Personalised Medicine in ANCA-Associated Vasculitis: The Role of Urine Soluble CD163

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Declaration

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Summary

In this thesis I have identified a highly sensitive and specific association between urinary sCD163 and active renal vasculitis in the clinical setting of renal vasculitis flare in both retrospective and prospective studies. To define clinical caveats in usCD163 interpretation, I have explored the influence of high-grade proteinuria on usCD163 interpretation. There is a strong biologic rationale for usCD163 in the monitoring of renal vasculitis activity as it is a marker of macrophages and is strongly expressed in glomerular crescents. In crescentic glomerulonephritis there is direct shedding of soluble CD163 protein from the glomerular crescent cell surface directly into the urinary space leading to elevated levels. usCD163 is easily measured by commercial ELISA with this thesis validating a diagnostic grade assay.

usCD163 is elevated in subtle renal vasculitis flare and is superior to uMCP-1: In collaboration with the Vasculitis Clinical Research Consortium usCD163 and uMCP-1 were measured in a serially sampled longitudinal multicentre cohort with clinically mild renal vasculitis. Both biomarkers were elevated in the presence of active renal vasculitis, with usCD163 displaying superior area under the curve than uMCP1, 0.794 and 0.687, respectively. usCD163 and uMCP1 correlated poorly with r² of 0.11, highlighted their differing roles in glomerular macrophage recruitment and activation. In subtle active renal vasculitis, the moderate clinical utility of each biomarker in isolation was enhanced by using usCD163 to exclude active vasculitis, and then grouping the “usCD163+/uMCP1+” and “usCD163/new proteinuria” as the two “Yes” nodes, giving a positive LR of 19. This decision tree approach increased diagnostic precision and incorporated proteinuria.

usCD163 is diagnostic of renal vasculitis flare: Prospective enrolment of patients with known AAV presenting with potential renal vasculitis flare was performed in a multicentre cohort. usCD163 was measured by diagnostic and research grade ELISAs. usCD163 was elevated in renal vasculitis flare with concentrations remaining low in renal vasculitis flare mimics such as sepsis, isolated hematuria and non-vasculitic acute kidney injury. usCD163 displayed exceptional biomarker characteristics in this setting with AUC of 0.95 with superiority to RBC casts, BVAS criteria and changes in serum creatinine. usCD163 displayed similar biomarker characteristics to the current “gold standard” of kidney biopsy. The use of a diagnostic grade sCD163 assay has enhanced the potential clinical translation of usCD163 as a diagnostic test for active renal vasculitis.
usCD163 is elevated in high-grade proteinuria but correction for urinary protein attenuates usCD163 concentrations in those without active renal vasculitis: Loss of integrity of the glomerular filtration barrier may lead to detection of serum sCD163 in urine. To address this diagnostically relevant potential caveat in usCD163 interpretation we studied usCD163 in (1) primary nephrotic syndrome and (2) renal AAV with and without proteinuria. In primary nephrotic syndrome usCD163 concentrations were hypothesised to be undetectable as there is no local source of sCD163 production, however there is extensive foot process effacement with potential for passage of serum sCD163 across the glomerular filtration barrier leading to detection in urine. In primary nephrotic syndrome with proteinuria >3.5g/day, usCD163 concentrations were elevated. This signal was subsequently attenuated when sCD163 concentrations were corrected for urine protein and albumin concentrations. In renal AAV usCD163 concentration was increased in remission AAV with persistent proteinuria compared to remission AAV without proteinuria, but concentrations remained significantly less than those with active renal vasculitis. The sCD163 ratio of serum to urine protein and albumin values was calculated to provide an estimate of local glomerular sCD163 production. Normalising usCD163 to total urine protein, albumin and sCD163 ratio of serum to urine protein and albumin values yielded similar results, with no significant difference between remission proteinuric and remission non-proteinuric subjects, but concentrations remained elevated in active renal vasculitis. The simple normalisation of usCD163 to total urine protein performing marginally better than the more complex fractional excretion of protein: sCD163 ratio with AUC values of 0.93 and 0.91, respectively. Therefore, in patients with nephrotic range proteinuria, normalisation of the usCD163 value to total urine protein is the method of greatest clinical utility for non-invasive identification of active renal vasculitis.

Validation of a Diagnostic Grade Test: A key step in the translation of usCD163 from research to diagnostic grade assay was collaboration with industry to develop a diagnostic usCD163 assay. We rigorously validated both commercially available research grade and in-house research grade assays and determined that the R&D Systems Duoset sCD163 assay had the most optimal operating characteristics for detection of active renal vasculitis. We then partnered with industry to develop a diagnostic grade usCD163 ELISA. The optimal research grade usCD163 assay was used to validate the diagnostic grade assay. This assay has received the CE marking, ISO 15189 and NEQAS accreditation allowing widespread adoption by clinical laboratories and is now commercially available.
Acknowledgements

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Publications, Presentations and Awards Arising from Thesis

Publications


Anticipated publications:


Moran SM, Goh T, Conlon N, Dunne J, Groarke E, Holian J, McLoughlin K, Molloy E, Wyse J, Little MA. usCD163 is an early predictor of treatment response in crescentic glomerulonephritis. Provisionally accepted by Frontiers in Immunology

Moran SM, Kretzler M, Little MA. The effect of Proteinuria on usCD163 interpretation.

Moran SM, O’Reilly V, O’Brien E, Preston RJ, Little MA. Soluble coagulation receptor levels in ANCA associated vasculitis.


