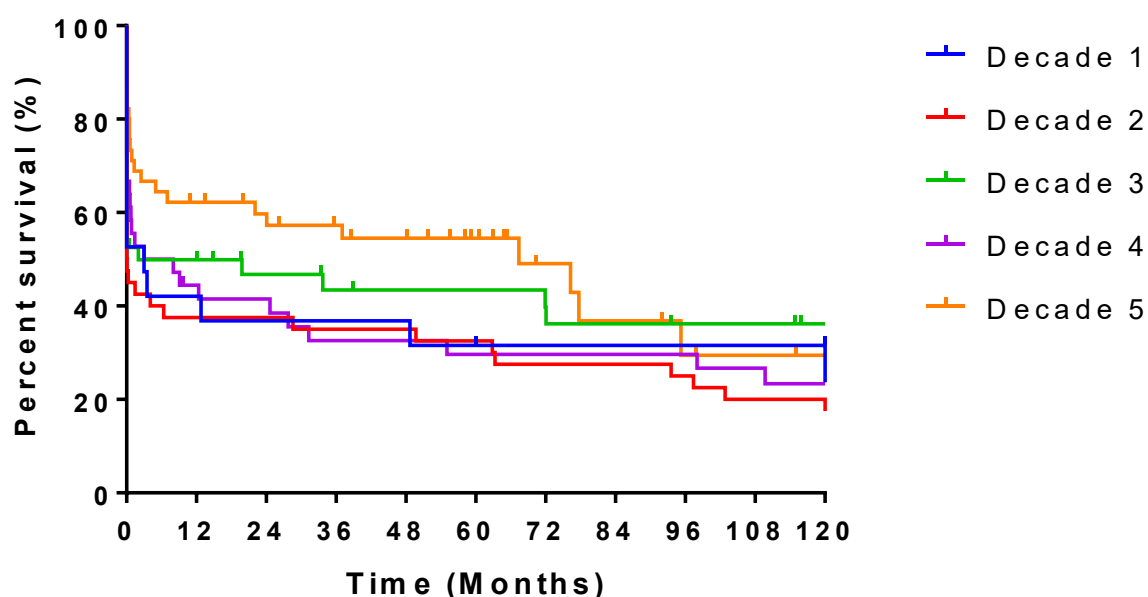


**Figure 5.9: Combined renal and patient survival according to decade of presentation**

Unadjusted Kaplan Meier curve describing a combined endpoint of renal and patient survival for the entire cohort of patients divided by decade of presentation. Decade 1: 1970-1980; Decade 2: 1980-1990; Decade 3: 1990-2000; Decade 4: 2000-2010; Decade 5: 2010-2020 (p=ns).

#### 5.4.5 Renal and patient survival according to age at presentation

Based on previous literature, we divided patients by according to age at presentation, into patients presenting below or above the age of 60. Figure 5.10 demonstrates no significant difference between the two groups of patients with regard to renal survival over 10 years. As expected and is illustrated in Figure 5.11, there was significantly improved patient survival in patients presenting under the age of 60 years.

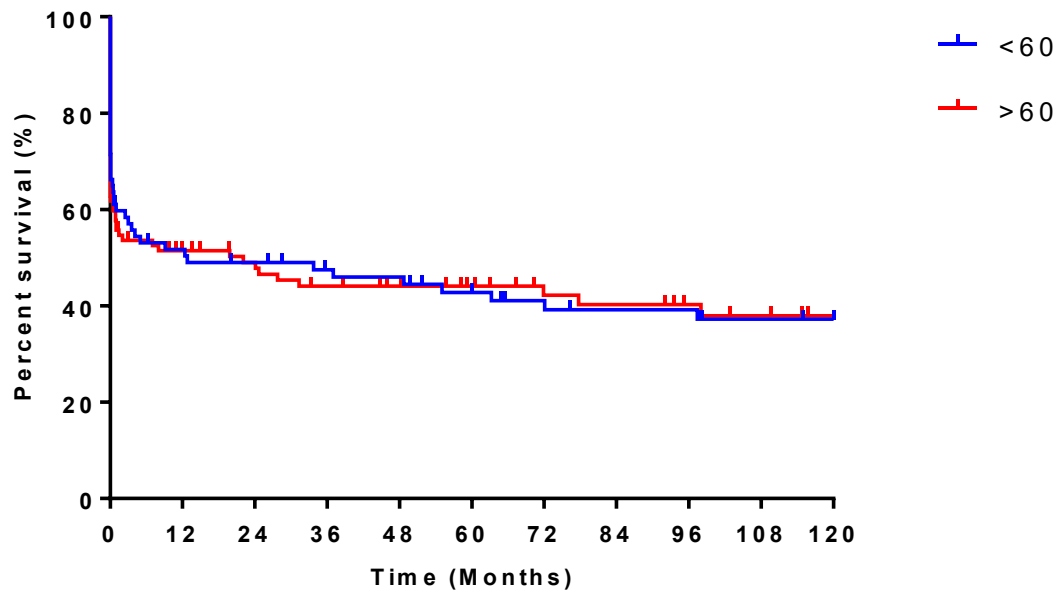


**Figure 5.10: Renal survival according to age.**

Unadjusted Kaplan-Meier survival curve demonstrating renal survival over a ten year period in patients who presented over and under the age of 60 years.

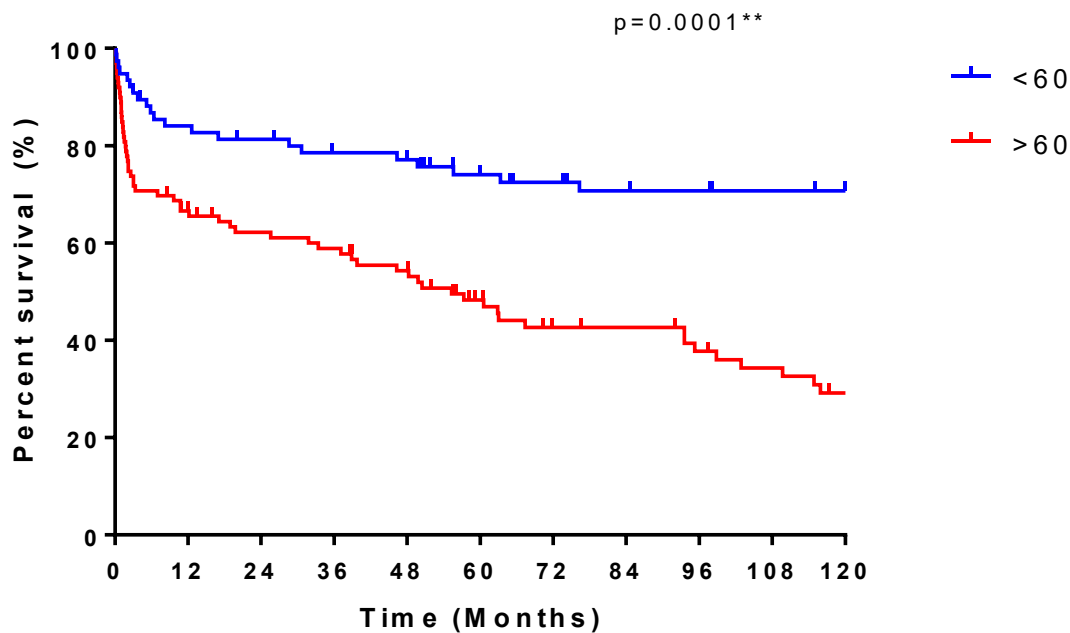


**Figure 5.11: Patient survival according to age.**

Unadjusted Kaplan-Meier survival curve demonstrating patient survival over a ten year period in patients who presented over and under the age of 60 years ( $p=0.0001$ ).

#### 5.4.6 Renal and patient survival according to ANCA status

Patients were also divided by ANCA type and survival compared. As can be seen in Figure 5.12, there was no significant difference in renal survival when patients were split according to ANCA type although there was a trend towards worse renal survival in the MPO positive patients. There was also no significant difference in patient survival.

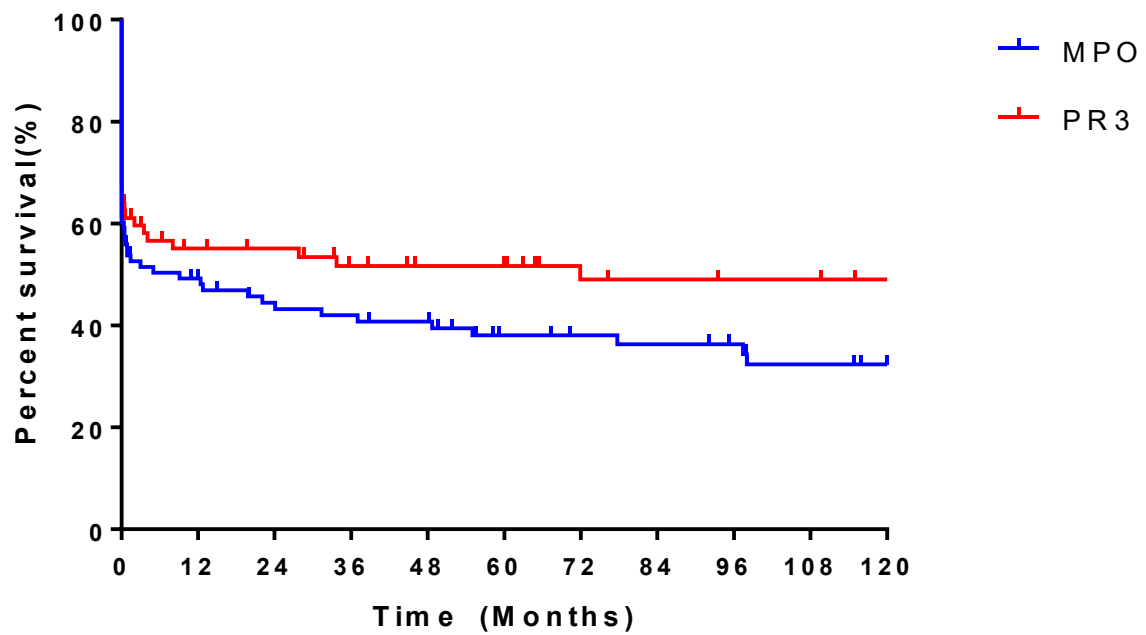


**Figure 5.12: Renal survival according to ANCA type**

Unadjusted Kaplan-Meier survival curve demonstrating renal survival over a ten year period in patients divided by MPO or PR3 positivity (p=ns).

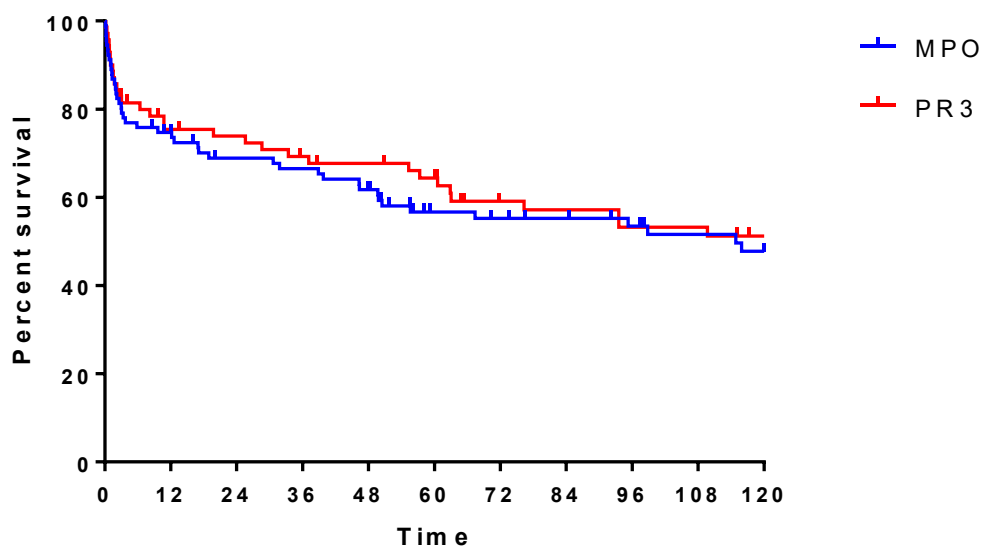


**Figure 5.13: Patient survival according to ANCA type.**

Unadjusted Kaplan-Meier survival curve demonstrating patient survival over a ten year period in patients divided by MPO or PR3 positivity (p=ns).

#### 5.4.7 Renal and patient survival divided by percentage normal glomeruli on biopsy.

We divided the entire cohort of patients by percentage normal glomeruli on biopsy, into three groups – 0-10, 11-24%, and > 25% normal glomeruli. We compared renal and patient survival according to percentage normal glomeruli on biopsy, and also used a composite endpoint of renal or patient death. Figure 5.14 demonstrates that there was a significantly improved renal prognosis in patients with >25% normal glomeruli when compared with patients with 0-10% normal glomeruli on biopsy. This was not the case for patient survival (Figure 5.15). Survival was not significantly better in the patients with a higher percentage of normal glomeruli, using the combined endpoint of renal and patient survival (Figure 5.16).

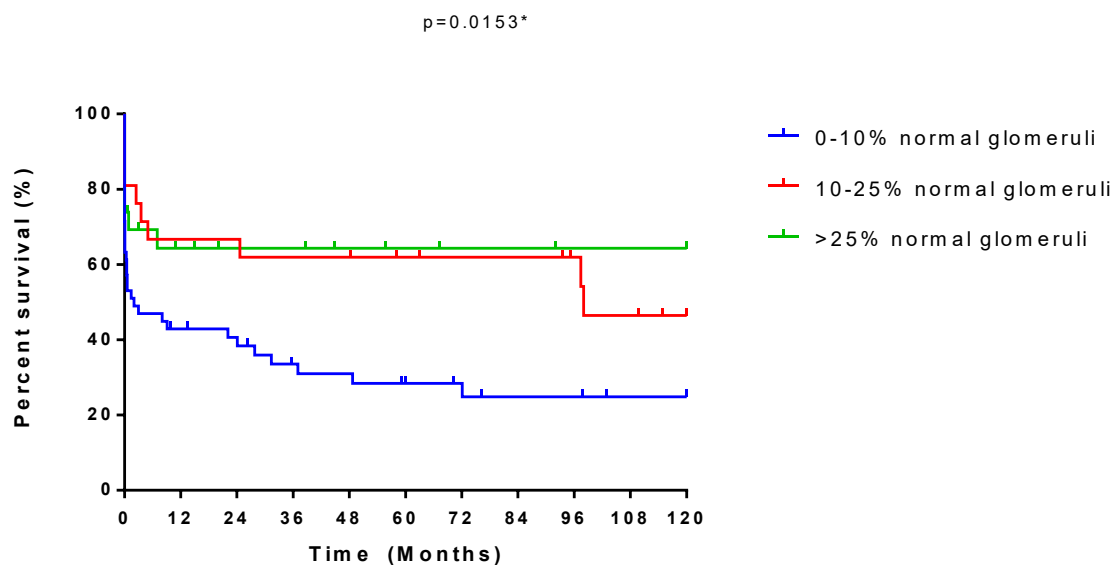


**Figure 5.14: Renal survival according to percentage normal glomeruli.**

Unadjusted Kaplan-Meier plot describing renal survival over 10 years in patients. There was a significantly better renal survival in patients who presented with a higher proportion of normal glomeruli on biopsy ( $p=0.0153$ ).

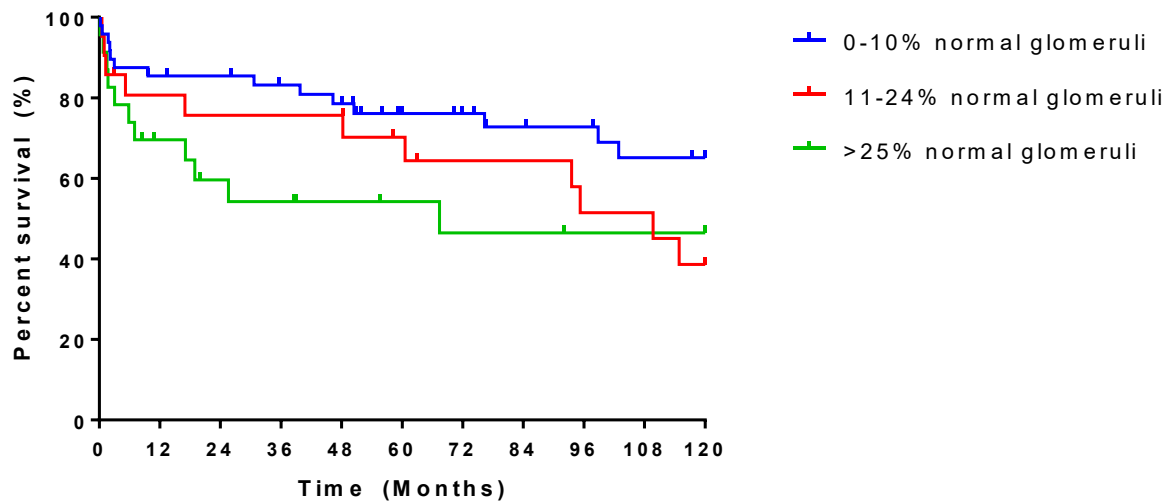


**Figure 5.15: Patient survival according to percentage normal glomeruli.**

Unadjusted Kaplan-Meier plot describing patient survival over 10 years in patients

There was not a significant difference in patient survival when patients were divided by percentage normal glomeruli on biopsy.

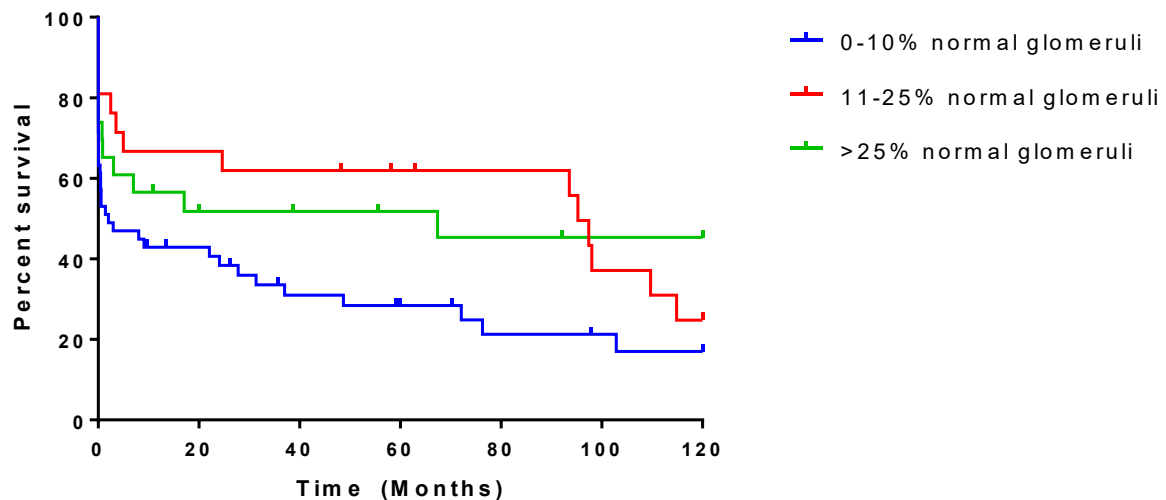


**Figure 5.16: Combined renal and patient survival according to percentage normal glomeruli.**

Unadjusted Kaplan-Meier plot describing a combined endpoint of renal and patient survival divided by % normal glomeruli. There is a trend towards worse survival in patients with a lower percentage of normal glomeruli when using a combined endpoint of renal and patient survival.

#### 5.4.8 Analysis of patients who presented dialysis dependent

One hundred and forty- six patients presented dialysis dependent. In Table 5.4 we compare the characteristics of those patients who had come off dialysis at one year, with those who had remained on renal replacement therapy. Sixty patients (59%) became dialysis independent at one year. Sixty percent of these patents were male. Patients who came off dialysis presented with a lower mean starting creatinine than those who did not (432  $\mu\text{mol/l}$  compared with 789  $\mu\text{mol/l}$ ).

|                                              | <b>Came off dialysis</b> | <b>Remained on dialysis</b> |
|----------------------------------------------|--------------------------|-----------------------------|
| Number (%)                                   | 60 (59)                  | 41 (40)                     |
| Male (%)                                     | 36 (60)                  | 22 (54)                     |
| Average age (yrs.)                           | 57.5                     | 57.5                        |
| MPO (%)                                      | 27 (45)                  | 20 (48)                     |
| PR3 (%)                                      | 27 (45)                  | 16 (40)                     |
| Starting Creatinine<br>( $\mu\text{mol/l}$ ) | 432                      | 789                         |
| <10% Normal Glom<br>N (%)                    | 17 (61)                  | 17 (71)                     |
| 10-24% Normal Glom<br>N (%)                  | 6 (21)                   | 4 (17)                      |
| >25 %Normal Glom<br>N (%)                    | 5 (18)                   | 3 (12.5)                    |
| Decade 1                                     | 2 (3)                    | 3 (7)                       |
| Decade 2                                     | 13 (22)                  | 6 (15)                      |
| Decade 3                                     | 18 (30)                  | 7 (17)                      |
| Decade 4                                     | 13 (22)                  | 13 (32)                     |
| Decade 5                                     | 14 (23)                  | 12 (29)                     |

**Table 5.4: Comparison of the 146 patients who presented dialysis dependent at presentation, divided by those who remained dialysis dependent at one year, and those who came off dialysis.**

45 patients did not survive until one year (31%). Decades of presentation; decade 1:1970-1980, decade 2: 1980-1990, Decade 3: 1990-2000, Decade 4: 2000-2010, Decade 5: 2010-2020

#### 5.4.9: Histological analysis

As shown above, full biopsy reports were difficult to obtain for all cases presenting in the earlier decades due to changes in recording of biopsy results over the years. In order to assess the impact of histological classification of biopsies, we included patients who presented from 2000. Eighty- one patients from our dialysis dependent cohort who presented from January 2000 were included. The demographics of these patients are listed in Table 5.5. Of these patients, 83% had been biopsied. Fourteen patients had not been biopsied mainly on account of their being treated immediately, usually due to pulmonary haemorrhage. Of the 67 patients who were biopsied, 58, (87%) of patients had more than 10 glomeruli for analysis, and hence qualified for classification using the Berden system. Figure 5.17 illustrates this. Figure 5.18 demonstrates the number of biopsies in each histological class. As expected for this degree of renal dysfunction, there were very few patients in the focal category.

| <b>Characteristic:</b>                              | <b>N</b>         |  |
|-----------------------------------------------------|------------------|--|
| Number of patients                                  | 81               |  |
| No of males – no. (%)                               | 46 (57)          |  |
| Mean age - yrs.                                     | 62.4             |  |
| MPO – no. (%)                                       | 43 (53)          |  |
| PR3 – no. (%)                                       | 32 (39.5)        |  |
| ANCA negative – no (%)                              | 6 (7)            |  |
| Mean follow up - months (range)                     | 79.7 (0.33-242)  |  |
| Mean presenting creatinine – $\mu\text{mol/L}$      | 616.4 (200-1543) |  |
| Number presenting dialysis<br>dependent – no. (%)   | 60 (74)          |  |
| Number presenting dialysis<br>independent – no. (%) | 21 (26)          |  |

**Table 5.5: Demographics of patients presenting after 2000**

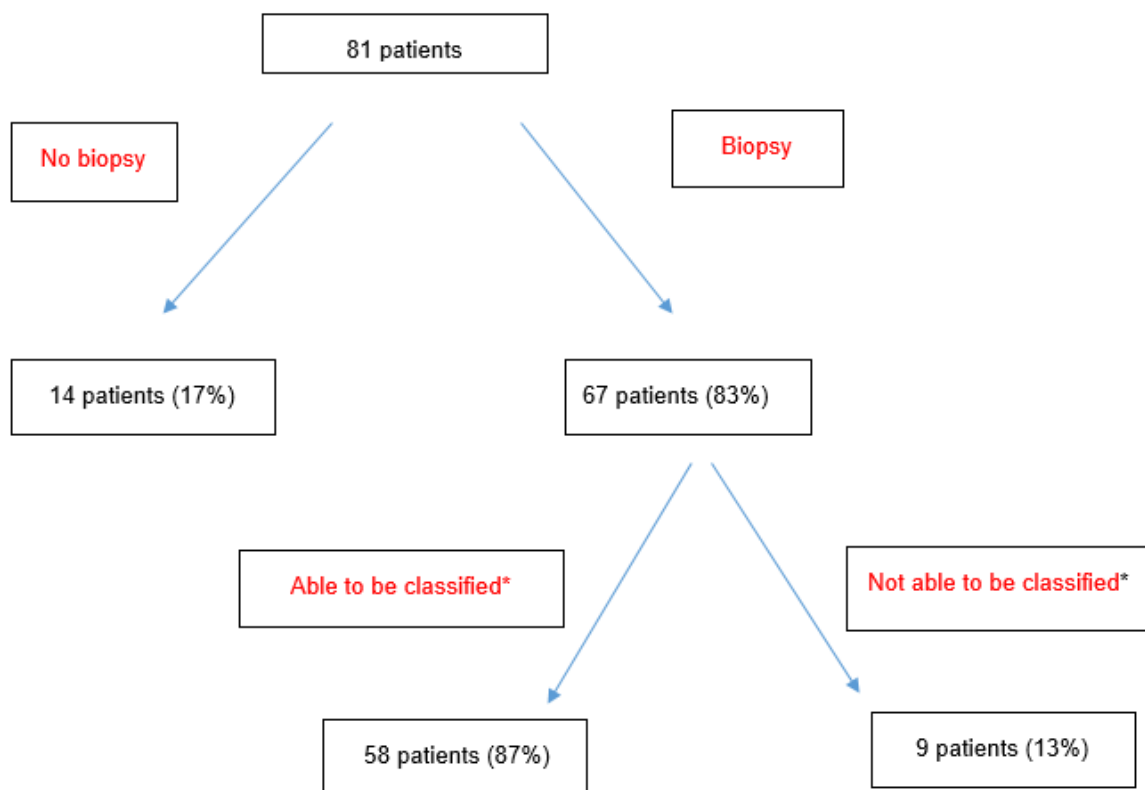


**Figure 5.17: Schematic of patients who presented post 2000.**

\*Patients were divided by histological class using the Berden classification. This was possible if greater than 10 glomeruli were available for analysis.

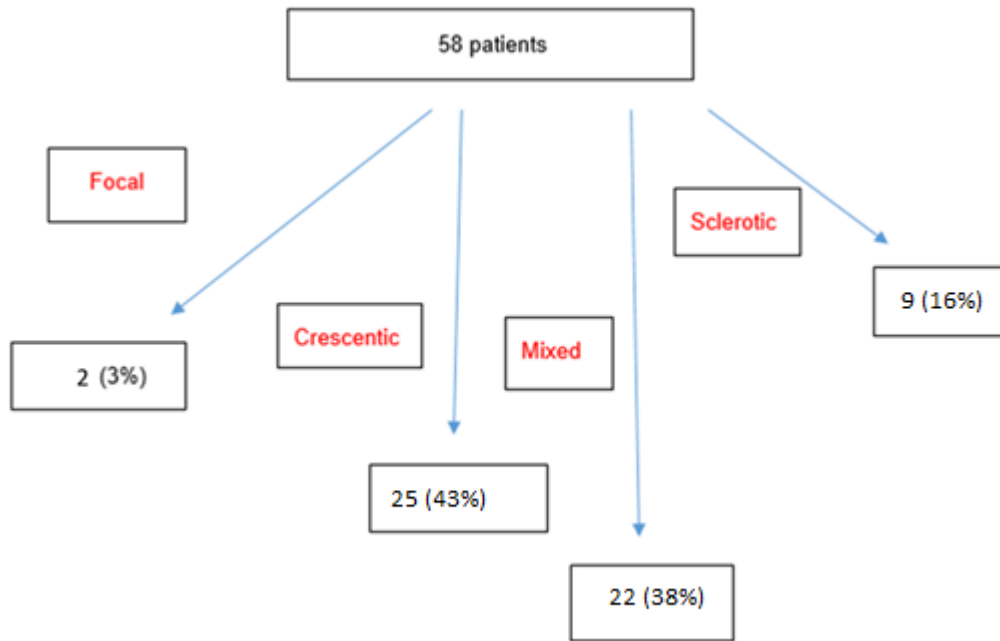


**Figure 5.18: Schematic to demonstrate classification of biopsies into histological class**

Biopsies were classified according to the Berden Classification.

