Use of a novel therapy for the treatment and understanding of autoimmune membranous nephropathy

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Abstract

Background: Membranous nephropathy (MN) is amongst the most common causes of adult nephrotic syndrome worldwide. For the majority of patients, it is an autoimmune condition associated with anti-PLA_2R autoantibodies. Despite recent advances in our understanding of the condition, much remains unknown and treatment has not changed in two decades. Here we use immunoadsorption therapy to directly remove IgG antibodies from patients with active autoimmune MN to induce remission.

Methods: Using Peptide-GAM immunoadsorption we treated 12 patients with autoimmune MN for five consecutive days. Primary outcome was reduction of anti-PLA_2R antibodies at day 14. Secondary outcomes were, change in anti-PLA_2R level compared to baseline, uPCR, eGFR, EQ5D and adverse events at months 3, 6 and 12. Immune system modelling was carried out using flow cytometry to study cell populations of B cells, PLA_2R specific B cells, T cell and monocytes.

Results: At week 2 (day 14), median antibody level increased from a baseline of 679 U/mL (IQR 191-1070) to 902 U/mL (IQR 522-2665). Three patients have completed follow up with the first patient becoming antibody negative at week two and remaining negative at last follow up. Using flow cytometry, we demonstrated evidence for a new pathway in the pathogenesis of the disease in the role of natural T Regs and the potential involvement of IgM antibodies.

Conclusion: Immunoadsorption therapy to directly remove the pathogenic anti-PLA_2R antibody has potential efficacy in autoimmune MN and could avoid the need for toxic medications. We have also described for the first time important new components in the disease pathway to help further our understanding of the underlying mechanisms.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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For Tess and Morley

Contributions

During my PhD. I have made the following academic contributions:

Published papers

Hamilton P, Kanigicherla D, Hanumapura P, Walz L, Kramer D, Fischer M, Brenchley P, Mitra S. Peptide GAM immunoadsorption therapy in primary membranous nephropathy (PRISM): Phase II trial investigating the safety and feasibility of peptide GAM immunoadsorption in anti-PLA_2R positive primary membranous nephropathy. ***J Clin Apher.*** 2018 Jun;33(3):283-290.

I wrote the protocol and manuscript. All authors were involved with the development of the protocol from the beginning and reviewed the manuscript throughout its preparation

Hamilton P, Kanigicherla D, Venning M, Brenchley P, Meads D. Rituximab versus the modified Ponticelli regimen in the treatment of primary membranous nephropathy: A Health Economic Model. ***Nephrol Dial Transplant***. 2018 Mar 29. doi: 10.1093/ndt/gfy049

I conceived the study together with Dr Kanigicherla, Dr Venning and Prof Brenchley. Dr Meads and I designed the model and carried out the data analyses. I wrote the draft manuscript. All authors discussed the results and implications and reviewed the manuscript at all stages.

Submitted manuscript

Hamilton P, Wilson F, Chinnadurai R, Sinha S, Singh M, Ponnusamy A, Hall P, Dhaygude A, Kanigicherla D, Brenchley P. The investigative burden of Membranous Nephropathy in the UK. Submitted to Clinical Kidney Journal September 2018.

I conceived the study together with Dr Kanigicherla and Prof Brenchley. Dr Wilson, Dr Chinnadurai, Dr Singh and I collected the data. Dr Wilson and I performed the data analyses. I wrote the draft manuscript. All authors discussed the results and implications and reviewed the manuscript at all stages.

Abbreviations

ß$_1$-AAB	ß$_1$-adreno-receptor autoantibodies
ACEi	Anticholinesterase inhibitor
AE	Adverse events
AKI	Acute Kidney Injury
ANCA	Anti-neutrophil cytoplasmic antibodies
ARB	Angiotensin II receptor blocker
ASFA	American Society for Apheresis
BCM	Body cell mass
BIS	Multifrequency electrical bioimpedance spectroscopy
BIVA	Bioelectrical impedance vector analysis
BNF	British National Formulary
BP	Blood pressure
CEAC	Cost-effectiveness Acceptability Curve
CI	Confidence Interval
CKD	Chronic Kidney Disease
Cm	Cell membrane capacitance
COX	Cyclo-oxygenase
CRF	Manchester Clinical Research Facility
CRP	C-Reactive Protein
CTLD	C-type lectin domain
DMARDS	Disease-modifying anti-rheumatic drugs
DVT	Deep Vein Thrombosis
ECW	Extracellular water
EBV	Epstein–Barr virus
ECG	Electrocardiograph
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked ImmunoSorbent Assay

EM	Electron Microscopy
eMIT	Department of Health electronic market information tool
ERT	Enzyme replacement therapy
ESR	Erythrocyte sedimentation rate
ESRD	End-stage renal disease
EQ5D	EuroQoL EQ5D Index Score
EVPI	Expected Value of Perfect Information
FACS	Fluorescence-activated cell sorting analysis
FBC	Full Blood Count
FSGS	Focal Segmental Glomerulosclerosis
GN	Glomerulonephritis
GWAS	Genome Wide Association Study
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
HR	Hazard Ratio
HRA	Health Research Authority
IA	Immunoadsorption
ICER	Incremental Cost-effectiveness Ratio
ICW	Intracellular water
IF	Immunofluorescence
Ig	Immunoglobulin
IgG	Immunoglobulin subclass G
INMB	Incremental Net Monetary Benefit
IV	Intravenous
IVIG	Intravenous immunoglobulin
LFT	Liver Function Test
LQR	Lower Quartile Range
MHRA	Medicines and Healthcare products Regulatory Agency

MINT	Manchester Institute of Nephrology & Transplantation
MMF	Mycophenolate Mofetil
MN	Membranous Nephropathy
MPO	Myeloperoxidase
mPR	Modified Ponticelli regime
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
NSAIDs	Non-steroidal anti-inflammatory drugs
OH	Overhydration
PBS	Phosphate-buffered saline
PLA2R	Phospholipase A2 receptor
PR3	Proteinase 3
PROM	Patient Reported Outcomes
PSA	Probabilistic Sensitivity Analysis
QALY	Quality adjusted life year
RA	Rheumatoid Arthritis
RCT	Randomly Controlled Trial
Re	Extracellular resistance
Ri	Intracellular resistance
RRT	Renal Replacement Therapy
SAE	Serious adverse events
SD	Standard Deviation
SF36	Medical Outcomes Survey Short Form – 36
SLE	Systemic lupus nephritis
SOP	Standard Operating Procedure
SUSAR	Serious Unexpected Adverse Event

TBW	Total body water
THSD7A	Thrombospondin Type-1 Domain-Containing 7A
TPE	Therapeutic plasma exchange
uPCR	Urinary Protein:creatinine ratio
UK	United Kingdom
UQR	Upper Quartile Range
VAT	Value Added Tax
VTE	Venous Thromboembolism

Introduction

1.1 Background

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in adults worldwide but despite this remains a rare disease. Incidence is estimated at 1.2 per 100,000 in European cohorts with a peak incidence in the 5th and 6th decades although it can affect any age, and has a slight male preponderance[1]. The classical presentation of the disease is with nephrotic syndrome, i.e. the tetrad of leg swelling, proteinuria and serum hypoalbuminaemia, with or without hypercholesterolaemia. A number of patients have also been known to present with venous thrombosis. This can be in the form of a deep vein thrombosis (DVT) and, not uncommonly as the first presentation of the disease, with acute kidney injury (AKI) as a result of a renal vein thrombosis. Hypercoagulopathy as a result of the loss of anti-thrombotic factors such as anti-thrombin III and plasminogen due to proteinuria, an increased level of factor VIII and fibrinogen, along with an increased platelet hyperaggregability has been noted in nephrotic syndrome whatever the cause. However, compared to other conditions that have a similar degree of proteinuria, MN has a relatively higher risk of venous thrombosis and its associated risks; the mechanism for this association has not been ascertained[2-4]. Clinically there are two distinct forms of MN, but these are histologically very similar and difficult to differentiate. Both require very different treatment strategies and therefore distinguishing between them is imperative. Primary MN accounts for the majority of patients (approximately 75-80%) and has now been shown to be an autoimmune disease. Secondary MN is caused by a multitude of medications, disorders and toxins (table 1.1), and its treatment is therefore aimed at the underlying condition[5].

Causes	
Malignancy	Carcinomas *Lung, GI tract, renal, prostate, breast, colon, ovary* Others *Lymphomas, Leukaemia, melanoma, mesothelioma, Wilm's tumour, Hepatic adenoma, Angiolymphatic hyperplasia, Schwannoma, Neural blastoma, Adrenal, ganglioneuroma*
Infections	Hep B & C, HIV, Syphilis, Malaria, Schistosomiasis, Filariasis, Enterococcal endocarditis, Hydatid disease, Leprosy
Medications	Penicillamine, gold, NSAID & COX-2 inhibitors, lithium, captopril, Myozyme enzyme replacement therapy, bucillamine, probenecid, trimethadione, clopidogrel
Toxins	Mercury, hydrocarbons, formaldehyde, lead, cadmium, hydrocarbons
Autoimmune-associated	SLE, RA, Graves' disease, Hashimoto's thyroiditis, Sjögren's syndrome, mixed connective tissue disorder, ankylosing spondylitis, Dermatomyositis, Ankylosing Spondylitis, Systemic Sclerosis, Myasthenia Gravis, Bullous Pemphigoid, Temporal Arteritis, Crohn's disease.
Alloimmune-associated	Transplant glomerulopathy, de novo MN, graft vs host disease
Miscellaneous	Sarcoidosis, Sickle Cell Disease, Polycystic kidney disease, alpha-1 anti-trypsin deficiency, Weber-Christian disease, Primary biliary cirrhosis, Systemic mastocytosis, Guillain-Barre syndrome, Urticarial vasculitis, Haemolytic-uremic syndrome, Dermatitis herpetiformis, Myelodysplasia

Table 1.1 Causes of secondary membranous nephropathy (MN)[5]. GI – gastrointestinal, CLL - chronic lymphocytic leukaemia, NSAID - non-steroidal anti-inflammatory drug, COX - cyclo-oxygenase, SLE - systemic lupus erythematous, RA – rheumatoid arthritis

The prognosis for patients with MN represents a spectrum of severity with a third of patients going into remission and remaining well throughout their life, whereas a third of patients will progress to end-stage renal disease (ESRD) necessitating the need for renal replacement therapy in the form of dialysis or renal transplantation. The remaining third will have a slowly progressive decline in renal function over years but remain well. Whether this heterogeneity in outcomes relates to the factors discussed below, such as the genetic link, remains to be seen[6-8].

If patients do reach ESRD and receive a renal transplant, it has been well demonstrated that this can provide a dramatic improvement to not only life expectancy but also quality of life[9-11]. However, this comes with the risk of recurrence of MN following transplantation (up to 34% of patients) despite the judicial use of immunosuppression and can lead to the loss of the graft in up to 50% of these cases. There is some evidence to suggest that receiving a transplant from a living related donor increases the risk of recurrence, but this is far outweighed by the complications associated with remaining on dialysis[12]. Current practice therefore, is to attempt to match HLA antigens as closely as possible to reduce the reliance on immunosuppression to minimise rejection.

For most patients, MN remains a relapsing and remitting disease, requiring lifelong follow up under the care of specialists in tertiary care. Despite being a rare condition, its chronicity, current standard treatments and their associated side-effects, the risk of ESRD, and disease recurrence means it is a disease that has a significant impact on both a patient's quality of life and a healthcare system with finite resources.

1.2 Diagnosis

Recent advances in biomarker research for MN have shown promising results but at present diagnosis requires biopsy confirmation. Histologically the disease is characterised by thickening of the glomerular basement membrane and spikes on silver staining. Immunofluorescence almost universally shows coarse granular immunoglobulin IgG and complement C3 deposition on the capillary wall. The use of electron microscopy (EM) will show sub-epithelial immune complex deposition (figure 1.1). It has become apparent over the years that the dominant IgG subclass found histologically (and for antibodies to PLA2R as described below) in primary MN is IgG4[13-15]. This appears to differ from secondary MN where IgG1 predominates[16]. IgG makes up a significant proportion of serum protein in humans contributing approximately 10-20% of circulating proteins. It can be further subdivided into four subclasses with differing effects. IgG4 is the least abundant of these subclasses and is generally found in response to allergens or in response to repeated exposure to an antigen[17].

New research findings suggest that there may be a class switch involved in primary membranous nephropathy. Here it has been shown that in early MN (stage I of the Ehrenreich & Churg scale) the predominant subclass of antibody is IgG1 but as the disease progresses this changes so that IgG4 predominates[16].

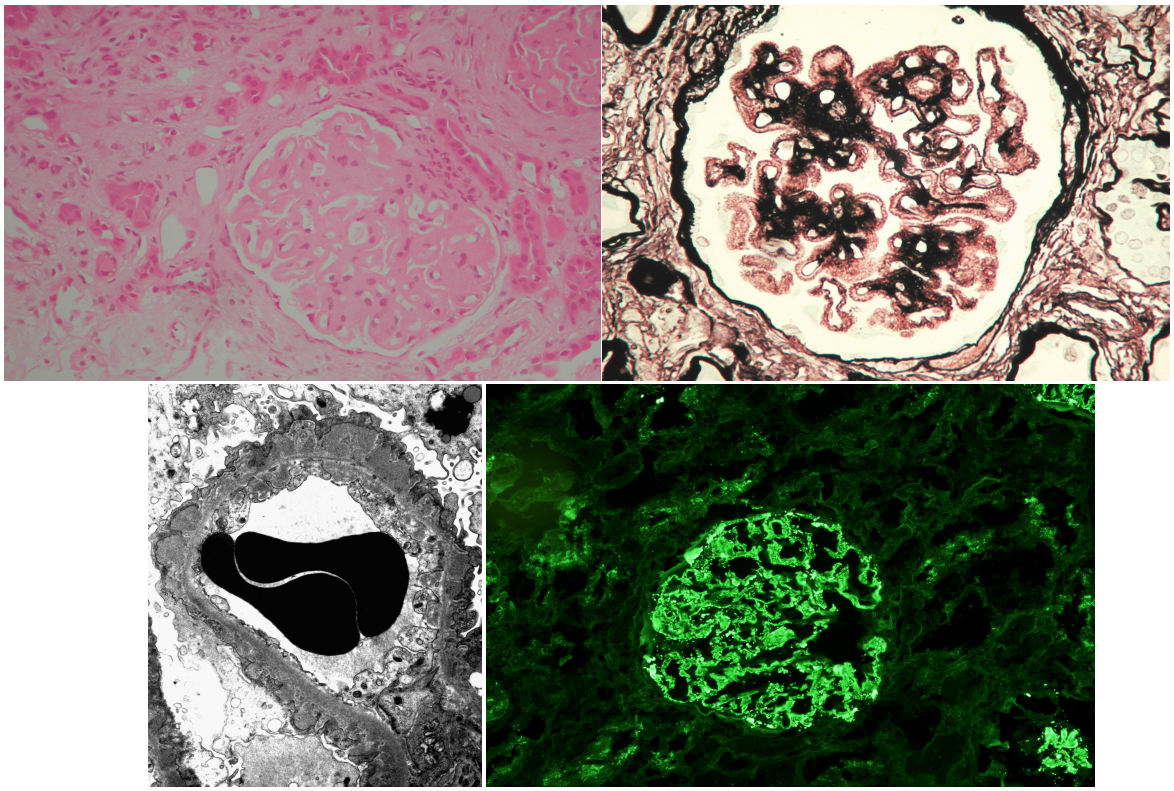

Figure 1.1 – Histological appearance of membranous nephropathy a) Haematoxylin and Eosin stain (H&E) showing marked capillary loop thickening b) Silver staining showing spikes c) Electron microscopy of MN showing sub-epithelial immune complex deposition d) Immunofluorescence showing IgG deposition on the capillary wall.
Figures courtesy of Dr Lorna Williams, Consultant Histopathologist,
Manchester University Hospitals Foundation Trust, UK

1.3 Prognosis

It is one of the idiosyncrasies of MN that up to a third of patients if left untreated will go into spontaneous remission within the first two years following diagnosis, and this potential for spontaneous remission has informed the current treatment options, especially for those patients without rapidly progressive renal decline[7]. The mainstay of treatment at present has a focus on the reduction of proteinuria with the use of an Angiotensin-converting-enzyme inhibitor (ACEi) or an Angiotensin receptor blocker (ARB), or immunosuppression if this fails[5]. It has also meant that for many studies, patients undergo six months of supportive care before they are eligible, in case any response to treatment seen is actually as a result of spontaneous remission. However, with the increasing use, understanding and monitoring of biomarkers such as anti-PLA_2R, treatments are likely to be less empiric in the future.

One of the difficulties presented to healthcare professionals and patients alike at the time of diagnosis is the variance and uncertainty in the disease prognosis. At present, it is currently not possible to accurately predict which patients will progress to ESRD and which patients will have a spontaneous remission. This is in part due to the lack of any specific widely available biomarker, although the use of proteinuria severity and renal dysfunction has been shown to at least provide some guidance on who may deteriorate[18-20].

An early attempt at developing a predictive model for primary MN showed that proteinuria at biopsy of over 3.5g/day was associated with renal decline. This work on the *Toronto Glomerulonephritis Registry* showed that an improvement in disease progression prediction could be attained with the use of persistent proteinuria as opposed to a single level at presentation. Here Pei *et al.* demonstrated that significantly raised proteinuria over a period of months was associated with a worse outcome. For patients with 8 g/day proteinuria for more than six months, were more than twice as likely as controls to develop

chronic renal impairment (defined as an eGFR of less than 60 ml/min/1.73m^2) with a positive predictive value and sensitivity of 66%. In contrast to this, patients with 8g/day of proteinuria per day but for less than six months only had a 12% chance of developing chronic renal impairment. However, use of the models developed by this group were only able to increase the probability of predicting if a patient will develop chronic renal insufficiency to 55-86% and this required the monitoring of proteinuria levels for a period of 9 to 18 months[19].

To reduce the time required to predict a patient's progression Cattran *et al.*, again using the *Toronto Glomerulonephritis Registry* but with validation from Italian and Finnish cohorts, developed a predictive model with an accuracy of 79-87%. This model used the maximum persistent proteinuria over a 6-month period and added in creatinine clearance at presentation and the rate of change in renal function over the six months. Again, as with the model described by Pei *et al.* this came with drawbacks. A patient would still have to be monitored for at least six months and potentially longer to include the period of maximum proteinuria[20]. With both of these methods of prediction, a patient with persistently high proteinuria who could be considered high risk is likely to miss out on early treatment and the benefits that this can confer[21].

The reverse of this is also true with patients presenting with proteinuria <4g/day, normal creatinine clearance and a renal function that remains stable over the 6-month period representing a group that has a good prognosis with only a low chance of progressive renal decline. Patients with normal creatinine clearance and stable renal function over the six month period but with proteinuria of between 4 and 8g per day, have a 55% chance of progressing to chronic renal impairment as defined above[20].

If severity of proteinuria on presentation does not accurately allow for prediction of disease progression, its reduction following this can give

reassurance that it is less likely. It is now known that even with an initially high level of proteinuria if a patient attains partial or complete remission, then their outcomes are generally very good[22,23]. This is true whether it is through treatment with immunosuppression, or through spontaneous improvement, and it is for this reason that reduction in proteinuria is the mainstay of treatment for the disease and the primary end-point in the majority of studies for primary MN.

1.4 Primary Membranous Nephropathy

Until recently primary MN was known as idiopathic MN as its cause remained unclear. It was generally a diagnosis of exclusion, once a patient had biopsy confirmation of MN and all causes of secondary MN had been ruled out. It was for a long time postulated to be an autoimmune disease, but the target antigen remained elusive. In the late 70s, work on the Heymann Nephritis rat model of experimental MN showed that circulating IgG antibodies could bind to the podocytes[24-26]. The target antigen was found to be megalin, but this was not present on human podocytes, so the search for the target antigen continued. It wasn't until 2009, almost 40 years later that this was discovered. Here Beck *et al.* used western blotting with MN patient sera, to show that antibodies bound to a 185kD protein band from glomerular extracts. This band was only seen in the primary MN group and not seen in normal patients or other proteinuric conditions including patients with secondary MN. Using mass spectrometry this band was found to contain the M-type phospholipase A2 receptor 1 (PLA2R)[15]. Since then, the increased interest and research into MN has led to the discovery of a second minor target antigen in Thrombospondin type-1 domain containing 7A (THSD7A)[27].

1.5 M-type phospholipase A2 receptor 1

The landmark paper by Beck *et al*. describing the discovery of autoantibodies to PLA2R found on human podocytes transformed our understanding of the MN disease process. Here was evidence that for the majority of patients with MN, the condition was, as had been postulated, an autoimmune disease[15].

PLA2R is a transmembrane receptor for Phospholipase A2, a protein from the mannose receptor family, one of four described in humans; Endo180, DEC205, Mannose Receptor (MR) and PLA2R[28-30]. As with all the mannose receptor family, the transmembrane glycoprotein has an extracellular component, in the case of PLA2R, this is made up of an N-terminal ricin rich domain, a fibronectin type II domain and 8 C-type lectin domains (CTLDs)[31]. In the kidney, it is found almost exclusively on the podocytes, but it has also been found on neutrophils and in the lung[32,33]. Its function in the kidney remains unknown, however, and how the anti-PLA_2R antibodies alter its normal function leading to proteinuria, if indeed that is what is part of the process, also remains unknown[15,34].

The predominant antibody to PLA2R is IgG and in particular IgG4, which is the major component of immune complex deposition in primary MN[13,14]. These immune complexes appear to form in the kidney with the IgG4 antibodies and the PLA2R antigen being co-localised, giving further evidence for the role of PLA2R in the disease process[35,36]. The fact that the complexes form in situ in the kidney may explain why some patients with biopsy-proven MN and clinical evidence for the disease are serum anti-PLA_2R negative. Debiec and Ronco showed in 2011 that there were a number of patients who were serum anti-PLA_2R negative but had detectable PLA2R in glomerular deposits. They did also find a few patients with no PLA2R in the glomerular deposits but who were serologically positive[37]. We know that anti-PLA_2R antibodies have a high affinity for PLA2R in the podocytes and it may be that a certain level of

deposition is required to overload the system before the anti-PLA_2R antibodies become serologically detectable[31].

Much of the excitement surrounding anti-PLA_2R is due to its apparent pathogenicity with the resultant potential for use as a biomarker and as a treatment target. Several studies have provided evidence for its pathogenicity showing that a high titre correlates with disease activity. For patients who go into remission either spontaneously or through the use of immunosuppression, the anti-PLA_2R level falls months before this becomes clinically apparent with a fall in proteinuria. If a patient relapses, this again is predated by a rise in antibody titres[38-43].

Outcomes can also be predicted with high titres predicting a worse outcome in regards to renal function and an improved outcome with low titres[38]. If treatment does not result in antibody negativity, then they are left with a high risk of relapse[34,40]. Ruggenenti *et al*. have shown similar results with a reduction in anti-PLA_2R levels strongly predicting remission and increasing titres following this, predicting relapse[42].

With the increasingly strong evidence for the involvement of anti-PLA_2R antibodies in the pathogenesis of primary MN, the focus has now shifted to trying to understand the antigen and its interaction with the autoantibody. Work carried out in Manchester has now determined the major epitope on the PLA2R antigen that is recognised by the anti-PLA_2R antibodies. Four different sized fragments of extracellular PLA2R (full-length N-C8, N-terminus to C-type lectin

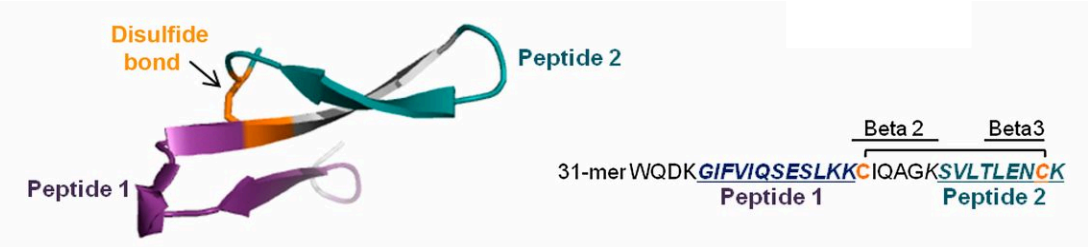

Figure 1.2 - 31mer peptide representing the major epitope on PLA2R for anti-PLA2R antibodies. Reprinted with kind permission from Fresquet *et al*. JASN 2015 Feb;26(2):302-13

domain (CTLD) 3 (N-C3), N-terminus to CTLD2 (N-C2) and a ricin rich domain) were used to investigate the reactivity of human anti-PLA_2R autoantibodies. It was found that the major epitope was located in the N-C3 region of the receptor. The antibodies were also found to bind with an equal affinity to the four different fragments, confirming the existence of a single epitope. The epitope itself is a 31-mer peptide made up of the beta-1 and beta-3 strands and encompassing the beta-2 strand (figure 1.2)[31].

Leading on from this the Manchester team also constructed a 3D model of the structure of the immune complex incorporating the extracellular N-C8 PLA2R and the autoantibody with the binding site (see figure 1.3)[31]. This work has been further confirmed by Kao *et al*. who found that the dominant epitope is in the N-terminal region as well, in particular in the region from the ricin rich domain through the fibronectin-like type to the CTLD1[44].

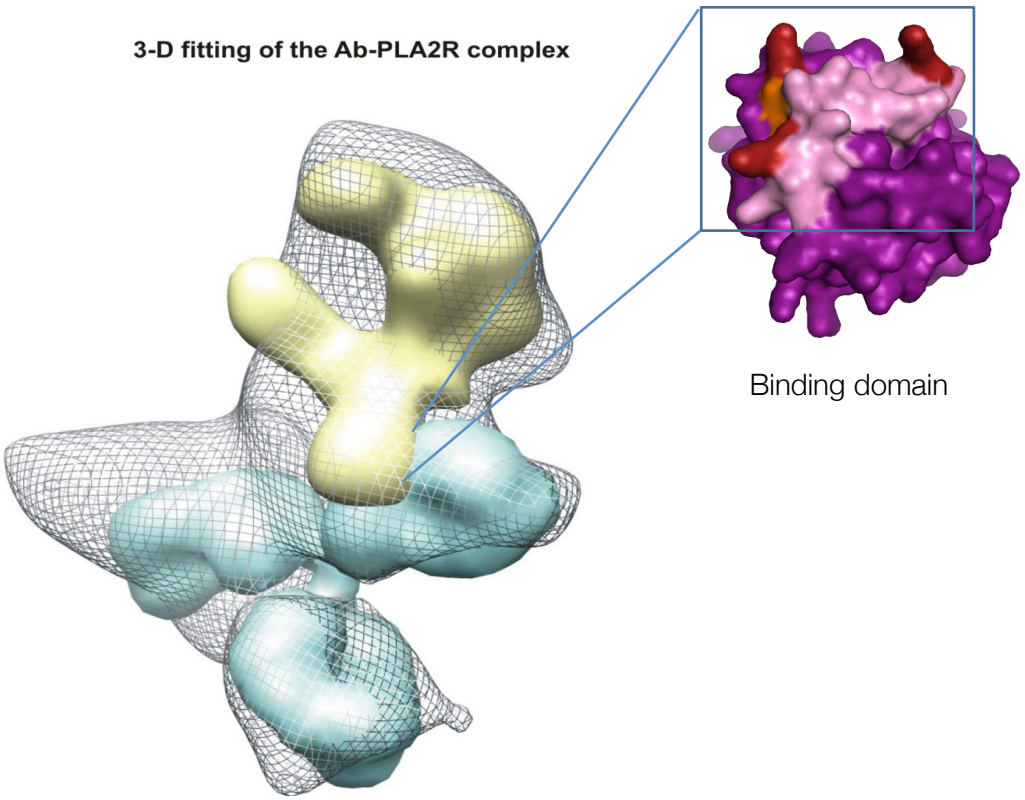

Figure 1.3 – 3D structure of PLA2R and the antibody immune complex with binding domain. Reprinted with kind permission from Fresquet M *et al*. JASN 2015 Feb;26(2)302-13

1.6 Thrombospondin Type-1 Domain-Containing 7A

The fact that anti-PLA_2R antibodies are found in approximately 70% of patients with primary MN raises a number of possibilities. It is known that some patients with secondary membranous can develop malignancies years after the diagnosis of MN and it may be that these patients fall into this category. Whether these patients represent a cohort who have two separate conditions and it is coincidental that one is known to cause the other is still up for debate. A second possibility is that there are more pathogenic antigens leading to the formation of autoantibodies than previously thought. In fact, for a small number of patients with primary MN, this seems to be the case.

Using western blotting, Thomas *et al.* discovered a glomerular protein of 250 kD in patients with anti-PLA_2R negative biopsy-proven membranous nephropathy. This corresponded to THSD7A, a protein found in the podocyte foot processes[27].

They went on to show that the predominant antibody to this antigen was IgG4 in keeping with a diagnosis of primary MN, and on histological staining, in a similar fashion to anti-PLA_2R, the immune complexes were co-localised with the antigen. Figure 1.4. Levels of the antibody were shown to correlate with disease activity, being higher in active disease and lower as the clinical manifestations of the disease improved. Interestingly, there appeared to be no statistical significance in the clinical presentation or demographics between the anti-PLA_2R positive and the anti-THSD7A positive patients, except for a slightly higher number of women in the anti-THSD7A group, although this is believed to be due to the small numbers involved.

This evidence suggests that for a minority of primary MN patients, approximately 2.5 - 5% in this study, a second unrelated and discrete antigen is involved with the pathogenesis of the disease[27]. Whether this all represents a separate disease and whether there are other minor antigens still to be

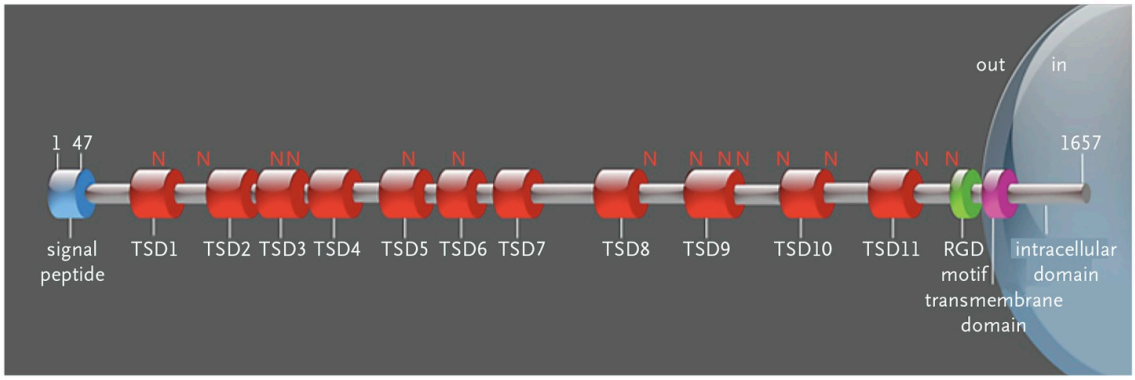

Figure 1.4 – Structure of THSD7A. Reproduced with permission from Tomas NM *et al.* N Engl J Med. 2014 Dec 11;371(24):2277-2287, Copyright Massachusetts Medical Society

discovered remains unknown as does the major epitope in THSD7A. However, for PLA2R, in addition to the major epitope in the CysR domain, evidence from the work of Fresquet *et al*. on the identification of the major epitope of PLA2R, showed that 10% of anti-PLA_2R positive sera reacted with an epitope at CTLD4-8. This suggests that there may be a further, as yet unidentified, antibody to this minor epitope[31]. This idea of epitope spreading has been suggested by Lambeau *et al.* who have defined additional epitopes in CTLD1 and CTLD7 domains[255].

1.7 Genetic association

Why some patients develop an autoantibody to PLA2R is still an unknown, but it does appear to have a strong genetic component. The first clue to the genetic basis of the disease was the discovery of the association with Human Leucocyte Antigen (HLA) - DR3 followed closely by the identification of familial clustering in 1984[45-47]. Following the discovery of the PLA2R antigen, researchers studying Korean and Chinese populations investigated the association of a number of single nucleotide polymorphisms (SNPs) known to be associated with PLA2R. They both found that a polymorphism at rs35771982 was significantly associated with primary MN. Interestingly, this polymorphism is located on CTLD1, in the region that was later found to contain an epitope in the antigen[31,48,49].

The major Genome-Wide Association Study (GWAS) in MN of 556 patients (French, Dutch and British) revealed two major loci of allelic association. The first is not unexpectedly on chromosome 6p21 within HLA-DQA1 gene, and the second is on chromosome 2q24 containing PLA2R1. For patients who were homozygous for these alleles, their odds ratio for having primary membranous nephropathy was 78.5[50]. This work has recently been validated in a study using genotype and HLA imputation alongside a GWAS in 323 patients with primary MN. Here the association of HLA-DQA1 and PLA2R1 with primary MN was confirmed, without detecting any other novel signals[51].

How these genetic markers modulate the risk of developing MN is unknown. The idea that the genetically restricted class II presentation of PLA2R peptides to affect the class switch to high-affinity IgG anti-PLA_2R is a theory to test.

1.8 The multi-hit hypothesis

Indicative of the rapid pace of research into primary MN since the discovery of the PLA2R antigen, we now have not only the clinical correlation of the antibody with disease activity but also the major epitope on the antigen and evidence for the genetic polymorphism located in the antigen itself. This, however, does not completely explain the development of the disease. The polymorphisms described in these studies are actually variants that are common to the general population. It seems likely that, similar to other autoimmune diseases such as IgA nephropathy, primary MN is a multi-hit disease. A patient with the polymorphism has a genetic predisposition but to develop the disease needs an external trigger.

There is now direct (unpublished) evidence that there exists a soluble form of PLA2R in normal healthy controls and the continued stability of this may stave off the development of the disease. Part of the peptide forming the epitope shares a sequence with a cell wall enzyme called D-alanyl-D alanine carboxypeptidase. This enzyme is common to a number of bacteria including Clostridia and raises the possibility that immune mimicry could lead to the loss of tolerance, therefore, starting the immunodysregulation seen in primary MN[28,31].

1.9 Treatment

In primary MN, disease activity is still measured by proteinuria level and renal excretory function despite the advances in anti-PLA_2R research. Proteinuria level has been shown to be not only a marker for remission when it is low but also predicts progression to ESRD when increased. If proteinuria reduces through either spontaneous remission or with treatment, then the risk of CKD progression also falls. It is for this reason that the main focus of treatment in primary MN is concerned with control of proteinuria, with or without the use of

immunosuppression, generally in the form of the Ponticelli regime (or calcineurin inhibitors if cyclophosphamide is not tolerated or is contraindicated). This regime of rotating high dose intravenous steroids and immunosuppression was first described in the mid-nineties and has been the recommended regime since[5,52-54]. Despite its success in treating the condition, the Ponticelli regime comes with a significant side effect burden, including an increased risk of infection, osteoporosis, diabetes mellitus, weight gain, haemorrhagic cystitis, infertility and malignancy[52]. It is this that has led many researchers to search for an alternative therapy including mycophenolate mofetil and tacrolimus but with little evidence to show an improvement in outcomes.

Rituximab is a monoclonal antibody against CD20, found on the B-cells, which leads to a reduction in B-cell numbers and has been used extensively in cancer therapy and autoimmune diseases since its introduction in the late 1990s. A number of case series and studies have shown the potential that Rituximab can have for primary MN, but so far there has only been one randomised controlled trial (RCT) [55-58]. Here it has been shown that compared to standard anti-proteinuric therapy, patients treated with rituximab show a greater reduction in anti-PLA_2R levels at month 3, followed by a later reduction in proteinuria, increase in serum albumin and are more likely to enter remission[58]. Combined with the high cost of the medication itself, its widespread use has been restricted in resource-limited, evidence-based healthcare systems, such as the NHS.

The use of many of these medications come with side effects that can be unpalatable to the patient and physician and the search for treatments with a reduced side-effect profile is ongoing. Treatments such as immunoadsorption (IA) allow for the controlled removal of antibodies without the side effects associated with immunosuppression. Immunoadsorption RCTs in MN though,

are non-existent and certainly not in the anti-PLA_2R era. Immunoadsorption is a method of removing specific circulating immunoglobulins from the blood and has been shown to remove over 80% of circulating IgG with a single session immunoadsorption of 2.5 plasma volumes, with albumin and antithrombin III almost unaffected[59]. With multiple sessions, this can rise to over 98%[60]. These are removed in the absorber through binding Peptide-GAM. Using two columns per machine, one regenerating whilst the other is removing antibodies; the process can occur indefinitely until the required level of antibody has been removed.

Post IA it appears that autoantibodies can be slow to re-emerge. Use in dilated cardiomyopathy for the removal of $ß_1$-adreno-receptor autoantibodies ($ß_1$-AAB) has shown that only a small minority of patients (0% in the first year and 15% by three years) will show an increase in significant $ß_1$-AAB autoantibodies[61,62].

To our knowledge, there has only been one publication using immunoadsorption for the treatment of MN[63]. In 1999 Esnault *et al.* successfully used IA for the treatment of various aetiologies of Nephrotic syndrome including four patients with MN[63]. Here they showed that not only is the procedure safe but that there was a significant improvement in proteinuria in all patients with membranous nephropathy (figure 1.5). The main side effect in this group of patients was headache, which resolved without sequelae. Since that time the treatment has been used in numerous other autoimmune conditions including Focal Segmental Glomerulosclerosis (FSGS)[64], systemic lupus nephritis (SLE)[65,66], ANCA-associated small vessel vasculitides[67,68], Anti-glomerular basement membrane antibody disease[69] and in renal transplantation[70-72].

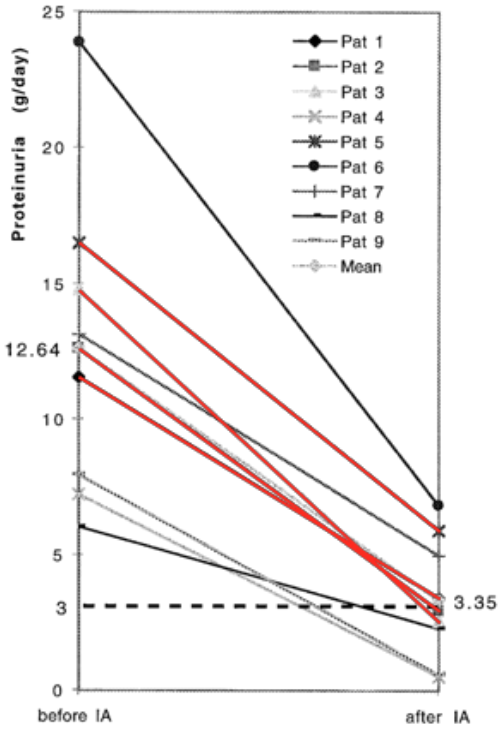

Figure 1.5 – Proteinuria response to immunoadsorption therapy in a range of nephrotic patients. Patients marked in red are membranous nephropathy patients. Reproduced with kind permission by Esnault *et al*. J Am Soc Nephrol. 1999 Sep;10(9):2014-7

In conditions such as SLE, the use of immunoadsorption can dramatically reduce the level of circulating immune complexes and autoantibodies leading to clinical improvement in even severe life-threatening SLE. These results have been shown with as little as two sessions within three days and repeated every three weeks if patients remain with active disease[65].

With the current understanding of primary MN's autoimmune process, the use of IA could provide the ability to rapidly remove the pathogenic antibodies leading to remission. Current IA machines can remove the different classes such as IgG4 with an increased specificity but cannot differentiate further than that. If IA is proved to work for primary MN, it may be possible to develop an IA column that is specific only for anti-PLA_2R, therefore allowing for an even more targeted and personalised treatment.

Hypothesis

The use of immunoadsorption therapy in autoimmune membranous nephropathy will result in a reduction of anti-PLA_2R autoantibodies leading to disease remission.

Aims

1 To investigate the safety and efficacy of immunoadsorption therapy in treating autoimmune membranous nephropathy

2 To study the kinetics of anti-PLA_2R antibodies in response to immunoadsorption therapy

3 To understand the pathogenesis of autoimmune membranous nephropathy using flow cytometry analysis

Immunoadsorption techniques and their current role in medical therapy

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2.1 Abstract

Immunoadsorption is an extracorporeal technique used for the removal of antibodies and molecules from the blood. A large number of different adsorbents are now available allowing for the non-selective removal of all subclasses of immunoglobulins such as IgG or more selective removal of disease-specific molecules such as lipoprotein(a) and CRP. This selectivity, coupled with its highly efficient removal of the molecule, along with a favourable side-effect profile, has made immunoadsorption an attractive option in a range of autoimmune diseases.

Here we discuss the mechanism and technique of immunoadsorption and review the current evidence and indications for its use.

2.2 Introduction

Immunoadsorption (IA) was developed in the 1990s as a method of extracorporeal removal of molecules from the blood, in particular molecules of the immune system. There are now a large number of devices/columns on the market, each with a different active component to which the molecule of interest attaches, allowing for selectivity in the molecules removed. This selectivity is one of immunoadsorption's significant advantages over other apheresis techniques, in that it negates the need for replacement of factors such as albumin and plasma. With the vast majority of IA systems directed against components of the immune system, its use has traditionally been in autoimmune conditions and transplantation, although new systems are increasingly being used for other indications such as sepsis. Figure 2.1.

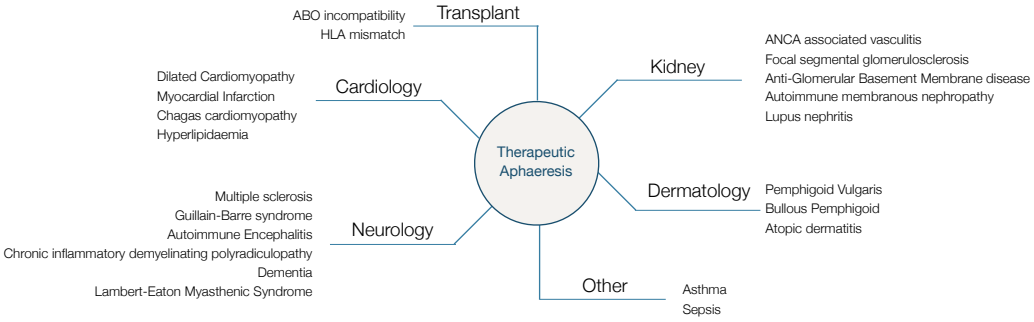

Figure 2.1 – Therapeutic apheresis and its indications

2.3 Procedure

Despite a large number of IA columns available the basic principle of the procedure is similar throughout. As with other extracorporeal therapies central venous access is required to ensure an adequate blood flow of approximately 100-150ml/min through the system. The system itself is a closed system using single-use tubing throughout, passing the blood from the central venous catheter to a plasma or cell separator, through the column, before combining with the blood components and back into the body via the central venous catheter. Figure 2.2.

The initial step in immunoadsorption is, therefore, the separation of plasma from the blood cells. Currently, there are a number of machines available for this; the Art Universal plasma separator (Fresenius Medical Care), Octo Nova plasma separator (Diamed Medizintechnik), COBE Spectra Apheresis system (Terumo), Plasmaflo OP plasma separator (Asahi Kasei Medical Co.) and the Comtec cell separator (Fresenius Medical Care).

The plasma then flows through to a second machine and into the immunoadsorption column. A number of machines are on the market for this stage of the procedure to monitor and regulate the plasma flow through the column; the Adsorption-Desorption-Automated system (ADAsorb, Medicap Clinic GmbH) being the most common dual column system in use today.

In dual column systems, the plasma passes through one column whilst the second column is being regenerated. Once the active column has been saturated, the plasma flow switches to the second column whilst the first column itself undergoes regeneration. This system allows for continuous treatment of the plasma with no theoretical upper limit on the number of plasma volumes that can be treated.

All columns share the same fundamental basics, with a matrix containing the molecule used to bind the required immunoglobulin. It is through this matrix

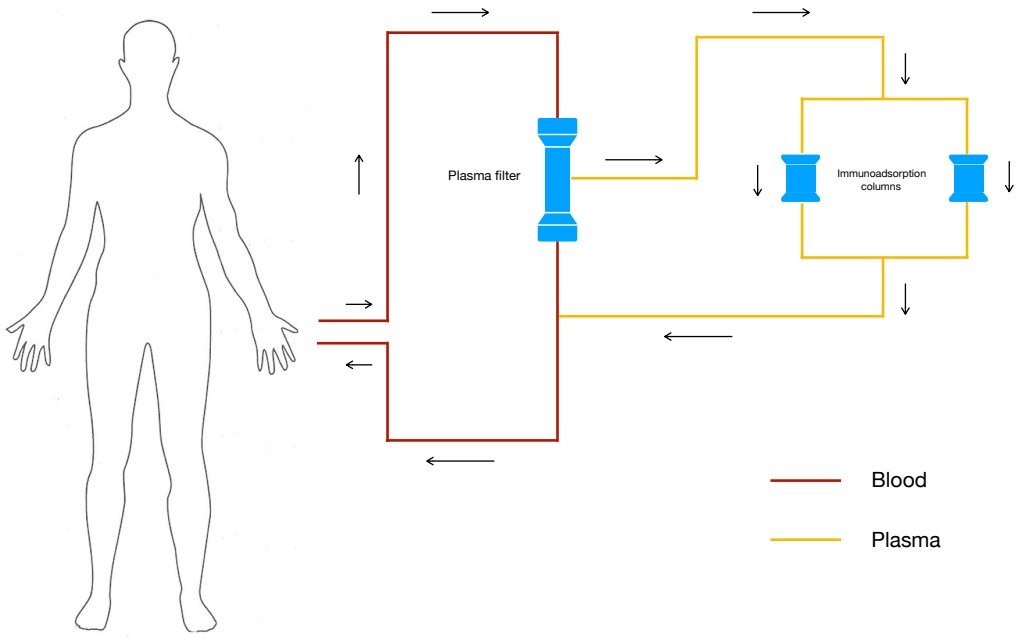

Figure 2.2 – Schematic of dual-column immunoadsorption. Blood first passes to plasma filter. Plasma then passes on to immunoadsorption column before returning to the patient. Schematic shown is a dual column system. As the plasma is passing through one column, the second column is being regenerated. Once the first column is saturated the flow switches to the second column whilst the first is then regenerated.

that the plasma flows with immunoglobulin binding as it passes. The binding molecule in each adsorber come from a number of different sources both synthetic and organic, and this heterogeneity adds to the versatility of the treatment. For example, Protein A is found in the cell wall of Staphylococcus Aureus and has been shown to bind immunoglobulins and in particular IgG with high affinity. It has the ability to bind all the subclasses of IgG, with minimal binding of other immunoglobulins[73]. The Globaffin adsorber, in contrast, uses a synthetic peptide (Peptide-GAM) to bind IgG with high affinity, and again, all subclasses[74]. Table 1.

Treatment prescriptions for immunoadsorption are based on plasma volumes with differing recommendations for each condition as discussed below. Depending on the condition being treated, sessions can be daily or intermittent, again discussed below for each indication. For most patients, plasma volume can be calculated using the Kaplan formula; estimated plasma volume = (0.065 x Weight(kg)) x (1 - Haematocrit) [75]. This formula, however, does assume a normal body mass index with decreasing accuracy for outliers. In these situations, particularly relevant in patients with nephrotic syndrome and morbid obesity, body composition monitoring may be of benefit to assess a patients normohydration/ideal body weight (IW). This can then be used in the Kaplan formula for a more accurate plasma volume:

Estimated plasma volume = (0.065 x IW(kg)) x (1 - Haematocrit).

All patients undergoing IA need anticoagulation. This usually takes the form of citrate sodium with IV calcium replacement. In our centre, we use 10ml 10% calcium gluconate for every 2L of plasma treated. Heparin can also be used as anticoagulation although generally in combination with sodium citrate and not as the sole agent.

Immunoadsorption type	Binding material	Available columns
Non-selective	Phenylalanine	Immunosorba PH
	Tryptophan	Immunosorba TR-350
	Dextran Sulphate	Selesorb
Semi-selective	Staphylococcal Protein A	Immunosorba
	Sheep anti-human Ig	Therasorb & Ig-Adsopak
	Peptide-GAM	Globaffin & Ligasorb
Selective	C1q	Miro
	ABO	Gylcosorb ABO & ABO Adsopak
	PDCM075 & PDCM349	Coraffin
	IgE	IgEnio
	CRP	PentraSorb CRP
	Cholesterol	DALI
	Lipoproteins and macromolecules	MONET
	LDL Cholesterol	Pocard LDL Lipopak
	Lipoprotein (a)	Pocard Lp (a) Lipopak
	Sepsis & septic shock	Pocard Toxipak

Table 1 Currently available Immunoadsorption columns

All columns are single patient use only. However, the number of times a column can be used differs from single-use, such as the Ligasorb (Fresenius Medical Care) up to 2 years for the Globaffin column (Fresenius Medical Care).

Due to the disposable single-use consumables and patient-specific columns along with the fact that there is no reliance on blood component replacement, the risk of blood-borne disease is minimal. However, there is still a theoretical risk of cross-infection, and pre-therapy screening for blood borne viruses is advisable.

Of note is the contraindication for the use of concomitant angiotensin-converting enzyme inhibitors (ACEi) with the use of columns using a native peptide such as tryptophan immunoadsorption[76]. This is due to the ACEi induced reduction of bradykinin metabolism following its release during IA. In columns using a synthetic peptide such as the Globaffin, this appears to be less of a concern and the use of ACEi is not contraindicated.

2.4 Immunoadsorption therapy prescription - example

Patient name	……
Date of Birth	……
Hospital Number	……
Primary disease for treatment	……
Dates of therapy	……
Frequency	Daily / weekly
Plasma volumes to treat	……
Weight	……kg
Plasma Volume (PV)	[Body weight (kg) x 0.065] x [1 – HCT] = …. L
Treatment volume	Plasma volumes to treat x PV = …… L
Flow rate	25ml / min (1.5L / hour)
Expected time	Treatment volume / 1.5L = …. hrs & …. mins
Anticoagulation	Citrate Sodium / Heparin Calcium infusion as per local guidelines
Name of prescriber	……
Signature of prescriber	……

2.5 Indications

2.5.1 Nephrology

2.5.1.1 Transplantation

As patients reach end-stage renal disease (ESRD) and require renal replacement therapy (RRT), dialysis can be a lifeline, but long-term outcomes remain poor. Renal transplantation can not only improve a patients' quality of life but also extend it beyond that of dialysis[9-11]. Traditionally renal transplantation matching has been based on a close Human Leukocyte Antigen (HLA) match and ABO compatibility. With an ever-increasing population reaching ESRD and necessitating RRT but with the continued donor kidney shortage, methods to allow for a relaxation of these matching criteria can greatly increase the uptake of renal transplantation[77].

ABO-incompatibility

Early attempts to use transplantation in the presence of ABO-incompatibility (ABOi) proved unsuccessful, and its use was contraindicated for many years due to the risk of hyperacute and acute allograft rejection[78-82]. The ABO blood group system was first described by Landsteiner in 1901[83]. Patients can have A, B, both or neither antigens on their erythrocytes along with antibodies to the antigens they don't possess. For example, patients with blood group A will have A antigens on their erythrocytes, and antibodies to B antigen (anti-B) in their plasma. Since the 1980s there has been an increased understanding of the underlying mechanisms of ABOi rejection. This rejection is triggered by the recognition by the recipient antibodies (anti-A or anti-B) of the corresponding A and/or B blood group antigen on the graft endothelium.

Earlier attempts at removing these antibodies to allow for ABOi transplantation involved intensive perioperative plasma exchange, splenectomy and judicious immunosuppression with resulting high mortality and morbidity but with little improvement in outcomes[78].

Given the anti-A/B blood group antigens are of the IgG, and IgM subclass, the use of immunoadsorption offers the ability to selectively remove these antibodies, and there is now strong evidence for its use with long-term follow up[78,84].

In 2001, Tyden *et al.* published a protocol utilising immunoadsorption and rituximab as an adjunct to standard triple therapy immunosuppression to significantly reduce the blood group antigens prior to transplantation. This regimen has now been used extensively, particularly in Europe, with excellent long-term outcomes, comparable to ABO-compatible transplantation[85-90].

HLA mismatch

In a similar manner to ABOi, recipient antibodies directed against donor HLA are a major cause of graft rejection[91,92]. Unfortunately, a large number of patients on the transplant waiting list will have these antibodies as a result of blood transfusions, pregnancy or previous transplants[93-96]. As with ABOi, the presence of these antibodies can reduce the chance of a patient receiving a transplant and increase time on the waiting list. Methods have therefore been sought to desensitise patients to improve their chances of a suitable match and to improve outcomes post-transplantation. Most strategies at present employ plasma exchange and IVIG with good results showing that the removal of these antibodies can confer a favourable outcome for the patient[97]. Given its more selective nature, IA offers an alternative to plasma exchange and has been used in a number of small studies with varying degrees of success.

In 1996, Higgins *et al.* used IA in 13 highly sensitised patients prior to transplantation. Three patients' grafts failed due to rejection, and six of the remaining ten patients had reversible episodes of rejection[70]. Since that time there have been a number of studies showing IA is a viable therapy for desensitisation prior to transplantation[98,99].

2.5.1.2 Autoimmune Membranous Nephropathy

Despite being a rare disease, autoimmune membranous nephropathy (MN) is among the most common causes of adult nephrotic syndrome worldwide[1,100-104]. In the majority of patients, it is associated with the M-Type Phospholipase 2 Receptor autoantibody (anti-PLA_2R), discovered in 2009[15]. Since that time there has been a tremendous increase in our understanding of the disease process although this has yet to translate into disease-specific therapies for the patient. At present, the current standard of care involves the use of a rotating regimen involving high dose steroids and cyclophosphamide over a six-month period, known as the Ponticelli regimen, and has been in use in various forms for almost 20 years[5,52,53]. This regimen was developed before the discovery of the anti-PLA_2R but with the belief that the condition was an autoimmune disease. It takes a blunderbuss approach to suppress the immune system with good clinical response but with a significant side-effect burden both in the short term and the long term.

The anti-PLA_2R antibody itself is an IgG antibody, and current evidence appears to suggest that it is a pathogenic antibody[15,38,41-43]. This makes it not only a useful biomarker for disease activity and response to treatment but potentially a target of treatment in itself.

Before the discovery of anti-PLA_2R, Esnault *et al.* use Protein A immunoadsorption on four patients with membranous nephropathy. All four

patients had an improvement in their proteinuria with minimal side-effects. However, the study only had a short follow up period of four weeks and no antibody data[63].

A clinical trial using the Fresenius Peptide GAM immunoadsorption column Globaffin has at the time of writing completed recruitment and treatment of 12 patients. The Globaffin column has a specificity for IgG antibodies of all subclasses and as such is expected to render the patients anti-PLA_2R negative. Follow up is ongoing but unpublished reports suggest that this is a promising new therapy for autoimmune membranous nephropathy with a drastically reduced side-effect burden when compared to the Ponticelli regimen[105].

2.5.1.3 Anti-Glomerular Basement Membrane disease

Anti-Glomerular basement membrane disease (anti-GBM), also known as Goodpasture's syndrome, is a rare, life-threatening autoimmune disease, typically presenting as rapidly progressive crescentic glomerulonephritis and lung haemorrhage. It is invariably fatal unless treated promptly with an intensive regime of immunomodulation with high dose steroids, immunosuppression and plasma exchange. With current treatment standards, mortality has improved although renal impairment remains a significant challenge[5,106]. Patients who are dialysis dependent on presentation unfortunately rarely recover renal function[5,107,108].

The disease is associated with the pathogenic anti-GBM autoantibodies which are directed against the glomerular basement membrane[109] and in particular the non-collagenous domain 1 (NC1) of α3 chain of type IV collagen. These antibodies are predominantly IgG, occasionally IgM, and can be readily detected in the circulation as well as being demonstrated along the glomerular

basement membrane on histology, a combined finding that is confirmation of the diagnosis[106].

Treatment strategies are aimed at the removal of the pathogenic antibody with oral prednisolone at the earliest clinical suspicion of the disease. Once a diagnosis has been confirmed, cyclophosphamide is started as is plasma exchange. Plasma exchange continues for 14 sessions or until the serum antibody is negative. If a patient goes into remission, unlike many other autoimmune diseases, patients rarely have a return of the antibody or relapse of the condition[5].

Given its superiority in removing antibodies compared to plasma exchange, immunoadsorption provides a promising alternative to the rapid reduction of the offending autoantibodies. Currently, there are no RCTs investigating the efficacy of IA versus standard of care, and for many years evidence was conflicting based on small case series from around the world using different adsorbers.

The first published treatment of Goodpasture's using IA was in 1985 by Bygren *et al.* using Protein A immunoadsorption resulting in a dramatic clinical improvement in a patient who had failed to respond to plasma exchange[110]. In four Chinese patients using Protein A IA, all saw a reduction in their antibody levels and resolution of their pulmonary haemorrhage. One patient managed to recover renal function to stop haemodialysis, but the three others remained dialysis dependent. All three of these had 100% crescent formation on biopsy[111]. However, two patients with dialysis-dependent anti-GBM disease treated with Protein A immunoadsorption by Esnault *et al.* showed no clinical improvement at all[112].

Two patients treated in Spain showed a reduction in the circulating antibody and improvement in respiratory symptoms but no renal improvement[113]. A Swedish study treating three patients with Goodpasture’s also showed no

clinical improvement using IA (Excorim, Sweden) although all patients were dialysis dependent on initiation of the treatment[114]. Two patients from Vienna were successfully treated using the TheraSorb adsorber, one of whom regained renal function despite presenting with 100% crescents on histology[115].

The largest series to date though, reveals some encouraging results. Biesenbach *et al.* treated ten consecutive patients using either the TheraSorb (Milteny Biotec, Germany) or the Immunosorba (Fresenius Medical Care, Germany), treating 2.5-3.0 PV per session. All patients had adjunctive prednisolone and cyclophosphamide. All ten patients were rendered anti-GBM antibody negative within nine sessions and with greater efficiency than demonstrated in PE. Two patients were initially treated with plasma exchange but switched to IA when the antibody failed to reduce. Clinical improvement was seen in both pulmonary haemorrhage and in renal impairment, with three of six patients who had initially presented with dialysis dependency managing to recover renal function. One patient died of fungal infection after the antibody had become negative, but otherwise, the safety profile was acceptable with no significant adverse events recorded[69].

2.5.1.4 Lupus Nephritis

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting multiple organs with up to 60% of patients having renal involvement (Lupus Nephritis) [116]. SLE is caused by a loss of immune tolerance leading to the production of autoantibodies, such as anti-double-stranded DNA (anti-dsDNA) autoantibodies, and the development of immune complexes[117-119]. The current standard of care is the use of intravenous cyclophosphamide therapy and is aimed at the inhibition of formation, and reduction of, these pathogenic antibodies[5].

There are now multiple case series, showing a favourable response to IA with a reduction in proteinuria and anti-dsDNA levels, and disease activity as characterised by the Systemic Lupus Activity Measure (SLAM) and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [65,66,120-127]. Many of these studies have treated patients with severe disease activity resistant to immunosuppression with very few side effects. As yet there are no RCTs investigating the use of IA versus immunosuppression alone or in combination. Despite this, the use of IA has shown promise as an alternative or adjunctive treatment in lupus nephritis in both the short and long term.

2.5.1.5 Focal Segmental Glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) is a histological diagnosis of a heterogeneous group of conditions. It is the most common cause of adult nephrotic syndrome in the US, and one of the most common causes worldwide and its incidence is rising[1,128]. It is separated into either primary or idiopathic FSGS or secondary FSGS. Secondary FSGS can be further subdivided into genetic, virus-associated, drug-induced or adaptive FSGS[129].

Given this heterogeneity, a sound pathogenic basis of the disease has been elusive. The initiation of the disease process undoubtedly follows a number of different routes, all with resultant podocyte injury. In primary FSGS an immunologic cause has long been suspected with a number of circulating factors now identified as potential candidates such as the IgG anti-CD40 autoantibody although further work is needed in this area[130].

Based on this supposition, the use of immunoadsorption both for primary disease and for recurrent disease post-transplant has been used with varying degrees of success[64,131].

Haas *et al.* used IA in five patients with native kidney disease and three patients with recurrent disease in their grafts. Six patients used protein-A IA (Immuno-adsorba, Excorim, Sweden) and two patients with an anti-IgG column (Ig-TheraSorb, Germany). Patients initially had five sessions within ten days at 2.5 plasma volumes per session. If proteinuria did not improve by more than 50% in this time they underwent another cycle. In four of the eight patients, proteinuria reduced by more than 50% although the mean time to relapse was only 21 days. Following relapse, patients had a further cycle of IA which did appear to provide a benefit with one patient having stable remission for 1.5 years and a second patient being stable for two years. However, of the two others who had initially responded, one became resistant to treatment, and the other lost his graft after three months[64].

LDL-apheresis has also shown some promise with reports from Japan suggesting it may have a role in not only reducing cholesterol, triglycerides and low-density lipoprotein but also proteinuria and an improvement in renal function[132-134]. This has led the ASFA to classify FSGS as a category III condition with grade 2C evidence (Optimum role of apheresis therapy is not yet established based on weak evidence, and decision making should be individualised) [135].

2.5.1.6 ANCA associated vasculitis

Antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is an autoimmune disorder affecting small vessels. It can involve any organ although has a predilection for the upper airways, lungs and kidneys. It is a chronic relapsing-remitting disease following the general pattern of many autoimmune diseases with a genetic component, environmental or infective

trigger and the formation of autoantibodies resulting in an immune cascade and subsequent injury[136,137].

Prior to the introduction of steroids and immunosuppression, the disease was invariably fatal[138]. Nowadays the vast majority of patients will survive, but given the judicious amounts of steroids and immunosuppression required for remission, many patients will have iatrogenic complications of the treatment itself[139-143].

The disease is associated with the formation of autoantibodies to either myeloperoxidase (MPO) or proteinase 3 (PR3) found on the granules of neutrophils and the lysosomes of monocytes in 90% of patients. As well as being a biomarker for the disease, there is evidence to suggest that it has at least some pathogenic features, particularly in animal models of the disease[136]. Along with this and the fact that it is an IgG antibody[144], a number of groups have investigated the use of immunoadsorption in the treatment of AAV. There does appear to be effective removal of the antibodies; however numbers in these studies are limited, there is concomitant use of immunosuppression and the results inconsistent[67,68,112,114].

2.5.2 Cardiology

2.5.2.1 Hyperlipidaemia

Familial Hypercholesterolaemia (FH) is an autosomal dominant genetic defect resulting in raised serum cholesterol and an increased risk of cardiovascular disease. Patients can present as either homozygous or heterozygous FH, with homozygous patients exhibiting a more severe phenotype. If left untreated patients with FH have a significantly increased in the risk of cardiovascular disease. The majority of patients exhibit a mutation in the LDL receptor, although mutations in the Apo B and proprotein convertase subtilisin/kexin type 9 genes have also been detected[145-147].

Initially patients should be treated with lifestyle changes and aggressive statin therapy; however, in many patients, this will not suffice. In the United Kingdom, the National Institute for Health and Care Excellence (NICE) suggests considering the use of IA for adults and young patients with homozygous familial hypercholesterolaemia (FH) and in heterozygous FH progressive, symptomatic coronary heart disease despite maximal medical therapy. This is generally on a weekly or biweekly regimen, and given the frequency, an arterio-vascular fistula is recommended[148].

In the United States (US), LDL-apheresis is approved for use by the Food and Drug Administration (FDA) in patients who have not responded to treatment after six months. In homozygous FH non-response is defined as patients with an LDL cholesterol of above 300mg/dL or a non-HDL-cholesterol level of above 330mg/dL. In heterozygous FH, non-response is defined as HDL-cholesterol above 300mg/dL and 0-1 risk factors. In patients with established coronary heart disease, cardiovascular disease or diabetes, an HDL-cholesterol level of above 160mg/dL is used[149].

Lipoprotein(a) is a plasma protein consisting of a low-density lipoprotein (LDL) covalently bonded to an apolipoprotein(a) molecule. Elevated lipoprotein(a) levels have consistently been reported as an association for increased risk of cardiovascular disease although much of this has been a causal link. However, given the weight of evidence for its involvement in cardiovascular disease, the European Atherosclerosis Society Consensus Panel on the treatment of lipoprotein(a) recommends treatment to ensure the serum level is below 50mg/dL[150]. Therapeutic agents are limited with the standard therapy being niacin, alone or in combination with statins, with little impact from lifestyle changes. In patients unresponsive to or intolerant of pharmacological solutions immunoadsorption provides an alternative therapy. European Atherosclerosis Society Consensus Panel also suggests considering IA therapy in young or middle-aged patients with progressive coronary disease and significantly raised plasma lipoprotein(a) levels[150]. In the US, apheresis is approved for use by the FDA in heterozygous FH patients unresponsive to medical therapy after six months with an LDL-cholesterol level of above 200 mg/dL and lipoprotein(a) above 50 mg/dL[149].

Homozygous FH is a category I condition whilst heterozygous FH is a category II condition with both having a grade 1A recommendation as per the American Society for Apheresis (ASFA) guidelines on the use of therapeutic apheresis in clinical practice. Lipoprotein(a) hyperproteinaemia is a category II condition with a 1B grade recommendation. A category I condition is a disorder in which apheresis is the accepted first-line therapy, and a category II condition is one in which apheresis is the accepted second-line therapy, as a stand-alone modality or as an adjunct to other treatment. A grade 1A recommendation is defined as a strong recommendation based on high-quality evidence and applicable to most patients without reservation. A grade 1B recommendation is a strong

recommendation based on moderate quality evidence and can be applied to the majority of patients in most circumstances[135].