Presentations


urine sCD163 is a biomarker of active Nephrotic Syndrome. Moran SM, Dooley D, Kretzler M, Little MA. American Society of Nephrology AGM New Orleans 2017

A Steroid free maintenance regime is an effective and sage treatment strategy in ANCA associated vasculitis and glomerulonephritis. Pepper RJ, Moran SM, Kelly D, Gopaluni P, Jayne D, Hamour S, Burns A, Little MA, Salama AD. 18th International ANCA and Vasculitis Workshop 2017, Tokyo, March 2017

De novo hypocomplementemic ANCA negative medium vessel vasculitis in a case of previously treated p ANCA positive microscopic polyangiitis. Murray S, Moran SM, Meyer N, Clarkson MR. 18th International ANCA and Vasculitis Workshop 2017, Tokyo, March 2017


Awards and Grants

ANCA Workshop Travel Grant, 2019

Ainsworth Scholarship, University College Cork, 2018

Postgraduate Travelling Scholarship in Medicine & Surgery, Trinity College Dublin, 2018

Sheppard Memorial Prize, Trinity College Dublin, 2018

Sir John Banks Medal in Medicine, Trinity College Dublin, 2018

American Society of Nephrology ASN Karen L. Campbell Travel Support Program for Fellows, 2017

Louise A. Fast Foundation Scholarship, University of Toronto, 2017

Vasculitis UK Travel Grant, 2017

Vasculitis Ireland Research Award, 2017

Brian Keogh Feschrift Award in Nephrology, Meath Foundation, 2016

National Specialist Registrar Academic Fellowship, Health Research Board Ireland, 2014
Chapter 1: Introduction
1.1. Introduction

ANCA associated vasculitis (AAV) is a group of autoimmune conditions characterised by pauci-immune small vessel vasculitis [1]. Three primary subtypes are recognised: microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA). Anti-neutrophil cytoplasmic antibody (ANCA) was discovered in the 1980s [2, 3]. AAV is associated with two putative auto-antibodies in 85-90% of cases: anti-myeloperoxidase antibody and anti-proteinase 3 antibody. GPA, MPA and EGPA have respective annual incidence rates of 2.1–14.4, 2.4–10.1 and 0.5–3.7 per million in Europe. AAV prevalence is estimated to be 46–184 per million[4-10].

Immunosuppressive therapy has revolutionised the care of patients with AAV. Glucocorticoids were introduced in 1948, cyclophosphamide in the 1960s and rituximab in the 2000s. These interventions have increased survival rates from 17% in untreated patients at two years, to 60-97% at five years [10-12]. In the modern era early mortality is primarily infection and treatment related, followed by vasculitis related. After one year the primary cause of mortality is cardiovascular disease [13].

AAV is sub-classified according to the extent and severity of organ involvement. Localised disease is classified as one site affected, for example the upper respiratory tract. Early systemic disease is classified as any disease without imminent vital organ failure. Generalised disease is classified as renal or other organ threatening. Severe disease is classified as renal or other vital organ failure, for example serum creatinine >500umol/l. Refractory disease is classified as progressive disease unresponsive to steroids and cyclophosphamide[14]. Clinical presentation is highly variable ranging from mild sinonasal disease to life threatening pulmonary hemorrhage and renal failure. When remission is attained it can last indefinitely or be punctuated by clinical flares. 10-30% of patients are refractory to initial treatment and up to 50% experience clinical relapse within five years[15]. AAV has a peak age incidence of 65-75 years with slight male predominance[16].

Glomerulonephritis (GN) related to AAV is the commonest form of new onset glomerulonephritis in adults greater than 50 years of age. Glomerulonephritis is the second commonest cause of end-stage kidney disease (ESKD) in the United Kingdom and third commonest in the USA[17]. ESKD is associated with significant morbidity, mortality and high annual healthcare costs. In Ireland the estimated annual cost of dialysis of €62,400 per year[18].
1.2. Classification

The 2012 Chapel Hill Consensus Conference Nomenclature of Vasculitis (CCHC) 2012 defines AAV as necrotizing vasculitis, with few or no immune deposits, predominantly affecting small vessels (for example capillaries, venules, arterioles and small arteries), associated with myeloperoxidase (MPO) ANCA or proteinase 3 (PR3) ANCA. However, a subset of patients with AAV do not have detectable ANCA using conventional serological assays. This may reflect an unknown autoantibody or epitope masking[19]. In clinical case description adding a prefix indicating ANCA reactivity is recommended. For example, MPO-ANCA, PR3-ANCA, ANCA-negative.[1].

1.2.1. Microscopic Polyangiitis

Microscopic polyangiitis (MPA) is characterised by necrotizing vasculitis, with few or no immune deposits and predominantly affects small vessels, for example capillaries, venules, or arterioles. However, necrotizing arteritis involving small and medium arteries may be present. Necrotizing glomerulonephritis is very common and pulmonary capillaritis often occurs. Granulomatous inflammation is characteristically absent.

1.2.2. Granulomatosis with polyangiitis

Granulomatosis with polyangiitis (GPA) is characterised by necrotizing granulomatous inflammation. This usually involves the upper and lower respiratory tract. Necrotizing vasculitis predominantly affects small-to-medium vessels, for example capillaries, venules, arterioles, arteries, and veins. Necrotizing glomerulonephritis commonly occurs.

1.2.3. Eosinophilic granulomatosis with polyangiitis

Eosinophilic granulomatosis with polyangiitis (EGPA) is characterised by eosinophil-rich and necrotizing granulomatous inflammation. Respiratory tract involvement with asthma and eosinophilia are common. Necrotizing vasculitis when present predominantly affects small-to-medium vessels. In contrast to MPA and GPA, only 30-40% of cases are ANCA positive[20]. ANCA positivity is more frequent when glomerular disease is present.
1.3. Autoantibodies

1.3.1. Anti-neutrophil cytoplasmic antibody

AAV is associated with ANCA specific for myeloperoxidase (MPO-ANCA), proteinase 3 (PR3 ANCA) or alternative autoantibodies. Using a combination of immunofluorescence and ELISA testing less than 10% of all patients are ANCA negative. Epitope masking is one of the potential aetiologies of ANCA negative AAV[19]. ANCA monitoring may be used for relapse risk prediction and prognostication. Those who no longer have detectable anti-neutrophil cytoplasmic antibodies have a reduced risk of relapse compared to those with persistently positive ANCA. The utility and prediction of relapse with ANCA titre changes has been extensively investigated. In those with renal involvement a rising ANCA titre is associated with an up to 11 times increased likelihood of relapse. [21-23]. Despite this well described association, persistent ANCA positivity or rising ANCA titres in clinical isolation are insufficient to alter clinical management[24, 25].