Figure 5.19 describes renal survival by histological class. There was no significant difference in renal survival demonstrated according to histological class over a 10 year period. Figure 5.20 illustrates patient survival according to histological class and there was also no significant difference in patient survival according to histological class. A combined endpoint of renal and patient death was used in Figure 5.2.1, and again, there was no difference seen between histological classes.

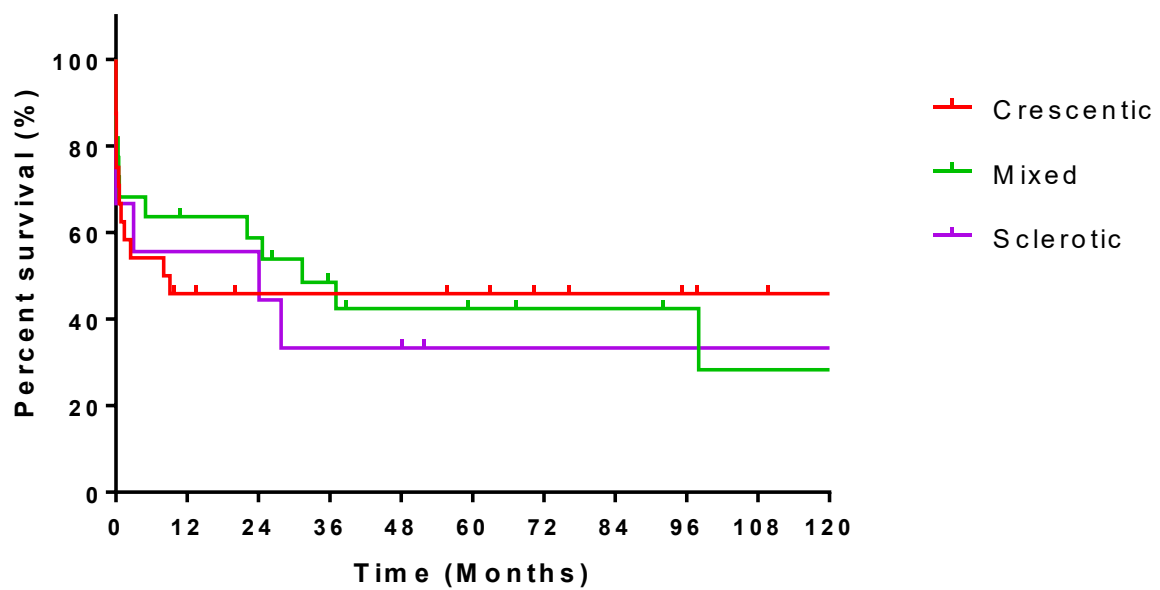


**Figure 5.19: Renal survival divided by histological class**

Unadjusted Kaplan-Meier plot describing renal survival over ten years as divided by histological class. The focal class was not included in this survival curve as there were only two patients in this category both of whom were censored for death before a year.

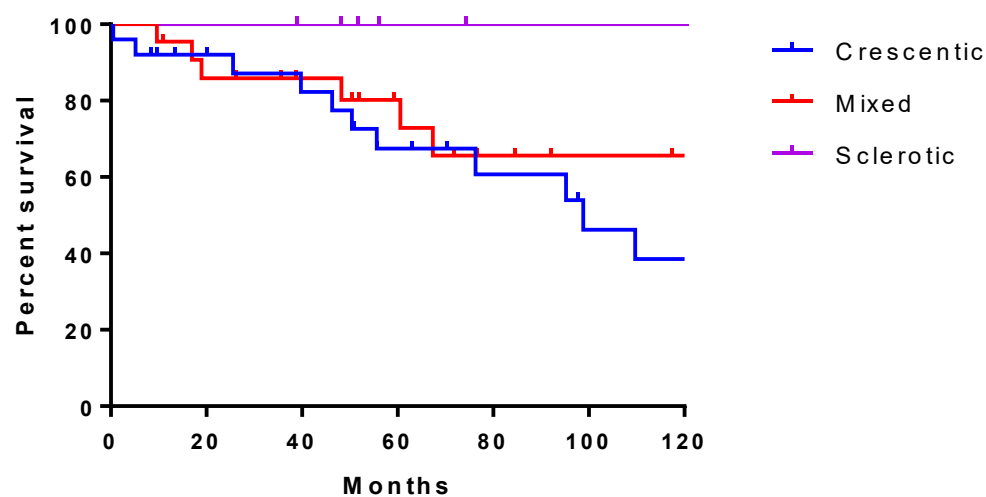


**Figure 5.20: Patient survival divided by histological class**

Unadjusted Kaplan-Meier plot describing patient survival over ten years as divided by histological class. There were only two patients in the focal category and they were therefore not included in this analysis.

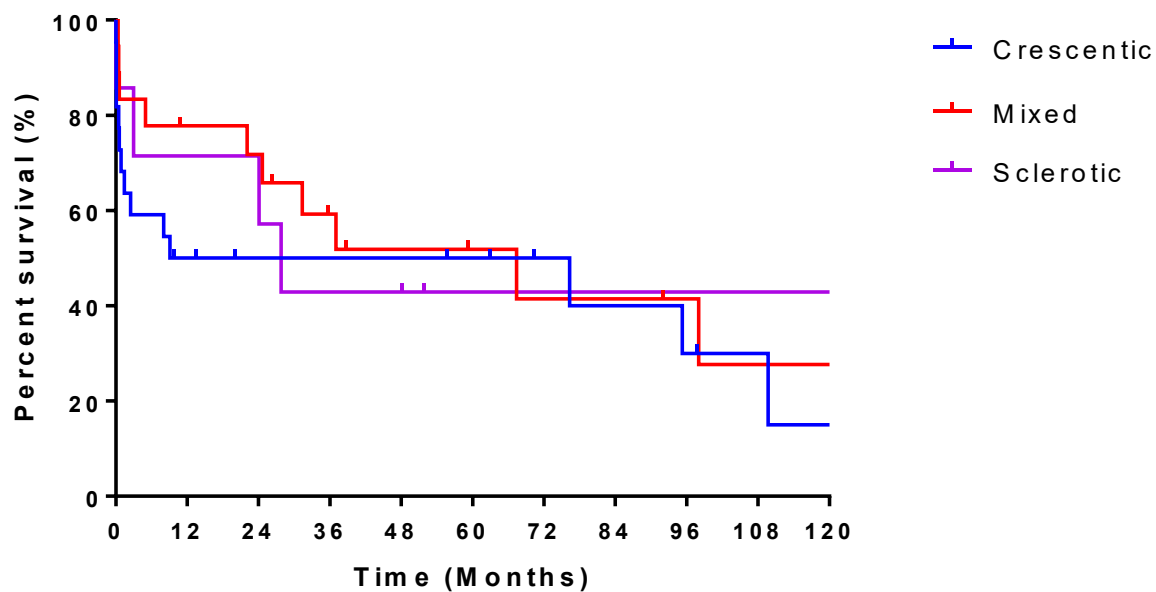


**Figure 5.21: Combined renal and patient survival divided by histological class**

Unadjusted Kaplan-Meier curve describing a combined endpoint of renal and patient survival over 10 years.

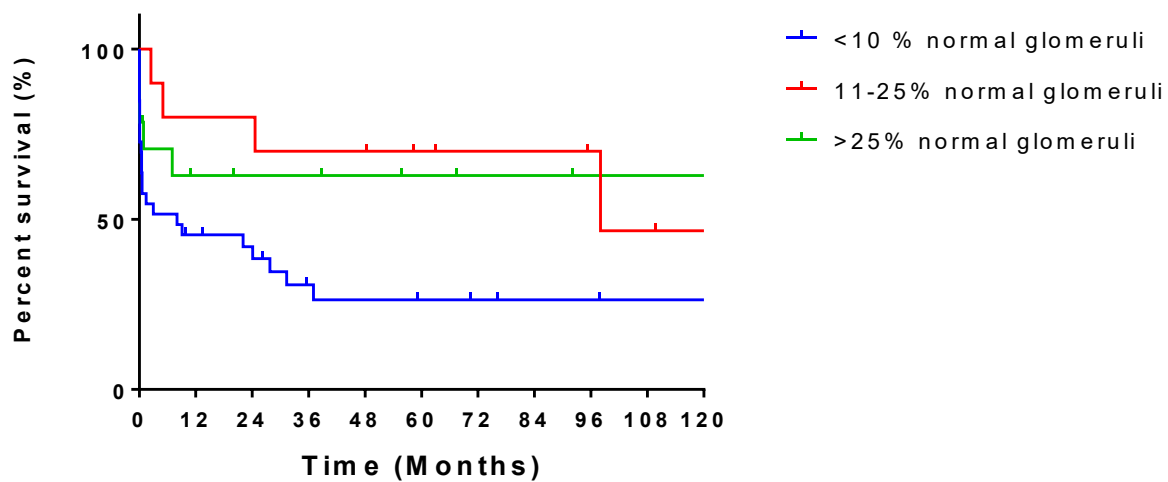


**Figure 5.22: Renal survival divided by percentage normal glomeruli.**

Unadjusted Kaplan-Meier curve illustrating renal survival by percentage normal glomeruli. Renal survival was significantly worse in patients who had <10% normal glomeruli on biopsy.

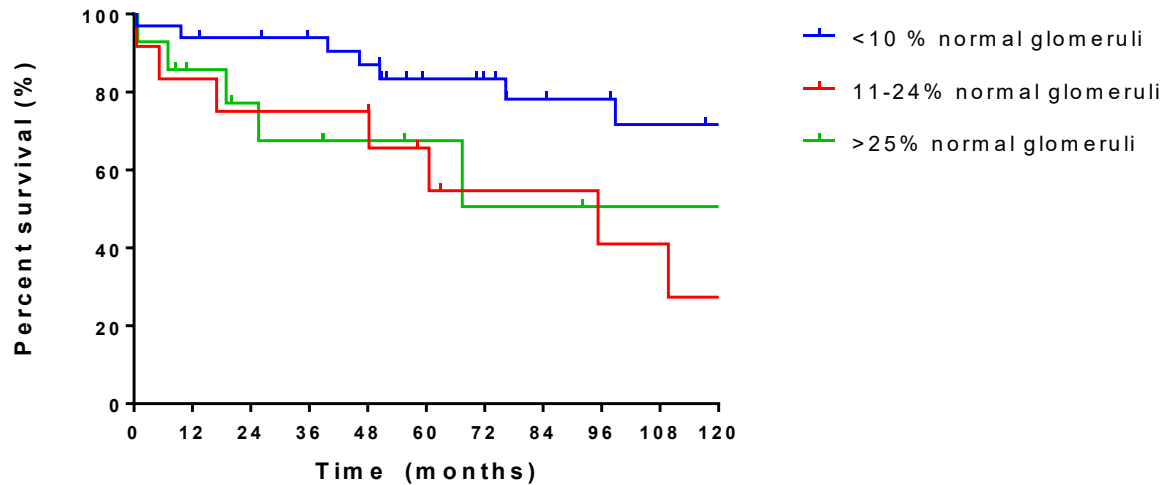


**Figure 5.23: Patient survival divided by percentage normal glomeruli**

Unadjusted Kaplan-Meier curve describing patient survival when divided by % normal glomeruli for patients presenting from 2000. There was improved patient survival in patients with <10% normal glomeruli.

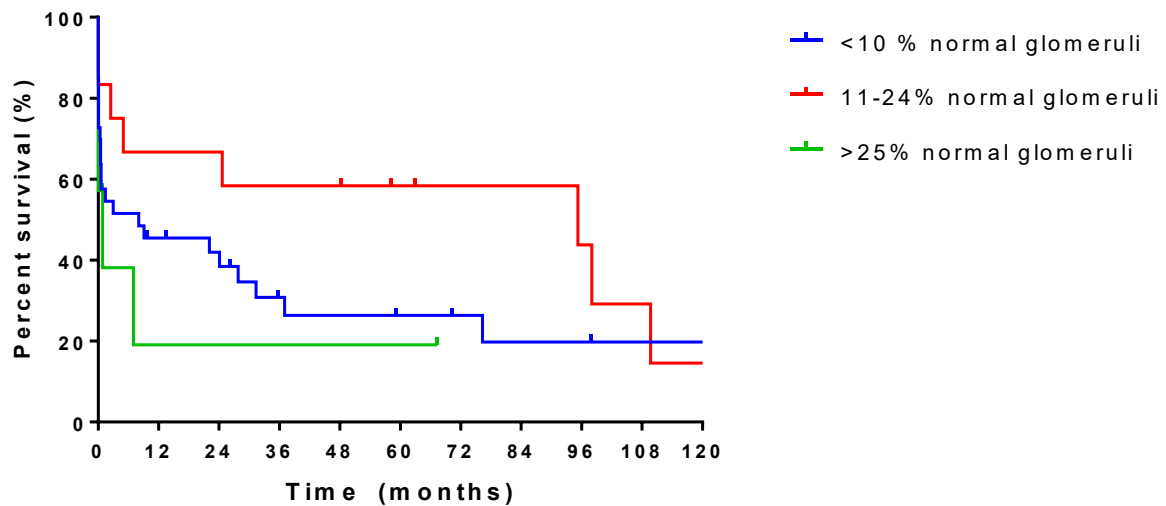


**Figure 5.24: Combined renal and patient survival divided by percentage normal glomeruli.**

Unadjusted Kaplan Meier curve describing survival using a combined endpoint of renal and patient death.

#### 5.4.10 Characteristics of patients who had presented dialysis dependent from 2000

After 2000, 60 patients presented dialysis dependent. 52 patients were alive at one year, 27 of these patients were independent of dialysis (52%).

|                                                 | <b>Came off dialysis</b> | <b>Did not come off dialysis</b> |
|-------------------------------------------------|--------------------------|----------------------------------|
| Number (%)                                      | 27 (52)                  | 25 (48)                          |
| Male (%)                                        | 16 (59)                  | 14 (56)                          |
| Average age (yrs.)                              | 60                       | 61.1                             |
| MPO (%)                                         | 12 (44)                  | 13 (52)                          |
| PR3 (%)                                         | 14 (52)                  | 9 (36)                           |
| Starting creatinine<br>( $\mu\text{mol/l}$ )    | 676                      | 721                              |
| Number without biopsy (%)                       | 8 (30)                   | 3 (12)                           |
| Patients biopsied                               | 19 (70)                  | 22 (88)                          |
| Biopsies excluded as not enough<br>glomeruli    | 3                        | 1                                |
| Number of patients with<br>interpretable biopsy | 16                       | 21                               |
| Focal (n)                                       | 0                        | 0                                |
| Crescentic n (%)                                | 8 (50)                   | 9 (43)                           |
| Mixed n (%)                                     | 4 (25)                   | 9 (43)                           |
| Sclerotic n (%)                                 | 4 (25)                   | 3 (14)                           |
| <10% Normal Glom n (%)                          | 11 (69)                  | 14 (66)                          |
| 10-24% Normal Glom n (%)                        | 2 (13)                   | 3 (14)                           |
| >25 %Normal Glom n (%)                          | 3 (19)                   | 3 (14) 0                         |

**Table 5.6: Comparison of patients who presented dialysis dependent from 2000 who came off dialysis at one year with those who remained dialysis dependent.**

## 5.5 Conclusions:

- In this chapter we describe the outcomes of a large cohort of patients with AAV and severe renal dysfunction, given uniform treatment including plasma exchange at a single centre.
- Both renal and patient outcomes were favourable when compared with other studies of this patient group.
- Fifty-nine percent of patients who presented dialysis dependent gained independent renal function at one year.
- The percentage normal glomeruli was predictive of renal survival and one of the factors associated with coming off dialysis at one year.
- Serum creatinine at presentation was also a factor associated with renal prognosis. Patients who did not regain independent renal function were shown to have a higher starting creatinine compared with those who did.
- The other factors assessed, including the Berden histological classification of patients, were not predictive of renal survival in this cohort.
- There was a striking difference in outcomes for dialysis dependent patients when compared to those who had a Scr of  $>500 \mu\text{mol/l}$  or an eGFR of  $<15 \text{ ml/min/1.73m}^2$  but who retained independent renal function. It may be relevant to analyse patients with severe renal function according to these criteria in future therapeutic trials.

## 5.6 Discussion:

The study described in this chapter has the advantages of representing a large patient cohort with severe renal dysfunction, from a single centre, treated in accordance with a uniform protocol including plasma exchange. One hundred and eighty AAV patients with severe renal involvement were included. Many of the published studies which did include patients with severe renal involvement report outcomes at one year. The mean follow-up time for this study was significantly longer, at 88 months. Only patients with newly diagnosed AAV were included (no relapsing disease), and this removed the potential confounder of previous immunosuppression for AAV.

Age has been demonstrated to be a prognostic factor in AAV. The mean age of patients included in this study was 59 years. Survival was compared for patients who presented over and under the age of 60 years. There was no difference in renal survival for these patients, however as expected, the younger patients had significantly improved patient survival.

MPO positive patients have been previously shown to experience a greater degree of long-term renal damage. In our cohort, 52% of patients were MPO positive, compared with 40% of patients who were PR3 positive. The remainder of the cohort was ANCA negative. When renal survival was compared between the PR3 and MPO positive patients, there was a trend towards better survival in the PR3 positive patients although this did not meet statistical significance. There was no difference in terms of patient survival.

The outcomes for this cohort in terms of both renal and patient survival at one and five years were comparable or better than the current literature. Almost 75% of patients

were alive at one year and 56% at five years. Survival with independent renal function was 64% at one year and 51% at five years. This is a positive result given their severe renal involvement and that 146 (80%) of these patients were dialysis dependent at presentation. These findings support the view that patients should be treated aggressively for their AAV despite presenting dialysis dependent, as there is a greater than 50% chance of recovering renal function when treated.

We divided patients into those who were dialysis dependent, and those who maintained independent renal function on admission. Eighty one percent of patients with this degree of renal dysfunction required dialysis within 72 hours of admission. There was a strikingly improved prognosis in those patients who presented independent of dialysis in terms of 1 and 5 year patient and renal survival. For patients who were not on dialysis, one year survival was 79% compared with 69% in the dialysis dependent cohort. At five years, patient survival was 74% for the dialysis independent patients, compared with 52% in the dialysis dependent. This difference was also seen for renal survival, with dialysis dependent patients having significantly worse renal survival at 1 and 5 years (59% and 52% respectively) compared with 96% and 74% in the dialysis independent group. Survival analysis over 10 years confirmed this difference in both patient and renal survival. This finding could have significant implications for both current and future studies as patients are not usually divided by dialysis independence at presentation and these patients may therefore require altered therapy. This is the first study to our knowledge, to separate patients in this manner.

Of the 146 patients who presented dialysis dependent, 60 (59%) became dialysis independent at one year. When comparing patients who came off dialysis with those

who remained dialysis dependent, there was no significant difference in terms of age or ANCA type. As described in other studies, starting creatinine was significantly higher in those who remained dialysis dependent than those who came off (789  $\mu\text{mol/l}$  compared with 432  $\mu\text{mol/l}$ ). There were only 34 patients who presented dialysis dependent in our study and further studies would need to be carried out to fully assess this difference and the possible reasons for this. A higher percentage of normal glomeruli was also associated with better renal survival. This is in accordance with current literature

Patients included in our study presented over a number of years. As these patients were treated in a single centre, they were all treated according to the same protocol. There were adjustments to this protocol however, including the addition of rituximab in 2011 and the change from oral to intravenous cyclophosphamide. In order to account for this, we assessed renal survival according to decade of presentation. There was no significant difference in renal survival according to decade of presentation.

Despite treatment for their AAV remaining fairly uniform over the years, there have been advances in terms of general medical care, and the comparison of patient survival across the decades accounts for this. There was a statistically significant improvement in patient survival for patients presenting between 2010 and 2020 compared with those presenting in the 1970s.

Although it was not possible to fully classify biopsies by the Berden classification for the entire cohort of patients, we were able to obtain results for the majority of patients who had been biopsied, with regards to percentage normal glomeruli. Percentage normal glomeruli were divided according to current literature including the Brix renal

risk score, into three groups, 0-10%, 11-24% and >25% normal glomeruli. There was significantly improved renal survival in patients with > 25% normal glomeruli. There was no improvement in patient survival, or using a combined endpoint of renal and patient death.

In order to compare patients by Berden histological class, we reviewed histology for patients who presented from January 2000. This included 81 patients, 83% of whom had been biopsied. The remaining patients had not been biopsied largely due to the need to commence urgent treatment, often for pulmonary haemorrhage. Of those who were biopsied, 87% could be classified according to the Berden classification. The remainder did contain enough glomeruli for analysis. As expected in a group with such severe renal involvement, there were only 2 biopsies which were classified as focal (over 50% normal glomeruli). These two patients had presented clinically with a number of pre-renal factors which contributed to their renal dysfunction. As there were such small numbers and both patients in this category died before one year, they were excluded from the survival analysis.

The majority of biopsies were classified as mixed or crescentic. In keeping with current studies, there was no significant difference in outcome, either renal or patient survival, according to histological class. Previous studies assessing the impact of histological features on patient outcome have suggested that those with a higher proportion of sclerotic glomeruli have a worse renal outcome and that there should be careful consideration of subjecting these patients to the potential risks of treatment. In our study, percentage of sclerotic glomeruli did not in fact affect outcome (although we accept that numbers were small). In practice, a biopsy specimen may not be representative of changes throughout the kidney in view of the fact that AAV causes

a focal and segmental glomerulonephritis and we suggest that this could explain our finding that patients with a greater number of sclerotic glomeruli did not experience worse outcomes. Our results are consistent with current studies which have concluded that histological class is not a prognostic factor in patients who present with severe renal involvement, and that these patients should be treated uniformly regardless of biopsy classification.

There is growing controversy over the role of plasma exchange in the treatment of patients with severe renal function particularly following the publication of the results of PEXIVAS. PEXIVAS has yet to report on the outcomes of patients who presented dialysis dependent. From our study, these patients have been successfully treated with a regimen including plasma exchange. From our results, it would be useful for patients to be divided according to dialysis dependence at presentation and that current studies could re-analyse outcomes according to this.

As with any retrospective analysis, particularly over so many years, there were limitations to our study. These include the possibility of missing some patients particularly in the earlier years if they were admitted for short periods to the intensive care unit. It was difficult to obtain precise information regarding possible side effects for all these patients but this work is ongoing.

Prospective studies which include this group of patients with severe renal involvement and perhaps divide them into those who present dialysis dependent and those who retain independent renal function are needed.



## Chapter 6: B cell survival factors and AAV

## 6.1 Introduction:

The pathogenicity of the B cell in AAV is now well established. As discussed in Chapter 1, there are a number of means by which B lymphocytes have been shown to contribute to the disease process. Possible mechanisms include the production of autoantibodies, interaction with T cells, and the release of inflammatory cytokines. It therefore follows that a reduction in B cells would serve a therapeutic function. Reduction in pathogenic B cells can occur by a number of mechanisms including increasing their apoptosis by binding to CD20, or by decreasing their survival by reduction in B cell survival factors.

Induction of apoptosis occurs following binding of the anti-CD20 agent RTX to the B cell. The first case of RTX being used for the treatment of AAV was described by Specks *et al* in 2001, who described its successful use in the treatment of a 66 year old man with severe relapsing GPA which was unresponsive to traditional therapies (175). A number of case reports and series describing the use of RTX alongside traditional therapies followed this report (176). Following two large randomised controlled trials, RAVE and RITUXVAS, RTX was approved by the FDA as an induction agent for the treatment of AAV in 2011 (40, 41). The discovery of a subgroup of B cells which are regulatory in nature (Bregs)(177) has highlighted the possible danger of widespread B cell depletion and the need to specifically target B cell subsets which are involved in pathogenesis.

As discussed previously, BlyS and APRIL are members of the TNF superfamily of proteins which have a central role in B cell homeostasis. Serum BlyS levels have been shown to be raised in patients with a number of autoimmune diseases including Sjogrens, SLE and rheumatoid arthritis (71, 178, 179). Studies have also found BlyS

levels to be raised in AAV (62). Neutrophils stimulated by ANCA have been shown to release BlyS (63) and BlyS levels have been found to be elevated in patients with active disease (63). Levels of BlyS have also been found to be elevated following treatment with RTX (63). It has been hypothesised that patients who have received B cell depletion therapy experience repopulation of their B cell compartment with B cells which, in a BlyS rich environment, have a tendency towards autoimmunity. This forms the rationale for the use of the anti-BlyS agent belimumab in combination with RTX for the treatment of AAV. APRIL binds to the receptors BCMA and TACI. Raised APRIL levels have been demonstrated in autoimmune conditions such as SLE (180, 181) as well as in a number of malignancies. Recombinant APRIL has been shown to co-stimulate B cells in vitro and in vivo but with less potency than BlyS (181). These findings make APRIL another possible therapeutic target.

One of the major challenges in the management of AAV remains the minimisation of treatment related toxicity whilst maintaining remission. Relapses occur often with non-specific symptoms and can be difficult to differentiate from septic and other complications. ANCA titre is not always an accurate indication of disease activity and alternative biomarkers are needed. There is the potential for both BlyS and APRIL to be used, in conjunction with ANCA titres and clinical status, as biomarkers for disease.

Intrarenal B cells have been demonstrated in a number of immune-mediated diseases including lupus, membranous nephropathies and renal transplant rejection (182, 183). Positive staining for BlyS and its three receptors, BR3, TACI and BCMA has also been demonstrated in renal biopsies of patients with lupus nephritis and shown to correlate with different categories of disease (184). Clusters of B cells have been demonstrated in kidney biopsies of patients with AAV using staining for CD20; however at the time

of writing, there is no literature describing staining for B cell survival factors and their receptors in patients with AAV.

The aim of this study was to assess the clinical significance of serum BlyS and APRIL levels in a large cohort of patients, treated uniformly at our centre. In addition we assessed for BlyS and its receptors by immunohistochemistry in a sample of our AAV patients.

## 6.2 Assessing the clinical significance of serum BlyS and APRIL

### 6.2.1 Experimental Design:

Ethical approval was granted by the West London National Research Ethics Service (NRES) committee. Patients were recruited for the study either when attending vasculitis outpatient clinic, or whilst admitted on the wards at Hammersmith Hospital. Written consent was given for the use of clinical data and samples. Plasma samples were obtained from patients in addition to routine bloods taken at their clinic attendances or during admission. Patient's clinical data at the time of sample collection were recorded. Patients with active disease, both new presentations and relapsing disease, and those in remission, were included. BlyS levels were measured on 76 patients, 20 with active disease and 56 in remission. Samples were also taken from 13 healthy volunteers. APRIL levels were measured in a subsequent experiment in 36 patients. The number of patients for whom APRIL levels could be obtained was limited by our supplies of ELISA kits for APRIL and amount of patient samples available following measurement of BlyS. Soluble BlyS and APRIL levels were detected using commercial ELISA kits for both BlyS and APRIL. Results were analysed with a plate reader at 450 nm. GraphPad prism 4.0 (GraphPad Software, San Diego, California, USA) was used for statistical analysis. To compare more than

2 groups, assuming non-parametric data, the Kruskal-Wallis test and Dunn's multiple comparison post- test were used. To compare 2 groups, the Mann-Whitney-U test was used. The non-parametric Spearman rank correlation analysis was used to assess correlation.