2.5.2.2 Dilated Cardiomyopathy

Dilated cardiomyopathy (DCM) is a progressive disease that is a major cause of heart failure worldwide with high mortality and morbidity. Despite treatment, it remains one of the main precipitants to heart transplants in adults[151,152]. In most patients the cause is unknown, but for a significant proportion, it is an autoimmune disease. After years of speculation that there was an autoimmune component to condition, a number of autoantibodies have now been discovered. Evidence now suggests that these autoantibodies, particularly ß1-adreno-receptor autoantibodies (ß1-AAB), are pathogenic in nature[153,154]. Given they are generally of the IgG class, removal of the antibody is particularly amenable to IA, and it has now been used successfully in DCM for over two decades with a significant body of evidence supporting its use. The first reported case series from 1996 used the Ig-TheraSorb (Baxter, Germany) column to treat eight patients with severe DCM and NYHA class II-IV[155].

Since that time a number of studies have reported on the benefits of IA in DCM both short and long term, with a reduction in circulating antibodies and with clinical improvement[61,62,156-161].

Dorfell *et al*. treated nine patients with NYHA class III or IV and ejection fraction < 25%, on five consecutive days with the Ig-TheraSorb (Baxter). Here there was a marked reduction in circulating antibody level and an improvement in the patients' dyspnoea. There was no improvement in LVEF in this study although this is likely due to the very short follow up[162]. A longer prospective case-control study with a one year follow up expanded on this earlier work. Here 34 patients with an NYHA class II or above significant LV dysfunction and

considered candidates for heart transplantation were enrolled. 17 patients received standard medical treatment whilst 17 received adjunctive IA for five consecutive days. ß1-AAB levels had a highly significant mean reduction of 93.2% at month three with no significant increase within the one year follow up. Antibody levels remained unchanged in the control group[61].

At one-year follow up there was also a marked improvement in the cardiac performance of patients in the control group with a significant increase in their LVEF and a reduction in the left ventricular internal diameter in diastole (LVIDd). At five years post-IA there was also a statistically significant improvement in survival for those patients in the treatment group compared to the control group [61].

Long-term data also suggests that the antibodies are slow to reappear. In a study of 108 patients, only 16 (14.6%) had detectable antibodies three years post-IA, and a further 9 (8.3%) had detectable antibodies after three years post-IA. In the majority of these patients (76%), the reappearance of the antibody correlated with a deterioration in their clinical symptoms. With this continued antibody remission there continues to be long-term clinical improvement. Some studies show a mortality rate similar to post-transplantation, although with a lower LVEF[62,154,156].

Many of these studies have utilised replacement intravenous immunoglobulins at the end of the IA treatment. There has been some suggestion that much of the benefits seen are due to this although there does appear to be a clinical and biochemical improvement without IVIG replacement[163].

IA use in dilated cardiomyopathy has a level II category and 1B grade recommendation as per the American Society for Apheresis (ASFA) guidelines on the use of therapeutic apheresis in clinical practice. A level II category is defined as a disorder in which apheresis is the accepted second-line therapy or first line in conjunction with other treatments. A grade 1B recommendation is

defined as a strong recommendation with moderate quality of evidence and can be applied to the majority of patients without reservation[135].

2.5.2.3 Myocardial infarction

Despite ever-increasing survival following acute MI, post-MI morbidity continues to present patients with a modest prognosis. Interest in the inflammatory response following an MI has gained traction in recent years and in particular the role C-reactive protein (CRP) plays in ongoing myocardial damage. Along with this, elevated CRP is a poor risk factor for all-cause mortality, major adverse cardiac events and recurrent MIs[164,165]. Experimental animal models have shown that inhibition of CRP following induced MI results in a smaller infarct area although this therapeutic molecule is still in early development and not yet humanised[166,167]. Immunoadsorption now offers the ability to remove CRP with specific adsorbers in early animal models suggesting a benefit. In a study of 10 pigs (5 receiving IA and five controls) with induced MI, those pigs who underwent IA had a reduction in the post-MI infarct size and preservation of their cardiac output as measured by LVEF[168]. Given these promising results, a clinical trial is now underway in Germany to investigate the benefit of using CRP-specific immunoadsorption in acute ST-elevation MI (STEMI). Unpublished interim analysis suggests that the therapy is safe and well tolerated post-STEMI with promising results on infarct size in relation to CRP reduction. The results of this study have the potential to change management following an MI and subsequent PCI with an improvement in patient morbidity and mortality long-term.

2.5.2.4 Chagas cardiomyopathy

Chagas disease, caused by Trypanosoma cruzi (*T. cruzi*), affects approximately 10 million people per year, predominantly in South America where it is endemic. Of those affected, many have no long-term sequelae, but up to 40% can develop Chagas Cardiomyopathy with arrhythmias, heart failure and an increased mortality[169,170]. The vast majority of patients with Chagas cardiomyopathy are known to possess IgG autoantibodies suggesting an autoimmune component to the disease with the potential to respond to IA therapy. A clinical trial is currently underway to investigate this.

2.5.3 Neurology

2.5.3.1 Multiple Sclerosis

Multiple sclerosis (MS) is the most common chronic inflammatory condition of the central nervous system (CNS) worldwide. It is characterised by demyelination of differing parts of the CNS (space) with different lesions appearing over time. A majority of patients present with visual loss due to optic neuritis although depending on where the lesion is can also present with symptoms such as limb weakness, sensory loss, ataxia or cognitive impairment[171-173]. It is estimated to affect 50-300 per 100,000 with approximately 2 million people diagnosed worldwide. It is generally a disease of early adulthood and given the impact on mobility and quality of life the disease confers, it represents a significant healthcare burden[174]. There are currently four recognised phenotypes of the condition. Many patients present with a single episode that resolves over time, known as a clinically isolated syndrome. Patients who then go on to have further episodes (relapses) are described as having remitting-relapsing MS. Approximately 15% of patients will present with a progressive disease course from onset known as primary progressive MS. The fourth category is the development over time of secondary progressive MS in a proportion of patients with relapsing-remitting MS. The pathogenesis of MS is still not clearly defined although genetic, lifestyle and autoimmune factors are all understood to play a role in the disease[172,173,175].

There are now a large number of approved disease-modifying medications for the treatment of MS with apheresis reserved for non-responders. In many national guidelines for the treatment of MS, TPE is considered a second-line therapy for steroid-resistant relapsing-remitting MS[176]. The American Society for Apheresis (ASFA) gives TPE for MS a category II (Disorders for which

apheresis is accepted as second-line therapy, either as a standalone treatment or in conjunction with other modes of treatment) based on grade 1B evidence (Strong recommendation, moderate quality evidence)[135]. As early as 1989, IA has been shown to be as effective as TPE in the treatment of MS with an ever-growing body of evidence to support its role[177-182]. However, given the lack of RCTs, there has been limited uptake of the therapy. This has led relapsing-remitting MS to be an indication for IA by the ASFA although the lack of RCTs has resulted in it being designated a category III disease with Grade 2C evidence (Optimum role of apheresis therapy is not established. Decision making should be individualised. Weak recommendation with low-quality or very low-quality evidence)[135].

2.5.3.2 Guillain-Barré Syndrome

Guillain-Barré syndrome (GBS) is one of the most common causes of acute polyneuropathy worldwide with an incidence of approximately 1 per 100,000. It is considered an autoimmune disease generally found in association with a preceding infection, initiating an immune cascade that results in an inflammatory demyelinating polyneuropathy or acute motor axonal neuropathy[183]. TPE has been used for a number of years with robust evidence. The ASFA have designated GBS a category I condition with grade 1A evidence (Disorders for which apheresis is accepted as first-line therapy, either as a primary standalone treatment or in conjunction with other modes of treatment. Strong recommendation, high-quality evidence)[135]. This has inevitably led researchers to consider IA in GBS.

Evidence for IA suggests that it is a treatment that should be considered as a viable alternative to TPE. Most published studies comparing IA to the standard of therapy, be it TPE, double filtration plasma exchange or IVIG have shown

that not only is safety comparable or better, but also efficacy is as comparable. This has led a number of researchers to suggest, given its safety record, that it should be considered instead of TPE as a first line treatment[184-187].

2.5.3.3 Autoimmune encephalitis

Autoimmune encephalitis is an acute neurological inflammatory condition now known to be caused by a variety of antibodies. Treatment therefore generally takes the form of immunomodulation using steroids, IVIG and TPE. As yet there are no randomised controlled trials investigating the efficacy of IA in autoimmune encephalitis and only retrospective trials.

Onugoren *et al.* treated 14 patients with autoimmune encephalitis caused by leucine-rich glioma inactivated 1 (LGI1), contactin-associated protein-2 (CASPR2), N-methyl-D-aspartate receptor (NMDAR), and intracellular glutamic acid decarboxylase (GAD) antibodies using either tryptophan and protein A adsorbers. Directly after follow up, nine patients (64%) had improved their Modified Rankin Scale (mRS) score by one or more point, and five (35%) became seizure free. At late follow-up, several months after IA therapy, 12 (86%) patients had improved mRS scores[188].

Köhler *et al.* treated 13 patients with antibodies to NMDAR, GAD, Lgl1 and γamino-butyric-acid (GABA) using tryptophan IA. 11 patients (85%) were noted to have a clinical improvement following IA with a good side effect profile[189].

In a prospective observational case-control study treating ten patients with tryptophan IA and eleven with TPE. 60% of patients in the IA group compared to 67% in the TPE showed a clinical improvement with a reduction of their mRS score of 1 or more points. There were more adverse events in the TPE group (3 in the TPE group and 0 in the IA group)[190].

A recent review analysed the published studies comparing IA (25 patients in total) to TPE therapy (57 patients), used alone or in combination with steroids. Here they found that 88% of patients improved following IA treatment with 77% of patients improving with TPE treatment. The effect seemed to be more pronounced for antibodies against the neuronal cell surface compared to intracellular antigens. It was also found to be the safer option with fewer side-effects[191].

Despite the lack of RCTs, the evidence for IA in autoimmune encephalitis is encouraging and would suggest that it should be considered as a therapy.

2.5.3.4 Chronic inflammatory demyelinating polyradiculoneuropathy

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is among the most common chronic neuropathies worldwide. Although the exact pathogenesis remains unknown, it is considered an autoimmune disorder directed against, and causing demyelination of, the myelin sheath. This results in progressive or relapsing distal and peripheral weakness. The condition has a multitude of phenotypes, and with this heterogeneity, many consider it a spectrum of disease, as opposed to a single disease[192]. Current treatment aims at immunomodulation with IVIG and steroids the first line therapy with consideration of TPE in non-responders. ASFA guidelines consider CIDP as a category I disorder for treatment with TPE (Disorders for which apheresis is accepted as first-line therapy, either as a primary standalone treatment or in conjunction with other modes of treatment) with grade 1B evidence (Strong recommendation, moderate quality evidence) [135]. Given the efficacy of TPE, a number of studies have investigated the use of IA in CIDP.

Galldiks *et al.* treated ten patients with CIDP unresponsive to standard therapy using a tryptophan-linked polyvinyl alcohol adsorber. Response as measured

by the inflammatory neuropathy cause and treatment disability (INCAT) score and improvements in strength, sensation and performance of activities of daily living. Improvements in the INCAT was seen in all but one of the patients. Four of the patients received long-term IA in an outpatient setting with clinical improvement. In three of these four patients, they had previously been treated with TPE and noted no clinical decline on switching to IA[193].

Zinman *et al.* conducted a randomised, single-blinded study investigating the efficacy of protein A immunoadsorption versus IVIG. Here they treated nine patients with high dose IVIG, four with low IVIG and five with IA. One patient in the high dose IVIG withdrew consent prior to treatment, and two patients in the low dose IVIG group died of illness not thought to be related to treatment. Six-month data was not available for one patient in the IA group and two in the IVIG arm. Two months following treatment, four patients (80%) in the IA group were considered responders compared to four out of eight (50%) in the IVIG arm. At six months, all four of the patients in the IA group were considered responders compared with three out of six in the IVIG group (100% versus 50%)[194].

More recently a prospective RCT investigating the efficacy and safety of IA versus TPE, again using the tryptophan-linked polyvinylalcohol adsorber. There were nine patients in each group with no significant differences in baseline characteristics. Clinical improvement was assessed using the INCAT score and the Medical Research Council (MRC) sum score. It was found that four patients (44.4%) in the TPE group responded to treatment compared to six patients (66.7%) in the IA group. In the IA group, 100% of the patients had an improvement in their MRC sum scores and four patients out of six patients (66.7%)[195].

Despite these small numbers, IA has shown promising results, especially when considering the majority of the patients included in the studies were patients

who had already failed standard therapy. The safety profile was comparable to TPE and IVIG, and albeit with limited study populations, appeared to be as, if not more, efficacious than the current standard therapy.

2.5.3.5 Dementia

Dementia represents an increasing problem for healthcare systems worldwide, exacerbated by an ageing problem. The most common form, Alzheimer's Disease, is characterised by the deposition of ß-amyloid plaques and neurofibrillary tangles. The exact cause of the disease remains unknown, and given the heterogeneous nature of the condition, it is likely to be multifactorial. There can also be some overlap in patients with both Alzheimer's disease and Vascular dementia, a disease resulting from damage to the vasculature of the brain. Research has suggested there can be an autoimmune component to some dementia patients with the discovery of autoantibodies against the $ß_1$-adrenergic receptor ($ß_1$-AR) and the $ß_2$-adrenergic receptor ($ß_2$-AR) present in up to 59% of dementia patients[196,197].

Hempel *et al.* treated eight patients with immunoadsorption; all patients were anti-$ß_1$-AR positive, and five were also anti-$ß_2$-AR positive. Patients treated for four consecutive days saw a reduction in anti- $ß_1$-AR levels of 96% compared to only 78% in those treated for two to three consecutive days. Those patients treated with four days of IA also saw a sustained elimination of antibody over the course of the study but in those treated for a shorter time period saw a rebound of the antibody level. Cognitive function was assessed using a range of tests including the Mini-Mental State Examination (MMSE), Alzheimer's Disease Assessment Scale (ADAS; cognitive and non-cognitive), Bayer Activities of Daily Living (Bayer-ADL), Clinical Global Impression Scale (CGI), Geriatric Depression Scale (GDS), and the Short Cognitive Performance Test

(SKT). They found that over the course of the study, those treated for four days had stabilisation of their cognitive function. Those treated for only two to three days suffered from declining cognition[198]. This is a limited study with a small number of patients, but its promise has led to a number of current studies to investigate further.

2.5.3.6 Lambert-Eaton Myasthenic Syndrome

Lambert-Eaton Myasthenic Syndrome (LEMS) is a rare autoimmune disease resulting in muscle weakness, autonomic dysfunction and areflexia. Up to 60% of patients with LEMS will also be found to have a carcinoma, with small cell lung cancer (SCLC) making up the vast majority of these patients. Pathogenic antibodies to voltage-gated calcium channels (VGCC) have been found in 80-90% of patients and up to 100% in patients with SCLC. Current therapy consists of 3,4-diaminopyridine as first line and treatment of any underlying malignancy. Second line treatment involves the addition of pyridostigmine to the 3,4-diaminopyridine or converting to Azathioprine and Prednisolone. In the case of severe weakness, TPE or IVIG can also be considered[199]. There are also a number of very small case series describing the use of IA in refractory LEMS.

Sauter *et al.* describe the case of a young man with rapidly progressive weakness, muscular atrophy and cerebellar dysfunction initially treated with thymectomy for presumed malignancy and pulsed prednisolone with some resolution of symptoms and a reduction in anti-VGCC antibodies titres. Further treatment with Azathioprine and IVIG was initiated with some improvement clinically although this was not sustained and corresponded with a rise in his antibody titre. IA was performed on three consecutive days every six weeks with a decrease in antibody level over this time and an improvement

symptomatically, especially in regards to gait[200]. Baggi *et al.* treated three patients unresponsive to immunosuppression and plasma exchange with IA. All patient showed clinical improvement with one patient regaining the ability to walk and one reaching pharmacological remission[201]. Batchelor *et al.* treated 13 paraneoplastic patients one of whom had LEMS characterised as bilateral ptosis and proximal limb weakness. They received a total of 6 IA sessions (two per week for three weeks) with a protein A adsorber. In the patient with LEMS, clinical improvement was seen with resolution of the ptosis and the recovery of muscle strength allowing her to climb stairs and walk unaided again. There was also a significant reduction in the anti-VGCC antibody titre from 458 pmol/L to 25 pmol/L[202]. Ishikawa *et al.* treated a 75-year-old man with gait disturbance and somnolence diagnosed as LEMS. Anti-VGCC titre was initially over 11,000 pmol/L, but the use of a phenylalanine adsorber column along with concomitant prednisolone resulted in a significant reduction in his antibody titre and subsequent clinical improvement[203].

There are currently no RCT or prospective trial data for the use of IA in LEMS. However, in patients who are non-responsive to standard therapy or in whom immunosuppression or TPE are contraindicated, there is limited data to suggest that IA can be considered an alternative.

2.5.4 Dermatology

2.5.4.1 Pemphigoid Vulgaris

Pemphigoid Vulgaris (PV) is a potentially fatal autoimmune blistering condition of the skin and mucous membranes. It is associated with pathogenic IgG autoantibodies to the desmosomal cadherins; desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3)[204-206]. Treatment and management of PV can be

challenging. Currently, treatment consists of oral steroids alone or in combination with dapsone and immunosuppression such as azathioprine, methotrexate or cyclophosphamide. This has dramatically improved survival, but there is significant morbidity as a result of the side-effects from these therapies[207].

A number of groups have now used IA with differing adsorbers and protocols. A tryptophan-linked polyvinylalcohol adsorber was used to treat seven patients with severe PV. There was a significant reduction in circulating antibodies and clinical improvement seen in the pemphigoid lesions and a reduction in steroid and immunosuppression required[208]. Protein A immunoadsorption has also been used with the first study describing its use in 2003. Here four patients were treated using IA as an additional treatment to steroids. All patients saw an improvement in their pemphigoid lesions and significant reduction in their antibody titres[209]. A further nine patients were treated with a modified protocol by Shimanovich *et al.* with a higher dose of adjunctive steroids and either azathioprine or mycophenolate mofetil. All patients showed a significant reduction in antibody levels and clinically, with remission reported up to 26 months after treatment[210]. Protein A immunoadsorption has also been used in combination with Rituximab and IVIG with positive results[211] and in patients with longstanding disease resistant to multiple therapiesf[212]. In the largest trial for IA in PV, IA was used in combination with Rituximab in 23 patients. 17 patients using Protein A IA (Immunosorba) and six patients using polyclonal anti-human IgG sheep antibodies coupled to sepharose (Thera-Sorb). IA was given more frequently than previous protocols with 1000mg Rituximab given on day 4 and day 24. This resulted in a significant reduction in antibody titres in all patients. At six months, 16 (70%) of the patients were in complete remission and five (22%) were in partial remission. A relatively low relapse rate

of 6 patients was seen over the follow-up period requiring either retreatment with IA, Rituximab or immunosuppression[213].

Given the antibodies to Dsg1 and Dsg3 are IgG, Eming *et al.* used the Globaffin (Fresenius, Germany), an IgG specific column to treat PV in four patients. All patients experienced a reduction in antibody levels of up to 70% and a marked improvement clinically[214]. Behzad *et al.* used the Globaffin column in combination with Rituximab in 10 difficult to control PV patients in a retrospective study. Six months after treatment, eight out of the ten patients were in remission, one had a partial response, and one patient did not respond at all[215]. In one study comparing adjunctive IA versus Rituximab therapy, antibody levels, clinical improvement as assessed by the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) and oral steroid doses all reduced faster in the IA group compared to the Rituximab group. However, there were more relapses in the IA group requiring further treatment[216].

Despite the evidence for IA in PV, an autoimmune disease with well-defined pathogenic IgG autoantibodies, its widespread adoption has been limited. This has been hampered by the small study numbers, lack of RCTs and multiple treatment protocols. Given this PV is a recommended indication for the use of IA by the ASFA where it is classified as a category III disease (Optimum role of apheresis therapy is not established) with 2C evidence (Weak recommendation, low-quality or very low-quality evidence)[135]. The British Association of Dermatologists guidelines for the treatment of pemphigus vulgaris also states that IA could be considered in patients unresponsive or intolerant to standard treatment[207].

2.5.4.2 Bullous Pemphigoid

Bullous Pemphigoid (BP) is an autoimmune condition resulting in the development of subepidermal blisters or bullae and is the most common of the autoimmune blistering conditions. It is caused by IgG autoantibodies directed against the BP180 and the BP230 antigens found in the hemidesmosomes. The mainstay of treatment is the use of topical or systemic steroids with or without oral immunosuppression[217,218]. In patient's refractory to this, IA has been used with varying success.

Herrero-González *et al.* used tryptophan IA to treat two patients with BP initially unresponsive to methylprednisolone, dapsone and in one patient, additional azathioprine and topical clobetasol propionate. Both patients saw dramatic improvement in their skin lesions after two weeks with all active lesions disappearing by six weeks[219]. Kasperkieicz *et al.* treated seven patients with severe disease using Protein A immunoadsorption. Here four patients had previously failed treatment with oral steroids, topical clobetasol propionate and either dapsone or mycophenolate mofetil and 3 were immunosuppression naïve. All patients saw a significant reduction in circulating antibodies and had no active lesions 1-3 months after therapy. Six of the seven patients remained in clinical remission at the end of follow up with two of the patients requiring no adjuvant medication[220]. Ino *et al.* used dextran sulfate conjugated cellulose columns to treat two patients who had not responded to steroids or dapsone. In one patient the lesions disappeared two weeks after treatment; however, in the second patient the skin lesions returned after six weeks and despite a second course of IA continued to have active blistering[221].

Given the pathogenicity of the IgG antibodies involved in BP and the positive results, albeit from limited published data, IA has the potential to provide adjunctive therapy in refractory BP.

2.5.4.3 Atopic Dermatitis

Atopic Dermatitis (AD) is a chronic inflammatory condition affecting up to 20% of the population[222]. It is characterised by recurrent pruritic eczematous lesions and generally presents in childhood. Its pathogenesis is not completely understood, exacerbated by the heterogeneous nature of the disease, but genetic, environmental and humoral factors are all associated with its development. The disease itself can be associated with other atopic and inflammatory conditions such as asthma, allergic rhinitis and inflammatory bowel disease. Far from being a typical type I hypersensitivity reaction as initially thought it now appears to be a complex combination of epidermal barrier dysfunction, T helper 2 (Th2) cell-mediated and IgE immune regulated pathways. The majority of patients show a raised serum IgE titre with some circumstantial evidence suggesting it plays a pathogenic role[223,224].

The first published study used IA in 12 patients with severe AD and total serum IgE levels of greater than 4500 kU/L. Patients saw a significant improvement in their mean Scoring Atopic Dermatitis (SCORAD), reducing from 78.6 ± 3.9 to 32.4 ± 3.5 at the end of the study at week 13. There were also significant improvements seen in the mean Eczema Area and Severity Index (EASI) and the pruritus score by the end of the study[225]. Since that time there has been a large number of patients treated in clinical trials with promising results[226-229]. Reich *et al*. treated 26 severe AD patients with IgE specific IA and 24 patients with a Pan-immunoglobulin IA. Both groups reported an equal improvement in their EASI scores with almost 50% of patients reporting a greater than 50% improvement. There were also improvements seen in the Dermatology Life Quality Index (DLQI), the SCORAD and the Patient-Oriented Eczema Measure (POEM). In this study, the IgE specific adsorber was better tolerated with less adverse events than the pan-immunoglobulin adsorber with similar clinical outcomes[229].

Given the weight of evidence now accumulating and the safety profile of the IgE specific adsorbers, IA should be considered in the case of AD unresponsive to standard care or in those in whom it is contraindicated.

2.5.5 Other

2.5.5.1 Asthma

Asthma is one of the world's most prevalent chronic diseases affecting an estimated 300 million people worldwide and rising. A variant of asthma, allergic asthma is classified as a type 1 hypersensitivity reaction. Here IgE binds to high-affinity FcεRI receptors on Mast cells and Basophils leading to degranulation and the release of inflammatory mediators. There is now increasing evidence that the incidence of IgE-mediated allergies is on the rise. In allergic asthma, as in other allergen related disease, the severity is progressive as patients come into contact with the allergen over time[230-232].

The IgEnio is a single use IgE specific adsorber developed by Fresenius Medical Care. The ESPIRA trial (Extracorporeal IgE Immunoadsorption in Allergic Asthma: Safety and Efficacy) is an RCT investigating the efficacy of IA in 14 adult patients with allergic asthma and raised IgE titres. Patients were treated for three cycles with each cycle consisting of 3 sessions. Mean IgE levels reduced by 87% per cycle for total IgE with similar reductions in IgE specific for seasonal and perennial allergens. A steady improvement in peak flow levels, overall allergy symptoms as assessed by the Visual Analogue Scale (VAS) and lung-specific symptoms were also seen. In the US, Omalizumab is only indicated in patients with an IgE titre of below 700 U/mL and in the EU below 1500 U/mL. Along with the clinical and biochemical improvements seen with the treatment, interestingly it also allowed three of the patients, who were previously ineligible for Omalizumab treatment due to their high titres, to qualify for Omalizumab treatment. Further work is needed given this is the first reported use of IA in allergic asthma, but the initial findings are promising[232].

2.5.5.2 Sepsis

Despite advances in the treatment and management, the incidence of sepsis is increasing worldwide, a result of an ageing population with ever more complex comorbidities. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) defines it as a life-threatening organ dysfunction caused by the dysregulated immune response to infection[233]. It has a mortality of approximately 20% with 5.3 million deaths a year globally and given the significant associated morbidity; it now constitutes a considerable economic and societal burden for healthcare systems worldwide[234].

When the body comes into contact with a pathogen, there is a complex proinflammatory and immune suppression at play. In sepsis, this balance is lost with an ever-increasing inflammatory response leading to organ dysfunction. Focus over the years on therapies that directly affect various participants in this immunoregulation has had inconsistent results with no real cross over to standard clinical practice. One component of this dysfunction is monocytic deactivation which has been shown to be influenced by factors such as lipopolysaccharide (LPS), interleukin-6 (IL-6) and complement-activation product 5a (C5a). This pathway was the subject of a case-control prospective study looking at the use of IA to selectively remove LSP, IL-6 and C5a in 11 adult patients (and 22 controls) with severe sepsis admitted to ICU. The treatment was well tolerated, and patients had no ongoing anticoagulation abnormalities following IA therapy. All three factors were markedly reduced following treatment in the IA group, in addition to which C-reactive protein (CRP) and fibrinogen were reduced to 27% and 36% of their initial values. There was no change to the inflammatory factors in the control group. Using a number of markers of disease severity, those patients in the treatment group showed a meaningful improvement compared to the control group. Number of days ventilated and the number of days in ICU were both significantly less in

the treatment group as was the amount of norepinephrine needed. There was a tendency to a reduction in the number needing renal replacement therapy although this was not statistically significant. Acute Physiology and Chronic Health Evaluation II (APACHE II), mean Sequential Organ Failure Assessment (SOFA), and mean Multiple Organ Failure (MOF) scores all improved significantly more in the treatment group compared to the control group[235].

This pilot study shows that IA appears to be safe and tolerated well in patients with severe sepsis with significant objective improvements as measured both biochemically and clinically.

2.6 Conclusion

Since its development over two decades ago, immunoadsorption therapy has proven to be a highly efficient method of removing antibodies with a remarkably safe side effect profile. As our understanding of autoimmune diseases increases, the range of conditions that are amenable to IA will also increase, and with the development of columns for more specific antibodies and molecules such as those for sepsis, its use can reasonably be expected to become more ubiquitous.

Peptide GAM Immunoadsorption therapy in Primary Membranous Nephropathy (PRISM)

Phase II trial investigating the safety and feasibility of Peptide GAM Immunoadsorption in anti-PLA_2R positive primary membranous nephropathy

Short form title: PRISM

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3.1 Abstract

Introduction

Membranous nephropathy (MN) is among the most common causes of nephrotic syndrome in adults worldwide. Most patients have primary MN (PMN), an autoimmune condition associated with the IgG anti-PLA_2R autoantibody. For patients with severe disease, standard of care continues to be a 6-month regime of rotating high dose steroids and immunosuppression that comes with a significant side-effect profile. Immunoadsorption is a relatively safe procedure for the extracorporeal removal of specific immunoglobulins without the need for medications.

Design

This is a Phase II multi-centre, single arm prospective clinical trial carried out across the Northwest region of the United Kingdom to assess the safety and clinical effectiveness of immunoadsorption therapy in PMN. 12 adult patients with biopsy-proven MN, nephrotic range proteinuria and serum anti-PLA_2R antibody titres of more than 170u/ml will undergo five consecutive daily sessions of immunoadsorption. Primary outcome is the reduction of serum anti-PLA_2R antibodies at day 14. Secondary outcomes are the safety and tolerability of immunoadsorption therapy in patients with primary MN at all-time points, reduction of serum anti-PLA_2R levels, proteinuria and improvement in renal function. Quality of life and cost-effectiveness of treatment will be assessed from a UK National Health Service perspective.

Discussion

With proven efficacy in removing IgG antibodies and its use as a relatively safe treatment option in a multitude of conditions, immunoadsorption has the potential to offer patients with primary MN a more directed therapy free from the short and long-term side-effects generally seen in this condition.

3.2 Introduction

Membranous nephropathy (MN) is among the most common causes of nephrotic syndrome in adults worldwide, second only to FSGS[1,100-104]. The majority of patients will remain stable with either complete remission or partial remission, but approximately 20-30% will progress slowly to end-stage renal disease necessitating the need for renal replacement therapy (RRT)[6,7,236-238].

MN has two distinct entities with primary or idiopathic MN (primary MN) now considered to be an autoimmune disease since the discovery of the M-type of phospholipase A2 receptor 1 (anti-PLA_2R) antibodies[15,38,39,50,239,240] and secondary MN caused by a multitude of disorders including but not restricted to malignancy, infection, autoimmune disease and drugs[5,241,242]. These two conditions have very different management priorities with the focus in secondary MN being the treatment of the underlying condition and in primary MN, the control of proteinuria with or without the use of immunosuppression. Although Rituximab, calcineurin inhibitors (CNI) such as ciclosporin and therapeutic plasma exchange have been used with varying success, treatment generally takes the form of the Ponticelli regime[57,243,244]. This regime of rotating high dose steroids and immunosuppression was first described over 20 years ago and has been the mainstay of treatment since. This regime, however, does come with a significant side effect burden including an increased risk of infection, osteoporosis, diabetes mellitus, weight gain, haemorrhagic cystitis, infertility and an increased risk of malignancy[5,52,53].

In 2009 Beck *et al.* showed that the majority of patients (70%) with idiopathic membranous nephropathy had IgG autoantibodies to M-Type Phospholipase A2 Receptor, the predominant subclass of which was IgG4 with smaller amounts of all other IgG subclasses[15,38]. There is now an increasing body of evidence for the apparent pathogenicity of the anti-PLA_2R antibodies, with the potential for its use as a biomarker and as a treatment target. High serum titres

correlate with active disease and low levels with remission[38]. For patients who go into remission either spontaneously or following immunosuppression, the anti-PLA_2R level falls months before this becomes clinically apparent with a fall in proteinuria[43,245]. If a patient relapses, this could be predated by a rise in antibody titres[42].

Immunoadsorption is a method of removing specific circulating immunoglobulins and has been shown to remove over 80% of circulating IgG in a single session immunoadsorption of 2.5 plasma volumes, with albumin and antithrombin III almost unaffected. With multiple sessions, this can rise to over 98%[59,60,246]. Post immunoadsorption it appears that autoantibodies can be slow to re-emerge. Removal of ß1-adreno-receptor autoantibodies (ß1-AAB) by immunoadsorption in Dilated Cardiomyopathy has shown that only a small minority of patients (0% in the first year and approximately 15-30% by three years) will show an increase in significant ß1-AAB autoantibodies[61,62,247].

To our knowledge, there has only been one published report of immunoadsorption treatment for the management of membranous nephropathy and none in the post-anti-PLA_2R era. In 1999 Esnault *et al.* successfully used Immunoadsorption for the treatment of various aetiologies of Nephrotic syndrome including four patients with membranous nephropathy. Here they showed that not only is the procedure safe but that there was a significant improvement in proteinuria in all patients with membranous nephropathy[63]. Since that time, the treatment has been used in numerous other autoimmune conditions including Focal Segmental Glomerulosclerosis (FSGS)[64], systemic lupus nephritis (SLE)[65,66], ANCA-associated small vessel vasculitides[67,68,114], Anti-glomerular basement membrane antibody disease[69] and in renal transplantation[60,70-72].

In conditions such as SLE, the use of immunoadsorption can dramatically reduce the level of circulating immune complexes and autoantibodies leading

to clinical improvement in even severe life-threatening SLE. These results have been shown with as little as two sessions within three days and repeated every three weeks if patients remain with active disease[65].

3.3 Rationale for study

The aim of this study is to investigate the potential for immunoadsorption to provide a more targeted disease control without the side effect burden in the longer term with use of high dose steroids and concurrent immunosuppression. With the safety profile of Immunoadsorption already shown and with evidence of benefit in membranous nephropathy[63], we propose to use the therapy in patients with significantly raised serum anti-PLA_2R titres and biopsy-proven membranous nephropathy.

3.4 Methods & Design

This is a multi-centre, single arm prospective clinical trial carried out across the Northwest of England. Immunoadsorption has been used in a multitude of conditions for many years with its safety and low side-effect profile being well documented. However, there is limited evidence for its use in primary MN. This is, therefore, a Phase II trial to assess the safety and clinical effectiveness of immunoadsorption therapy in patients with primary MN. The trial is being funded by Fresenius Medical Care Deutschland GmbH and is sponsored by Central Manchester University Hospitals NHS Foundation Trust (CMFT). All study visits will be carried out at the National Institute for Health Research (NIHR)/Wellcome Trust Clinical Research Facility at the CMFT.

3.4.1 Primary Objective

The primary objective is the reduction in serum anti-PLA_2R titres to normal range at day 14. All samples will be tested using the Enzyme-Linked immunosorbent assay (ELISA) for anti-PLA_2R antibodies as described by Kanigicherla *et al*[38].

3.4.2 Secondary Objectives

The safety and tolerability of immunoadsorption therapy in patients with primary MN will be assessed throughout the study at every follow-up visit. All other secondary endpoints will be reported at Day 14, 28, 56, 84, 168 and 365. These will be the reduction in proteinuria and improvement in renal function as measured by uPCR and serum creatinine level respectively. The reduction and pattern of serum anti-PLA_2R titres will be investigated using prospective blood tests peri-therapy and at time points as above. This is particularly important as

it is possible for a rebound of anti-PLA_2R following treatment which may impact on the primary objective. By prospectively measuring the anti-PLA_2R levels throughout the follow up this will help our understanding of the pathogenesis of the disease. Further kinetic modelling of anti-PLA_2R production will also involve the daily collection of urine whilst on Immunoadsorption therapy. Disease activity, adverse events (AEs) and serious adverse events (SAEs) are based on physician assessment. Serious adverse events are taken as any adverse events requiring hospital admission, prolonged hospital stay or intravenous (IV) antibiotic therapy outside of protocol. Quality of life measures will be assessed using the EuroQol five dimensions questionnaires (EQ5D) as completed by the patient[248]. The cost-effectiveness of treatment (using Incremental cost-effectiveness ratio) to be based on NHS reference costs and patient-reported personal and societal costs.

3.4.3 Inclusion Criteria

Both newly diagnosed and relapsing patients' adult patients (above 18 years old) with renal biopsy confirmed Membranous Nephropathy within the last 3 years. Persistent active disease despite 6 months of supportive care using an angiotensin-converting-enzyme inhibitor (ACEi) or angiotensin receptor blockers (ARB). Active disease defined as uPCR more than 300mg/mmol or 24-hour urinary protein of more than 3.5g/1.73m^2; and patients with serum anti-PLA_2R titres above 170 u/ml. Disease severity that in the physicians' view, warrants treatment prior to completion of 6 months supportive care.

All patients must have up to date Haemophilus and Pneumococcal vaccinations and are to be able to provide informed consent.

3.4.4 Exclusion Criteria

Patients are to be excluded if there is any evidence of secondary membranous nephropathy and if they have an estimated Glomerular Filtration Rate (eGFR) of less than 20ml/min. In order to ensure that patients have not had any artificial regulation of their immune system prior to the study they must not have received any treatment with steroids or immunosuppression (including but not limited to cyclophosphamide, Mycophenolate mofetil or azathioprine) or Biologics (including but not limited to Rituximab or belimumab) within 6 months of screening. Patients must also not have had Therapeutic Plasma Exchange within 28 days of screening or previous renal transplantation. Patients can also be excluded if they have a co-morbidity, which in physicians' view would preclude the patient from treatment with immunoadsorption or if they are pregnant at the time of screening.

3.4.5 Ethics, Informed Consent, and Safety

The study has full ethical approval from the NHS Health Research Authority Research Ethics Committee (REC reference 16/NW/0560). All study sites also have local Research and Development approval. All patients must provide written informed consent before any study procedures are carried out.

The Data Monitoring Committee will meet monthly to evaluate the results and safety.

Therapy will be stopped in the case of allergic reaction to the column, extracorporeal therapy or any other adverse or serious adverse event deemed by the safety committee to warrant withdrawal of treatment.

3.4.6 Treatment failure

Patients will not have immunosuppression as part of this study. However, this study is in addition to their usual routine care which will continue uninterrupted during the follow-up phase. The clinical datasets will capture all care received and any change in treatment. If a patient requires further immunosuppression as per their parent team, this will be recorded as a treatment failure, and all subsequent data will be analysed separately.

3.4.7 Recruitment

This study will recruit patients from across the Northwest of England. All treatment and study visits will take place at the MCRF located at the Manchester Royal Infirmary. Salford Royal Hospital (SRFT), Royal Preston Hospital (RPH), Royal Liverpool and Broadgreen University Hospitals Trust (RLBUHT), Bradford Teaching Hospitals NHS Foundation Trust (BTHFT), and University Hospitals of North Midlands (UHNM) will operate as Patient Identification Centres at which patients can be identified. Screening will then take place at the MCRF. These hospitals cover an ethnically diverse population of approximately 10 million providing a good representative cross-section of the UK population at large.

Patients are identified at weekly renal departmental multi-disciplinary biopsy meeting in each hospital. Here all patients with a biopsy are presented and management options discussed. Patients with membranous nephropathy as a differential will also have an anti-PLA_2R serum test. Any patient, who has an anti-PLA_2R antibody titre consistent with the inclusion criteria and a biopsy confirming membranous nephropathy, will be approached for screening and consent after discussion with their physician regarding their suitability.

3.4.8 Study population

Sample size has been determined on pragmatic grounds based on patient-level data from our centre.

For n=12, a difference equivalent to 0.9 of the intra-patient standard deviation can be detected (80% power for a paired t-test, 5% sig level). Using the log of the standard deviation for proteinuria, this gives a difference in log(proteinuria) of 0.45, therefore allowing detection of an improvement in log(proteinuria) of >0.41, i.e. a reduction from a (geometric) mean of 5.2g/1.73m^2 to 2.0g/1.73m^2.

This study will involve patients with nephrotic syndrome with proteinuria of greater than 3.50g/1.73m^2. The hypothesis underlying the study is that Immunoadsorption therapy will lead to the removal of anti-PLA$_2$R and therefore lead to improvement in the level of proteinuria. Based on the numbers of patients who attend our centre and allowing for the power calculation above to detect an improvement in proteinuria we aim to screen 20 patients and to recruit and complete treatment in 12 patients.

3.5 Risks, burdens and benefits

Immunoadsorption has been shown to be safe over the last two decades of use. The main complications include low BP, allergic reaction to the filter/column and a reduction in serum calcium levels. Patients will be closely monitored throughout by nursing staff specially trained in the use and provision of immunoadsorption, and medical backup will be provided at all times.

Vascath insertion is a safe procedure but does come with recognised complications. Patients will be informed of the need for vascath insertion prior to agreeing to participate in the study and consent will be obtained prior to insertion. The procedure will be carried out under aseptic conditions with the use of real-time ultrasound (USS) guidance.

We are testing the idea that using immunoadsorption will improve the patients' antibody level and thereby disease activity and quality of life. A subgroup of patients with MN will have a spontaneous remission by six months, international guidelines on the treatment of autoimmune membranous nephropathy reflect this with the suggestion that treatment with immunosuppression only starts after six months unless patients clinically deteriorate or have a rapid decline in their renal function. In accordance with this guidance, patients will only be started on immunoadsorption if they have remained stable but clinically and biochemically active disease for six months. As treatment with the immunoadsorption is only for five days, if the patient remains active and is required to start on immunosuppression as per the patient's medical team, there will be no clinically significant delay in treatment.

3.6 Filtration/Adsorption Device

For this study, we will be using the Peptide GAM Immunoadsorption (GLOBAFFIN Fresenius Medical Care Deutschland GmbH). This uses two systems, the Art Universal and ADAsorb®. The ADAsorb® became commercially available in 2002 and the Art Universal in 2005. As this system is already commercially available and we are not using it outside of its recommended remit, we have not applied for MHRA approval.

The Art Universal (Fresenius Medical Care) Hemoadsorption System is intended for performing adsorption treatments or plasma fractionation for the selective or semi-selective elimination of undesired components in the patient's blood or plasma.

The ADAsorb® (Medicap Clinic GmbH) is a secondary system for controlling and monitoring adsorbers for extracorporeal apheresis. The system uses microprocessors to monitor all predetermined adsorption and desorption

parameters of the adsorbers. It is operated in conjunction with a plasma separation system.

This is a dual column system allowing for continuous immunoadsorption to the required plasma volume clearance. Whilst one column is actively involved in immunoglobulin adsorption, the other is being desorbed. Once the active adsorption column has become saturated, the plasma flow is diverted to the newly refreshed column, allowing for the saturated column to start the desorption process. Peptide GAM immunoadsorption specifically removes IgG, and in particular IgG1, IgG2 and IgG4 (and to a lesser extent IgG3) and has been chosen for this study as anti-PLA_2R antibodies have been shown to be IgG with a predominant IgG4 subclass[15,38,249,250].

3.7 Treatment

Patients will report to the CRF on the Monday of the first treatment, where following confirmation of patients desire and suitability to proceed with the study will undergo observations, blood and urine tests. A femoral or jugular vascath (Double lumen blood access catheter; Medcomp, Harleysville, PA, USA) will then be inserted under local anaesthetic and real-time ultrasound guidance, which remains in situ for the duration of immunoadsorption. The patient will undergo daily Immunoadsorption for five days. The multiracial visual inspection catheter tool observation record (mr ViCTor) score will be assessed daily for signs of infection[251]. In order to allow for adequate treatment, we will aim to achieve pump speeds of 100-150ml/min. If patients are unable to complete five days consecutively, they will be allowed to complete five sessions within seven days. If, however, the treatment is deferred for more than 48 hours the patient will need to have an extra session to ensure the adequate removal of antibody. In this case, the patient will

receive six sessions in 8 days. Patients will have close monitoring throughout and repeat blood and urine tests daily. During this period patients will also collect daily 24-hour urine samples. Once the treatment period is complete, the vascath will be removed, and patients will enter a follow-up period.

Patients will have follow-up weekly for the first month. For months 2 & 3 patients will be followed up at two weekly intervals, reducing to monthly until the end of the one-year follow-up period.

All patients at the start of treatment will have biochemical and clinical evidence of nephrotic syndrome including oedema. For this reason, in order to accurately determine a patient's plasma volume, all patients will undergo Bioimpedance measurement (BCM) at screening. We will then use BCM derived normohydration weight (Ideal Weight (IW)) to calculate the Plasma Volume using Kaplan formulae; Estimated Plasma Volume = (0.065 x IW(kg)) x (1 - Haematocrit)[75]. In order to ensure adequate antibody clearance, we treat 2.5 plasma volumes daily with a maximum treatment of 1.5L plasma/hour. Anticoagulation will be provided using a combination of heparin and citrate sodium with continuous IV calcium replacement as per local guidelines throughout the treatment sessions (10ml 10% Calcium Gluconate for every 2L of plasma treated). Serum calcium levels will be assessed at the beginning, mid-point based on plasma volume and end of therapy.

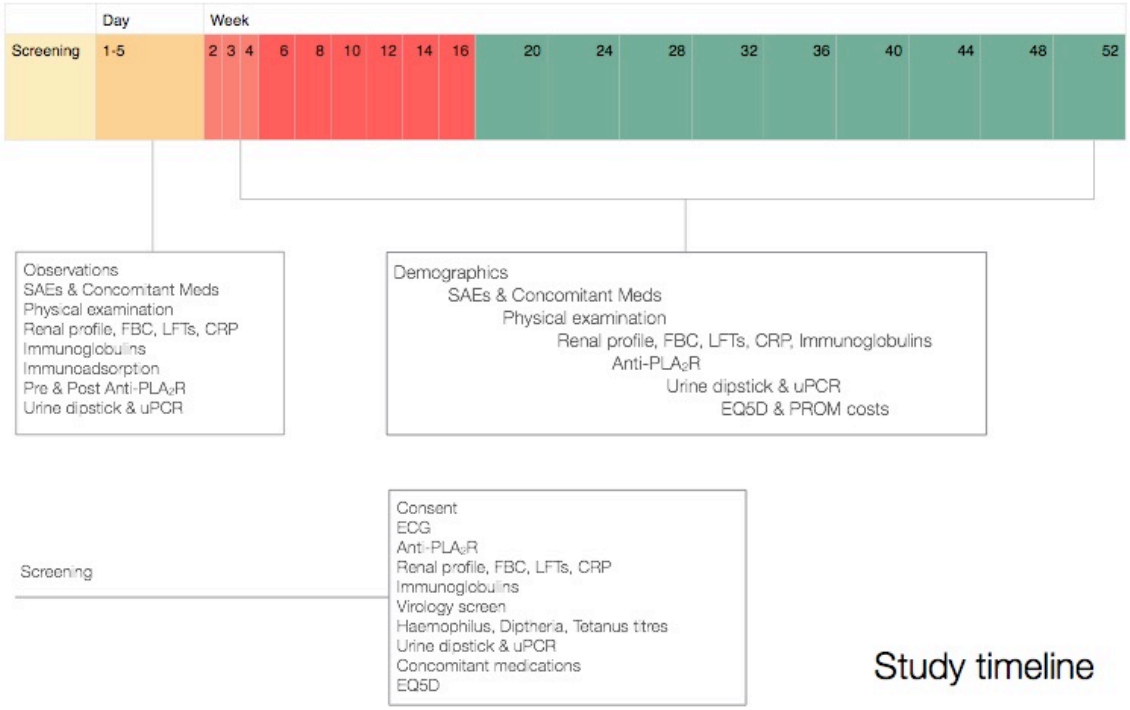

Figure 3.1 – PRISM study timeline

3.8 Statistical analysis

Demographics of patients will be presented. Simple descriptive statistics and survival analysis will be used to evaluate the primary and secondary outcome measures. Further analysis will involve the development of multivariate risk models using Cox proportional hazard regression to account for all clinically appropriate and statistically significant factors. The normal level of serum anti-PLA_2R taken as < 40 u/mL. Analysis will occur at each time point and will include total level of anti-PLA_2R, eGFR and proteinuria as well as mean change in level over time.

Health economic analysis to include calculation of outcomes cost per quality-adjusted life year (QALY), and the societal and personal cost of treatment. Costs of healthcare resource use to be obtained from the NHS reference costs and the Personal Social Services Research Unit (PSSRU) Unit Costs of Health and Social Care. The cost of medication will be taken from the Drugs and Pharmaceutical electronic market information (eMit) or the British National Formulary (BNF).

All analysis will be conducted using the R statistical program[252].

3.9 Conclusion

Primary MN is a rare disease but remains one of the most common causes of nephrotic syndrome worldwide. The current standard of care is based on a regime of high dose steroids and immunosuppression that was developed over 20 years ago and comes with a significant side-effect profile. The last decade of research into primary MN, however, has resulted in the discovery of the IgG anti-PLA_2R antibody and the increasing evidence for its pathological involvement in the development of the disease. With its proven efficacy in removing IgG antibodies and its long history as a relatively safe treatment option in a multitude of conditions, immunoadsorption has the potential to offer patients with primary MN a more directed therapy free from the short and long-term side-effects generally seen in this condition.

3.10 Declarations

Ethics approval and consent to participate

The study has full ethical approval from the NHS Health Research Authority Research Ethics Committee (REC reference 16/NW/0560). All study sites also have local Research and Development approval.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

LW, DK and MF are employees of Fresenius Medical Care Deutschland GmbH. PH has received an honorarium from Fresenius Medical Care Deutschland GmbH.

Funding

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Authors' contributions

All authors have been involved with the development of the protocol from the beginning and have reviewed the manuscript throughout its preparation

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Phase II trial investigating the safety and feasibility of Peptide GAM Immunoadsorption in anti-PLA_2R positive autoimmune membranous nephropathy. The PRISM trial.

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4.1 Introduction

Membranous nephropathy is a significant cause of nephrotic syndrome worldwide, with the majority of patients presenting with an autoimmune variant associated with the anti-PLA_2R autoantibody[1,15,100-104]. The current standard of care is a regime of high dose steroids rotating with cyclophosphamide over a six-month period[52,53]. This regime has been in use for over 20 years and was initially developed with the aim of eliminating the as then unknown antibody, by suppressing the immune system globally. Whilst this regime has improved mortality and renal outcomes, its significant side effects have left many patients with limitations on their quality of life.

Since the anti-PLA_2R antibody was discovered in 2009, there has been a dramatic increase in our understanding of the underlying disease pathology[15]. The antibody is now known to be a predominantly IgG3 subclass although the other subclasses are also represented albeit to a lesser extent[38]. An increasing body of circumstantial evidence suggests that not only is it a highly sensitive biomarker but is also pathogenic in nature[38,41-43].

Immunoadsorption (IA) is an extracorporeal therapy for the efficient removal of antibodies without the need for factor replacement. It has been in use for over 20 years for a variety of autoimmune conditions. Due to its directed manner, its side effect profile is minimal, making it an attractive alternative to empirical immunosuppression. A previous study investigating the use of IA in nephrotic syndrome, including four patients with membranous nephropathy, showed a significant reduction in proteinuria although this returned to baseline after only one month[63]. This study was limited with no long-term data on outcomes.

This study was in the pre-anti-PLA_2R era, and our knowledge of the condition has increased markedly since then. Given our current understanding of the disease, we carried out a study to investigate the effect of using IA on autoimmune membranous nephropathy.

4.2 Methods

The full trial protocol is presented in chapter 3. Further details and brief overview below.

4.2.1 Patients

The PRISM trial was a phase II prospective multicenter single-arm trial using Peptide-GAM immunoadsorption therapy for the treatment autoimmune membranous nephropathy. We aimed to recruit 12 adult patients with biopsy-proven, anti-PLA_2R positive membranous Nephropathy from the Northwest of England. Biopsies were to be within three years of the start of treatment, and anti-PLA_2R titres were required to be above 170 u/mL on the Manchester ELISA as described below. Other inclusion criteria included active disease despite six months of supportive care including Angiotensin-converting enzyme inhibitors (ACEi) or Angiotensin receptor blockers (ARB), or disease severity that in the physicians view warranted treatment prior to completion of 6 months supportive care. Active disease was defined as uPCR greater than 300mg/mmol or 24-hour urinary protein greater than 3.5g/1.73m^2. All patients were required to be up to date with Haemophilus and Pneumococcal vaccinations and able to provide informed consent.

Exclusion criteria consisted of any evidence of causes of secondary membranous nephropathy or any co-morbidity, which in physicians' view, would preclude patient from treatment with immunoadsorption. Patients with an eGFR of less than 20ml/min, previous renal transplant or therapeutic plasma exchange within the last 28 days were also excluded.

Any treatment with steroids or immunosuppression (including but not limited to cyclophosphamide, MMF or azathioprine) and Biologics (including but

limited to Rituximab or belimumab) within six months of screening or pregnant at the time of screening were also exclusion criteria.

4.2.2 Immunoadsorption

All treatment took place at the Manchester Clinical Research Facility (MCRF). Prior to treatment, access was obtained via a femoral or right internal jugular catheter (Double lumen blood access catheter; Medcomp, Harleysville, PA, USA) under local anaesthesia and full ultrasound guidance. A chest x-ray was then carried out for patients with a right internal jugular catheter. Following this, patients had daily immunoadsorption for five days (or 5 sessions within seven days if unable to complete consecutive days). If treatment was deferred for more than 48 hours, the patient would be required to have an extra session (i.e. six sessions in 8 days). Once the treatment period was complete, the vascath was removed, and patients entered the follow-up period as described below. Blood was separated into plasma using the Art Universal Haemadsorption System. Peptide-GAM immunoadsorption was carried out using a dual column system with Globaffin columns and the ADAsorb machine for column monitoring (figure 4.1). All columns and machines were provided by Fresenius Medical Care. For each session, 2.5 plasma volumes were treated. Anticoagulation during treatment comprised of heparin and sodium citrate. Due to the risk of hypocalcaemia with the use of sodium citrate, calcium was replaced using 10ml 10% calcium gluconate for every 2L of plasma treated.

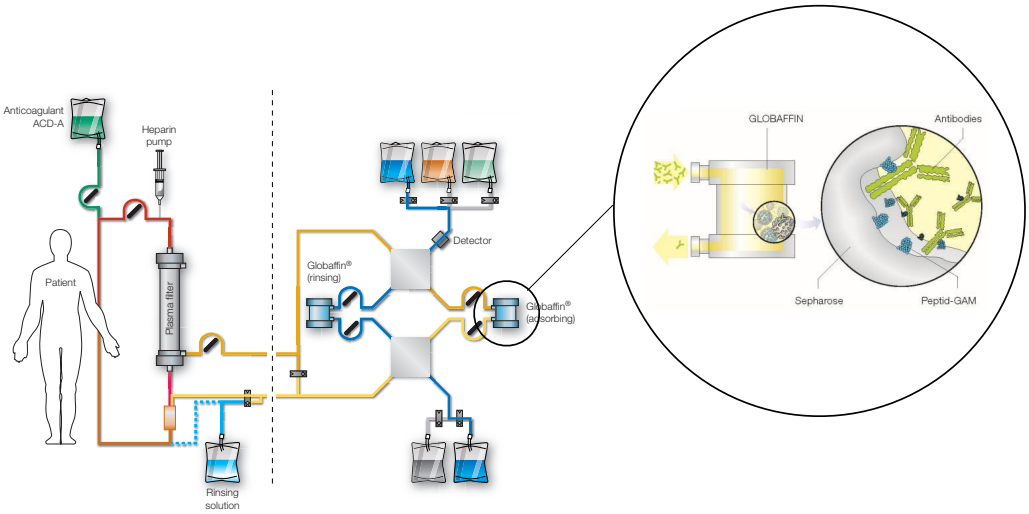

Figure 4.1 – Peptide-GAM immunoadsorption. A dual column system with Globaffin columns and the ADAsorb machine for column monitoring. Used with kind permission from Fresenius Medical Care

4.2.3 Follow up

Once treatment was completed patients were followed up for a total of one year. For the first three weeks after treatment, this follow up is weekly, then every two weeks until week 16. After this, follow up is monthly until last follow up. At each follow-up, patients are asked if they are happy to continue in the trial. Adverse events and concomitant medications are then reviewed. Following this, vital signs and a physical examination is performed. Bloods tests were performed at each visit with full blood count, renal profile, ESR and CRP being sent for analysis directly whilst 30ml of whole blood was biobanked (10ml for anti-PLA_2R testing and 20ml separated into PBMCs). Urine was collected for uPCR which is analysis in the central laboratories, and 30ml was biobanked for future analysis. Total serum immunoglobulins were tested at week 4, 8, 12, 24 and 52. At the same time points 24-hour urine collection and EQ5D were also carried out.

4.2.4 Outcomes

The primary outcome was a reduction of anti-PLA_2R levels to normal at day 14. Secondary outcomes were change in anti-PLA_2R level compared to baseline, uPCR, eGFR, EQ5D and adverse events at months 3, 6 and 12.

4.2.5 Analysis

Demographics and descriptive statistics are presented as number and percentage for categorical variables, and median and interquartile range for continuous variables. Significance estimated using chi-squared for categorical variables and Kruskal–Wallis one-way analysis of variance for nonparametric continuous variables. Subgroup analysis was also carried out on patients

above and below 70 years old. Time to antibody response was taken as the date of 50% or more reduction of anti-PLA_2R compared to baseline on two consecutive follow up appointments or any reduction in antibody level on three consecutive occasions. Analysis of time to antibody response carried out using Kaplan Meier method of estimation and Cox proportional hazards regression. All analysis carried out in R statistical program 3.5.1[252].

4.2.6 ELISA

All anti-PLA_2R tests were carried out in the Manchester Institute of Nephrology and Transplantation using the Manchester ELISA[38] and were carried out on samples that had been previously biobanked and stored at -80°C.

A 96 well flat-bottomed ELISA plate was coated with 100µl per well of sodium bicarbonate buffer, containing recombinant PLA_2R at 25µl/ml and left for 18 hours at 4°C before discarding the contents.

The plate was then blocked using 100µl SuperBlock (Thermo Fisher Scientific, Cramlington, UK) for 2 hours at 4°C before again discarding the contents. 100µl of SuperBlock with 0.1% Tween 20 was added to each well along with patient serum in a dilution of 1:100 (each patient sera had duplicate wells). Each plate also contained a standard curve quality control dilution series (1:3000; 1:1000; 1:313; 1:111; 1:37; 1:12). Duplicated background wells containing only 100µl SuperBlock with 0.1% Tween 20 were also included. Plates were kept at room temperature for two hours on a plate shaker at medium speed and then washed nine times in PBS with 0.1% Tween 20.

Following this, 100µl of anti-human IgG-HRP conjugate (Jackson ImmunoResearch, Newmarket, UK) was added to each well in a dilution of 1:25,000 in SuperBlock and incubated for 2 hours at 4°C. As previously, the

contents of the plates were discarded and washed nine times in PBS with 0.1% Tween 20.

In each well, 100µl 3,3′,5,5′-Tetramethylbenzidine (TMB) enzyme substrate (Sigma Aldrich) was added and allowed to develop for five minutes. The reaction was then stopped using H_2SO_4.

The plates were read using the Softmax software Molecular Devices (Sunnyvale, CA).

See General laboratory methods section for SOP.

4.2.7 Quality of Life

Quality of life (QoL) outcomes were assessed using the globally validated EuroQol-5D 3 level version (EQ5D-3L) [248,253]. The EQ5D measure of health care was chosen for the study as it is the most widely used quality of life measure worldwide, is well validated and is a simple to use a questionnaire that only takes five minutes to complete. Its generic nature allows it to be used for comparison across diseases and the utility value obtained through its use is the predominant quality of life score for cost-effectiveness analysis. See Appendix for EQ5D.

The EQ5D is comprised of a one-page questionnaire on five health state dimensions comprising mobility, self-care, usual activities, pain/discomfort and anxiety/depression. For each question, patients are asked to choose one of three options that best describes how they feel. This allows each health state to be analysed separately or combined to give an overall utility value of health. Level 1 describes ‘no problems’; level 2 describes ‘some problems’ and level 3 describes ‘extreme problems’. For each questionnaire a single utility value can then be calculated, with 1 being the best imaginable health state, 0 being

dead, and less than zero is a quality of life considered to be worse than death, i.e. coma.

A second page adds a further dimension to the questionnaire with the EQ5D visual analogue scale, a vertical continuous scale from 0 to 100. Patients mark on this scale how they feel overall with 0 being the worst imaginable health state and 100 being the best imaginable health state.

EQ5D data were initially analysed by calculating the proportion of patients at each level in each separate dimension and at each time point. A single utility score was calculated and presented a median value (IQR) for each time point. Further analysis was based on Paretian principles. Here, the EQ5D health state was compared to the previous time point and allocated one of four outcomes; no change, better, worse or mixed. If a patient was better on one or more dimension and no worse in any of the other dimensions, this was deemed to be a 'better' health state. If a patient was worse in one or more dimensions and no better in all others, this was deemed a 'worse' health state. 'No change' meant that all dimensions remained the same and a 'mixed' health state was one in which one or more was better and one or more was worse[254]. Subgroup analysis based on age group was carried out for all EQ5D analysis.

4.2.8 Ethics

The study received full ethical approval from the NHS Health Research Authority Research Ethics Committee (REC reference 16/NW/0560), and each study site also provided local Research and Development approval. All patients were required to give written informed consent before any study procedures were carried out and their continued participation was checked at each visit prior to visit procedures being carried out. The trial is registered on ClinicalTrials.gov, Identifier: NCT03255447.

4.3 Results

4.3.1 Demographics

Thirteen patients were screened and consented. One patient went into spontaneous remission prior to the start of treatment and was therefore removed from the trial. Twelve patients (11 male and 1 female) completed treatment successfully, and three have now completed a one-year follow-up. Median follow up was 229.5 days (IQR 156.0-310.0) and the median time from biopsy to the start of treatment was 177.5 days (IQR 106.25-400.75). All twelve patients completed their week 12 follow up, and ten patients had completed their week 24 follow up.

Median age at treatment was 68 years (IQR 53-73). There were seven patients (58.3%) below 70 years old with a median age at treatment of 53 (IQR 47-66), and 5 (41.7%) patients over the age of 70 with a median age at treatment of 73 (IQR 73-81).

Ten patients were naïve to steroids and immunosuppression prior to treatment, and two patients had failed the modified Ponticelli regimen. One of these patients (PRISM07) completed the full course over six months prior to screening and had remained significantly nephrotic with worsening uPCR and antibody level. The other patient (PRISM04) was unable to complete the course due to leucopenia and sepsis over one year prior to screening. It was felt at the time that he had no other treatment options and was being considered for medically induced nephrectomy.

All patients were taking either an ACEi or ARB, and nine patients (75%) were taking a statin at screening and the start of treatment. At the time of screening, eight patients (66.7%) had hypertension, and two patients (16.7%) had diabetes mellitus.

		Total population	Less than 70 years old	Over 70 years old	Sig.
n		12 (100.0)	7 (58.3)	5 (41.7)	
Age		68 [53, 73]	53 [47, 66]	73 [73, 81]	0.004
Gender	Female	1 (8.3)	0 (0.0)	1 (20.0)	0.860
	Male	11 (91.7)	7 (100.0)	4 (80.0)	
Bx to IA	Days	177.50 [106.25, 400.75]	209.00 [115.50, 412.50]	132.00 [110.00, 201.00]	0.685
Follow up	Days	229.50 [156.00, 310.00]	212.00 [135.00, 271.50]	275.00 [192.00, 352.00]	0.329
Previous IS	No	10 (83.3)	6 (85.7)	4 (80.0)	1.000
	Yes	2 (16.7)	1 (14.3)	1 (20.0)	
Cyclo	No	10 (83.3)	6 (85.7)	4 (80.0)	1.000
	Yes	2 (16.7)	1 (14.3)	1 (20.0)	
HTN	No	4 (33.3)	4 (57.1)	0 (0.0)	0.147
	Yes	8 (66.7)	3 (42.9)	5 (100.0)	
Diabetes	No	10 (83.3)	6 (85.7)	4 (80.0)	1.000
	Yes	2 (16.7)	1 (14.3)	1 (20.0)	
ACEi	No	1 (8.3)	0 (0.0)	1 (20.0)	0.860
	Yes	11 (91.7)	7 (100.0)	4 (80.0)	
ARB	No	11 (91.7)	7 (100.0)	4 (80.0)	0.860
	Yes	1 (8.3)	0 (0.0)	1 (20.0)	
Statin	No	3 (25.0)	1 (14.3)	2 (40.0)	0.735
	Yes	9 (75.0)	6 (85.7)	3 (60.0)	
BMI	kg/m^2	28.95 [25.25, 31.35]	30.90 [27.30, 34.65]	24.50 [23.70, 29.40]	0.062
BP - Sys	mm/Hg	159.00 [137.75, 176.25]	140.00 [129.50, 157.50]	177.00 [174.00, 191.00]	0.019
BP - Dia	mm/Hg	80.00 [75.50, 88.25]	88.00 [77.00, 89.00]	76.00 [74.00, 82.00]	0.121
Anti-PLA_2R	U/mL	702.50 [206.25, 1089.75]	679.00 [309.00, 762.50]	1006.00 [159.00, 1341.00]	0.570
uPCR	mg/mmol	908.00 [591.25, 1172.25]	844.00 [617.00, 1092.00]	972.00 [571.00, 1379.00]	0.685
Albumin	g/L	21.50 [18.00, 22.25]	21.00 [18.50, 22.00]	22.00 [18.00, 23.00]	0.622
Creatinine	umol/L	134.50 [112.25, 166.75]	131.00 [107.50, 146.00]	139.00 [128.00, 208.00]	0.465
eGFR	$mL/min/1.73m^2$	46.50 [31.25, 59.50]	50.00 [44.50, 62.00]	32.00 [27.00, 48.00]	0.291
Sodium	mmol/L	142.00 [138.50, 144.25]	142.00 [138.00, 142.00]	144.00 [139.00, 145.00]	0.508
Potassium	mmol/L	4.20 [4.00, 4.78]	4.10 [4.00, 4.50]	4.60 [4.10, 5.10]	0.413
ESR	mm/1stHr	42.50 [32.25, 57.75]	45.00 [33.00, 54.50]	40.00 [34.00, 57.00]	0.871

Table 4.1 - Demographics. Continuous variable given in median (interquartile range) and categorical variables presented in number (%). IS - immunosuppression; ACEi - angiotensin-converting-enzyme inhibitor; ARB - angiotensin receptor blocker; BMI - body mass index; Bx – Biopsy; BP - blood pressure; Sys – systolic; Dia – diastolic; Cyclo – cyclophosphamide; HTN – Hypertension; SAEs - serious adverse events; AEs - adverse events; uPCR - urinary protein:creainine ratio; CRP - C-Reactive Protein; ESR - erythrocyte sedimentation rate; IA - immunoadsorption

Patient	Baseline uPCR	Weight	BMI	ECW	PV	Total PV treated
PRISM01	382	176kg	56.8	35.0L	3.96	11.1
PRISM02	352	85.0kg	29.8	21.8L	2.56	10.8
PRISM03	691	70.0kg	23.7	22.2L	2.73	11.1
PRISM04	972	81.4kg	28.5	31.5L	3.44	10.9
PRISM05	558	87.3kg	28.5	24.1L	3.25	11.8
PRISM06	971	78.0kg	30.9	21.9L	2.58	11.5
PRISM07	462	87.3kg	25.5	27.7L	3.52	11.4
PRISM09	2213	104.8kg	32.7	26.5L	3.06	11.8
PRISM10	548	73.4kg	24.5	24.5L	3.13	12.0
PRISM11	1492	53.9kg	21.6	19.8L	2.07	12.2
PRISM12	1132	109.6kg	36.6	27.8L	3.50	12.5
PRISM13	663	73.2kg	28.6	17.9L	2.60	12.5

Table 4.2 - Plasma volumes treated per patient. uPCR - urinary protein:creatinine ratio; BMI - Body Mass Index; ECW - Extracellular water; PV - Plasma volume. uPCR given in mg/mmol. BMI given in kg/m^2. ECW and PV given in litres.