1.3.2. Other autoantibodies

Additional auto-antibodies have been described in AAV, including lysosome-associated membrane protein-2 (LAMP-2), moesin, plasminogen and pentraxin-3. LAMP-2 autoantibodies have been identified in 80-90% of those presenting with AAV, become undetectable with immunosuppressive therapy and recur in clinical relapse[26]. Potential caveats of LAMP2 monitoring include detection rates in control groups of up to 16% and lower frequency of autoantibody detection in replication studies [27]. Anti-moesin antibodies have been associated with renal damage but their presence has not been validated in non-Japanese populations nor have they been correlated with relapse[28]. Plasminogen antibodies are present in 18% with active disease and 2% of those in remission[29, 30]. Pentraxin-3 antibodies have been reported in up to 50% of ANCA negative AAV[31].
1.4. Renal AAV

Pauci-immune necrotizing and crescentic glomerulonephritis (GN) is the characteristic pattern of AAV-associated glomerulonephritis. The classical histopathological lesion is of localised vessel wall fibrinoid necrosis. In contrast to crescentic glomerulonephritis of other aetiologies there is a paucity of immunoglobulin and complement deposition within the glomeruli[32]. On electron microscopy neutrophil degranulation, micro thrombosis and subendothelial edema are seen but electron dense deposits are absent. [33]

Clinical presentation with glomerulonephritis can range from asymptomatic microscopic haematuria with preserved glomerular filtration rate to dialysis requiring acute kidney injury. In those who present with acute kidney injury requiring dialysis, up to 50% will regain independent renal function[34]. Renal transplantation is an option for those who develop end-stage kidney disease with sustained clinical remission for at least 12 month period. [35] In those who present with AAV initially involving other organ systems, over time greater than 70% of all patients will develop renal involvement with glomerulonephritis [29, 30].

A classification system was devised by Drs. Berden and Bajema based on the extent of glomerular crescents and glomerulosclerosis on light microscopy[33]. Within this classification the following subclasses were described: focal class has ≥50% normal glomeruli and has the most favorable prognosis, sclerotic class has ≥50% globally sclerosed glomeruli and has the least favorable prognosis, crescentic class has ≥50% glomeruli with crescents, and has an intermediate prognosis and mixed class has a heterogenous glomerular phenotype with no predominant pathological pattern and carries an intermediate prognosis.
1.5. Clinical Course

1.5.1. Relapsing disease

AAV is a chronic disease characterised by periods of relapse and remission. Up to 30% of patients experience a relapse within the first three years after induction of remission[36, 37]. Clinical and laboratory features that have been associated with increased relapse risk include PR3-ANCA positivity, ANCA titre, upper respiratory tract involvement, nasal carriage of staphylococcus aureus, absence of renal disease, sclerotic Berden biopsy classification and withdrawal of glucocorticoid therapy [21, 23, 38, 39]. There are no consensus criteria for the diagnosis of renal relapse. In clinical practice a combination of serum creatinine, urine sediment analysis for haematuria, proteinuria and red blood cell casts are used. These biomarkers are limited by their lack of sensitivity for the detection of active renal inflammation. Renal biopsy is the gold standard for diagnosis of crescentic glomerulonephritis. Biopsy interpretation is however limited by the focal nature of histological findings, with potential for false negative results in the absence of crescentic glomerular lesions. Clinical trials use the Birmingham vasculitis activity score (BVAS/WG) with disease relapse defined by one major or three minor disease activity items[40, 41]. Major BVAS/WG renal items include red blood cell casts and rise in serum creatinine greater than 30%[40].

1.5.2. Refractory Disease

A subset of patients with AAV are refractory to conventional immunosuppressive induction regimens. Refractory AAV definitions include both disease progression and inadequate disease control despite adequate induction therapy. Refractory AAV is a retrospective diagnosis with potential for significant organ damage accruing prior to its identification. Prior to confirming a diagnosis of refractory disease, the exclusion of vasculitis mimics such as infection or malignancy is recommended. Refractory disease is more common in those who are PR3-ANCA positive. [42]

The European League Against Rheumatism (EULAR) defines refractory disease as[43]:

- Unchanged or increased disease activity in acute AAV after four weeks of treatment with standard therapy in acute AAV

OR
- Lack of response (defined as <50% reduction in the disease activity score (Birmingham Vasculitis Activity Score (BVAS) or BVAS/Wegener’s granulomatosis (WG)), after 6 weeks of treatment

OR

- Chronic, persistent disease defined as presence of at least one major or three minor items on the BVAS disease activity score after >12 weeks of treatment

1.6. Treatment

The goal of AAV treatment is to achieve clinical remission and prevent potentially fatal end organ damage. Induction and maintenance treatment regimen recommendations in AAV are informed by randomised controlled trials[44-49]. There is, however, a clinical need to identify those in whom more and less intensive immunosuppressive treatment is required. This treatment is underpinned by the observation that the primary cause of death in the first year is infection followed by active vasculitis.
Figure 1. Algorithm to describe the management of newly diagnosed ANCA-associated vasculitis. Dashed lines indicate alternative or supplementary action to consider. From EULAR/ERA-EDTA recommendations for the management of ANCA-associated vasculitis[50].