### 6.2.2 Results: Measurement of serum BlyS levels

Seventy-six patients were included in this study, of whom 38 were positive for anti-PR3 antibody and 17, for anti- MPO antibody. The remainder of patients were ANCA negative. Fifty percent of patients were female and the average age at time of testing was 60 years. Twenty of these patients (26%) had active disease at the time of testing and 56 were in remission. Thirteen healthy controls were recruited from colleagues both in the Trust and the laboratory. Despite an attempt to age match the healthy controls to patients, the average age of our healthy controls was 50 compared with 60 for the patients, and this was recognised as a limitation of our study. Serum BlyS concentrations were measured for these patients as shown in Figure 6.3.1. It can be seen that BlyS concentrations were significantly higher in all AAV patients (both active and in remission), when compared with healthy controls. Levels were also significantly raised in patients with active disease compared with those in remission.

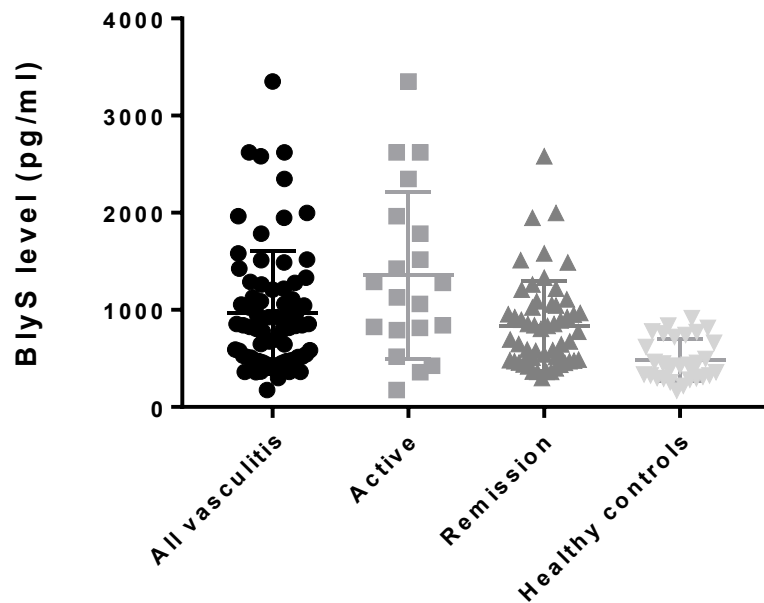


**Figure 6.1: Graph to show serum BlyS concentrations in patients in active AAV vs those in remission vs healthy controls.**

There is a statistically significant difference between the groups. Kruskal-Wallis test  $p < 0.0001$ . There was no correlation between serum BlyS concentrations and CRP or creatinine at the time the sample was taken, Figures 6.2 (a) and 6.2 (b). BlyS concentrations were not related to ANCA titre, Figure 6.2 (c) or ANCA type, Figure 6.2 (d).

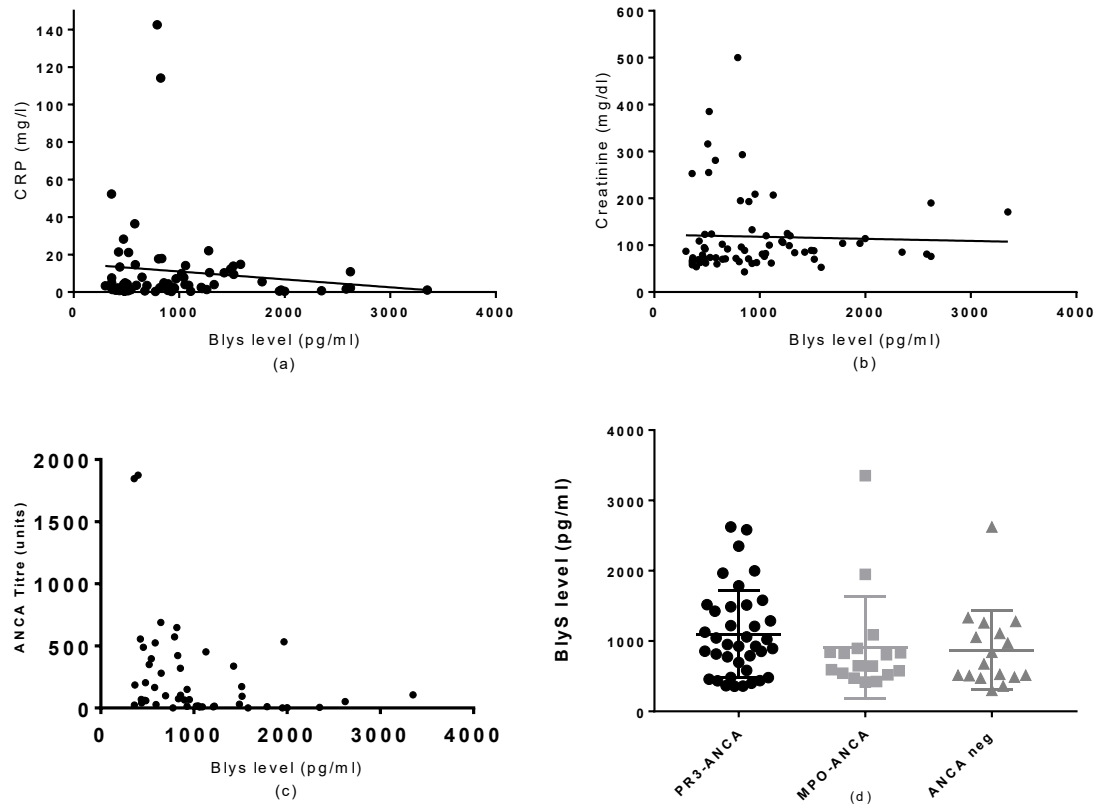


**Figure 6.2: Correlation between serum BlyS and crp, serum creatinine, ANCA titre and BlyS level.**

(a) Graph to assess correlation between serum BlyS level and CRP. There was no significant correlation shown. (B) Graph to assess correlation between serum BlyS level and serum CRP. There was no significant correlation found. (c) Graph to assess correlation between Serum BlyS level and ANCA titre. There was no significant correlation found. (d) Comparison between BlyS levels in patients who were MPO positive, PR3 positive, and ANCA negative. There was no significant difference found.

We also divided all patients included in our study into those who had been previously treated with rituximab and those who had not. As demonstrated in Figure 6.3 there was a statistically significant increase in serum BlyS levels in those who had been treated with rituximab in the past ( $p < 0.0001$ ). The average time from last dose of rituximab was 16.8 months.

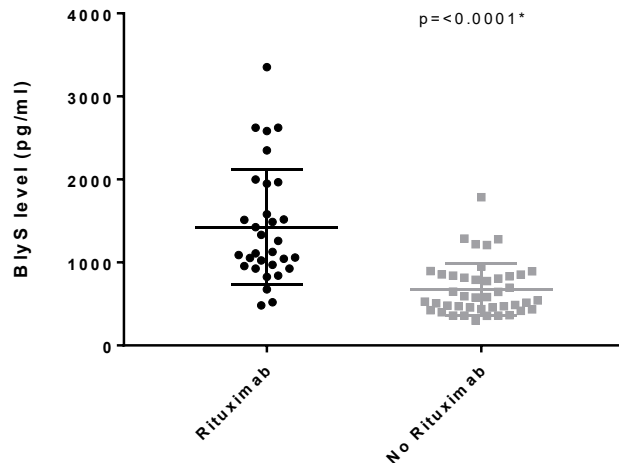


**Figure 6.3: Graph to show all patients included in this study divided according to treatment with rituximab.**

Serum BlyS levels were significantly increased in rituximab patients, ( $p < 0.0001$  Mann Whitney test).

### 6.2.3 Results: Measurement of serum APRIL levels

We carried out testing of serum APRIL levels in a group of 34 patients. Fifty five percent of patients were male and the average age at the time of obtaining a sample was 58 years. These patients included 10 active patients and 24 remission patients. Ten healthy control patients were also used for comparison. Twenty- eight patients had a diagnosis of GPA, one EGPA and 5 MPA. Nineteen patients were positive for PR3 and 9 for MPO. Six patients were ANCA negative. As demonstrated in Figure 6.5, serum APRIL levels were significantly increased in AAV patients compared with healthy controls, and patients with active disease had significantly higher APRIL levels than those in remission.

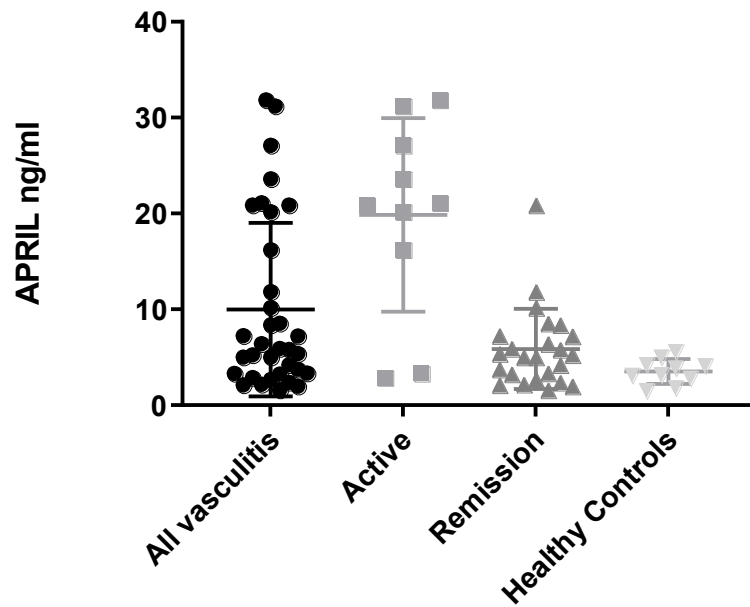


**Figure 6.5: Graph to show comparison between serum APRIL levels in patients with active AAV compared with those in remission and healthy controls.**

There was significant difference between the groups,  $p=0.011$  (Kruskal-Wallis test). Median values and standard errors are represented.

In contrast to serum BlyS levels, there was no significant difference in serum APRIL levels according to treatment with rituximab. This is demonstrated in Figure 6.6.

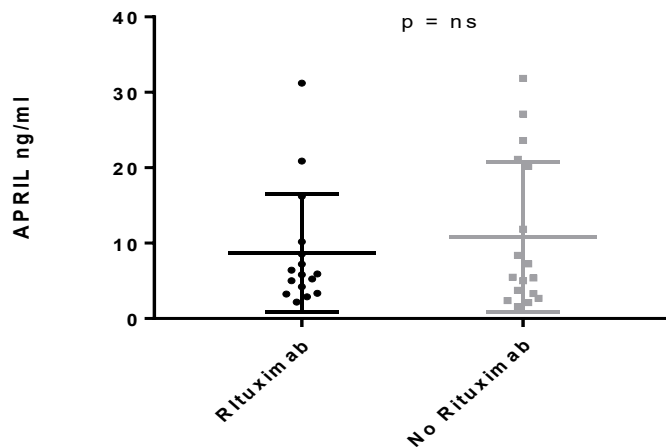


**Figure 6.6: Graph to show the effect of rituximab treatment on serum APRIL levels.**

There was no significant difference demonstrated for all patients who had been treated with rituximab when compared with patients treated with other immunosuppressive medications ( $p=ns$ , Mann-Whitney test).

There was no correlation found between serum APRIL levels and CRP (Figure 6.7a).

There was however a correlation between serum APRIL and creatinine level, with higher levels of serum APRIL correlating with higher creatinine values (Figure 6.7b).

There was no correlation with ANCA titre (Figure 6.7c); however there was a significantly higher serum APRIL level in patients with either MPO or PR3 positivity compared with those who were ANCA negative (Figure 6.7d)

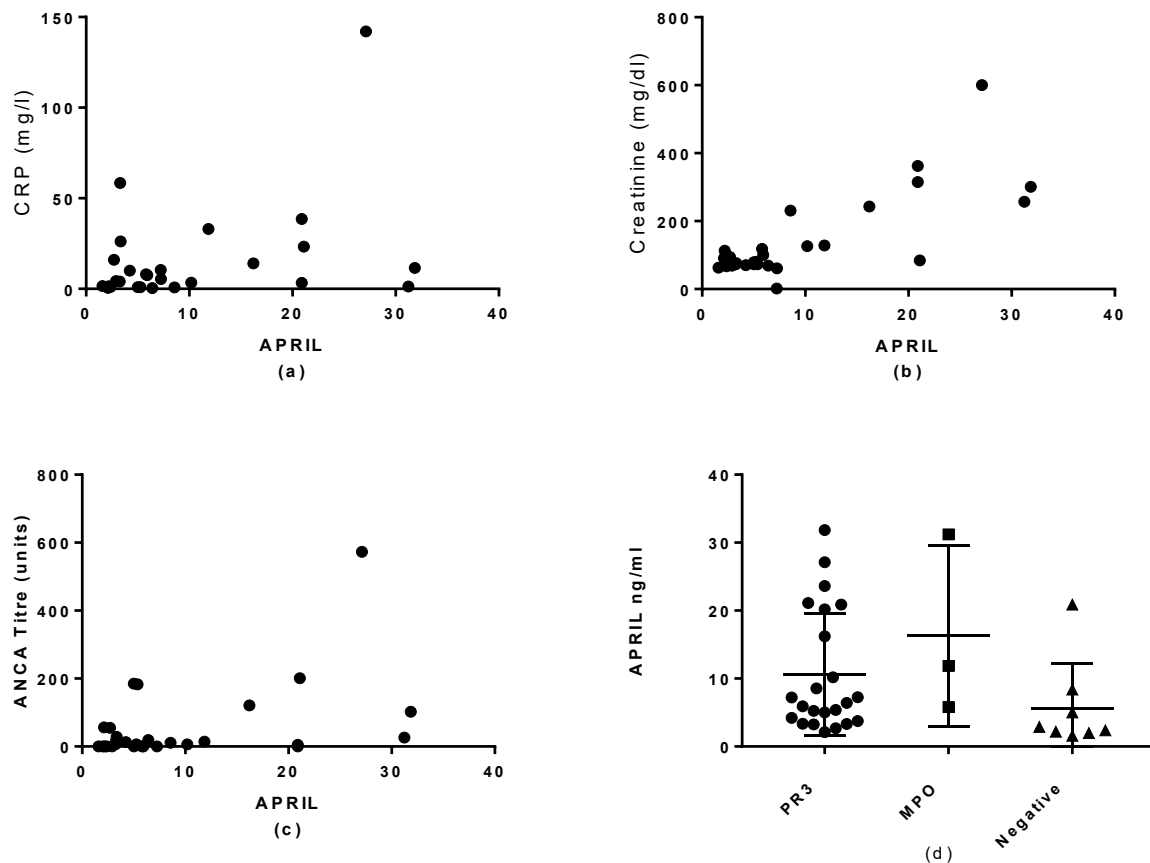


**Figure 6.7: Graphs to assess correlation between serum APRIL levels and crp, serum creatinine, ANCA titre and ANCA type.**

(a) Graph to assess correlation between serum APRIL levels and CRP. There was no significant correlation shown. (b) Graph to assess correlation between serum BlyS level and serum creatinine ( $\mu\text{mol/l}$ ). There was a significant correlation found with higher APRIL levels at higher levels of serum creatinine ( $p=0.004$ , Spearman Rank correlation). (c) Graph to assess correlation between Serum APRIL levels and ANCA titres. There was no significant correlation found. (d) Comparison between serum APRIL levels in patients who were MPO positive, PR3 positive, and ANCA negative. There was a significant difference between the groups ( $p=0.04$ , Mann-Whitney U)

There were some patients for whom it was possible to assess both serum APRIL and BlyS levels from the same serum sample. There was no correlation seen between serum APRIL and BlyS levels (Figure 6.8).

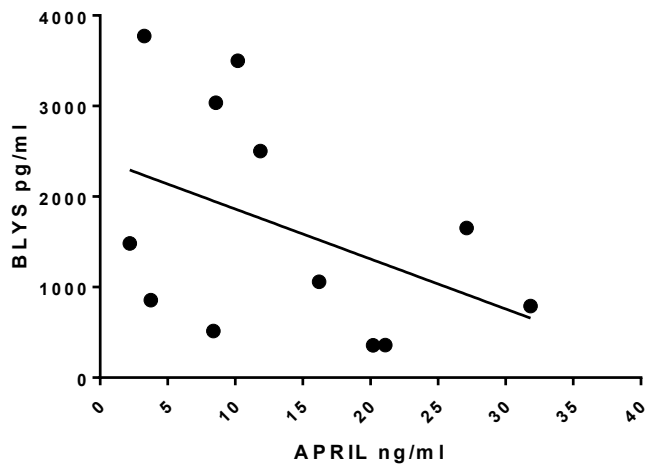


**Figure 6.8 Graph to demonstrate correlation between BlyS and APRIL levels.**

There was no significant correlation seen in our patients.

In ten patients, we were able to measure consecutive BlyS levels at 0 and 6 months and to correlate these with disease activity. It is clearly demonstrated in Figure 6.4 that BlyS levels were related to disease activity. In patients whose disease status did not change over the six month periods, the lines on the graph remained relatively flat with no significant change in BlyS levels. Patient 1 became active at six months having been in remission at time 0, and this was accompanied by a significant rise in serum BlyS level. Patients 2 and 3 went into remission at six months and the graph illustrates a drop in their serum BlyS levels.

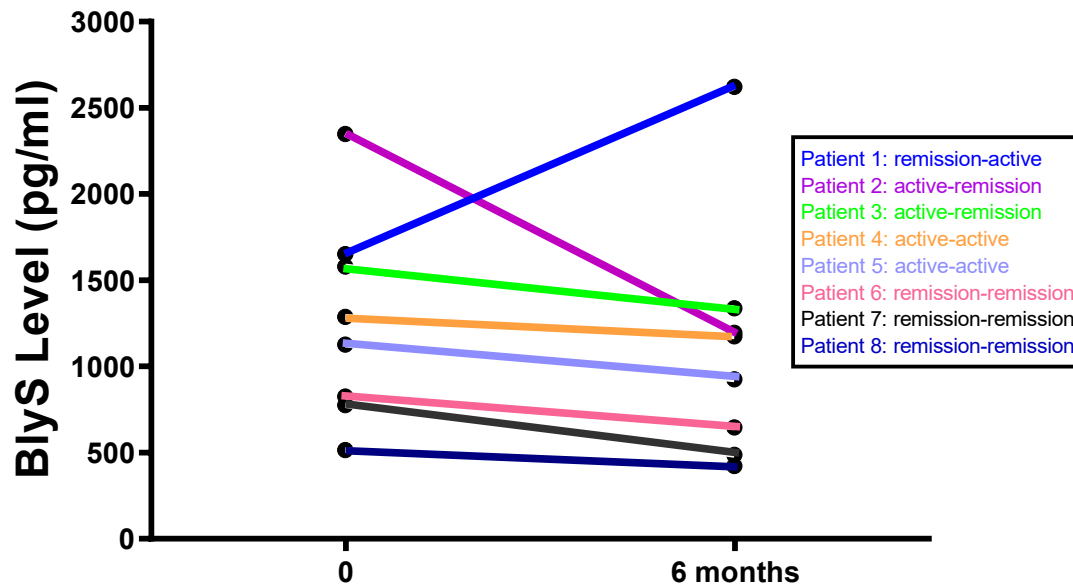


**Figure 6.9: Graph demonstrating sequential BlyS levels in ten AAV patients.**

Levels were taken at 0 and 6 months. It can be seen that BlyS levels correspond with disease status at the various time-points described in the key.

#### 6.2.4 Conclusions from this study:

- Serum BlyS levels are raised in patients with AAV compared with healthy controls.
- Serum BlyS levels are raised in patients with active disease
- Serum BlyS levels are raised following treatment with rituximab
- Serum BlyS could represent a useful therapeutic target in AAV
- Serum APRIL levels are raised in patients with AAV compared with healthy controls.
- Serum APRIL levels are raised in patients with active disease
- Rituximab does not cause rise in serum APRIL levels
- Serum APRIL could represent a useful therapeutic target in AAV

## 6.3 Assessment of BlyS and its receptors in renal biopsy specimens of patients with AAV.

### 6.3.1 Experimental Design

We carried out a descriptive study on 10 renal biopsy samples from patients with AAV. Immunohistochemistry for BlyS, its receptors (TACI, BCMA, BAFFR) and CD20 was carried out. Samples were scored according to percentage of cells with positive expression. Table 6.1 lists patients for whom biopsy specimens were provided and their histological classification by the International classification as described in Chapter 1.

| Patient Number | Histological Classification |
|----------------|-----------------------------|
| 1              | PR3 focal                   |
| 2              | PR3 focal                   |
| 3              | MPO focal                   |
| 4              | MPO crescentic              |
| 5              | MPO crescentic              |
| 6              | MPO crescentic              |
| 7              | PR3 crescentic              |
| 8              | MPO mixed                   |
| 9              | MPO sclerotic               |
| 10             | MPO mixed                   |

**Table 6.1: Patients included by Histological Classification**

### 6.3.2 Results:

Representative images for both glomerular and interstitial staining have been included for each factor for each patient where possible. Images for Patient 1, a patient with PR3 focal AAV are shown in Figure 6.10. For this patient, it can be seen that there was BlyS staining in both glomeruli and the interstitium (Figure 6.10 A and B). BAFFR staining was also present in both the glomeruli and the interstitium (Figure 6.10 C and D). CD 20 staining was clearly demonstrated in the interstitium (Figure 6.10 E and F) and TACI staining was primarily glomerular (Figure 6.10 G and H).

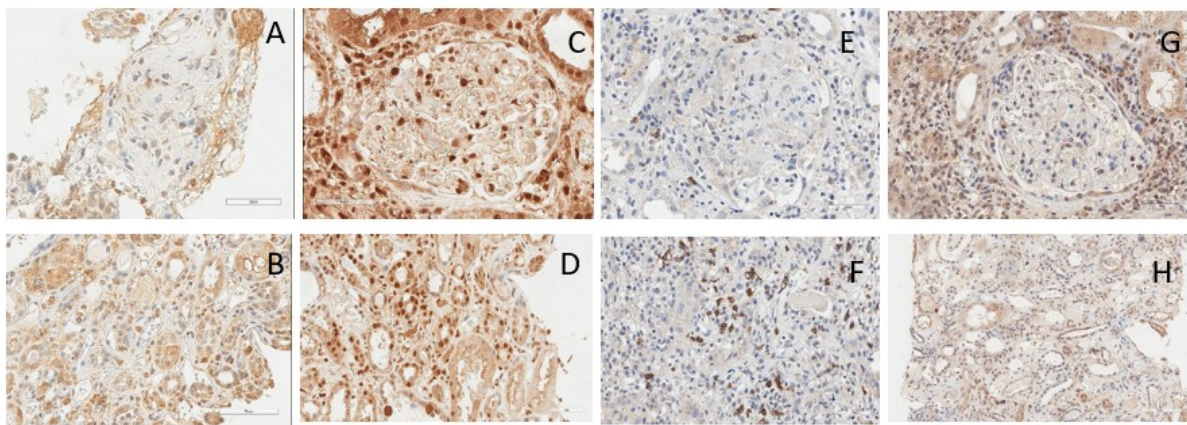


**Figure 6.10: Patient 1**

A: BlyS glomerular staining; B: BlyS interstitial staining; C: BAFFR glomerular staining; D: BAFFR interstitial staining; E: CD20 glomerular staining; F: CD20 interstitial staining; G: TACI glomerular staining; H: TACI interstitial staining. \*BCMA staining was not suitable for analysis.

Patient 2 was also a patient with PR3 positive focal AAV. BlyS and BAFF R staining were not possible to analyse for this patient. There was very little BCMA staining in either the glomeruli or interstitium (Figure 6.11 A and B). Similar to Patient 1, CD20 positivity was primarily in the interstitium and spared glomeruli (Figure 6.11 C and D).

There was some TACI staining in both the glomeruli and interstitium (Figure 6.11 E and F).

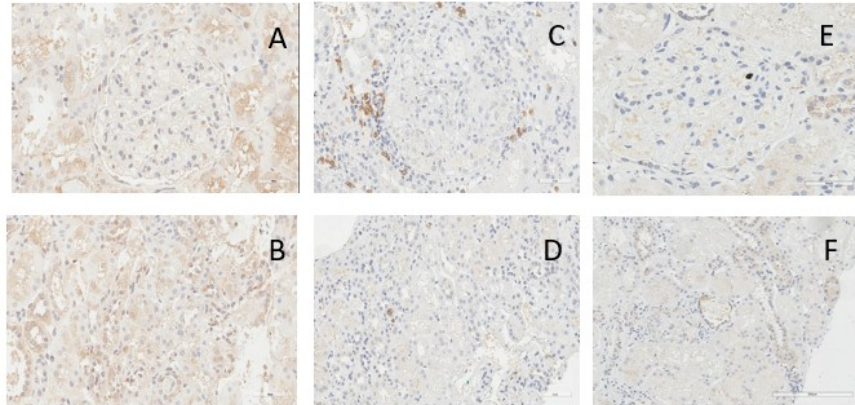


**Figure 6.11: Patient 2:**

A: BCMA glomerular staining. B: BCMA interstitial staining; C: CD20 glomerular staining; D: CD20 interstitial staining; E: TACI glomerular staining; F: TACI interstitial staining. \*BAFF and BAFF R staining not suitable for analysis.

Patient 3 was also classified as having focal disease by the Berden classification but was MPO positive. Images for this patient are shown in Figure 6.12. It can be seen that BlyS staining was weak but primarily glomerular (Figure 6.12 A and B). BAFF R staining was also predominantly glomerular with the highest concentration in the crescent (Figure 6.12 C). BCMA staining was positive in the glomerulus (Figure 6.12 E) and negative in the interstitium (Figure 6.12 F). As for the patients above, CD20 staining was very clear and in highest concentration surrounding the glomerulus and in the interstitium (Figure 6.12 G and H). Again, TACI staining was primarily glomerular (Figure 6.12 I and J).

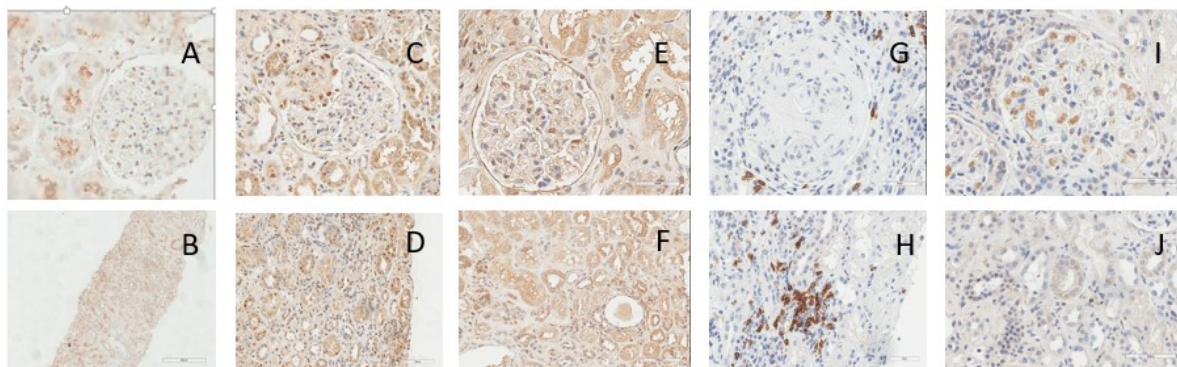


**Figure 6.12 Patient 3.**

A: BlyS glomerular staining; B: BAFF interstitial staining; C: BAFF R glomerular staining; D: BAFF R interstitial staining; E: BCMA glomerular staining; F: BCMA interstitial staining; G: CD20 glomerular staining; H: CD20 interstitial staining; I: TACI glomerular staining; J: TACI interstitial staining.