The median anti-PLA_2R level at the start of treatment was 703 U/mL (IQR 207-1090), the median uPCR level was 908 mg/mmol (IQR 591-1172), and the median serum albumin level was 22g/L (IQR 18-22). The median serum creatinine level was 135μmol/L (IQR 112-167) giving a median eGFR of 47mL/min/1.73m^2 (IQR 31-60).

The only statistically significant difference at baseline between those above and those below the age of 70 was renal function, with those under the age of 70 having a lower starting eGFR. Detailed demographics are presented in table 4.1.

4.3.2 Treatment

All 12 patients completed five sessions of IA within 7 days aiming for 2.5PV per session (plasma volumes treated are presented in table 4.2). Two patients missed one treatment during the treatment week due to mechanical faults with the machine, and both had their fifth session on the Saturday (day 6). One patient (PRISM02) failed treatment with increasing antibody level, proteinuria and worsening renal function. The decision was therefore made to start him on immunosuppression in the form of steroids and cyclophosphamide. Due to this PRISM02 was removed from outcome analysis in order to allow for outcomes based only on IA therapy to be assessed.

4.3.3 Clinical results

4.3.3.1 Anti-PLA_2R

At the end of treatment, the median level of serum antibody showed a reduction of 86.6% (IQR 83.1-91.5) compared pre-treatment on day 1. Table 4.3 & figure 4.2.

At week 2 (day 14), median antibody level increased from a baseline of 679 U/mL (IQR 191-1070) to 902 U/mL (IQR 522-2665). At week 12 the overall median level had reduced to 391 U/mL (IQR 266-4480). It increased to 508 U/mL (IQR 360-5150) at week 24 and reduced again to 138 U/mL (IQR 78-198) at week 52.

Patients less than 70 years old started to show an improvement in their anti-PLA_2R levels at week 12 with their median level reducing to 391 U/mL (IQR 265-2059). For patients above the age of 70, the median level did not improve until week 24, with a median level at week 12 of 2914 U/mL (IQR 282-11648) and 439 U/mL (IQR 330-7881) at week 24. Data was available for only two patients at week 52, one in each age group (as PRISM02 removed from the analysis). The median anti-PLA_2R level for the patient in the younger age group was 18 U/mL compared to 258 U/mL in the patient over the age of 70. Figure 4.3. Table 4.4 for results.

Treatment day	Antibody		Total IgG	
	Median	IQR	Median	IQR
Day 1 pre	0.00	0.00-0.00	100.00	100.00-100.00
Day 1 post	47.1	32.8-63.0		
Day 2 pre	30.3	16.3-44.7	11.38	8.86-13.04
Day 2 post	68.4	58.0-81.0		
Day 3 pre	37.9	20.3-48.1	9.36	7.55-13.87
Day 3 post	74.8	72.9-78.6		
Day 4 pre	54.8	44.6-60.2	8.31	6.56-9.43
Day 4 post	81.5	75.6-86.0		
Day 5 pre	58.3	46.7-63.5	7.48	5.53-8.31
Day 5 post	86.6	83.1-91.5		

Table 4.3 - median (IQR) percentage reduction in serum anti-PLA_2R antibody and total serum IgG compared to pre-treatment levels on day 1

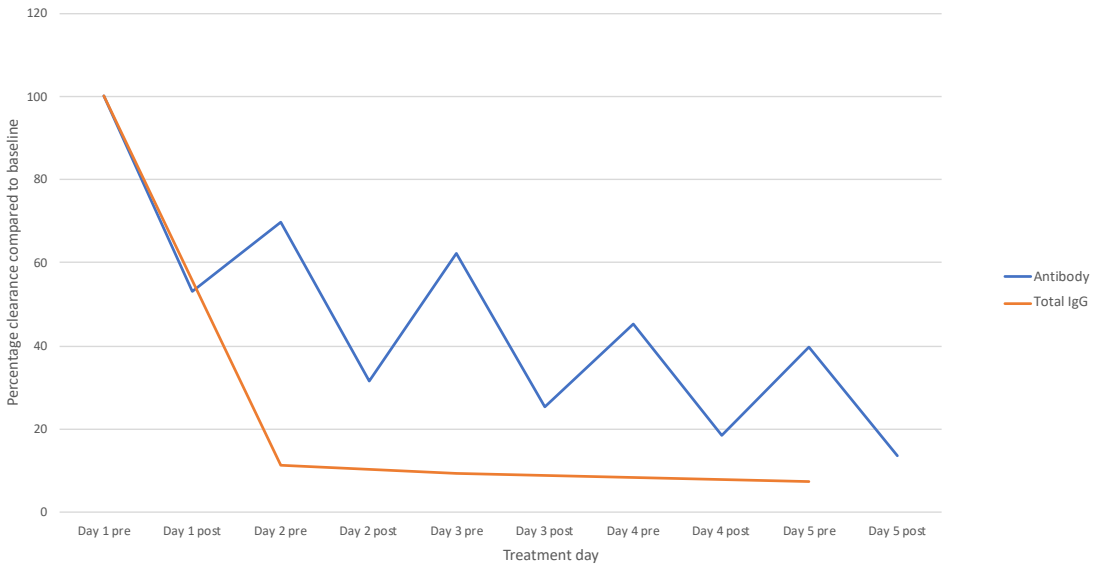

Figure 4.2 – Antibody clearance during treatment week. Percentage antibody change from baseline during immunoadsorption treatment week.

4.3.3.2 Proteinuria

Patients remained nephrotic until after week 24. At week 24 the median uPCR was 779mg/mmol (IQR 473-941) dropping to 396mg/mmol (IQR 263- 528).

At baseline, patients over the age of 70 had a higher median uPCR compared to those under the age of 70 although the difference was not significant; 832mg/mmol (IQR 655-1102) and 663mg/mmol (IQR 510-1052) respectively.

For patients under the age of 70 years old, the uPCR showed a continuous downward trend with a median level of 643mg/mmol (IQR 471-679) at week 12, 493mg/mmol (IQR 473-941) at week 24 and the level was 131mg/mmol at week 52. Patients above the age of 70 years old had an initial deterioration in their proteinuria with a median level of 1043mg/mmol (IQR 908-1262) at week 12. At week 24 this had improved back to baseline with a median level of 835mg/mmol (IQR 700-1043). At week 52 this had improved further to 660mg/mmol.

4.3.3.3 Albumin

Serum albumin levels showed an overall improvement compared to baseline. At week 12 rising to 28g/L (IQR 27-30) at week 52.

For patients above the age of 70, there was an initial increase from baseline with a median level at week 12 of 24g/L (IQR 19-26). The level then remained stable with median serum albumin of 24mg/mmol (IQR 21-24) at week 24 and a level of 25g/L at week 52.

In patients below the age of 70, in serum albumin there was a non-significant difference compared to baseline with a median level of 21g/L (IQR 19-24) at week 12, 23g/L (IQR 22-27) at week 24 and 31g/L at week 52. There was no significant difference between the age groups.

	Follow up	Overall	Above 70 years old	Less than 70 years old	Sig.
n (%)		11 (100)	4 (36.4)	7 (63.6)	0.008
Anti-PLA_2R	0	679.00 [190.50, 1070.00]	750.00 [150.50, 6415.75]	679.00 [309.00, 762.50]	0.850
	2	902.00 [521.50, 2665.00]	876.00 [685.75, 6201.50]	1188.00 [522.00, 2665.00]	1.000
	12	391.00 [265.50, 4480.00]	2914.00 [281.75, 11647.50]	391.00 [265.00, 2058.50]	0.571
	24	508.00 [360.00, 5150.00]	439.00 [330.25, 7881.00]	1530.00 [360.00, 5150.00]	0.806
	52	138.00 [78.00, 198.00]	258.00 [258.00, 258.00]	18.00 [18.00, 18.00]	0.317
Creatinine	0	138.00 [107.50, 179.50]	173.50 [124.50, 214.50]	131.00 [107.50, 146.00]	0.345
	12	150.50 [107.25, 205.75]	186.50 [140.75, 235.50]	126.50 [107.25, 165.25]	0.522
	24	145.00 [135.00, 199.00]	180.00 [128.25, 237.50]	145.00 [135.00, 187.00]	0.624
	52	105.00 [100.50, 109.50]	96.00 [96.00, 96.00]	114.00 [114.00, 114.00]	0.317
eGFR	0	45.50 [31.25, 53.00]	27.00 [25.50, 29.50]	50.00 [45.50, 60.50]	0.030
	12	40.00 [26.50, 62.50]	27.00 [24.00, 38.00]	51.50 [39.00, 62.50]	0.283
	24	32.00 [32.00, 49.00]	29.00 [24.25, 40.25]	42.00 [32.00, 49.00]	0.319
	52	63.00 [61.00, 65.00]	67.00 [67.00, 67.00]	59.00 [59.00, 59.00]	0.317
uPCR	0	691.00 [553.00, 1052.00]	831.50 [655.25, 1102.00]	663.00 [510.00, 1051.50]	0.571
	12	650.00 [558.00, 1014.50]	1042.50 [908.00, 1261.50]	643.00 [471.00, 678.50]	0.089
	24	779.00 [473.00, 941.00]	834.50 [700.00, 1042.50]	493.00 [473.00, 941.00]	0.624
	52	395.50 [263.25, 527.75]	660.00 [660.00, 660.00]	131.00 [131.00, 131.00]	0.317
Albumin	0	20.00 [19.00, 21.50]	21.50 [18.50, 22.75]	20.00 [19.00, 20.50]	0.340
	12	22.00 [18.50, 25.50]	24.00 [18.75, 26.00]	21.00 [18.50, 23.50]	0.635
	24	23.00 [22.00, 25.00]	23.50 [20.75, 24.25]	23.00 [22.00, 27.00]	0.805
	52	28.00 [26.50, 29.50]	25.00 [25.00, 25.00]	31.00 [31.00, 31.00]	0.317
IgG	0	3.27 [1.86, 4.74]	4.33 [2.73, 5.47]	3.16 [1.86, 3.96]	0.571
	12	5.34 [3.42, 6.75]	5.94 [5.25, 6.89]	3.59 [3.14, 6.54]	0.345
	24	6.03 [4.51, 6.96]	5.83 [5.31, 6.03]	6.76 [3.65, 7.55]	0.655
	52	9.54 [8.06, 11.02]	6.58 [6.58, 6.58]	12.50 [12.50, 12.50]	0.317
ESR	0	45.00 [36.50, 58.50]	48.50 [38.50, 71.50]	45.00 [33.00, 54.50]	0.571
	12	46.00 [35.50, 75.00]	57.50 [43.50, 82.75]	39.00 [30.50, 63.50]	0.298
	24	56.50 [38.50, 86.00]	43.00 [38.50, 64.00]	70.00 [40.00, 89.00]	0.655
	52	39.50 [39.25, 39.75]	39.00 [39.00, 39.00]	40.00 [40.00, 40.00]	0.317
EQ5D	0	1.00 [0.83, 1.00]	0.93 [0.84, 1.00]	1.00 [0.90, 1.00]	0.827
	12	1.00 [0.83, 1.00]	0.83 [0.74, 0.89]	1.00 [1.00, 1.00]	0.080
	24	1.00 [0.69, 1.00]	0.94 [0.79, 1.00]	1.00 [0.69, 1.00]	0.788
	52	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	1.00 [1.00, 1.00]	NaN
VAS	0	75.00 [70.00, 80.00]	75.00 [67.50, 80.00]	75.00 [70.00, 87.50]	0.584
	12	77.00 [72.50, 85.00]	73.00 [65.00, 79.50]	78.00 [76.00, 85.00]	0.345
	24	77.00 [64.00, 90.00]	78.50 [70.25, 82.50]	75.00 [64.00, 93.00]	0.806
	52	75.00 [67.50, 82.50]	90.00 [90.00, 90.00]	60.00 [60.00, 60.00]	0.317

Table 4.4 - results at day 1 (week 0) and weeks 12, 24 and 52 for all patients except PRISM02

All values given as median (IQR) unless otherwise stated. eGFR - estimated glomerular filtration rate; IgG - immunoglobulin G; ESR - erythrocyte sedimentation rate; VAS - Visual acuity score

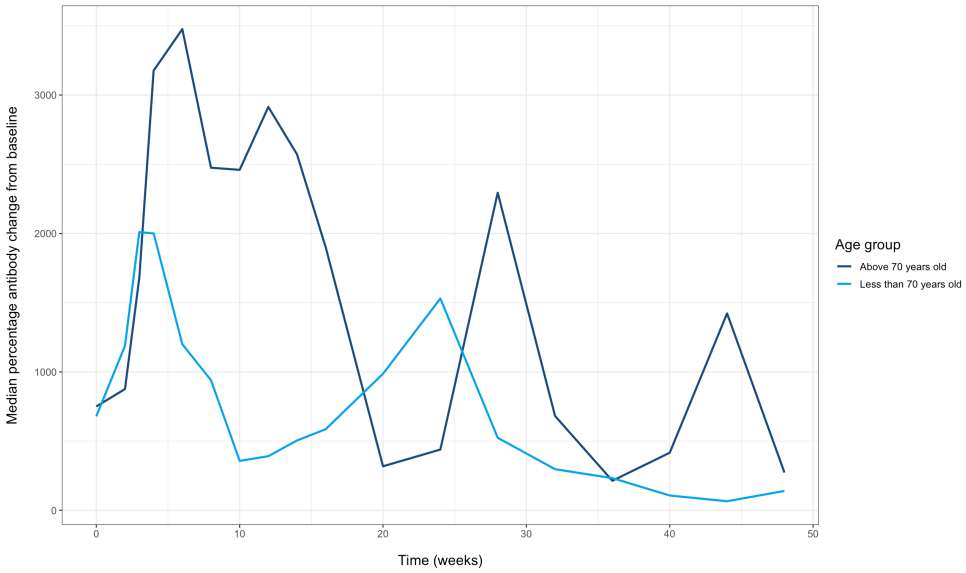

Figure 4.3 – Anti-PLA_2R change in response to immunoadsorption. Median percentage anti-PLA_2R change compared to baseline by age group.

4.3.3.4 Renal function

At week 12, there was a deterioration in renal function with a median eGFR of 40mL/min/1.73m^2 (IQR 27-63). At week 24 the eGFR dropped to 32mL/min/1.73m^2 (IQR 32-49) before increasing to 63mL/min/1.73m^2 (IQR 61-65) at week 52. Baseline eGFR was 45mL/min/1.73m^2 (IQR 31-53). For patients above the age of 70 the baseline eGFR was 27mL/min/1.73m^2 (IQR 26-30), and for those below the age of 70, it was 50mL/min/1.73m^2 (IQR 46-61), a significant difference with a p-value of 0.030.

For patients above the age of 70 years old, the renal function remained stable for week 12 and 24 with an eGFR of 27mL/min/1.73m^2 (IQR 24-38) and 29mL/min/1.73m^2 (IQR 24-40) respectively, before increasing to 67mL/min/1.73m^2 at week 52.

For patients below the age of 70, the renal function remained stable from baseline to week 12 with a median eGFR of 52mL/min/1.73m^2 (IQR 39-63). At week 24 the renal function had deteriorated to an eGFR of 42mL/min/1.73m^2 (IQR 32-49) before improving by week 52 with an eGFR of 59mL/min/1.73m^2.

4.3.3.5 Time to response

Using the definition that time to response is an antibody level of less than 50% of baseline on two consecutive occasions, the overall median time to antibody response was 28 weeks (IQR 11-40). For patients older than 70 years old the median time to antibody response was 30 weeks (IQR 16-43), and in patients younger than 70 years old it was 28 weeks (IQR 11-34). There was no statistical significance between these groups with a log-rank score of 0.909. See Figure 4.4.

With response defined as any improvement in antibody on three consecutive occasions, the overall median time to response was 8 weeks (IQR 4-21). For

patients over the age of 70 this was 21 weeks (12-31) and for patients younger than 70 years old, the median time to response was 4 weeks (4-11). This, however, was not statistically significant with a log-rank score of 0.184. Figure 4.5.

Univariable analysis as described in the methods revealed no statistically significant variables (table 4.5).

	< 50% of baseline on two occasions		Antibody reduction three occasions	
	HR (95% CI)	P value	HR (95% CI)	P value
Age group	1.10 (0.20-6.07)	0.909	2.74 (0.54-13.88)	0.224
Time to treatment from biopsy	1.00 (1.00-1.01)	0.379	1.00 (0.99-1.00)	0.41
Antibody neg post treatment	0.30 (0.03-2.56)	0.269	0.85 (0.20-3.59)	0.829
Percentage antibody clearance	1.03 (0.90-1.18)	0.654	1.04 (0.94-1.16)	0.426
Hypertension	1.44 (0.26-7.94)	0.672	0.60 (0.14-2.56)	0.489
Diabetes	6.91 (0.96-49.87)	0.055	0.62 (0.08-5.03)	0.652
uPCR	1.00 (1.00-1.00)	0.611	1.00 (1.00-1.00)	0.746
Albumin	0.91 (0.71-1.15)	0.421	1.11 (0.90-1.35)	0.333
Sodium	1.11 (0.87-1.42)	0.411	0.89 (0.72-1.10)	0.283
Potassium	1.17 (0.36-3.83)	0.791	1.56 (0.63-3.89)	0.339
Anti-PLA_2R	1.00 (1.00-1.00)	0.293	1.00 (1.00-1.00)	0.323
CRP	0.77 (0.44-1.35)	0.365	0.52 (0.23-1.16)	0.109
ESR	1.03 (0.99-1.07)	0.123	0.97 (0.93-1.01)	0.089

Table 4.5 - Univariate analysis of time to antibody response.
uPCR - urinary protein:creatinine ratio; CRP - C-reactive protein; ESR - Erythrocyte sedimentation rate

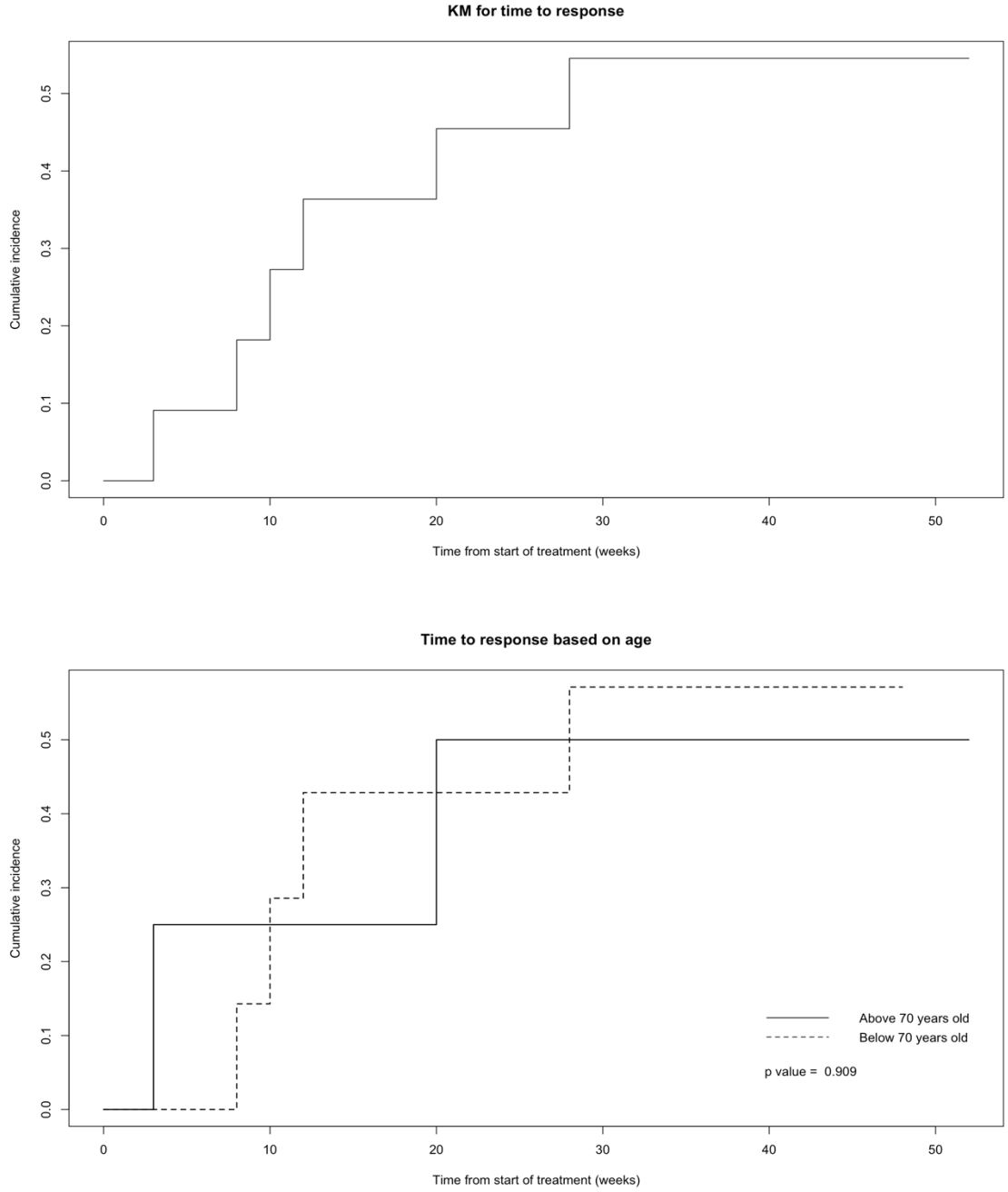

Figure 4.4 – Time to response. Cumulative incidence plot for time to response as defined by an antibody level of less than 50% of baseline on two consecutive occasions. Top plot is total population. Bottom plot is by age group.

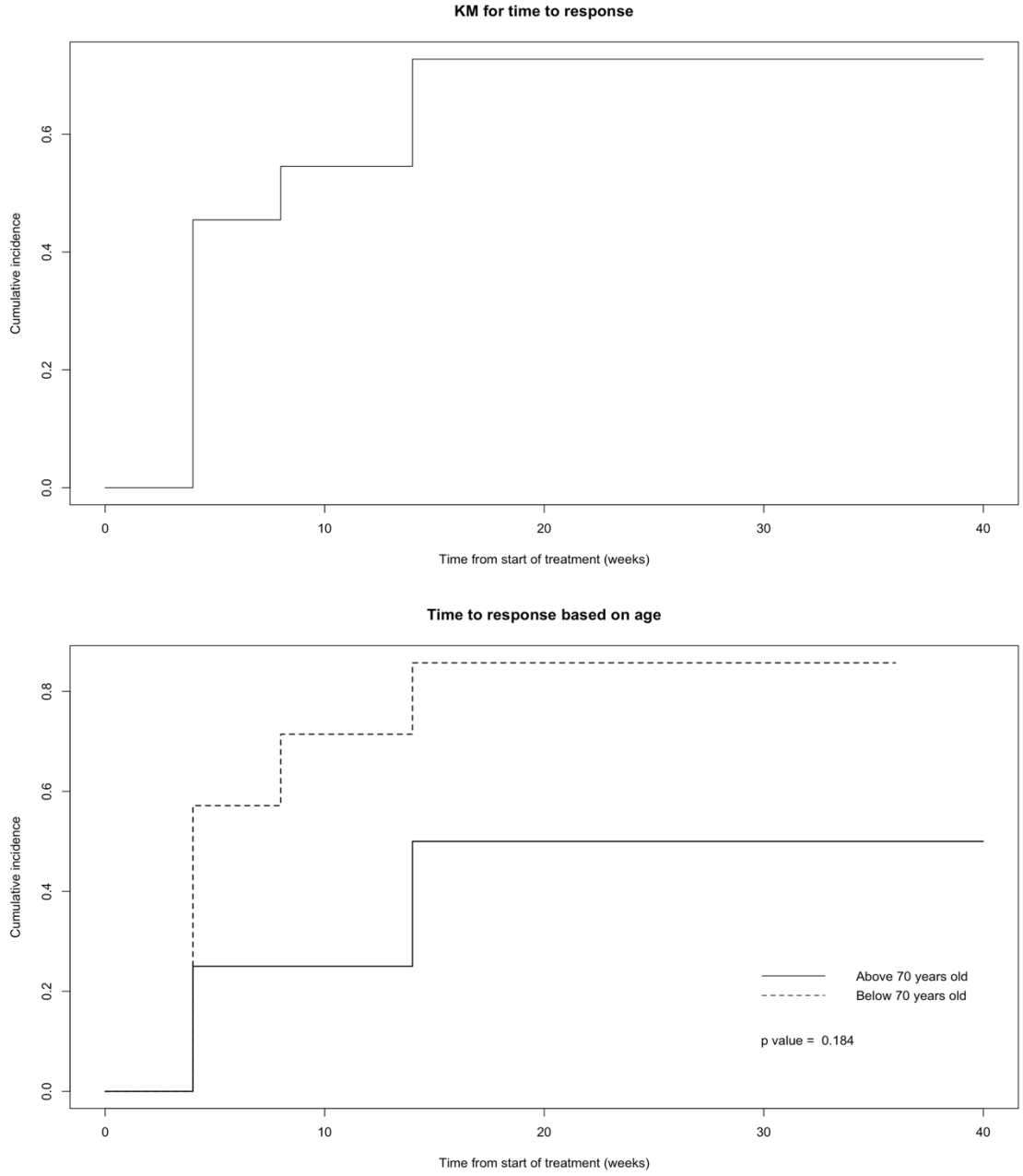

Figure 4.5 – Time to response. Cumulative incidence plot for time to response as defined by any improvement in antibody on three consecutive occasions. Top plot is total population. Bottom plot is by age group.

4.3.3.6 QoL

The overall median EQ5D utility values did not change, being 1.00 at each time point. For patients less than 70 years old this was also the case. Patients over the age of 70 however started with a lower median utility value of 0.93 (IQR 0.84-1.00), this decreased to 0.83 (IQR 0.74-0.89) at week 12, before rising to 0.94 (IQR 0.79-1.00) at week 24 and a utility score of 1.00 at week 52. There was no statistical significance between the groups.

The overall Visual Acuity Score (VAS) remained stable at each time point, starting at a median of 75 (IQR 70-80), rising slightly to 77 (IQR 73-85) at week 12 and 77 (IQR 64-90) at week 24. At week 52 this had decreased slightly to a value 75. For patients under the age of 70, the median VAS pre-treatment was 75 (IQR 70-88), rising to 78 (IQR 76-85) before reducing to 75 (IQR 64-93) at week 24 and 60 at week 52. In contrast, for those patients over the age of 70, the median VAS was 75 (IQR 68-80) pre-treatment, decreasing slightly to 73 (IQR 65-80) at week 12 before rising to 79 (IQR 70-83) at week 24 and a utility score of 90 at week 52.

Using Paretian principles, it was found that at week 12, 72.7% of patients had no change in their quality of life or were better, as assessed by the EQ5D. At week 24, this had risen to 88.9% and by week 52 was 100%. For patients above the age of 70 years old, only 25% of patients had no change, none were better, 50% were worse, and 25% were mixed. By week 24, 100% of patients reported that there was no change or they were better. At week 52, the one patient reported that they had no change to their quality of life. For patients under the age of 70, 100% reported no change or better at week 12. At week 24, 80% of patients reported no change whilst one patient (20%) reported that they were worse. At week 52, the one patient reported no change to their quality of life. Table 4.6.

Follow up	Health state change	Overall (n = 11)	< 70 yrs old	> 70 yrs old	Sig.
Week 12	No change	7 (63.6)	6 (85.7)	1 (25.0)	0.063
	Better	1 (9.1)	1 (14.3)	0 (0.0)	
	Worse	2 (18.2)	0 (0.0)	2 (50.0)	
	Mixed	1 (9.1)	0 (0.0)	1 (25.0)	
Week 24	No change	6 (66.7)	4 (80.0)	2 (50.0)	0.155
	Better	2 (22.2)	0 (0.0)	2 (50.0)	
	Worse	1 (11.1)	1 (20.0)	0 (0.0)	
	Mixed	0 (0.0)	0 (0.0)	0 (0.0)	
Week 52	No change	2 (100.0)	1 (100.0)	1 (100.0)	1.000
	Better	0 (0.0)	0 (0.0)	0 (0.0)	
	Worse	0 (0.0)	0 (0.0)	0 (0.0)	
	Mixed	0 (0.0)	0 (0.0)	0 (0.0)	

Table 4.6 - Change in EQ5D scores based on the Pareto principle.

			Overall (n = 12)			Less than 70 years old (n = 7)			Over 70 years old (n = 4)		
Dimension	Wk	n	1	2	3	1	2	3	1	2	3
Mobility	0	11	10 (90.9)	1 (9.1)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	12	11	9 (81.8)	2 (18.2)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)
	24	9	7 (77.8)	2 (22.2)	0 (0.0)	4 (80.0)	1 (20.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	52	2	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
Self-care	0	11	11 (100.0)	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
	12	11	10 (90.9)	1 (9.1)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	24	9	8 (88.9)	1 (11.1)	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	52	2	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
Activities	0	11	9 (81.8)	2 (18.2)	0 (0.0)	6 (85.7)	1 (14.3)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	12	11	8 (72.7)	3 (27.3)	0 (0.0)	6 (85.7)	1 (14.3)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)
	24	9	5 (55.6)	4 (44.4)	0 (0.0)	3 (60.0)	2 (40.0)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)
	52	2	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
Pain	0	11	9 (81.8)	2 (18.2)	0 (0.0)	5 (71.4)	2 (28.6)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)
	12	11	9 (81.8)	2 (18.2)	0 (0.0)	6 (85.7)	1 (14.3)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	24	9	6 (66.7)	3 (33.3)	0 (0.0)	3 (60.0)	2 (40.0)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	52	2	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
Anxiety	0	11	9 (81.8)	2 (18.2)	0 (0.0)	6 (85.7)	1 (14.3)	0 (0.0)	3 (75.0)	1 (25.0)	0 (0.0)
	12	11	8 (72.7)	3 (27.3)	0 (0.0)	6 (85.7)	1 (14.3)	0 (0.0)	2 (50.0)	2 (50.0)	0 (0.0)
	24	9	6 (66.7)	3 (33.3)	0 (0.0)	3 (60.0)	2 (40.0)	0 (0.0)	3 (75.0)	1 (20.0)	0 (0.0)
	52	2	2 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)

Table 4.7 - EQ5D dimensions by follow up and age group. All results given in number (%). Wk - week

When analysed separately, overall each dimension saw an increase in the proportion of level 2 responses (some problems) at weeks 12 and 24 compared to baseline. By week 52 all patients described 'no problems' (level 1) for each dimension. In patients under the age of 70, the biggest changes were seen in activities, anxiety and pain. In patients over the age of 70, the biggest changes were in mobility, activities and anxiety. Table 4.7.

4.3.3.7 SAEs / AEs

All 12 patients experienced adverse events during the trial with 83 in total. Of these 5 (6.0%) were deemed to be related to the immunoadsorption. These five adverse events consisted of lightheadedness for one patient, headaches in two patients, a viral URTI in one patient and fatigue in one patient. None of these adverse events were deemed serious and did not result in treatment being interrupted or aborted. All resolved with no sequelae. There was no difference in the number of adverse events by age group.

During treatment, the most common adverse event was isolated mild pyrexia not associated with any evidence of infection or sepsis. All four occurred overnight and had resolved by the morning with no sequelae. The next most common adverse event was a headache. In all cases, this was well controlled with paracetamol and did not affect subsequent treatments. Following treatment, the most common adverse events were viral URTIs followed by LRTI. For the LRTI, four patients required oral antibiotics and responded well to a single course. Two patients required a short course of oral glucocorticoids along with the antibiotics (one patient with asthma and one patient with COPD). Again, both responded well to the single course. Table 4.8.

There have been four serious adverse events in four patients, none of which were deemed to be related to the treatment. PRISM01 had previously suffered

with lower limb cellulitis and had been admitted for intravenous antibiotics on a number of occasions prior to entry into the trial. During the follow-up period, he again developed lower limb cellulitis and required admission for IV antibiotics to which he responded well. During the treatment week, PRISM04 developed a recurrence of his urinary retention secondary to benign prostatic hypertrophy, for which he had previously been hospitalised. He was catheterised and admitted for IV antibiotics for presumed urinary tract infection. He was discharged home three days later with no catheter and has been well since. PRISM11 developed shortness of breath after stopping her furosemide after week 28 follow up. She was found to have pulmonary oedema and was also treated medically for a non-ST elevation myocardial infarction (NSTEMI).

	Adverse event	Number of events	Number of patients experiencing event
During treatment	Hypokalaemia	1	1
	Hyperkalaemia	1	1
	Headache	3	3
	Pyrexia	4	4
	Felt hot with no pyrexia	1	1
	Cramp	2	1
	Vasovagal	3	2
	Nausea / vomiting	4	2
	Diarrhoea	2	2
	Dry skin	1	1
	Femoral line pain	1	1
	Dizziness	2	1
	Post-micturition hypotension	1	1
	Viral URTI	2	2
	Dry cough	2	2
	Right knee pain	1	1
	Allergy to dressing	1	1
Post-treatment	Viral URTI	6	5
	LRTI	4	4
	Flash eye burn	1	1
	Cellulitis	1	1
	Lipoma	1	1
	Nasal lumpectomy	1	1
	Contact dermatitis	1	1
	Lower limb lump	1	1
	Upper limb pins and needles	1	1
	Infective exacerbation of	1	1
	Dry mouth	1	1
	Dry eyes	1	1
	Nausea / vomiting	2	1
	Vasovagal	1	1
	Easy bruising	1	1
	Dizziness	3	2
	Short of breath	1	1
	Diarrhoea	1	1
	Lethargy / fatigue	2	2
	Lumbar back pain	2	2
	Folate deficiency	1	1
	Shoulder MSK pain	1	1
	Felt hot - no recorded pyrexia	1	1
	Cramp	3	3
	Viral gastroenteritis	2	2
	Haemorrhoids	1	1
	Constipation	1	1
	Mechanical fall	3	1
	Forearm laceration - traumatic	1	1
	Headache	1	1
	Allergy to dressing	1	1
	Painful red eye - likely	1	1
	Right sided loin pain	1	1

Table 4.8 - number of adverse events and patients experiencing each event, during treatment and following treatment.

4.4 Discussion

Treatment for autoimmune membranous nephropathy at present uses empirical immunosuppression with the aim of eliminating a pathogenic antibody. Management has changed little in over 20 years, and there remains no directed therapy for autoimmune membranous nephropathy[5,52,53].

Previous studies, with various immunosuppressive agents, have shown the importance of removing the anti-PLA_2R antibody on response to the treatment[41,42]. Accumulating evidence now suggests that the anti-PLA_2R antibody is particularly pathogenic making it an attractive target for novel treatments[38,41-43]. The advantage of immunoadsorption therapy over alternative apheresis techniques is its highly efficient removal of specific molecules and immunoglobulins. In this study we have used peptide-GAM immunoadsorption, developed to target IgG, with the aim of directly removing the anti-PLA_2R antibody in patients with autoimmune membranous nephropathy.

All patients completed the full five sessions of immunoadsorption and saw a significant decrease in their total IgG and anti-PLA_2R levels. Between treatments, there was a rebound of the anti-PLA_2R level, but over the week the overall level continued to reduce. The treatment itself was well tolerated with only mild adverse events attributed to the procedure, none which impacted on the treatment.

In general, the treatment was found to be safe, none of the SAEs were deemed to be as a result of, or exacerbated by, the treatment. Many of the adverse events could be expected in the normal population such as LRTI in patients with COPD or asthma. As this was a single-arm study, we have not compared the adverse event rate to a control group. However, many of the adverse events can be accounted for by the patient's co-morbidities, and all were

considered to be mild with no deviation from the protocol or treatment required.

All patients experienced a significant increase in their antibody level in the week following treatment, and this was accompanied by an increase in uPCR and also a decrease in the quality of life scores reported by the patient. Following this, the antibody levels appeared to stabilize for a number of months before beginning to reduce in patients who seem to respond to the treatment. Throughout this time, even though proteinuria showed a general improvement, the patients remained nephrotic. This is not unexpected given that previous studies have shown a lag time between reduction in antibody levels and clinical response. Reassuringly, despite the continued nephrotic range proteinuria and the raised antibody level, overall the renal function, serum albumin and ESR showed an improvement at each time point. Total IgG started to improve within the first two weeks and had almost normalized at week 12 although it did take to week 24 for the majority of patients to be within the normal range.

The first patient treated became antibody negative from week 10 and this continued until his last follow up. In fact, 15 months after treatment and over a year from becoming antibody negative, he remains in partial remission by proteinuria and continues to have an antibody of less than 10 U/mL. This patient has remained steroid and immunosuppression naïve and continues to remain clinically well.

After successfully completing treatment, PRISM02 continued to see a deterioration in his renal function, antibody and proteinuria. This eventually led to his lead physician starting immunosuppression in the form of steroids and pulsed cyclophosphamide. This was associated with normalisation of his anti-PLA_2R levels, but he also suffered from significant complications of the treatment. He developed marked weight gain and anaemia, along with a

prolonged hospital admission for viral and atypical pneumonia. This was further complicated with the development of multiple peripheral pulmonary emboli.

The third patient to complete follow up did so without normalisation of his antibody level although on a slow downward trend. Clinically he remains stable with no plans for further treatment at present.

There certainly does appear to be a difference in response to treatment based on age although this is not statistically significant, albeit with small sample numbers. Patients below the age of 70 seem to respond to treatment earlier with a more marked improvement in proteinuria and serum albumin.

This apparent difference between the age groups would fit with our current understanding of the disease pathogenesis. It has already been shown that the epitope itself shares an amino acid sequence with common pathogens such as Clostridium; repeated interaction with which in susceptible individuals, could potentially lead to loss of tolerance and the development of pathogenic autoantibodies[31]. Recent evidence suggests that this is a gradual process resulting in an ever more severe phenotype. In younger patients, the antibody was directed against a limited portion of the PLA_2R protein (only the cysteine-rich domain epitope) whilst in older patients, the anti-PLA_2R antibody demonstrated activity to the whole protein (cysteine-rich, C-type lectin domain 1, and C-type lectin domain 7 domains). With this increasing spread of antibody activity came a deterioration in clinical outcomes, suggesting that as the immune response develops activity over time to the whole PLA_2R extracellular epitope, the disease becomes more severe and difficult to treat[255]. Certainly, in our cohort, patient 2 who failed immunoadsorption therapy and required further immunosuppression was in the older age group, and despite there not being statistical significance, the trend clinically is that the younger patients seem to be responding earlier than the older patients.

This study does have a number of limitations. It is a single arm prospective study with all the bias inherent in such a trial given there is no control arm to compare against. The sample size is small with only one female participant. As autoimmune membranous nephropathy is a rare disease, given our narrow inclusion criteria (patients with significantly raised antibody titres and proteinuria but otherwise well), and due to time limitations, it was necessary to recruit all patients who fit the criteria, and therefore we were unable to design out these biases. Here we have presented, outcome data at 6 months, with the majority of patients yet to complete their one year follow up. It is apparent that the antibody reduction following immunoadsorption therapy is a slowly progressive decline with the clinical response following afterwards. It is likely that a prolonged follow up will be required to fully assess the benefits of IA, particularly in the event of antibody recurrence. It is reassuring that PRISM01 responded and not only became antibody negative but has had a sustained antibody remission for over a year.

4.5 Conclusion

Here we have shown that immunoadsorption can successfully reduce the anti-PLA_2R antibody in patients with autoimmune membranous nephropathy and can do so safely and has the potential to provide an alternative treatment for the disease without the need for steroids or immunosuppression.

Immune system modelling in autoimmune membranous nephropathy using flow cytometry

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5.1 Introduction

Flow cytometry is a laboratory technique that uses a device to identify the number and size of cells or particles in a given sample. All flow cytometry devices follow a similar basic principle although as the technology evolves, the range of particles that can be assessed per sample is growing rapidly. The technique itself involves a number of components working in tandem; the fluidics, optics and detection.

In order for the sample to be analysed, a single particle at a time is delivered to the optics. The sample is directed into the flow of sheath fluid which then passes through to the interrogation point. Here the laser is fired into the stream of particles and interacts with each individual cell. This interaction is then picked up by the detector and recorded. Computer software is then used to display and analyse these interactions[256,257].

At its most basic, the flow cytometry can detect the size and the granularity of each particle which is the displayed as forward scatter (size of the cell) and side scatter (internal complexity / granularity of the cell) [256,257]. This is useful for taking a macro view of the sample allowing for the ability to differentiate between different types of cells such as in the immune system, differentiating between lymphocytes and granulocytes. However, for more nuanced analysis a further level of preparation and detection is required using fluorescence. With the addition of fluorochromes to the sample which will tag the cell in question, using flow cytometers with multiple wavelength detectors allows for the identification and quantification of a range of cell subsets from the sample. Here, light is absorbed by the fluorochrome at a particular wavelength specific to that compound. This absorption of light leads to excitation of the molecule with an electron rising to a higher state from the ground state. This quickly decays releasing a photon which can then be detected. This means that the more detectors used in the device, the higher the number of fluorochromes

that can be used and the more specific cells can be identified[256-258]. A consideration with increasing fluorochromes used, however, is the issue of spectral overlap. Each fluorochrome will emit over a range of wavelengths with some overlap. In order to adjust for this, and necessary prior to any analysis, a method of subtracting a portion of one fluorochromes signal from another is used, known as colour compensation[256].

Once the raw data has been obtained, generally as an FCS 3.0 format, a dedicated computer program is required for the analysis. Given the heterogeneity of samples used in flow cytometry and the vast array of available combinations of fluorochromes available there is no commonly agreed upon strategy for analysis. The software allows for the display of the data with dot plots and histograms. Dot plots allow for two fluorochromes to be displayed against each other, whilst the histogram will display only one fluorochrome. Using these outputs, regions can then be captured and identified, known as gating. Multiple plots can be connected in tandem from the whole plot or from a specific gate within the plot, meaning cells defined by a number of fluorochromes can be identified. From these plots, statistics such as the number and proportion of the cells of interest can then be obtained.

The ability to use multiple fluorochromes on a single sample means that particular cell populations can now be readily identified. In the case of B cells, the proportion of subsets such as memory B cells, naïve B cells and plasmablasts can be investigated, making it powerful tool for the modelling of the immune system over time.

The use of immunoadsorption for autoimmune MN allows for a unique opportunity to study the change in the various components of the immune system over time, without using immunomodulating medications such as steroids and immunosuppression. Here we have used flow cytometry at four

time points following immunoadsorption therapy to model the immune system's response to treatment.

5.2 Methods

Flow cytometry was carried out on all 12 patients who underwent IA, and 9 healthy controls using frozen PBMCs (see appendix for PBMC separation and thawing SOPs). Healthy volunteers were recruited from the Manchester Renal Biobank 2016-2012 for which full ethics committee and R&D approval has been granted (REC reference 16/NW/0119). For the patients treated with IA, analysis was carried out on samples taken at day 1 pre-treatment, and weeks 4, 10 and 16.

For all samples, the FC500 MPL flow cytometer (Beckman Coulter, California, United States of America) was used. It uses a single blue 488nm argon laser with 5-colour analysis. Fluorochromes used were Fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), Electron coupled dye (ECD, also known as Phycoerythrin-Texas Red), R-phycoerythrin-Cyanin 5.5 (PC5.5) and R-phycoerythrin-Cyanin 7 (PC7). Each lymphocyte subset was analysed using a separate panel as per table 5.1 and full SOP in appendix allowing for further classification of each cell population using standard cell markers[259] (figure 5.1).

In the PLA_2R panel, we used a P28mer PLA_2R peptide (KGIFVIQSESLKKCIQAGKSVLTLENCK) with PC7 fluorochrome (the dominant B cell epitope located in the CysR domain[31]) and a scrambled peptide of this amino acid sequence (KVQKEAGCSVKGLIKSKIENLSCTFQIL) as a control. Both peptides were synthesised by Proimmune Ltd, Oxford, UK.

Full minus one analysis was carried out on two patients at all four time points and eight healthy controls. Full minus one analysis included B cell panel minus CD19, CD27, CD38 and IgD; T cell panel minus CD25, CD45 and Cd127; anti-PLA_2R panel minus PLA_2R antigen; and the monocytes panel minus CD56. A negative control for two patients at all four time points was also carried out with no fluorochromes added. See appendix for results, gating, full minus one and negative control output plots. Quality control was carried out daily using

flow check beads as per SOP. All analysis and gating carried out in Flowing Software 2.5.1[260].

5.2.1 Statistical analysis

All results are the percentage of cells per region and presented as median (interquartile range). Significance tested using chi-squared for categorical variables, and Kruskal–Wallis one-way analysis of variance (ANOVA) for nonparametric continuous variables. Linear regression used to investigate the change over time in the patient cohort, with multivariable modelling to adjust for age at treatment, antibody level, change in antibody level compared to baseline and change in antibody level compared to previous time point. All statistical analysis carried out R statistical programme 3.5.1 [252].

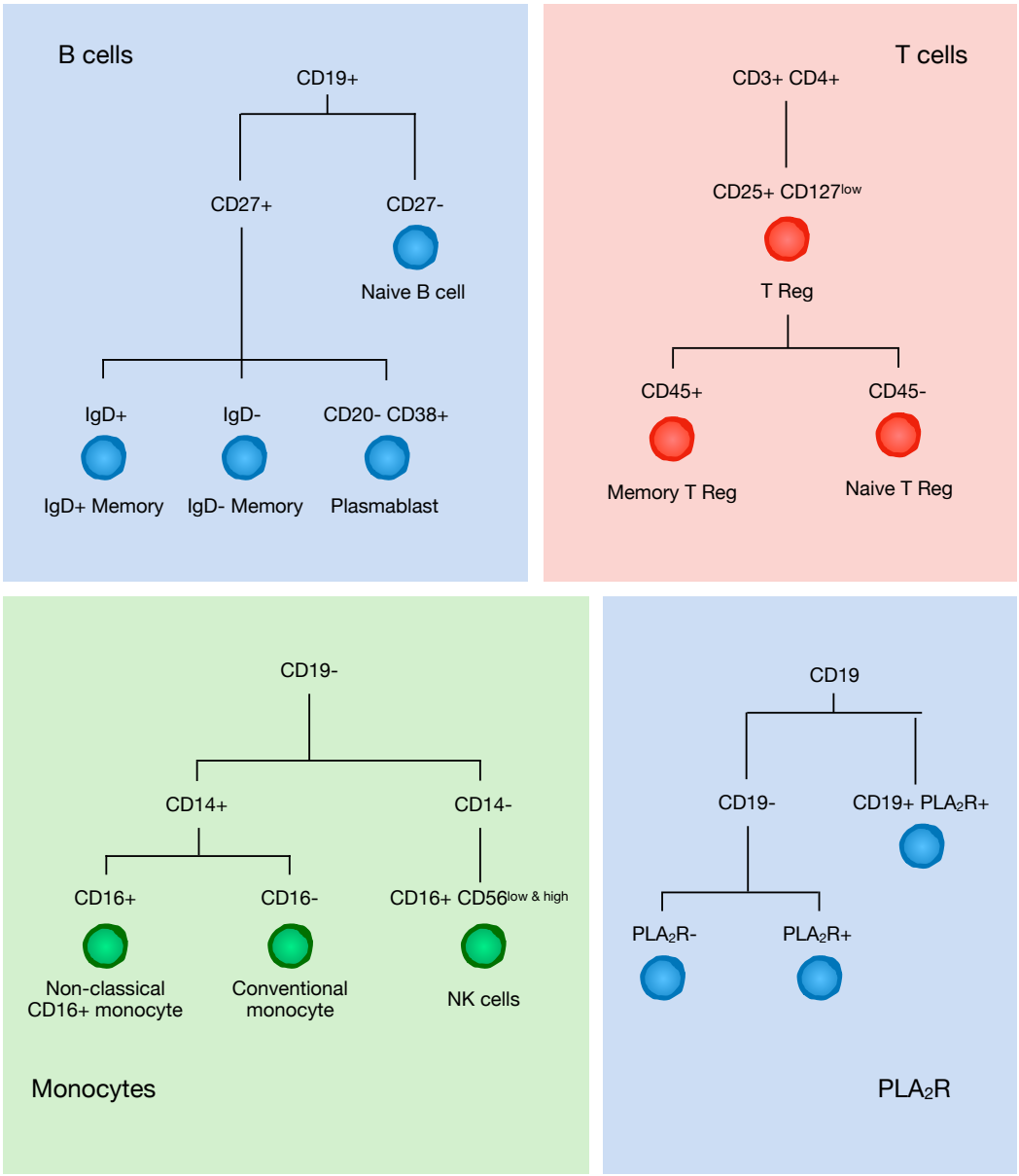

Figure 5.1 – Cell population subsets. The markers used to identify each cell population in the four panels used for flow cytometry

Fluorochrome	B Cells	T Cells	anti-PLA_2R B Cells	Monocytes
FITC	Anti-human IgD	CD45	CD19	CD45
PE	CD27	CD127	CD27	CD14
ECD	CD38	CD25	CD38	CD16
PC5.5	CD20	CD4	CD20	CD56
PC7	CD19	CD3	PLA_2R antigen	

Table 5.1 - Fluorochromes used for each marker in lymphocyte subset panels

5.3 Results

There were nine healthy volunteers ranging in age from 25 to 62 years old, with 5 males and 4 females, and 12 patients. See Table 5.2 and figures 5.2 – 5.4 and appendix for full results.

5.3.1 B cells

In the B cell panel, the only significantly different cell population at day 1 between the healthy volunteers and the patients was IgD+ memory B cells. Here the control group had a median percentage of region cells of 1.76 (IQR 7.39-17.37), and the patients had a median percentage of 2.77 (IQR 0.08-5.91), with a p-value of 0.027. Despite it not being significant, there does also appear to be a tendency towards a lower level of IgD- memory B cells in the patient population as seen in the boxplot (figure 5.2). Over time, again despite no statistical significance demonstrated, there appears to be a reduction in naïve B cells and an increase in IgD- memory B cells as seen in figure 5.3.

5.3.2 PLA2R

In the anti-PLA_2R panel, the only cell population with a significant difference was the CD19- PLA_2R- B cell population. For this population, the control group had a median percentage per region of 90.19 (IQR 89.78-92.53), and the patient group had a median percentage per region of 70.36 (IQR 65.84-85.48), with a p-value of 0.003). Figure 2. Over time there did not appear to be any change in the PLA_2R positive B cell population as seen in figure 5.3 and no significance shown, with both univariable and multivariable linear regression (figure 5.4).

There was a demonstrated response to the scrambled peptide in the PLA_2R panel. However, this response was lower than the PLA_2R p28mer peptide, likely

		Healthy (n = 9)	Patient (n = 12)	Sig.
		B Cells		
IgD- memory b cell	Day 1	19.84 [11.69, 24.93]	19.88 [12.39, 83.08]	0.722
	Week 4		18.86 [6.87, 91.95]	
	Week 10		16.71 [12.47, 40.15]	
	Week 16		65.66 [15.70, 92.49]	
IgD+ memory	Day 1	11.76 [7.39, 17.37]	2.77 [0.08, 5.91]	0.027
	Week 4		1.36 [0.00, 12.30]	
	Week 10		6.81 [0.00, 14.42]	
	Week 16		0.07 [0.01, 6.73]	
Naive	Day 1	53.18 [51.22, 62.25]	49.15 [0.39, 67.40]	1.000
	Week 4		25.94 [0.13, 48.46]	
	Week 10		47.16 [0.59, 64.15]	
	Week 16		7.67 [0.14, 58.47]	
Plasmablasts	Day 1	10.06 [6.95, 16.22]	7.94 [6.94, 23.50]	0.546
	Week 4		6.25 [4.72, 7.92]	
	Week 10		5.84 [3.99, 9.88]	
	Week 16		7.70 [5.64, 9.70]	
		PLA2R panel		
CD19- PLA2R+	Week 1	2.18 [1.39, 4.33]	0.36 [0.00, 1.91]	0.153
	Week 4		0.00 [0.00, 0.51]	
	Week 10		0.46 [0.00, 2.50]	
	Week 16		0.00 [0.00, 0.00]	
CD19+ PLA2R+	Week 1	4.04 [3.56, 7.45]	0.60 [0.00, 7.88]	0.317
	Week 4		0.00 [0.00, 0.40]	
	Week 10		0.57 [0.00, 4.40]	
	Week 16		0.00 [0.00, 0.00]	
CD19- PLA2R-	Week 1	90.19 [89.78, 92.53]	70.36 [65.84, 85.48]	0.003
	Week 4		70.64 [63.55, 83.09]	
	Week 10		71.34 [64.25, 78.20]	
	Week 16		68.94 [62.70, 84.43]	
		T Cells		
T Reg	Week 1	4.39 [3.98, 4.74]	4.12 [2.90, 5.51]	0.65
	Week 4		3.24 [2.29, 4.46]	
	Week 10		4.08 [2.98, 4.68]	
	Week 16		3.81 [3.23, 5.62]	
Naïve T Regs	Week 1	20.27 [16.62, 60.31]	51.78 [18.80, 64.25]	0.643
	Week 4		58.07 [46.98, 88.38]	
	Week 10		67.84 [45.59, 89.06]	
	Week 16		53.82 [18.62, 87.68]	
Memory T Regs	Week 1	79.73 [39.69, 83.38]	48.22 [35.75, 81.20]	0.643
	Week 4		41.93 [11.62, 53.02]	
	Week 10		32.16 [10.94, 54.41]	
	Week 16		46.18 [12.32, 81.38]	
		Monocytes		
CD16 monocytes	Week 1	0.54 [0.49, 1.29]	0.83 [0.76, 1.91]	0.209
	Week 4		0.56 [0.36, 0.79]	
	Week 10		0.70 [0.18, 1.10]	
	Week 16		0.72 [0.53, 1.17]	
Conventional monocytes	Week 1	1.87 [1.45, 2.30]	3.10 [2.55, 4.90]	0.063
	Week 4		4.91 [2.83, 7.88]	
	Week 10		3.42 [2.40, 3.87]	
	Week 16		2.76 [1.92, 4.45]	
NK cells	Week 1	5.46 [2.90, 5.67]	3.99 [2.25, 7.13]	0.79
	Week 4		2.72 [0.90, 5.09]	
	Week 10		2.72 [1.75, 3.56]	
	Week 16		2.87 [2.17, 4.25]	
		Anti-PLA_2R antibody		
Antibody	Day 1		702.50 [206.25, 1089.75]	
	Week 4		2710.00 [652.00, 6870.00]	
	Week 10		1529.50 [245.75, 9052.50]	
	Week 16		611.50 [298.75, 3472.50]	

Table 5.2 - Flow cytometry results. All presented in median and interquartile range unless otherwise stated

representing non-specific staining and background noise. See appendix for full results.

5.3.3 T cells

There were no significant differences noted between the control group and the patient group in the T cell panel. There was no statistical significance demonstrated; however, in the patient group, there appears to be a lower proportion of memory T Regs and a higher proportion of Naïve T Regs compared to the control group. Over time it appears that the T Regs decrease directly after treatment and then start to rise, and memory T Regs follow a similar pattern to the T Regs with a fall after treatment at week 4 and then week 10 before starting to rise at week 16. Naïve T Regs appear to rise after treatment before decreasing at week 16. Figure 5.3.

5.3.4 Monocytes

There were no statistically significant differences demonstrated between the patient and control group for any of the monocyte cell subsets. There was a tendency towards a higher level of conventional monocytes and a lower proportion of NK cells in the patient's population, although these were not statistically significant. Over time the proportion of conventional monocytes did not appear to change. The proportions of both the CD16+ monocytes and NK cells reduced following treatment before starting to rise from week 10 to week 16. Figure 5.3.

5.3.5 Linear regression

Linear regression revealed no significant change in cell subset populations over time at univariate level or in relation to antibody level or change in antibody level, either compared to baseline or to the previous time point. There was no

significant change in cell populations when adjusted for age. Table 5.3 and figure 5.4.

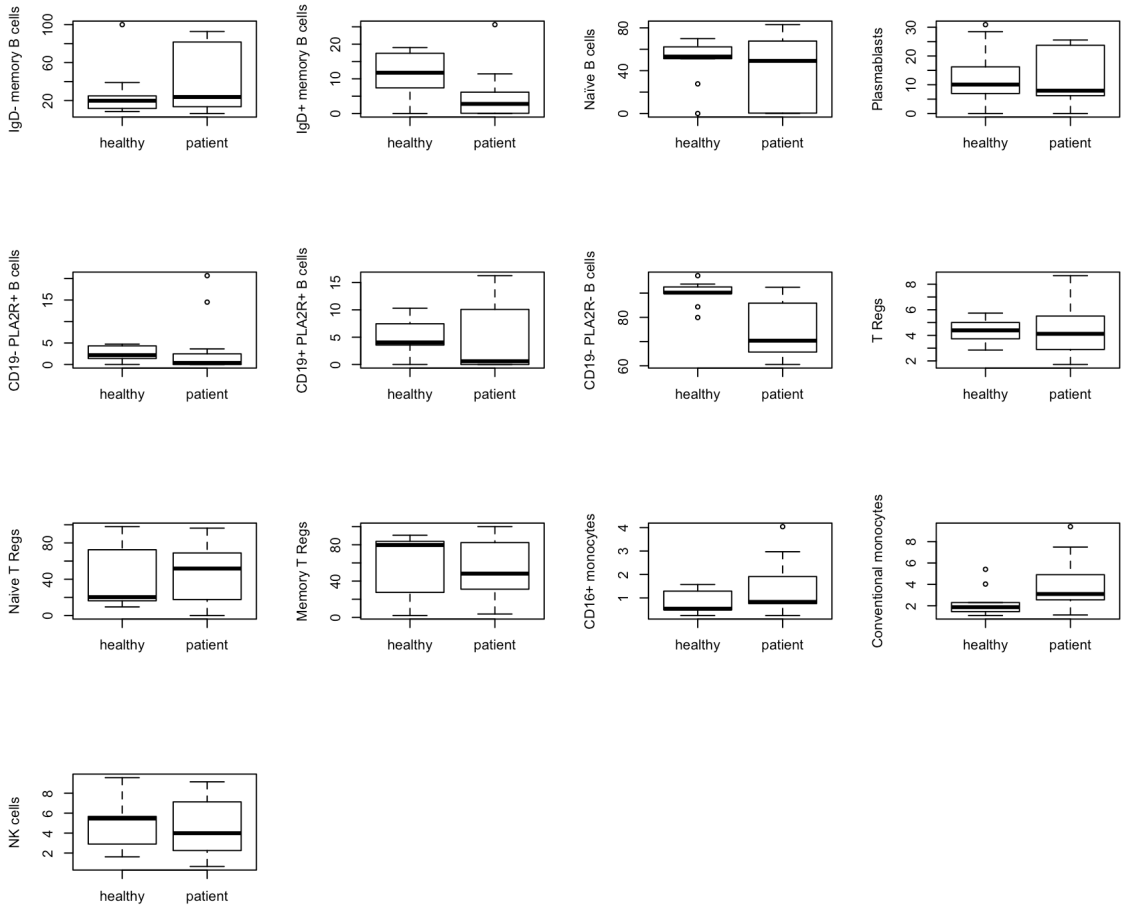

Figure 5.2 - Boxplots for healthy versus control group at baseline. Box plots showing each cell subset. Box showing median and interquartile range with minimum and maximum shown as error bars

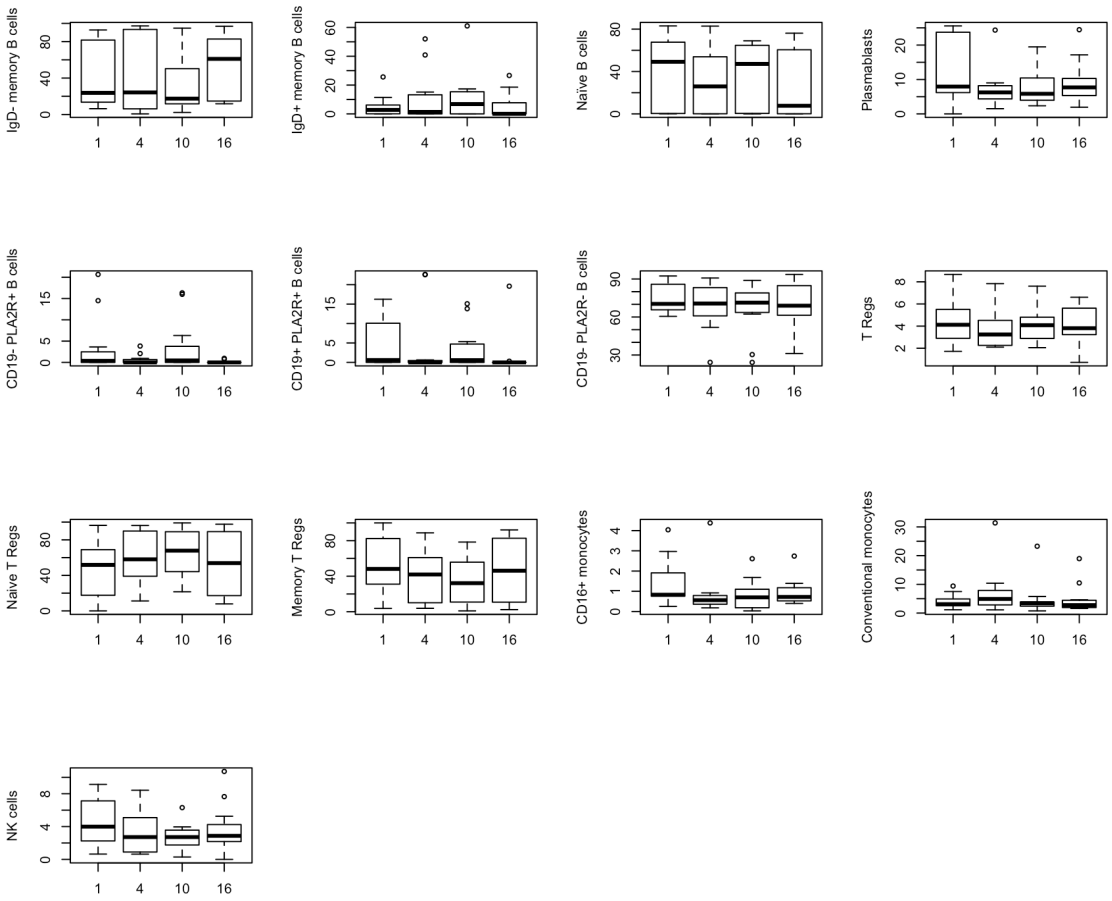

Figure 5.3 – Change in cell subset over time. Box plots showing change in each cell subset over time in patients only. Box showing median and interquartile range with minimum and maximum shown as error bars.