1.6.1. Cyclophosphamide

Cyclophosphamide remains first line therapy alongside rituximab for remission induction of new onset organ or life threatening AAV in conjunction with corticosteroids[51]. Initial treatment regimens in the 1970s included cyclophosphamide at a dose of 2mg/kg/day[52, 53]. Due to clinical concern regarding toxicity from high dose oral regimens, a pulsed intravenous regimen was therefore devised and assessed in the CYCLOPS study[49]. CYCLOPS compared cyclophosphamide either intravenously or orally until clinical remission was attained and then continued for a further three months when all were transitioned to maintenance therapy with azathioprine. The median time taken to achieve remission was 3 months (IQR, 0.5-8 months) for both groups. There was no difference in primary end points of time to remission or proportion of patients achieving remission. Those who received intravenous cyclophosphamide received a significantly lower cumulative dose. Long term follow up of the CYCLOPS cohort revealed no differences in survival, renal function or adverse events between the two arms, although the proportion of patients with at least one relapse was higher in those individuals treated with pulsed cyclophosphamide, there were no differences in survival, renal function at the end of the study or adverse events between the two arms[54]. Adverse events associated with cyclophosphamide include malignancy, bone marrow suppression, infection, malignancy, reduced ovarian reserve, ovarian failure leading to premature menopause and male infertility[42, 54-61].

1.6.2. Rituximab

Rituximab is an alternate first line therapy for remission induction for new onset organ or life threatening AAV in conjunction with corticosteroids[50]. Its efficacy has been assessed in two randomised controlled trials in combination with glucocorticoids: RAVE ((Rituximab for the Treatment of Wegener's Granulomatosis and Microscopic Polyangiitis) and RITUXVAS (an international, randomised, open label trial comparing a rituximab-based regimen with a standard cyclophosphamide/azathioprine regimen in the treatment of active, ‘generalised’ ANCA associated vasculitis) [46, 48]. In these studies, rituximab was dosed at 375 mg/m2 of body surface area, once a week for four infusions. In both trials, rituximab was non-inferior to cyclophosphamide with a signal of increased efficacy for relapsing disease in RAVE. Rituximab is
effective for maintenance of remission with lower relapse rates compared to azathioprine[62, 63]. Adverse events associated with rituximab include infusion reactions, late onset neutropenia, infection, malignancy, progressive multifocal leukoencephalopathy[64].

1.6.3. Glucocorticoids

Corticosteroids form a critical component of induction therapy and have been a cornerstone of therapeutic regimens since their development[12]. Evidence regarding optimal dosing is continuously evolving. Treatment is generally commenced at 1mg/kg body weight to a maximum of 80mg daily[50]. Reduced-dose corticosteroids are non-inferior to traditional dosing in severe AAV with respect to death or ESKD, with less serious infections at one year (incidence rate ratio 0.69) [65]. EULAR/ERA-EDTA guidelines recommend a dose of between 7.5-10mg after 12 weeks of treatment but in practice this is often only achieved by five months. Steroid sparing and avoidance strategies are considered in populations at high risk of steroid related complications[66]. Given the associated complications of therapy including but not limited to infection, type II diabetes mellitus, weight gain, gastric ulceration, osteopenia, cataract and glaucoma there has been a concerted effort to safely reduce cumulative dosing[67].

1.6.4. Plasma Exchange

Plasma exchange (PLEX) rapidly depletes circulating ANCA and has been used in the management of severe AAV to reduce end organ damage. The MEPEX trial compared 3g of IV methylprednisolone to 7 sessions of PLEX in addition to oral cyclophosphamide and prednisolone in 140 patients[45]. The PLEX arm had reduced progression to ESKD by 24% but there was no difference in death or other outcomes at one year. Subsequent meta-analysis with 378 patients from 9 randomised controlled trials (RCTs) expanded on these results, showing a reduction in ESKD with RR of 0.64, but not mortality with RR 1.01[68].

PEXIVAS was a randomized trial of 704 patients designed to evaluate the use of plasma exchange and two regimens of oral glucocorticoids in patients with severe AAV, defined as estimated glomerular filtration rate of <50 mls/min or diffuse pulmonary hemorrhage. Patients were randomly assigned to undergo seven plasma exchanges within 14 days after randomization or no plasma exchange. The use of plasma exchange did not reduce the incidence of death or ESKD with rates of 28.4% in the plasma-exchange group and in 31.0% in the control group, respectively[65].
1.6.5. Additional Treatment Options

Methotrexate and mycophenolate mofetil are used for non-organ threatening vasculitis[69, 70]. The role of the alternative complement pathway in AAV has been demonstrated in animal models and ex vivo experiments[71-73]. Avacopan, an oral C5a receptor inhibitor has demonstrated in phase II clinical trials efficacy, safety and steroid sparing potential. Phase III trials are underway [74].

1.6.6. Maintenance Treatment

General strategies include combination of low dose glucocorticoids plus one of the following: azathioprine, rituximab, methotrexate or mycophenolate mofetil. Duration of maintenance therapy remains controversial with general minimal duration of treatment of 24 months but recent evidence of reduced relapse rates with azathioprine continued to 4 years[75]. TAPIR (The Assessment of Prednisone in Remission Trial) is an ongoing clinical trial investigating the optimal duration of corticosteroid therapy in disease remission (ClinicalTrials.gov Identifier: NCT01933724).


Adverse events related to AAV therapy are highly prevalent and are the leading cause of mortality in the first year following diagnosis. One-year mortality is 11.1% with 59% of deaths related to immunosuppressive therapy associated adverse events and 14% of deaths caused by active vasculitis. Primary early drivers of mortality are infection and leucopenia. Little et al devised a cumulative adverse event scoring system and identified that those with a cumulative burden greater than 7 have a probability of death of 53.1% by 12 months compared to a 5% probability of death in those with those with a score of less than 7[13]. Additional risk factors for death include reduced GFR and advanced age.