Figure 6.13 shows representative images for patient 4, a patient with AAV classified as crescentic by the Berden classification. BlyS staining was primarily glomerular and within the crescent seen in Figure 6.13A. There was minimal staining BlyS staining in the interstitium. BAFF R staining was also primarily glomerular (Figures 6.13 C and D) with its highest concentrations in the area of the crescent. There was minimal BCMA staining for this patient as seen in Figure 6.13 E and F. Weak TACI staining was also seen mainly in the glomerulus (Figure 6.13 G and H).

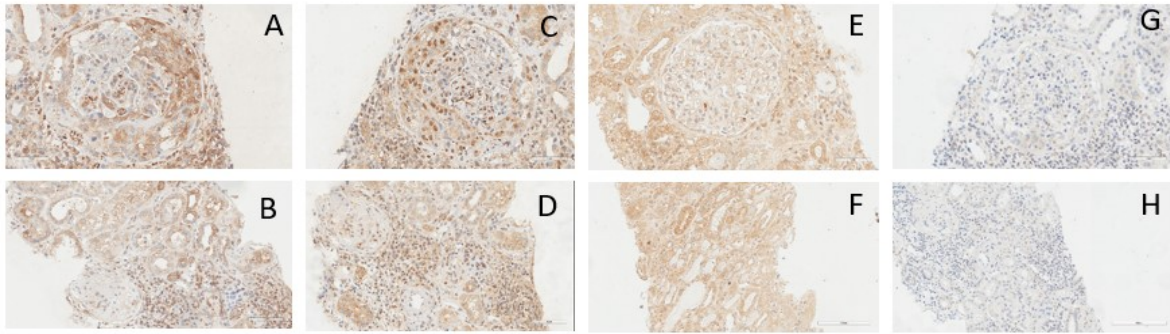


**Figure 6.13 Patient 4.**

A: BlyS glomerular staining; B: BlyS interstitial staining. C: BAFF-R glomerular staining; D: BAFF-R interstitial staining; E: BCMA glomerular staining; F: BCMA interstitial staining. G: TACI glomerular staining, H: TACI glomerular staining.

Patient 5 was also a patient with MPO positive AAV classified as crescentic. Representative images are seen in Figure 6.14. It is demonstrated that BlyS staining is present in the glomerulus again, primarily at the site of crescent formation (Figures 6.14 A and B). Despite there being significant background in the BAFF R staining, it can be seen that this too was found mainly in the glomerulus, concentrated around the crescent (Figure 6.14 C and D). Again, CD20 was localised to the interstitium (Figure 6.14 E and F), and TACI staining was primarily glomerular (Figures 6.14 G and H).

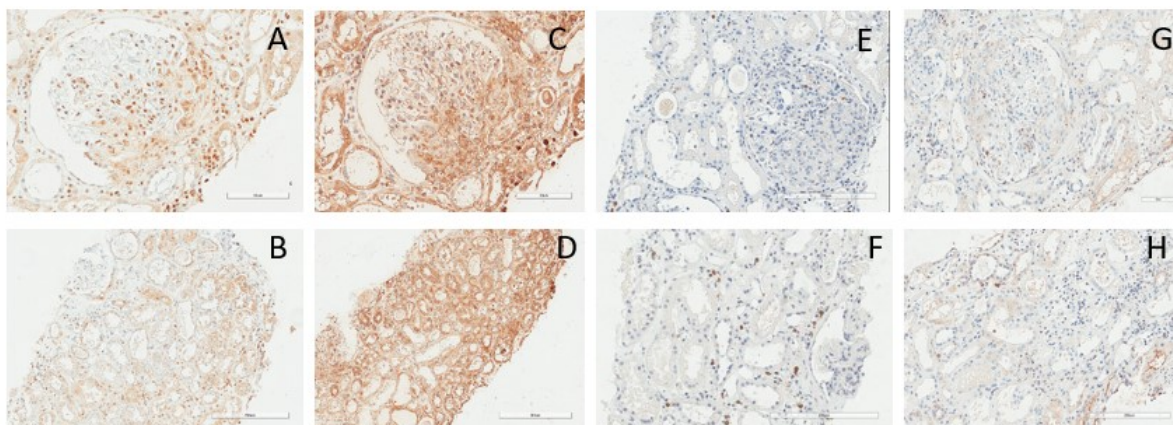


**Figure 6.14 Patient 5**

A: BlyS glomerular staining; B: BlyS interstitial staining; C: BAFFR glomerular staining; D: BAFFR interstitial staining; E: CD20 glomerular staining; F: CD20 interstitial staining; G: TACI glomerular staining; H: TACI interstitial staining. \*BCMA staining was not possible.

Patient 6 was a further patient who was MPO positive and had crescentic disease. The images for this patient can be seen in Figure 6.15. BlyS staining was difficult to assess for as the first slide contained sclerotic glomeruli (Figure 6.15 A) and there was no staining seen in the interstitium (Figure 6.15B). BAFF R staining was primarily glomerular as seen in Figure 6.15 C and D. There was no significant TACI staining seen (Figure 6.15 E and F).

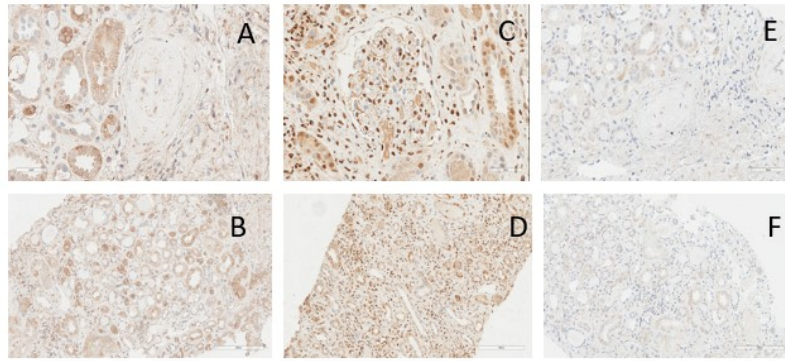


**Figure 6.15 Patient 6**

A: BlyS glomerular staining. B: BlyS interstitial staining; C: anti-BAFF R glomerular staining; D: anti-BAFF R interstitial staining; E: anti-TACI glomerular staining; F: anti-TACI interstitial staining. \*CD20 and BCMA staining not possible due to insufficient number of slides available.

The images for patient 7, a PR3 positive patient with crescentic AAV are shown in Figure 6.16. There was no significant BlyS staining seen (Figures 6.16 A and B). BAFF R was positive in both the glomerulus and interstitium (Figures 6.16 C and D). There was no BCMA staining seen (Figures 6.16 E and F), and strongly positive CD20 staining in the interstitium (Figures 6.16 G and H).

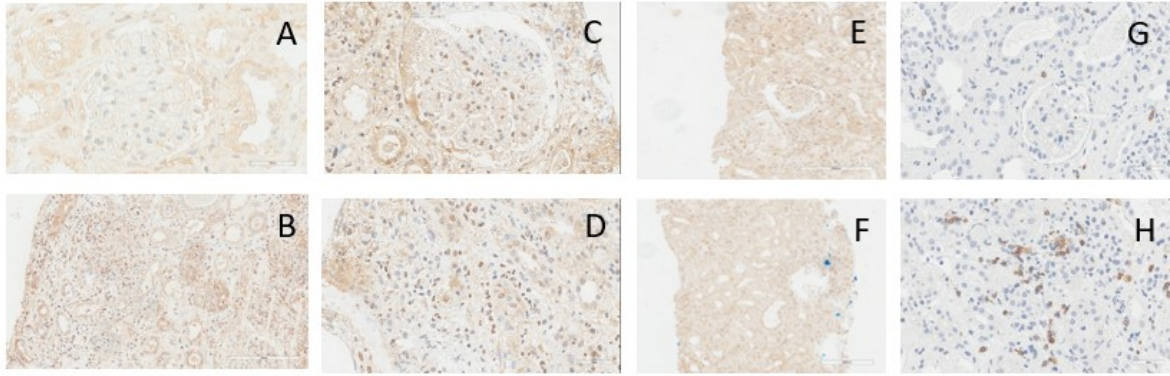


**Figure 6.16 Patient 7.**

A: BlyS glomerular staining; B: BlyS interstitial staining; C: BAFF R glomerular staining; D: BAFF R interstitial staining; E: BCMA glomerular staining; F: BCMA interstitial staining; G: CD20 glomerular staining; H: CD20 interstitial staining.

Patient 8 had PR3 positive disease and a Berden classification of mixed AAV. As seen in Figure 6.17 A and B, there was no significant BlyS staining. BAFF R staining was strongly positive in the glomeruli but not the interstitium (Figures 6.17 C and D). CD20 staining was confined to the intersitium and was mild in comparison to the previous patients (Figures 6.17 E and F). Due to background staining, it was difficult to assess for BCMA positivity.

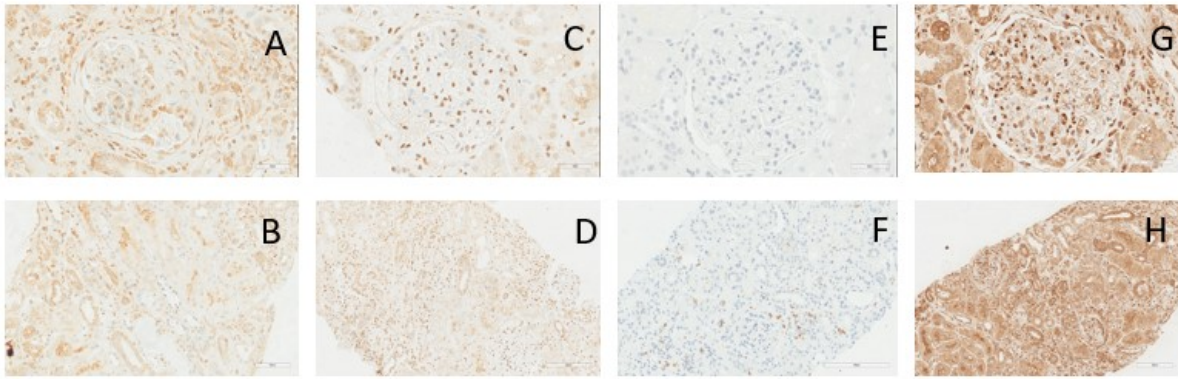


**Figure 6.17 Patient 8**

A: BlyS glomerular staining; B: BlyS interstitial staining; C: anti-BAFFR glomerular staining; D: BAFFR interstitial staining; E: CD20 glomerular staining; F: CD20 interstitial staining; G: BCMA glomerular staining; H: BCMA interstitial staining.

Figure 6.18 shows representative images for patient 9, an MPO positive patient with sclerotic AAV. It can be seen that BlyS staining is mild and concentrated mainly in the glomerulus (Figures 6.18 A and B). There was strongly positive BAFFR staining found primarily in the glomerulus (Figures 6.18 C and D). CD 20 staining was again seen in the interstitium (Figure 6.18 E and F). TACI Staining was minimal and found predominantly in the glomerulus.

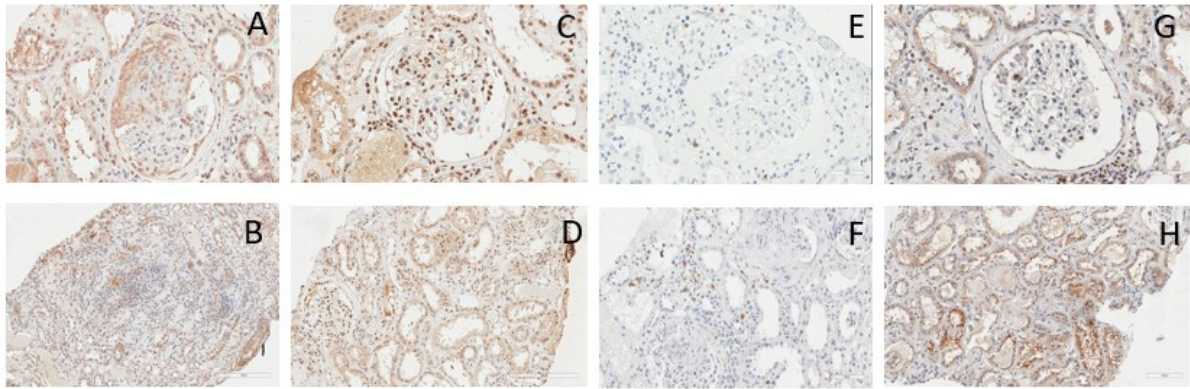


**Figure 6.18 Patient 9**

A: BlyS glomerular staining; B: BlyS interstitial staining; C: BAFFR glomerular staining; D: BAFFR interstitial staining; E: CD20 glomerular staining; F: CD20 interstitial staining; G: TACI glomerular staining; H: TACI interstitial staining.

Figure 6.19 shows images for patient 10 who was MPO positive with a Berden classification of mixed. BAFF R staining is seen to be primarily glomerular (Figures 6.19 A and B). BCMA staining was also primarily glomerular (Figures 6.19 C and D). TACI staining was mild and positive both in the glomerulus and interstitium (Figures 6.19 E and F).

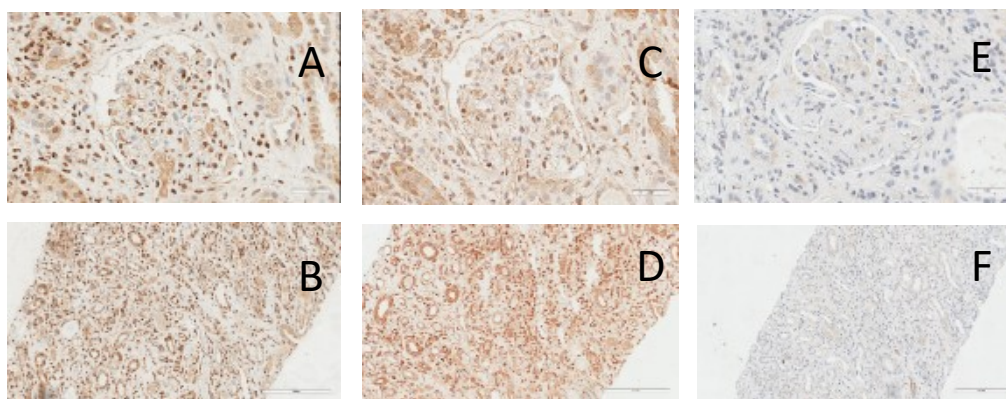


**Figure 6.19: Patient 10**

(A) BAFF R glomerular staining, (B) BAFF R glomerular staining, (C) BCMA glomerular staining, (D) BCMA interstitial staining, (E) TACI glomerular staining, (F) TACI interstitial staining.

### 6.3.3 Analysis

Table 6.2 includes the values for percentage positivity of the different stains for each patient using the Aperio Image Scope.

|               |                                | Anti BAFF |      | Anti BAFF<br>R |      | Anti BCMA |      | Anti TACI |      | Anti CD20       |      |
|---------------|--------------------------------|-----------|------|----------------|------|-----------|------|-----------|------|-----------------|------|
| Patient<br>No | Histological<br>classification | Glom      | TI   | Glom           | TI   | Glom      | TI   | Glom      | TI   | Glom            | TI   |
| 1             | PR3 focal                      | na        | na   | 2.91           | 0.4  | 1.26      | 0.45 | 0.13      | 0.14 | 0.16            | 0.25 |
| 2             | PR3 focal                      | 0.09      | 0.07 | 0.24           | 0.33 | 0.05      | 0.02 | na        | na   | 0.16            | 0.25 |
| 3             | MPO focal                      | 0.21      | 0.22 | 0.55           | 0.07 | 3.07      | 1.96 | na        | na   | 0.01            | 0.11 |
| 4             | MPO<br>crescentic              | Na        | Na   | 2.2            | 1.55 | 5.66      | 3.14 | 0.19      | 0.11 | 0.07            | 2.33 |
| 5             | MPO<br>crescentic              | 1.01      | 0.56 | 6.55           | 3.58 | 15.62     | 19.4 | 1.04      | 2.14 | 0.08            | 0.48 |
| 6             | MPO<br>crescentic              | 0.84      | 0.66 | 14.5           | 13.8 | na        | na   | 1.55      | 0.81 | 0.156           | 0.38 |
| 7             | PR3 mixed                      | 0.21      | 0.56 | 0.69           | 0.51 | 0.08      | 0.13 | 0.03      | 0.02 | Faulty<br>slide |      |
| 8             | MPO mixed                      | na        | na   | 6.75           | 9.76 | 0.758902  | 8.59 | 0.11      | 0.01 | na              | na   |
| 9             | MPO sclerotic                  | 0.04      | 0.08 | 0.29           | 0.12 | 1.15      | 5.09 | 0.12      | 0.02 | na              | na   |
| 10            | MPO mixed                      | 0.01      | 0.03 | na             | na   | 0.04      | 0.2  | 0.09      | 0.08 | 0.01            | 0.21 |

**Table 6.2: Percentage positive staining.** The numbers in the table are representative of the percentage positive staining of each section calculated using the Aperio Image Scope.

Glom: Glomerular staining. TI: Tubulointerstitial staining.

Figure 6.20 is a heat-map representing the percentage positivity in the glomeruli of each stain divided by classification of patient. There appears to be a trend towards increased staining in the crescentic biopsies. Figure 6.21 is a heat-map representation for the interstitium and also appears to show an increased positivity of staining in the crescentic patients.

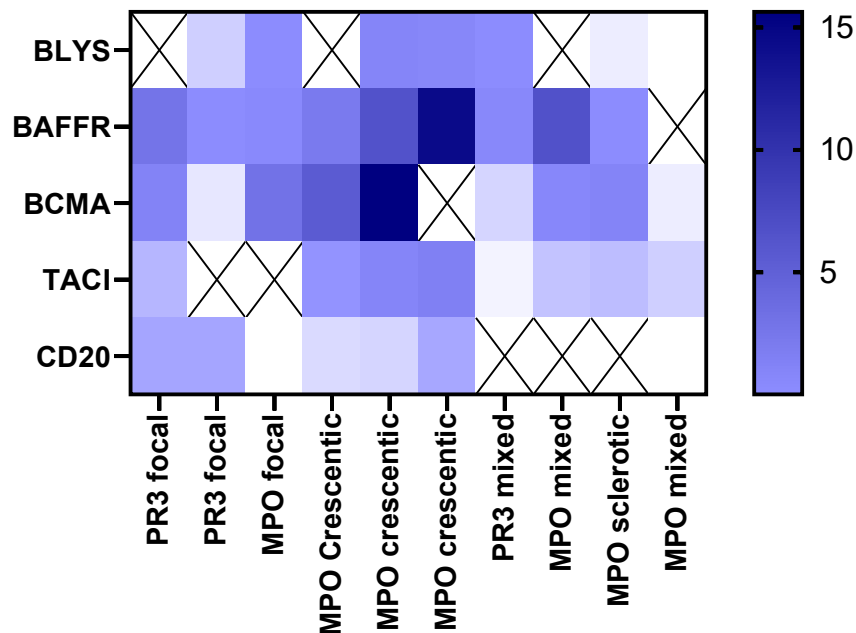


Figure 6.20: Heatmap to show percentage glomerular positivity of each stain versus classification of patient

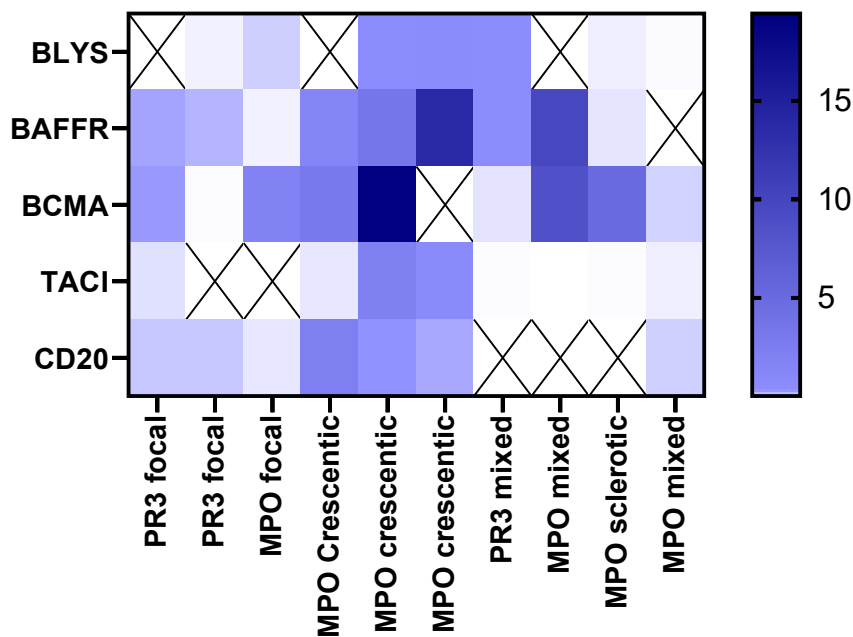


Figure 6.21: Heatmap to show percentage interstitial positivity of each stain vs classification of patient.

#### 6.4 Discussion:

Serum BlyS and APRIL levels had not been previously measured at our centre. We carried out this study with the primary objective of assessing our ability to measure these levels reliably and accurately in patients with AAV, and to assess their clinical significance. Patients were recruited as they attended clinic and when identified as in patients on the ward. As such, only 20 patients with active disease were included. The primary aim had been to assess BlyS levels. Following the success of this study, APRIL levels were also measured. There was a significant increase in BlyS levels in patients with AAV and in particular with active disease. Levels were also increased following rituximab treatment, in keeping with the hypothesis discussed earlier of CD20 mediated depletion of B cells resulting in increased production of BlyS and an

environment favouring the development of autoimmunity. Serum APRIL levels were also raised in AAV compared with healthy controls; however, in contrast with serum BlyS, there was no increase seen in patients who had been treated with rituximab. This could be in keeping with the mechanism of action of APRIL acting primarily on BCMA receptors and promoting survival of plasma cells which are not CD 20 dependent. It is difficult to draw significant conclusions, however, due to the small numbers of patients for whom APRIL levels were measured. We recognise the limitations of our studies and that our conclusions are limited by the small numbers and retrospective nature of the work. Based on these conclusions, further work is planned. This would include a prospective study, assessing levels of serum BlyS and serum APRIL at set time points including at initial diagnosis, 3, 6 and 12 months post. This would provide highly informative data regarding correlations with disease activity and treatment, and would enable a direct comparison of BlyS with APRIL. It would also be useful to assess the ability of these factors to predict relapse and to assess correlation with timing of B cell depletion, and to carry out flow cytometry to assess B cell subsets at the varying time points. This study suggests there could be a role for B cell survival factors as a biomarker for disease, if used in conjunction with traditional markers of disease activity including CRP and ANCA titre. Serum APRIL levels would be of particular utility in patients who had been treated with rituximab previously.

In clinical practice, it would be useful to create a profile of a patient's peripheral B cell survival factors and combine these results with findings on renal biopsy. We have shown that it is possible to stain for BlyS and its receptors in the renal biopsies of patients with AAV. We had access to only a small sample of patient biopsies. Particular stains were not successful for some patients and we were limited by having no extra slides on which to repeat experiments. Due to numbers we only attempted to

stain for BlyS, CD 20 and the three BlyS receptors. APRIL staining was not attempted. Staining for CD20 was successful in 7/10 patients and in keeping with other studies, was more strongly positive in the tubulointerstitium than the glomeruli. BlyS and its receptors were detected as shown below however it was difficult to compare such a small sample for positivity. From the heat-maps in figures 6.20 and 6.21 it appeared that patients with a histological diagnosis of crescentic disease had increased expression of B cells, BlyS and its receptors. Further studies would include assessment of a larger sample of patients and would help determine if there was any difference in expression of B cell survival factors when biopsies are divided by histological classification, in a similar manner to lupus nephritis (184) Assessment of APRIL staining would also ideally be undertaken. Further studies would include correlation of a patient's staining for B cell survival factors and receptors, with peripheral levels with the aim of creating a BlyS profile which could inform about the utility of agents such as Belimumab in treating disease. Following treatment with Belimumab it would also be useful to assess both BlyS levels and correlate with levels and biopsy findings prior to treatment.

With the development of newer inhibitors of B cell survival factors and the now widespread usage of rituximab in both induction and maintenance of remission the studies carried out will provide a basis for further work assessing the clinical significance of B cell survival factors in AAV.

## Chapter 7 – Anti-BlyS antibody as a novel treatment for experimental autoimmune vasculitis.

## 7.1 Introduction and Aims:

As discussed in Chapter 1, a role for B cell survival factors in the pathogenesis of AAV has been established. BlyS levels have been demonstrated to be elevated in many autoimmune diseases, including AAV. Chapter 6 describes the clinical significance of BlyS and APRIL in a cohort of patients with AAV at our centre. Both BlyS and APRIL were demonstrated to be elevated in the context of active disease. BlyS levels were also found rise following B cell depletion with rituximab. The complex interplay between B cell depletion and re-population in an environment rich in B cell survival factors and favouring autoimmunity has yet to be fully understood.

There are a number of complex factors which impact on a patient's response to therapy including behavioural and environmental factors. There have been clinical trials of BlyS inhibitors in multiple autoimmune diseases in humans with varying results (185). The use of an animal model to assess a therapeutic agent enables such external factors to be minimized. As discussed in Chapter 1, EAV is the animal model which most closely represents AAV and is, in contrast to other models involving passive transfer of antibodies, a true model of autoimmunity, with the WKY rat producing its own anti-MPO antibodies. There have been multiple successful *in vivo* studies of BlyS inhibitors in mice, mainly with lupus-like diseases, but also with a variety of other conditions including experimental autoimmune encephalitis (EAE)(121).

This chapter describes two studies in which we assessed the effect of a mouse chimeric anti-BlyS monoclonal antibody (mAb), 10F4, on the pathogenesis of EAV. As discussed in Chapter 1, the treatment of AAV involves both induction and maintenance therapy and we aimed to assess the utility of this compound for both

stages of treatment. In the first experiment, the antibody was administered upon induction of disease with the aim of assessing the impact on the rats developing the typical disease manifestations of EAV. The model of EAV was developed in the renal laboratory at Imperial College by Mark Little *et al* and there is significant experience of its use. The optimal dosage of the compound 10F4 was not clear, and this was assessed by one group of rats receiving high and one low dose treatment. The third group were treated in an identical fashion but received an isotype control antibody. From this first study, we determined that the optimal dosage for this experiment was the higher dose of the antibody, and this was therefore used for the second study.

The aim of the second experiment was to assess the impact of the anti-Blys mAb on established disease and more closely mimicking the clinical situation. In this experiment, treatment commenced on week 4, which is characteristically the week by which proteinuria and haematuria reach their peak in EAV, before starting to fall as part of the normal course of the disease.

We thus carried out two studies with the aim of assessing the effect of the anti-BlyS mAb as an induction agent from onset of disease and once disease has been established.

## 7.2 Methods

The methods used were consistent for both experiments apart from the immunisation protocol. The general methods for both experiments will be detailed here. The protocol for administration of the anti-BlyS mAb for both experiments will also be described.

### 7.2.1 Ethics

Animals were handled according to the directives of the United Kingdom Animals (Scientific Procedures) Act 1986.