	Univariable	Antibody level	Antibody change	Change from baseline
IgD- Memory B cells	0.505	0.600	0.096	0.732
IgD+ Memory B cells	0.910	0.550	0.089	0.800
Naïve B cells	0.498	0.574	0.081	0.727
Plasmablasts	0.437	0.563	0.079	0.719
CD19- PLA_2R+	0.294	0.663	0.078	0.691
CD19+ PLA_2R+	0.286	0.646	0.080	0.680
CD19- PLA_2R-	0.501	0.599	0.070	0.710
T Regs	0.999	0.526	0.090	0.771
Naïve T Regs	0.669	0.537	0.069	0.682
Memory T Regs	0.669	0.537	0.069	0.682
CD16+ Monocytes	0.352	0.629	0.083	0.738
Conventional Monocytes	0.887	0.546	0.071	0.646
NK cells	0.480	0.567	0.087	0.658

Table 5.3 - linear regression for univariable, and multivariable adjusting for actual antibody level, change in antibody compared to baseline and change in antibody level compared to previous time point

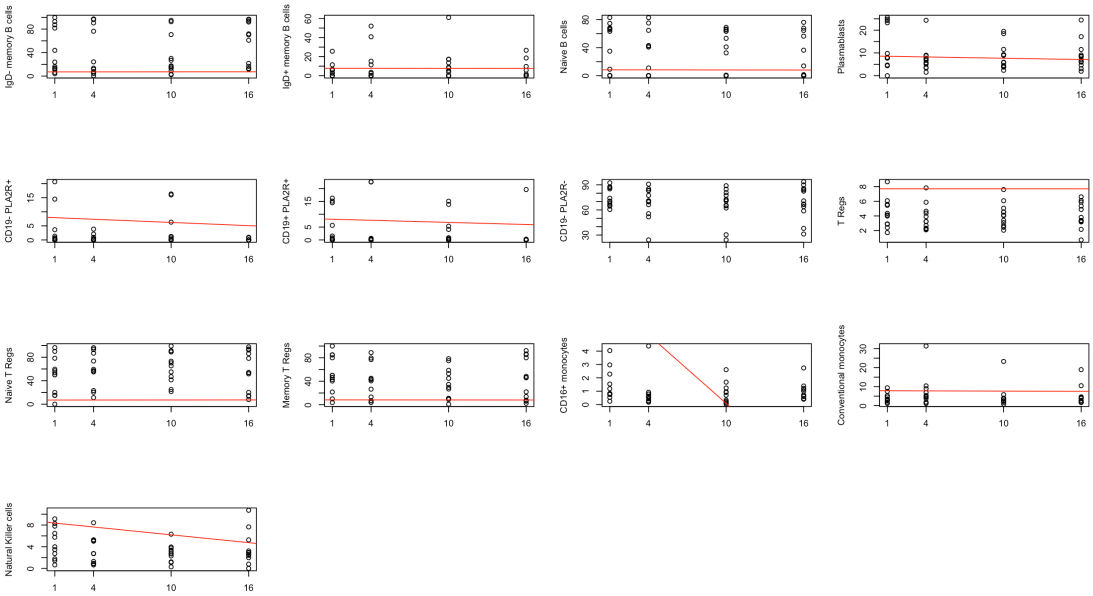

Figure 5.4 - Univariate linear regression for cell subset with time in weeks on x-axis.

5.4 Discussion

As the technology evolves, flow cytometry is becoming an ever more powerful tool for the study of the immune system. Here we have used the technique to attempt to begin to understand the mechanisms at play following immunoadsorption in the absence of any immunomodulating medications. The limited sample size meant there are few statistically significant results. However, there are a number of promising emerging patterns from the data, in particular with the PLA_2R and T cell panels.

A recent study using patients enrolled in the GEMRITUX trial showed that patients had lower proportions of IgD- and IgD+ memory B cells, T Regs and a higher proportion of naïve B cells at baseline compared to healthy donors[261]. In our cohort, we also found that there was a lower proportion of IgD+ memory B cells in the patient group but a similar level of IgD- memory B cells albeit with a much larger range. For the Naïve B cells and T Regs, the medians were very similar between the patients and control group but with a much larger range in the patient cohort.

One of the striking differences between our patient group and the control group at baseline is that there does not seem to be any statistical difference in PLA_2R positive B cells, with a number of volunteers in the control group showing a relatively high proportion of these cells (figure S5.6 in appendix). This seemingly counterintuitive result, in fact, appears to add weight to the importance of loss of tolerance in the disease process. Fresquet *et al.* have shown that an amino acid sequence which is part of the dominant epitope in the CysR region of the PLA_2R antigen is also found in the cell wall of some species of clostridia[31]. Further searches have shown that this peptide sequence is found in a number of other common pathogens such as Pseudomonas and Saccharomyces cerevisiae. Given this shared sequence of amino acids (SVLTLENC), it could be expected during the development of normal natural

immunity to a range of pathogens, developing IgM antibodies to this linear peptide sequence is common, entirely normal and beneficial to the host. The risk of developing an autoimmune pathology only arises then, if a patient has the genetic makeup (pathological alleles of DQA1 and PLA2R) required to present PLA_2R T cell peptides to their immune system. Only with the permissive genetic background and continued exposure to the pathogen or environmental trigger, causing immune processing of PLA_2R, will class switching occur from IgM to IgG, and therefore allowing the development of pathogenic high-affinity antibodies. In our PLA_2R panel, the healthy control group showed a significant level of PLA_2R positive B cells. As per figure S5.6, this was most striking in BIO-013, a fit and well 33-year-old female who takes no regular medications. A current ongoing and unpublished project being carried out in our lab is the development of an IgM anti-PLA_2R ELISA. When we analysed BIO-013 on a sample taken at the same time as that used in our flow cytometry experiment, she showed a highly positive result. Although it cannot be proven in the current flow cytometry experiment, it would appear to suggest that there is a high likelihood that the B cells seen in the healthy population may, in fact, be IgM positive B cells as opposed to IgG positive.

A further dimension to immune regulation and loss of tolerance is the role that T reg cells play and how they are a potential mechanism for the suppression of pathogenic antibodies. In other conditions such as autoimmune thyroiditis, it has been shown that the presence of circulating T cell antigen-specific thyroglobulin maintains a level of natural T Regs and inhibits the development of the disease[262].

The relapsing and remitting nature of autoimmune membranous nephropathy and the phenomenon of spontaneous remission indicates that at some level there must be an immune mechanism capable of suppressing the anti-PLA_2R antibodies, much like that found in autoimmune thyroiditis. Another ongoing

study, again unpublished, in our lab has identified a number of healthy controls without the prerequisite HLA-DQA1 or PLA2R1 genes needed to develop autoimmune MN, who have a detectable level of circulating soluble PLA_2R using mouse anti-PLA_2R as the capture antibody. There is the potential that these circulating soluble-PLA_2R antigens are active in maintaining a functioning level of T Regs to suppress class switching and downregulate the pathogenic antibody level. If natural T Regs did indeed have a role in keeping the pathogenic IgG anti-PLA_2R antibodies suppressed, the expectation would be that in times of active disease the levels would be low. The opposite would also be true with high levels in times of remission or just before remission or response to treatment. The T cell panel used for the patient cohort does start to show a pattern of T Regs change over time, a pattern that appears to support the theory above, especially when taken in the context of antibody level. At week 4 follow up, the T Regs level have dropped to their lowest point, this is also at the same time point at which the anti-PLA_2R is at its highest. The proportion of T Regs then show an increase at both week 10 and week 16 follow up, just as the antibody level is decreasing. In the study by Rosenzwajg *et al.*, in patients who responded to treatment they observed a lower proportion of T Regs baseline compared to those who did not respond to treatment. They also noted that in patients with no response to treatment, there was no increase in T Regs following treatment, however in patients who went on to respond, there was a significantly higher proportion of T Regs at day 8 compared to baseline[261].

One of the limitations of this study has been the short follow up time. Many of the patients in the trial have seen no reduction in their antibody until week 20 onwards meaning that any change in their immune system would not be picked up at the early time points employed in this study. This has also meant that at present we have been unable to separate the patient group into responders

versus non-responders. At present, there is no discernible pattern from the results to suggest which patients may or may not respond to the immunoadsorption therapy although as the follow-up continues over the coming year, this will become apparent. For this study, we have chosen four fixed time points that we felt had the best chance of capturing a change in the immune system cell populations. In order to accurately model the kinetics of the immune system, it will be necessary to carry out further analysis at more time points, guided by the antibody level and clinical response to treatment as opposed to predetermined time points. As discussed earlier, the current PLA_2R panel is unable to differentiate between IgM positive and IgG positive B cells, an important distinction to make in order to understand the disease pathogenesis.

Despite the lack of statistically significant results, partially as a result of small sample sizes and also due to the short follow up times, this study has shown some important emerging patterns. This is the first study to be conducted using flow cytometry to model the immune system in autoimmune membranous nephropathy following a treatment with no lasting immunomodulatory effects. We have demonstrated for the first time that not only do patients with the condition have PLA_2R positive B cells, but so do a proportion of healthy volunteers. It is likely that these are in fact IgM positive B cells and represent a normal response to common pathogens. In patients genetically susceptible to autoimmune MN, self-antigen processing and a loss of tolerance is required to allow for the eventual development of IgG secreting B cells. Prior to this, and in people who do not develop the disease, it is likely natural T Regs play a role in maintaining tolerance. Here we have shown T Regs will decrease in tandem with a raised anti-PLA_2R level and increase when the antibody level drops. Not only has this the potential, if proved with further analysis over the full PRISM study follow up, to provide more answers to how

the disease develops and the role loss of tolerance has in it, but can also provide a means to help prognosticate on future response to treatment.

Bioimpedance analysis in autoimmune membranous nephropathy in response to immunoadsorption therapy

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6.1 Introduction

Oedema is one of the defining characteristics of the nephrotic state and autoimmune membranous nephropathy. The traditional and most widely used approach to the assessment of oedema and fluid overload is the use of clinical signs and observations such as blood pressure. The inherent variability and inaccuracy of such a method has led to the development of more objective techniques to assess body fluid. Multifrequency electrical bioimpedance spectroscopy (BIS) using the principle that electric currents of differing frequencies will transverse different tissues at varying speeds. This allows for a greater degree of accuracy and less variability than clinical assessment alone. For this reason, the ease of use and low cost had made the emergence of bioimpedance and increasingly used tool in the assessment of fluid status in renal patients, particularly in the case of end-stage renal disease.

Despite the advantages of bioimpedance, little data exists for its use in adult nephrotic syndrome particularly in patients with membranous nephropathy or in regards to immunoadsorption therapy[263]. Here we use bioimpedance spectroscopy to accurately assess body composition in active adult autoimmune membranous nephropathy and investigate its response to immunoadsorption therapy.

6.2 Methods

All patients had BIS testing within two weeks of starting treatment, and one patient (PRISM13) had further testing before and after each IA session. All monitoring was carried out using the BCM-Body Composition Monitor (Fresenius Medical Care, Germany), which uses whole body bioimpedance spectroscopy via the hand-to-foot electrode method to estimate the compartmental fluid distribution and body mass composition (figure 6.1). In this technique, an electric current is passed from two electrodes placed on the wrist to two further electrodes on the ipsilateral foot, and a range of frequencies are measured from 5kHz to 1000kHz, with 50 in total. Different types of body tissue have different conducting properties and exhibit a different reactance and resistance to high and low frequencies of electric current. An electric current at high frequencies is able to pass through all tissues, and its impedance can be used to calculate total body water (Intracellular water (ICW) and Extracellular water (ECW)). At low frequencies, a current is unable to pass through the cell membranes, and the impedance at these frequencies can be used to calculate water content in the ECW space. The various impedance measurements and measurements of the phase angle (reflecting time delay caused by crossing a cell membrane) from multifrequency BIS are plotted in a curve using the Cole-Cole algorithm. Using this methodology, the BCM module will produce the following outputs: Absolute overhydration index (L), relative overhydration index (percentage overhydration compared to body weight, OH/ECW), total body water (TBW), intracellular water (ICW), extracellular water (ECW), body cell mass (BCM), intracellular resistance (Ri), extracellular resistance (Re) and cell membrane capacitance (Cm). Biochemical variables collected at the same time included anti-PLA2R titres, serum albumin, sodium, potassium, CRP, ESR and uPCR. Vital signs including blood pressure, height and weight were also recorded.

Bioimpedance vector analysis (BIVA) was carried out using the method as described by, and using the free open source software provided by, Piccoli *et al*[264,265]. Output from the BCM-Body Composition Monitor does not include resistance and reactance, therefore in order to carry out BIVA, these were calculated using the below equations where Z_50 is Impedance at 50kHz and Phi_50 is the Phase angle at 50kHz. Reference population used for the BIVA method was white males aged between the ages of 16 and 85 years old, with a BMI ranging from 16 to 31 kg/m^2 [266].

$$Resistance = [Z\_50] \times Cos\left(\frac{[Phi\_50]}{180}\pi\right)$$

$$Reactance = [Z\_50] \times Sin\left(\frac{[Phi\_50]}{180}\pi\right)$$

Descriptive statistics for continuous variables presented as mean and standard deviation, and number and percentage for categorical variables. Correlations calculated using the Pearson correlation coefficient. All analysis carried out in R statistical software version 3.4.3[252].

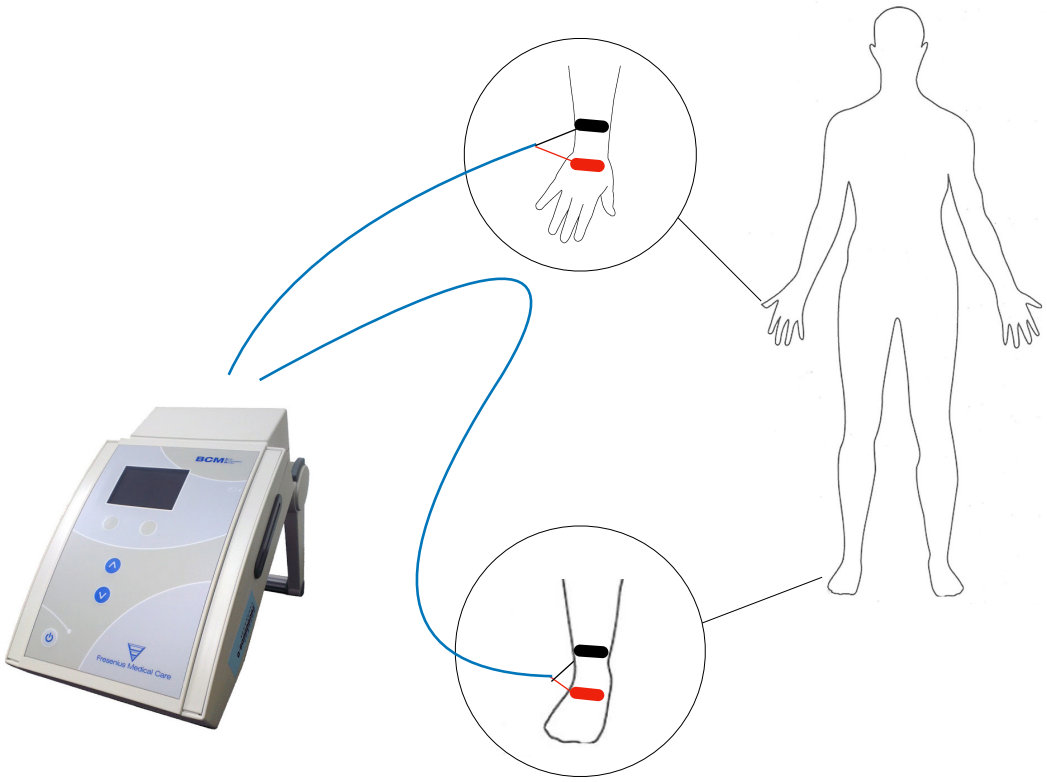

Figure 6.1 - BCM schematic. Two electrodes on the wrist and hand and two electrodes on ipsilateral foot.

6.3 Results

There was a total of 13 patients included in the study, 2 (15%) females and 11 (85%) males with a mean age 64 (SD 14.45). All were significantly nephrotic with a mean uPCR of 869.31 mg/mmol (SD 414.69). Table 6.1 for full demographics.

6.3.1 Total body water

The mean total body water was 49.58L (SD 10.97) which correlated with 58.63% of the total weight. The mean intra and extracellular water were 25.33L (SD 6.10) and 24.22L (SD 5.56) respectively. Intracellular water was therefore 48.96% of the total body water, and extracellular water was 50.99%. The mean overhydration was 4.33L (SD 3.03) giving a mean overhydration relative to weight of 17.57% (SD 10.47).

6.3.2 Bioimpedance measurements

The mean extracellular resistance and intracellular resistance were 381.32 Ohms (SD 71.33) and 1063.43 Ohms (SD 292.36) respectively, whilst the mean cell membrane capacitance was 2.19 nF (SD 0.91). The mean phase angle at 50 KHz was 5.03^0 (SD 1.29).

6.3.3 Body composition at baseline

For the three-component model of body composition; the mean adipose tissue mass was 27.48Kg (SD 28.45) which was 31.8% of the total body weight; the lean tissue mass mean was 18.89 (SD 3.11), 63.2% of the total weight and the

mean overhydration was 4.33 (SD 3.03), corresponding to 5.0% of the total weight.

For standard measures of body composition, the mean total fat was 20.18Kg (SD 20.89) corresponding to a mean of 23.3% of total body weight. Fat-free mass

Parameter	Mean (SD)
Number	13
Sex	
Female n(%)	2 (15%)
Male n(%)	11 (85%)
Age	63.62 (14.45)
Weight (Kg)	87.43 (31.01)
Normohydration Weight (Kg)	83.09 (31.64)
Height (cm)	170.00 (9.24)
BMI (Kg/m2)	30.20 (8.96)
Anti-PLA_2R antibody (U/mL)	2326.62 (5854.26)
uPCR (mg/mmol)	869.31 (414.69)
Albumin (g/L)	20.85 (4.98)
Sodium (mg/mmol)	141.08 (3.57)
Potassium (mg/mmol)	4.35 (0.65)
CRP (mg/L)	2.23 (1.69)
ESR (mm/1stHr)	49.69 (25.32)
BP Systolic (mmHg)	162.54 (30.22)
BP Diastolic (mmHg)	80.92 (11.20)
Total body water (L)	49.58 (10.97)
Total body water / weight (%)	58.63 (8.80)
Overhydration (L)	4.33 (3.03)
Relative overhydration (%)	17.57 (10.47)
Extracellular water (L)	24.22 (5.56)
Intracellular water (L)	25.33 (6.10)
Relative extracellular water (%)	48.96 (4.07)
Relative intracellular water (%)	50.99 (4.10)
Extracellular resistance (Ohms)	381.32 (71.33)
Intracellular resistance (Ohms)	1063.43 (292.36)
Cell membrane capacitor (nF)	2.19 (0.91)
Body Cell Mass	32.78 (8.58)
Phase angle 50 kHz (degrees)	5.03 (1.29)
Lean Tissue Mass (Kg)	54.71 (13.04)
Relative Lean Tissue Mass (%)	65.32 (14.23)
Adipose tissue mass	27.48 (28.45)
Lean Tissue Index (kg/m^2)	18.89 (3.11)
Fat (Kg)	20.18 (20.89)
Relative fat (%)	20.53 (11.96)

Table 6.1 – Demographics and baseline characteristics. All results are given in mean (SD) unless otherwise stated.

	3 Compartment Model	Body composition	Intra/Extracellular water
Fat	-	23.3	-
Adipose tissue mass	31.8	-	-
Relative overhydration	5.0	5.0	-
Lean tissue mass	63.2	-	-
Extracellular water	-	28.0	48.9
Intracellular water	-	29.2	51.1
Bone	-	14.5	-

Table 6.2 - Proportion of body components for three different models. The 3-compartment model consists of normally hydrated adipose tissue, normally hydrated lean tissue and overhydration. The standard body composition consists of fat, relative overhydration, intra and extracellular hydration and bone. The third model is the proportion of intracellular and extracellular water relative to total body weight. All values given in percentages. Includes all patients prior to IA treatment.

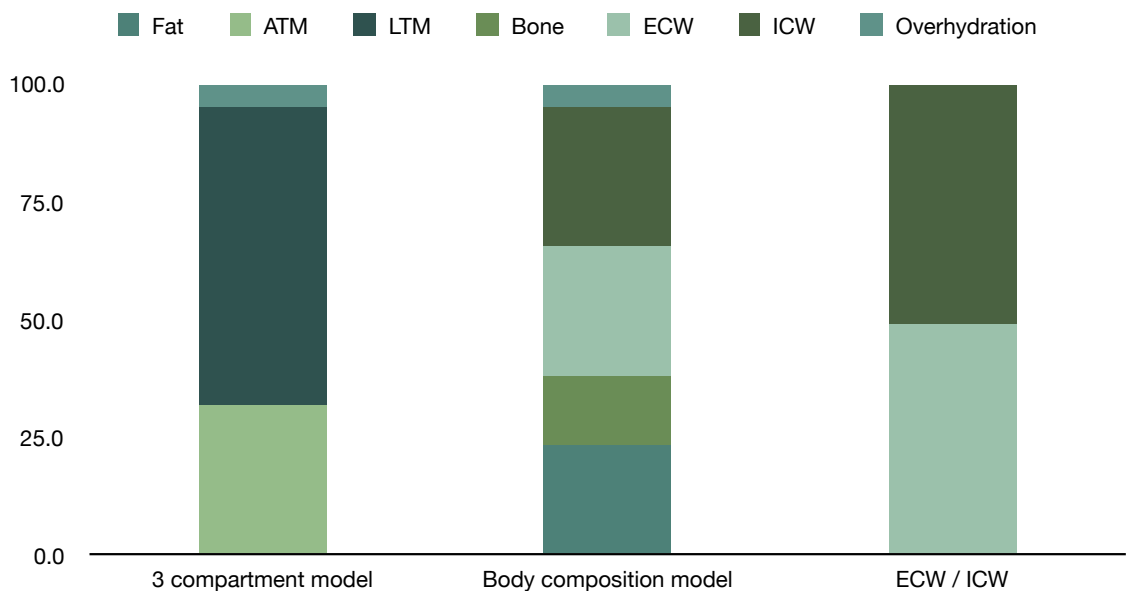

Figure 6.2 - Body composition based on 3-compartment model and standard body composition as a proportion of overall weight. ECW versus ICW as a proportion of total body water. ATM - adipose tissue mass. LTM - Lean tissue mass. ECW - extracellular water. ICW - intracellular water.

therefore, made up of intracellular and extracellular water, overhydration, bone and minerals. The intracellular water was 29.2% of the total body weight with 25.33L (SD 6.10) whilst the extracellular water contributed 28.0% of the total body weight with 24.22L (SD 5.56). Overhydration made up 5.0% of the total body weight, with the remaining 14.5% of the total body weight consisting of bone and minerals. Tables 6.1 and 6.2 and figure 6.2.

6.3.3.1 Correlation analysis

Intracellular resistance has the strongest negative correlations, particularly with body cell mass (-0.87), cell membrane capacitance (-0.91), total body water (-0.81) and intracellular water (-0.91). Figures 6.3 and 6.4.

6.3.3.2 Relative overhydration

For relative overhydration, the strongest positive correlations were seen with anti-PLA_2R levels (0.61), age (0.61) and ESR (0.66). The strongest negative correlations were seen with cell membrane capacitance (-0.63) and extracellular resistance (-0.62). With serum albumin, the correlation coefficient was -0.50, and with uPCR it was 0.43.

6.3.3.3 Total body water

The strongest correlations seen with TBW were with ICW (0.95), ECW (0.93) and BCM (0.82). With albumin and anti-PLA_2R antibody levels, there was a small negative correlation of -0.37 and -0.38 respectively. For ESR the correlation was -0.10.

6.3.3.4 Extracellular Water

For ECW, the strongest positive correlations were seen with TBW as above, BCM (0.61) and ICW (0.77). The strongest negative correlations were with extracellular resistance (-0.80) and intracellular resistance (-0.60). With albumin, the correlation coefficient was -0.44, for anti-PLA_2R antibody levels it was -0.23 and with uPCR it was 0.00.

6.3.3.5 Intracellular water

The strongest positive correlations with ICW were with total body water as above, body cell mass (0.92) and cell membrane capacitance (0.85). The strongest negative correlation was with intracellular resistance for which the correlation coefficient was -0.91. For serum albumin, the correlation coefficient was -0.26, with anti-PLA2R antibody levels it was -0.48 and for uPCR it was -0.13.

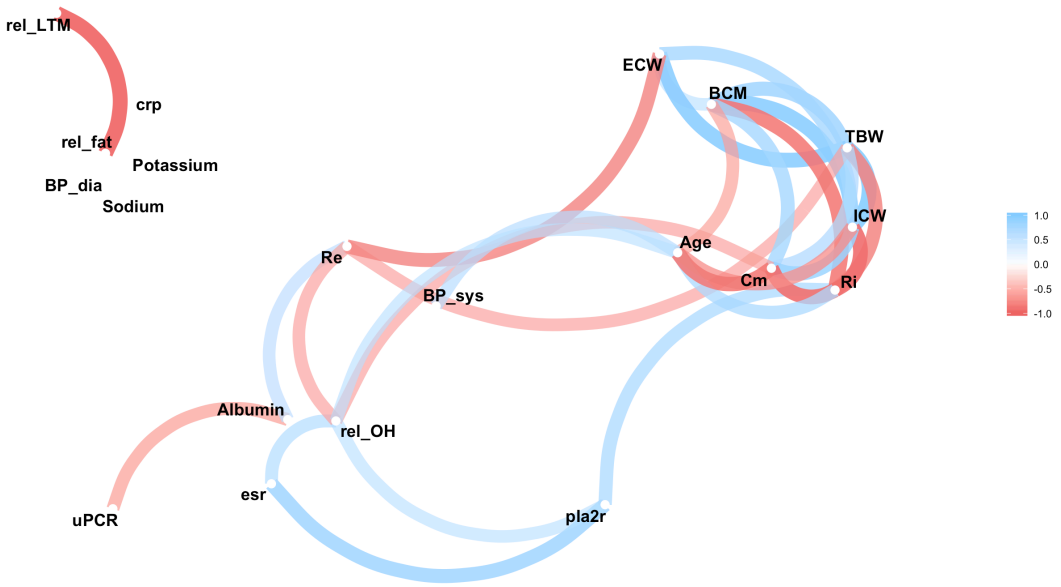

Figure 6.3 - Correlation plot using clustering. The closer each variable is to each other the higher the relationship while the opposite is true for widely spaced variables. Red lines represent a negative correlation, and blue lines represent a positive correlation. The line shade and thickness represent the strength of the relationship. The minimum correlation shown with a connection is 0.6

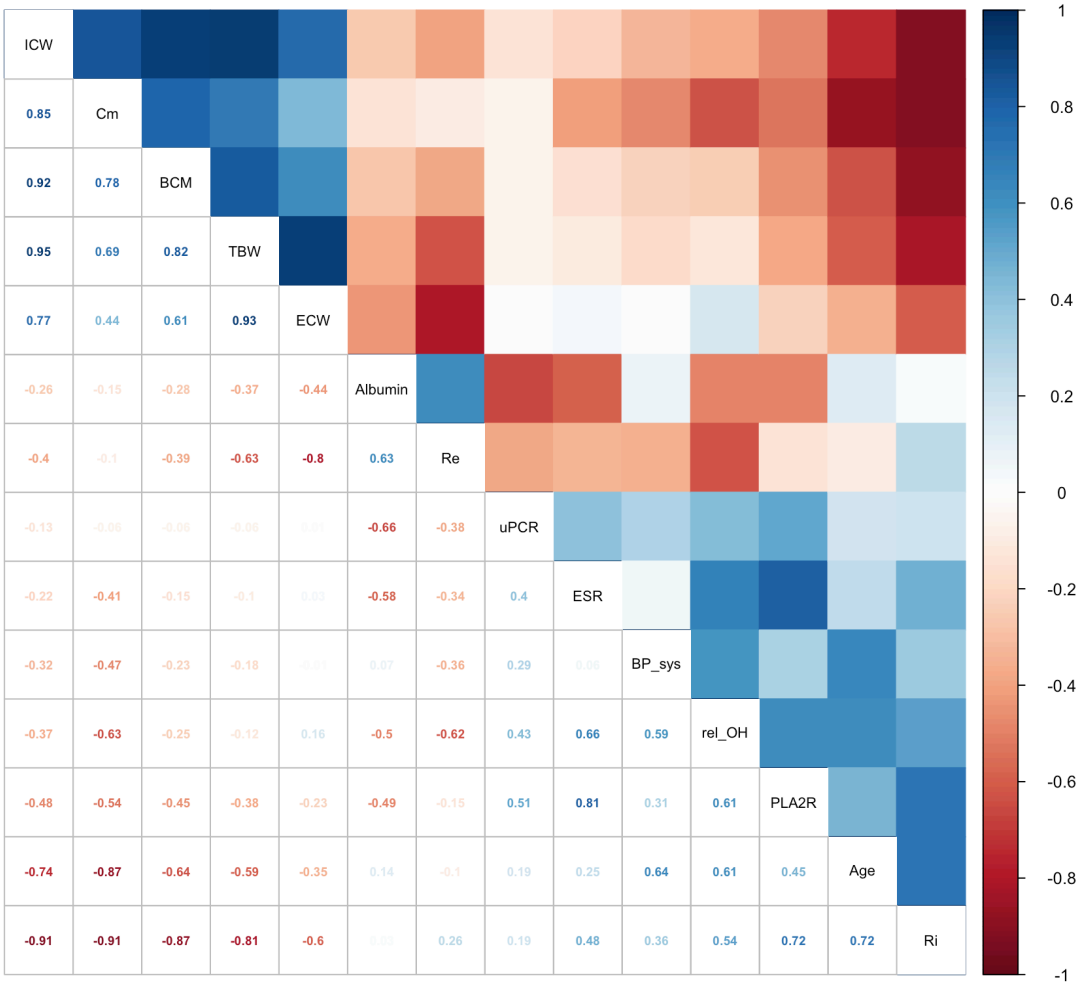

Figure 6.4 – Correlation heatmap - only including variables with a correlation with one or more other variables that is greater than 0.6. Colour of square in upper panel represents the strength and direction of correlation; blue is positive and red is negative. Bottom panel shows corresponding r correlation. All patients prior to treatment.

6.3.4 Body composition in response to immunoadsorption

The proportion of ECW and ICW remained static throughout the treatment week; however total body weight increased progressively (figure 6.5). Extracellular resistance and cell membrane capacitance both decreased during the treatment whereas in intracellular resistance remained stable (figure 6.6). Albumin remained stable throughout the treatment week whilst uPCR improved. Overhydration increased, particularly in the first two days (figure 6.7).

6.3.4.1 Total Body Water

In response to IA therapy, TBW showed a strong positive correlation with ECW (0.89), relative overhydration (0.75), CRP (0.77) and serum albumin (0.62). There were negative correlations with relative fat levels (-0.92), systolic blood pressure (-0.75), uPCR (-0.8), ESR (-0.79), cell membrane capacitance (-0.87), extracellular resistance (-0.87), serum potassium (-0.93) and anti-PLA_2R antibody titres (-0.90). Figures 6.8 & 6.9.

6.3.4.2 Extracellular water

The strongest positive correlations for ECW were with TBW as above and with relative overhydration which had a correlation coefficient of 0.97. Strong negative correlations were seen with uPCR (-0.93), ESR (-0.98), cell membrane capacitance (-0.99), extracellular resistance (-1.00), serum potassium (-0.98) and anti-PLA_2R antibody titres (-0.95).

6.3.4.3 Intracellular water

ICW showed strong positive correlations with relative lean tissue mass (0.98) and body cell mass (0.99). Strong negative correlations were seen with intracellular resistance (-1.00) and relative fat (0.55). Only weak correlations were seen with uPCR, ESR and anti-PLA_2R; 0.27, 0.37 and 0.1 respectively.

6.3.4.4 Relative overhydration

The strongest positive correlation for relative overhydration was with total body weight, with a correlation coefficient of 0.75. Strong negative correlations were seen with uPCR (-0.92), ESR (-0.99), cell membrane capacitance (-0.97), anti-PLA_2R antibody titres (-0.90), extracellular resistance (-0.98) and serum potassium (-0.93). The correlation coefficient for serum albumin was 0.38.

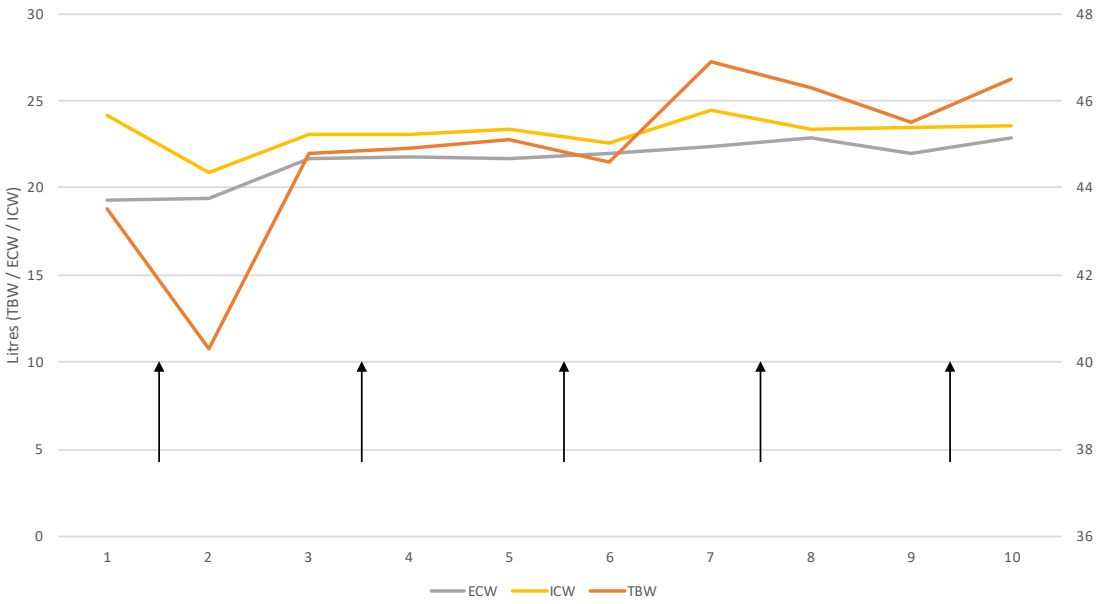

Figure 6.5 - TBW, ECW and ICW in response to IA. Arrows indicate IA treatment. ECW – Extracellular water. ICW – Intracellular water. TBW – Total body water.

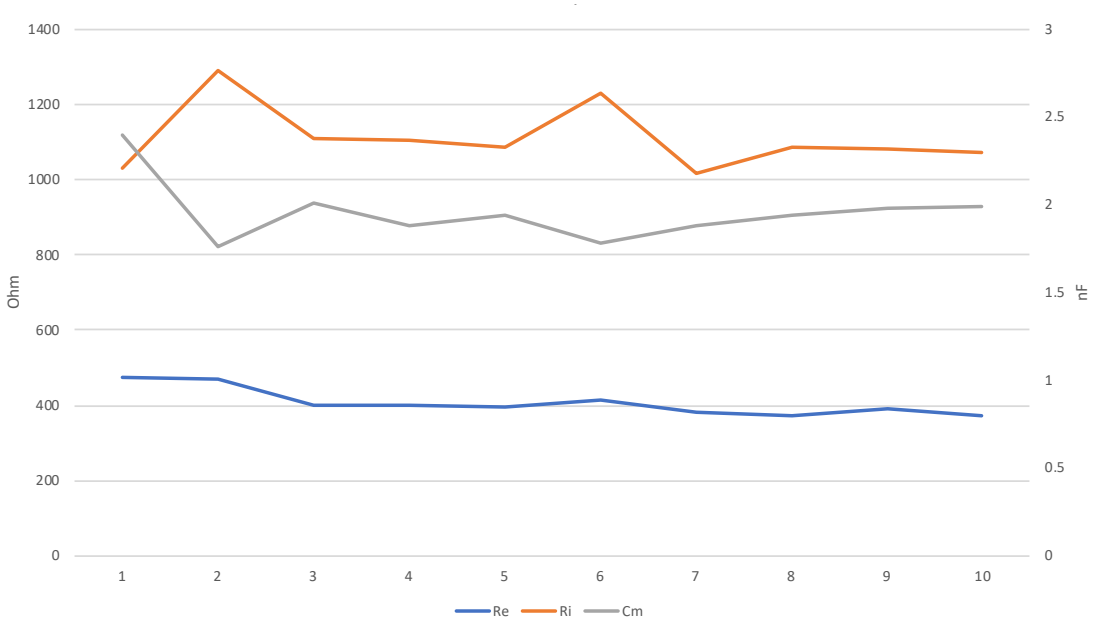

Figure 6.6 - Re Ri and Cm response to IA. Re – extracellular resistance. Ri – intracellular resistance. Cm – cell membrane capacitance.

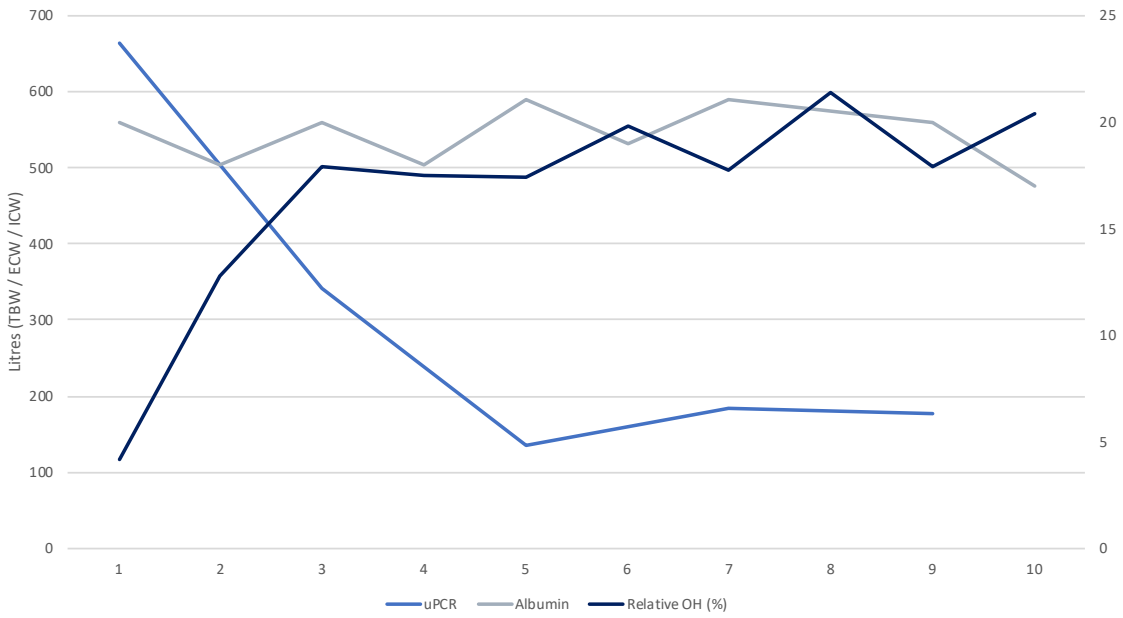

Figure 6.7 - uPCR, albumin and relative OH in response to IA. OH - overhydration

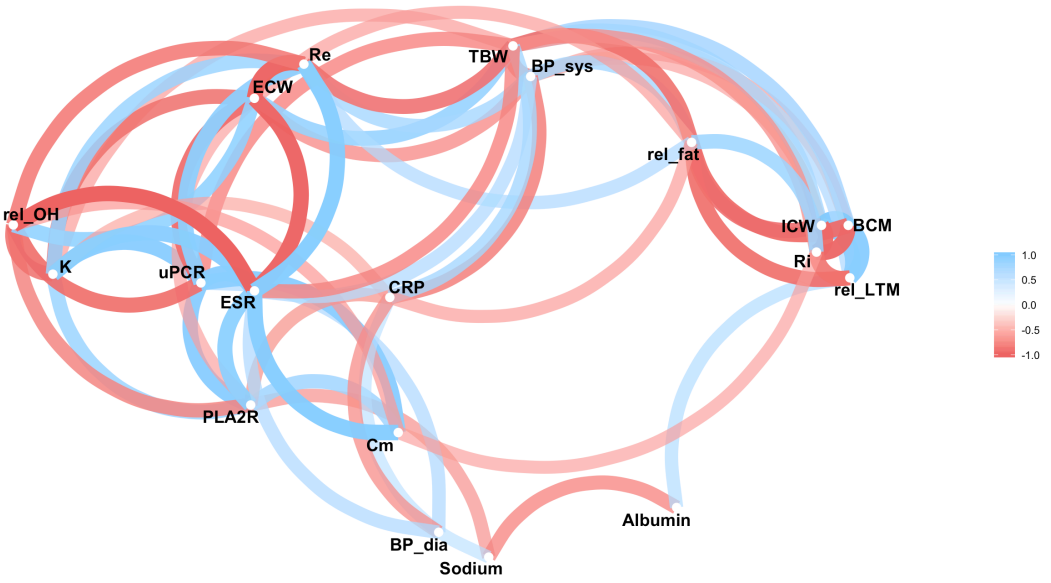

Figure 6.8 – Correlation cluster for immunoadsorption therapy

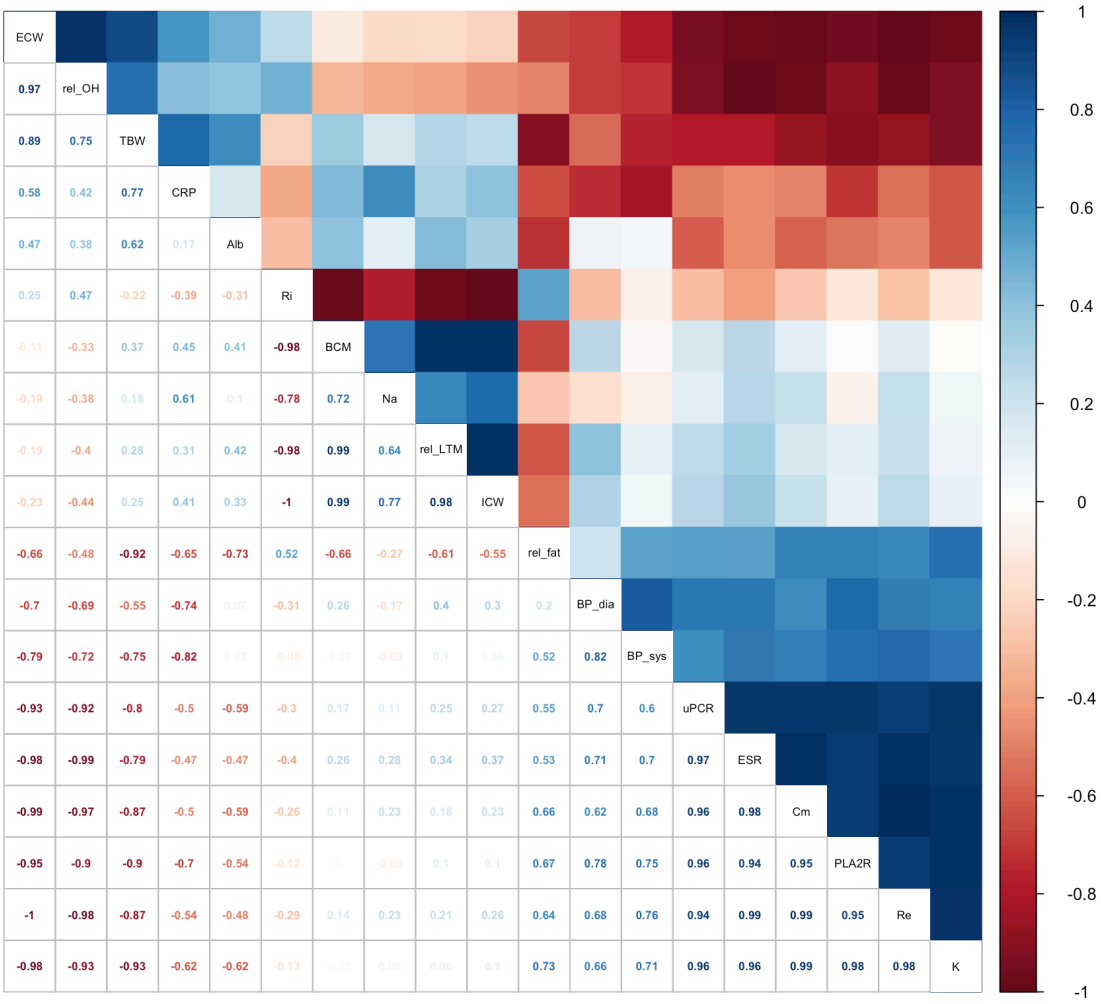

Figure 6.9 - Correlation chart for IA therapy

6.3.5 Bioimpedance Vector Analysis

We analysed all 13 patients who consented to the PRISM trial including PRISM08 who went into spontaneous remission and did not receive treatment. 12 of the 13 patients (92%) were outside the 50% tolerance ellipse, 11 patients (85%) were outside the 75% tolerance ellipse, and 9 patients (69%) were outside the 95% tolerance ellipse. All patients were at the lower pole indicating increased body fluid compared to the reference population. The one patient who was within the normal range was PRISM08 who was going into spontaneous remission at the time of assessment. Figure 6.10.

Figure 6.11 shows the BIVA plot for PRISM13 in response to immunoadsorption therapy. Day 1 pre and post-IA are shown within the 50% tolerance ellipse as compared to the reference population. As the treatment week continues, the patient moves further out towards the lower pole of the reference range ellipse, indicating an increase in overhydration.

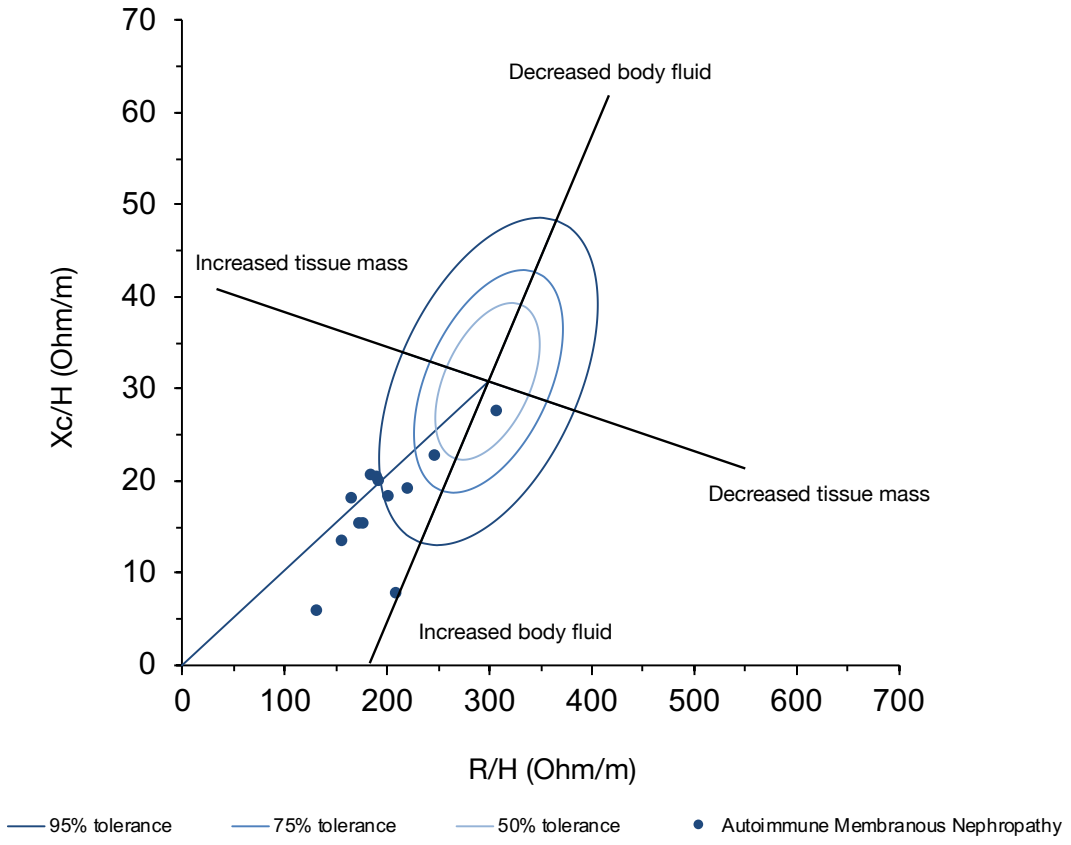

Figure 6.10 - BIVA plot with all 13 consented patients

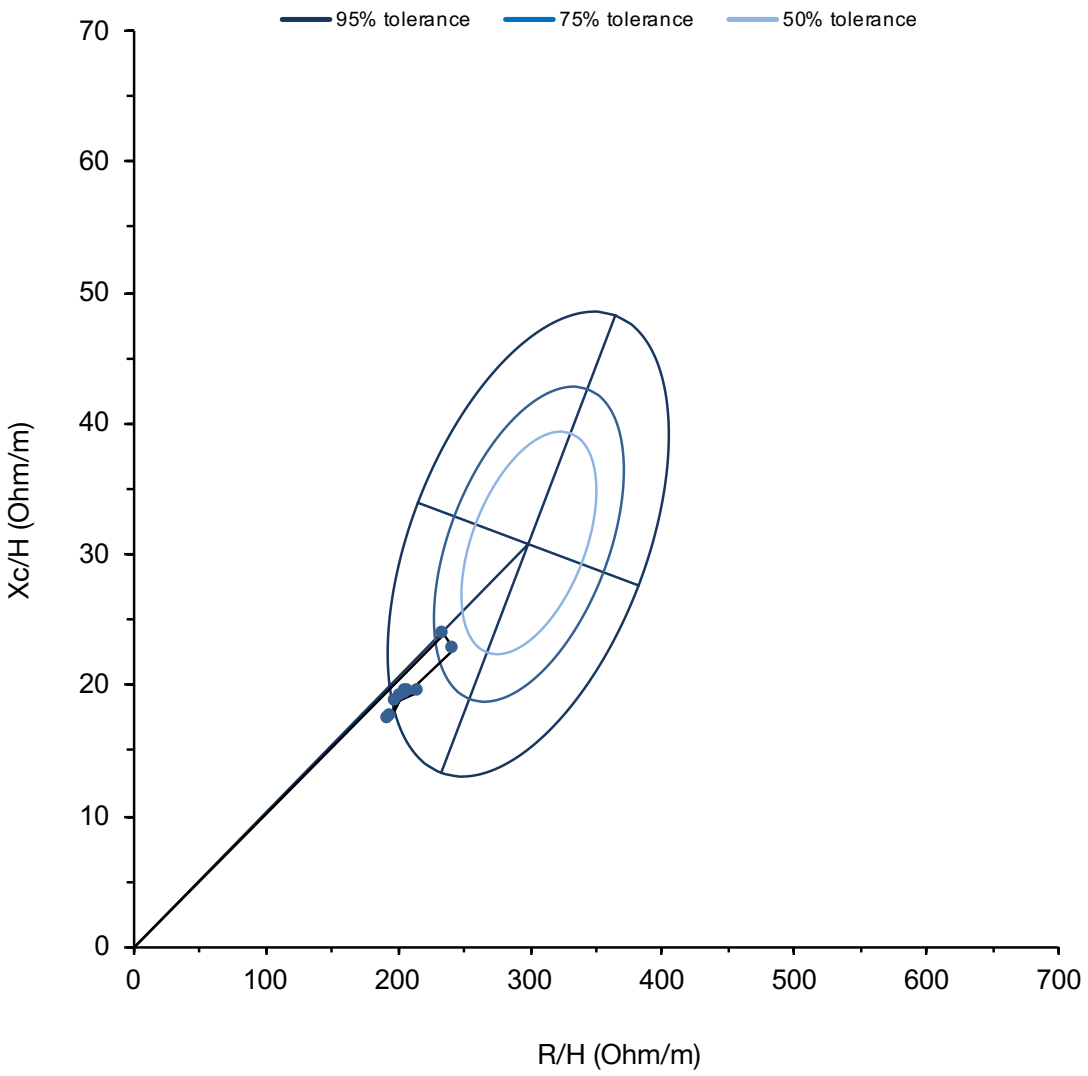

Figure 6.11 - BIVA path plot showing change in BIVA for patient 13 in response to immunoadsorption with 5 sessions in one week. Quadrants as per figure 6.10.

6.4 Discussion

Bioimpedance monitoring allows for a quick, low-cost and non-invasive assessment of fluid status and body composition. Here we describe for the first time the body composition of adult patients with active autoimmune membranous nephropathy and the effect immunoadsorption therapy has on the compartments.

The principal component of body mass is water, contributing approximately 65% to the body weight, of which approximately 30% is extracellular water[278]. Here we show that in our cohort of patients that there is a higher degree of extracellular water with a mean of 49%. BIVA analysis also shows the degree to which our cohort differs from the normal population with the majority of our patients outside of the 95% tolerance ellipse in the direction indicative of fluid overload. The relative overhydration as calculated by the bioimpedance analysis would appear to suggest that it is correlated with the severity of disease as measured by the anti-PLA_2R levels, ESR and to a lesser extent serum albumin, with only a weak correlation seen with proteinuria.

With immunoadsorption one of the striking features quantified with the bioimpedance monitoring is the increase in fluid overload seen over the week of treatment. As part of the therapy, fluid is used as the end of the procedure to help wash the blood back into the body from the circuit. In patients without nephrotic syndrome, this presents little problem as it will be processed by the kidney and excreted as necessary. Indeed, many reports on the peri-procedural complications are of hypotension. In our cohort, however, many of the complications could be considered as a result of fluid gains such as hypertension and headaches. BIVA analysis during the treatment week shows that the patient actually started within the 75% tolerance ellipse of the reference population, but as the week progressed and he received more immunoadsorption, he moved further out towards the lower pole of the

tolerance ellipse. The relative overhydration, however, does stabilise after day 1 as seen in figure 6.6 and 6.11. In concert with this increase, the uPCR was decreasing precipitously, particularly in the first two days. It is conceivable that this reduction in proteinuria has allowed for an improvement in fluid balance handling by the kidneys given the resulting increased intravascular osmotic pressures, although the serum albumin remained static throughout. At day 3 of treatment, due to the increasing fluid overload, the patient required an increased in his diuretics, a factor that is likely to be contributing to the stabilisation of the overhydration seen on bioimpedance monitoring. During the treatment week, the relative overhydration had a higher correlation with extracellular water as opposed to intracellular reflecting its importance in oedema and fluid overload. Pertinent to patients with autoimmune membranous nephropathy, it was strongly correlated with the anti-PLA_2R antibody and ESR although the correlation is negative. This may be as a result of the fluid overload described above as a result of the procedure. With small sample sizes and data points, it has not been possible to carry out multivariable modelling and therefore has not been possible to elicit whether this apparent counterintuitive result is correct or a result of the immunoadsorption itself. As expected there is a strong correlation seen between the inflammatory response as seen in ESR, the anti-PLA_2R autoantibody and with proteinuria.

6.5 Conclusion

Patients with autoimmune membranous nephropathy have significantly increased extracellular water and overhydration compared to the normal population. This overhydration appears to have a relatively strong correlation with markers of disease activity in the ESR and anti-PLA_2R antibodies.

When patients with autoimmune membranous nephropathy undergo immunoadsorption therapy, despite a fall in the anti-PLA_2R antibodies, proteinuria and ESR, and a stable serum albumin, there was an increase in overhydration. This increase in overhydration has not proven to have had any impact on the treatment itself, and all patients completed the full session prescribed, but is an important consideration for future therapy. It is likely that this is due to the immunoadsorption itself and for patients with membranous nephropathy who are nephrotic it would be prudent to consider an increase in any diuretics at an early stage. Despite this, immunoadsorption remains a safe procedure, with only minimal side-effects noted, even in highly nephrotic patients with fluid overload.

Discussion

7.1 Summary

Autoimmune MN has experienced a step change in our understanding of the disease pathogenesis since the discovery of the anti-PLA_2R autoantibody in 2009[15], however, there is much that still remains unknown. Despite the advances seen over the last decade, the management of the disease remains an empirical treatment based on a regimen first introduced over two decades ago. There is as yet no disease-specific therapy or alternative to glucocorticoids and immunosuppression in mainstream use.

This body of work represents a multifaceted approach to the understanding and management of autoimmune MN. By looking at the disease from a number of aspects, we have attempted to not only understand the disease from an immunological viewpoint but to also understand the challenges of the disease from its diagnosis to its management. IA therapy has been in use for a number of years and has previously been used in membranous nephropathy. However, with the understanding of the disease at that time, the outcomes were not encouraging, so further use was abandoned. Since that time the identification and characterisation of the anti-PLA_2R antibody was able to inform the basis for the multi-center clinical trial that we have carried out. As with any healthcare system worldwide, there are limited resources and as such access to any treatment or therapy is increasingly dependent on its cost-effectiveness. We have therefore also undertaken the most comprehensive cost-effectiveness study for the management of MN yet undertaken comparing Rituximab versus the modified Ponticelli regimen. This will form the basis of the cost-effectiveness of IA therapy once the clinical trial is complete. With increasing evidence for the pathogenicity of anti-PLA_2R and its use as a

biomarker, we also looked at the effect that its use has on reducing the burden of investigations a patient must undergo. We found that as the use of anti-PLA_2R increases, there is a trend towards a reduction in the cost and number of tests carried out. These results indicate the need for further work to describe the most efficient and cost-effective use of resources for the diagnosis of MN. Ultimately this will lead to less invasive investigations for patients and allow for more timely diagnosis and treatment.

In tandem with the clinical trial, the advantage of IA's lack of adjunctive immunosuppression, allowed us to carry out further work to understand the underlying mechanisms of the disease. As with all autoimmune diseases, the eventual clinically apparent symptoms are the end result in a journey of multiple steps, the so-called multi-hit hypothesis. We know that there is a strong genetic component in the development of the disease, with patients homozygous for both the HLA-DQA1 and PLA2R1 genes are almost 80 times more likely to develop the disease than patients who do not[50]. What we still don't know is whether the possession of these genes in itself guarantees the development of the disease. It's likely that a further trigger (likely environmental) is required to progress to the disease state. As shown by Fresquet *et al.* and following further study using the Basic Local Alignment Search Tool (BLAST)[279], there is an amino acid sequence found in the PLA_2R epitope shared with a range of commonly encountered pathogens[31]. Development of the normal natural immunity requires the production of antibodies, including IgM, to linear peptides in a whole range of epitopes. With this beneficial protective immunity, circulating IgM antibodies to the PLA_2R p28mer peptide can, in fact, be a normal occurrence. The presence of these antibodies in patients without the genetic predisposition to the disease would just be an expected variant of normal. It is in those patients who do have the genetic predisposition to developing the disease, that the presence

of IgM antibodies with the ability to recognise the p28mer will have the potential to progress to the disease state to generate a high-affinity IgG response. Once this occurs, and there is recognition of the podocyte PLA_2R epitope there begins a positive reinforcement with ever-increasing affinity. The exact nature of how patients eventually develop a pathogenic IgG antibody remains elusive. However, here we have, for the first time, shown that in a control group of healthy volunteers and a patient group with active disease there is a PLA_2R antigen positive B cell population in both. This is coupled with an ongoing unpublished study showing a level of circulating anti-PLA_2R IgM antibodies in these normal healthy patients. This requires further work, but it is the first evidence for an antibody class switch in autoimmune MN.

A characteristic of autoimmune MN is the heterogeneity shown in prognosis and its waxing and waning nature over time. A proportion of patients will undergo a phenomenon of spontaneous remission, and in patients with a more severe phenotype, it is not unusual for them to follow a relapsing and remitting course. Many patients, when they first come to medical attention, will describe self-limiting episodes many months or years prior to their diagnosis that are likely to be nephrotic states and the first signs of the disease. This suggests that far from being a continuously progressive immunological process, particularly in light of the pathogenicity of the autoantibody, that there are natural mechanisms at play attempting to maintain a balance. Work in other autoimmune diseases such as autoimmune thyroiditis have proven the existence of antigens capable of maintaining a population of natural T Regs and thereby keeping pathogenic antibodies suppressed[262]. Using flow cytometry following immunoadsorption we have shown that as the anti-PLA_2R antibody rises in the weeks following treatment, there is a reduction in the natural T Regs. Following this, as the level of T Regs starts to rise there is a corresponding fall in the antibody level. Taken in tandem with unpublished

work that is ongoing showing a measurable level of circulating soluble PLA_2R in healthy controls, this would appear to show that a similar process to autoimmune thyroiditis is taking place in autoimmune MN.

This theory is at the basis of the clinical trial that we carried out. If there is a circulating antigen keeping the anti-PLA_2R antibody suppressed, the removal of the pathogenic antibody in active disease would allow the rebalancing of the immune system with an increase in the suppressive antigen and an increase in T Regs. Without the use of immunosuppression or glucocorticoids, in this regards immunoadsorption is a treatment analogous to spontaneous remission. Previous attempts to use IA in MN showed an initial improvement of proteinuria although this quickly returned to normal. Follow up was short, however, and at the time the anti-PLA_2R antibody had not been discovered[63]. If the long-term response to IA is dependent on the re-emergence a circulating antigen and natural T Regs it would conceivably take a number of months for this to occur. We know from studies using various agents, that a clinical response based on proteinuria takes a number of months following the reduction in the circulating antibody.

We demonstrated the efficacy of immunoadsorption in the reduction of serum antibody level with a median reduction of 87% over five consecutive sessions in a week. This treatment was well tolerated with only mild adverse events that had no impact on the treatment itself and no serious adverse events related to the therapy. Peri-treatment there is a demonstrable increase in fluid overload and hypertension counter to other conditions for which IA is used. The most common side effect reported is usually hypotension. In our cohort of nephrotic patients however, the extra intravenous fluid given during the treatment cannot be excreted as normal. This is an important consideration, at odds with other

conditions, an increase in a patient's diuretics may be necessary early in the treatment course.

The therapy has shown promise with the first patient treated becoming antibody negative at week 10 and remaining so until last follow up 15 months following treatment. Clinically his proteinuria reduced to below his baseline level, and he was in partial remission as defined by KDIGO[5].

Unfortunately, the second patient treated did not fare so well, and a deterioration in antibody level, proteinuria and renal function, the decision to start him on IV Cyclophosphamide was made. This patient does give a concise view of the current standard of care and the need for a more directed treatment. Following treatment with steroids and cyclophosphamide, his antibody dropped precipitously followed by his proteinuria. However, his quality of life has been drastically reduced with a long hospital admission due to viral pneumonia, atypical bacterial pneumonia and the development of multiple peripheral pulmonary emboli. He now has significant weight gain, anxiety, anaemia and is unable to work full time.

Fitting with the theories discussed above, any improvement that has been seen in the antibody level has taken a number of months to become evident, up to and over six months in some cases. What appears to be a factor in response is age, the older the patient, the longer it takes to respond. PRISM02 who failed the treatment altogether and was started on immunosuppression was in the older age group, whether this was a driving factor cannot be conclusively determined as yet. The phenomenon of epitope spreading would certainly lend itself to this observation, with older patients exhibiting a more severe phenotype of the disease with antibodies recognising more than one epitope.

7.2 Future work

At present, given the long timeline for a response to IA therapy, it is not possible to distinguish between responders and non-responders. The last patient will have his final one-year study follow up in April 2019. As patients come to the end of their follow up, they will be invited to participate in the Manchester Renal Biobank. This will allow for the continued collection of samples, both blood and urine, and the continued review of medical notes. Why some patients respond to the treatment and others do not, is pertinent not just to the clinical efficacy of the therapy but also will go some way to understanding the disease in more detail. Does epitope spreading play a role, do patients need to be treated to negativity as in anti-GBM disease, and to what extent does a patient's genetics contribute? An ELISA is currently under development to test epitope spreading in our patients, to determine whether the antibodies are directed against the CysR, the CysRC1 and CysRC1C7 regions. Whether those patients with antibodies to the CysRC1C7 region have worse outcomes compared to those to just the CysR region remains to be seen. Full tissue typing of each patient will also be carried out to determine if this is implicated in how well patients respond to treatment.

All patients have not responded to the IA therapy, with one treatment failure already. Further work will be required to optimise the treatment regimen. With almost 90% reduction in antibody level after 5 sessions we have shown the therapy works to remove the antibody efficiently. However, not all patients became antibody negative (including PRISM02 who failed treatment), and some were rendered antibody negative earlier than others. It may be that a certain threshold of antibody level must be reached in order for the immune system to respond in a beneficial manner. If this is the case, a more personalised regimen may be possible with regular monitoring of the antibody, and patients only receiving the immunoadsorption they require. This could

lead to some patients receiving fewer sessions allowing for a cost saving. The initial cost of IA therapy is expensive, although the columns themselves are single patient columns but can be used for up to two years, with storage at 4°C between uses, drastically reducing the cost if retreatment is required. As part of the PRISM study, all healthcare contact and equipment costs have been recorded, along with patient-reported costs and quality of life measures in the form of the EQ-5D. This will allow us to perform cost-effectiveness analysis and allow for a comparison with current standard of care and Rituximab as published and described in the appendix[267].

The completion of the clinical trial follow-up and the emergence of a response group and a non-response group will allow a more targeted modelling of the immune system. Flow cytometry at time points related to the antibody level and over a prolonged follow up may start to show clinically relevant patterns, particularly when subgroup analysis is carried out using responders versus non-responders. New flow cytometry panels will also need to be set up in order to assess IgG and IgM PLA_2R +ve B cells in both our patient cohort and healthy volunteers. Validation of the ELISA for IgM anti-PLA_2R antibodies and for soluble PLA_2R will also provide further evidence for the loss of tolerance required for disease progression.