Overall, 53% of patients develop infections requiring antimicrobial therapy and 28% are hospitalized for treatment of these infectious complications[59]. The commonest sites of infections are pulmonary and upper respiratory tract. Staphylococcus aureus is the most commonly identified pathogen at 41% of positive cultures[76].
Cancer incidence is historically 1.6–2.4 times higher than the general population with increases in bladder cancer and leukemia. Notably these estimates are based on immunosuppressive strategies from the 1970s and 1980s with prolonged high dose courses of cyclophosphamide. Current estimates of cancer incidence are 1.58 for cancers at all sites, 1.30 for cancers at all sites excluding non-melanoma skin cancer[60].

1.6.8. Duration of treatment

Treatment of AAV usually comprises intense immunosuppression over approximately 3 -6 months with cyclophosphamide or infusion over 2-4 weeks with rituximab with subsequent corticosteroid de-escalation. Clinical tools to guide the duration and intensity of therapy include urine dipstick, serum creatinine level and ANCA titres. Recent work by our group has shown that after initiation of induction therapy nadir urine protein and creatinine levels are reached after a median of 322 and 88 days respectively[77]. Persistent hematuria is present in 40% at time of switch from induction to maintenance immunosuppression [14]. These findings suggest that at the point of de-escalation of immunosuppression, kidney function is generally still improving by conventional measures. These measures are therefore inadequate to guide to timing of switch from induction to maintenance therapy.

1.7. Tools for Detection of Active Renal Vasculitis

1.7.1. Current non-invasive clinical tools

Current clinical tools for detection of active renal inflammation include serum creatinine, hematuria, proteinuria and red blood cell (RBC) casts. See figure 1.2. However, these biomarkers lack sensitivity for detection of early renal structural and functional loss and do not differentiate active vasculitis from flare mimics such as sepsis, urinary tract infections, and adverse drug reactions.

1.7.1.1. Urinalysis

Persistent hematuria post-induction therapy is present in up to 42% of patients at six months post-induction therapy and can persist for years despite apparent clinical remission[77, 78]. Persistent hematuria post-induction therapy portends an increased risk of renal relapse but in isolation remains non-specific. Persistent proteinuria has not been identified as a predictive of relapsing
disease[79]. Red blood cell cast assessment is a non-invasive method of assessing red blood cell morphology but in clinical practice is fraught with challenges limiting its application including inter-operator variability, time sensitivity and lack of availability outside centres of expertise. [80].

Figure 1.2. Time to nadir creatinine / PCR after treatment for ANCA vasculitis from Oomatia et al. [77]

1.7.1.2. Serum biomarkers

Serum creatinine changes do not distinguish active renal vasculitis from other causes of renal dysfunction. Due to compensatory mechanisms such as hyperfiltration and variability in muscle mass, a change in serum creatinine may only occur once substantial irreversible scarring has occurred. In one large series, 14% of patients with normal renal function had 25-49% fibrosis and 5% had more than 50% fibrosis on biopsy[81]. Inflammatory markers such as C-reactive protein are limited by lack of specificity, with elevation in potential vasculitis mimics including infection[82].

1.7.1.3. ANCA

Severe disease flares are unlikely in the absence of detectable ANCA but remain possible[23, 44, 83]. ANCA measurement has limited value in identifying relapse as rising titers have been reported in up to 40% of patients without new or worsening disease activity[24]. Post-induction therapy
many patients remain ANCA positive despite clinical remission. The significance of a rising ANCA titre or the degree of rise is controversial with meta-analyses concluded that a rising ANCA titre in AAV is only modestly predictive of future disease relapse regarding the significance of a rising ANCA titre. Retrospective studies have shown that a rising ANCA titre is more predictive of relapse in those with renal AAV than in those with extra-renal AAV with a hazard ratio of 11.09[23]. It is notable, however, that less than 50% of these patients experienced a relapse within 12 months making decisions regarding therapeutic modifications increasingly challenging [24, 84].

1.7.1.4. Risk stratification

Risk stratification is required to determine optimal disease monitoring, choice and duration of immunosuppression in the management of AAV. Clinical features associated with increased relapse risk include anti-PR3 positivity, GPA, serum creatinine less than 100umol/L, cardiac involvement and rising ANCA titre with renal MPA. Prior treatment exposures that are associated with increased relapse risk include: iv compared to oral cyclophosphamide induction, mycophenolate mofetil induction and azathioprine compared to rituximab maintenance therapy[37, 85].

1.7.1.5. Patient global assessment tool

Increase in the patient global assessment tool has been shown to precede the detection of active vasculitis by at least three months[86].

1.7.2. Kidney Biopsy

EULAR guidelines suggest that “a positive biopsy is strongly supportive of a diagnosis of vasculitis and they recommend biopsies to assist in establishing a new diagnosis and for further evaluation for patients suspected of relapsing vasculitis” (level of evidence 3, grade of recommendation C)[50].

Histopathological evidence of vasculitis is considered the gold standard for diagnosis. However, diagnostic yield is organ dependent. The highest yield is from kidney biopsy with evidence of renal involvement with crescentic glomerulonephritis and/or extra-capillary proliferation in 91% of those with active renal vasculitis [87]. Thus, despite kidney biopsy being the historic gold standard, there are significant limitations with false negative rate of up to 9%[87].
Kidney biopsy is invasive with hemorrhage in up to 11% and major bleeding requiring blood transfusion or embolization in up to 0.9% and estimated per procedure healthcare costs of $1394-1800 USD[88-91]. Sequential biopsies are uncommon in routine clinical practice, of those recruited to a randomised controlled trial of AAV induction therapy only 44% of those with renal flare underwent diagnostic confirmation with a kidney biopsy[46, 47]. Factors that are associated with increased biopsy risk include advanced age, reduced glomerular filtration rate (GFR), hypertension and those receiving plasma exchange all of which are more likely to occur in those with AAV [92]. Kidney biopsy is not required for diagnosis of renal AAV flare in clinical guidelines or trial protocols[50, 93]. Alternate clinical tools such as Birmingham vasculitis activity score (BVAS) that have been adopted in clinical trials are not diagnostic criteria, and were designed for use as standardised scoring systems based on expert opinion[40].