### 7.2.2 Animals

Female WKY rats (Charles River, UK) were purchased at 6-8 weeks of age, weighing 100-120 grams. The animals were cared for in accordance with institutional guidelines, with free access to water and a normal laboratory diet.

### 7.2.3 BlyS inhibitor and Isotype control antibody

The BlyS inhibitor 10F4, a murine chimeric monoclonal antibody to BlyS, was provided by Human Genome Sciences. The isotype control antibody was ANO2-mIgG2A/kappa, obtained from Human Genome Sciences.

### 7.2.4 Immunisation

Immunisation of animals was carried out with human MPO at a concentration of 5 mg/kg. This was prepared in Complete Freund's Adjuvant with the addition of Mycobacterium butyricum (4.5 mg/ml) and injected intramuscularly. Five hundred milligrams of pertussis toxin was administered intraperitoneally at day 0 and day 2 (Figure 7.1).

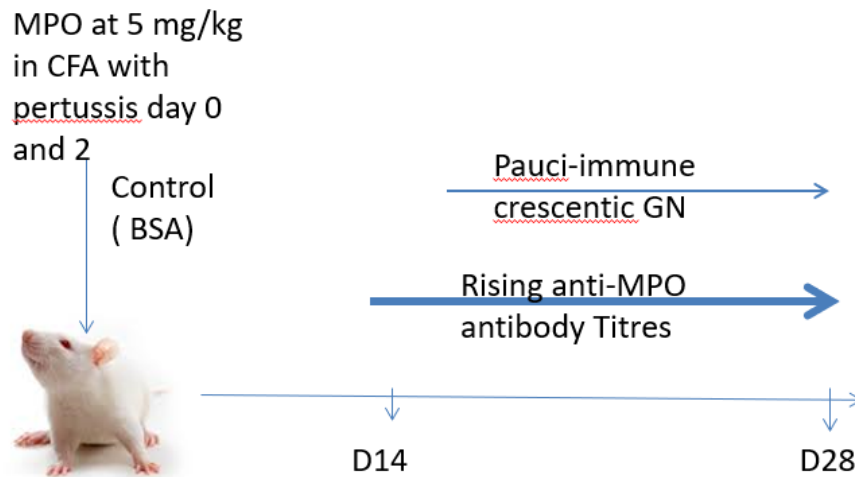


**Figure 7.1: Protocol for induction of EAV**

The treatment schedules for both experiments are described below and summarized in Figures 7.2 and 7.3. Animals were sacrificed at week 6.

#### *7.2.4.1 Experiment 1:*

Rats were treated with either isotype control or low or high dose anti-BlyS mAb at days 1 and 5 of the first week, followed by a weekly dose of maintenance therapy. High dose therapy was administered at 5000 mcg/mAb/kg intraperitoneally on days 1 and 5 for induction, and was followed by maintenance therapy of 1500 mcg/mAb/kg intraperitoneally weekly from week 3. Induction with low dose therapy was administered at a dose of 1500 mcg/mAb/kg on days 1 and 5, followed by 750 mcg/mAb/kg weekly from week 3 as maintenance (Figure 7.2).

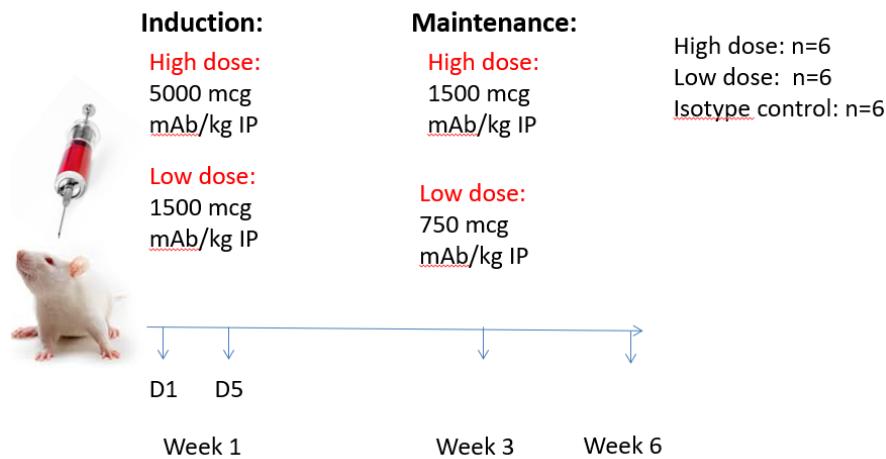


**Figure 7.2: BlyS inhibitor experiment 1.**

#### 7.2.4.2 Experiment 2:

Experimental autoimmune vasculitis was induced as per protocol in 10 female WKY rats. Baseline haematuria and proteinuria were assessed and rats were then randomized into receiving either an anti-BlyS antibody or an isotope control antibody. The antibody was administered at a dose of 5000 mcg mAb/kg by intraperitoneal injection on days 1 and 4 of week 4. From week 5, 1500 mcg mAb/Kg was administered weekly. Four age matched female WKY rats were kept in the same environment as healthy controls (Figure 7.3).

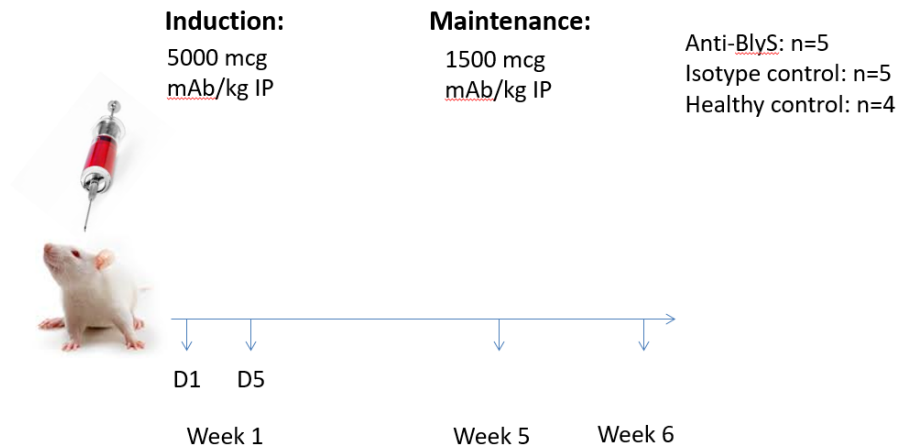


**Figure 7.3: BlyS inhibitor experiment 2**

#### 7.2.5 Urine collection and analysis

Proteinuria and haematuria were assessed using multicolour dipstick analysis (Bayer Diagnostics, Bridgend, London). Proteinuria was quantified using the sulphosalicylic acid method for precipitation of protein, as described in Chapter 2.

#### 7.2.6 Immunohistochemistry

Kidney sections were stained with haematoxylin and eosin, period acid-Schiff and Jones methanamine silver stain and were assessed for glomerular injury. Fifty glomeruli were counted per animal and the percentage abnormal calculated.

Glomerular macrophage infiltration was assessed by immunostaining using a mAb to CD68 (clone ED1, Serotec, Oxford) by the following method. Four micrometre formalin-fixed paraffin embedded sections were rehydrated. Antigen retrieval was carried out by heating the slides in a microwave in tri-sodium citrate buffer (pH6) for

15 min. Following washing, slides were incubated in 0.03% hydrogen peroxide for 10 min. The slides were incubated with a mouse mAb to ED1 (Serotec, Oxford), at a dilution of 1:500 in PBS, at room temperature for 1 h. Slides were incubated with an HRP labelled anti-mouse polymer (DAKO Ltd UK) for 30 min at room temperature and developed in 3'3 diaminobenzidine (Dako Ltd) for 10 min. They were then counterstained with haematoxylin and dehydrated. After drying, the slides were mounted in De Pex medium (BHD laboratory supplies) and analysed for the number of positively stained cells in 10 sequential glomerular cross sections using image analysis software (Image Pro Plus, Media Cybernetics, Bethesda, MD).

#### 7.2.7 Immunofluorescence: This was carried out with Gurjeet Bhargal.

As described in Chapter 2, slides were thawed and rinsed in PBS, then blocked in 20% rabbit serum (Dako) for 30 min. Slides were washed three times in PBS, then incubated with a secondary rabbit anti-rat IgG FITC (1:100) for 30 min, washed again, mounted in PBS/Glycerol (Citifluor) and inspected immediately by fluorescence microscopy. Automated image analysis was used to calculate fluorescence intensity throughout each glomerulus, in arbitrary units, and expressed as the mean intensity per glomerulus for each animal.

#### 7.2.8 Anti-MPO antibody detection by direct ELISA

Wells were coated with commercially available human MPO (Calbiochem). Serum samples were diluted to 1/100 concentration with PBS/T and 1% BSA, and were added to the plates. A rabbit anti- IgG alkaline phosphatase conjugate (Sigma) diluted to 1/1000 with PBS/T and BSA was added, and samples incubated for 1 h. One hundred

µl of SIGMA FAST p-nitrophenyl phosphate were applied to each well. Plates were read at 405nm on a plate reader.

#### 7.2.9 B-cell Elispot

The B cell ELISpot was used to quantify antigen-specific antibody producing cells. Elispot filter plates (Multiscreen HTS 96 well filter plates, Merck Millipore) were coated with human MPO (50µg/ml) and incubated at 4°C overnight. After sacrifice of animals, spleens were dissected and kept on ice in cold Hanks balanced salt solution (HBSS). Splenocytes were isolated by passing the tissue through a 100µm cell strainer with cold, sterile PBS. Red blood cells were lysed using a commercially available red cell lysis buffer (Ebioscience). Cells were re-suspended to a concentration of  $5 \times 10^6$ /ml, 100 µl of cell suspension were added per well and plates incubated at 37°C in 5% CO<sub>2</sub> for 48 h. A biotinylated rabbit anti-rat IgG secondary antibody was used (1:250; Dako) followed by extravidin-ALP conjugate (1:100; Sigma). Plates were developed in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP-NBT) solution (Sigma) and the reaction stopped with distilled water. Plates were dried and spots per well quantified using an Elispot plate reader and software (ELISpot 4.0, Autoimmun Diagnostika, Strassberg, Germany).

#### 7.2.10 Assessment of lung injury

Pulmonary surfaces were inspected upon sacrifice of the animals and a visual pulmonary haemorrhage score given according to the number of visible petechiae. Lungs were graded as zero, one (<10 petechiae), two (>10 petechiae), three (>20 petechiae) or four (massive haemorrhage).

### 7.2.11 Analysis

Data were analysed and graphs plotted using Microsoft Excel and Graph Pad Prism version V (Graph pad Software San Diego, CA). Statistical analysis was performed using a one way ANOVA, Kruskal-Wallis and Spearman rank correlation. Differences were considered significant if  $P < 0.05$ .

## 7.3 Results

### 7.3.1 Experiment 1: The effect of an anti-BlyS monoclonal antibody in the prevention of EAV.

#### *7.3.1.1 Assessment of haematuria*

Consistent with our experience of the model of EAV, there was development of haematuria with a peak at five weeks (Figure 7.4). Treatment with the anti-BlyS antibody resulted in a dose dependent reduction in haematuria as demonstrated in Figure 7.4. This reduction was statistically significant ( $p < 0.001$ , 2 way ANOVA) and there was no haematuria detectable in high dose antibody treated rats at six weeks.

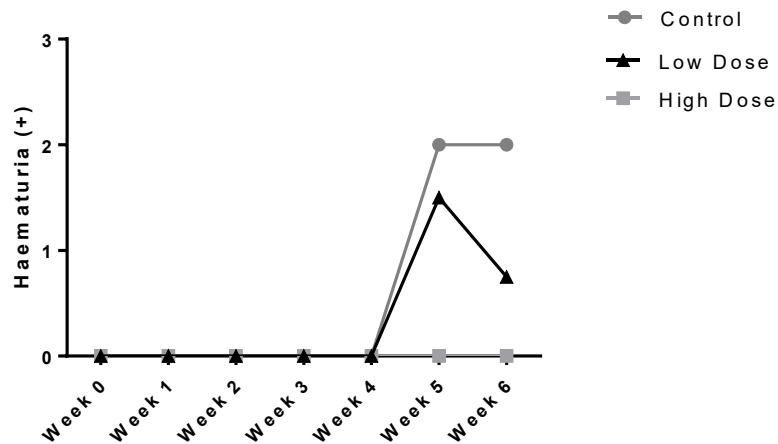


**Figure 7.4 Dipstick haematuria by week in EAV prevention study**

Assessment of dipstick haematuria by week following induction of EAV. At week 6 there was a statistically significant reduction in haematuria in the high dose antibody treated group, ( $p < 0.0001$ , 2 way ANOVA).

#### 7.3.1.2 Assessment of proteinuria

In keeping with the EAV model, proteinuria became evident between weeks 4 and 5. In this experiment, as shown in Figure 7.5, there was a statistically significant dose dependent reduction in dipstick proteinuria with treatment ( $p < 0.0001$ , 2 way ANOVA). Figure 7.6 shows proteinuria over a 24 hour period as quantified by the sulphosalicylic acid method described in Chapter 2. Again there was a dose dependent reduction in proteinuria from 5 weeks and there was a statistically significant difference between the groups ( $p = 0.02$ , 2 way ANOVA).

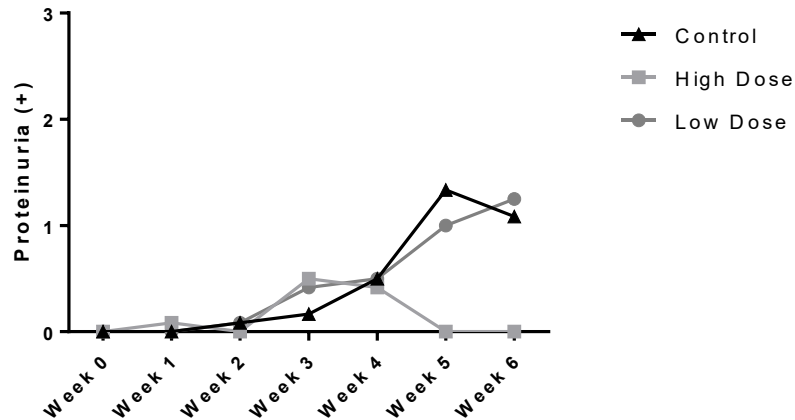


**Figure 7.5: Dipstick proteinuria by week in EAV prevention study**

Assessment of dipstick proteinuria by week following induction of EAV. At week 6 there was a statistically significant reduction in proteinuria in the high dose antibody treated group,  $p=0.0049$  (Kruskal-Wallis).

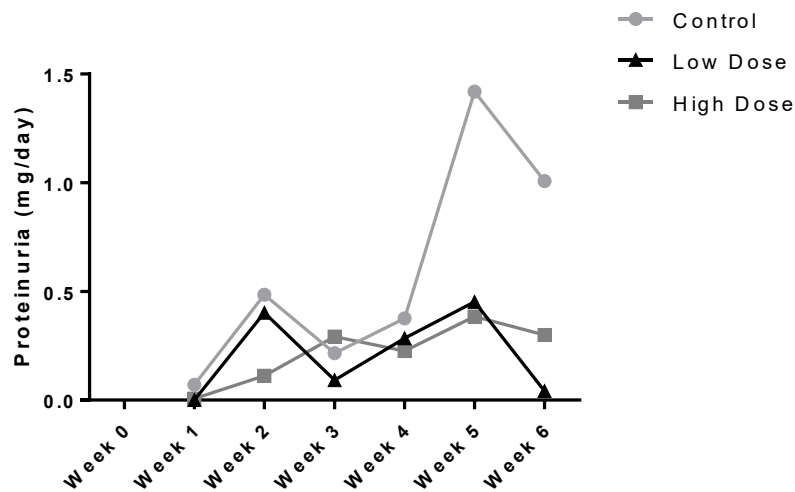
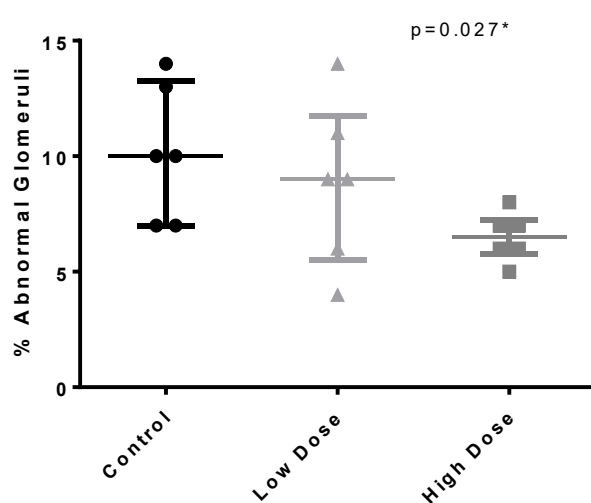


**Figure 7.6: Proteinuria per day measured using the sulphosalicylic acid method.**

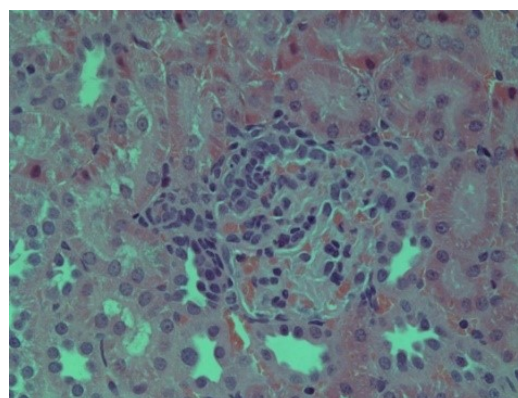
There was a statistically significant dose dependent reduction in proteinuria measured in mg per day across the group by week 6 ( $p=0.02$ , 2 way ANOVA).

### 7.3.1.3 Histological injury

In terms of histological abnormalities, the percentage of abnormal glomeruli seen by light microscopy, was significantly reduced in the high dose antibody treated group at six weeks ( $p=0.027$ ) (Figure 7.7). Using immunostaining with a monoclonal antibody to CD68 as a marker for macrophage infiltration, there was a marked reduction in the mean percentage of CD68+ cells per glomerular cross section in the animals treated with the anti-BlyS antibody, particularly in the high dose antibody treated group ( $p=0.04$ )(Figure 7.8).



(a)



(b)

**Figure 7.7: Histological injury**

(a) Histological injury was assessed by the percentage of abnormal glomeruli per kidney cross section assessed using light microscopy in the anti-BlyS treated and vehicle treated animals at 6 weeks shown as median +/- IQR.  $P=0.027$ , Mann-Whitney U test (b) Example of glomerular damage.

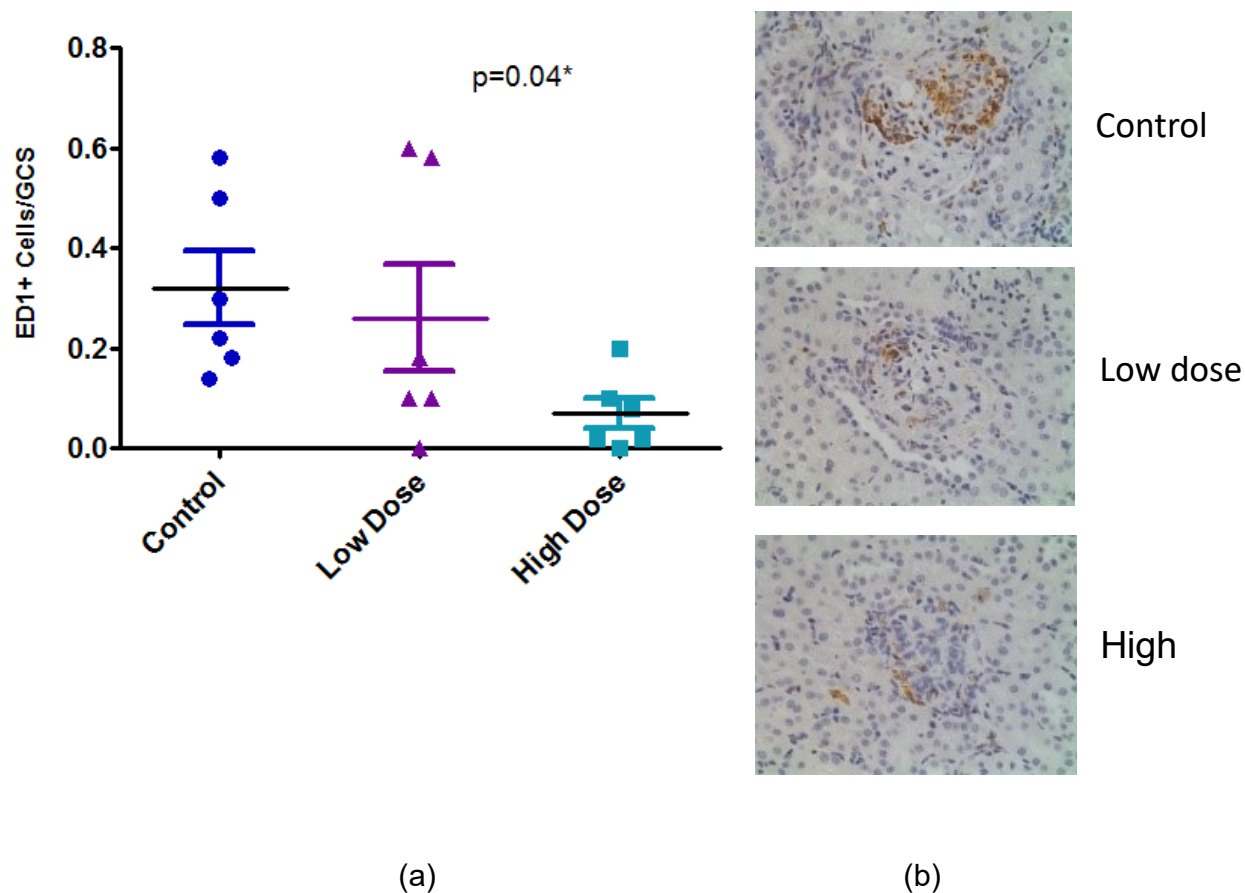


**Figure 7.8: Macrophage infiltration in EAV anti-BlyS study**

(a): The mean percentage of ED1+ cells per glomerular cross section (GCS) was measured and taken to be representative of degree of macrophage infiltration. Data were analysed using image analysis software. Data shown as median  $\pm$  IQR, \* $p=0.04$ . Kruskal Wallis (b): Photomicrographs demonstrating representative immunoperoxidase stains for ED1+ cells in the three groups at 6 weeks, with haematoxylin counterstain, x 400 magnification.

#### 7.3.1.4 Immunofluorescence

There was no significant difference in intensity of renal immunofluorescence when comparing the untreated and antibody treated animals which was low in all groups. This is keeping with the finding that EAV is known to be a “pauci immune” disease (Figure 7.9 a). Representative images for immunofluorescence in each group are shown in Figure 7.9b.  $p=0.10$  (Kruskal Wallis).

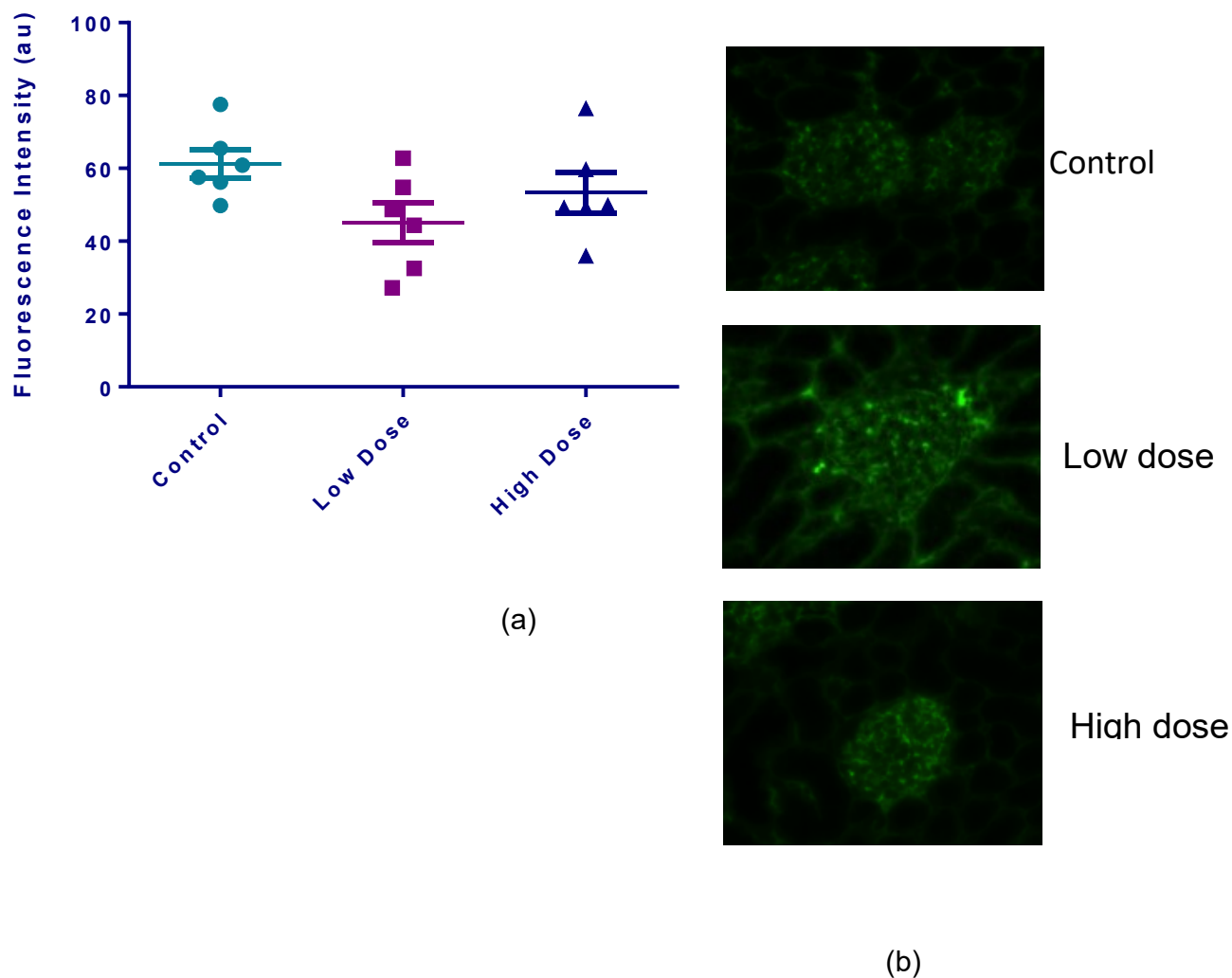
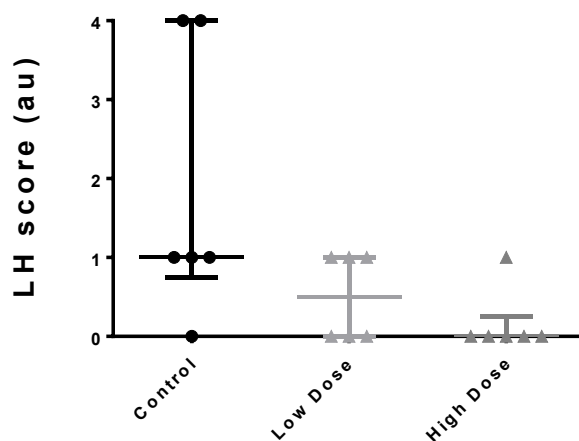


**Figure 7.9: Direct immunofluorescence for deposited IgG in glomeruli following administration of the anti-BlyS antibody in EAV.**

(a) Quantification of fluorescence intensity for deposited rat IgG, acquired using automated image analysis software, in each treatment group at week 6. No difference between groups was observed by the Kruskal-Wallis test. Data shown as median  $\pm$  IQR. (b) Photomicrographs showing representative fluorescence images in each group. Anti-rat IgG FITC, x400 magnification.