7.3 Conclusion

Current understanding for the pathogenesis of autoimmune MN has grown exponentially over the last decade. There remain unknowns, and as yet the science has not translated into more targeted novel therapies. By using immunoadsorption therapy to directly remove the pathogenic antibody and treat autoimmune membranous nephropathy, we have shown it has the potential to be used in the management of the disease without the need for toxic medications. We have also described for the first time important new components in the disease pathway to help further our understanding of the condition.

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Appendix

General laboratory methods

Separation of PBMCs from whole blood

Reagents required

All reagents should be at room temperature

20mls of EDTA blood – 2x 10ml tubes

Lymphoprep

PBS with 2% FCS

CTL-Wash buffer (this needs to be made up using RPMI)

CTL-Freezing media (this needs to be made up and filter sterilised)

Method

1 The whole blood is transferred to a 50ml tube using a pastette and diluted up to 40ml using PBS/BSA

2 Pipette 15mls of lymphoprep in 50ml Sepmate tube directly into central hole in Sepmate insert

3 Layer 20mls of diluted blood onto lymphoprep in Sepmate tube by pipetting down the side of the tube

4 Centrifuge at 1200g for 20 mins with the brake on

5 Pour off top layer into a 50 ml tube. This should be done in one smooth action, and the tube should not be left inverted for longer than 2 secs

6 Make up the volume to 35ml with PBS/FCS

7 Centrifuge at 300g for 10 mins with the brake off

8 Pour off top layer into a 50 ml tube

9 Flick the bottom of the 50ml tube containing the cells to loosen the pellet and resuspend the cells in 35mls PBS/FCS

10 Centrifuge at 300g for 10 mins with the brake on

11 Repeat steps 8 to 10 once more this time discarding the supernatant

12 Flick the bottom of the 50ml tube containing the cells to loosen the pellet. This time resuspend the pellet in 10mls of CTL-Wash buffer

13 Count the cells

14 Centrifuge at 300g for 10 mins with the brake on

15 Resuspend the cells in CTL-Cryo C to give a cell concentration of 2×10^7

16 Add an equal volume of CTL-Cryo A+B slowly over 2 mins

17 Place 1ml of cell suspension in freezing medium into a cryotube and label

18 Place the cryotubes into a -80°C freezer overnight

19 The cells should be stored in the vapour phase nitrogen store the next day

Manchester Anti-PLA_2R ELISA

1 Coat a 96 well flat-bottomed ELISA plate with 100µl per well of sodium bicarbonate buffer, containing recombinant PLA_2R1 ($rPLA_2R$) at 25µl/ml

2 Leave overnight (4 hours minimum) at 4°C

3 Discard contents.

4 Add 100µl SuperBlock (Thermo Fisher Scientific, Cramlington, UK)

5 Leave for 2 hours at 4°C before again discarding contents.

6 Add 100µl of SuperBlock with 0.1% Tween 20 to each well along with patient serum in a dilution of 1:100 (each patient sera had duplicate wells).

Each plate to also contain a standard curve quality control dilution series (1:3000; 1:1000; 1:313; 1:111; 1:37; 1:12).

To also contain a duplicated background wells containing only 100µl SuperBlock with 0.1% Tween 20.

7 Leave at room temperature for two hours on a plate shaker at medium speed

8 Wash thoroughly nine times in PBS with 0.1% Tween 20.

9 Add100µl of anti-human IgG-HRP conjugate (Jackson ImmunoResearch, Newmarket, UK) to each well in a dilution of 1:25,000 in SuperBlock

10 Leave for 2 hours at 4°C.

11 Discard the contents of the plates

12 Wash nine times in PBS with 0.1% Tween 20.

13 Add 100µl 3,3′,5,5′-Tetramethylbenzidine (TMB) enzyme substrate (Sigma Aldrich) to each well and allowed to develop for five minutes.

14 Add 100µl H_2SO_4 to stop the reaction

15 Read using the Softmax software Molecular Devices (Sunnyvale, CA).

Thawing cryopreserved PBMCs

1 Thaw the cells under hot tap until only a small ice crystal is left in the tube

2 Mix by inversion

3 Using pastette aspirate all the medium from the cryovial and slowly drip the cell suspension into a 15ml tube containing 10mls of cold RPMI (CTL-AAW)

4 Rinse the cryovial out using 1ml of RPMI (CTL-AAW)

5 Spin the cells at RTP for 10mins @ 330g (rapid acceleration max brake)

6 Remove the supernatant using a fine tipped pastette, flick the tube gently to loosen the cell pellet (take care not to create too many bubbles) and resuspend the cells in 2mls of warmed RPMI (CTL-AAW) and mix by inversion

7 Count cells

8 Spin the cells @ RTP for 10mins @ 330g (rapid acceleration max brake)

9 Remove the supernatant using a fine tipped pastette and resuspend the cells in CTL-Test Medium at a suitable the cell concentration for the test (if the cell count is not available, resuspend in 1ml of CTL-Test and add the missing volume before plating)

NB the PBMCs should be used immediately in the test

FC500 flow cytometry

Pre-run checks

1 Check there is sufficient sheath fluid for the run

2 Check the waste fluid and empty if necessary

3 Open the front of the machine and check there is enough cleanse fluid in the reservoir. If not then fill up to the first step

4 Log in to the computer (user name = transplant.lab, password = transplant) leave the computer logged on for at least 10 minutes.

5 Log in as renal research (password = research#). The password requires changing every 30 days, when asked to change the password please make a note of the new password and

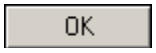
6 Start the program double clicking on the MXP icon, check that the cytometer powers up and both red lights are lit on the cytometer interface box. Leave for at least 40 minutes before running any samples.

7 Initialise the cytometer by pressing the  button. This will grey out indicating that the machine is busy.

Cleanse & wash

1 Four tubes 1 of bleach (1ml of Milton fluid and 1ml distilled water) and 3 tubes of 2ml distilled water. Place in positions 1, 2, 3 and 4 in an 8x5 tube plate with the notch in the top left-hand corner and place in the plate holder and close the lid.

2 Press on the plate icon  and clear the plate. From the menu that appears on the left of the screen click on the panel tab select cleanse panel and drag over to the plate. Check that well positions 1 to 4 are

highlighted click on 

, you will be asked to save the panel, choose cleaning.tdf to save and overwrite.

3 Press the play icon  . The 

icon will grey out indicating that the machine is busy. This process will take approximately 30 minutes.

Flowcheck

1 Take the flowcheck beads out of the fridge 30 minutes prior to running (PC7 (770/488).

2 Briefly mix the beads and dispense 10 drops of flowcheck fluorospheres (in transplant's fridge in post PCR) and 5 drops of flowcheck 770 fluorospheres (renal research fridge cell culture) into a tube.

3 Press on the plate icon and clear the plate. From the menu that appears on the left of the screen click on 'research qc flowcheck' and drag over to the plate. Check that well position 1 is highlighted and press the play icon 

4 If the flowcheck is successful, the FC500 is ready to run the samples.

Laser Channels

FL1=FITC

FL2=PE

FL3=ECD (energy coupled dye + PE)

FL4=PC5.5 (PE + cyanine 5.5.)

FL5=PC7 (PE + cyanine 7)

Staining the cells

1 100µl of whole blood into tube add the antibodies directly into the blood as directed in the table below

2 Briefly spin mix and leave to incubate for 30 mins in the dark

3 Add 1ml of versalyse to each tube, briefly mix and then incubate for 20 mins in the dark

4 Add 1ml of PBS check at this point that the solution is bright, cherry red in colour

5 Centrifuge at 300g for 5 minutes

6 Remove as much of the supernatant as possible using a pipette/pastette

7 Add 1ml of PBS, briefly mix and place into the cytometer

Frozen cells should be at a concentration of 5 million per ml and use 20µl of cell suspension per test

MARKER	DYE	SUGGESTED USE	CAT Nº
B CELL PANEL			
IgD	FITC	20UL	B30652
CD27	PE	20UL	B96790
CD38	ECD	10ul	A60792
CD20	PC5.5	10ul	B23134
CD19	PC7	10UL	IM3628
T-Reg			
CD45	FITC	20UL	A07782
CD127	PE	20UL	B49220
CD25	ECD	10UL	6607112
CD4	PC5.5	10UL	B16491
CD3	PC7	10UL	737657
MONOCYTES			
CD45	FITC	20ul	A07782
CD14	PE	20ul	A07764
CD16	ECD	10ul	B49216
CD56	PC5.5	10ul	B49189
PLA2R			
CD19	FITC	20ul	A07768
CD27	PE	20ul	B49220
CD38	ECD	10ul	A60792
CD20	PC5.5	10ul	B23134
PLA2R Antigen	PC7	20uL	
FLOWCHECK FLUOROSPHERES			6605359
PC7 (770/488) SET UP KIT			737664

Table S1.1 – flow cytometry panels and reagents used

Rituximab versus the modified Ponticelli regimen in the treatment of Primary Membranous Nephropathy: A health economic model

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Summary

Treatment for primary membranous nephropathy remains cyclophosphamide-based (the Ponticelli regimes) since the 1980s, despite its high side-effect burden. Newer therapies such as Rituximab show promise but are expensive. We undertook a cost-effectiveness analysis of overall administration costings; based on UK NHS prices to compare Rituximab with than the modified Ponticelli regime, the current standard of care.

Abstract

Background

Membranous Nephropathy is among the most common causes of nephrotic syndrome worldwide, with a high healthcare burden. Treatment using the modified Ponticelli regimes (mPR) has remained the standard of care for decades, but newer therapies such as Rituximab offer promising results with reduced side effects. The cost of this treatment, however, is perceived as a barrier to widespread use; especially in resource-limited healthcare systems.

Methods

We developed a decision-analytic model to estimate the cost-effectiveness of Rituximab versus the mPR from the perspective of the National Health Service in the UK over a one-year, five-year and lifetime horizon. Primary outcome is the cost-effectiveness of Rituximab vs mPR at five-years post-treatment. Secondary outcomes are cost-effectiveness at 1 and 10 years post-treatment and over a lifetime.

Results

At one-year post-treatment, Rituximab therapy dominates mPR. At five years post-treatment, Rituximab therapy is cheaper than the Ponticelli regime but at a loss of 0.014 QALYs with an ICER of £95,494.13. Over a lifetime, Rituximab remains the cheaper option with an incremental cost of -£5251.03 but with a reduced quality of life (incremental QALY of -0.512) giving an ICER of £10,246.09.

Conclusions

Our analysis indicates that Rituximab has the potential to be a cost-effective treatment in the short and medium term despite the high single dose cost. This evaluation suggests that further research is warranted and highlights the need for a high-quality clinical trial to confirm the efficacy and cost-effectiveness of Rituximab versus the current standard of care.

Introduction

Membranous nephropathy (MN) is one of the most common causes of adult nephrotic syndrome worldwide with a high healthcare burden in which approximately 20% of patients progress to end-stage renal disease (ESRD)[1,2].

MN has two distinct entities with primary MN (PMN) now considered to be an autoimmune disease since the discovery of the M-type of phospholipase A2 receptor 1 (anti-PLA_2R) antibodies[3-7].

In PMN, disease activity and prognosis are still measured by proteinuria level and renal excretory function with the risk of renal decline falling in the presence of a reduction in proteinuria[6,10-14]. A key marker of treatment efficacy in PMN is, therefore, control of proteinuria, with or without immunosuppression[9]. Such immunosuppression is generally a combination of alkylating agents and steroids, as used in studies by Ponticelli *et al*[15-18]. This regime of rotating high dose intravenous steroids and immunosuppression was first described in 1984 and has been the mainstay of treatment since[15]. Initially using Methylprednisolone and Chlorambucil, it was later modified to include Methylprednisolone and Cyclophosphamide[15-18]. Despite its treatment success, the modified Ponticelli regime (mPR) bears a significant side effect profile, including an increased risk of infection, osteoporosis, diabetes mellitus, weight gain, haemorrhagic cystitis, infertility and malignancy[16]. This led many researchers to search for alternative therapies including tacrolimus and Mycophenolate Mofetil but with little evidence to show any improvement in outcomes[19-23].

Rituximab has been used extensively in cancer therapy since the late 1990s and more recently for autoimmune diseases. A number of case series and studies have demonstrated potential in PMN but so far randomised controlled trials (RCT) have been scarce[24-28]. This, combined with the high cost of the medication itself, has restricted its widespread use in resource-limited,

evidence-based, healthcare systems such as the National Health Service in the UK (NHS).

We developed a decision-analytic model to estimate the cost-effectiveness of Rituximab therapy versus the standard of care, namely the modified Ponticelli regime for the treatment of primary MN.

Methods

A cost-effectiveness analysis was carried out using a stochastic cohort Markov model developed using standard methods[29], conducted from the perspective of current practice in the UK NHS at 2015 prices.

The primary outcome was the cost-effectiveness of Rituximab versus mPR at five-years post-treatment. Secondary outcomes were cost-effectiveness at one and ten-years post-treatment and over a lifetime. A literature search revealed no studies directly comparing Rituximab versus mPR, and therefore data was taken from the only studies of sufficient size to afford representative outcome assessment as described below. The analysis employed the cost-utility framework where the main measure of benefit is the quality-adjusted life year (QALY) and with analysis outcomes presented in terms of incremental cost-effectiveness ratios (ICER) of cost per QALY gained.

Choice of comparator

Here we have used the mPR which is the standard of treatment as per the KDIGO guidelines having established that the majority of UK renal centres use versions of the mPR as described by Ponticelli *et al.* and Jha *et al*[9,17,18,30].

Model Structure

The model was developed in consultation with an expert panel including physicians, health economists and clinical scientists, and was identical for each treatment arm (see figure S1.1).

For the treatment phase, all patients were assumed to experience active disease and costs were calculated from the papers described below. Following the treatment phase, patients could transition to (persistent) active disease, partial remission or complete remission. Health states then included sustained remission, relapse, ESRD (conservative management, haemo- or peritoneal

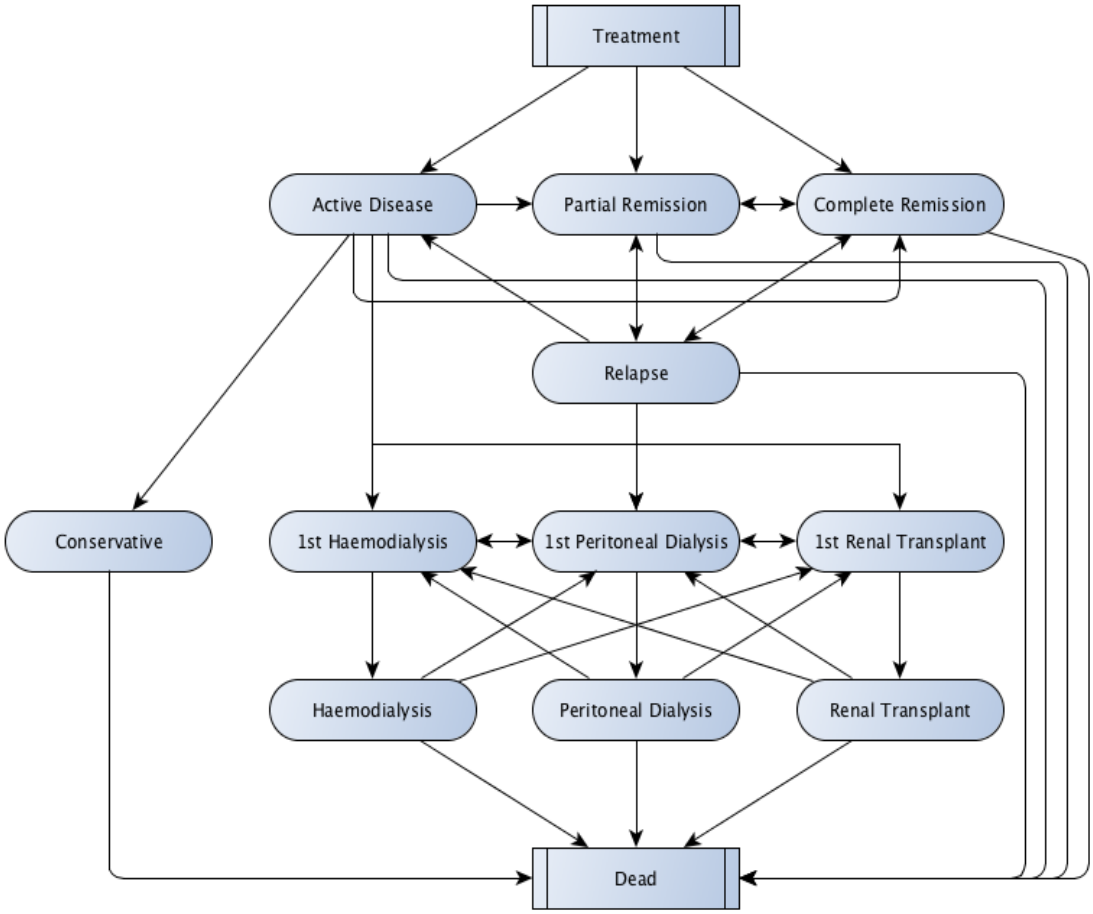

Figure S1.1 – Model structure

dialysis and renal transplant) or death. Following the initial treatment phase, patients transitioned between health states on three-monthly cycles over a lifetime horizon.

PMN is generally considered a disease of middle age with the median age of patients with PMN at diagnosis is 53 years old; we, therefore, extended the lifetime over an additional 47 years corresponding to a maximum survival of 100 years old[31].

Parameter values

Model parameter values and effectiveness of the interventions were based on the most robust data available for each arm; Jha *et al.* for the mPR arm and Ruggenenti *et al.* for the Rituximab arm[18,26]. Jha *et al.* was a prospective RCT comparing the mPR with supportive care, in biopsy-proven adults (>16 years old) with nephrotic syndrome for more than 6 months duration and less than 2 months of treatment with either steroids or immunosuppression. There was a total of 93 patients completing the study, 47 receiving the mPR with oral cyclophosphamide and IV Methylprednisolone.

Ruggenenti *et al.* published an observational study describing 100 consecutive patients, considered to be at high risk of progressing to ESRD or to develop significant cardiovascular complications of their nephrotic syndrome, treated with Rituximab and no control group. It involved two distinct regimes; initially, patients received Rituximab in four weekly doses of 375mg/m^2. However, as many patients on this regime were found to be B cell deplete after only the first dose of Rituximab, all subsequent patients from 2005 onwards were changed to a titrated regime. Prior to inclusion in the trial, 32 patients had received treatment with alternative immunosuppression. 20 of these did achieve partial remission prior to relapsing and necessitating treatment. The remaining 12 never achieved remission prior to starting Rituximab. Of the 100

patients described in the study, 71 received a single 375mg/m^2 dose of Rituximab and only received a second dose if their serum B cells were more than 5 cells/mm^3. The cost of treatment in the Rituximab arm was therefore calculated using the same proportion of treatments (with corresponding outcomes) as in this study. This resulted in 29% of the total cost of treatment being taken as the cost of the initial four doses of 375mg/m^2 Rituximab regime and 71% as the cost of the B-cell titration regime.

These papers were also chosen for their similar observational period allowing for a similar evaluation of care; however partial and complete remission were defined slightly differently (table 1), Jha *et al.* having more stringent remission criteria. In practice, there is a cohort of patients that spontaneously remit, but the majority will remain nephrotic and therefore require treatment. Both these studies, as in clinical practice, have included patients with biopsy-proven membranous nephropathy and significant proteinuria warranting immunosuppression. Both studies have a male predominance reflecting clinical practice and the mean age at presentation was older in the study as described by Ruggenenti *et al.* Jha *et al.* was carried out in India, and Ruggenenti *et al.* was carried out in Italy, two differing healthcare systems. However, both studies were carried out using standard methods and are comparable to use in the UK[18,26]. See table S2.1.

Probabilities

Transition probabilities from the treatment phase to active disease, complete remission, partial remission, relapse and death were taken from the literature as above (Jha *et al.* and Ruggenenti *et al*[18,26]). Here there was an assumption of constant hazards based on survival at a single time point. If a patient developed ESRD, they transitioned into the renal replacement pathway, which

	Jha *et al*.	Ruggenenti *et al*.
Country	India	Italy
Cohort size	47	100
Median follow up	11 years (range 10.5 – 11)	29 months (range 6 – 121)
Age in years – mean ± SD	38.0 ± 13.6	51.5 ± 5.9
Gender		
Male – n (%)	30 (63.8)	72 (72)
Female – n (%)	17 (36.2)	28 (28)
Disease state definitions		
Active disease	Proteinuria ≥ 3.5g/d or Proteinuria ≥ 2.5g/d & serum albumin < 2.5g/dl with oedema and hyperlipidaemia	Proteinuria ≥ 3.5g/d
Partial remission	Proteinuria < 2.0g/d or ≥ 50% reduction from baseline	Proteinuria < 3.0g/d & ≥ 50% reduction from baseline
Complete remission	Proteinuria < 0.2g/d	Proteinuria < 0.3g/d & ≥ 50% reduction from baseline
Relapse	Not defined	Proteinuria ≥ 3.5g/d after partial or complete remission
Adverse events – n (%)		
During infusion		
Allergy	0 (0)	8 (8)
Bronchial wheezing	0 (0)	10 (10)
Cutaneous rash	0 (0)	1 (1)
Hypotension	0 (0)	1 (1)
Stroke	0 (0)	3 (3)
TIA	0 (0)	2 (2)
Acute MI	1 (1)	3 (3)
Cancer	0 (0)	3 (3)
Respiratory tract infection	3 (6)	0 (0)
Urinary tract infection	5 (11)	0 (0)
Pyomyositis	1 (2)	0 (0)
Disseminated tuberculosis	1 (2)	0 (0)
Thrombosis	3 (0)	0 (0)
Deaths	1 (1)	4 (4)

Table S2.1 – Comparison of trials used for model

includes conservative management. Transition probabilities after ESRD have been obtained from the UK Renal Registry (2014)[32]. Death rates were taken as those described in the study arms. At the end of the study follow up, UK Office of National Statistics (ONS) data was used to provide a baseline mortality rate[33]. For patients in active disease, the death rate obtained from the ONS data was added to the transition probability from the studies. Once in partial or complete remission, death rate was taken as that in the ONS only. Death rates once in ESRD were taken from the UK Renal Registry.

Costs

Healthcare resource use included all healthcare contact, hospital stays, medication and serious adverse event (SAEs) episodes described in each publication. The cost of relapse was taken as the cost of treatment but without SAEs. Costs for each hospital/healthcare contact and SAEs were taken from the NHS reference costs 2014 to 2015[34]. Standard Deviation estimated using S = Q3–Q1 / 1.35 [35]. The cost of medication was taken from the Drugs and Pharmaceutical electronic market information (eMit) or from the British National Formulary 2015 if not available[36,37]. For medications for which the dose is based on Body Surface Area we used 1.79m^2[,38]. Maintenance therapy was not costed. Standard deviation of costs is not provided by the BNF, so these were taken to be half the mean. (Table S2.2, S2.3 & S2.4). See supplementary material for table with disaggregated costs of treatment stage for reference case and regimes used in sensitivity analysis.

Utility/Quality of life

For many patients, the presenting symptoms that bring them to the notice of healthcare professionals, and ultimately to the diagnosis of PMN, is that of the

Medication	Dose	Pack size	Treatment Dose	Mean Value (£)	SD (£)	Source
IV Methylprednisolone	1000mg	1 pack	1000mg	11.04	5.90	DFN009 eMIT
Prednisolone tablets	5mg	100 tablets	35mg	4.39	0.26	DFC045 eMIT
PO Cyclophosphamide	50mg	100	140mg	82.00	41.00	BNF
IV Cyclophosphamide	1000mg	1 vial		9.41	5.56	DHA014 eMIT
Rituximab	10mg/ml	10mL vial	375mg/m^2	174.63	87.32	BNF
		50mL vial		873.15	436.58	BNF
Basiliximab	20mg	1 vial		842.38	421.19	BNF
IV Hydrocortisone	100mg/mL	1mL amp	100mg	1.08	0.54	BNF
		5mL amp	500mg	4.89	2.45	BNF
Paracetamol	500mg	100 tablets	1000mg	0.52	0.29	DDM003 eMIT
Ondansetron	8mg	10 tablets	8mg	1.06	5.89	DDF029 eMIT
IV Chlorphenamine	10mg/1ml	5 ampoules	10mg	22.80	3.52	DCI002 eMIT
PO Mesna	400mg	10 tablets	400mg	42.90	21.45	BNF
IV Mesna	100mg/ml	4mL vial	200mg	3.95	1.98	BNF
Normal Saline	1000ml	1 bag	1000ml	0.80	0.40	BNF

Table S2.2 – Cost of medication. All medications oral unless otherwise stated. All doses based on weight of 70kg patient. All prices based on dose and pack size. SD – Standard deviation. eMIT – Department of Health electronic market information tool accessed on 30th June 2016 and costs correct to December 2015[36]. Prices given in eMIT are excluding VAT therefore taken as 20%. BNF – British National Formulary accessed on 30th April 2015[37]. Standard Deviations for BNF meds taken as Mean / 2 as they are not provided.

Health Service	Mean Value	LQR	UQR	SD	Source
Delivery of Chemo (1st)					
Simple Parenteral	257.00	136.00	311.00	129.63	SB12Z NHS ref costs
Complex & Infusional	414.00	250.00	521.00	200.74	SB14Z NHS ref costs
Subsequent chemo	362.00	230.00	413.00	135.56	SB15Z NHS ref costs
AVF, Graft or Shunt DC	1910.66	1334.41	2342.81	746.96	YQ42Z NHS ref costs
PD associated procedure DC	1268.00	503.00	1815.00	971.85	LA05Z NHS ref costs
Nephrology clinic	160.00	110.00	185.00	55.56	WF01A 361 NHS ref costs
Transplant clinic	358.00	220.00	493.00	202.22	WF01A 102 NHS ref costs
Haemodialysis					
CKD via AVF at base	166.00	143.00	176.00	24.44	RENALCKD LD02A NHS ref costs
Peritoneal Dialysis					
Automated PD	71.00	50.00	67.00	12.59	RENALCKD LD12A NHS ref costs
Renal Transplant					
Cadaver NHB	12,845.93	10,179.00	14,250.00	3015.56	LA01A NHS ref costs
Cadaver HB	12,434.09	12,904.00	14,450.00	1145.19	LA02A NHS ref costs
Live donor	13,828.19	9996.00	17,756.00	5748.15	LA03A NHS ref costs
Pre-transplant work-up					
Live donor	1205.75	958.00	1559.00	445.19	LA11Z NHS ref costs
B Cell subsets	5.00	2.00	7.00	3.70	DAPS06 NHS ref costs

Table S2.3 – Cost of healthcare provision. All costs given in British Pound Sterling. NHS ref costs – National Health Service reference costs 2014 – 2015[34]. LQR – Lower Quartile Range. UQR – Upper Quartile Range. IP – Inpatient. OP – outpatient. DC – Day case. AVF – Arterioventricular Fistula. PD – Peritoneal Dialysis. AKI – Acute Kidney Injury. CKD – Chronic Kidney Injury. NHB – Non-Heart Beating donor. HB – Heart beating donor. Sat – Satellite unit. CAPD – Continuous Ambulatory Peritoneal Dialysis. Complex & Infusional – Complex Parenteral and prolonged infusion treatment. Standard Deviation estimated using S = Q3 – Q1 / 1.35 from Cochrane Handbook from Systematic Reviews and Interventions 2008[35].

Complication	Mean	LQR	UQR	SD	Source	Notes/Assumptions
				Jha *et al.*		
Resp Infections	1540.00	1255.00	1685.00	318.52	DZ22Q NHS ref costs	Unspecified acute LRTI (0-1)
UTI	1503.00	1233.00	1659.00	315.56	LA04S NHS ref costs	Kidney/UTI – no intervention (0-1)
Gluteal Abscess	1358.00	960.00	1557.00	442.22	HD26G NHS ref costs	MSK signs or symptoms (0-3)
Bact. Meningitis	2339.00	1561.00	2638.00	797.78	AA22G NHS ref costs	Nervous system infections (0-4)
Pulmonary Tb	2650.00	1702.00	3131.00	1058.52	DZ14J NHS ref costs	Pulmonary, pleural, other Tb
Septicaemia	1993.00	1586.00	2224.00	472.59	WJ06J NHS ref costs	Sepsis (0-1)
DVT	1362.00	992.00	1491.00	369.63	YQ51E NHS ref costs	DVT (0-2)
				Ruggenenti *et al.*		
Acute MI	1505.00	1205.00	1701.00	367.41	EB10E NHS ref costs	Actual/Suspected MI (0-3)
Stroke	2348.00	1803.00	2597.00	588.15	AA35F NHS ref costs	Stroke (0-3)
TIA	1253.00	978.00	1393.00	307.41	AA29F NHS ref costs	TIA (0-4)
Lung cancer	3047.00	2063.00	3610.00	1145.93	DZ17R NHS ref costs	Resp. neoplasm (0-5)
Breast cancer	3357.00	1504.00	4554.00	2259.26	JA12F NHS ref costs	Malignant - intervention (0-2)
Prostate Ca	2268.00	1469.00	2660.00	882.22	LB06M NHS ref costs	Prostate Ca – intervention (0-1)

Table S2.4 – Cost of AEs and SAEs. All costs given in British Pounds. NHS ref costs – National Health Service reference costs 2014 – 2015[34]. LQR – Lower Quartile Range. UQR – Upper Quartile Range. CC Score in parenthesis. TIA – Transient Ischaemic Attack. Tb – Tuberculosis. UTI – Urinary tract infection. DVT – Deep vein thrombosis. All costs taken as non-elective short stay. Standard Deviation estimated using SD = Q3 – Q1 / 1.35 from Cochrane Handbook from Systematic Reviews and Interventions 2008[35].

nephrotic syndrome, namely oedema, increasing shortness of breath and fatigue. Currently, there is limited data available on the quality of life (or utility) for patients with PMN, therefore utility values for active disease were taken as that of active nephrotic syndrome, given these are the main symptoms a patient will experience when their disease is active[39]. For patients with partial or complete remission, we used age and sex-matched EQ-5D UK population norms[40]. Once patients reached ESRD, utility values were estimated using SF-6D values from Wyld *et al*. converted to utility scores[41,42]. (Table S2.5).

Cost-effectiveness analysis

All costs are presented as mean cost per patient. Expected costs and QALYs were estimated for each arm and, where appropriate, ICERs calculated (derived from the incremental cost of treating with Rituximab and the incremental QALY). ICERs below the £20,000 threshold would indicate that Rituximab is considered cost-effective as set by National Institute for Health and Care Excellence (NICE) standards[43]. Following NICE guidelines, half-cycle correction was conducted, and a discount rate of 3.5% per annum was applied to all outcomes incurred beyond one year[43].

Incremental Net Monetary Benefit (INMB)

INMB's were calculated using the incremental QALY, the incremental cost and the Lambda, which in this case is £20,000, as per NICE guidelines[43]. A positive value indicates that Rituximab therapy is cost-effective and therefore the preferred option when compared with the mPR.

Deterministic Sensitivity Analysis

We performed one-way sensitivity analysis on a range of parameters to assess the impact of each parameter on the outcome of the model at five-years post-

Utility	Mean	LCI	UCI	SD / SE	Source	Notes
Complete remission	0.860	0.630	1.000	0.230	Kind *et al.*	Age & Sex matched
Partial remission	0.860	0.630	1.000	0.230	Kind *et al.*	
Active disease	0.738	0.422	1.000	0.317	Liborio *et al.*	SF36 converted to EQ5D
ESRD	0.800	0.650	0.940	0.030	Wyld *et al.*	CKD (pre-treatment)
Conservative	0.620	0.360	0.890	0.090	Wyld *et al.*	SF36 converted to EQ5D
Haemodialysis	0.680	0.530	0.820	0.020	Wyld *et al.*	SF36 converted to EQ5D
Peritoneal dialysis	0.710	0.590	0.820	0.020	Wyld *et al.*	SF36 converted to EQ5D
Renal transplant	0.820	0.740	0.900	0.040	Wyld *et al.*	SF36 converted to EQ5D
Dead	0.000	0.000	0.000	0.000		

Table S2.5 – Quality of life utility values. ESRD – End-stage renal disease. Partial remission and Complete Remission taken as the same.

treatment as described by the INMB. For sensitivity analysis of the costs, these were altered, the quality of life and transition probabilities remaining unchanged. For sensitivity analysis of the transition probabilities, the costs remained unchanged. Exact alterations to costs and probabilities are given below.

Rituximab regimes

The study described by Ruggenenti *et al.* used to inform the Rituximab arm in our model utilised two different regimes as described in the methods section. We therefore carried out a sensitivity analysis based on all patients in the Rituximab arm receiving the original regime consisting of four weekly infusions of 375mg/m^2 Rituximab. We then carried out the analysis based on all patients in the Rituximab arm receiving the B cell titrated regime, i.e. a single 375mg/m^2 dose of Rituximab with a second dose if their serum B cells were subsequently more than 5 cells/mm^3. For both of these, the costs in the Ponticelli arm remained unchanged. Further sensitivity analysis was carried out using the recently reported RCT described by Dahan *et al*[27]. Here patients in the treatment arm were given 2 doses of 375mg/m^2 Rituximab on days 1 and 8. For this analysis, only the costs in Rituximab arm of the model were changed, and all outcomes remained the same.

Ponticelli regimes

The mPR uses low-cost medications but requires multiple hospital admissions to receive steroid infusions. Therefore, to assess the impact that drug delivery has on the overall cost, we performed a sensitivity analysis with patients only receiving oral prednisolone and no IV Methylprednisolone, with cyclophosphamide remaining unchanged. We also assessed how a change in the cyclophosphamide regime might affect the overall cost by carrying out a

sensitivity analysis using pulsed monthly cyclophosphamide for 6 months with adjunctive oral prednisolone (with no IV methylprednisolone) as described by Kanigicherla *et al*[44]. The costs for the Rituximab arm remained unchanged for both of these analyses.

Other

To assess how the cost of drug delivery itself affects the model outcomes we performed a sensitivity analysis with an increase and decrease in the cost of the delivery of an infusion in a day-care setting by 20% and on the cost of the medication itself (Rituximab and Cyclophosphamide). For the cost of infusion delivery, the cost was altered in both arms. For the cost of medication, the cost was altered in each arm and analysed separately.

In order to provide consistency, the cost of cancer in the original analysis was taken as the cost for the least severe form of the disease as per the NHS reference costs[34]. To assess whether the cost of cancer impacts on the results we used the cost for the most severe form of the various cancers as reported in the NHS reference costs[34] for the sensitivity analysis.

Given the known uncertainty in the quality of life measures available we performed a sensitivity analysis on this by altering the utility value of partial remission to be the same as active disease instead of complete remission. This was changed in both arms simultaneously.

Transition probabilities

To investigate the impact of the transition probabilities on outcomes, we performed a number of analysis including altering the death rate to be equal in both arms, the chance of developing ESRD and needing RRT to be equal in both arms and the rate of relapse to be equal in both arms. We analysed the effect of treatment efficacy by altering the transition probabilities of going from the treatment phase to either active disease, partial remission or complete

remission by making them equal in both arms. We then altered the chance of transitioning from active disease to remission so that it was equal in both arms. We altered all transition probabilities to be equal in both arms with no change to costs or utility values. We also increased and decreased the probability, by 20%, of going into remission in the Rituximab arm and keeping the Ponticelli arm unchanged. We then performed the same analysis by altering the transition probability in the Ponticelli arm and kept the Rituximab arm unchanged.

Probabilistic Sensitivity Analysis

A probabilistic sensitivity analysis (PSA) was conducted with 10,000 Monte Carlo simulations based on random draws of all parameter values simultaneously from probability distributions. This provided 10,000 estimates of costs and QALYs, which were used to generate 10,000 ICERs and incremental net monetary benefit (INMB) estimates and allowed us to estimate the level of parameter uncertainty in the analysis. These simulated analyses were plotted on a cost-effectiveness plane and a cost-effectiveness acceptability curve (CEAC)[45]. The CEAC indicates the probability that Rituximab is cost-effective versus mPR across a range of willingness to pay per QALY gain thresholds[46]. The higher the probability, the lower the uncertainty is in the model and decision.

Validation

We employed a number of tests to ensure the model was valid as possible although, given the nature of the disease and lack of clinical trials, we were unable to perform a full validation. Validation was carried out using recognised techniques[47]. Face validation was carried out with each aspect of the model design, data sources, formulae and eventual results reviewed and discussed by

a panel of experts including clinicians, clinical scientists and health economists. Internal validation was performed using deterministic sensitivity analysis and testing whether changes in model inputs led to changes in outputs in the expected direction - for example by increasing the SAE / AE risks for Rituximab we expected the cost-effectiveness of that intervention would be reduced. Verification of the code was performed by one clinician and two separate and independent health economists.

As there are no other health economic or epidemiological models or RCTs in this area, cross-validation, external validation and predictive validation were not possible.

Results

Incremental Cost-Effectiveness Ratio

At five years post-treatment, Rituximab therapy is cheaper than the Ponticelli regime but at a loss of 0.014 QALYs. Here the ICER is £95,494.13 (incremental cost -£1,355.82 and incremental QALY -0.014). At one-year post-treatment, Rituximab therapy dominates mPR. At 10 years post-treatment, Rituximab remains the cheaper option with an incremental cost of -£2,201.37. With an incremental QALY of -0.091, the ICER is £24,256.91. Over a lifetime the ICER was £10,246.09, obtained from the incremental per-patient cost of -£5,251.03 and incremental QALY of -0.512. See supplementary material for frequency of patients in each disease state at five-years post-treatment with corresponding costs and QALYs. See table S2.6.

Figure S1.2 - cost-effectiveness plane showing incremental costs versus incremental QALY at one-year, five-year and over a lifetime. Threshold line at £20,000 per QALY for 10,000 PSA simulations. At one-year and five-year post treatment the majority of simulated ICERs are in the right-hand side of the plane indicating Rituximab is more effective. There is a majority of patients in the lower half of the plane indicating that at five-years post-treatment, Rituximab therapy is cheaper. The vast majority are below the £20,000 per QALY threshold set by NICE as the acceptable limit for the cost-effectiveness[43]. Over a lifetime the majority of patients are in the left lower quadrant showing that Rituximab therapy is cheaper but less effective.

	Deterministic Sensitivity Analysis				Probabilistic Sensitivity Analysis			
	Incremental Cost	Incremental QALY	ICER	INMB	Incremental Cost	Incremental QALY	ICER	INMB
1-year	-£748.20	0.002	Rituximab Dominates	£785.44	-£761.19	0.001	Rituximab Dominates	£777.54
5-years	-£1,355.82	-0.014	£95,494.13	£1,071.86	-£1,383.61	-0.014	£101,665.93	£1,111.42
10-years	-£2,201.37	-0.091	£24,256.91	£386.32	-£2,217.16	-0.092	£24,222.17	£386.47
Lifetime	-£5,251.03	-0.512	£10,246.09	-£4,998.79	-£5,228.58	-0.612	£2,198.07	-£7,016.21

Table S2.6 - Results for both probabilistic and deterministic sensitivity analysis at one, five and ten years post-treatment and over a lifetime. Lambda taken as £20,000. QALY – Quality-adjusted life year. ICER – Incremental cost-effectiveness ratio. INMB – Incremental net monetary benefit.

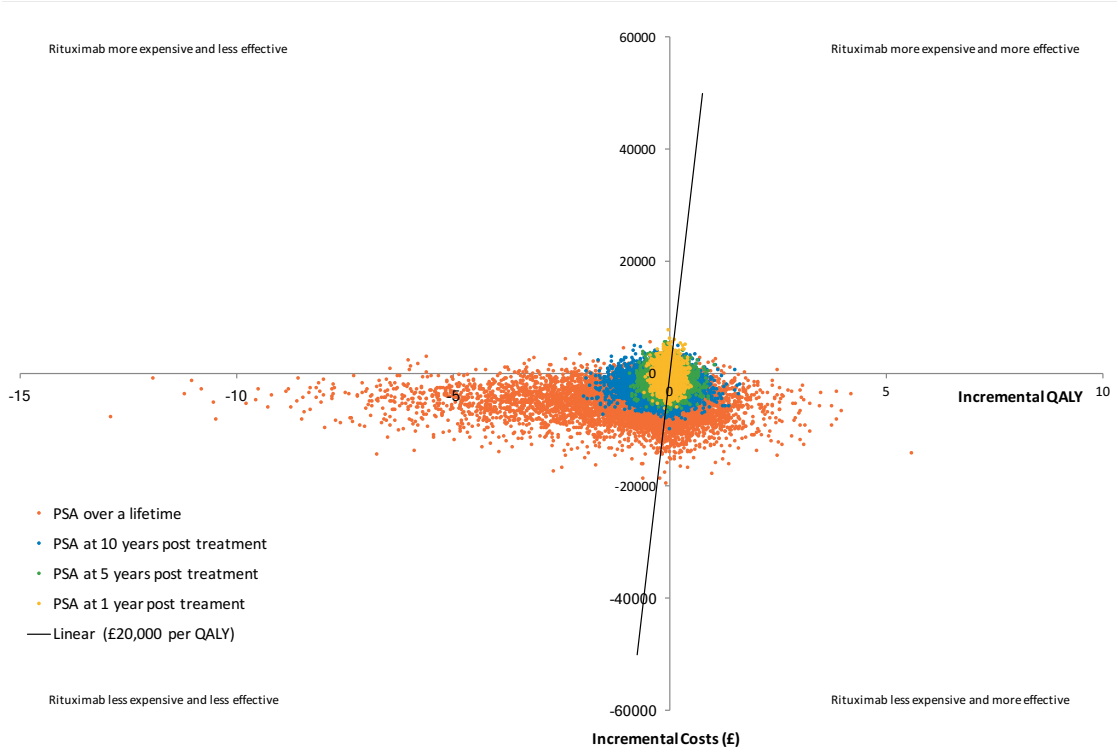

Figure S1.2 - cost-effectiveness plane

Cost

At five-years post-treatment the cost for the mPR was -£13,116.65 and the cost for the Rituximab regime was £11,760.83, showing that the mPR is more expensive than Rituximab with an incremental cost of -£1,355.82. At one-year post-treatment, the cost of mPR and Rituximab was £8,676.10 and £7,927.90 respectively giving an incremental cost of -£748.20. At ten-years post-treatment, the cost of mPR was £17,834.30 and for Rituximab was £15,632.93, indicating that Rituximab continues to be cheaper with an incremental cost of -£2,201.37. Over a lifetime the cost of mPR is £29,943.80 compared to £24,692.77 for the mPR; an incremental cost of -£5,251.03. See table S2.6.

QALY

The QALY gains for mPR and Rituximab were 3.712 and 3.697 respectively at five-years post-treatment, 0.952 and 0.954 respectively at one-year, 6.603 and 6.513 respectively at ten-years, and 14.162 and 13.650 respectively over a lifetime. Therefore, at one-year Rituximab confers QALY benefits over mPR but this is reversed by five-years and continues over a lifetime.

Incremental Net Monetary Benefit

At one-year, five-year and ten-year post-treatment the incremental net monetary benefit (INMB) of Rituximab therapy is £785.44, £1,071.86 and £386.32 respectively, indicating Rituximab is more cost-effective. Over a lifetime the INMB is -£4,998.79 showing mPR is the more cost-effective option. See table S2.6.

Deterministic Sensitivity Analysis

Constrained to address outcomes with a mixed-protocol Rituximab analysis the sensitivity analysis confirms that a major driver of cost for Rituximab was the number of infusions required. The original four-dose regime is too expensive at five-years post-treatment, but for the B cell titrating regime and the regime described by Dahan *et al*[27], at five-years post-treatment, Rituximab is the cost-effective option. The other major drivers of cost-effectiveness in the Rituximab arm were death rate and the probability of reaching remission.

For the mPR arm, the main driver of the cost appears to be the frequency of infusions with removal of the cost of IV methylprednisolone resulting in the mPR being more cost-effective at five-years post-treatment. The use of pulsed monthly IV cyclophosphamide alongside daily oral Prednisolone (again without IV Methylprednisolone) also resulted in the mPR being the most cost-effective at five-years post-treatment. See figure S1.3 for full tornado plot of sensitivity analysis.

Cost-effectiveness acceptability curve

Figure S1.4 - CEAC for the comparison based on the 10,000 PSA simulations. It shows the likelihood that Rituximab is cost-effective compared to mPR over a range of willingness-to-pay (WTP) per QALY gain threshold values (Lambda). At a lambda of £20,000 Rituximab has a 64% chance of being the cost-effective option at five-years post-treatment. At a threshold of £30,000, this falls to 61%. This reflects the fact that Rituximab is the cheaper option at this time point but with a slightly reduced QALY.

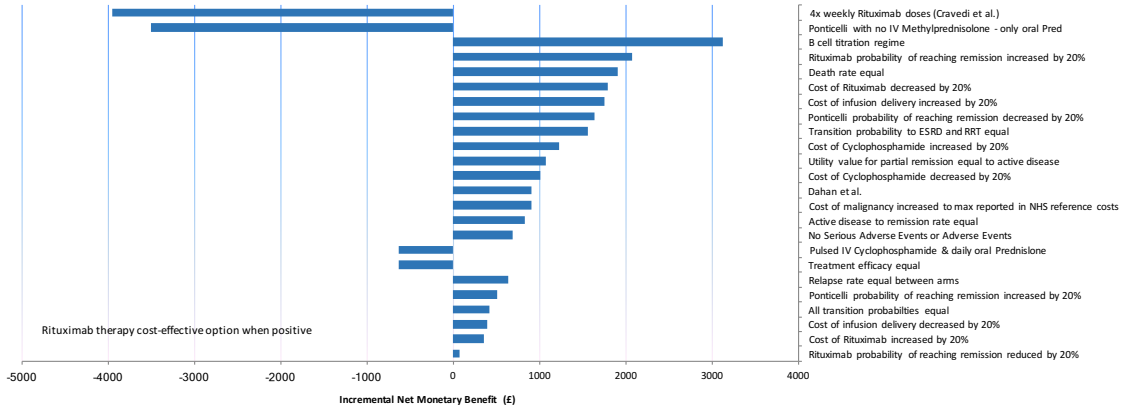

Figure S1.3 - Sensitivity analysis tornado plot

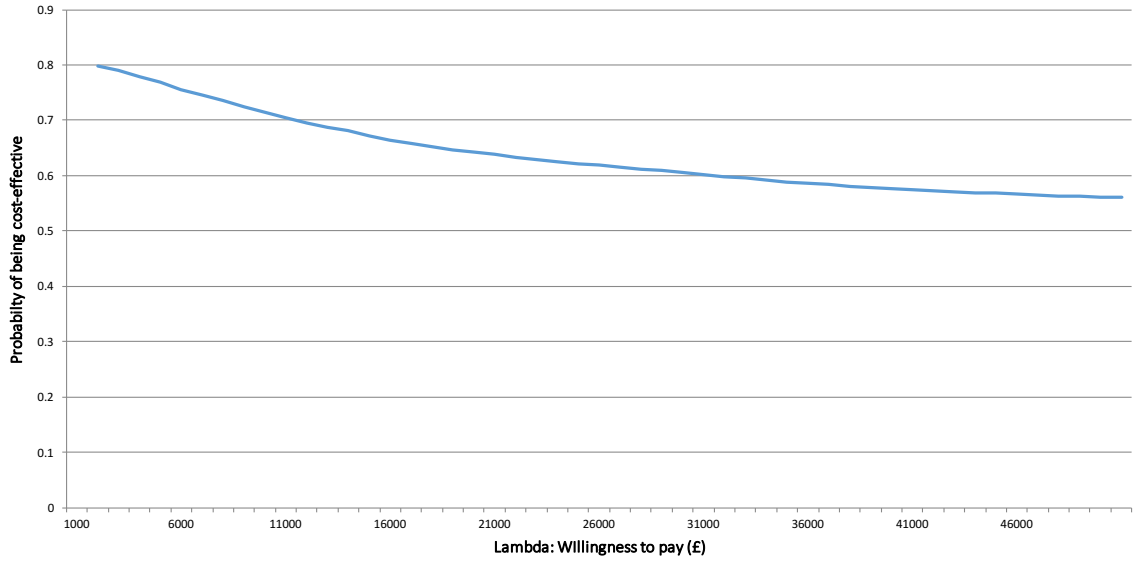

Figure S1.4 – Cost-effectiveness acceptability curve

Threshold analysis

In order for Rituximab to be the most cost-effective option over a lifetime, threshold analysis shows that the transition probability for treatment to active disease, partial remission and complete remission would have to change from 0.51250 to 0.61706, from 0.28500 to 0.22387 and from 0.20250 to 0.15907 respectively. Alternatively, the transition probability for active disease to death and partial remission to death for Rituximab would have to change from 0.00315 to 0.00136 and from 0.00680 to 0.00225 respectively.

Threshold analysis to determine the cost at which Rituximab represents the cost-effective option over a lifetime showed that due to the disparity in QoL there is no price at which it is cost-effective over a lifetime.

Discussion

The NHS, as with healthcare systems around the world, endeavours to provide the best care possible, with limited resources, for its ageing population and increasingly complex patients. This has resulted in NICE, the regulatory body, considering not only the health benefits of therapies but also their economic impact.

Rituximab has become increasingly important in the treatment of a range of autoimmune conditions[48-58]. Its attraction lies in its more directed immunoregulation and reduced side effect profile as compared to other immunosuppressants. Its single dose cost, however, has limited its use in conditions such as MN, especially where there is a paucity of evidence from RCTs available.

With this lack of RCTs but with good evidence that Rituximab can provide a benefit for patients in a number of trials and case series[24-28], we constructed a Markov model to assess its cost-effectiveness when compared to the standard of care, i.e. the mPR. Using costs from the UK NHS, we found that at every time point analysed Rituximab was the cheapest option, and this was especially true if using the B-cell titration regime. At one-year post-treatment, the QALY was better using Rituximab than the mPR, but over a lifetime this reduced with the mPR providing an increment of approximately half a QALY. However, Rituximab may still represent value for money given the cost savings are so high for every QALY lost.

It appears that the main driver of cost for the mPR is the frequency of infusions, adding cost to an inexpensive medication such as Methylprednisolone. This is also true for Rituximab, with the original regime, in which patients have four doses, proving less cost-effective[25]. In the B-cell titration regime[24], patients continue to have a good response to treatment but with fewer infusions making it consistently more cost-effective.

The reduction in quality of life for Rituximab over time is in part associated with the slightly increased risk of death and to a lesser extent the higher risk of relapse after Rituximab. Our model, however, is a conservative estimate for the quality of life benefits from Rituximab, as we do not take into account late complications associated with the therapies. It is well documented that there is an increased risk of malignancy many years after treatment with Cyclophosphamide[59]. Rituximab, in contrast, appears to have fewer complications and no indication of an increased risk of malignancy. Our model does not capture the quality of life associated with the provision of treatment, such as early onset side effects, notably nausea in cyclophosphamide, or with the number of visits. With the reduced side effect profile and reduced hospital visits needed for Rituximab therapy one could deduce that this would contribute to an improved quality of life although this is not possible to prove in this model.

This is the most comprehensive estimate of the cost-effectiveness of treatment for PMN to date, but it does come with limitations. The spread of results on the scatterplot for the PSA at the lifetime horizon indicates significant uncertainty in the results with the robustness of data available degenerating over time. This highlights the need for further good quality long-term prospective research comparing these therapies. Another limitation is that this evaluation was based on a naive comparison, if other single arm or cohort study data becomes available, it may be that an indirect comparison would then be feasible.

Due to the paucity of RCTs investigating the efficacy of Rituximab in PMN we opted to base the Rituximab arm on the largest data series available for its use in this condition. This is a prospective observational study with all the limitations this confers on the data such as patient selection and centre bias, but it remains the most robust data available.

This and the Jha study used to inform the model are international studies (Italy and India), but for precision, our model is costed to the UK health system. At present, there are no large-scale clinical trials published using Rituximab in a UK population, and there have been no large clinical trials in the UK using Cyclophosphamide for the treatment of PMN.

Another limitation has been the assignment of utility values to the disease. There is good validated data for population norms, but renal specific quality of life data is scarce. This meant for active disease and RRT we had to convert SF-36 scores to utility values using standard methods[39-42].

PMN can be a slowly progressing disease with many patients following a relapsing and remitting pattern over a number of years. Here we used only the rates for transition to ESRD and RRT as described in the two papers. This is likely to have underestimated the degree to which patients progressed to ESRD over a lifetime due to the relatively short follow up time of the studies. Given the uncertainty already apparent in the model over a lifetime, it adds further evidence for the need for long term RCTs in PMN.

This model has only included the cost of therapy at a tertiary level. It was beyond the scope of the study to assess the overall societal cost, and there is likely to be a significant cost to patients, families and carers in the form of lost days of work, travel costs, equipment costs. The cost of primary healthcare contact has also not been included in this model.

Conclusion

Rituximab has shown promise as a therapy for PMN in a number of studies, but the high cost of the medication has proven to be a barrier to its widespread acceptance. Here we have constructed the most detailed economic model yet for the treatment of PMN and show that Rituximab is not more expensive than the gold standard treatment and is cheaper over a lifetime. This work highlights the uncertainty surrounding PMN treatment with the small number of RCTs available to guide practitioners and commissioning bodies. Based on the evidence available, the longer-term effectiveness of Rituximab in PMN needs further evaluation, and importantly, long-term trials comparing Rituximab with cyclophosphamide-based therapy should be undertaken to help establish the most cost-effective management of the condition.

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Parameter	Distribution
Costs	Gamma
Utilities	Beta
Initial treatment transition probabilities	Dirichlet
ESRD transition probabilities	Dirichlet
All other transition probabilities	Beta

Table (supplementary material) – PSA distributions by parameter.

	Ponticelli arm		Rituximab arm	
Cost per disease state (£)	Mean	SD	Mean	SD
Initial treatment	£5105.72	£491.59	£4691.15	£941.80
Relapse	£1168.83	£343.70	£1129.26	£454.16
Active disease	£346.67	£88.32	£346.67	£88.32
Partial remission	£160.00	£60.00	£160.00	£60.00
Complete remission	£80.00	£42.43	£80.00	£42.43
ESRD/Conservative	£346.67	£88.32	£346.67	£163.56
1st Haemodialysis	£6951.67	£377.68	£6951.67	£377.68
Haemodialysis	£6474.00	£171.18	£6474.00	£171.18
1st Peritoneal Dialysis	£6795.75	£500.24	£6795.75	£500.24
Peritoneal Dialysis	£6478.75	£134.40	£6478.75	£134.40
1st Renal Transplant	£5215.85	£2938.59	£5215.85	£2938.59
Renal Transplant	£358.00	£140.00	£358.00	£140.00
Dead	£0.00	£0.00	£0.00	£0.00

Table (supplementary material) – cost per disease in British pounds. Initial treatment cost is per year. All other costs are quarterly. SD – standard deviation. ESRD – end-stage renal disease.

Year 5		Frequency			Costs			QALYs	
n = 1000	Ponticelli	Rituximab	Incremental	Ponticelli	Rituximab	Incremental	Ponticelli	Rituximab	Incremental
Active disease	525.524	329.032	-196.492	£158760.84	£99400.59	-£59360.25	337.977	211.609	-126.369
PR	235.725	334.689	98.965	£32867.26	£46666.00	£13798.75	162.138	230.209	68.071
CR	194.073	266.131	72.057	£13529.89	£18553.40	£5023.51	133.489	183.053	49.563
Relapse	9.214	6.324	-2.890	£9385.45	£6223.27	-£3162.18	5.926	4.067	-1.859
Initial HD	5.325	5.564	0.240	£32256.61	£33708.05	£1451.44	3.155	3.297	0.142
Initial PD	0.320	0.325	0.005	£1897.82	£1926.98	£29.16	0.198	0.201	0.003
Initial Trans	3.411	3.726	0.314	£15506.19	£16934.97	£1428.78	2.438	2.662	0.225
HD	0.003	0.003	0.000	£14.76	£15.54	£0.78	0.002	0.002	0.000
PD	0.002	0.002	0.000	£10.61	£10.87	£0.25	0.001	0.001	0.000
Transplant	0.007	0.007	0.001	£2.08	£2.29	£0.20	0.005	0.005	0.000
ESRD	0.145	0.152	0.007	£43.83	£45.95	£2.11	0.078	0.082	0.004
Dead	20.990	48.230	27.240	£0.00	£0.00	£0.00	0.000	0.000	0.000

Table (supplementary table) – Frequency of patients in each disease state with total number of patients = 1000. Cost in British pounds and QALY measure of each disease state. All results at the end of year 5 post-treatment. PR – partial remission. CR – complete remission. HD – haemodialysis. PD – peritoneal dialysis. Trans – transplant. ESRD – end-stage renal disease.

	Ponticelli arm	Rituximab arm	B cell titration regime	Cravedi regime	No IV Methylprednisolone	Pulse IV Cyclo & daily Pred
Total cost of treatment	£5105.72	£4691.15	£3344.46	£7988.22	£2066.13	£3995.85
Cost of drug	£345.65	£2358.23	£1324.28	£4889.64	£249.06	£111.77
Cost of drug delivery	£2943.00	£750.36	£444.17	£1500.00	£0.00	£2067.00
Prophylactic medications	£0.00	£18.23	£10.24	£37.80	£0.00	£0.00
Clinic attendance	£1386.67	£1386.67	£1386.67	£1386.67	£1386.67	£1386.67
SAEs & AEs	£430.40	£174.12	£174.12	£174.12	£430.40	£430.40

Table (supplementary material) – disaggregated costs for each treatment arm and regimes used for sensitivity analysis.

The investigative burden of Membranous Nephropathy in the UK

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Abstract

Background

Membranous Nephropathy (MN) represents two distinct disease entities. Primary MN is now recognized as an autoimmune condition associated with the anti-PLA2R antibody, and secondary MN occurs in tandem with malignancy, infection, drug therapy and other autoimmune conditions. Prior to the development of accessible ELISAs, the diagnosis of MN was one of exclusion. We reviewed the investigative burden for patients in the anti-PLA2R era.

Methods

Patients from 3 UK centres with a diagnosis of MN between 2009 and 2014 were identified. We compared patients who had a positive anti-PLA2R test within 6 months of biopsy to those who had no test or a negative test. Records were reviewed for investigations which took place 6 months prior to, and 6 months following, the biopsy date to see if these were normal or identified a secondary cause of MN.

Results

184 patients were included. 80 had no test, 66 had a negative anti-PLA2R test, and 38 had a positive test within 6 months of diagnosis. In 2012, 46.5% of patients had an anti-PLA2R test rising to 93.3% in 2014. From 2012 to 2014 the number of screening tests dropped from 10.03 to 4.29 and the costs from £497.92 to £132.94.

Conclusion

Since its introduction, a progressively higher proportion of patients diagnosed with MN had an anti-PLA2R test. This has led to a reduction in the number of screening tests and the cost of investigations carried out. The anti-PLA2R test has the potential to reduce this burden as its use becomes more widespread.

Introduction

Membranous nephropathy (MN) is among the most common causes of nephrotic syndrome in adults worldwide[1,100-104]. For decades it has been a histological diagnosis with two distinct entities; primary or autoimmune membranous nephropathy (PMN) and secondary MN. Despite their histological similarities, the pathogenesis and treatments differ greatly, meaning that differentiating between the two conditions is essential. Secondary MN is associated with a multitude of conditions such as malignancy, viral infections such as Hepatitis B & C, medications, other autoimmune conditions such as Lupus and toxins[5,241,242]. As such, the management is aimed at treating the underlying condition. PMN, originally known as idiopathic membranous nephropathy, has always been considered an autoimmune disease although the offending antibody remained elusive until the discovery of antibodies to the M-type phospholipase receptor 1 (anti-PLA_2R) in 2009[15,38,39,50,239,240]. This antibody is found in approximately 75% of patients with PMN and given its high affinity for podocytes is likely to be found on renal biopsy in a proportion of seronegative patients[15,31]. Soon after this the first quantitative anti-PLA_2R enzyme-linked immunosorbent assay (ELISA) test was developed in Manchester and became available across the Northwest of England towards the end of 2011[38]. Since then a commercial anti-PLA_2R has been developed and is now readily available internationally. Prior to the development of these ELISAs though, PMN was a diagnosis of exclusion. Given the association of secondary MN with malignancy and given the disease itself is generally a disease of middle age and older, many patients undergo a number of invasive procedures in order to rule out neoplastic disease. At present, there is no universally accepted consensus on the investigative pathway for primary or secondary MN. In patients with PMN, this results in many procedures performed, with normal findings, at a cost not only to the

patient terms of quality of life but also a societal cost to healthcare systems with limited resources.

With the anti-PLA_2R test becoming more ubiquitous, we hypothesised that the introduction of anti-PLA_2R testing leads to a modification in the investigative pathway for MN patients.

Methods

Patients with biopsy-proven membranous nephropathy between 2009 and 2014, from three large teaching hospitals in the Northwest of England covering a population of approximately 7 million were identified.

Day zero was taken as the date of renal biopsy. Records were reviewed for the investigations which took place 6 months prior to, and 6 months following the biopsy date to see if these were normal or identified a secondary cause of MN. Investigations included viral and autoimmune screens, x-rays, computed tomography (CT) scans, magnetic resonance imaging (MRI), positron emission tomography (PET) scans, ultrasound scans, upper and lower gastrointestinal (GI) endoscopies and cystoscopies. Investigations were excluded if they were not performed in relation to the diagnosis of primary versus secondary MN.

Records were also interrogated to determine if a patient had an anti-PLA_2R test and at what date. The result was only included if the sample was also taken within 6 months of the date of biopsy. A positive anti-PLA_2R test was taken as greater than 40 u/mL for the ELISA and a titre of more than 1:10 for the Euroimmun Indirect immunofluorescence test (IIFT). A negative ELISA was taken as less than 40 u/mL and a titre of 1:10 or less for the Euroimmun IIFT [38].

Costs were assigned to each investigation in pounds sterling and taken from the National Health Service (NHS) reference costs 2015-16[18]. For chest and

abdominal x-rays, the costs were taken from the NHS England National Tariff 2015-16[19]. The cost of anti-PLA_2R testing was not included. (Table S3.1).

For each patient, a total cost was determined for the investigations they underwent using the resource costs as above. Mean cost and number of investigations with 95% confidence intervals were calculated with standard bootstrapping using 10,000 samples with replacement[268,269].

The number of investigations and the cost of investigations per year were then analysed based on the presence of a positive anti-PLA_2R versus a negative test or no sample taken. Significance was calculated using students t-test and defined as less than 0.05. All analyses were carried out in R statistical software version 3.4.3[21].

Investigation	Mean value	LQR	UQR	Source
Hepatitis B	6.42	4.02	7.65	DAPS06 NHS ref costs
Hepatitis C	6.42	4.02	7.65	DAPS06 NHS ref costs
HIV	6.42	4.02	7.65	DAPS06 NHS ref costs
RF	6.42	4.02	7.65	DAPS06 NHS ref costs
ds-DNA	6.42	4.02	7.65	DAPS06 NHS ref costs
ANA	6.42	4.02	7.65	DAPS06 NHS ref costs
Complement	6.42	4.02	7.65	DAPS06 NHS ref costs
PSA	1.18	0.78	1.39	DAPS04 NHS ref costs
ANCA	6.42	4.02	7.65	DAPS06 NHS ref costs
TFTs	1.18	0.78	1.39	DAPS04 NHS ref costs
Chest x-ray	25.00			National tariff
Abdominal x-ray	25.00			National tariff
CT Head	93.93	65.19	115.59	RD20A NHS ref costs
CT Thorax	102.50	70.75	134.97	RD21A NHS ref costs
CT Abdomen	102.50	70.75	134.97	RD21A NHS ref costs
CT TAP	120.70	88.30	138.91	RD26Z NHS ref costs
MRI	145.14	113.26	173.53	RD01A NHS ref costs
PET	798.20	430.64	1213.54	RN07A NHS ref costs
OGD	352.21	322.20	432.22	FZ60Z NHS ref costs
Colonoscopy	371.27	236.45	521.90	FZ51Z NHS ref costs
Sigmoidoscopy	207.69	152.04	247.24	FZ54Z NHS ref costs
USS abdomen	50.62	38.54	60.44	RD40Z NHS ref costs
Cystoscopy	151.71	101.68	175.50	LB72A NHS ref costs

Table S2.1 - Cost of investigations. All costs in British Pound Sterling. NHS ref costs- National Health Service reference costs 2015-2016[18]. National tariff- National Health Service non-mandatory currencies and prices 2015-2016[19]. LQR- Lower Quartile Range. UQR- Upper Quartile Range. HIV- Human immunodeficiency virus. RF- Rheumatoid factor. dsDNA- double-stranded deoxyribonucleic acid. ANA- antinuclear antibody. ANCA- anti-neutrophil cytoplasmic antibody. PSA- prostate-specific antigen. CT- computed tomography. MRI- magnetic resonance imaging. PET- Positron emission tomography. OGD- oesophagogastroduodenoscopy. USS - ultrasound scan.

Results

There was a total of 184 patients identified across the three hospitals with a mean age at diagnosis of 58 years. The majority of patients were male with 117 patients (64%). 80 (43%) patients did not undergo anti-PLA_2R testing within 6 months of the date of biopsy. 104 (57%) patients did have an anti-PLA_2R within 6 months of the date of biopsy; 66 (63% of those tested) had a negative test, and 38 (37% of those tested) had a positive test. See table S3.2 for full demographics. Of the 184 patients included in the study, 21 (11.4%) were confirmed as secondary MN. Of these 21 patients, 9 were tested for anti-PLA_2R, and all were negative.