The clinical challenge facing Physicians is the balance of competing risks of inadequate or excess immunosuppression based on clinical estimation of current glomerular inflammation. There is a disconnect between the EULAR recommendations and expert clinical practice as evidenced by the 44% renal biopsy rate in those enrolled in a clinical trial of induction therapy.

1.7.3. Urinary Biomarkers

1.7.3.1. Urine CD25

Urine CD25, a T cell interleukin-2 alpha receptor (sIL-2Ra) has increased expression on CD4+ effector memory T cells during active AAV compared to healthy controls[94]. Soluble CD25 is shed from T cells after activation and is increased in serum of AAV patients with active disease and correlated with vasculitis disease activity with levels falling in remission[95, 96].

A collaboration between our group and the University of Groningen showed that usCD25, ssCD25 and usCD163 levels were elevated in active renal vasculitis and fell in remission. Recursive partitioning models were developed, the optimal model selected a combination of usCD25, ssCD25 and usCD163. This model outperformed each biomarker or traditional clinical tools for detection of active renal vasculitis in isolation with sensitivity of 84.7%, specificity 95.1%. See figure 1.3.

Further study of sCD25 was not conducted as part of this thesis as a combination of both serum and urine sCD25 were required to improve detection of active renal vasculitis in addition to
usCD163. The additional requirement of a paired serum sample limits the translational potential of this work into clinical practice.

Figure 1.3 sCD25 and sCD163 utility in the diagnosis of AAV[97].

1.7.3.2. Urine monocyte chemoattractant protein-1

Urinary monocyte chemoattractant protein-1 is a chemokine that promotes monocyte recruitment to areas of inflammation. uMCP-1 has been shown to be increased in AAV at diagnosis and fall throughout therapy[74, 98, 99]. Elevated uMCP-1 levels have also been associated with poor prognosis and relapse[100]. Tam et al previously showed potential utility for uMCP1 in active renal vasculitis with some indication that the levels then fall in remission. This biomarker is currently being used as a secondary outcome measure in clinical trials of AAV, although it has not entered routine clinical practice[74]. Its levels have been shown to be increased in diabetic nephropathy and other forms of primary glomerulonephritis. [101] uMCP-1 does however carry a number of physicochemical limitations most notably its instability at room temperature and need for rapid processing. These characteristics have hampered its translation from bench to biomarker used in routine clinical practice.
1.7.3.3. Urine soluble CD163

See section 1.9.

1.7.4. Serological biomarkers

Calprotectin is expressed in neutrophils and monocytes. It is secreted during inflammation, allowing measurement in serum. Calprotectin is upregulated in many inflammatory disorders including AAV. Pepper et al. showed that during periods of remission calprotectin levels increased in those who subsequently relapsed compared with those with sustained remission. Failure to suppress serum calprotectin by month 2 or 6 post induction therapy is associated with a higher rate of relapse in PR3-ANCA vasculitis treated with rituximab. Calprotectin therefore may be a useful predictor of relapse risk[102, 103].

Plasma levels of C3a, C5a, soluble C5b-9 and Bb are increased in active compared to remission AAV[104]. The same authors also reported a positive correlation between urine Bb and serum creatinine and an inverse correlation between urine Bb and percentage normal glomeruli on renal biopsy.

A biomarker panel designed to assess angiogenesis, tissue repair and repair in those enrolled in the RAVE study identified MMP-3, TIMP-1 and CXCL13 as discriminators between active and remission AAV[105]. In both diabetic and non-diabetic glomerular disease, chemokines are implicated in kidney injury. In addition to contributions to the systemic immune responses implicated in the immunopathogenesis of GN, chemokines also contribute to local kidney tissue injury; production by local and infiltrating cells contributes directly to parenchymal inflammation and injury via pathways such as oxidative stress[106, 107]. The MMPs are endopeptidases that contribute to modulation of chemokine induced injury through complex and reciprocal effects on regulation of inflammatory pathways. These endopeptidases are also important regulators of matrix degradation[108].

1.8. Urine soluble CD163

1.8.1. CD163 Background
CD163 is a monocyte/macrophage specific scavenger receptor for haemoglobin-haptoglobin complexes and is expressed solely on macrophages and monocytes [109]. Its soluble form (sCD163) is present in high concentrations in serum[110]. Its biological function is in innate immune defence by reversibly binding bacteria and free haemoglobin in the circulation to allow immune system processing. Membrane CD163 has a short cytoplasmic tail, a single transmembrane domain and a large ectodomain consisting of nine scavenger receptor cysteine-rich scavenger receptor class B domains[111]. CD163 expression is limited to macrophages and monocytes. The soluble form (sCD163) is cleaved from the cell surface by the action of ADAM17/TACE (a disintegrin and metalloproteinase 17/TNFα converting enzyme) in the setting of proinflammatory stimuli[112].

1.8.2. Serum sCD163

Following ectodomain shedding, the extracellular portion of CD163 circulates in blood of healthy individuals as a soluble protein at a level of 0.7–3.9mg/mL[113]. Relatively high levels of soluble CD163 can be found in the plasma with elevation in both acute and chronic inflammatory states. Highest reported concentrations are seen in clinical syndromes of macrophage excess such as macrophage activation syndrome/hemophagocytic lymphohistiocytosis as well as fulminating liver failure due to hemophagocytosis. Increased plasma levels have been reported in bacterial sepsis, malaria, hepatitis, rheumatoid arthritis, scleroderma, crohn’s disease, coeliac disease, diabetes mellitus, obesity, atherosclerosis, HIV and Gaucher’s disease [113-126].