### 7.3.1.5 Pulmonary haemorrhage

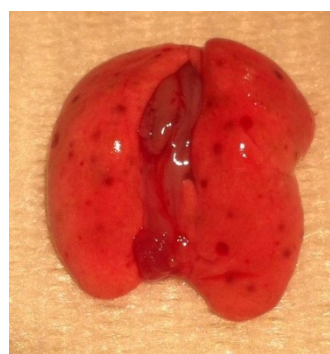
The anti-BlyS antibody treated animals had significantly less severe lung disease when quantified using a visual scoring system based on macroscopic inspection of lung surfaces ( $p=0.05$ ) (Figure 7.10).



(a)



Example of macroscopic appearance of lungs in the high dose antibody treated group.



Example of macroscopic appearance of lungs in the control group.

(b)

**Figure 7.10 Assessment of pulmonary haemorrhage**

(a) Assessment of pulmonary haemorrhage using the visual assessment score. Data are presented as a scatter plot with medians, ( $p=0.05$ , Kruskal-Wallis test). (b) Representative macroscopic images of lungs of animals in the high dose antibody treated group and those in the control group.

#### 7.3.1.6 Measurement of B cell Elispot

B cell Elispot was carried out to enumerate MPO-specific B splenic B cells. Figure 7.11 demonstrates a significant decrease in MPO specific splenic B cells in all antibody treated animals. Both the low and high dose antibody treated group had significantly lower numbers of antigen specific splenic B cells at six weeks.

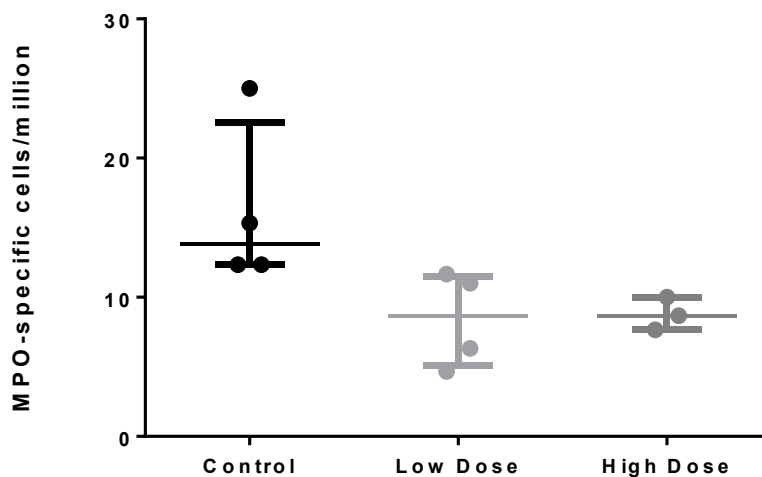


**Figure 7.11: Assessment of MPO specific cells by B cell Elispot.**

There was a significant reduction in MPO specific cells in the antibody treated groups. ( $p=0.01$ , Kruskal-Wallis test).

#### 7.3.1.7 Assessment of circulating ANCA titres by direct ELISA

Figure 7.12 demonstrates ANCA titres at weeks 2 and 4 detected by direct ELISA. At week 2 there was a statistically significant difference between ANCA titres in the isotype control treated and high dose antibody treated groups ( $p=0.001$ , ANOVA with Bonferroni's correction). There was also a statistically significant reduction in the ANCA titres in the low dose antibody group compared with the isotype control,  $p=0.001$ , ANOVA with Bonferroni's correction). At week 4 there was a reduction in antibody titres in the BlyS treated animals but this did not meet statistical significance.

There was technical problem with the samples for week 6 making them uninterpretable.

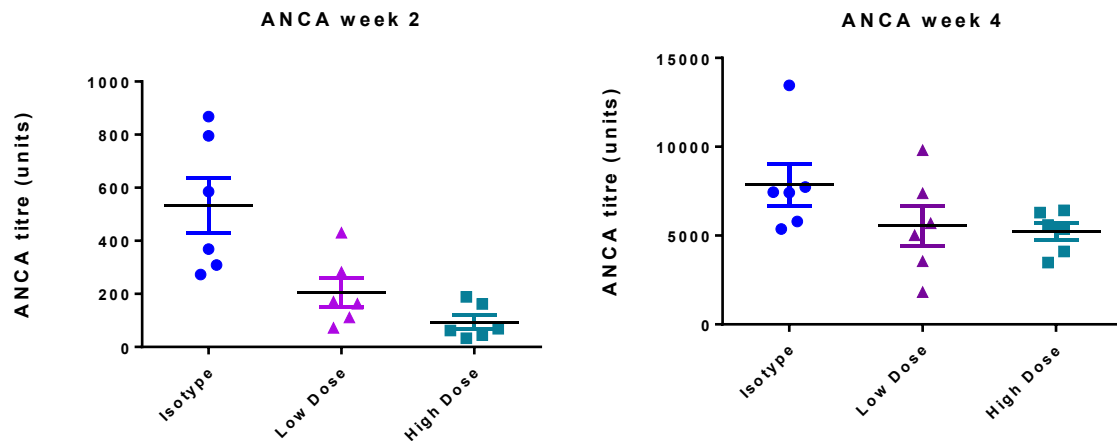


**Figure 7.12: Assessment of circulating ANCA titres by direct ELISA, comparison of anti-MPO levels across the groups**

There was reduction in circulating ANCA titres by direct ELISA at both 2 and 4 weeks. At 2 weeks this was statistically significant. (One way ANOVA,  $p=0.0036$ ).

Figure 7.12 demonstrates ANCA titres at weeks 2 and 4 detected by direct ELISA. At week 2 there was a statistically significant difference between ANCA titres in the isotype control treated and high dose antibody treated groups ( $p=0.001$ , ANOVA with Bonferroni's correction). There was also a statistically significant reduction in the ANCA titres in the low dose antibody group compared with the isotype control,  $p=0.001$ , ANOVA with Bonferroni's correction). At week 4 there was a reduction in antibody titres in the BlyS treated animals but this did not meet statistical significance. There was technical problem with the samples for week 6 making them uninterpretable.

### 7.3.1.8 Haematological indices

The effect of the anti-BlyS antibody on the peripheral blood count of the rats was assessed. There was a reduction in leucocyte count in the antibody treated animals, but preservation of platelet count and haemoglobin (Figure 7.13).

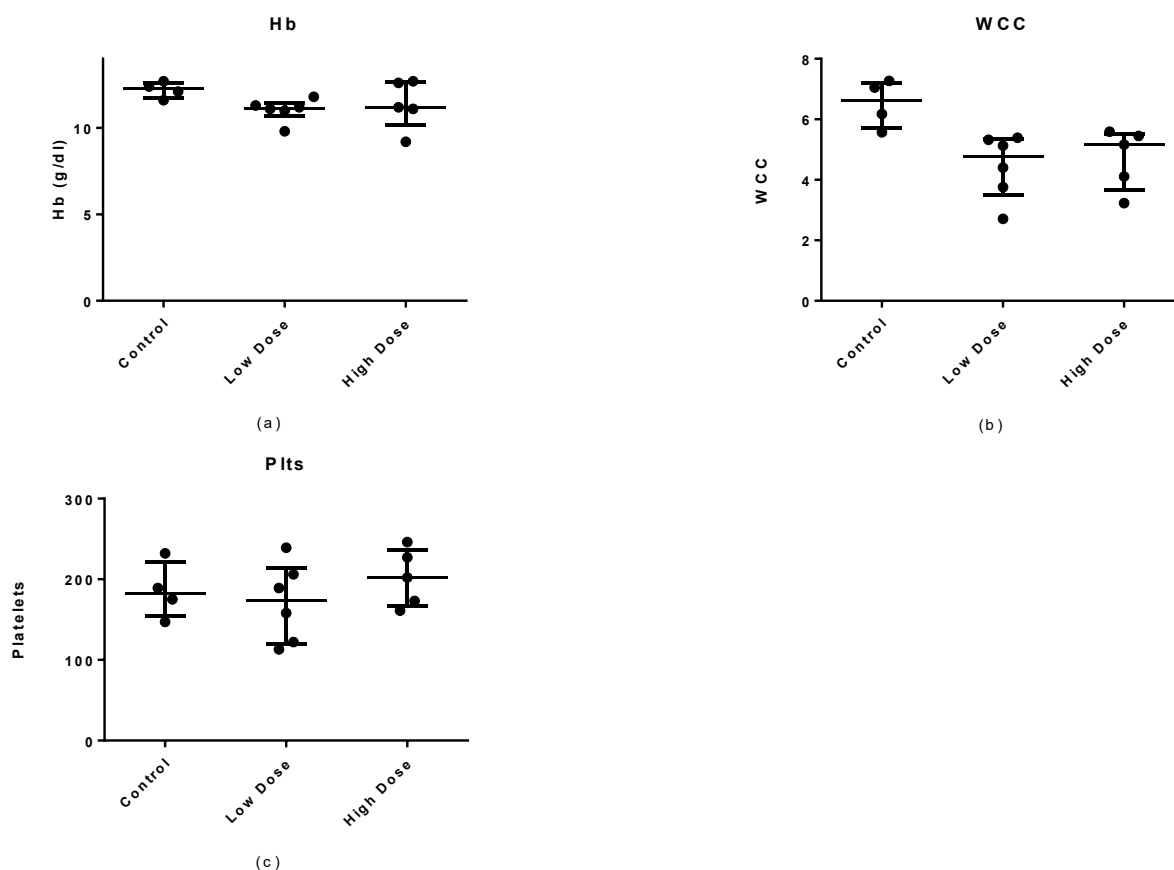


**Figure 7.13: Assessment of haematological indices across treatment groups.**

(a) Haemoglobin concentration ( $p=ns$ , Mann-Whitney U Test); (b) White cell count (WCC), ( $p<0.01$ , Mann-Whitney U Test); (c) Platelet count ( $p=ns$ , Mann-Whitney U Test). Data shown as median  $\pm$  IQR.

#### *7.3.1.9 Conclusion and brief discussion: Experiment 1*

In summary, treatment with high dose anti-BlyS mAb 10F4 results in significant reduction in both renal and pulmonary injury in EAV.

Both low dose and high dose anti-BlyS antibody treatment resulted in reduction of anti-MPO antibody production from splenocytes on week 6 and clear reduction in circulating anti-MPO antibody at week 2.

#### *7.3.2 Experiment 2: The effect of an anti-BlyS monoclonal antibody in treating EAV after onset of the disease.*

The aim of the second experiment was to assess if treatment with the anti-BlyS mAb (10F4) was effective following the onset of EAV and thereby in the treatment of established disease. The methods and immunisation protocol used were as described for Experiment 1.

##### *7.3.2.1 Summary of treatment groups:*

- 1) Anti-BlyS antibody treated group (n=5): EAV + treatment with high dose anti-BLYS mAb (10F4) from week 4. Final assessment of histology at week 6
- 2) Isotype control antibody treated group (n=5): EAV + treatment with isotype control antibody from week 4. Final assessment of histology at week 6.
- 3) Healthy control rats (n=4).

##### *7.3.2.2: Assessment of haematuria*

In keeping with the model of EAV where haematuria usually peaks at 4-5 weeks, haematuria peaked at 4 weeks (Figure 7.14). There was no significant reduction in

haematuria in the anti-BlyS antibody treated group over the course of the 6 weeks (Figure 7.14).

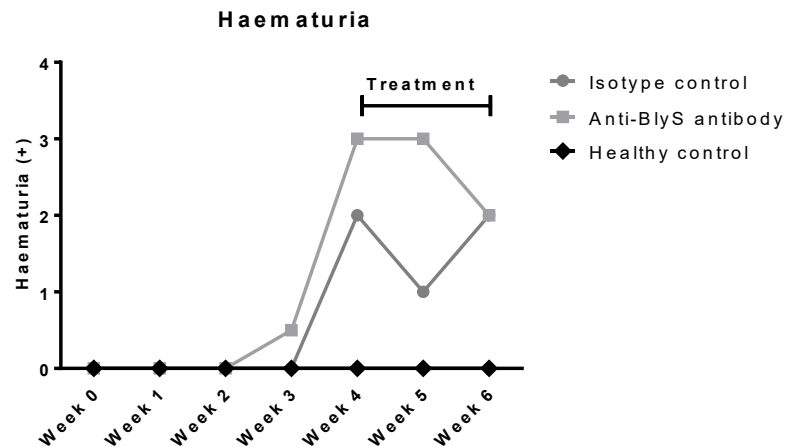


**Figure 7.14: Assessment of haematuria, by week, following the induction of EAV.**

Haematuria was assessed using multicolour dipstick analysis p=ns.

### 7.3.2.3 Assessment of proteinuria

Proteinuria was detectable from week 4 with the same median values in both the isotype and anti-BlyS antibody treated group before they received treatment. Following anti-BlyS antibody treatment, the median dipstick proteinuria was lower than the isotype control group at both weeks 5 and 6, but the difference did not reach statistical significance (Figure 7.15).

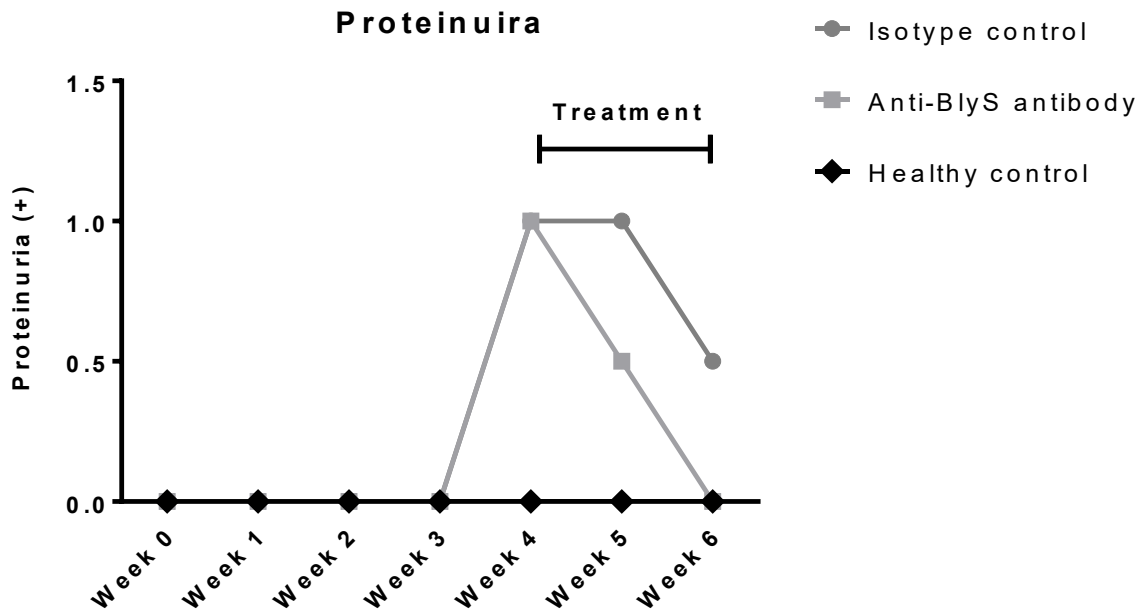


**Figure 7.15 Assessment of proteinuria, by week, following the induction of EAV.**

Proteinuria was measured using multicoloured dipstick analysis. There was no significant difference across groups, (p=ns).

#### 7.3.2.4: Quantification of proteinuria

Twenty-four hour urine, measured using the sulphosalicylic acid method for protein precipitation, showed that the isotype control group had persistent proteinuria (peak at 5 weeks). The anti-BlyS antibody treated group had less proteinuria (median 0 range (0-0.35) mg/day) than the isotype antibody treated group (1.3 (0.2-3.6) mg/day) but this did not reach statistical significance (Figure 7.16).

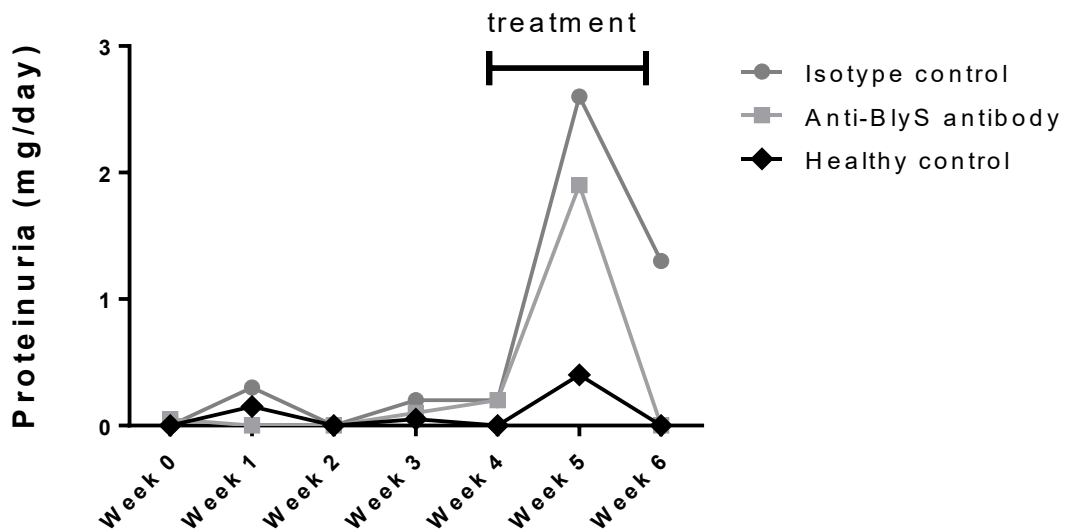


**Figure 7.16 Proteinuria by week as measured using the sulphosalicylic acid method for protein precipitation.**

Proteinuria was reduced in the anti-BlyS treated rats at weeks 5 and 6 but this did not reach statistical significance.

#### *7.3.2.5 Pulmonary haemorrhage*

There was no difference in pulmonary haemorrhage in the anti-BlyS antibody treated group when compared with the isotype control antibody treated group (Figure 7.17).

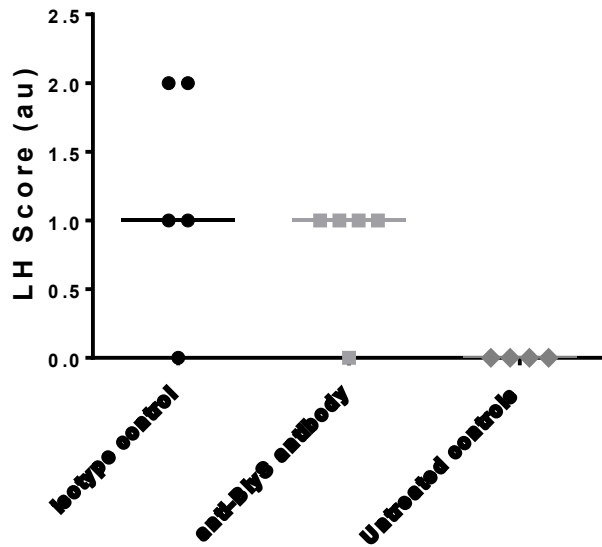


**Figure 7.17 Assessment of pulmonary haemorrhage score using the visual assessment score,**

p=ns. Data are presented as a scatter plot with medians.

#### 7.3.2.6 Circulating anti-MPO antibodies

There was a 49% reduction in circulating anti-MPO antibodies in the anti-BlyS antibody treated rats, in comparison to the isotype control treated rats by week 6 ( $p=0.0003$ , one way ANOVA (Figure 7.18)).

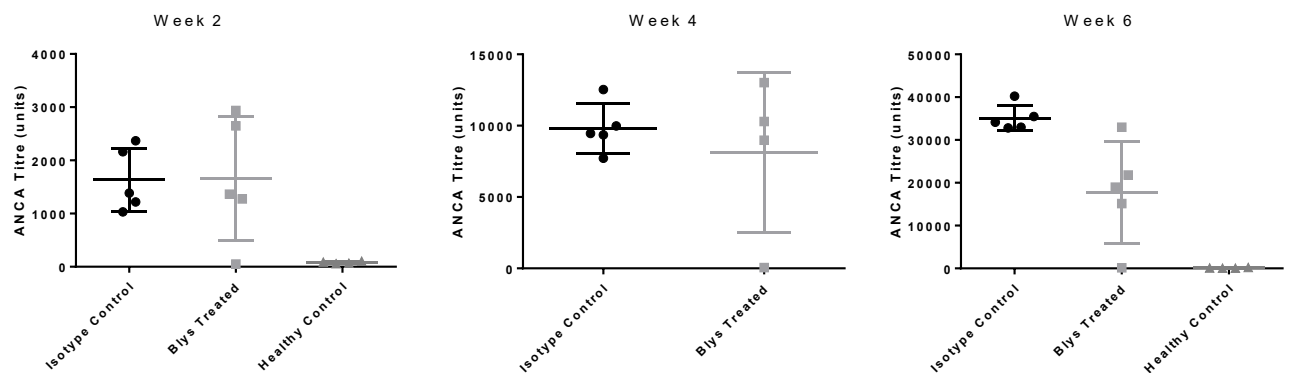


**Figure 7.18: Assessment of circulating ANCA titres by direct ELISA at weeks 2, 4 and 6.**

Data are presented as mean and SEM. There were no data available for the normal rats at week 4 due to a problem with samples. There a significant reduction in ANCA titres between the isotype control and anti-BlyS treated rats at week 6 ( $p=0.0003$ , Two way ANOVA).

#### 7.3.2.7 Renal histology

There was no significant difference found in terms of percentage abnormal glomeruli, across the groups (figure 7.19).

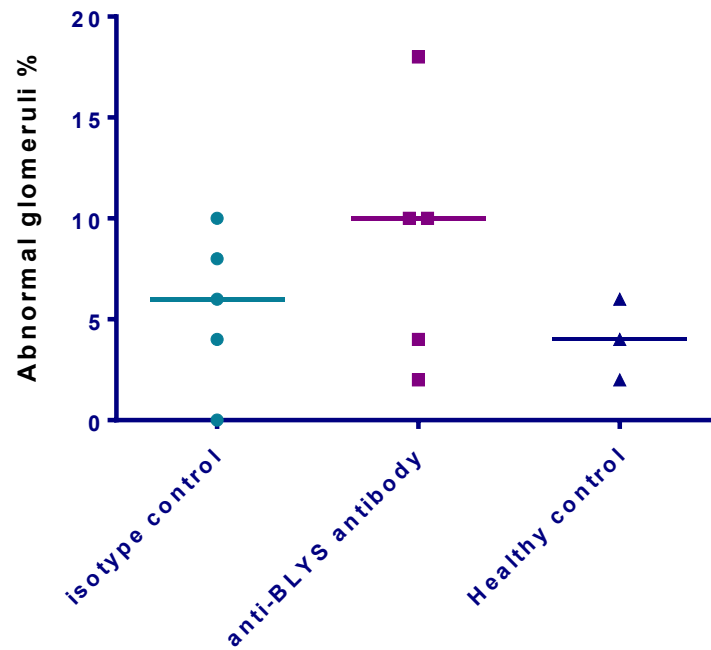


**Figure 7.19 Histological injury**

This was assessed by the percentage of abnormal glomeruli per kidney cross section assessed using light microscopy in the anti-BlyS treated and vehicle treated animals at 6 weeks shown as median +/- IQR. p=ns

#### 7.3.2.8 Macrophage infiltration

There was no significant difference found in macrophage infiltration as measured by CD68 staining, according to treatment group (Figure 7.20).

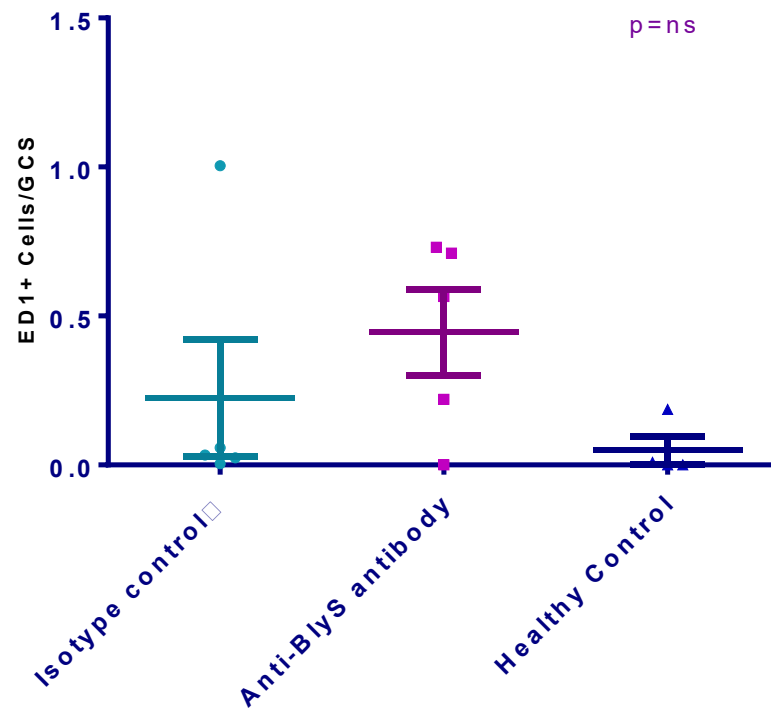


**Figure 7.20: Macrophage infiltration.**

The mean percentage of ED1 + cells per glomerular cross section (GCS) was measured and taken to be representative of degree of macrophage infiltration. Data were analysed using image analysis software. Data shown as median +/- IQR, \*p=0.04, Mann Whitney U test. Note: Missing data on glomerular histology for one rat in the normal group, because of a technical problem.

#### 7.3.2.9 Conclusion and brief discussion, Experiment 2:

Treatment with the anti-BlyS antibody from week 4 appeared to result in a greater reduction in haematuria and proteinuria than treatment with the isotype control. This was however, not statistically significant and a longer experiment would be of value to test this further.

There was a 49% reduction in circulating anti-MPO antibodies at week 6 in the anti-BlyS antibody treated group when compared with the isotype control treated group ( $p < 0.05$ ).

There was no significant effect on renal histology. Longer duration of treatment may have been needed to demonstrate this effect.

## 7.4 Overall conclusion and discussion:

In our study, there was a significant improvement in EAV upon administration on induction of the anti-BlyS antibody, 10F4, particularly at high dose. This was demonstrated by a reduction in haematuria, proteinuria, glomerular damage and macrophage infiltration. There was also a reduction in autoantibody production in splenocytes and in the circulation by weeks 2 and 4. In addition, there was marked reduction in pulmonary haemorrhage in the anti-BlyS treated group. These findings provide further evidence for the pathogenicity of BlyS in AAV and in particular its role in initiation of the disease process. The study assessing late treatment did not show a significant reversal in glomerular damage. There was no significant reduction in haematuria or proteinuria and no improvement in histology, either in terms of percentage glomerular injury, or macrophage infiltration. There was also no improvement in pulmonary haemorrhage. The only positive result of administration of the anti-BlyS agent in this study was a significant reduction in circulating MPO antibodies in the anti-BlyS treated animals at 6 weeks. Whilst it is possible that there was not sufficient duration of treatment to improve the disease process, as antibody was only administered weekly from weeks 4-6, it is also possible that the mechanism of action of BlyS inhibition is mainly in the prevention of the initiating disease process and that once disease is established, as in the second experiment, pathogenicity is primarily T cell mediated. BlyS inhibition therefore may not reverse the disease process despite a reduction in MPO antibody production.