Frequency of anti-PLA_2R testing

In 2011 when the anti-PLA_2R test became available locally, it was only tested in 8 out of 20 (40%) patients diagnosed with MN. Since that time there has been a steady increase in the number of patients tested for anti-PLA_2R within 6 months of their biopsy with 93.3% of patients having the test in 2014. See table S3.3 and figure S2.1.

Number of investigations

There were a total of 1230 investigations performed in all patients of which only 20 were positive and led to a diagnosis of secondary MN. From 2011 onwards, there is a reduction in the number of investigations performed in anti-PLA_2R seropositive patients. In 2012, the first full year of anti-PLA_2R availability, there was a mean of 6.85 tests (95% CI 5.61-8.09) per patient in those with no anti-PLA_2R testing or a negative test. In the seropositive group, the mean number of tests was 6.59 (95% CI 4.9-8.2). This difference was not statistically significant; p-value 0.823. In 2014, the mean number of tests performed per

Parameter		No Anti-PLA_2R	Negative Anti-PLA_2R	Positive Anti-PLA_2R	Total
Patients	n (%)	80 (43%)	66 (36%)	38 (21%)	184 (100%)
Age at diagnosis	Mean (SD)	59 (15.58)	57 (15.64)	57 (13.19)	58 (15.10)
Gender	Female	32 (40%)	24 (36%)	11 (29%)	67 (36%)
	Male	48 (60%)	42 (64%)	27 (71%)	117 (64%)
Hepatitis B	Negative Test	38 (48%)	28 (42%)	13 (34%)	79 (43%)
	No test	42 (52%)	38 (58%)	25 (66%)	105 (57%)
Hepatitis C	Negative Test	38 (48%)	28 (42%)	12 (32%)	78 (42%)
	No test	42 (52%)	38 (58%)	26 (68%)	106 (58%)
Hiv	Negative Test	17 (21%)	20 (30%)	12 (32%)	49 (27%)
	No test	63 (79%)	46 (70%)	26 (68%)	135 (73%)
Rheumatoid factor	Negative Test	31 (39%)	17 (26%)	8 (21%)	56 (30%)
	No test	48 (60%)	48 (73%)	30 (79%)	126 (68%)
	Positive Test	1 (1%)	1 (2%)	0 (0%)	2 (1%)
Anti-dsDNA	Negative Test	44 (55%)	44 (67%)	26 (68%)	114 (62%)
	No test	35 (44%)	22 (33%)	12 (32%)	69 (38%)
	Positive Test	1 (1%)	0 (0%)	0 (0%)	1 (1%)
Ana	Negative Test	61 (76%)	53 (80%)	29 (76%)	143 (78%)
	No test	18 (22%)	12 (18%)	9 (24%)	39 (21%)
	Positive Test	1 (1%)	1 (2%)	0 (0%)	2 (1%)
Complement (c3/c4)	Negative Test	60 (75%)	48 (73%)	27 (71%)	135 (73%)
	No test	19 (24%)	17 (26%)	11 (29%)	47 (26%)
	Positive Test	1 (1%)	1 (2%)	0 (0%)	2 (1%)
PSA	Negative Test	11 (14%)	13 (20%)	10 (26%)	34 (18%)
	No test	68 (85%)	53 (80%)	28 (74%)	149 (81%)
	Positive Test	1 (1%)	0 (0%)	0 (0%)	1 (1%)
ANCA	Negative Test	61 (76%)	50 (76%)	31 (82%)	142 (77%)
	No test	19 (24%)	16 (24%)	7 (18%)	42 (23%)
TFTs	Negative Test	30 (38%)	18 (27%)	20 (53%)	68 (37%)
	No test	50 (62%)	48 (73%)	18 (47%)	116 (63%)
CXR	Positive Test	3 (4%)	1 (2%)	0 (0%)	4 (2%)
	Negative Test	39 (49%)	33 (50%)	21 (55%)	93 (51%)
	No test	38 (48%)	32 (48%)	17 (45%)	87 (47%)
AXR	Negative Test	4 (5%)	1 (2%)	1 (3%)	6 (3%)
	No test	76 (95%)	65 (98%)	37 (97%)	178 (97%)
CT Head	Negative Test	3 (4%)	2 (3%)	3 (8%)	8 (4%)
	No test	77 (96%)	64 (97%)	35 (92%)	176 (96%)
CT Thorax	Positive Test	0 (0%)	1 (2%)	0 (0%)	1 (1%)
	Negative Test	6 (8%)	1 (2%)	5 (13%)	12 (7%)
	No test	74 (92%)	64 (97%)	33 (87%)	171 (93%)
CT Abdomen	Positive Test	0 (0%)	1 (2%)	0 (0%)	1 (1%)
	Negative Test	2 (2%)	1 (2%)	1 (3%)	4 (2%)
	No test	78 (98%)	64 (97%)	37 (97%)	179 (97%)
CT TAP	Positive Test	2 (2%)	1 (2%)	0 (0%)	3 (2%)
	Negative Test	20 (25%)	15 (23%)	9 (24%)	44 (24%)
	No test	58 (72%)	50 (76%)	29 (76%)	137 (74%)
MRI	Negative Test	1 (1%)	1 (2%)	1 (3%)	3 (2%)
	No test	79 (99%)	65 (98%)	37 (97%)	181 (98%)
PET	Positive Test	0 (0%)	1 (2%)	0 (0%)	1 (1%)
	No test	80 (100%)	65 (98%)	38 (100%)	183 (99%)
OGD	Positive Test	0 (0%)	1 (2%)	0 (0%)	1 (1%)
	Negative Test	11 (14%)	8 (12%)	4 (11%)	23 (12%)
	No test	69 (86%)	57 (86%)	34 (89%)	160 (87%)
Colonoscopy	Positive Test	1 (1%)	0 (0%)	0 (0%)	1 (1%)
	Negative Test	7 (9%)	8 (12%)	5 (13%)	20 (11%)
	No test	72 (90%)	58 (88%)	33 (87%)	163 (89%)
Sigmoidoscopy	Negative Test	0 (0%)	0 (0%)	1 (3%)	1 (1%)
	No test	80 (100%)	66 (100%)	37 (97%)	183 (99%)
USS Abdomen	Negative Test	41 (51%)	33 (50%)	20 (53%)	94 (51%)
	No test	39 (49%)	33 (50%)	18 (47%)	90 (49%)
Cystoscopy	Negative Test	0 (0%)	1 (2%)	3 (8%)	4 (2%)
	No test	80 (100%)	65 (98%)	35 (92%)	180 (98%)

Table S3.2 – Demographics. N (%) unless otherwise stated. HIV - Human Immunodeficiency Virus, ANA - Antinuclear antibody, PSA - prostate-specific antigen, ANCA - Antineutrophil cytoplasmic antibodies, TFTs - Thyroid function tests, CXR - Chest X-Ray, AXR - Abdominal Xray, CT - computed tomography scan, CT TAP - CT Thorax, Abdomen & Pelvis, MRI - Magnetic Resonance Imaging, PET - Positron emission tomography scan, OGD - oesophagogastroduodenoscopy, USS - Ultrasound scan

patient in the seropositive group had reduced to 4.29 tests (95% CI 2.6-6.1) in comparison to 9.01 in seronegative patients; this represented a significant difference (95% CI 6.6-11.02, p-value of 0.019). See table S3.4.

Cost of investigations

The total cost of investigations within 6 months of biopsy for all patients was £39,177.83, of this £5,533.04 was spent on investigations with a result leading to a diagnosis of secondary MN. In patients with no anti-PLA_2R testing or a negative result the cost of investigations remained relatively stable over the years, £220.27 (95% CI 137.93-315.77) in 2009 and £244.11 (95% CI 109.88-429.97) in 2014. In patients with a positive anti-PLA_2R, the cost of investigations reduced each year from its introduction going from £497.92 (95% CI 89.83-909.00) in 2011 to £132.94 (95% CI 29.66-309.44) in 2014, although the difference in cost per year was not significant between the groups. See table S3.4.

Year of biopsy	Number of patients	No Anti-PLA_2R	Anti-PLA_2R tested
2009	39	39 (100.0)	0 (0.0)
2010	28	28 (100.0	0 (0.0)
2011	20	12 (60.0)	8 (40.0)
2012	43	23 (53.5)	20 (46.5)
2013	39	15 (38.5)	24 (61.5)
2014	15	1 (6.7)	14 (93.3)

Table S3.3 – number of patients per year of biopsy. Number of patients who did and did not have an anti-PLA_2R test within 6 months of the date of biopsy. N (%).

Year of diagnosis	No test / Anti-PLA_2R negative	Anti-PLA_2R positive	Sig.
	Cost of tests (£)		
2009	220.27 (137.93-315.77)	NA (NA-NA)	NA
2010	216.93 (120.46-328.56)	NA (NA-NA)	NA
2011	227.07 (85.92-392.93)	497.92 (89.83-909.00)	0.363
2012	161.16 (106.45-227.11)	226.39 (111.68-369.71)	0.414
2013	225.64 (107.82-395.67)	218.88 (107.62-383.89)	0.946
2014	244.11 (109.88-429.97)	132.94 (29.66-309.44)	0.405
	Number of investigations		
2009	6.87 (5.90-7.82)	NA (NA-NA)	NA
2010	6.89 (5.57-8.18)	NA (NA-NA)	NA
2011	4.57 (2.75-6.62)	10.03 (5.00-14.5)	0.164
2012	6.85 (5.61-8.09)	6.59 (4.90-8.20)	0.823
2013	6.44 (5.04-7.88)	8.08 (6.21-9.71)	0.177
2014	9.01 (6.60-11.2)	4.29 (2.60-6.10)	0.019

Table S3.4 – Mean (95% confidence intervals) for number of tests and cost of tests based on year of biopsy and anti-PLA_2R test status – no test or seronegative versus seropositive.

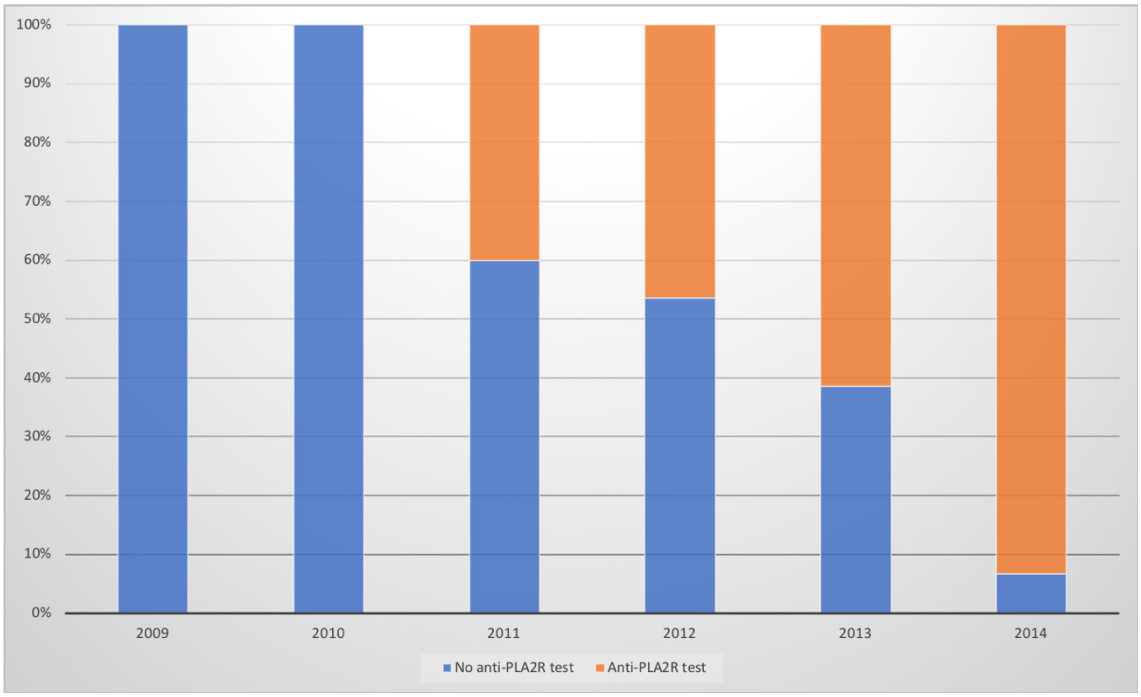

Figure S2.1 – Proportion of MN patients with anti-PLA_2R testing

Discussion

The majority of patients with a histological diagnosis of membranous nephropathy will have primary MN, an autoimmune disease in which 70-80% are anti-PLA_2R positive[15]. Since its discovery in 2009 our understanding of the condition has vastly improved, with evidence suggesting the pathogenic nature of the antibody[38,41-43]. This, coupled with its relative absence in secondary MN[270] makes it a valuable biomarker not only for disease activity but also for diagnosis.

Prior to the development of the anti-PLA_2R blood test, the diagnosis of PMN was one of exclusion at a cost to patients and the healthcare system. In our cohort the vast majority of investigations carried out for this reason were negative, a use of resources that is considerable given MN is one of the most common causes of adult nephrotic syndrome worldwide[1,100-104].

Here we show use of the test has increased over the years with a higher proportion of our patients with a tissue diagnosis of MN undergoing concomitant anti-PLA_2R testing; 93% of patients in 2014 compared to only 46.5% in 2012. Along with increased use of anti-PLA_2R testing, there is a corresponding reduction in the number of other investigations being carried out and a reduction in the cost of investigations.

Approximately a third of patients with a diagnosis of PMN will go into spontaneous remission, most within the first year[271]. For this reason and along with the complications associated with immunosuppression, patients have traditionally been treated with supportive care through inhibition of the renin-angiotensin-aldosterone system (RAAS) for 6 months before considering immunosuppression[5]. However, in the anti-PLA_2R era, a more proactive management may be warranted. It has now been shown that seronegative patients or those with a low anti-PLA_2R are more likely to go into spontaneous remission and less likely to suffer from renal decline[38,272]. Conversely, patients

with a high anti-PLA_2R at diagnosis are more likely to have disease progression, worsening renal function and higher levels of proteinuria[38,43,272]. The reduction of anti-PLA_2R and subsequent reduction in proteinuria has been shown to improve outcomes following treatment in a number of studies[42,43,245]. It has also long been shown that achieving either partial or complete remission leads to better long-term outcomes[23,273]. There is still some debate however around the benefits of early immunosuppression. In a randomised controlled trial (RCT), early immunosuppression did appear to lead to remission quicker than postponing immunosuppressive therapy with a similar adverse event profile. At the end of the 6 year follow up, 86% of patients in the early immunosuppression group had achieved remission compared to 67% in the late treatment group. However, there was no statistical difference in serum creatinine, albumin or proteinuria[21]. Given the relatively short follow up time in respect to the long disease course of MN, over time one could speculate that a difference may have been observed. This study was also carried out in the pre-anti-PLA_2R era when disease severity was based on proteinuria. By utilising the anti-PLA_2R titre, those patients with high levels who are unlikely to go into spontaneous remission and have a higher chance of disease progression could have a shorter time to treatment without the need to wait for unnecessary invasive investigations.

As use of the anti-PLA_2R test becomes more widespread and physician confidence in its ability to differentiate primary from secondary MN and to prognosticate disease progression increases, it has the potential to radically change management practice. As seen in our study, patients traditionally undergo a large number of invasive investigations in order to rule out pathology, and the majority of these understandably come back with nothing abnormal detected. Not only is the cost to the patients' quality of life a consideration but also the cost to the healthcare system, with the use of

resources that could be diverted elsewhere. This is especially true given that the cost of the anti-PLA_2R test, currently offered in the UK by the Protein Reference Unit in Sheffield, is £25.81 per sample. This makes it cheaper than many of the investigation's patients are currently subjected to.

Our study does have a number of limitations, in particular, the likely underestimate of investigations carried out. In the Greater Manchester and Preston, renal medicine operates in a hub and spoke manner, with specialist renal departments centralised in large teaching hospitals and patients transferred or referred in from smaller satellite units around the region. This does mean that some investigations may well have been carried out in the satellite unit before the patient's transfer of care and although the majority of these investigations would be expected to be low-cost tests such as biochemistry, there may be a number of scans and endoscopies that may not have been accounted for.

The number of positive anti-PLA_2R tests in our cohort was lower than reported in other studies with most reporting in the region of 70-80% of MN patients[15,39,245]. There were however a large number of patients in the earlier years of its use that were not tested. As the test became more ubiquitous over time the percentage of positive samples more reflected the literature. For example, in 2014, there were 14 anti-PLA_2R tests, of which 10 were positive, representing 71% of the patients.

The use of anti-PLA_2R is not infallible with a number of case reports identifying patients with secondary MN and a raised anti-PLA_2R[274-277]. Whether this is coincidental given that patients in the age group most affected by MN are also at risk of malignancy is yet to be proven conclusively. Each patient still needs a careful and thorough history and examination and investigation as appropriate.

Saying this, as the anti-PLA_2R test becomes commonplace in patients with nephrotic syndrome, as shown here its use can help to reduce the burden of

investigation for both the patient and society and its use should be included in future management guidelines and research.

Conflict of Interest

The results presented in this paper have not been published previously in whole or part, except in abstract format.

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Flow cytometry results

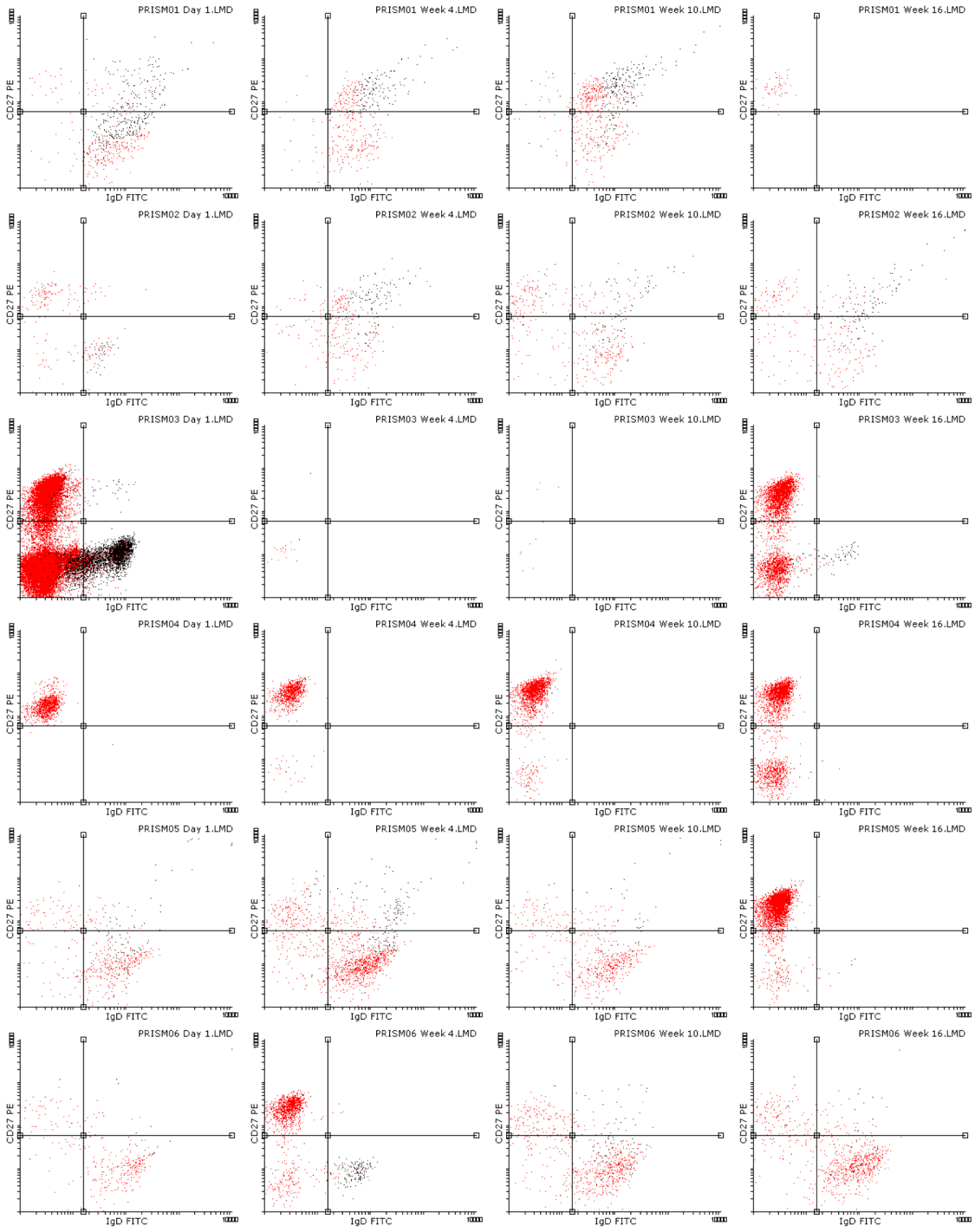


Figure S5.1a - PRISM01 - PRISM06. B cell panel showing gating for CD27 vs anti-human IgD cells. Bottom right is CD27- IgD+ representing Naive B cells. Top left is CD27+ IgD- representing IgD- memory B cells. Top right is CD27+ IgD+ representing IgD+ memory B cells.

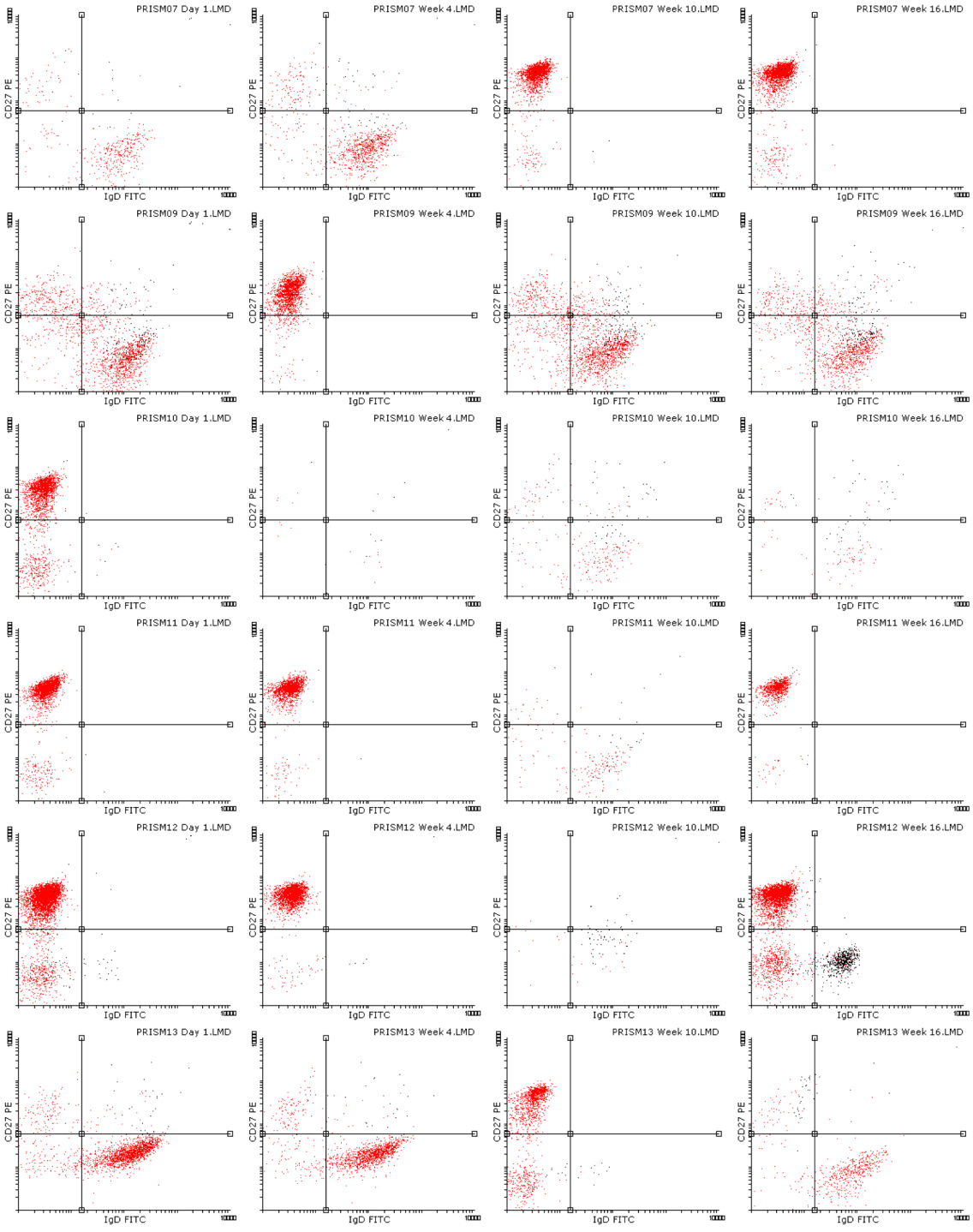


Figure S5.1b - PRISM07 – PRISM13. B cell panel.

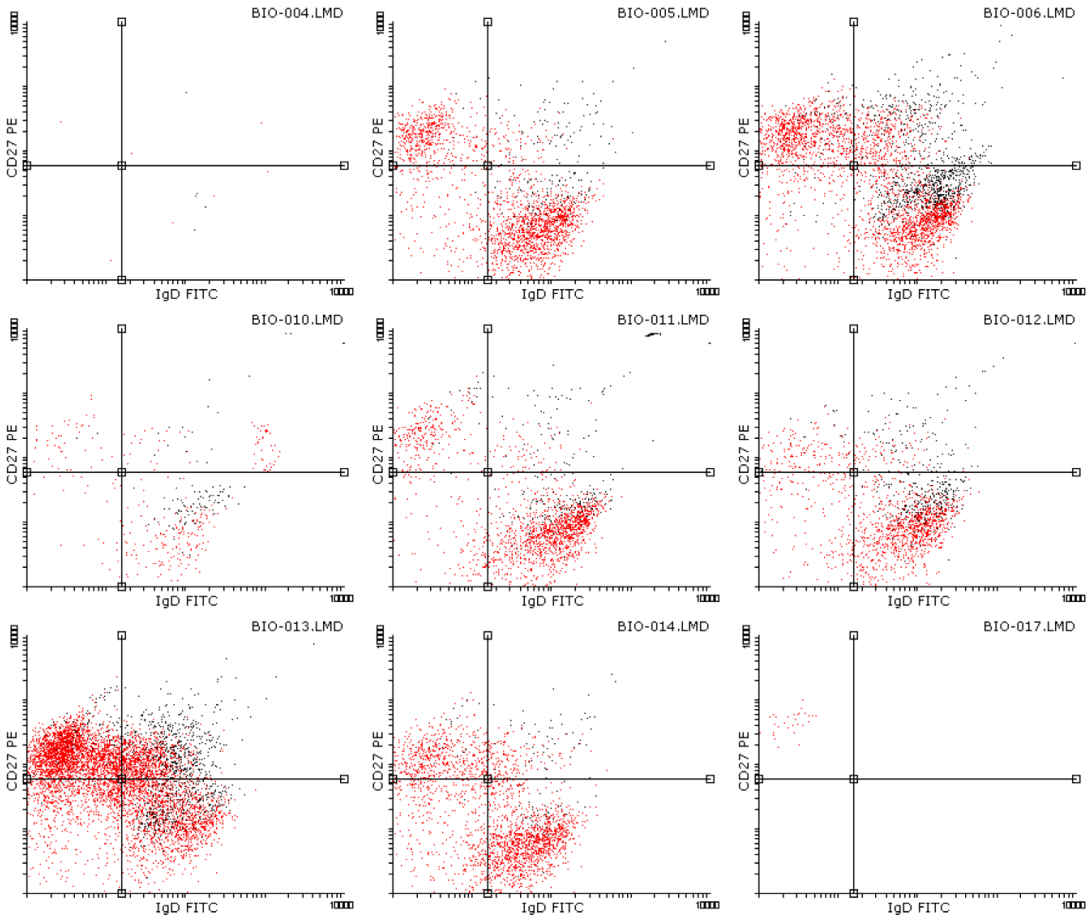


Figure S5.2 - B cell panel CD27 versus IgD in control group.

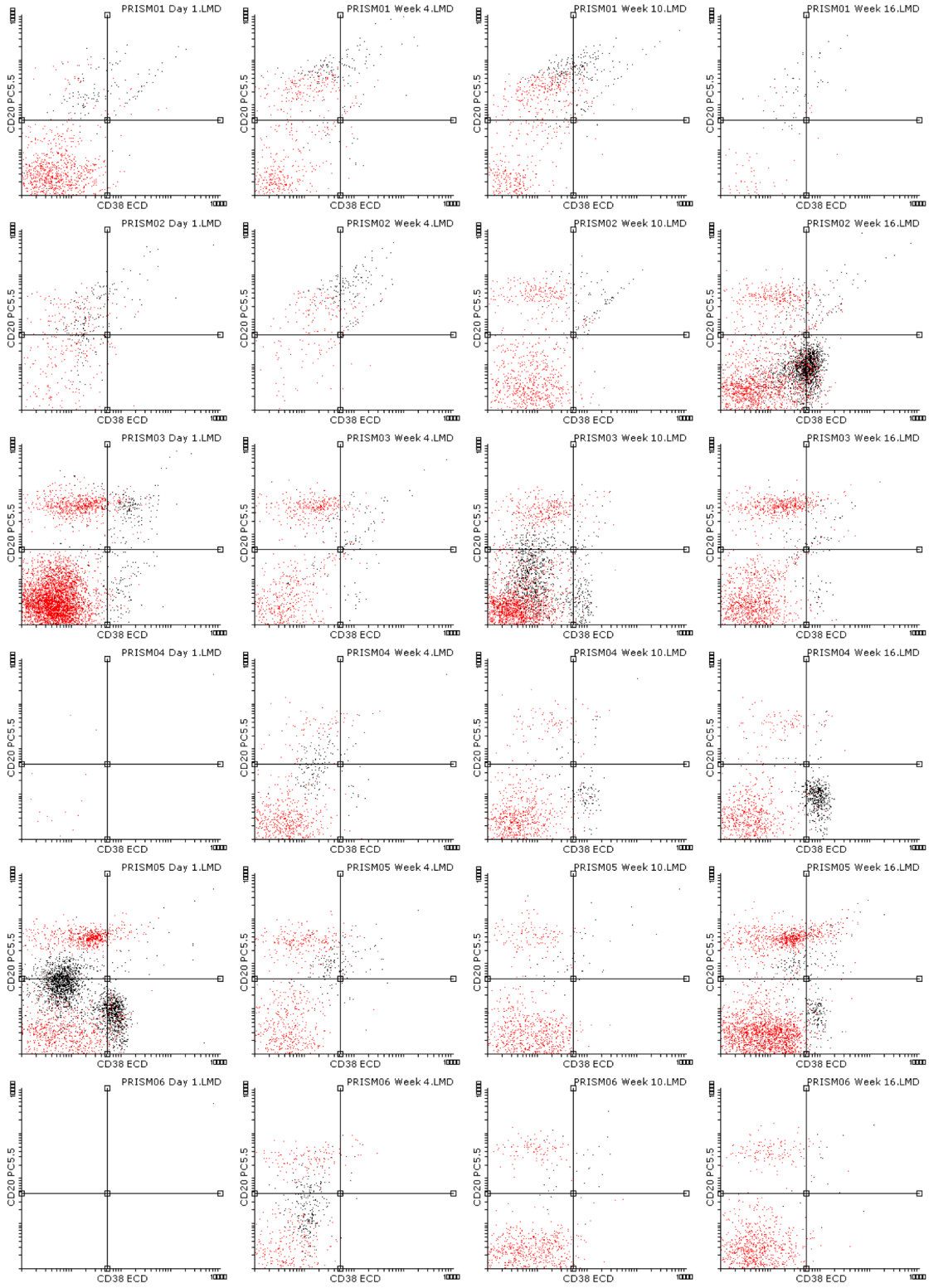


Figure S5.3a - PRISM01 - PRISM06. B cell panel CD20 versus CD38 in patient group. Top right panel is CD38+ CD27+ cells representing Plasmablasts.

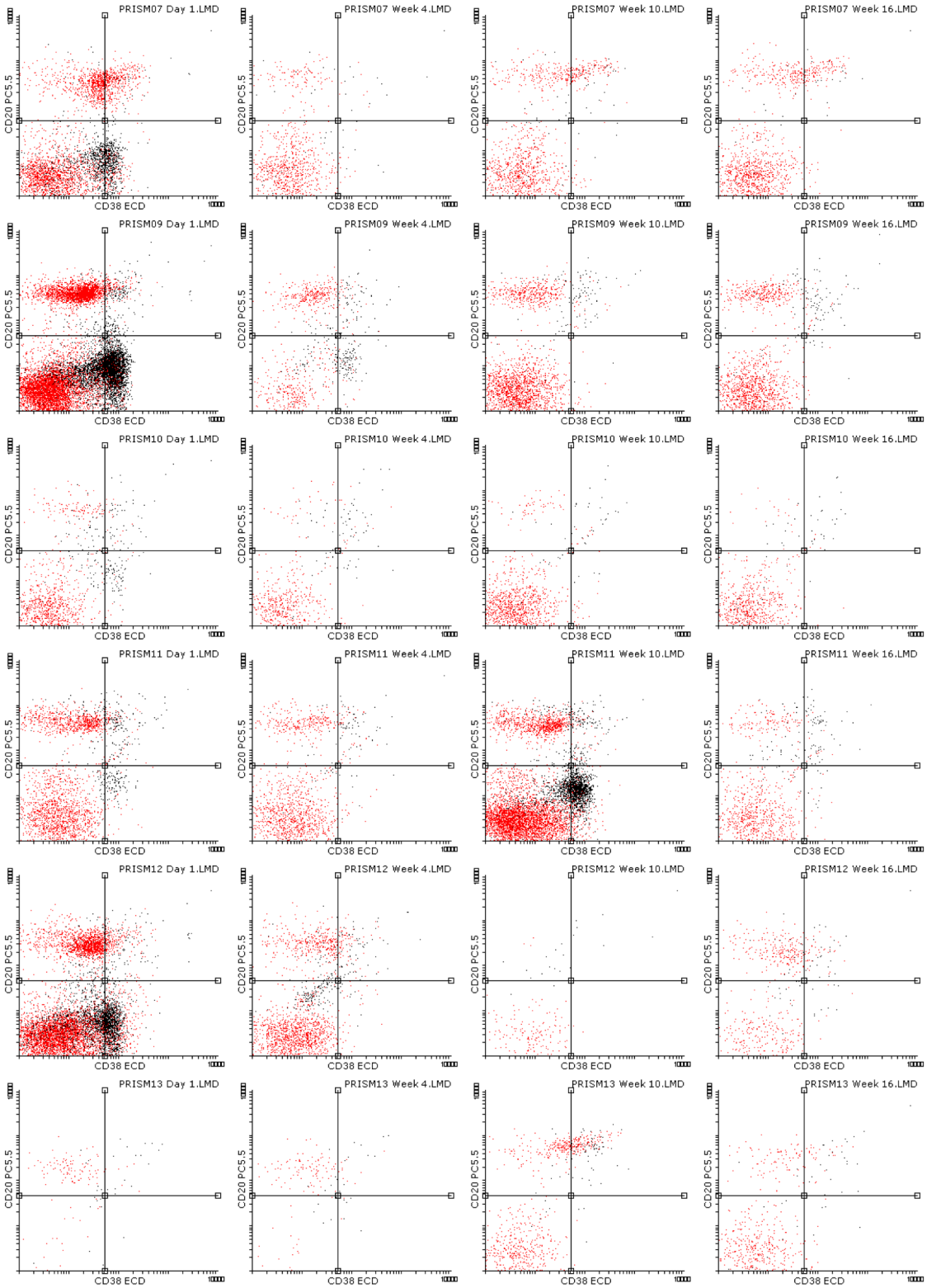


Figure S5.3b - PRISM07 - PRISM013. B cell panel CD20 versus CD38 in patient group.

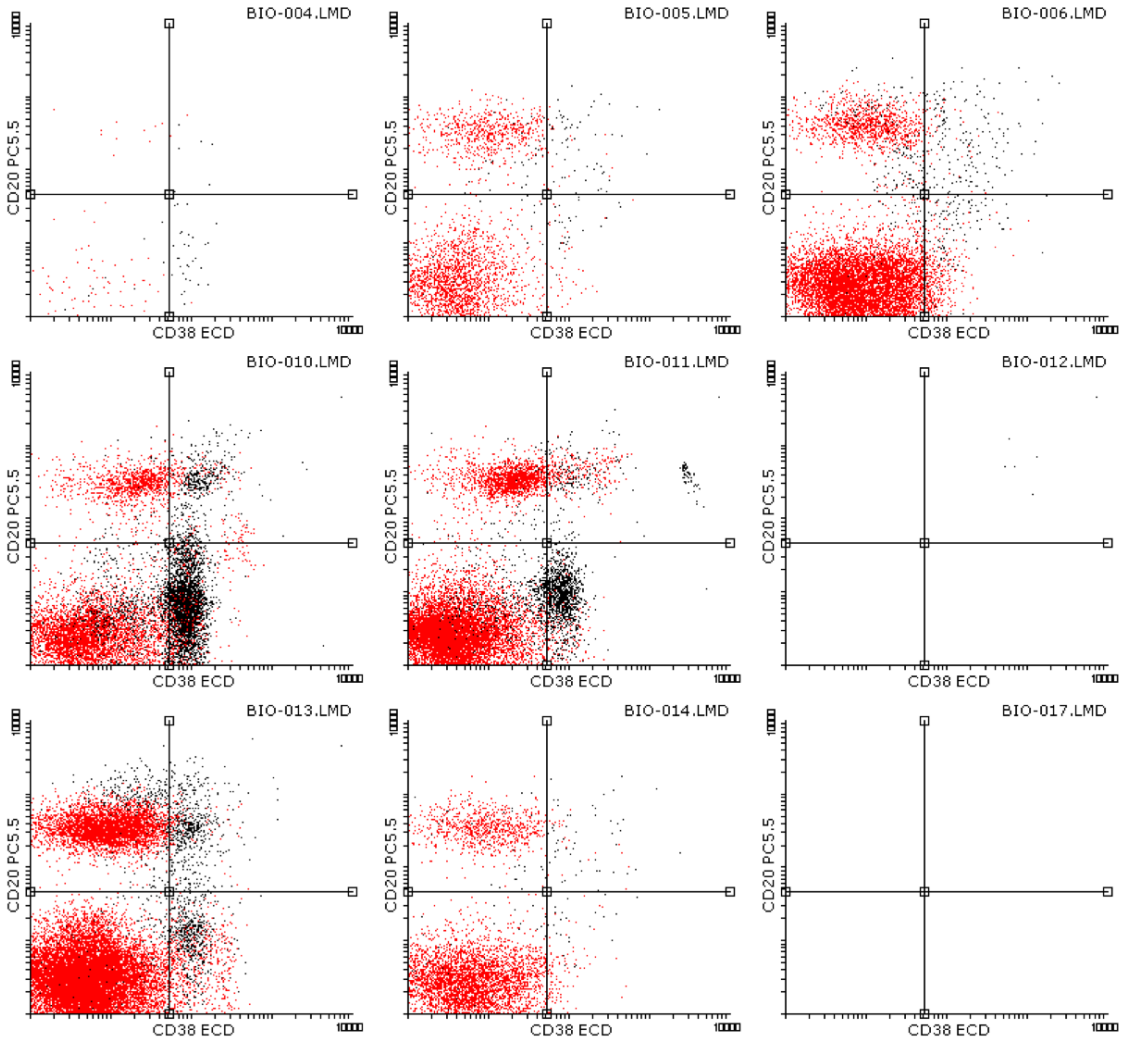


Figure S5.4 - B cell panel CD20 versus CD38 in control group.

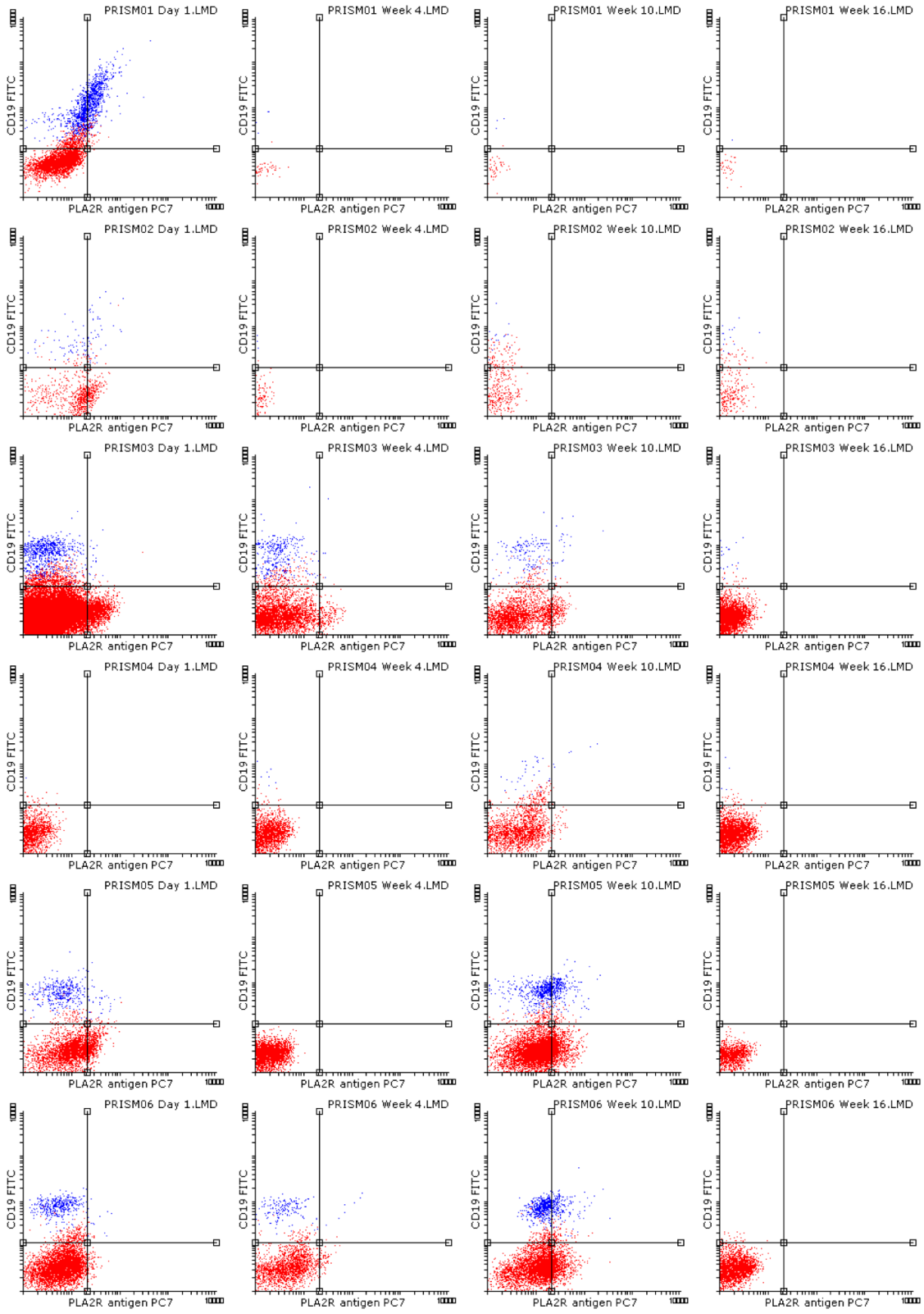


Figure S5.5a - PRISM01 - PRISM06. PLA2R panel CD19 versus PLA2R. Top right panel is CD19+ PLA2R+ and bottom right is CD19- PLA2R+.

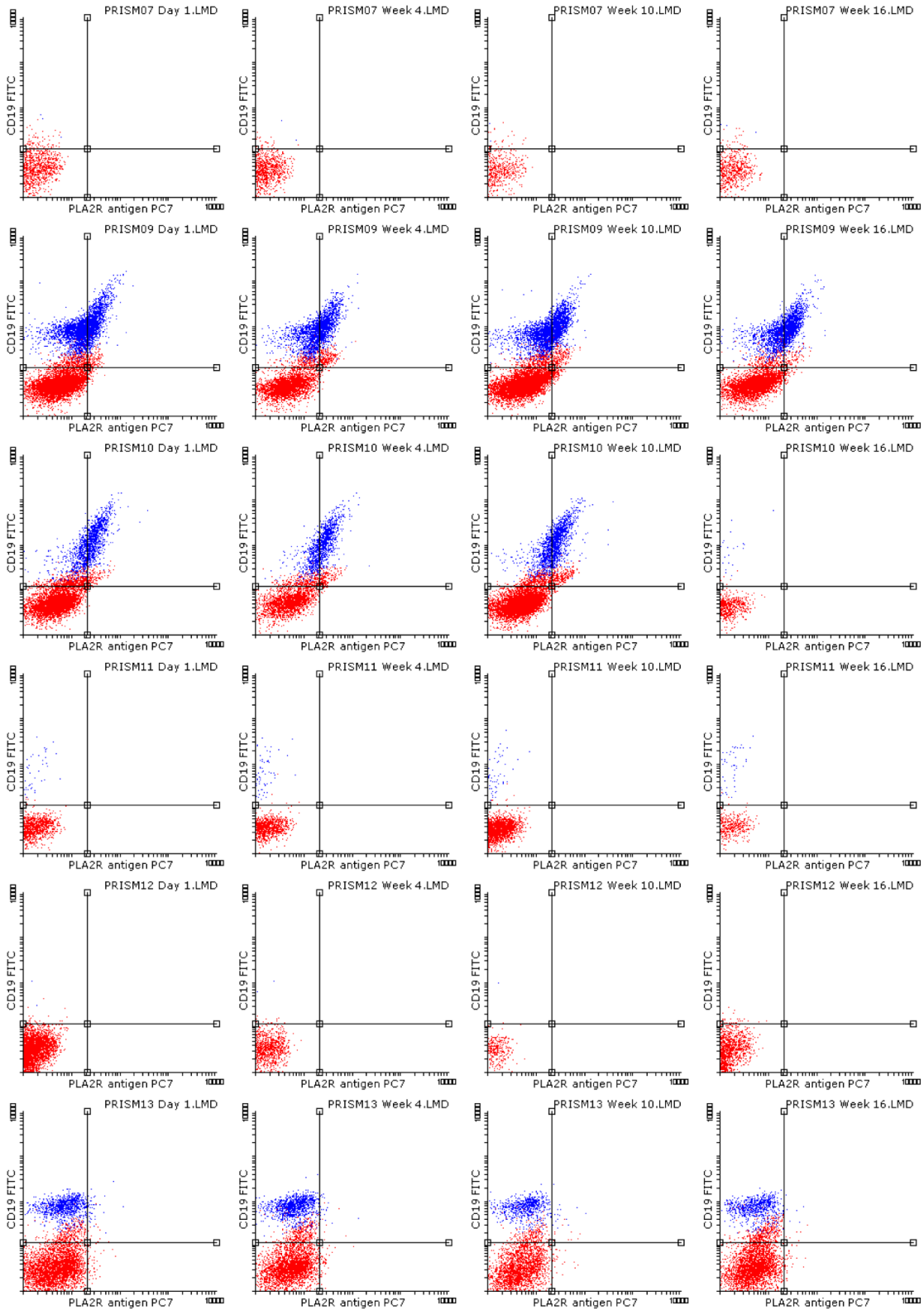


Figure S5.5b - PRISM07 - PRISM13. PLA2R panel.

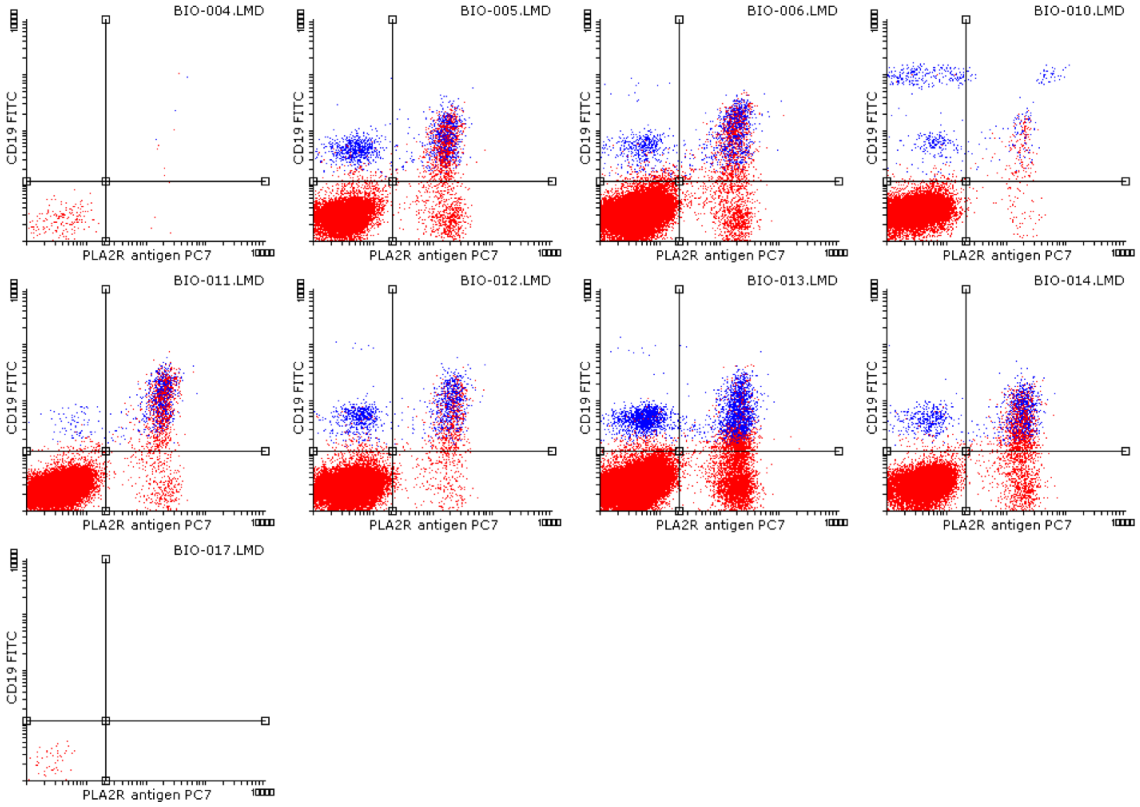


Figure S5.6 – PLA_2R panel in control group

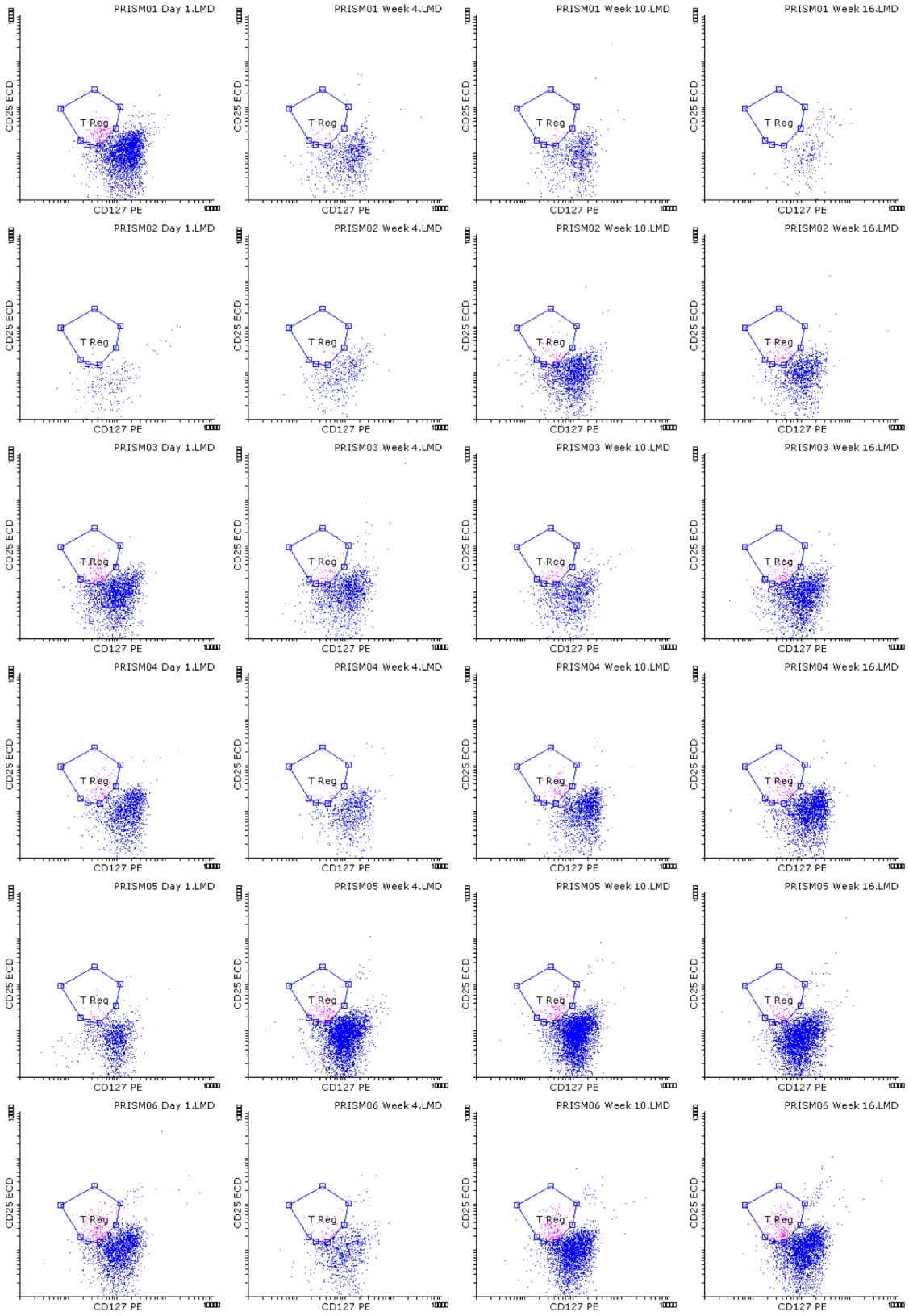


Figure S5.7a – PRISM01-PRISM06. T cell panel with CD25 versus CD127. Gated region represents T Regs - CD4+ CD25+ CD127+ low.

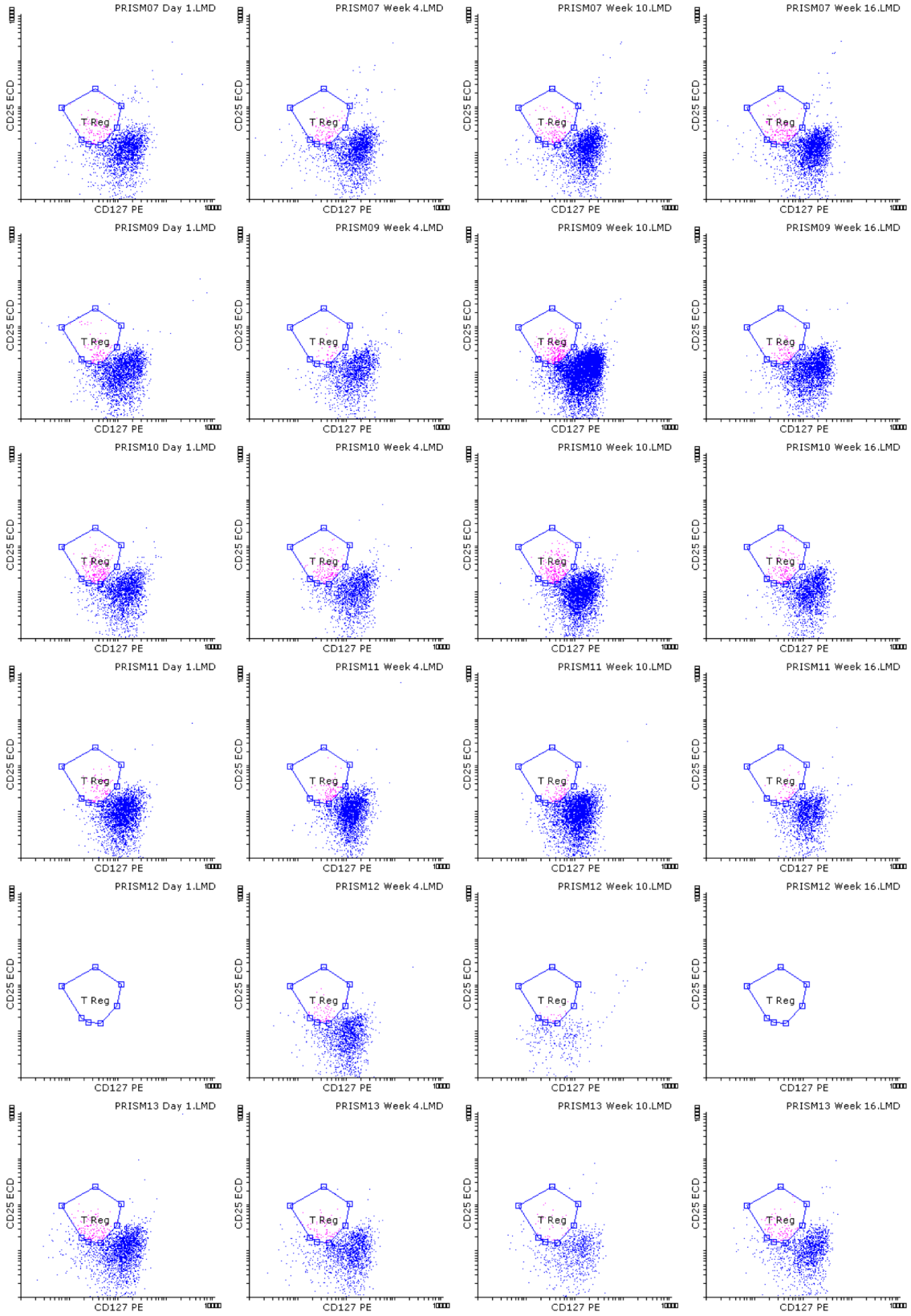


Figure S5.7b – PRISM07-PRISM13. T cell panel.

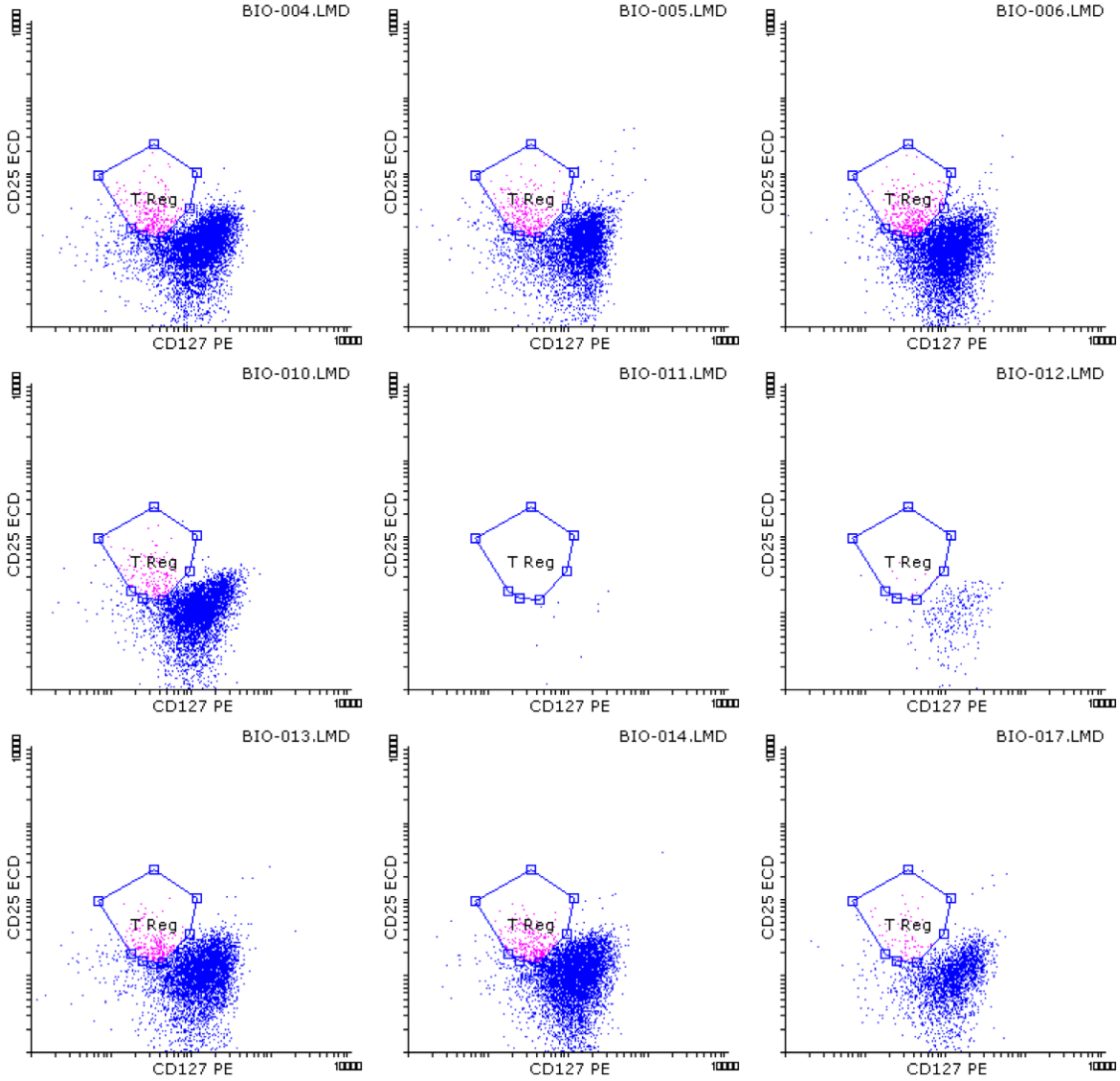


Figure S5.8 – Control group. T cell panel.

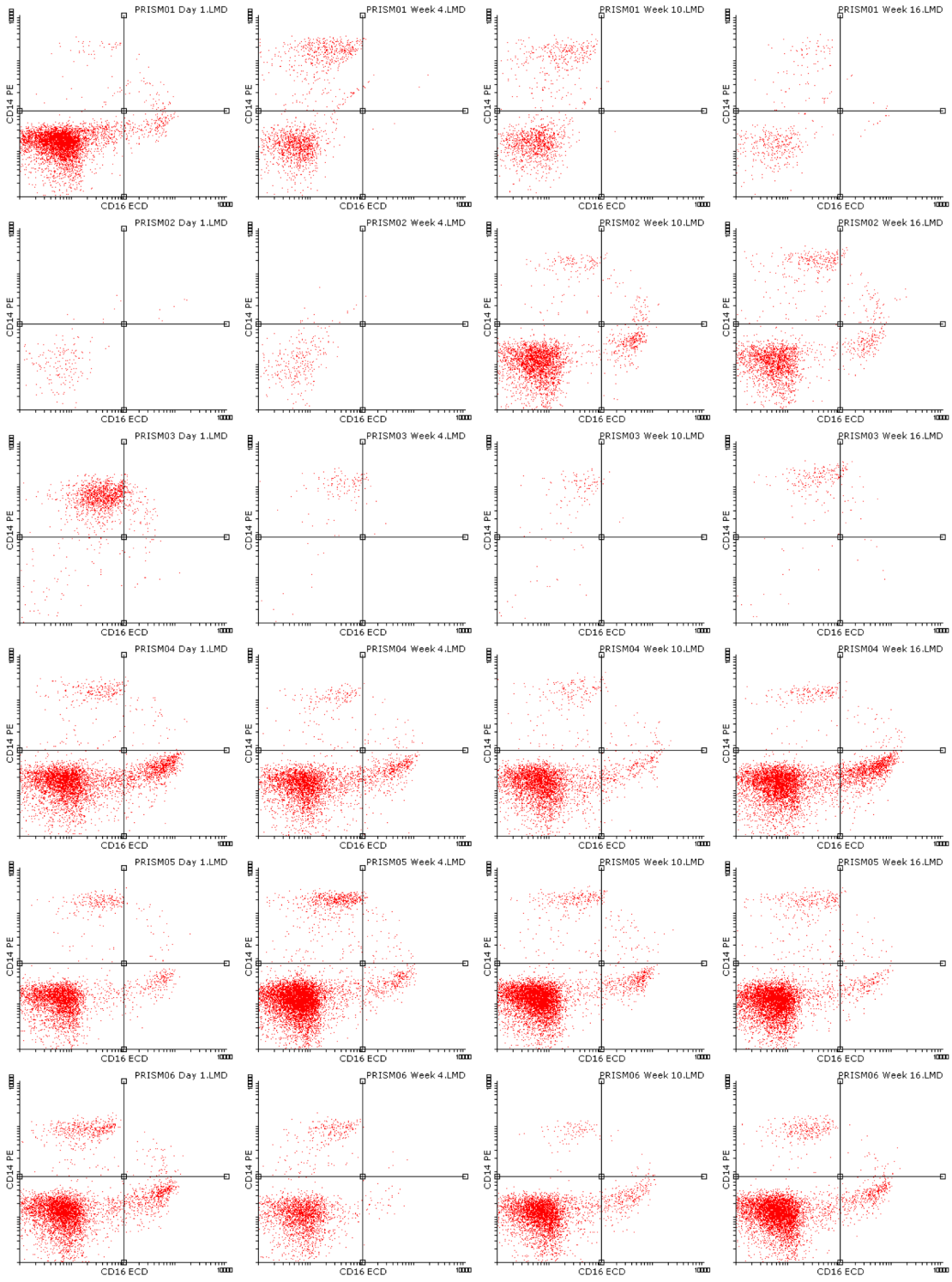


Figure S5.9a - PRISM01 - PRISM06. Monocytes panel showing CD14 versus CD16. Top right is CD14+ CD16+ representing CD16+ monocytes. Top left panel is CD14+ CD16- representing conventional monocytes.