1.8.3. Urine Soluble CD163

1.8.3.1. usCD163 in healthy population

In healthy individuals usCD163 level in urine is very low as it does not cross an intact glomerular filtration barrier due to its high molecular weight of (130 KDa). CD163 is not expressed in normal human glomeruli, non-inflammatory kidney disease and expression is limited to the tubulointerstitial space in interstitial nephritis.[127]

1.8.3.2. Macrophages are the predominant cell in crescentic glomerulonephritis

The histological hallmark of renal vasculitis is crescentic glomerulonephritis (CGN). Macrophages are the most frequent cell type in glomerular crescents[128, 129]. Crescents are histologically defined as two or more layers of proliferating cells in Bowman's space[32]. CGN has a variety of
potential aetiologies including AAV, anti-glomerular basement membrane disease, lupus nephritis, IgA vasculitis and cryoglobulinemia.

For macrophages in communication with Bowman’s space, CD163 is shed into the urinary space and is thus measurable in urine. In the presence of glomerular macrophage excess usCD163 levels are elevated in urine where it shows potential as a biomarker to identify patients with active glomerular inflammation. See figure 1.4.

![Figure 1.4](image)

*Figure 1.4. Schematic of the proposed pathway for appearance of sCD163 in the urine of patients with crescentic glomerulonephritis.*[130]

### 1.8.3.3. *usCD163 in ANCA-associated vasculitis*

In active renal vasculitis at diagnosis our group have shown in a multi-centre cohort elevated levels of usCD163 with a diagnostic cut off of >300ng/mmol creatinine with sensitivity of 83%, specificity of 96% and positive likelihood ratio of 20.8. Additionally, this paper showed detection of usCD163 early in the disease course of the rat model experimental autoimmune vasculitis. Micro dissected
glomeruli from patients with AAV showed marked elevations of CD163 RNA. Immunohistochemical staining of human AAV showed strong glomerular and interstitial staining. See figure 1.5.

Figure 1.5. CD163 is highly expressed in the kidneys of patients with vasculitis. (A) RNA was extracted from micro dissected glomerular and tubulointerstitial compartments from patients with diabetic nephropathy (DN), minimal change disease (MCD), IgA nephropathy (IgA), FSGS, MGN, lupus nephritis (SLE), and ANCA vasculitis. The degree of expression of the CD163 gene compared with micro dissected healthy control kidney was determined by Affymetrix microarrays. Bars represent fold changes compared with the respective controls. (****q<0.01%; q<5%) (b) Paraffin-embedded human kidney sections from patients with vasculitis were stained for CD163 protein by immunohistochemistry and scored blind according to the location of cells with each of five regions: (1) within regions of fibrinoid necrosis or crescent formation, (2) within regions of apparently normal glomeruli, (3) in the periglomerular region, (4) within tubules, and (5) in the interstitial compartment. (C) CD163 scores in each of the respective five regions stratified by clinical diagnosis (upper graphs) and antibody specificity (lower graphs), (P<0.05; **P<0.01; ***P<0.001). (D–I) Images depict representative low power (×40 magnification) views of healthy control (D) and vasculitic (E) kidney, alongside high power (×400) views of healthy control kidney (F), a glomerulus with mild vasculitic injury (G, arrow), a severely affected glomerulus with established crescent formation (H, arrow), and a glomerulus with a fibrous crescent from previous vasculitic injury (I, arrow, ×200). MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis. [127]
1.8.4. Physicochemical Properties of sCD163

sCD163 is highly stable in both blood and urine. In whole blood it is stable for 24 and 48 hours at room temperature and 4°C, respectively, while in plasma it is stable for weeks at 4°C and several years at −20°C[113]. Similarly, urine sCD163 is stable for at least 1 week at room temperature[127]. This stability infers that variations in the collection and processing of samples are unlikely to be a determining factor in the assay result. This represents a significant advantage over MCP-1, which degrades quickly. Additionally, elevated usCD163 levels are not a surrogate marker of haematuria as our group confirmed by spiking blood into urine samples from healthy controls as it does not lead to elevated sCD163 levels until that the spiked blood is at 25% V/V. See figure 1.6.

Figure 1.6 Physicochemical properties of usCD163. (A) Six samples of known sCD163 level were stored for 24 and 168 hours (one week) at room temperature, 4°C and -20°C. The % change in sCD163 level from samples stored in parallel at -80°C was plotted against time; mean±SEM. (B). Samples were subjected to between 1 and 6 freeze-thaw cycles and sCD163 level determined by ELISA; mean±SEM. (C). Healthy control urine was spiked with serial dilutions of blood and the level of sCD163 was determined by ELISA. The + symbols indicate those dilutions at which the urine appeared macroscopically blood stained. The dotted line indicates the upper limit of normal as determined in
1.8.4.1 Measurement of sCD163

usCD163 is easily and reliably measured by enzyme linked immunoabsorption assay (ELISA). An in house assay established at Aarhus University in 2002 has been used extensively in plasma studies and provides stable results over time using a set of calibrators and control samples. The reported inter-assay coefficient of variation of the ELISA test is 6.6%-7.4%[131].

Moller and colleagues first identified the hemoglobin scavenger receptor (HbSR/CD163), an interleukin-6- and glucocorticoid-regulated macrophage/monocyte receptor for uptake of haptoglobin-hemoglobin complexes in 2002[110]. Plasma sCD163 was identified using immunoprecipitation and immunoblotting. An in house sandwich ELISA was developed and measured in haematology inpatients (n=140) and healthy controls (n=130). Elevated sCD163 levels were present in leukemias, most notably pre-treatment and sepsis[110]. Highest reported levels of plasma sCD163 are in hemophagocytosis and macrophage activation syndrome (MAS) with emerging evidence for its role in the diagnosis of MAS [116, 132, 133]. These conditions are characterised by macrophage accumulation in bone marrow and liver. sCD163 has been proposed a diagnostic criterion for MAS[133, 134]. Increased plasma levels have also been reported in other acute inflammatory conditions such as sepsis, tuberculosis, malaria, HIV, hepatitis [120, 126, 135, 136]. In chronic inflammatory conditions such as atherosclerosis, rheumatoid arthritis, scleroderma and coeliac disease [117, 118, 137-139].