This finding is in keeping with studies using the MPO transfer model in mice (88). It has been shown in passive transfer models, that administering MPO to genetically intact mice can result in the development of anti-MPO antibodies but that these

antibodies are not sufficient to cause disease. In the MPO transfer model it has been demonstrated that the use of a low dose anti-GBM antibody can cause neutrophil influx and MPO deposition without disease manifestations. Using this model, depletion of CD4<sup>+</sup> T cells in the effector phase significantly improved disease. Mice deficient in B cells developed a similar disease phenotype to those with B cells, again suggesting that pathogenicity was B cell independent. Further experiments included the pre-immunisation of B cell deficient mice with MPO to induce MPO specific CD4<sup>+</sup> T cells. This resulted in significant disease which was prevented by T cell depletion (186).

Both our studies were limited by the number of animals available per experiment, and the quantity of antibody we were provided with. As EAV is characteristically a mild disease model, it would also be useful to repeat these experiments in a similar but more severe model of autoimmune vasculitis. Induction with MPO and a subnephritogenic dose of anti-GBM antibody serves as a model of “double positivity” and has more severe glomerulonephritis. It would be extremely useful to assess the effect of the BlyS inhibitor on B cell subsets. Rat B cell surface markers are not currently well defined and it would be helpful to carry out flow cytometry for intracellular staining for markers of regulatory B cells including IL10. It would also be useful to carry out T cell studies. Once a reputable rat B cell depletion agent becomes available and a BlyS ELISA, validated in the rat, it would also be of value to assess the effect of B cell depletion on BlyS levels in the rat model.

There have been several clinical trials of BlyS inhibitors in autoimmune diseases in humans. The most successful of these trials have been in SLE (123, 124) following which the BlyS inhibitor belimumab (Benlysta) was the first new drug to be licensed in the treatment of this condition in over 40 years. Patients with lupus nephritis were

excluded from these trials and the effect of belimumab in these patients was assessed in the BLISS-LN trial (The Efficacy and Safety of Belimumab in Patients with Active Lupus Nephritis trial) (187). This trial revealed that patients treated with belimumab in addition to standard therapy had a greater renal response than those treated with standard of care alone (187). In AAV, BREVAS assessed the efficacy of belimumab used alongside azathioprine to maintain remission (126). There was no benefit demonstrated. COMBIVAS is currently recruiting to assess the benefit of rituximab used in combination with belimumab in PR3 AAV.

There are a number of confounding factors involved in human studies and further work with animal models such as EAV will be helpful in elucidating further information regarding mechanisms of pathogenicity in AAV, and potential treatment targets.

To the best of our knowledge, this is the first study to demonstrate the successful treatment of an autoimmune model of vasculitis with an anti-BlyS therapeutic agent.

## 7.5 Acknowledgments:

I would like to thank my colleague Dr. McAdoo who carried out the *in vivo* experiments with me and also Dr. Predecki, Gurjeet Bhangal and Dr. Tempest-Roe who kindly assisted me with the experimental work and analysis.

## Chapter 8: Discussion and Future Work

As discussed in Chapter 1, the overall aim of this project was to assess factors predictive of prognosis in our cohort of patients with AAV at the Hammersmith Hospital, and to assess the clinical significance of B cell survival factors. The overall aims of this study are summarised below:

## 8.1 Aims of the study

There were a number of aims of this study including:

- To assess factors affecting outcome in a cohort of over 500 patients with AAV at our centre at Hammersmith Hospital.
- To address clinical and histological factors affecting outcome in a smaller subgroup of patients and carry out a validation study of the international histological classification system described by Berden *et al* in 2010.
- To assess factors affecting outcome in patients with severe renal disease.
- To assess the clinical significance of the B cell survival factors BlyS and APRIL in AAV.
- To assess the use of a BlyS inhibitor *in vivo* using the rat model of AAV, EAV.

The Hammersmith Hospital (HH) is a tertiary referral centre for AAV. Prior to my commencement of this work, there was no united database for all the AAV patients previously or currently under follow up. In order to undertake this project I compiled a database of over 500 patients with AAV who had been under the care of HH over a 40 year period. This involved collation of laboratory, clinical and histopathological data

which had been collected using a variety of programmes and recorded both in paper notes and electronic systems such as microfilm. The main limitations of this part of the study were its retrospective nature, size and timescale. This project did however have the advantage of including a large number of patients at a single centre, diagnosed and treated in a consistent fashion and followed up over a significant period of time. Other large studies of outcome in AAV for example the study by Flossman *et al*, included similar numbers of patients but these patients were from a number of different centres and not treated in a consistent manner. An additional advantage to this project was the ethnic diversity of the population served by HH. The main factors found to be contributory to the overall survival of our patients, were age and requirement for renal replacement therapy at presentation. Advancing age and requirement for RRT were associated with worse survival. This was consistent with the literature. In addition we found decade of diagnosis to be a significant factor determining survival. It is likely that early diagnosis facilitated by ANCA testing, advances in immunotherapy and improved general medical supportive care have contributed to this. These findings are discussed fully in Chapter 3.

Having established the general factors affecting overall prognosis in our cohort of patients and with the knowledge that treatment with immunosuppression results in significant morbidity and mortality, we wanted to assess more specific factors predictive of renal and patient outcome, with the aim of being able to deliver patient specific immunosuppressive therapy and give patients a more accurate prognosis. Histopathological features had been previously reported as correlating with renal prognosis in AAV and formed the basis of the international classification system for AAV proposed by Berden *et al* in 2010. This classification is described in Chapter 4. There were a number of validation studies of this classification system, results of which

varied, and one of the aims of our study was to validate the system in a subgroup of our patients. One hundred and four patients with biopsy proved AAV and with more than 10 glomeruli available for analysis, were included in our study. Upon multivariate analysis, the histological classification was not found to be predictive of renal outcome following adjustment for other factors including age, baseline eGFR and tubular atrophy. In terms of other factors, age, presenting eGFR and percentage normal glomeruli were found to be important prognostic factors. These findings were in keeping with other studies in the literature. There was also a correlation between a greater degree of tubular atrophy and poorer renal outcome. With PR3-ANCA and MPO-ANCA vasculitis increasingly being thought of as separate disease entities, it was interesting to find that patients with PR3-ANCA had significantly better renal prognosis in our cohort of patients. The conclusion reached from this validation study was that the international histological classification is predictive of renal outcome in AAV (as shown in univariate analysis), but should be used in conjunction with other established parameters such as tubular atrophy and percentage normal glomeruli. This work has been published in NDT(140). Further work would include a prospective study including a larger number of patients.

Patients with severe renal dysfunction are frequently excluded from studies in ANCA vasculitis. We wanted to assess the factors affecting long term patient and renal outcomes in these patients. These results are shown in Chapter 5. Patients were included if they presented with an eGFR of  $<15 \text{ ml/min/1.73m}^2$  or were dialysis dependent. One hundred and eighty patients with severe renal dysfunction were included. Outcomes were assessed separately for patients who presented dialysis dependent vs those who presented with an eGFR of  $<15 \text{ ml/min/1.73m}^2$  but were dialysis independent. Despite being a group with severe renal dysfunction and hence

a poor prognosis, both renal and patient outcomes were favourable when compared with other studies of this patient group. Fifty- nine percent of patients who presented dialysis dependent gained independent renal function at one year. As for the previous study assessing histological factors predictive of outcome, percentage normal glomeruli was predictive of renal survival in this patient group and was a factor associated with becoming dialysis independent at one year. Again, starting serum creatinine was also associated with renal prognosis even at these higher levels. Patients who did not regain independent renal function at one year were found to have a higher starting creatinine than those who did. The Berden classification was not found to be predictive of survival in this cohort of patients. An extremely interesting finding was the difference in outcome for patients who were dialysis dependent on presentation compared with those who had an eGFR  $<15$  ml/min/1.73m<sup>2</sup> but were dialysis independent. Patients who presented dialysis independent had a significantly improved prognosis in terms of 1 and 5yr patient and renal survival. This improved survival was confirmed over a 10 year period. Major trials in AAV have previously reported outcomes based on these patients being grouped together. This is the first study to our knowledge where patients have been separated in this manner. This may have significant implications for both current and future studies as these patients may require altered therapy. This study had the limitations associated with being a retrospective analysis with the possibility of not capturing all data for some patients particularly in the earlier years if they were admitted for short periods to the intensive care units. Prospective studies including this group of patients with severe renal dysfunction, divided according to dialysis dependence and independence, are needed.

With the pathogenicity of the B cell in AAV having become well established and the need for more directed therapy, Chapter 6 describes a study carried out to assess the clinical significance of the B cell survival factors BlyS and APRIL, in a cohort of patients at our centre. Serum levels in patients with both active disease and in remission, were assessed, and in addition, an assessment was made of BlyS and its receptors in renal biopsies of a group of AAV patients. As described in Chapter 6, serum BlyS levels were found to be elevated in patients with AAV compared with healthy controls. Levels were raised in patients with active disease compared with those in remission, and also following treatment with rituximab. It has been hypothesised that B cell depletion following administration of rituximab results in re-population of B cells in a BlyS rich environment which favours the development of autoimmunity. This has important implications given the widespread usage of rituximab in both induction and maintenance of disease remission. APRIL levels were also found to be elevated in patients with active disease however there was no rise following rituximab therapy. This could be because APRIL acts primarily on receptors on plasma cells which are not affected by CD20 mediated depletion. These findings suggest clinical significance of APRIL and BlyS in AAV both as potential biomarkers of disease and therapeutic targets. Current biomarkers include ANCA and inflammatory markers such as CRP. In the clinical setting it is notoriously difficult to differentiate between infective complications and relapsing disease. There are some patients who have a persistently elevated ANCA titre which does not reflect disease status. This implies a role for both BlyS and APRIL to potentially form part of a panel of biomarkers for disease. A descriptive study was also carried out on 10 biopsy samples from patients with AAV. Staining for CD20 in renal tissue is well established. Staining by immunohistochemistry for BlyS and its receptors BAFFR, TACI and BCMA had not

been carried out previously in our laboratory, and at the time of writing had not been carried out in patients with AAV. Expression of BlyS and its receptors has been reported in the haematology literature and for patients with SLE. We decided to attempt to detect BlyS and its receptors in a small sample of patients with AAV. Although limited by the number of slides we could use which were not in clinical use, we were able to demonstrate very clear CD 20 staining particularly in the interstitium of patients with AAV. Varying degrees of staining were demonstrated for BlyS, BAFF R, TACI and BCMA and examples of these are shown in Chapter 6. Having shown it is possible, future work would ideally include a prospective study obtaining serum BlyS and APRIL levels at baseline along with a staining profile for CD20, BlyS, APRIL and their receptors on renal biopsy and producing a B cell profile for each patient. This could guide potential therapy with anti-B cell and anti-B cell survival factor agents including both rituximab and belimumab. Blys and APRIL levels could be taken at regular intervals and correlated with clinical and biological features of relapse.

Following the finding that BlyS is of clinical significance in AAV, Chapter 7 describes experiments carried out to assess effect of a BlyS inhibitor, 10F4 on EAV, the in vivo model of AAV. There was significant improvement in EAV upon administration on induction of the anti-BlyS antibody 10F4 particularly at high dose. This was evidenced by a reduction in haematuria, proteinuria, glomerular damage and macrophage infiltration, as well as a reduction in pulmonary haemorrhage and decreased production of autoantibodies both anti-MPO antibodies in splenocytes, and peripheral anti-MPO antibodies at weeks 2 and 4. The second experiment assessed late treatment from weeks 4 to 6 and did not show a significant improvement in glomerular injury. There was however a reduction in circulating anti MPO antibodies at six weeks. These findings suggest a role for Blys inhibition in the early treatment of AAV and to

our knowledge this was the first study demonstrating successful treatment of an autoimmune model of vasculitis with an anti-BlyS therapeutic agent. These experiments were primarily limited by the amount of BlyS inhibitor available to us. Further work would include repeating these experiments in larger numbers of rats and also, in the late treatment experiment for a longer duration. As discussed in Chapter 7, it would also be useful to repeat these experiments once there is a reliable B cell depletion agent in the rat and a standardised ELISA for measuring BlyS levels in the rat.

## 8.2 Concluding remarks

The work in this thesis suggests that there are a number of factors affecting prognosis and outcome in AAV. One of the greatest challenges in the management of patients with AAV is to balance the risk of side effects of immunosuppressive therapy with the risks of the disease process itself especially given its relapsing remitting nature. Further work on prognostic factors, including histological factors, and outcome, would enable a patient specific plan for immunosuppression to be formulated. Studies are on-going to evaluate more directed therapy for AAV and this includes B cell directed therapy. With the finding that rituximab may deplete B cells of a regulatory nature in addition to those involved in pathogenesis, more studies targeting B cell survival factors are needed. Here, we have demonstrated the clinical significance of BlyS and its presence, along with its receptors, in renal tissue. The clinical significance of APRIL has also been shown. In vivo work has demonstrated the potential value of an anti-BlyS agent as an induction agent for EAV, the rat model of AAV and this supports the trial of anti-BlyS agents in human disease.

## References:

1. Jennette JC. Overview of the 2012 revised International Chapel Hill Consensus Conference nomenclature of vasculitides. *Clin Exp Nephrol*. 2013;17(5):603-6.
2. Kemna MJ, Cohen Tervaert JW, Broen K, Timmermans S, van Paassen P, Damoiseaux J. Seasonal Influence on the Risk of Relapse at a Rise of Antineutrophil Cytoplasmic Antibodies in Vasculitis Patients with Renal Involvement. *J Rheumatol*. 2017;44(4):473-81.
3. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, et al. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet*. 1985;1(8426):425-9.
4. Luqmani RA, Suppiah R, Grayson PC, Merkel PA, Watts R. Nomenclature and classification of vasculitis - update on the ACR/EULAR diagnosis and classification of vasculitis study (DCVAS). *Clin Exp Immunol*. 2011;164 Suppl 1:11-3.
5. Watts RA, Robson J. Introduction, epidemiology and classification of vasculitis. *Best Pract Res Clin Rheumatol*. 2018;32(1):3-20.
6. Watts RA, Gonzalez-Gay MA, Lane SE, Garcia-Porrúa C, Benthall G, Scott DG. Geoepidemiology of systemic vasculitis: comparison of the incidence in two regions of Europe. *Ann Rheum Dis*. 2001;60(2):170-2.
7. Cao Y, Schmitz JL, Yang J, Hogan SL, Bunch D, Hu Y, et al. DRB1\*15 allele is a risk factor for PR3-ANCA disease in African Americans. *J Am Soc Nephrol*. 2011;22(6):1161-7.
8. Mahr A, Guillevin L, Poissonnet M, Ayme S. Prevalences of polyarteritis nodosa, microscopic polyangiitis, Wegener's granulomatosis, and Churg-Strauss syndrome in a French urban multiethnic population in 2000: a capture-recapture estimate. *Arthritis Rheum*. 2004;51(1):92-9.
9. Pearce FA, Lanyon PC, Grainge MJ, Shaunak R, Mahr A, Hubbard RB, et al. Incidence of ANCA-associated vasculitis in a UK mixed ethnicity population. *Rheumatology (Oxford)*. 2016;55(9):1656-63.
10. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med*. 2012;367(3):214-23.
11. Jagiello P, Gencik M, Arning L, Wiczorek S, Kunstmann E, Csernok E, et al. New genomic region for Wegener's granulomatosis as revealed by an extended association screen with 202 apoptosis-related genes. *Hum Genet*. 2004;114(5):468-77.
12. Gomez-Puerta JA, Gedmintas L, Costenbader KH. The association between silica exposure and development of ANCA-associated vasculitis: systematic review and meta-analysis. *Autoimmun Rev*. 2013;12(12):1129-35.
13. Rihova Z, Maixnerova D, Jancova E, Pelclova D, Bartunkova J, Fenclova Z, et al. Silica and asbestos exposure in ANCA-associated vasculitis with pulmonary involvement. *Ren Fail*. 2005;27(5):605-8.
14. Hogan SL, Cooper GS, Savitz DA, Nylander-French LA, Parks CG, Chin H, et al. Association of silica exposure with anti-neutrophil cytoplasmic autoantibody small-vessel vasculitis: a population-based, case-control study. *Clin J Am Soc Nephrol*. 2007;2(2):290-9.
15. Csernok E, Lamprecht P, Gross WL. Clinical and immunological features of drug-induced and infection-induced proteinase 3-antineutrophil cytoplasmic antibodies and myeloperoxidase-antineutrophil cytoplasmic antibodies and vasculitis. *Curr Opin Rheumatol*. 2010;22(1):43-8.

- 16.Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG. Association of chronic nasal carriage of *Staphylococcus aureus* and higher relapse rates in Wegener granulomatosis. *Ann Intern Med*. 1994;120(1):12-7.
- 17.Stegeman CA, Tervaert JW, de Jong PE, Kallenberg CG. Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med*. 1996;335(1):16-20.
- 18.Booth AD, Almond MK, Burns A, Ellis P, Gaskin G, Neild GH, et al. Outcome of ANCA-associated renal vasculitis: a 5-year retrospective study. *Am J Kidney Dis*. 2003;41(4):776-84.
- 19.Joshi L, Tanna A, McAdoo SP, Medjeral-Thomas N, Taylor SR, Sandhu G, et al. Long-term Outcomes of Rituximab Therapy in Ocular Granulomatosis with Polyangiitis: Impact on Localized and Nonlocalized Disease. *Ophthalmology*. 2015;122(6):1262-8.
- 20.Mukhtyar C, Guillevin L, Cid MC, Dasgupta B, de Groot K, Gross W, et al. EULAR recommendations for the management of primary small and medium vessel vasculitis. *Ann Rheum Dis*. 2009;68(3):310-7.
- 21.Merkel PA, Aydin SZ, Boers M, Direskeneli H, Herlyn K, Seo P, et al. The OMERACT core set of outcome measures for use in clinical trials of ANCA-associated vasculitis. *J Rheumatol*. 2011;38(7):1480-6.
- 22.Mukhtyar C, Lee R, Brown D, Carruthers D, Dasgupta B, Dubey S, et al. Modification and validation of the Birmingham Vasculitis Activity Score (version 3). *Ann Rheum Dis*. 2009;68(12):1827-32.
- 23.Kitching AR, Anders HJ, Basu N, Brouwer E, Gordon J, Jayne DR, et al. ANCA-associated vasculitis. *Nat Rev Dis Primers*. 2020;6(1):71.
- 24.Guillevin L, Pagnoux C, Seror R, Mahr A, Mouthon L, Le Toumelin P, et al. The Five-Factor Score revisited: assessment of prognoses of systemic necrotizing vasculitides based on the French Vasculitis Study Group (FVSG) cohort. *Medicine (Baltimore)*. 2011;90(1):19-27.
- 25.Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. *Kidney Int*. 1992;41(2):375-83.
- 26.Savage CO, Pottinger BE, Gaskin G, Pusey CD, Pearson JD. Autoantibodies developing to myeloperoxidase and proteinase 3 in systemic vasculitis stimulate neutrophil cytotoxicity toward cultured endothelial cells. *Am J Pathol*. 1992;141(2):335-42.
- 27.Radford DJ, Savage CO, Nash GB. Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion. *Arthritis Rheum*. 2000;43(6):1337-45.
- 28.Hewins P, Williams JM, Wakelam MJ, Savage CO. Activation of Syk in neutrophils by antineutrophil cytoplasm antibodies occurs via Fcγ receptors and CD18. *J Am Soc Nephrol*. 2004;15(3):796-808.
- 29.McAdoo SP, Predecki M, Tanna A, Bhatt T, Bhangal G, McDaid J, et al. Spleen tyrosine kinase inhibition is an effective treatment for established vasculitis in a pre-clinical model. *Kidney Int*. 2020;97(6):1196-207.
- 30.Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest*. 2002;110(7):955-63.
- 31.Little MA, Smyth L, Salama AD, Mukherjee S, Smith J, Haskard D, et al. Experimental autoimmune vasculitis: an animal model of anti-neutrophil cytoplasmic autoantibody-associated systemic vasculitis. *Am J Pathol*. 2009;174(4):1212-20.

32. Little MA, Smyth CL, Yadav R, Ambrose L, Cook HT, Nourshargh S, et al. Antineutrophil cytoplasm antibodies directed against myeloperoxidase augment leukocyte-microvascular interactions in vivo. *Blood*. 2005;106(6):2050-8.
33. Bansal PJ, Tobin MC. Neonatal microscopic polyangiitis secondary to transfer of maternal myeloperoxidase-antineutrophil cytoplasmic antibody resulting in neonatal pulmonary hemorrhage and renal involvement. *Ann Allergy Asthma Immunol*. 2004;93(4):398-401.
34. Silva F, Specks U, Sethi S, Irazabal MV, Fervenza FC. Successful pregnancy and delivery of a healthy newborn despite transplacental transfer of antimyeloperoxidase antibodies from a mother with microscopic polyangiitis. *Am J Kidney Dis*. 2009;54(3):542-5.
35. Hagen EC, Daha MR, Hermans J, Andrassy K, Csernok E, Gaskin G, et al. Diagnostic value of standardized assays for anti-neutrophil cytoplasmic antibodies in idiopathic systemic vasculitis. EC/BCR Project for ANCA Assay Standardization. *Kidney Int*. 1998;53(3):743-53.
36. Boomsma MM, Stegeman CA, van der Leij MJ, Oost W, Hermans J, Kallenberg CG, et al. Prediction of relapses in Wegener's granulomatosis by measurement of antineutrophil cytoplasmic antibody levels: a prospective study. *Arthritis Rheum*. 2000;43(9):2025-33.
37. Joshi L, Lightman SL, Salama AD, Shirodkar AL, Pusey CD, Taylor SR. Rituximab in refractory ophthalmic Wegener's granulomatosis: PR3 titers may predict relapse, but repeat treatment can be effective. *Ophthalmology*. 2011;118(12):2498-503.
38. Han WK, Choi HK, Roth RM, McCluskey RT, Niles JL. Serial ANCA titers: useful tool for prevention of relapses in ANCA-associated vasculitis. *Kidney Int*. 2003;63(3):1079-85.
39. Jayne DR, Gaskin G, Pusey CD, Lockwood CM. ANCA and predicting relapse in systemic vasculitis. *QJM*. 1995;88(2):127-33.
40. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, et al. Rituximab versus cyclophosphamide for ANCA-associated vasculitis. *N Engl J Med*. 2010;363(3):221-32.
41. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, et al. Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis. *N Engl J Med*. 2010;363(3):211-20.
42. Jayne DR, Gaskin G, Rasmussen N, Abramowicz D, Ferrario F, Guillevin L, et al. Randomized trial of plasma exchange or high-dosage methylprednisolone as adjunctive therapy for severe renal vasculitis. *J Am Soc Nephrol*. 2007;18(7):2180-8.
43. Xu PC, Cui Z, Chen M, Hellmark T, Zhao MH. Comparison of characteristics of natural autoantibodies against myeloperoxidase and anti-myeloperoxidase autoantibodies from patients with microscopic polyangiitis. *Rheumatology (Oxford)*. 2011;50(7):1236-43.
44. Roth AJ, Ooi JD, Hess JJ, van Timmeren MM, Berg EA, Poulton CE, et al. Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis. *J Clin Invest*. 2013;123(4):1773-83.
45. Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci U S A*. 1990;87(11):4115-9.
46. Savage CO, Gaskin G, Pusey CD, Pearson JD. Myeloperoxidase binds to vascular endothelial cells, is recognized by ANCA and can enhance complement dependent cytotoxicity. *Adv Exp Med Biol*. 1993;336:121-3.
47. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007;176(2):231-41.

48. Geetha D, Jefferson JA. ANCA-Associated Vasculitis: Core Curriculum 2020. *Am J Kidney Dis*. 2020;75(1):124-37.
49. Radford DJ, Luu NT, Hewins P, Nash GB, Savage CO. Antineutrophil cytoplasmic antibodies stabilize adhesion and promote migration of flowing neutrophils on endothelial cells. *Arthritis Rheum*. 2001;44(12):2851-61.
50. Prendecki M, Pusey CD. Recent advances in understanding of the pathogenesis of ANCA-associated vasculitis. *F1000Res*. 2018;7.
51. Manenti L, Urban ML, Maritati F, Galetti M, Vaglio A. Complement blockade in ANCA-associated vasculitis: an index case, current concepts and future perspectives. *Intern Emerg Med*. 2017;12(6):727-31.
52. Merkel PA, Jayne DR, Wang C, Hillson J, Bekker P. A randomized, double-blind, active-controlled, Phase 3 study to evaluate the safety and efficacy of avacopan, a C5a receptor inhibitor, in patients with ANCA-associated vasculitis treated concomitantly with rituximab or cyclophosphamide/azathioprine. *JMIR Res Protoc*. 2020.
53. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, et al. Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells. *Immunology*. 2010;130(1):64-73.
54. Chavele KM, Shukla D, Ketepe-Arachi T, Seidel JA, Fuchs D, Pusey CD, et al. Regulation of myeloperoxidase-specific T cell responses during disease remission in antineutrophil cytoplasmic antibody-associated vasculitis: the role of Treg cells and tryptophan degradation. *Arthritis Rheum*. 2010;62(5):1539-48.
55. Free ME, Bunch DO, McGregor JA, Jones BE, Berg EA, Hogan SL, et al. Patients with antineutrophil cytoplasmic antibody-associated vasculitis have defective Treg cell function exacerbated by the presence of a suppression-resistant effector cell population. *Arthritis Rheum*. 2013;65(7):1922-33.
56. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, et al. Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. *Nephrol Dial Transplant*. 2010;25(7):2209-17.
57. Tanna A, Tam FW, Pusey CD. B-cell-targeted therapy in adult glomerulonephritis. *Expert Opin Biol Ther*. 2013;13(12):1691-706.
58. Mauri C, Blair PA. Regulatory B cells in autoimmunity: developments and controversies. *Nat Rev Rheumatol*. 2010;6(11):636-43.
59. Todd SK, Pepper RJ, Draibe J, Tanna A, Pusey CD, Mauri C, et al. Regulatory B cells are numerically but not functionally deficient in anti-neutrophil cytoplasm antibody-associated vasculitis. *Rheumatology (Oxford)*. 2014;53(9):1693-703.
60. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, et al. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity*. 2010;32(1):129-40.
61. Bunch DO, Mendoza CE, Aybar LT, Kotzen ES, Colby KR, Hu Y, et al. Gleaning relapse risk from B cell phenotype: decreased CD5+ B cells portend a shorter time to relapse after B cell depletion in patients with ANCA-associated vasculitis. *Ann Rheum Dis*. 2015;74(9):1784-6.
62. McClure M, Gopaluni S, Jayne D, Jones R. B cell therapy in ANCA-associated vasculitis: current and emerging treatment options. *Nat Rev Rheumatol*. 2018;14(10):580-91.
63. Holden NJ, Williams JM, Morgan MD, Challa A, Gordon J, Pepper RJ, et al. ANCA-stimulated neutrophils release BLyS and promote B cell survival: a clinically relevant cellular process. *Ann Rheum Dis*. 2011;70(12):2229-33.