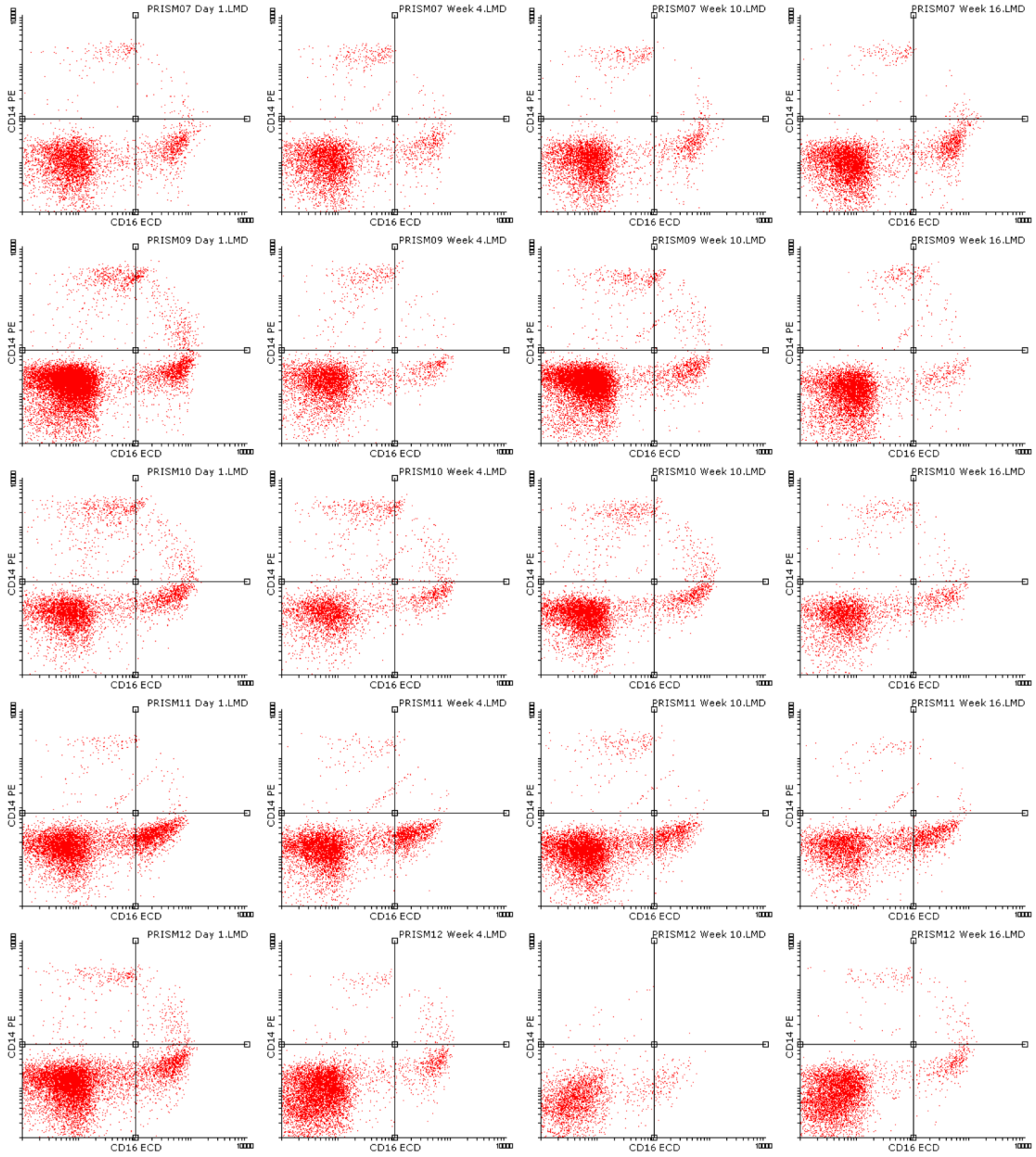


Figure S5.9b - PRISM07 – PRISM12. Monocytes panel.

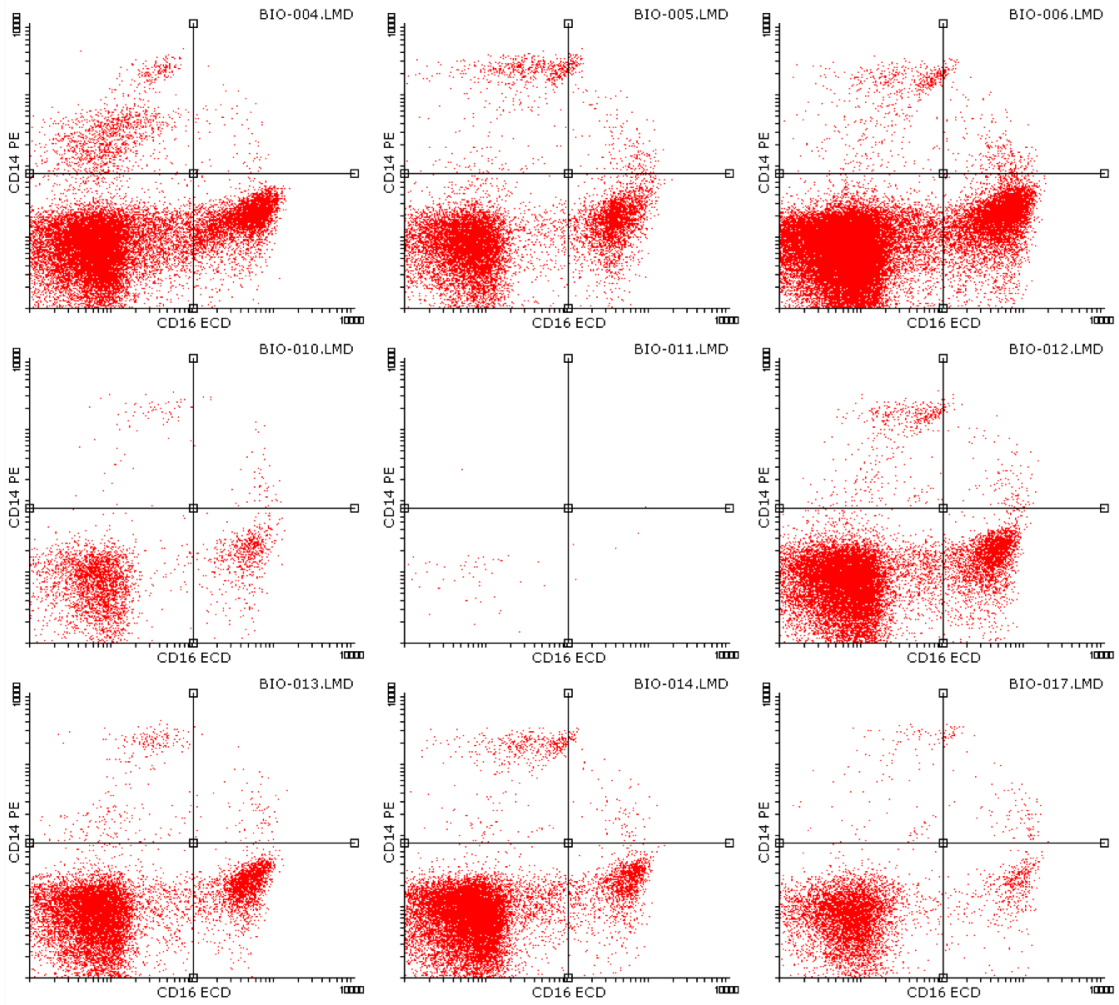


Figure S5.10 – Monocyte panel for control group. CD14 versus CD16.

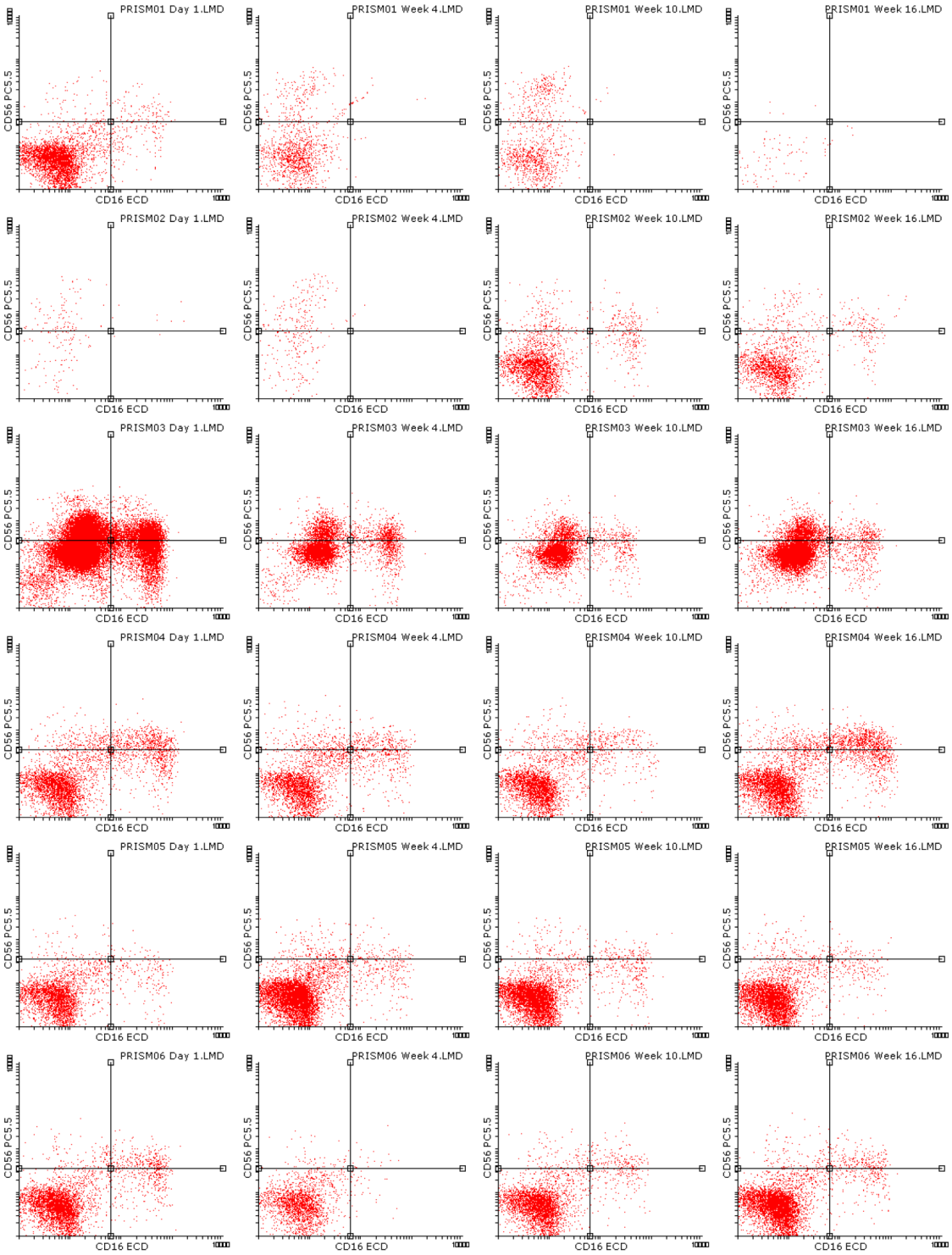


Figure S5.11a – PRISM01-PRISM06. Monocyte panel for NK cells. CD56 versus CD16. Top right quadrant represents NK cells (CD16+ CD56+)

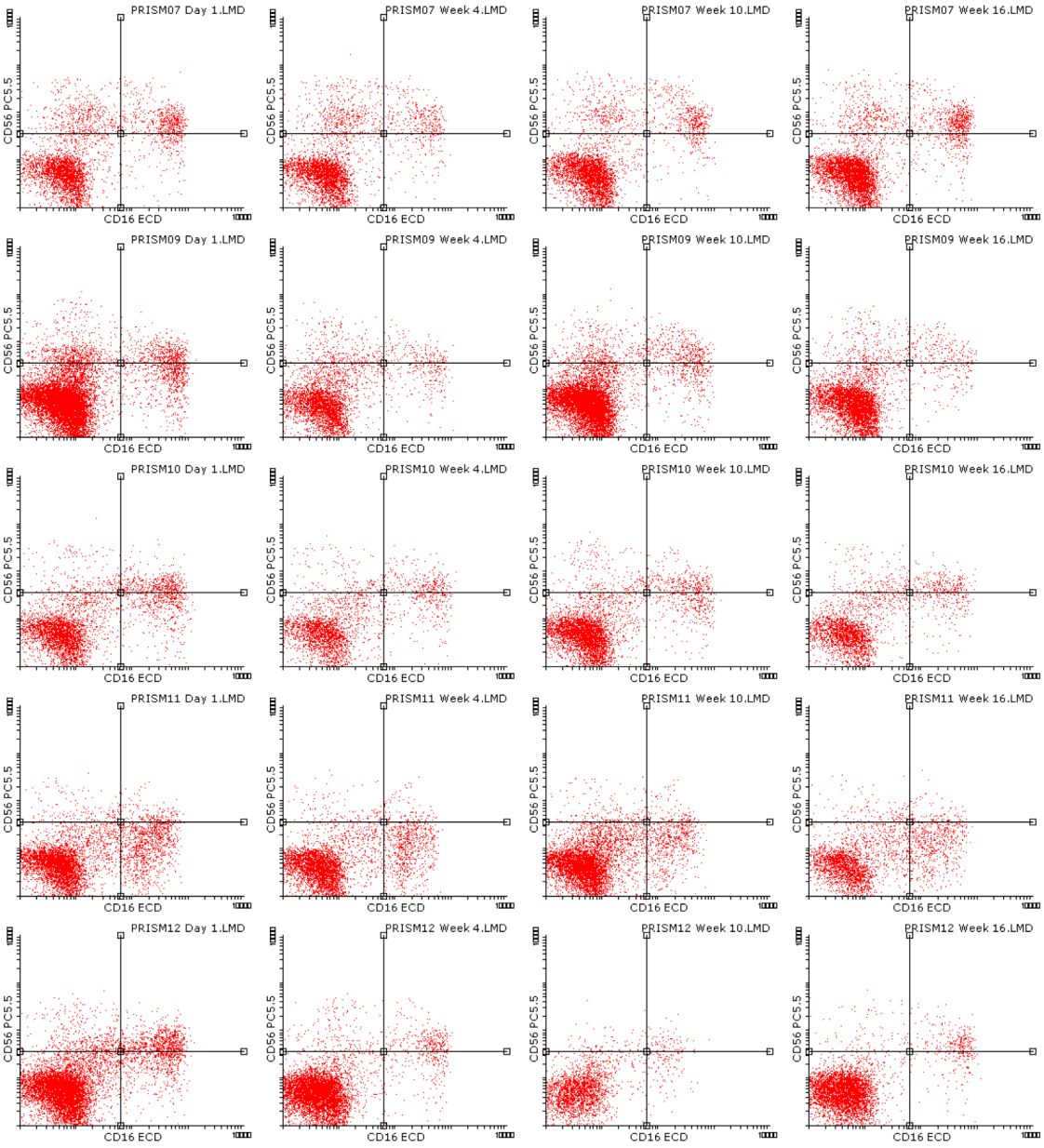


Figure S5.11b – PRISM07-PRISM12. Monocyte panel. CD56 versus CD16.

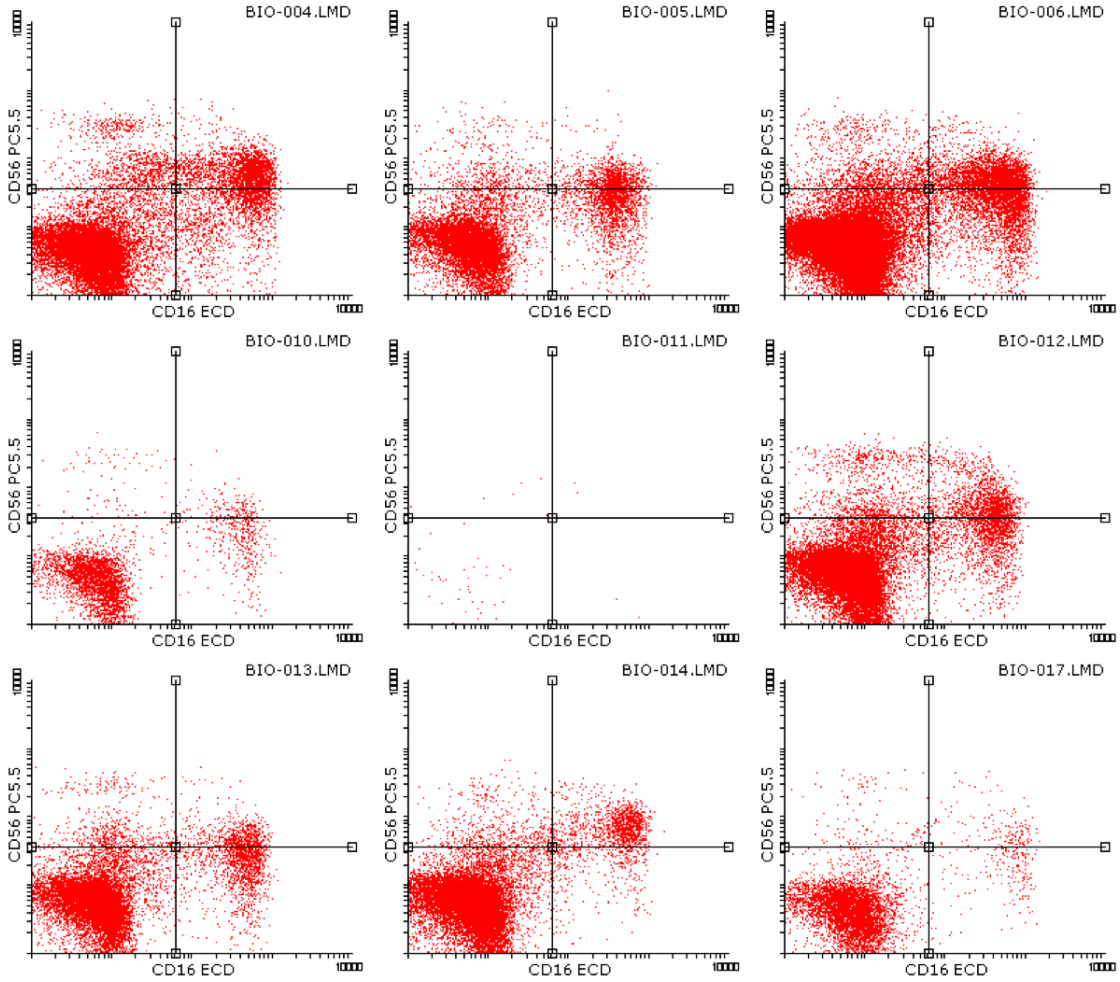


Figure S5.12 – Monocyte panel for control group. CD56 versus CD16

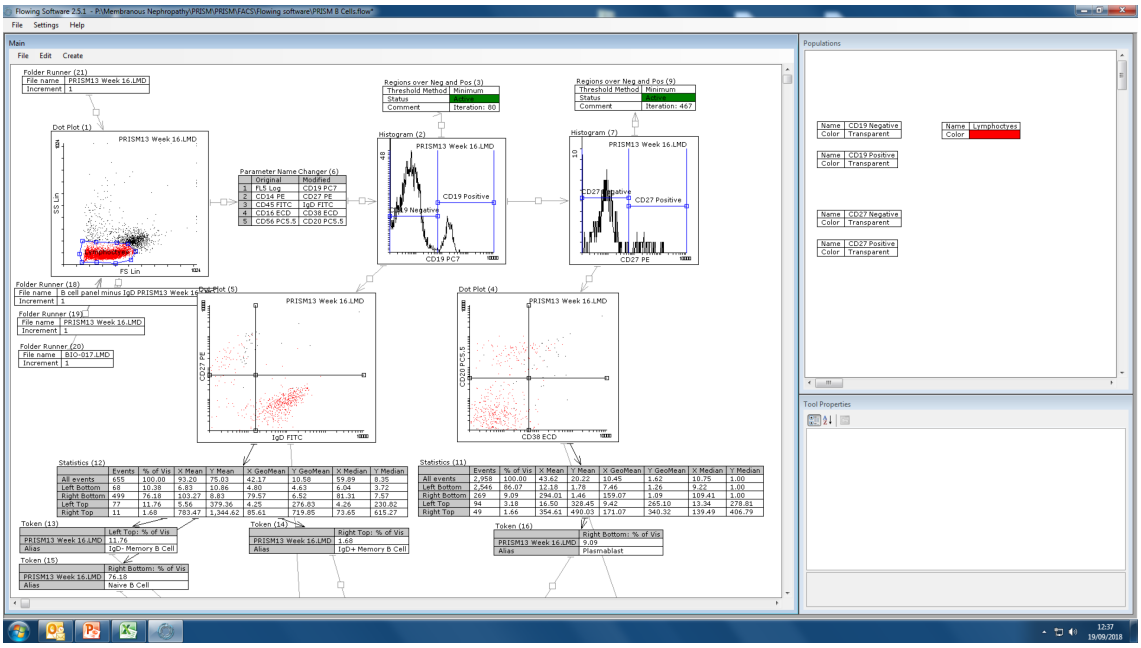


Figure S5.13 – B cell gating using flowing software

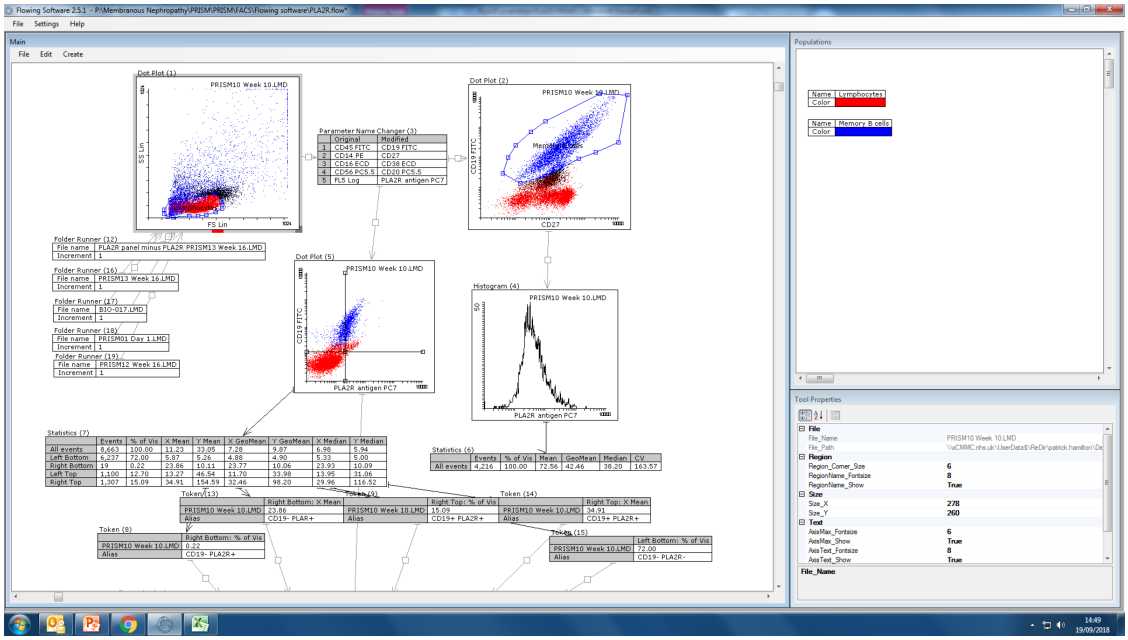


Figure S5.14 – PLA_2R panel gating strategy using flowing software

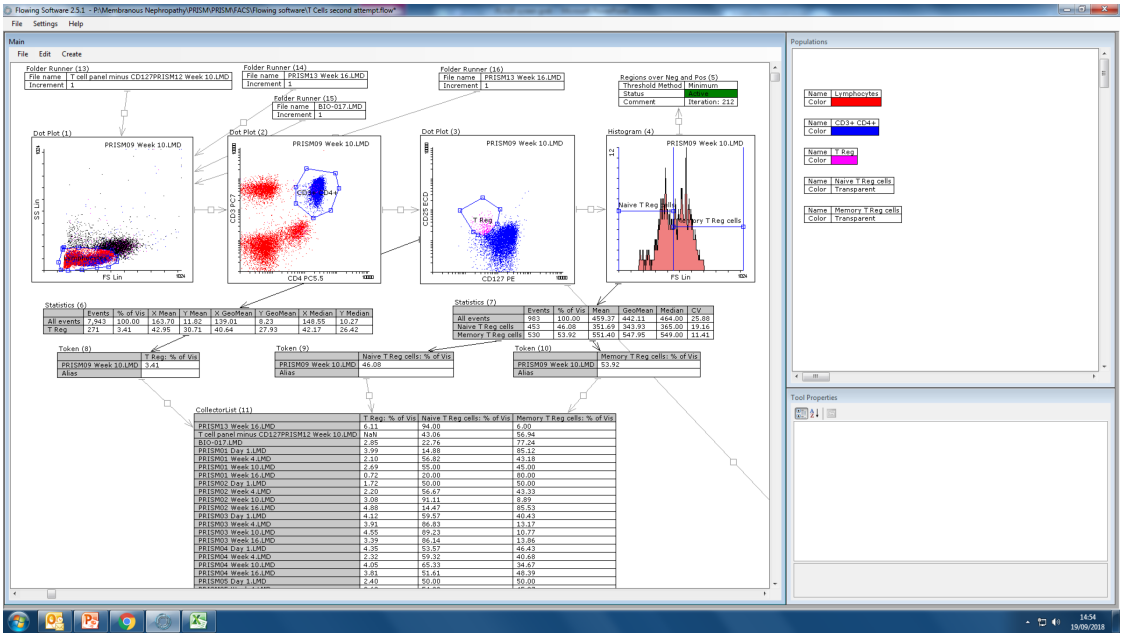


Figure S5.15 – T cell gating strategy using flowing software

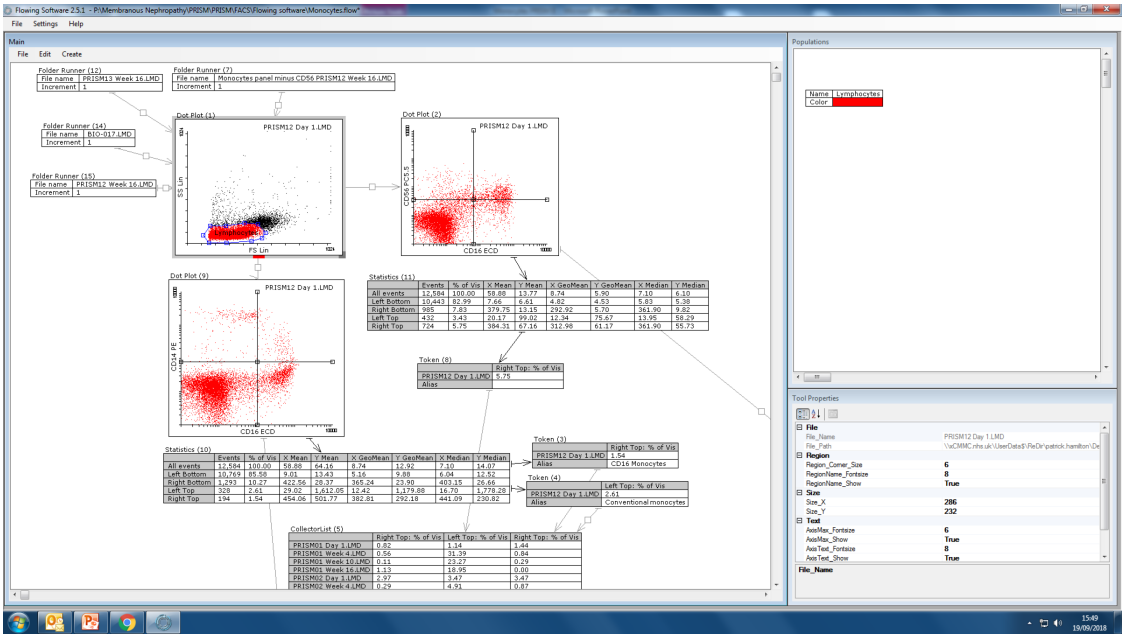


Figure S5.16 – Monocyte panel gating strategy using flowing software

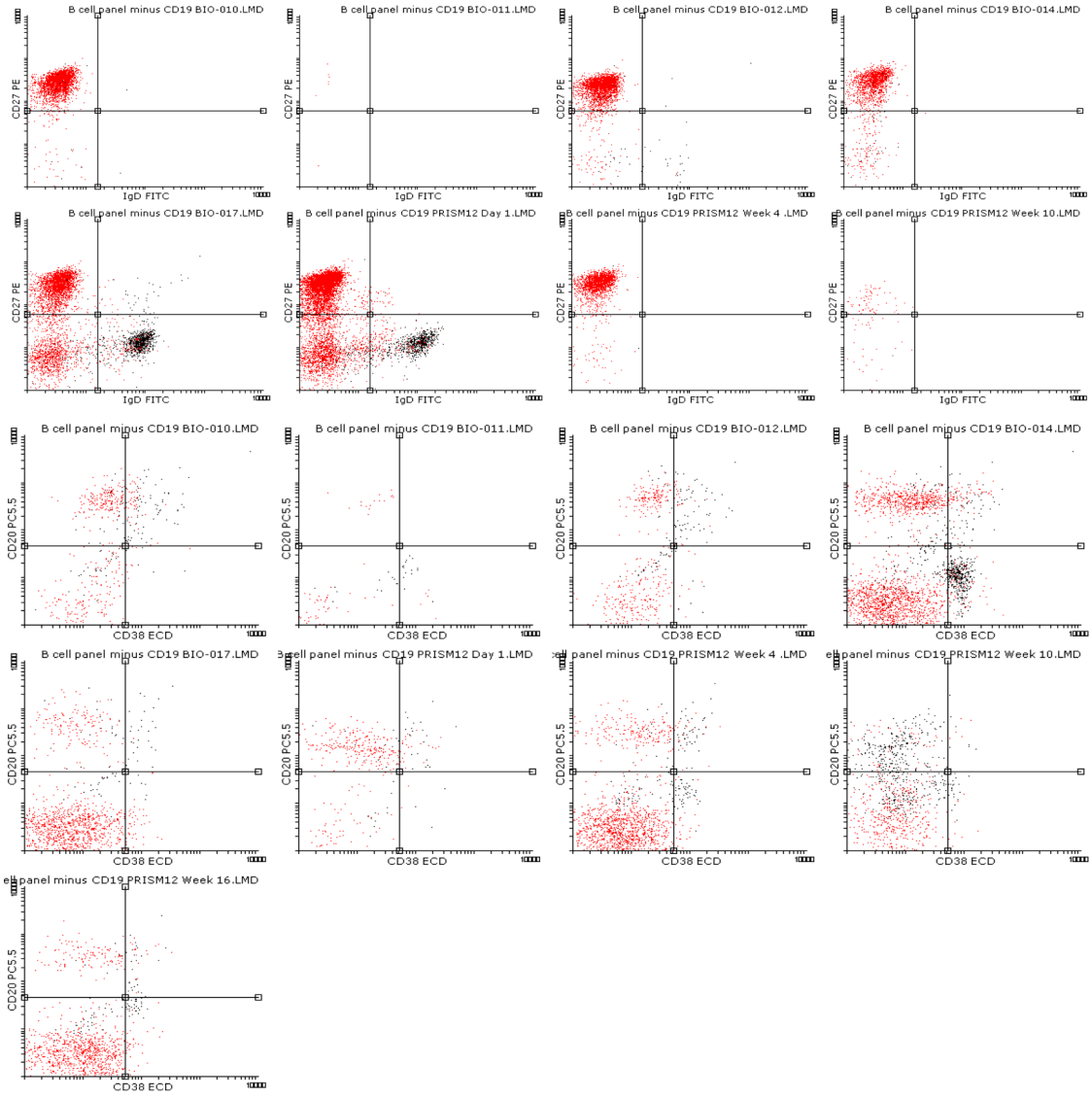


Figure S5.17 – B cell full minus CD19

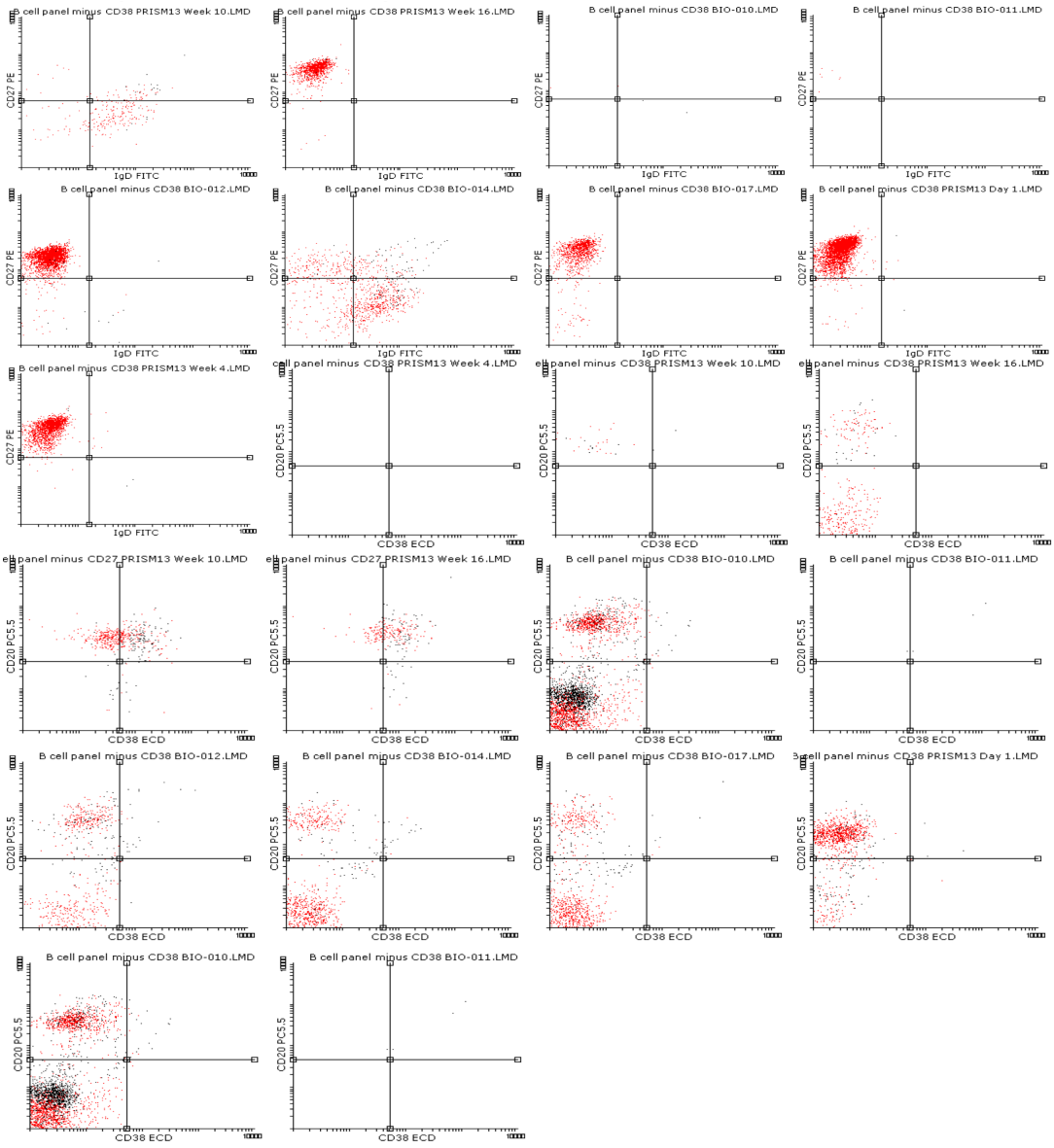


Figure S5.18 – B cell full minus CD38

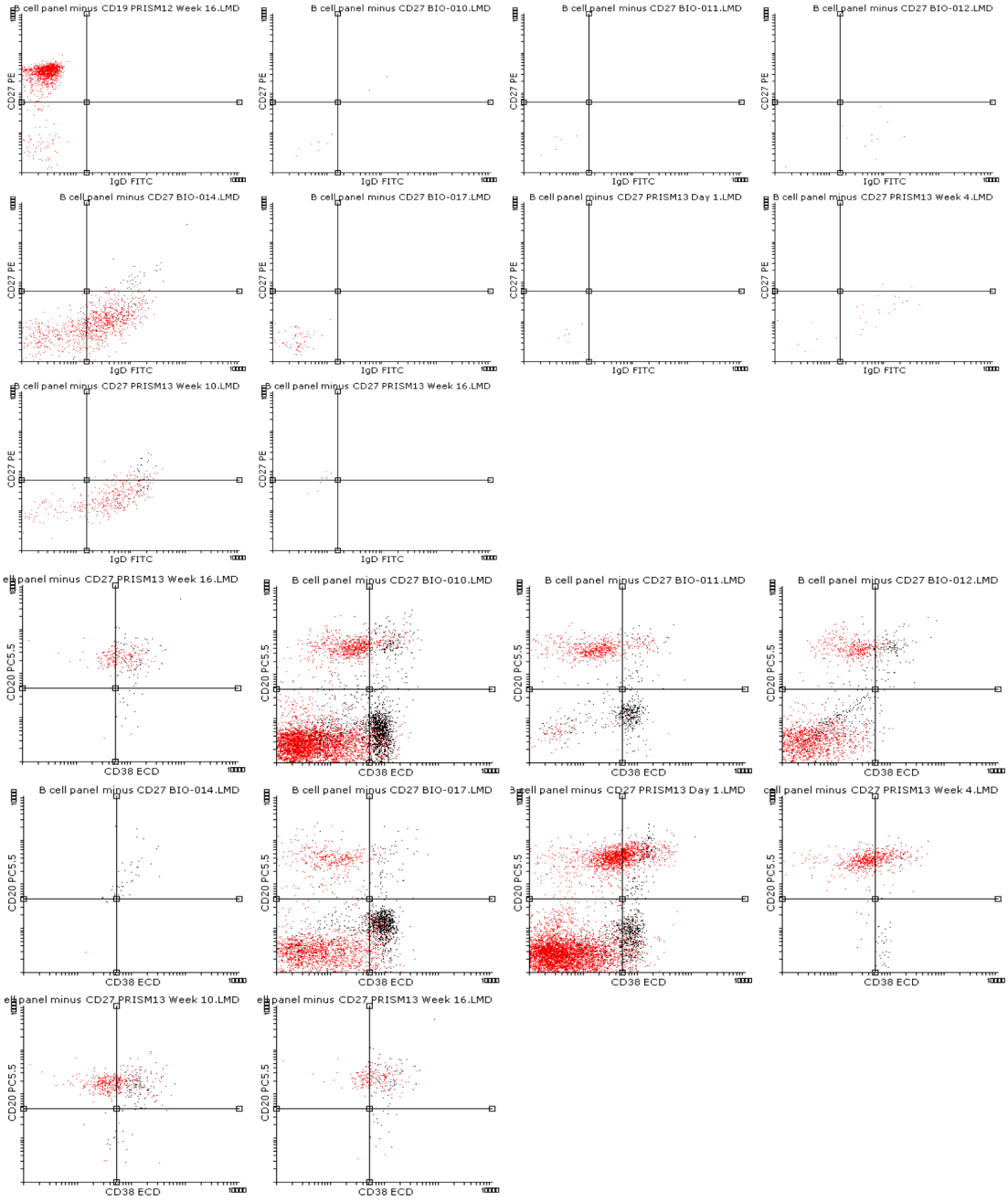


Figure S5.19 – B cell full mins CD27

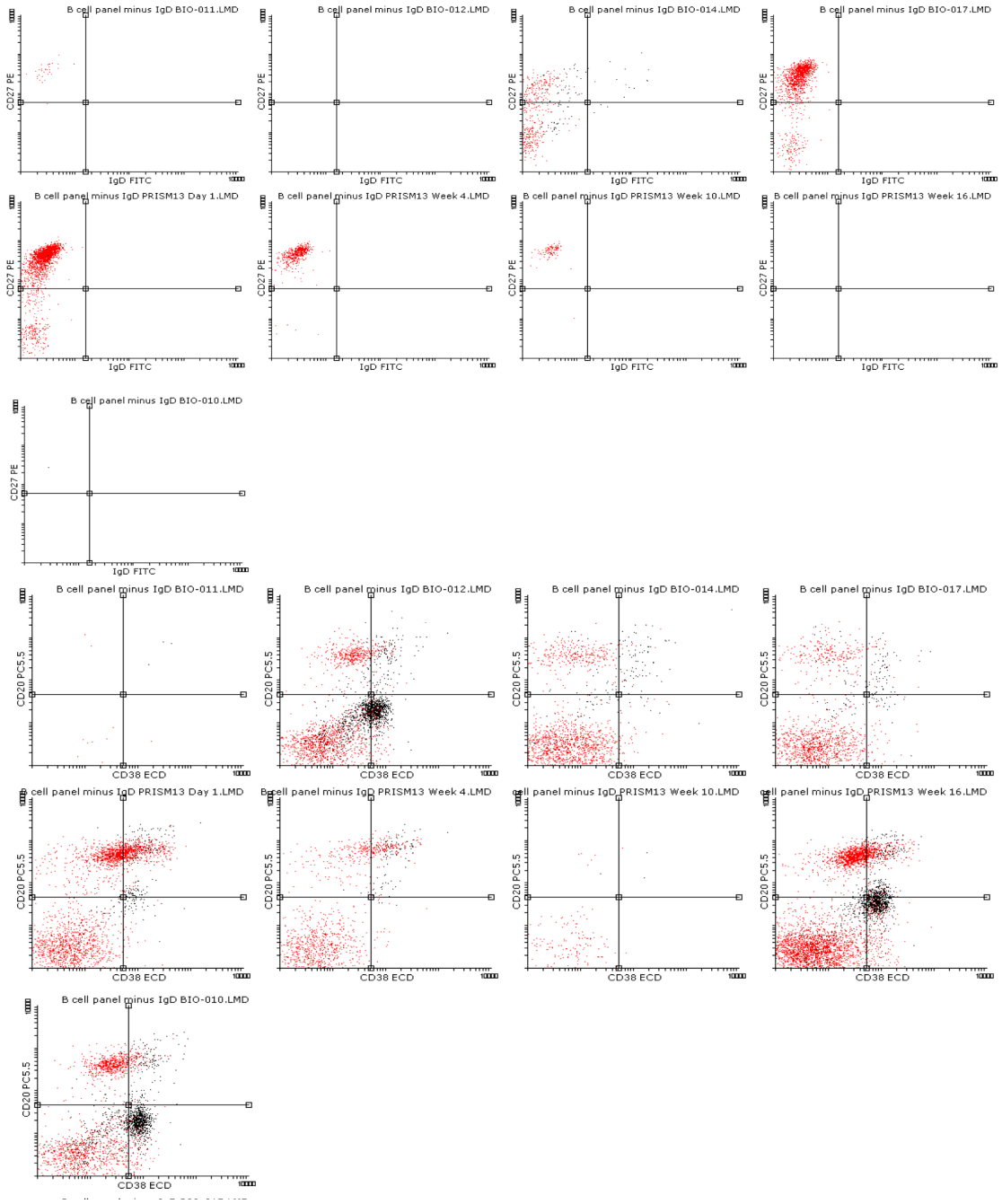


Figure S5.20 – B cell full minus IgD

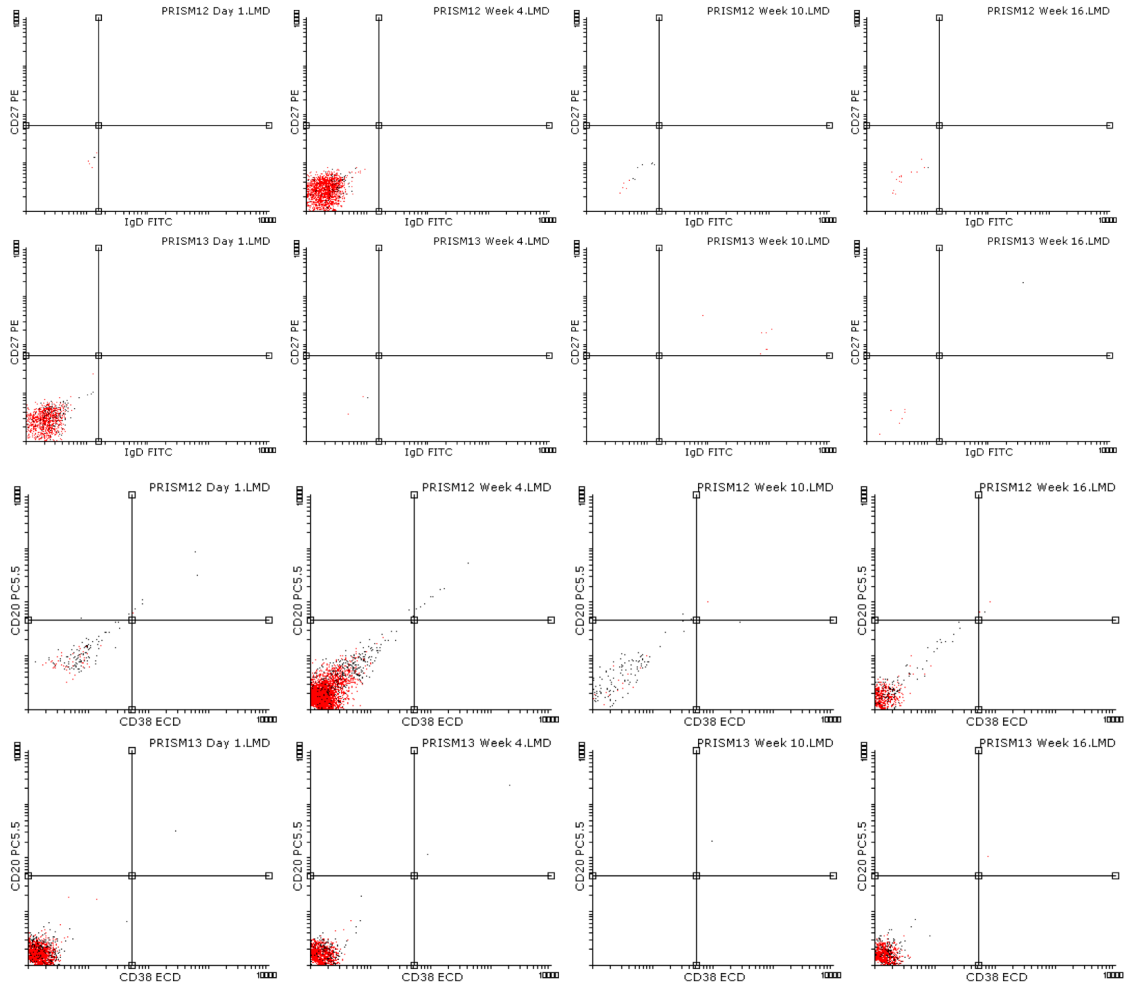


Figure S5.21 – B cell panel with no reagents added

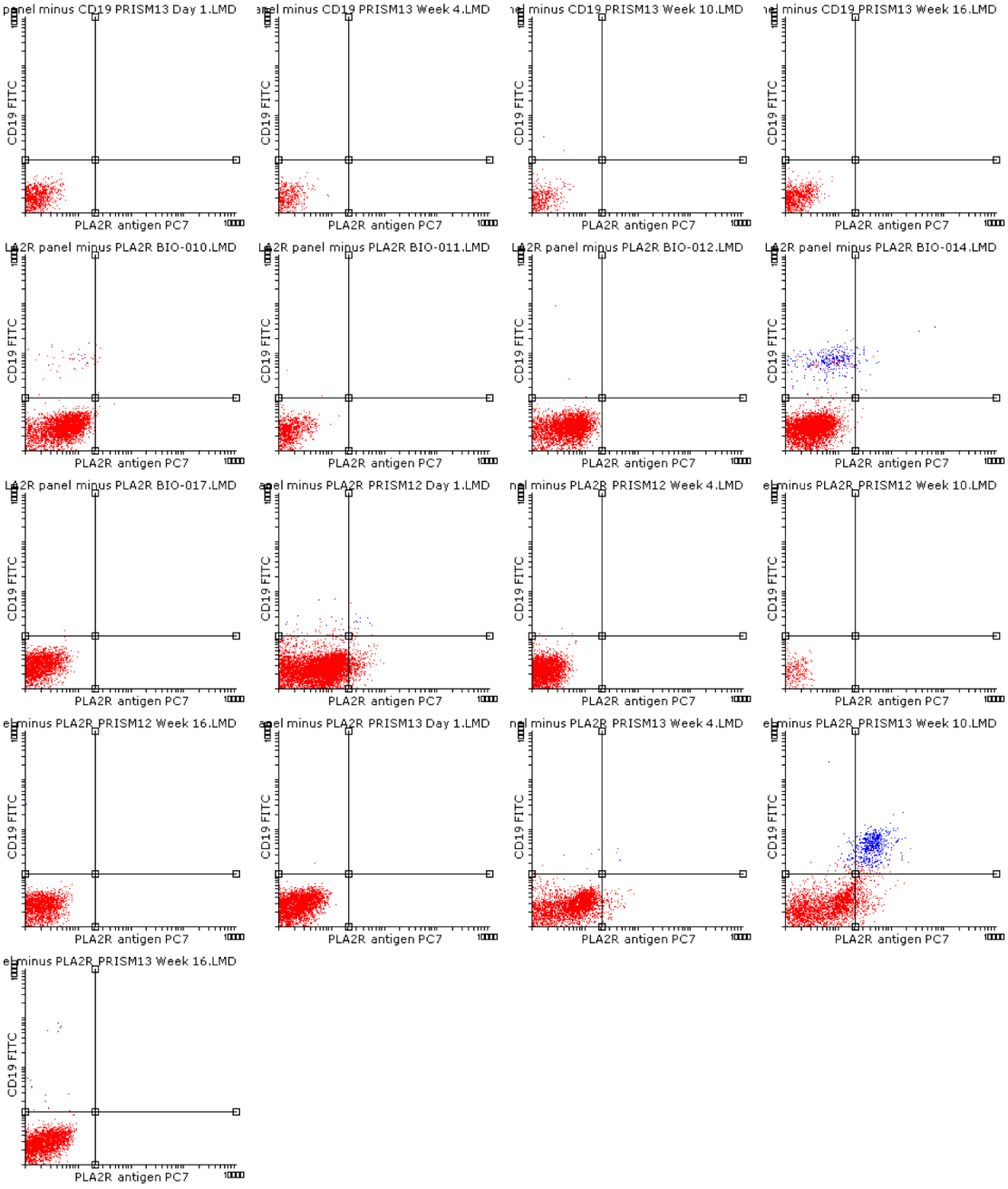


Figure S5.22 – PLA_2R panel full minus CD19 and PLA_2R

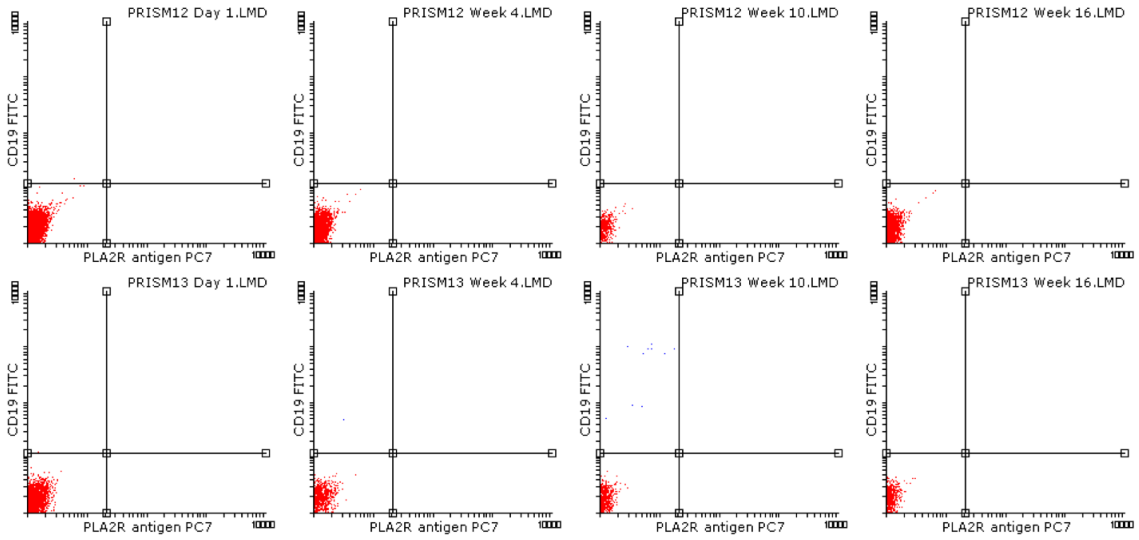


Figure S5.23 – PLA_2R panel with no reagents added

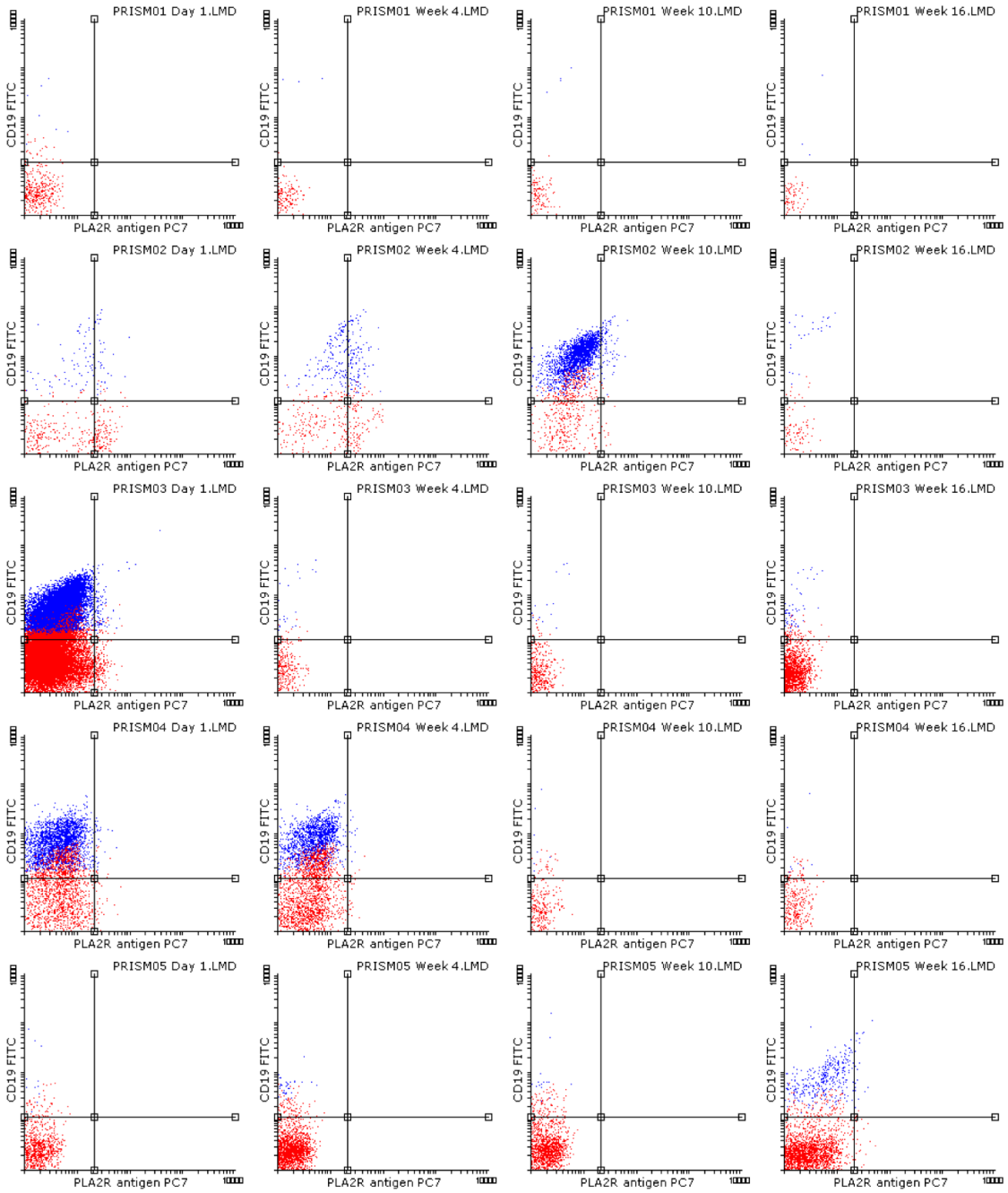


Figure S5.24a – PRISM01 - PRISM05. PLA2R panel using scrambled PLA2R antigen showing less interactions compared to full PLA2R antigen.

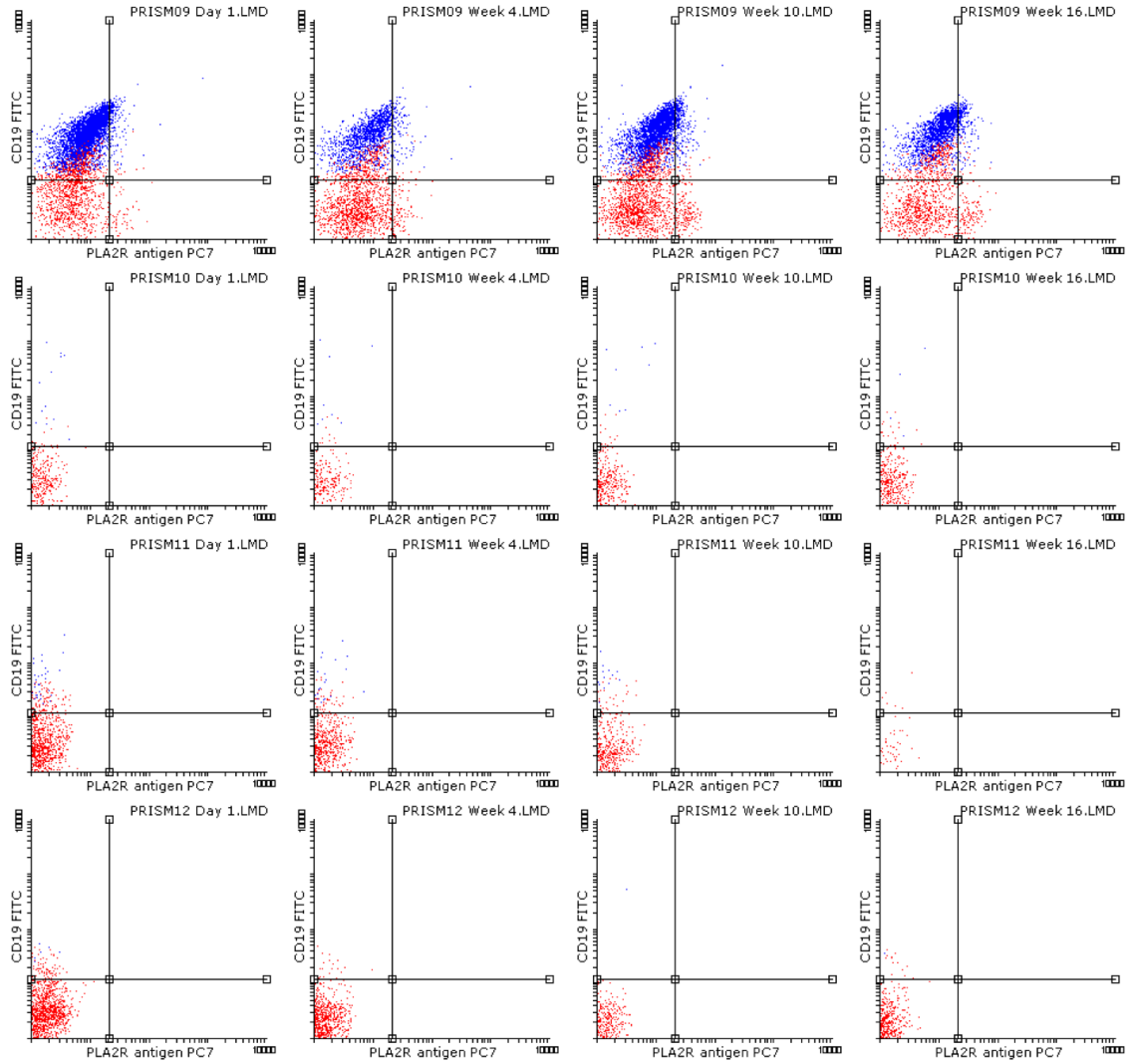


Figure S5.24b – PRISM09 – PRISM12. PLA_2R panel with scrambled antigen

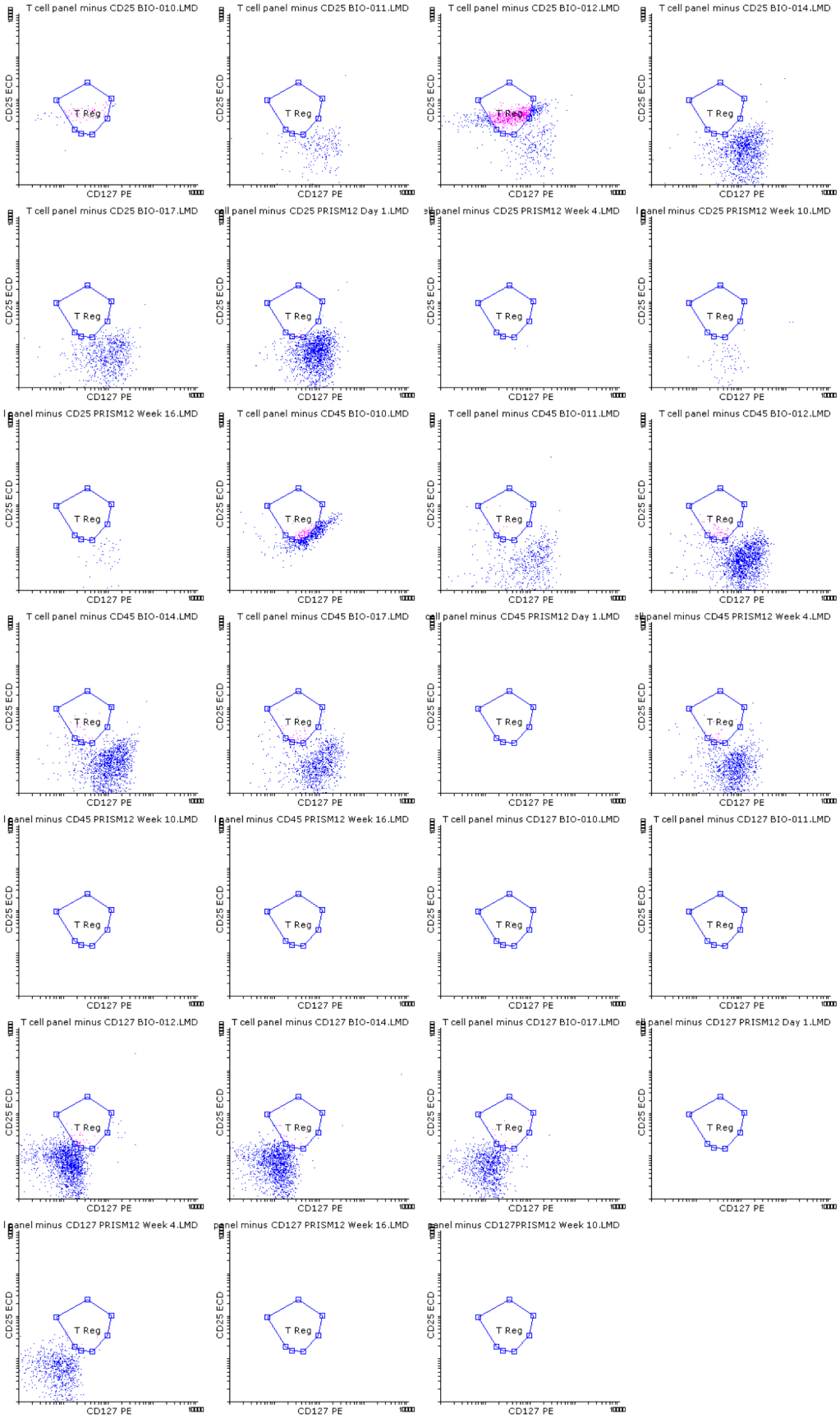


Figure S5.25 – T cell full minus CD25 / CD45 / CD127

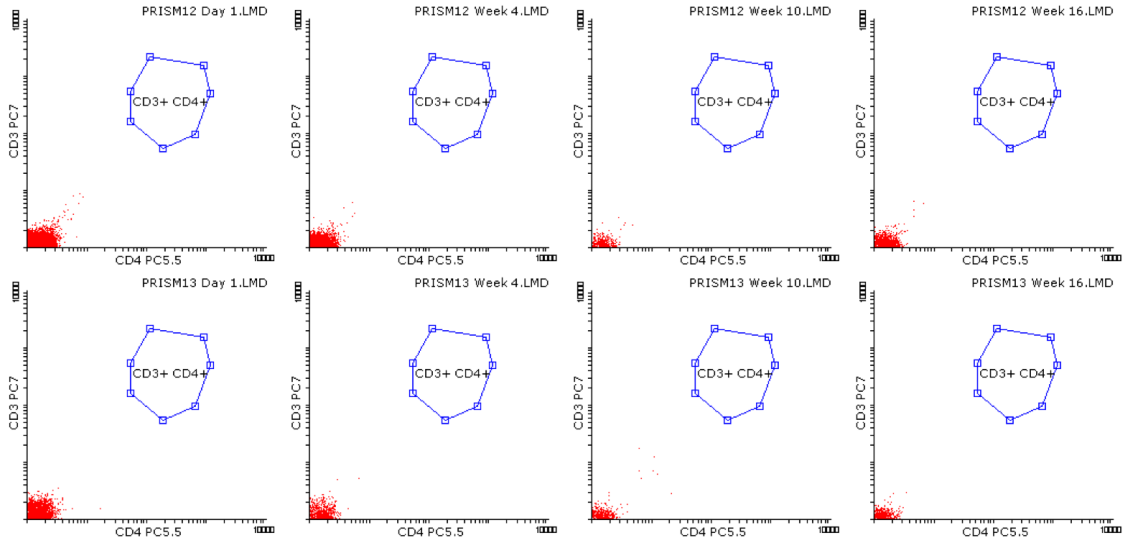


Figure S5.26 – T cell panel with no reagents added

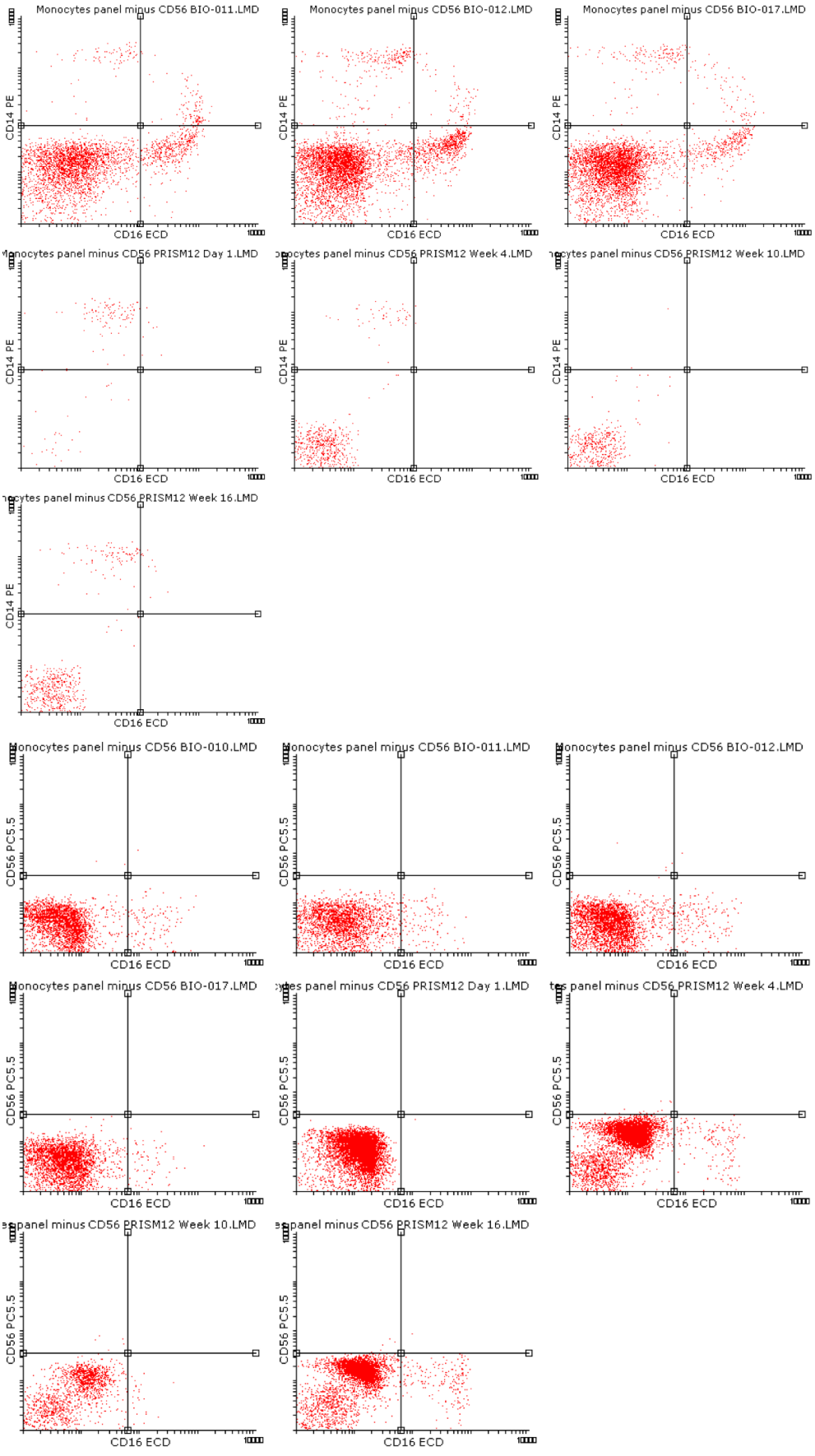


Figure S5.27 – Monocyte panel full minus CD56

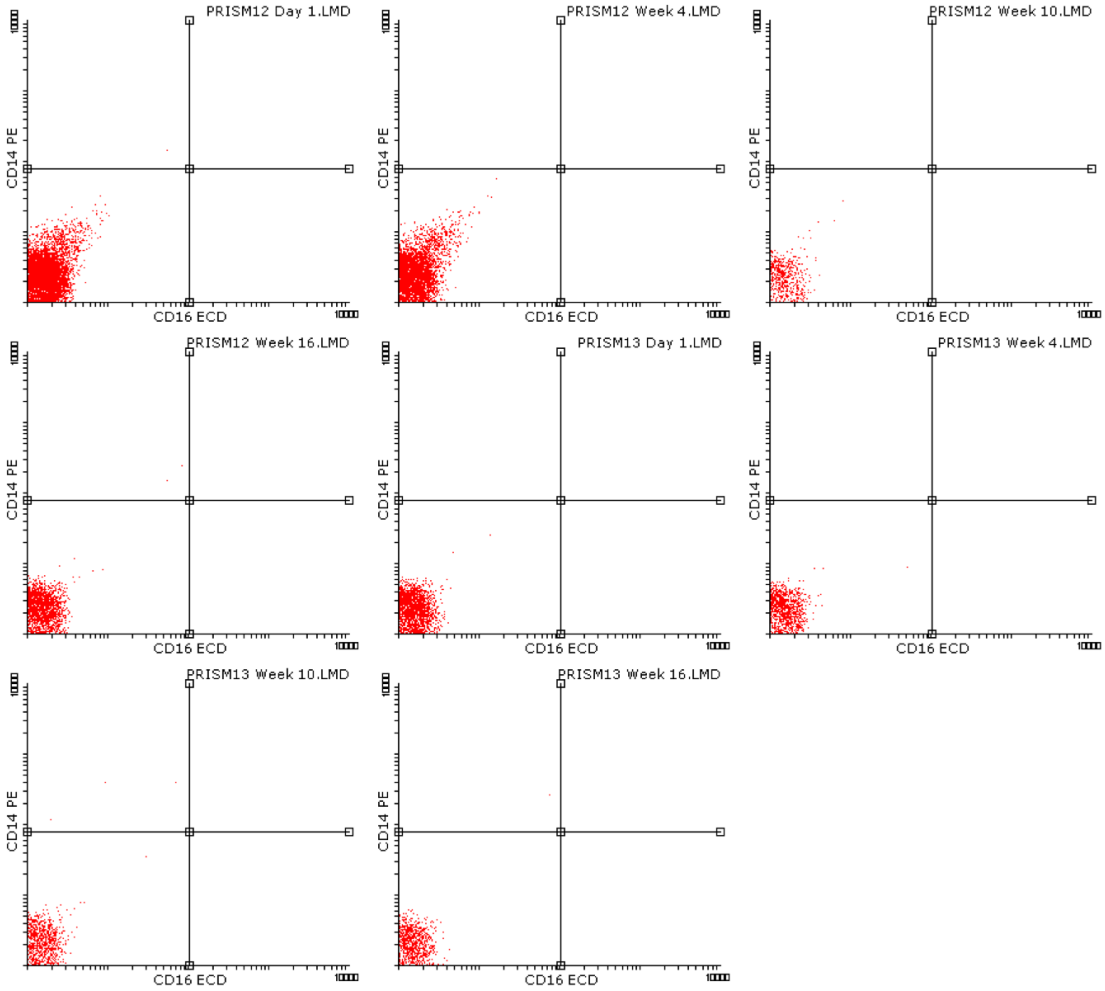


Figure S5.28 – Monocyte panel with no reagents added

Manchester University
NHS Foundation Trust

Greater Manchester Therapeutic Apheresis Service (GMTAS)

Service Proposal

Background

Immunoadsorption is a method of removing specific circulating immunoglobulins with albumin and anti-thrombin III almost unaffected. This directed nature of the therapy provides a treatment strategy with high efficacy and a low side effect profile without the needs for medications. This has resulted in its use in a multitude of autoimmune diseases across a range of specialities such as dilated cardiomyopathy, bullous pemphigoid, multiple sclerosis, myasthenia gravis, Focal Segmental Glomerulosclerosis (FSGS), systemic lupus nephritis (SLE), ANCA-associated small vessel vasculitides, Anti-glomerular basement membrane antibody disease and in renal transplantation.

In conditions such as SLE, the use of immunoadsorption can dramatically reduce the level of circulating immune complexes and autoantibodies leading to clinical improvement in even severe life-threatening SLE. Use in Dilated Cardiomyopathy for the removal of ß1- adreno-receptor autoantibodies (ß1-AAB) has shown that only a small minority of patients (0% in the first year and 15% by 3 years) will show an increase in significant ß1-AAB autoantibodies.

The Manchester Institute of Nephrology & Transplantation currently provides therapeutic Immunoadsorption not only for the renal department itself, but also for the renal transplantation department, currently the largest single centre unit in the UK, and also for the Endocrinology department. We are at present also undertaking a clinical trial investigating the novel use of Peptide GAM immunoadsorption therapy for Autoimmune Membranous Nephropathy, the first use of the machine in the UK and in this condition.

Rationale

With current treatment for a range of autoimmune diseases generally taking the form of empiric chemotherapy designed to suppress the immune system, and with our understanding of these conditions through research increasing, the ability to offer a more directed therapy with a reduced side-effect burden becomes ever more possible. Given the recent advances in many autoimmune conditions and an increasing body of evidence showing benefit, IA has the ability to provide a treatment without the need for steroids or immunosuppression along with a reduced side-effect profile.

Service need

Many of the conditions that can benefit from therapeutic aphaeresis are rare diseases spread across a range of specialities. This results in a situation where individual patients may be geographically and clinically isolated, meaning that access to therapeutic aphaeresis is not possible due to its perceived limited use. However, across a region such as the Northwest with a large population, there is a large unmet need and demand for therapy. This centralised service to allow specialities to have a single point of access.

Patient need

Therapeutic aphaeresis provides its treatment benefit through its regulation of the immune system and given the nature of the diseases it can treat, many of the standard therapies come with significant side effects and contra-indications. Aphaeresis has the potential to not only offer a more targeted therapy with a reduced side effect profile but an alternative treatment in refractory disease.

THERAPEUTIC APHAERESIS INDICATIONS

PLASMA EXCHANGE	IMMUNOADSORPTION	LIPID APHAERESIS
ANCA Associated Vasculitis	Antibody-mediated rejection	Hyperlipidaemia
Focal Segmental Glomerulosclerosis	Dilated Cardiomypothy	
Anti-GBM disease	Pemphigus Vulgaris	
Antibody-mediated rejection	Atopic dermatitis	
Lupus Cerebritis	Guillain-Barre syndrome	
Multiple Sclerosis	Myasthenia Gravis	
HUS / TTP	Autoimmune Encephalitis	

Other indications currently undergoing clinical trials using immunoadsorption include

Primary Membranous Nephropathy (at the MRI)

Chagas Cardiomyopathy

Myocardial Infarction

Vascular dementia

Benefit

The ability to provide an alternative safe and effective treatment for patients who are either intolerant or unresponsive to current standard of care at present is limited due to the cost implications of aphaeresis therapy. However, by centralising the service with a single access referral and treatment pathway, this will allow a cost-effective provision of therapy within the NHS framework to a multitude of specialities and patients.

Objective

Given the expertise and infrastructure already in place in the Manchester Royal Infirmary, we will expand this service to provide therapeutic immunoadsorption to a number of specialities in which the treatment has been shown to provide benefit.

Current and proposed activity

Current

Lipid Aphaeresis

All Lipid aphaeresis now carried out in CAPD.

There are 5 patients on regular treatment. Three patients have weekly sessions, and two patients have fortnightly sessions.

Therapeutic Plasma Exchange

January to June 2017

A total of 12 patients were treated a total of 114 times.

Conditions treated: ANCA associated vasculitis (AAV)
Focal Segmental Glomerulosclerosis
Anti-GBM disease
Antibody-mediated rejection
Lupus cerebritis

Immunoadsorption

Immunoadsorption currently being trialled in 12 adult patients with autoimmune membranous nephropathy in the PRISM[1] trial run from the NIHR / Wellcome Trust Clinical Research Facility at the Manchester Royal Infirmary.

Plasma double filtration

Offered for AAV, Goodpasture's, ABO incompatible transplantation, ABO antibody-mediated transplant rejection, cryoglobulinaemia and Myeloma.

[1]Phase II trial investigating the safety and feasibility of Peptide GAM Immunoadsorption in anti-PLA2R positive autoimmune membranous nephropathy.

Proposed

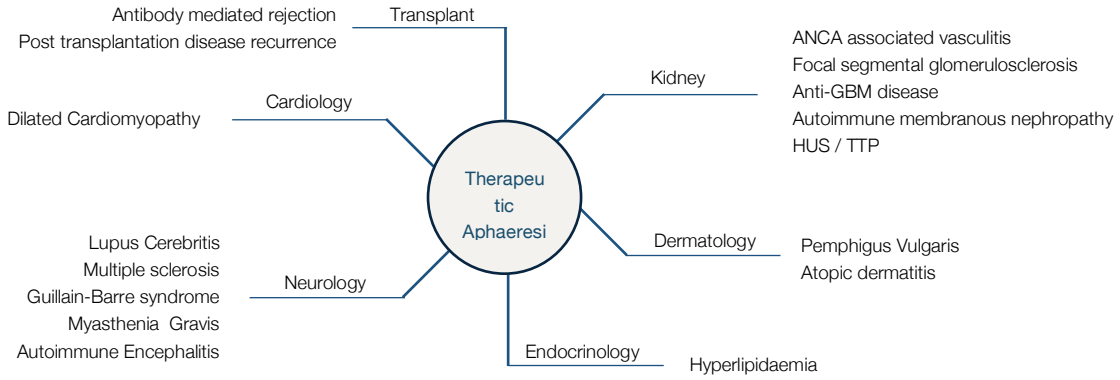
Along with the established aphaeresis techniques such as TPE and LDL aphaeresis, we propose to increase the scope and use of immunoadsorption for use in conditions from Renal, Cardiology, Dermatology, Endocrinology and Neurology.

Stakeholders

Renal	Dr Sandip Mitra, Prof Paul Brenchley, Dr Patrick Hamilton, Prasanna Hanumapura
Dermatology	Dr Helen Young
Endocrinology	Dr Handrean Soran
Cardiology	Dr Forzia Ahmed
Neurology	TBC
Fresenius	Dr Mortiz Fischer

Business model

TBC



Costs

NHS National schedule of reference costs 2015-2016[1]

SA13A Single Plasma Exchange, Leucopheresis or Red Cell Exchange, 19 years and over

£505 (IQR £335 - £602)

Lipid aphaeresis

Current Lipid aphaeresis costs £28,000 per patient per year

Same cost for each patient no matter the frequency

Lines/consumables	£472 + VAT = £566
Total cost for 4L	£575

Plasma Exchange

Lines/consumables	£172 + VAT = £206
	4.5% Albumin(500ml) –£ 30.38 + VAT
	Octaplas -£55 + VAT
Total cost for 4L	£520 if we use 4.5% HAS (incl VAT & Miscellaneous)
	£1520 if we use octaplas (incl VAT & miscellaneous)

Plasma double filtration

Lines/consumables	£472 + VAT = £566 + 2lts of 4.5% HAS
Total cost for 4L	£700

[1]NHS Reference Costs 2015 to 2016. https://www.gov.uk/government/publications/ nhs-reference-costs-2015-to-2016

Immunoadsorption pricing proposal

Part Number	Description	UOS	Patient Numbers			
			1-2	3-4	5-6	7+
			Pricing Band			
			A	B	C	D
f00004856	GLOBAFFIN Columns	1	£5,250.00	£4,500.00	£3,750.00	£3,000.00
9798191	Kit ADAsorb GLOBAFFIN	1	£556.50	£477.00	£397.50	£318.00
9797413	ART Adasorb DB01/04	2	£553.00	£474.00	£395.00	£316.00
9797310	Fraction Bags	10	£79.50	£74.50	£69.50	£64.50
9798151	PBS	4	£180.00	£178.00	£176.00	£174.00
TMD000031	ACD-A	8	£81.20	£69.60	£58.00	£46.40
9798171	Glycene Buffer	2	£98.00	£96.00	£94.00	£92.00

Example cost for treating a single patient for 4 sessions

Part Number	Description	UOS	Patient Numbers			
			1-2	3-4	5-6	7+
			Pricing Band			
			A	B	C	D
f00004856	GLOBAFFIN Columns	2	£10,500.00	£9,000.00	£7,500.00	£6,000.00
9798191	Kit ADAsorb GLOBAFFIN	4	£2,226.00	£1,908.00	£1,590.00	£1,272.00
9797413	ART Adasorb DB01/04	2	£1,106.00	£948.00	£790.00	£632.00
9797310	Fraction Bags	1	£79.50	£74.50	£69.50	£64.50
	TOTAL COST		£13,911.50	£11,930.50	£9,949.50	£7,968.50

Summary

This service will allow a single point of access for therapeutic aphaeresis, a therapy of proven benefit but limited use, given the rare nature and multi-disciplinary spread of these conditions. By centralising the service for the region it provides a treatment with high setup costs, running and high staff skill levels in an efficient and cost-effective manner to patients with difficult to control disease.

The long-term benefits of treating conditions that can otherwise lead to significant co-morbidities and resource use (both NHS and societal) with minimal complications and side-effects adds to its potential and need. Along with this, and as demonstrated by the current PRISM trial, this therapy provides a number of research pathways in all specialities to not only understand the efficacy of therapy but to also help understand disease pathogenesis without the use of immunoregulatory medications. This offers the potential to develop more targeted treatments with a reduced side effect burden and a better quality of life for patients at the same time as generating extra funding streams. With the expertise and support already available at the Manchester Royal Infirmary, in both a clinical and research capacity, and through close links with industry, Manchester has the potential to be not only the national leader for aphaeresis therapy but also an International leader in research and development.

Central Manchester University Hospitals NHS
NHS Foundation Trust

Peptide GAM Immunoadsorption therapy in Autoimmune Membranous Nephropathy

Dr Patrick Hamilton, Dr Durga Kanigicherla, Dr Sandip Mitra, Prof Paul Brenchley
Manchester Institute of Nephrology and Transplantation, Manchester Royal Infirmary, Manchester

Protocol Version	1.2
Long title	Phase II trial investigating the safety and feasibility of Peptide GAM Immunoadsorption in anti-PLA2R positive autoimmune membranous nephropathy.
Indication	Autoimmune Membranous Nephropathy
Investigational product	Fresenius Globaffin®
Trial Sponsor	Central Manchester University Hospital Foundation Trust
Funder	Fresenius SE & Co. KGaA
Study Centre	NIHR/Wellcome Trust Clinical Research Facility
Principle Investigator	Dr Sandip Mitra
Study period	24 months
Trial Registration	TBC
REC reference	16/NW/0560

MINT

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Sub-investigator	Dr Durga Kanigicherla Consultant Nephrologist Renal Department Manchester Royal Infirmary Oxford Road Manchester M13 9WL durga.kanigicherla@cmft.nhs.uk
Sub-investigator	Dr Patrick Hamilton Renal Research Registrar Manchester Institute of Nephrology and Transplantation Manchester Royal Infirmary Oxford Road Manchester M13 9WL patrick.hamilton@cmft.nsh.uk Phone: 0161 276 7987

Senior Statistician	Dr Stephen Roberts Centre for Biostatistics Institute of Population Health 1st Floor, Jean McFarlane Building University of Manchester Manchester M13 9PL steve.roberts@manchester.ac.uk Phone: 0161 275 5192

Abbreviations

ACEi	Anticholinesterase inhibitor
AE	Adverse events
ANCA	Anti-neutrophil cytoplasmic antibodies
ARB	Angiotensin II receptor bloc
CIC	Circulating Immune Complex
COX	Cyclo-oxygenase
eGFR	Estimated Glomerular Filtration Rate
ESRD	End-stage renal disease
EQ5D	EuroQoL EQ5D Index Score
FSGS	Focal Segmental Glomerulosclerosis
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin subclass G
IMN	Idiopathic / Autoimmune Membranous Nephropathy
IV	Intravenous
NSAIDs	Non-steroidal anti-inflammatory drugs
PLA2R	Phospholipase A2 receptor
PROM	Patient Reported Outcomes
RA	Rheumatoid Arthritis
RRT	Renal Replacement Therapy
SAE	Serious adverse events
SF36	Medical Outcomes Survey Short Form – 36
SLE	Systemic lupus nephritis
uPCR	Urinary Protein:creatinine ratio
VTE	Venous Thromboembolism
CRF	Wellcome Trust Clinical Research Facility

Background

Membranous nephropathy (MN) is among the most common causes of nephrotic syndrome in adults worldwide, second only to FSGS[1-8]. The majority of patients will remain stable with either complete remission or partial remission but approximately 20% will progress slowly to end stage renal disease necessitating the need for renal replacement therapy (RRT)[9-11].

MN has two distinct entities with primary or idiopathic MN (IMN) now considered to be an autoimmune disease since the discovery of the M-type of phospholipase A2 receptor 1 (anti-PLA2R) antibodies[12-15] and secondary MN caused by a multitude of disorders including but not restricted to malignancy, infection and drugs (see table 1)[16,17]. These two conditions have very different management priorities with the focus in secondary MN being the treatment of the underlying condition and in IMN, the control of proteinuria with or without the use of immunosuppression, generally in the form of the Ponticelli regime. This regime of rotating high dose steroids and immunosuppression was first described in the mid-nineties and has been the mainstay of treatment since. This regime however does come with a dramatic side effect burden including an increased risk of infection, osteoporosis, diabetes mellitus, weight gain, haemorrhagic cystitis, infertility and an increased risk of malignancy[18].

In 2009 Beck et al showed that the majority of patients with idiopathic membranous nephropathy had IgG autoantibodies to M-Type Phospholipase A2 Receptor, the predominant subclass of which was IgG4 with smaller amounts of all other IgG subclasses[19]. Immunoadsorption is a method of removing specific circulating immunoglobulins and has been shown to remove over 80% of circulating IgG with a single session immunoadsorption of 2.5 plasma volumes, with albumin and antithrombin III almost unaffected[20]. With multiple sessions this can rise to over 98%[21]. Post Immunoadsorption it appears that autoantibodies can be slow to re-emerge. Use in Dilated Cardiomyopathy for the removal of β_1- adreno-receptor autoantibodies (β_1-AAB) has shown that only a small minority of patients (0% in the first year and 15% by 3 years) will show an increase in significant β_1-AAB autoantibodies[22,23].

To our knowledge there has only been one publication using immunoadsorption for the treatment of membranous nephropathy. In 1999 Esnault et al successfully used Immunoadsorption for the treatment of various aetiologies' of Nephrotic syndrome

including four patients with membranous nephropathy[24]. Here they showed that not only is the procedure safe but that there was a significant improvement in proteinuria in all patients with membranous nephropathy. Since that time the treatment has been used in numerous other autoimmune conditions including Focal Segmental Glomerulosclerosis (FSGS)[25], systemic lupus nephritis (SLE)[26,27], ANCA-associated small vessel vasculitides[28-30], Anti-glomerular basement membrane antibody disease[31] and in renal transplantation[32-35].

In conditions such as SLE, the use of immunoadsorption can dramatically reduce the level of circulating immune complexes and autoantibodies leading to clinical improvement in even severe life threatening SLE. These results have been shown with as little as two sessions within three days and repeated every three weeks if patients remain with active disease[26].

What has not been studied however is the role that immunoadsorption has in membranous nephropathy in the anti-PLA2R era. What role can it have in the removal of IgG4 and levels of anti-PLA2R titres? If the levels are reduced do they recur? What is the role of soluble Anti-PLA2R and circulating immune complexes in the disease process? And what effect does this have on disease activity.

The aim of this study is to answer these questions in the hope that it will allow for a more targeted disease control without the side effect burden long term and high dose steroids and immunosuppression can confer on a patient. With the safety profile of Immunoadsorption already shown and with some evidence for its benefit in membranous nephropathy we propose to use the therapy in patients with significantly raised serum anti-PLA2R titres and biopsy proven membranous nephropathy.

STUDY DESIGN

Primary Outcome

Reduction in serum anti-PLA2R titres to normal range

Secondary Outcomes and measures

Safety and tolerability of Immunoadsorption therapy

To determine the effect on soluble Anti-PLA2R levels

Kinetic modelling of anti-PLA2R production

Reduction in circulating immune complex levels

Analysis of T and B cell regulatory cells and molecules

Reduction in Proteinuria level

Reduction in creatinine / increase in eGFR

To determine the effect on disease activity

To determine the effect on Quality of life measures (EQ5D & SF36)

Cost-effectiveness of treatment (Incremental cost-effectiveness ratio)

Primary Endpoint

The primary endpoint is the reduction in serum anti-PLA2R titres at day 14

Secondary endpoints

Secondary endpoints will be reported at Day 14, 28, 56, 84, 168 and 365. These will be the reduction in proteinuria and improvement in renal function as measured by uPCR and serum creatinine level respectively. The reduction and pattern of serum anti-PLA2R titres, circulating immune complex levels and the effect on soluble Anti-PLA2R levels will be investigated using prospective blood tests peri-therapy. Kinetic modelling of anti-PLA2R production will also involve the daily collection of urine whilst on Immunoadsorption therapy. Disease activity, AEs and SAEs to be based on physician assessment. Serious adverse events are taken as any adverse events requiring hospital admission, prolonged hospital stay or intravenous (IV) therapy outside of protocol. Quality of life measures will be assessed using patient completed EQ5D and SF36. Incremental cost effectiveness to be based on NHS reference costs and patient reported personal and societal costs.

Inclusion Criteria

Biopsy confirmed Primary Membranous Nephropathy within the last 3 years

Active disease despite 6 months of supportive care including ACEi or ARB

- Active disease defined as uPCR > 300mg/mmol or 24 hour urinary protein > 3.5g/1.73m^2

Disease severity that in the physicians view warrants treatment prior to completion of 6 months supportive care

Anti-PLA2R titre > 170 u/ml

Haemophilus and Pneumococcal vaccinations up to date

Above the age of 18

Able to provide informed consent

Exclusion criteria

Evidence of causes of secondary membranous nephropathy

eGFR < 20ml/min

Treatment with steroids or immunosuppression (including but not limited to cyclophosphamide, MMF or azathioprine) and Biologics (including but limited to Rituximab or belimumab) within 6 months of screening

Therapeutic Plasma Exchange within 28 days of screening

Previous renal transplantation

Co-morbidity, which in physicians' view, would preclude patient from treatment with immunoadsorption.

Pregnant at time of screening

Treatment description

Recruitment will be carried out at approved sites and consent to be carried out in the Manchester Royal Infirmary Clinical Research Facility (CRF). Patients will report to the CRF on the Monday of the first treatment, where following confirmation of patients desire and suitability to proceed with the study, will undergo observations, blood and urine tests as outlined below. A femoral vascath (Double lumen blood access catheter; Medcomp, Harleysville, PA, USA) will then be inserted under local anaesthetic, which remains in situ for the week. Patient will then undergo daily Immunoadsorption for five days. If patients are unable to complete five days consecutively they will be allowed to complete 5 sessions within 7 days. If however the treatment is deferred for more than 48 hours the patient will need to have an extra session to ensure the adequate removal of antibody. In this case the patient will receive 6 sessions in 8 days. . Patients will have close monitoring throughout and repeat bloods and urine tests daily. During this period patients will also collect daily 24 hour urine samples. Once the treatment period is complete, the vascath will be removed and patients will enter a follow up period as described below.

There is a relative contraindication of ACEi therapy and Immunoadsorption with a reported increased bradykinin release. Therefore at consent all patients to be converted to ARB. If patients are unable to tolerate ARB therapy then to stop ACEi 48 hours prior to Immunoadsorption and can restart following completion of Immunoadsorption.

Study Duration

Patients will have follow up weekly for the first month. For months 2 & 3 patients will be followed up at two weekly intervals, reducing to monthly until the end of the one year follow up period. All follow ups will be conducted at the CRF.

Population Size

We aim to recruit 12 patients from across the North of England.

Screening

Review inclusion and exclusion criteria

Confirm patient agrees to continue with study participation

Consent

Pulse, BP, temperature, oxygen saturations & respiration rate

Height and Weight & demographics recorded

Physical assessment

Bio-impedance

Concomitant meds

ECG

Pregnancy test

EQ5D

Full blood count, renal profile, LFTs, anti-PLA2R, soluble anti-PLA2R, circulating immune complex level, coagulation screen, immunoglobulin's, autoimmune screen, virology screen

Haemophilus, tetanus, diphtheria, pneumococcal titres

Urine dipstick

uPCR

Biologic specimens for BioBank

Day 1 - 5

Day 1	Confirm patient agrees to continue with study participation
	Pulse, BP, temperature, oxygen saturations & respiration rate
	Height and Weight & demographics recorded
	Physical assessment
	Concomitant meds
	ECG
	Pregnancy test
	EQ5D & PROM costs
	Vascath insertion
	Full blood count, renal profile, LFTs, CRP, ESR, anti-PLA2R, soluble anti-PLA2R, circulating immune complex level, coagulation screen, G&S
	Biologic specimens for BioBank before (3x 10ml EDTA & 15ml urine) and after treatment (1x 10ml EDTA)
	Urine dipstick and uPCR
	24 hour urine collection
	Bio-impedance pre and post treatment
	Immunoadsorption
	Neutralised eluate sampling:
	First five cycles into one bag
	150ml in separate bags at plasma volume 1.0, 1.5, 2.0 & 2.5
	Serum anti-PLA2R sampling at plasma volume 1.0, 1.5, 2.0 & 2.5
	Serum calcium and Immunoglobulins post treatment
	Provide 24 hour urine collection bottle prior to discharge for use on Day 2

Day 2	Confirm patient agrees to continue with study participation
	Pulse, BP, temperature, oxygen saturations & respiration rate
	Physical assessment
	Full blood count, renal profile, LFTs, CRP, ESR, anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Biologic specimens for BioBank before (1x 10ml EDTA & 15ml urine) and after treatment (1x 10ml EDTA)
	Urine dipstick and uPCR
	Process 24 hour urine sample from Day 1
	24 hour urine collection for Day 2
	Treatment with Immunoadsorption
	Serum calcium and Immunoglobulins post treatment
	Provide 24 hour urine collection bottle for use on Day 3

Day 3	Confirm patient agrees to continue with study participation
	Pulse, BP, temperature, oxygen saturations & respiration rate
	Physical assessment
	Full blood count, renal profile, LFTs, CRP, ESR, anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Biologic specimens for BioBank before (1x 10ml EDTA & 15ml urine) and after treatment (1x 10ml EDTA)
	Urine dipstick and uPCR
	Process 24 hour urine sample from Day 2
	24 hour urine collection for Day 3
	Treatment with Immunoadsorption
	Serum calcium and Immunoglobulins post treatment
	Provide 24 hour urine collection bottle for use on Day 4

Day 4	Confirm patient agrees to continue with study participation
	Pulse, BP, temperature, oxygen saturations & respiration rate
	Physical assessment
	Full blood count, renal profile, LFTs, CRP, ESR, anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Biologic specimens for BioBank before (1x 10ml EDTA & 15ml urine) and after treatment (1x 10ml EDTA)
	Urine dipstick and uPCR
	Process 24 hour urine sample from Day 3
	24 hour urine collection for Day 4
	Treatment with Immunoadsorption
	Serum calcium and Immunoglobulins post treatment
	Provide 24 hour urine collection bottle for use on Day 5

Day 5	Confirm patient agrees to continue with study participation
	Pulse, BP, temperature, oxygen saturations & respiration rate
	Physical assessment
	Full blood count, renal profile, LFTs, CRP, ESR, anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Biologic specimens for BioBank before (3x 10ml EDTA & 15ml urine) and after treatment (1x 10ml EDTA)
	Urine dipstick and uPCR
	Process 24 hour urine sample from Day 4
	24 hour urine collection for Day 5
	Treatment with Immunoadsorption
	Serum calcium and Immunoglobulins post treatment
	Provide 24 hour urine collection bottle prior to discharge for use on Day 8
	Remove Vascath
	Discharge home if remains well after 2 hours observation

Day 6 – 28

Patients to have weekly review by physician at CRF

Observations	Pulse, BP, temperature, oxygen saturations & respiration rate
	Weight
Bloods	Anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Full blood count, renal profile, LFTs, Immunoglobulins, CRP and ESR
	Biologic specimens for BioBank (3x 10ml EDTA & 15ml urine)
Urine	Urine dipstick
	uPCR
	24 hour urine on day 8 and 28
PROM	EQ5D on day 28
	Personal & Societal costs on day 28

Physical assessment including AEs, SAEs and concomitant meds

Day 29 - 84

Patients to have two-weekly review by physician at CRF

Observations	Pulse, BP, temperature, oxygen saturations & respiration rate
	Weight
Bloods	Anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Full blood count, renal profile, LFTs, Immunoglubulins, CRP and ESR
	Biologic specimens for BioBank (3x 10ml EDTA & 15ml urine)
Urine	Urine dipstick
	uPCR
	24 hour urine on day 56 & 84
PROM	EQ5D on day 56 & 84
	Personal & Societal costs on day 56 & 84

Physical assessment including AEs, SAEs and concomitant meds

Day 85 - 365

Patients to have monthly review by physician at CRF

Observations	Pulse, BP, temperature, oxygen saturations & respiration rate
	Weight
Bloods	Anti-PLA2R, soluble anti-PLA2R, circulating immune complex level
	Full blood count, renal profile, LFTs, Immunoglubins, CRP and ESR
	Biologic specimens for BioBank (3x 10ml EDTA & 15ml urine)
Urine	Urine dipstick
	uPCR
	24 hour urine on day 168 & 365
PROM	EQ5D on day 168 & 365
	Personal & Societal costs on day 168 & 365

Physical assessment including AEs, SAEs and concomitant meds

		Day					Week																
Test	Screening	1	2	3	4	5	2	3	4	6	8	10	12	14	16	20	24	28	32	36	40	44	48	52
Renal Biopsy	✓																							
Consent	✓	✓																						
Pregnancy test	✓	✓																						
Observations		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Height		✓																						
Weight		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
PE		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ECG	✓	✓																						
Anti-PLA2R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
S. anti-PLA2R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CICs	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Renal profile	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Full blood count	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
CRP		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
ESR		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Coag screen	✓	✓					✓																	
LFTs	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Group & Save		✓																						
AIS	✓																							
Immunoglobulins	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Haemophilus	✓																							
Tetanus	✓																							
Diptheria	✓																							
Pneumococcal	✓																							
Virology screen	✓																							
Urine dipstick	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
uPCR	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
24 hour urine		✓	✓	✓	✓	✓	✓		✓		✓		✓				✓							✓
SAEs		✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Con meds	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
EQ5D	✓	✓					✓		✓		✓		✓				✓							✓
PROM Costs		✓					✓		✓		✓		✓				✓							✓
Immunoadsorption		✓	✓	✓	✓	✓																		
30ml EDTA (3x10ml) – pre treatment	✓	✓				✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Freshly voided urine (15ml)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
10ml EDTA (Pre Treatment)			✓	✓	✓																			
24 hour urine			✓	✓	✓	✓	✓		✓		✓		✓				✓							✓
Neutralised eluate*		✓			✓																			
10ml EDTA as per eluate		✓			✓																			
10ml EDTA post treatment		✓	✓	✓	✓	✓																		

Table 1 – Time and events table. PE – Physical Assessment. S. anti-PLA2R – Soluble anti-PLA2R. CICs - circulating immune complexes. LFTs – Liver function tests. SAEs – Serious Adverse Events. *First five cycles in one bag. Then PV 1 / 1.5 / 2.0 / 2.5. 30ml EDTA on day 1 and 5 treatment must be kept at room temperature and be transported to the lab on the day. Freshly voided urine and 24hour urine can be chilled.

Data collection and storage

Data will be collected using Case Report Forms (see appendix). All data will be stored on a secure database using Microsoft Access, held on hospital computers, and only accessible to the research team with the use of unique usernames and passwords. The information will be fully anonymised with the allocation of unique study numbers for each patient.

Data will be archived for 15 years under the auspices of the Central Manchester University Hospitals NHS Foundation Trust standard archiving policy.

Sample analysis and storage

Routine bloods and urine tests (including Renal profile, Full blood count, CRP, ESR, coagulation screen, LFTs, group & save, autoimmune screen, immunoglobulins, Haemophilus, Tetanus, Diptheria, Pneumococcal, virology screen and uPCR) will be analysed centrally in the CMFT biochemistry, microbiology and immunology labs. Serum Anti-PLA2R, soluble anti-PLA2R, circulating immune complexes and 24 hour urine will be analysed in the MINT labs. All Serum, Urine and DNA will be stored in the MINT Biobank (see appendix for sample processing standard operating procedures (SOP)).

Sample size

Sample size has been determined on pragmatic grounds based on patient level data from our centre.

For n=12, a difference equivalent to 0.9 of the intra-patient standard deviation can be detected (80% power for a paired t-test, 5% sig level). Using the log of the standard deviation for proteinuria, this gives a difference in log(proteinuria) of 0.45, therefore allowing detection of an improvement in log(proteinuria) of >0.41, i.e. a reduction from a (geometric) mean of 5.2g/1.73m^2 to 2.0g/1.73m^2.

This study will involve patients with biochemical nephrotic syndrome with a proteinuria of greater than 3.50g/1.73m^2, we believe that immunoadsorption therapy will lead to remission and therefore lead to a dramatic improvement in the level of proteinuria. Based on numbers of patients who attend our centre and allowing for the power calculation above to detect a large improvement in proteinuria we aim to recruit 12 patients to complete the study.

Data analysis

Demographics of patients will be presented. Simple descriptive statistics and survival analysis will be used to evaluate the primary and secondary outcome measures. Further analysis will involve the development of multivariate risk models using Cox proportional hazard regression to account for all clinically appropriate and statistically significant factors.

Health economic analysis to include calculation of outcomes cost per quality adjusted life year (QALY), and the societal and personal cost of treatment.

Publication and dissemination of results

Irrespective of whether positive or negative, we will publish the results obtained in peer-reviewed journals, present at national and international conferences and publish on the webpages of the MN RADAR Rare Disease group (www.rarerenal.org.uk).

Safety monitoring

The Data Monitoring Committee will meet monthly to evaluate the results and safety.

Adverse Events

Definitions

Adverse Events (AE)	Any untoward medical occurrence in a subject during the study; it does not have to be as a direct result of the Immunoadsorption. It could therefore be any unfavourable and/or unintended sign (including abnormal laboratory findings), symptom, or disease temporally associated with the use of Immunoadsorption. This definition includes intercurrent illnesses or injuries, and exacerbation of pre-existing conditions.
Serious Adverse Event (SAE)	Any untoward medical occurrence that results in: • Death • Is life-threatening • Requires or prolongs hospitalization. • Results in persistent or significant disability or incapacity. • Is a congenital anomaly or birth defect. • Is an important and significant medical event that, based on appropriate medical judgment, may jeopardize the patient and/or may require medical or surgical intervention to prevent one of the other outcomes defining serious. • Malignancies • Infections requiring IV antibiotics
Serious Unexpected Adverse Event (SUSAR)	An SAE that is considered possibly related to the Immunoadsorption and is unexpected.

Reporting of Adverse Events

All AEs, SAEs and SUSARs to be recorded on the adverse event clinical report form and in the Microsoft Access Database.

Reporting of Pregnancy

Any pregnancies that occur in female subjects or partners of male study subjects must be reported within 24 hours of awareness

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Appendix

Reference ranges

Patient Information leaflet

Patient Consent Form

Clinical Report Form (To be included at a later date)

Adverse Event report form (To be included at a later date)

Patient Reported Outcome Measures	EQ5D
	Patients' use of Health and Support Services
Standard Operating Procedures	Processing of Samples
	Nucleon DNA extraction Kit 44100 (SOP)
	Biologic samples for Biobank

Reference Ranges

Test	Range	Units
	Full blood count	
White blood cells	4.0 - 11.0	$x10^9/L$
Red blood cells	4.50 - 6.00	$x10^{12}/L$
Haemoglobin	130 - 180	g/L
Haematocrit	0.400 - 0.520	Ratio
Mean cell volume	80 - 98	fl
Mean cell haemoglobin	27.0 - 33.0	pg
Mean cell haemoglobin conc.	320 - 365	g/L
Platelets	150 - 400	$x10^9/L$
Neutrophils	1.80 - 7.50	$x10^9/L$
Lymphocytes	1.00 - 4.00	$x10^9/L$
Monocytes	0.20 - 1.00	$x10^9/L$
Eosinophils	0.00 - 0.40	$x10^9/L$
Basophils	0.00 - 0.10	$x10^9/L$
	Standard renal profile	
Calcium	2.20 - 2.60	mmol/L
Phosphate	0.7 - 1.4	mmol/L
Alkaline phosphatase	30 - 130	U/L
Albumin	34 - 48	g/L
Sodium	133 - 146	mmol/L
Potassium	3.5 - 5.5	mmol/L
Urea	3.5 - 7.4	mmol/L
Creatinine	59 - 104	umol/L
Estimated GFR		$ml/min/1.73m^2$
Bicarbonate	19 - 28	mmol/L
Adjusted calcium	2.20 - 2.60	mmol/L
Glucose		mmol/L
	Hepatic profile	
Alanine transaminase	5 - 40	U/L
Total protein	60 - 80	g/L
Bilirubin	0 - 22	umol/L
C-reactive protein	0.3 - 5.0	mg/L
ESR	0 - 5	mm/1stHr
	Coagulation Profile	
PT Patient	12.5 - 15.3	Seconds
APTT	24.6 - 34.9	Seconds

Urine protein/creatinine ratio		
Urine p/c ratio	1 - 20	mg/mmol
Immunology Screen (Serum)		
Anti-PLA2R	< 40	u/mL
ANCA	Pos / Neg	
MPO	0 - 0.9	AI
PR3	0 - 0.9	AI
Anti-GBM	0 - 0.9	AI
C4	0.14 - 0.39	g/L
C3	0.62 - 1.6	g/L
IgG	6.0 - 16.0	g/L
IgA	0.8 - 4.0	g/L
IgM	0.5 - 2.0	g/L
Anti-nuclear antibody	Pos / Neg	
Centromere	0 - 0.9	AI
SS-A Antibody	0 - 0.9	AI
SS-A52 Antibody	0 - 0.9	AI
SS-A60 Antibody	0 - 0.9	AI
SS-B Antibody	0 - 0.9	AI
RNP 68	0 - 0.9	AI
Anti Sm	0 - 0.9	AI
SMRNP Antibody	0 - 0.9	AI
Ribosomal P	0 - 0.9	AI
Chromatin	0 - 0.9	AI
Jo-1	0 - 0.9	AI
Scl-70	0 - 0.9	AI
Centromere	0 - 0.9	AI
IgG ds-DNA Antibody	0 - 13.9	iu/mL
Virology Screen		
Hepatitis B surface antigen	Pos / Neg	
Hepatitis C antibody	Pos / Neg	
HIV 1+2 antibody and P24 antigen	Pos / Neg	
Varicella-Zoster IgG	Pos / Neg	
CMV IgG	Pos / Neg	
EBV Serology	Pos / Neg	

All reference ranges taken from Central Manchester University Hospitals laboratory protocols as stated in laboratory handbook

Central Manchester University Hospitals NHS
NHS Foundation Trust

Patient Information Sheet

Peptide GAM Immunoadsorption therapy in Autoimmune Membranous Nephropathy

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. If you do take part you will be given a copy of this information sheet and a signed consent form to keep.

What is the purpose of this study?
There is currently no treatment that targets Membranous Nephropathy directly and many of the treatments available today come with side effects. The part of the immune system that is involved with the development of the disease is now known (anti-PLA2R antibody) and this allows us to develop treatments that target this, with the potential to provide more control of the disease and to reduce the side effects for the patient. The aim of this study is to investigate the benefit of using a therapy called immunoadsorption in Membranous Nephropathy. Immunoadsorption is a therapy that has been around for a long time that cleans the blood of specific parts of the immune system, and in particular the parts that are involved in the development of Membranous Nephropathy. We therefore believe that by using this therapy we will be able to control the disease and avoid the need for immunosuppression.

Why have I been chosen?
We are asking patients who have a diagnosis of Autoimmune Membranous Nephropathy as confirmed on a biopsy and with a high level of Anti-PLA2R in their blood. We are interested in patients who have had treatment already for at least 6 months but still have active disease.

Do I have to take part?
No. It is up to you to decide whether or not to take part. If you decide to take part it will not affect your usual standard of care and you are free to withdraw at any point. If you decide to take part we will ask you to sign a consent form and you will receive a copy of this and the Patient Information Sheet.

What will happen to me if I take part?
We will ask you to come to the Manchester Clinical Research Facility (MCRF) at the Manchester Royal Infirmary (MRI) where we will do routine observations such as blood pressure, temperature, height and weight. We will perform Bio-impedance at the screening visit in order to calculate how much free water is in your body. This is a painless routine test that takes approximately 10 minutes and involves attaching two electrode stickers to your wrist and two to your foot. We will also do a tracing of your heart. We will ask you to donate (gift) blood samples and a urine sample. This will be to send to the laboratory for assessment of your kidney function, liver function, blood count, immune system tests and the ability of your blood to clot. We will also send your urine to the lab to see how much protein is in it. If you are a woman of childbearing age we will perform a pregnancy test. Some of your blood

will be sent to the Manchester Institute of Nephrology & Transplantation labs in the MRI where they will be analysed for tests specific to Membranous Nephropathy. You will also see a Doctor who will do a physical examination and ask you about your medical history and medication list (please bring your medication list with you).

Once all of this is complete the Doctor will need to insert a plastic tube into your groin (usually on the right hand side although sometimes on the left). This is done in the MCRF and the Doctor will use local anaesthetic so that it does not hurt. This is what we will use in order to give you the treatment. Once this has been inserted we will start the treatment. To do this your blood is taken from the plastic tube in your groin and it goes through a machine where it is cleaned before going back into your body through the plastic tube. The whole treatment takes about four hours. You will need to have this every day for five days and so that we can monitor you will need to be an inpatient in the CRF. If for some reason you cannot complete five days in a row we can defer one or two sessions to the week afterwards. You will be closely monitored throughout and will see the doctor regularly throughout this period. We will take blood tests from the tube daily and we will ask you to donate (gift) a urine sample every day. You will also have a daily examination with the Doctor, where he/she will also ask you about any untoward events and medications. We will ask you to fill out some simple questionnaires to help us to know how you feel about the treatment and the cost that you and your family/friends have incurred because of your disease. The treatment can sometimes make the calcium in your blood low, so we will check this throughout the treatment and once it has finished. It is easily treated by giving you extra calcium whilst you are on the Immunoadsorption via the machine.

Once the course of treatment is complete you will be discharged home. We will then ask you to come back to the MCRF for a review over the next year. We will ask you to come back weekly for the first month, this then reduces to twice per month for months 2 & 3 and then monthly until the end of the follow up period. At these clinic appointments we will ask you to donate (gift) blood tests and a urine sample. You will also see the Doctor where he will perform an examination and ask you about any untoward events and review your medications. We will ask you to fill out the same questionnaires as before.

We will study your case notes to learn more about your disease history and any treatments that you have had in the past. We will keep a record of the information from the study and all of this information will be anonymised so that no one can identify you. This data will be used to look for factors that can contribute to the disease. In the future we may like to do further research into kidney disease and we would like to ask your permission to use the samples and data for these studies as long as they are approved by the ethics committee. Taking part in this study will not affect the treatment you receive from your regular medical team. Your medical team will be kept up to date with all results from the study.

What are the possible benefits of taking part?

We believe that this treatment will result in remission and control the disease without the need for immunosuppression or steroids. This is not certain though and that is why we are doing the study, there is a chance that the treatment will not work in which case you will receive the standard treatment from your medical team. We will constantly review participant's response to the Immunoadsorption to ensure that you are not put at risk.

What are the possible disadvantages to taking part?

We do not anticipate that there will be any side effects from taking part in this study however there is a small risk of complications associated with the vascath including small blood clots and infection. You will be closely monitored throughout the study to ensure there is as little chance of this happening as possible.

Will my taking part in this study be kept confidential?
Yes, your identity will remain confidential to your local care team when you consent to enter the study. You will be allocated a study number so that your data and samples are anonymised for use by the research team. When the results of the study are reported and published, your name will not be released and it will not be possible to identify your results or any other individual patient's results. We ask your permission to inform your GP that you are taking part in the study and it may be necessary for the hospital regulatory authorities to review this study to confirm that the research has been conducted properly. In the future it may be important for your medical team to know that you were a part of this study in order to guide treatment and so we will keep a record for 3 years of your details and involvement.

What will happen to the results of this study?
We expect to start to get results in the next year. We will publish all the results in medical journals and present them at national and international conferences. You will not be identified in any of these publications or presentations. We will publicise the research studies amongst local kidney patient groups and on relevant websites. We expect that the results of this study will have a beneficial impact on the way that Doctors manage patients with Membranous Nephropathy. We will also provide a summary of the results to all participants of the study on completion.

What will happen to the samples that I donate for research?
We are asking you to donate (gift) samples of blood and urine for research into kidney disease. The samples will be stored in the Renal Research Labs of the Manchester Royal Infirmary. If you withdraw from the study, any identifiable data or tissue already collected with consent will be retained and used in the study. No further samples or data will be collected.

The Renal Research Labs have obtained ethics committee approval for the Manchester Institute of Nephrology & Transplantation (MINT) Bio Bank, which will store blood, DNA, tissue and urine on patients with kidney disease. We are asking your consent to store any remaining samples left over from the study in the MINT Bio Bank for future studies on kidney disease. In this event your samples will be fully anonymised so that no one can identify the patient that they came from. Research benefits from collaborative projects both nationally and internationally. We are also asking for your consent to use any leftover samples in future national or international studies that MINT obtains ethical approval for. Again your samples will be fully anonymised so that the patient who donated them cannot be identified.

Who is funding the research?
This study is being funded by Fresenius Healthcare. It is sponsored by Central Manchester University Hospitals NHS Foundation Trust.

Who has reviewed this study?
This study has been approved by a central ethics committee. This project will be subject to monitoring by CMFT R&D Department for compliance to all national and local research governance schemes.

Complaints or harm
If you have any concerns and/or complaints about this study and wish to contact someone independent of the research team, please contact your Patient Advisory Liaison Service (PALS) through your local hospital.

Contact Information or if you wish to know more about the study

Dr Patrick Hamilton	0161 276 7987	patrick.hamilton@cmft.nhs.uk
Dr Sandip Mitra	0161 276 6509	sandip.mitra@cmft.nhs.uk
Prof Paul Brenchley	0161 276 6323	paul.brenchley@manchester.ac.uk

Central Manchester University Hospitals NHS
NHS Foundation Trust

CONSENT FORM

Patient Number _____

Title Peptide GAM Immunoadsorption therapy in Autoimmune Membranous Nephropathy

Chief Investigator Dr Sandip Mitra

		Please initial box
1)	I confirm that I have read and understand the information sheet dated 30/01/2017 (Version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2)	I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3)	I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records	☐
4)	I agree to my GP being informed of my participation in the study.	☐
5)	I agree to take part by donating (gifting) blood (DNA and serum) and urine samples for medical research as described in the Patient Information Sheet (version 1.2).	☐
6)	I agree to these samples being stored for use in National/International Research Collaborations on kidney disease now and in the future.	☐
7)	I agree to my anonymised clinical data being used in the research project (your identity will not be known outside of your clinical care team and you will not be identifiable in any research publication).	☐
8)	I agree to take part in the above study.	☐

Name of Participant	Signature of Participant	Date
Name of person taking consent	Signature of person taking consent	Date

EuroQol 5D-5L

By placing a tick in one box in each group below, please indicate which statements best describe your own health state today.

Mobility

I have no problems in walking about ❑

I have some problems in walking about ❑

I am confined to bed ❑

Self-Care

I have no problems with self-care ❑

I have some problems washing or dressing myself ❑

I am unable to wash or dress myself ❑

Usual Activities (*e.g. work, study, housework, family or leisure activities)*

I have no problems with performing my usual activities ❑

I have some problems with performing my usual activities ❑

I am unable to perform my usual activities ❑

Pain/Discomfort

I have no pain or discomfort ❑

I have moderate pain or discomfort ❑

I have extreme pain or discomfort ❑

Anxiety/Depression

I am not anxious or depressed ❑

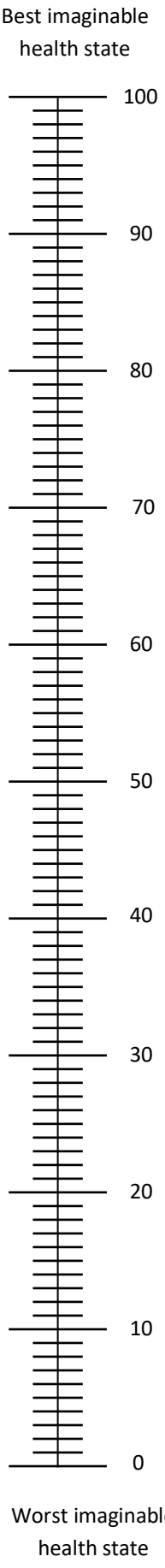
I am moderately anxious or depressed ❑

I am extremely anxious or depressed ❑

To help people say how good or bad a health state is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your health state is today

Your own health state



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Patients use of Health and Support Services

Peptide GAM Immunoadsorption therapy in Autoimmune Membranous Nephropathy

This questionnaire asks you about the health services you have used and anything you have had to buy since your last follow up in the study

This information is completely confidential and will be anonymised before being used in the study. We will ask you about any services that you have used whilst in the trial such as visits to your GP or visits from the District Nurse. It also asks you about travel expenses associated with these and also whether you have had to buy any extra equipment as a result of your disease. Please fill it out ot the best of your ability and if you need any help please get in touch with the study team who will be more than happy to help.

Many thanks

The set of questions below ask about the type and number of health care services you have used in the past X months and about any costs you have incurred due to your illness and treatment.

Question 1 – Have you used any of these services since your last trial follow up and if so how many times?

Type of service	Have you used the service in the **last X months**? Please tick (✓) Yes or No	Total number of face to face contacts in the **last X months**	Total number of contacts by telephone or email in the **last X months**
GP, surgery visit	Yes ☐ No ☐		
GP, home visit	Yes ☐ No ☐		
District nurse or health visitor	Yes ☐ No ☐		
Occupational therapist	Yes ☐ No ☐		
Physiotherapist	Yes ☐ No ☐		
Social worker	Yes ☐ No ☐		
Counsellor	Yes ☐ No ☐		
Home help or care worker	Yes ☐ No ☐		
Psychiatrist or psychologist	Yes ☐ No ☐		
Other - for example: day centre, food delivery service, lunch club Please specify below:	Yes ☐ No ☐		

Question 2 – Have you used any of these hospital based or residential care services since your last trial follow up and if so how many times?

(please do not complete the shaded squares)

Type of service	Which services have you used **in the last X months**? Please tick (✓) Yes or No		Total number of days spent in hospital/ nursing home **in the last X months**	Total number of visits **in the last X months**
Hospital inpatient stay	Yes ☐	No ☐		
Hospital day centre	Yes ☐	No ☐		
Hospital outpatient clinic	Yes ☐	No ☐		
Hospital accident and emergency department	Yes ☐	No ☐		
Nursing/ residential home	Yes ☐	No ☐		
Hospice	Yes ☐	No ☐		

Travel costs and additional expenses

We need to find out what **you** have spent on travel and other items or expenses you incurred **as a result of your illness and treatment.**

Question 3 – Since the last time you filled out this questionnaire, how much do you think you have spent on travel as a result of your illness and its treatment? (for example, this might include travel costs to visit your GP, hospital, therapist or day centre)

If you have not spent anything on travel please tick the box here: ❑

Please estimate your spending on travel **in the last X months** (e.g. fares for public transport, taxis and car park fees) £'s	If you have used your **own car**, approximate number of miles travelled **in the last X months**
£ ☐☐☐	☐☐☐ Miles

Question 4 – Please describe any large (£50 or more) expenses that you personally have had to meet because of your illness and treatment since the last time you filled out this questionnaire:

Description of Item	Cost to you £'s
i.	£ ☐☐☐☐
ii.	£ ☐☐☐☐
iii.	£ ☐☐☐☐

Question 5 – Employment and support

Description of Item		
If you were in employment before you started treatment, how much time have you taken off work due to your health and getting treatment?	.....hour(s)	
Have you received help or support from family or friends? *(please tick one)*	Yes ☐	No ☐
If 'Yes', how long in total did they spend helping you?	.....hour(s)	
If they took any time off work to provide you with help or support, how long was this in total over **the last X months**?	.....hour(s)	

Processing of samples

Enter patient name, date of birth, date of collection and hospital site in the log book and assign unique study identifier.

Label all samples with unique study identifier

Sample labelling format

Centre ID 30092015	e.g. MRI for Manchester Royal Infirmary	MRI- 001
Patient No	e.g. 001	
Date	DDMMYYYY	

Blood

The blood tubes will be spun at 2000rpm for 10 minutes (see operating instructions for Jouan centrifuge).

Plasma will be aspirated from the cell pellet and aliquoted as outlined above and frozen at -70C.

The cell pellet with be retained at -20^{O}C for DNA extraction.

Urine

Transfer the urine to a 15ml centrifuge tube. Spin the urine tube @ 2000 rpm for 10mins at room temperature.

Divide into aliquots 6 X 200µl + the remaining into 1.5ml tubes Mark tube caps with RED permanent marker.

Label and store at 80^{O}C

DNA Extraction

DNA extraction using nucleon kit no 44100 SOP

Nucleon DNA extraction Kit 44100

Use for whole blood or cell pellet from approximately 10ml blood. For cell pellet make up to 10ml with PBS.

1. Add up to 6ml sample to a 15ml polypropylene centrifuge tube and add 8ml of reagent A and mix quickly.
2. Rotate for 5 minutes at room temperature.
3. Centrifuge at 2500 rpm (2200g) in the Jouan for 5 minutes, pour off supernatant into an autoclave bag containing 25g Virkon, supported by a beaker. Wipe the external thread and inside the cap of the centrifuge tube if any liquid is visible and vortex.
4. Add a further 4ml reagent A to the cell pellet. Vortex or mix for up to 1 minute to disperse the pellet, then repeat spin at 2500 rpm for 5 minutes and again pour off the supernatant, then wipe the centrifuge tube and cap.
5. Vortex the cell pellet to resuspend and break up. Add 500µl reagent B and incubate the sample at 370C for 10 minutes in a water bath until completely dissolved. Vortex if necessary.
6. Add 175µl of reagent C and mix by inverting at least 7 times.
7. Add 150µl Nucleon resin drop-wise to the top of the sample with quick further mixing and spin at 4100 rpm for 4 minutes.
8. Transfer the supernatant to a clean 15ml centrifuge tube, measure the volume using a 1ml Finpipette and add an equal volume of propan-2-ol (iso-Propanol).
9. Mix gently by inversion to precipitate the DNA pellet
10. Centrifuge at 4300 rpm (3800g) for 5 minutes, pour off the supernatant and retain the DNA pellet.
11. Add 2 ml 70% Ethanol, pipette with a Pastette to dislodge the pellet from the bottom of the tube, cap and wash by inverting several times.
12. Pipette off the excess Ethanol using a fine tipped pastette and stand for several minutes for any remaining Ethanol to drain to the bottom of the tube, repeat the removal of excess Ethanol and allow the pellet to air dry for 10 minutes, then add 400µl DEPC distilled water or water for injection and shake to suspend the pellet. Leave at room temperature overnight to dissolve the pellet.

12 Shake to ensure the pellet has dissolved then gently pipette the solution and transfer to a screw top microtube for storage.

Biologic samples for BioBank

DAY 1

AM (pre treatment)

30mls EDTA – to go to Shelley/Patrick – 5 ml EDTA to be sent to Genetics for B-Cell line

5ml EDTA to go to Renal lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY PRE D1

P (X= patient ID) cell pellet to be saved for DNA extraction

20ml for PBMC prep to be labelled PRISM 0X DDMMYYY (save the plasma from the separation)

PBMC for FACS analysis

Freshly voided urine – 15 ml to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured PRISM 0X DDMMYYY U PRE D1

PM (post treatment)

Eluate – Neutralised eluate

The first 5 bags of eluate will be brought to the Renal lab each to be weighed

Transfer 150mls from each bag into 3X 50ml tubes and spin. Once spun to be aliquoted into 6X 250µl up to 7 1.5ml and the remainder to be put into fresh 50ml tubes and stored labelled (tubes lids will be left clear)

to be labelled PRISM 0X DDMMYYY E D1 PV0.5, PV1, PV1.5, PV2 AND PV2.5 respectively. Whatever is left should be frozen in the bag.

10ml EDTA to go to renal lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY P POST D1

Cell pellet to be saved for DNA extraction

DAY 2

AM (pre treatment)

We will receive 10mls to go to lab for processing – 2mls to be placed in a 5ml EDTA tube and frozen for tissue typing the rest to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY PRE D2

Cell pellet to be saved for DNA extraction

Freshly voided urine – 15 ml to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured PRISM 0X DDMMYYY U PRE D2

24 hour urine collection container to be weighed, note weight in the log book. Transfer into 4X 50ml tubes and spin. Once spun to be aliquoted into 6X 250µl up to 7 1.5ml lids to be coloured red to be labelled and the remainder to be put into fresh 50ml tubes and stored labelled PRISM 0X DDMMYYY 24U D2

The 24hour urine container to be emptied into the sluice and either weigh the empty container or an empty urine container note weight in the log book.

PM (post treatment)

10ml EDTA to go to lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY P POST D2

DAY 3-4

AM (pre treatment)

We will receive 10mls to go to lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY PRE D3 and D4

Freshly voided urine – 15 ml to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured PRISM 0X DDMMYYY U D3 and D4

24 hour urine collection container to be weighed, note weight in the log book. Transfer into 4X 50ml tubes and spin. Once spun to be aliquoted into 6X 250µl up to 7 1.5ml lids to be coloured red to be labelled and the remainder to be put into fresh 50ml tubes and stored labelled PRISM 0X DDMMYYY 24U D2

The 24hour urine container to be emptied into the sluice and either weigh the empty container or an empty urine container note weight in the log book.

labelled PRISM 0X DDMMYYY 24U D3 and D4

PM (post treatment)

10ml EDTA to go to lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY P POST D3 and D4

DAY 5

AM (pre treatment)

We will receive 30mls EDTA – 20 to go to Shelley/Patrick for PBMC prep to be labelled PRISM 0X DDMMYYY PRE D5

10ml EDTA to go to lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY PRE D5 P (X= patient ID)

PBMC for FACS analysis

Freshly voided urine – 15 ml to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured PRISM 0X DDMMYYY U PRE D5

PM (post treatment)

10ml EDTA to go to lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY P POST D5

WEEK 2 ONWARDS

We will receive 30mls EDTA – 20ml to go to Shelley/Patrick – 10ml EDTA to go to Renal lab for processing – to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured blue to be labelled

PRISM 0X DDMMYYY PRE W2, W3……W52

Freshly voided urine – 15 ml to be spun down 6X 250µl up to 7 1.5ml aliquots lids coloured red PRISM 0X DDMMYYY U W2, W3……W52

24 hour urine collection - container to be weighed, note weight in the log book. Transfer into 4X 50ml tubes and spin. Once spun to be aliquoted into 6X 250µl up to 7 1.5ml lids to be coloured red to be labelled and the remainder to be put into fresh 50ml tubes and stored

labelled PRISM 0X DDMMYYY W2, W3……W52

The 24hour urine container to be emptied into the sluice and either weigh the empty container or an empty urine container note weight in the log book.