64. Gavin AL, Ait-Azzouzene D, Ware CF, Nemazee D. DeltaBAFF, an alternate splice isoform that regulates receptor binding and biopresentation of the B cell survival cytokine, BAFF. *J Biol Chem.* 2003;278(40):38220-8.
65. Cancro MP, D'Cruz DP, Khamashta MA. The role of B lymphocyte stimulator (BLyS) in systemic lupus erythematosus. *J Clin Invest.* 2009;119(5):1066-73.
66. Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. *Proc Natl Acad Sci U S A.* 2009;106(33):13945-50.
67. Baker KP. BLyS--an essential survival factor for B cells: basic biology, links to pathology and therapeutic target. *Autoimmun Rev.* 2004;3(5):368-75.
68. Yan M, Wang H, Chan B, Roose-Girma M, Erickson S, Baker T, et al. Activation and accumulation of B cells in TACI-deficient mice. *Nat Immunol.* 2001;2(7):638-43.
69. Thien M, Phan TG, Gardam S, Amesbury M, Basten A, Mackay F, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity.* 2004;20(6):785-98.
70. Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, et al. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med.* 1999;190(11):1697-710.
71. Cheema GS, Roschke V, Hilbert DM, Stohl W. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum.* 2001;44(6):1313-9.
72. Krumbholz M, Specks U, Wick M, Kalled SL, Jenne D, Meinl E. BAFF is elevated in serum of patients with Wegener's granulomatosis. *J Autoimmun.* 2005;25(4):298-302.
73. Bader L, Koldingsnes W, Nossent J. B-lymphocyte activating factor levels are increased in patients with Wegener's granulomatosis and inversely correlated with ANCA titer. *Clin Rheumatol.* 2010;29(9):1031-5.
74. Nagai M, Hirayama K, Ebihara I, Shimohata H, Kobayashi M, Koyama A. Serum levels of BAFF and APRIL in myeloperoxidase anti-neutrophil cytoplasmic autoantibody-associated renal vasculitis: association with disease activity. *Nephron Clin Pract.* 2011;118(4):c339-45.
75. Pitzalis C, Jones GW, Bombardieri M, Jones SA. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nat Rev Immunol.* 2014;14(7):447-62.
76. Zhao Y, Odell E, Choong LM, Barone F, Fields P, Wilkins B, et al. Granulomatosis with polyangiitis involves sustained mucosal inflammation that is rich in B-cell survival factors and autoantigen. *Rheumatology (Oxford).* 2012;51(9):1580-6.
77. Parsa R, Lund H, Georgoudaki AM, Zhang XM, Ortlieb Guerreiro-Cacais A, Grommisch D, et al. BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis. *J Exp Med.* 2016;213(8):1537-53.
78. Weidner S, Neupert W, Goppelt-Strube M, Rupprecht HD. Antineutrophil cytoplasmic antibodies induce human monocytes to produce oxygen radicals in vitro. *Arthritis Rheum.* 2001;44(7):1698-706.
79. Xiao H, Hu P, Falk RJ, Jennette JC. Overview of the Pathogenesis of ANCA-Associated Vasculitis. *Kidney Dis (Basel).* 2016;1(4):205-15.
80. Tarzi RM, Liu J, Schreiner S, Hill NR, Page TH, Cook HT, et al. CD14 expression is increased on monocytes in patients with anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitis and correlates with the expression of ANCA autoantigens. *Clin Exp Immunol.* 2015;181(1):65-75.

- 81.Haller H, Eichhorn J, Pieper K, Gobel U, Luft FC. Circulating leukocyte integrin expression in Wegener's granulomatosis. *J Am Soc Nephrol*. 1996;7(1):40-8.
- 82.Weidner S, Carl M, Riess R, Rupperecht HD. Histologic analysis of renal leukocyte infiltration in antineutrophil cytoplasmic antibody-associated vasculitis: importance of monocyte and neutrophil infiltration in tissue damage. *Arthritis Rheum*. 2004;50(11):3651-7.
- 83.Zhao L, David MZ, Hyjek E, Chang A, Meehan SM. M2 macrophage infiltrates in the early stages of ANCA-associated pauci-immune necrotizing GN. *Clin J Am Soc Nephrol*. 2015;10(1):54-62.
- 84.Salama AD, Little MA. Animal models of antineutrophil cytoplasm antibody-associated vasculitis. *Curr Opin Rheumatol*. 2012;24(1):1-7.
- 85.Huugen D, van Esch A, Xiao H, Peutz-Kootstra CJ, Buurman WA, Tervaert JW, et al. Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int*. 2007;71(7):646-54.
- 86.Jennette JC, Xiao H, Falk R, Gasim AMH. Experimental models of vasculitis and glomerulonephritis induced by antineutrophil cytoplasmic autoantibodies. *Contrib Nephrol*. 2011;169:211-20.
- 87.Xiao H, Schreiber A, Heeringa P, Falk RJ, Jennette JC. Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol*. 2007;170(1):52-64.
- 88.Ruth AJ, Kitching AR, Kwan RY, Odobasic D, Ooi JD, Timoshanko JR, et al. Anti-neutrophil cytoplasmic antibodies and effector CD4+ cells play nonredundant roles in anti-myeloperoxidase crescentic glomerulonephritis. *J Am Soc Nephrol*. 2006;17(7):1940-9.
- 89.Gan PY, Steinmetz OM, Tan DS, O'Sullivan KM, Ooi JD, Iwakura Y, et al. Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis. *J Am Soc Nephrol*. 2010;21(6):925-31.
- 90.Shochet L, Holdsworth S, Kitching AR. Animal Models of ANCA Associated Vasculitis. *Front Immunol*. 2020;11:525.
- 91.Heeringa P, Little MA. In vivo approaches to investigate ANCA-associated vasculitis: lessons and limitations. *Arthritis Res Ther*. 2011;13(1):204.
- 92.Little MA, Bhangal G, Smyth CL, Nakada MT, Cook HT, Nourshargh S, et al. Therapeutic effect of anti-TNF-alpha antibodies in an experimental model of anti-neutrophil cytoplasm antibody-associated systemic vasculitis. *J Am Soc Nephrol*. 2006;17(1):160-9.
- 93.Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, et al. Copy number polymorphism in *Fcgr3* predisposes to glomerulonephritis in rats and humans. *Nature*. 2006;439(7078):851-5.
- 94.Schonermark U, Gross WL, de Groot K. Treatment of ANCA-associated vasculitis. *Nat Rev Nephrol*. 2014;10(1):25-36.
- 95.Chen M, Yu F, Zhang Y, Zhao MH. Antineutrophil cytoplasmic autoantibody-associated vasculitis in older patients. *Medicine (Baltimore)*. 2008;87(4):203-9.
- 96.Little MA, Nightingale P, Verburgh CA, Hauser T, De Groot K, Savage C, et al. Early mortality in systemic vasculitis: relative contribution of adverse events and active vasculitis. *Ann Rheum Dis*. 2010;69(6):1036-43.
- 97.de Groot K, Harper L, Jayne DR, Flores Suarez LF, Gregorini G, Gross WL, et al. Pulse versus daily oral cyclophosphamide for induction of remission in antineutrophil cytoplasmic antibody-associated vasculitis: a randomized trial. *Ann Intern Med*. 2009;150(10):670-80.

98. McAdoo SP, Medjeral-Thomas N, Gopaluni S, Tanna A, Mansfield N, Galliford J, et al. Long-term follow-up of a combined rituximab and cyclophosphamide regimen in renal anti-neutrophil cytoplasm antibody-associated vasculitis. *Nephrol Dial Transplant*. 2019;34(1):63-73.
99. Walsh M, Casian A, Flossmann O, Westman K, Hoglund P, Pusey C, et al. Long-term follow-up of patients with severe ANCA-associated vasculitis comparing plasma exchange to intravenous methylprednisolone treatment is unclear. *Kidney Int*. 2013;84(2):397-402.
100. Jones RB, Hiemstra TF, Ballarin J, Blockmans DE, Brogan P, Bruchfeld A, et al. Mycophenolate mofetil versus cyclophosphamide for remission induction in ANCA-associated vasculitis: a randomised, non-inferiority trial. *Ann Rheum Dis*. 2019;78(3):399-405.
101. Jayne D, Rasmussen N, Andrassy K, Bacon P, Tervaert JW, Dadonienė J, et al. A randomized trial of maintenance therapy for vasculitis associated with antineutrophil cytoplasmic autoantibodies. *N Engl J Med*. 2003;349(1):36-44.
102. Hiemstra TF, Walsh M, Mahr A, Savage CO, de Groot K, Harper L, et al. Mycophenolate mofetil vs azathioprine for remission maintenance in antineutrophil cytoplasmic antibody-associated vasculitis: a randomized controlled trial. *JAMA*. 2010;304(21):2381-8.
103. Guillevin L, Pagnoux C, Karras A, Khouatra C, Aumaitre O, Cohen P, et al. Rituximab versus azathioprine for maintenance in ANCA-associated vasculitis. *N Engl J Med*. 2014;371(19):1771-80.
104. Charles P, Terrier B, Perrodeau E, Cohen P, Faguer S, Huart A, et al. Comparison of individually tailored versus fixed-schedule rituximab regimen to maintain ANCA-associated vasculitis remission: results of a multicentre, randomised controlled, phase III trial (MAINRITSAN2). *Ann Rheum Dis*. 2018;77(8):1143-9.
105. Chen M, Jayne DRW, Zhao MH. Complement in ANCA-associated vasculitis: mechanisms and implications for management. *Nat Rev Nephrol*. 2017;13(6):359-67.
106. Xing GQ, Chen M, Liu G, Heeringa P, Zhang JJ, Zheng X, et al. Complement activation is involved in renal damage in human antineutrophil cytoplasmic autoantibody associated pauci-immune vasculitis. *J Clin Immunol*. 2009;29(3):282-91.
107. Gou SJ, Yuan J, Wang C, Zhao MH, Chen M. Alternative complement pathway activation products in urine and kidneys of patients with ANCA-associated GN. *Clin J Am Soc Nephrol*. 2013;8(11):1884-91.
108. Jayne DRW, Bruchfeld AN, Harper L, Schaier M, Venning MC, Hamilton P, et al. Randomized Trial of C5a Receptor Inhibitor Avacopan in ANCA-Associated Vasculitis. *J Am Soc Nephrol*. 2017;28(9):2756-67.
109. Predecki M, McAdoo SP. New Therapeutic Targets in ANCA-associated Vasculitis. *Arthritis Rheumatol*. 2020.
110. Haas J, Wurthwein C, Korporal-Kuhnke M, Viehoveer A, Jarius S, Ruck T, et al. Alemtuzumab in Multiple Sclerosis: Short- and Long-Term Effects of Immunodepletion on the Peripheral Treg Compartment. *Front Immunol*. 2019;10:1204.
111. Walsh M, Chaudhry A, Jayne D. Long-term follow-up of relapsing/refractory anti-neutrophil cytoplasm antibody associated vasculitis treated with the lymphocyte depleting antibody alemtuzumab (CAMPATH-1H). *Ann Rheum Dis*. 2008;67(9):1322-7.
112. Schmitt WH, Hagen EC, Neumann I, Nowack R, Flores-Suarez LF, van der Woude FJ, et al. Treatment of refractory Wegener's granulomatosis with antithymocyte globulin (ATG): an open study in 15 patients. *Kidney Int*. 2004;65(4):1440-8.
113. Bonelli M, Scheinecker C. How does abatacept really work in rheumatoid arthritis? *Curr Opin Rheumatol*. 2018;30(3):295-300.

114. Langford CA, Monach PA, Specks U, Seo P, Cuthbertson D, McAlear CA, et al. An open-label trial of abatacept (CTLA4-IG) in non-severe relapsing granulomatosis with polyangiitis (Wegener's). *Ann Rheum Dis*. 2014;73(7):1376-9.
115. Wierda WG, Kipps TJ, Mayer J, Stilgenbauer S, Williams CD, Hellmann A, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. *J Clin Oncol*. 2010;28(10):1749-55.
116. McAdoo SP, Bedi R, Tarzi R, Griffith M, Pusey CD, Cairns TD. Ofatumumab for B cell depletion therapy in ANCA-associated vasculitis: a single-centre case series. *Rheumatology (Oxford)*. 2016;55(8):1437-42.
117. Dorner T, Goldenberg DM. Targeting CD22 as a strategy for treating systemic autoimmune diseases. *Ther Clin Risk Manag*. 2007;3(5):953-9.
118. Wallace DJ, Gordon C, Strand V, Hobbs K, Petri M, Kalunian K, et al. Efficacy and safety of epratuzumab in patients with moderate/severe flaring systemic lupus erythematosus: results from two randomized, double-blind, placebo-controlled, multicentre studies (ALLEVIATE) and follow-up. *Rheumatology (Oxford)*. 2013;52(7):1313-22.
119. Novikov P, Moiseev S, Bulanov N, Shchegoleva E. Bortezomib in refractory ANCA-associated vasculitis: a new option? *Ann Rheum Dis*. 2016;75(1):e9.
120. Hiepe F, Radbruch A. Plasma cells as an innovative target in autoimmune disease with renal manifestations. *Nat Rev Nephrol*. 2016;12(4):232-40.
121. Huntington ND, Tomioka R, Clavarino C, Chow AM, Linares D, Mana P, et al. A BAFF antagonist suppresses experimental autoimmune encephalomyelitis by targeting cell-mediated and humoral immune responses. *Int Immunol*. 2006;18(10):1473-85.
122. Hartung HP, Kieseier BC. Atacicept: targeting B cells in multiple sclerosis. *Ther Adv Neurol Disord*. 2010;3(4):205-16.
123. Navarra SV, Guzman RM, Gallacher AE, Hall S, Levy RA, Jimenez RE, et al. Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial. *Lancet*. 2011;377(9767):721-31.
124. Furie R, Petri M, Zamani O, Cervera R, Wallace DJ, Tegzova D, et al. A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2011;63(12):3918-30.
125. Stohl W, Hiepe F, Latinis KM, Thomas M, Scheinberg MA, Clarke A, et al. Belimumab reduces autoantibodies, normalizes low complement levels, and reduces select B cell populations in patients with systemic lupus erythematosus. *Arthritis Rheum*. 2012;64(7):2328-37.
126. Jayne D, Blockmans D, Luqmani R, Moiseev S, Ji B, Green Y, et al. Efficacy and Safety of Belimumab and Azathioprine for Maintenance of Remission in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis: A Randomized Controlled Study. *Arthritis Rheumatol*. 2019;71(6):952-63.
127. Berger JR, Malik V, Lacey S, Brunetta P, Lehane PB. Progressive multifocal leukoencephalopathy in rituximab-treated rheumatic diseases: a rare event. *J Neurovirol*. 2018;24(3):323-31.
128. Berden AE, Ferrario F, Hagen EC, Jayne DR, Jennette JC, Joh K, et al. Histopathologic classification of ANCA-associated glomerulonephritis. *J Am Soc Nephrol*. 2010;21(10):1628-36.
129. Flossmann O, Berden A, de Groot K, Hagen C, Harper L, Heijl C, et al. Long-term patient survival in ANCA-associated vasculitis. *Ann Rheum Dis*. 2011;70(3):488-94.

130. Berti A, Cornec D, Crowson CS, Specks U, Matteson EL. The Epidemiology of Antineutrophil Cytoplasmic Autoantibody-Associated Vasculitis in Olmsted County, Minnesota: A Twenty-Year US Population-Based Study. *Arthritis Rheumatol.* 2017;69(12):2338-50.
131. Eriksson P, Jacobsson L, Lindell A, Nilsson JA, Skogh T. Improved outcome in Wegener's granulomatosis and microscopic polyangiitis? A retrospective analysis of 95 cases in two cohorts. *J Intern Med.* 2009;265(4):496-506.
132. Hilhorst M, Wilde B, van Paassen P, Winkens B, van Breda Vriesman P, Cohen Tervaert JW, et al. Improved outcome in anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis: a 30-year follow-up study. *Nephrol Dial Transplant.* 2013;28(2):373-9.
133. Rhee RL, Hogan SL, Poulton CJ, McGregor JA, Landis JR, Falk RJ, et al. Trends in Long-Term Outcomes Among Patients With Antineutrophil Cytoplasmic Antibody-Associated Vasculitis With Renal Disease. *Arthritis Rheumatol.* 2016;68(7):1711-20.
134. Stratta P, Marcuccio C, Campo A, Sandri L, Messuerott A, Colla L, et al. Improvement in relative survival of patients with vasculitis: study of 101 cases compared to the general population. *Int J Immunopathol Pharmacol.* 2008;21(3):631-42.
135. Flossmann O. Risks of treatments and long-term outcomes of systemic ANCA-associated vasculitis. *Presse Med.* 2015;44(6 Pt 2):e251-7.
136. Jennette JC. L17. What can we expect from the revised Chapel Hill consensus conference nomenclature of vasculitis? *Presse Med.* 2013;42(4 Pt 2):550-5.
137. Savage CO, Winearls CG, Evans DJ, Rees AJ, Lockwood CM. Microscopic polyarteritis: presentation, pathology and prognosis. *Q J Med.* 1985;56(220):467-83.
138. Holle JU, Gross WL, Latza U, Nolle B, Ambrosch P, Heller M, et al. Improved outcome in 445 patients with Wegener's granulomatosis in a German vasculitis center over four decades. *Arthritis Rheum.* 2011;63(1):257-66.
139. Takala JH, Kautiainen H, Leirisalo-Repo M. Survival of patients with Wegener's granulomatosis diagnosed in Finland in 1981-2000. *Scand J Rheumatol.* 2010;39(1):71-6.
140. Tanna A, Guarino L, Tam FW, Rodriguez-Cubillo B, Levy JB, Cairns TD, et al. Long-term outcome of anti-neutrophil cytoplasm antibody-associated glomerulonephritis: evaluation of the international histological classification and other prognostic factors. *Nephrol Dial Transplant.* 2015;30(7):1185-92.
141. Booth AD, Almond MK, Burns A, Ellis P, Gaskin G, Neild GH, et al. Outcome of ANCA-associated renal vasculitis: a 5-year retrospective study. *Am J Kidney Dis.* 2003;41(4):776-84.
142. Watts RA, Scott DG. Epidemiology of the vasculitides. *Curr Opin Rheumatol.* 2003;15(1):11-6.
143. Bajema IM, Hagen EC, Hermans J, Noel LH, Waldherr R, Ferrario F, et al. Kidney biopsy as a predictor for renal outcome in ANCA-associated necrotizing glomerulonephritis. *Kidney Int.* 1999;56(5):1751-8.
144. Vergunst CE, van Gulp E, Hagen EC, van Houwelingen HC, Hauer HA, Noel LH, et al. An index for renal outcome in ANCA-associated glomerulonephritis. *Am J Kidney Dis.* 2003;41(3):532-8.
145. Hauer HA, Bajema IM, van Houwelingen HC, Ferrario F, Noel LH, Waldherr R, et al. Renal histology in ANCA-associated vasculitis: differences between diagnostic and serologic subgroups. *Kidney Int.* 2002;61(1):80-9.
146. Bajema IM. Pathological classification of anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. *Clin Exp Immunol.* 2011;164 Suppl 1:14-6.

147. Berden AE, Jones RB, Erasmus DD, Walsh M, Noel LH, Ferrario F, et al. Tubular lesions predict renal outcome in antineutrophil cytoplasmic antibody-associated glomerulonephritis after rituximab therapy. *J Am Soc Nephrol*. 2012;23(2):313-21.
148. Working Group of the International Ig ANN, the Renal Pathology S, Roberts IS, Cook HT, Troyanov S, Alpers CE, et al. The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility. *Kidney Int*. 2009;76(5):546-56.
149. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, et al. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int*. 2004;65(2):521-30.
150. Iwakiri T, Fujimoto S, Kitagawa K, Furuichi K, Yamahana J, Matsuura Y, et al. Validation of a newly proposed histopathological classification in Japanese patients with anti-neutrophil cytoplasmic antibody-associated glomerulonephritis. *BMC Nephrol*. 2013;14:125.
151. Chang DY, Wu LH, Liu G, Chen M, Kallenberg CG, Zhao MH. Re-evaluation of the histopathologic classification of ANCA-associated glomerulonephritis: a study of 121 patients in a single center. *Nephrol Dial Transplant*. 2012;27(6):2343-9.
152. Hilhorst M, Wilde B, van Breda Vriesman P, van Paassen P, Cohen Tervaert JW, Limburg Renal R. Estimating renal survival using the ANCA-associated GN classification. *J Am Soc Nephrol*. 2013;24(9):1371-5.
153. Quintana LF, Perez NS, De Sousa E, Rodas LM, Griffiths MH, Sole M, et al. ANCA serotype and histopathological classification for the prediction of renal outcome in ANCA-associated glomerulonephritis. *Nephrol Dial Transplant*. 2014;29(9):1764-9.
154. Bjornekleit R, Sriskandarajah S, Bostad L. Prognostic Value of Histologic Classification of ANCA-Associated Glomerulonephritis. *Clin J Am Soc Nephrol*. 2016;11(12):2159-67.
155. Ford SL, Polkinghorne KR, Longano A, Dowling J, Dayan S, Kerr PG, et al. Histopathologic and clinical predictors of kidney outcomes in ANCA-associated vasculitis. *Am J Kidney Dis*. 2014;63(2):227-35.
156. Unlu M, Kiremitci S, Ensari A, Ozluk Y, Kilicaslan I, Ozdemir BH, et al. Pauci-immune necrotizing crescentic glomerulonephritis with crescentic and full moon extracapillary proliferation: clinico-pathologic correlation and follow-up study. *Pathol Res Pract*. 2013;209(2):75-82.
157. Chen YX, Xu J, Pan XX, Shen PY, Li X, Ren H, et al. Histopathological Classification and Renal Outcome in Patients with Antineutrophil Cytoplasmic Antibodies-associated Renal Vasculitis: A Study of 186 Patients and Metaanalysis. *J Rheumatol*. 2017;44(3):304-13.
158. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. *Arthritis Rheum*. 2013;65(1):1-11.
159. Hamour S, Salama AD, Pusey CD. Management of ANCA-associated vasculitis: Current trends and future prospects. *Ther Clin Risk Manag*. 2010;6:253-64.
160. Mansfield N, Hamour S, Habib AM, Tarzi R, Levy J, Griffith M, et al. Prolonged disease-free remission following rituximab and low-dose cyclophosphamide therapy for renal ANCA-associated vasculitis. *Nephrol Dial Transplant*. 2011;26(10):3280-6.
161. de Lind van Wijngaarden RA, Hauer HA, Wolterbeek R, Jayne DR, Gaskin G, Rasmussen N, et al. Clinical and histologic determinants of renal outcome in ANCA-associated vasculitis: A prospective analysis of 100 patients with severe renal involvement. *J Am Soc Nephrol*. 2006;17(8):2264-74.
162. Lionaki S, Blyth ER, Hogan SL, Hu Y, Senior BA, Jennette CE, et al. Classification of antineutrophil cytoplasmic autoantibody vasculitides: the role of antineutrophil cytoplasmic autoantibody specificity for myeloperoxidase or proteinase 3 in disease recognition and prognosis. *Arthritis Rheum*. 2012;64(10):3452-62.

163. Franssen CF, Stegeman CA, Kallenberg CG, Gans RO, De Jong PE, Hoorntje SJ, et al. Antiproteinase 3- and antimyeloperoxidase-associated vasculitis. *Kidney Int.* 2000;57(6):2195-206.
164. Jennette JC. Rapidly progressive crescentic glomerulonephritis. *Kidney Int.* 2003;63(3):1164-77.
165. Li ZY, Gou SJ, Chen M, Zhao MH. Predictors for outcomes in patients with severe ANCA-associated glomerulonephritis who were dialysis-dependent at presentation: a study of 89 cases in a single Chinese center. *Semin Arthritis Rheum.* 2013;42(5):515-21.
166. Lee T, Gasim A, Derebail VK, Chung Y, McGregor JG, Lionaki S, et al. Predictors of treatment outcomes in ANCA-associated vasculitis with severe kidney failure. *Clin J Am Soc Nephrol.* 2014;9(5):905-13.
167. Weidanz F, Day CJ, Hewins P, Savage CO, Harper L. Recurrences and infections during continuous immunosuppressive therapy after beginning dialysis in ANCA-associated vasculitis. *Am J Kidney Dis.* 2007;50(1):36-46.
168. Lionaki S, Hogan SL, Jennette CE, Hu Y, Hamra JB, Jennette JC, et al. The clinical course of ANCA small-vessel vasculitis on chronic dialysis. *Kidney Int.* 2009;76(6):644-51.
169. Geetha D, Hruskova Z, Segelmark M, Hogan J, Morgan MD, Caverio T, et al. Rituximab for treatment of severe renal disease in ANCA associated vasculitis. *J Nephrol.* 2016;29(2):195-201.
170. Pusey CD, Rees AJ, Evans DJ, Peters DK, Lockwood CM. Plasma exchange in focal necrotizing glomerulonephritis without anti-GBM antibodies. *Kidney Int.* 1991;40(4):757-63.
171. Gaskin G, Pusey CD. Plasmapheresis in antineutrophil cytoplasmic antibody-associated systemic vasculitis. *Ther Apher.* 2001;5(3):176-81.
172. Pepper RJ, Chanouzas D, Tarzi R, Little MA, Casian A, Walsh M, et al. Intravenous cyclophosphamide and plasmapheresis in dialysis-dependent ANCA-associated vasculitis. *Clin J Am Soc Nephrol.* 2013;8(2):219-24.
173. Westman K, Flossmann O, Gregorini G. The long-term outcomes of systemic vasculitis. *Nephrol Dial Transplant.* 2015;30 Suppl 1:i60-6.
174. Brix SR, Noriega M, Tennstedt P, Vettorazzi E, Busch M, Nitschke M, et al. Development and validation of a renal risk score in ANCA-associated glomerulonephritis. *Kidney Int.* 2018;94(6):1177-88.
175. Specks U, Fervenza FC, McDonald TJ, Hogan MC. Response of Wegener's granulomatosis to anti-CD20 chimeric monoclonal antibody therapy. *Arthritis Rheum.* 2001;44(12):2836-40.
176. Eriksson P. Nine patients with anti-neutrophil cytoplasmic antibody-positive vasculitis successfully treated with rituximab. *J Intern Med.* 2005;257(6):540-8.
177. Mauri C. Regulation of immunity and autoimmunity by B cells. *Curr Opin Immunol.* 2010;22(6):761-7.
178. Zhang J, Roschke V, Baker KP, Wang Z, Alarcon GS, Fessler BJ, et al. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J Immunol.* 2001;166(1):6-10.
179. Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, et al. The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjogren's syndrome. *Ann Rheum Dis.* 2003;62(2):168-71.
180. Koyama T, Tsukamoto H, Miyagi Y, Himeji D, Otsuka J, Miyagawa H, et al. Raised serum APRIL levels in patients with systemic lupus erythematosus. *Ann Rheum Dis.* 2005;64(7):1065-7.

181. Marsters SA, Yan M, Pitti RM, Haas PE, Dixit VM, Ashkenazi A. Interaction of the TNF homologues BLYS and APRIL with the TNF receptor homologues BCMA and TACI. *Curr Biol*. 2000;10(13):785-8.
182. Cohen SB. B-cell depletion for rheumatic diseases: where are we? *MedGenMed*. 2005;7(2):72.
183. Martins HL, Silva C, Martini D, Noronha IL. Detection of B lymphocytes (CD20+) in renal allograft biopsy specimens. *Transplant Proc*. 2007;39(2):432-4.
184. Neusser MA, Lindenmeyer MT, Edenhofer I, Gaiser S, Kretzler M, Regele H, et al. Intrarenal production of B-cell survival factors in human lupus nephritis. *Mod Pathol*. 2011;24(1):98-107.
185. Lenert A, Lenert P. Current and emerging treatment options for ANCA-associated vasculitis: potential role of belimumab and other BAFF/APRIL targeting agents. *Drug Des Devel Ther*. 2015;9:333-47.
186. Gan PY, Holdsworth SR, Kitching AR, Ooi JD. Myeloperoxidase (MPO)-specific CD4+ T cells contribute to MPO-anti-neutrophil cytoplasmic antibody (ANCA) associated glomerulonephritis. *Cell Immunol*. 2013;282(1):21-7.
187. Furie R, Rovin BH, Houssiau F, Malvar A, Teng YKO, Contreras G, et al. Two-Year, Randomized, Controlled Trial of Belimumab in Lupus Nephritis. *N Engl J Med*. 2020;383(12):1117-28.