There are a number of commercially available research grade assays available and one clinical grade assay has been developed by Euroimmun. The most frequently cited usCD163 assays are R&D systems Duoset ELISA kit and IQ products Macro163. There remains a lack of assay standardisation which limits the development of clinically relevant reference ranges. An international standardisation of commercial assays based on traceable calibrators is needed[130].

usCD163 measurement is relatively cheap with an estimated cost €40 per sample and urine sample procurement is non-invasive.

1.8.4.2 Feasibility of translation into clinical practice

The ease of measurement, stability in urine, strong physiological basis and clear clinical utility make sCD163 a logical candidate for validated introduction into clinical practice.
1.9. Statistical Methodology in Risk Prediction

The term risk factor was first used in description of the initial Framingham study in 1961[140]. Risk factors have been including in risk prediction models. The most commonly used measure to quantify the improvements a biomarker adds to risk prediction is the c statistic (area under the curve). This is however an imperfect measure with challenges including the difficulty interpreting the usually small changes in this statistic and its relationship to magnitude of improvement to the performance of the baseline model[141-143]. The concept of risk reclassification was introduced to assess how subjects are classified differently when alternate models are used[144]. The net reclassification index is an attractive concept as it reports reclassification which is more abstractly more clinically relevant. There are however issues with NRI. It does not allow weighting for the importance of different outcomes i.e. false negative but instead bases the on how common the outcome of interest is[145]. Decision tree analysis incorporates clinical and research variables in an unbiased manner to estimate the key nodes which lead to the outcome of interest.

1.9.1. Biomarker Performance Terminology

Key terms referenced in this thesis include[146]:

<table>
<thead>
<tr>
<th>Disease Present</th>
<th>Disease Absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Positive</td>
<td>a</td>
</tr>
<tr>
<td>Test Negative</td>
<td>c</td>
</tr>
<tr>
<td>(a + c) = total number of patients with disease</td>
<td>(b + d) = total number of patients without disease</td>
</tr>
</tbody>
</table>

Table 1.1: 2x2 factorial design table

Sensitivity: the proportion of people with disease who will have a positive result \((a/(a+c))\).

Specificity: the proportion of people without the disease who will have a negative result \((d/(b+d))\).

Positive predictive value (PPV): the proportion of people with a positive test result who actually have the disease \((a/(a+b))\).

Negative predictive value (NPV): the proportion of people with a negative test result who do not have disease \((d/(c+d))\).

Positive likelihood ratio (PLR): the ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease. \(PLR = \frac{\text{Sensitivity}}{(1-\text{Specificity})}\)
Negative likelihood ratio (NLR): the ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease. \[ NLR = \frac{(1 - \text{Sensitivity})}{\text{Specificity}} \]

1.10. Current unmet clinical needs in renal vasculitis management

1.10.1. Rapid diagnosis of renal flare in the setting of known vasculitis.

Most forms of RPGN follow a relapsing and remitting course. In particular, in established ANCA-vasculitis and lupus nephritis, the treating physician is often faced with a clinical decision when faced with non-specific symptoms and change in kidney function. In this setting, the serological markers used in diagnosis have limited utility, so the physician must rely on detection of urinary blood, protein and casts, in association with non-specific inflammation markers such as c-reactive protein. Kidney biopsy, an invasive, costly test with potential morbidity may be required. A simple non-invasive test that would distinguish active glomerulonephritis from mimics, such as ischemic renal injury, interstitial nephritis, other de-novo renal injury or urine infection, would be valuable in clinical practice.

1.10.2. Personalised guidance of immunosuppressive therapy intensity using a biomarker providing direct evidence of glomerular macrophage activation.

Tracking the degree of glomerular inflammation and destruction during a course of cytotoxic induction treatment is currently using a “one-size fits all” approach, or approximating response based on urine parameters and excretory kidney function. A non-invasive point of care test could potentially allow optimisation of treatment duration, personalise treatment intensity with the goal of minimising unnecessary exposure and ensuring complete resolution of inflammation prior to de-escalation to maintenance therapy. Unmet clinical needs include a non-invasive method of diagnosing renal vasculitis flare and a tool for monitoring renal inflammation during periods of intense immunosuppressive therapy.
1.11. Establishment of the Rare Kidney Diseases Biobank

As AAV is a rare disease, multicentre collaboration is vital to achieve sufficient patient numbers to allow study of various phases of disease. Professor Little has developed the Science Foundation of Ireland funded Rare Kidney Disease Biobank, a national collaborative network of Nephrology and Rheumatology centres, which is linked to the UKIVAS registry of UK vasculitis patients. The biobank was established within Trinity College Dublin in 2012. During my PhD I established Cork University Hospital as a centre for recruitment and in 2013/14 personally recruited over 90 vasculitis patients. To date almost 700 vasculitis patients have been recruited. This research framework provides the basis for two prospective and one retrospective study as described in this thesis. See figure 1.7.

Figure 1.7: RKD Recruitment throughout Ireland. Dark green: Nephrology, Orange: Rheumatology, Light green: Immunology. Solid lines represent vasculitis recruits, hatched lines represent healthy control recruits.
Chapter 2: Methods
2.1. Introduction

In this chapter I will discuss in detail the usCD163 assays used, the rationale for their selection and future directions for a diagnostic grade test. Additionally, I will discuss two statistical methods I learned and applied in this thesis: the derivation of optimal diagnostic cut-off points and decision tree analysis.

2.1.1. usCD163 Measurement

A key component of this work is the experimental assay selected to measure our protein of interest urine soluble CD163. At the time of writing there were four commercially available assays, one in-house academic assay and one industry prototype utilised [97, 113, 127, 147, 148]. CD163 has three known isoforms. Soluble CD163 is formed by cleavage from amino acid at position 41. Only one manufacturer was willing to share details of which isoforms were detected by their assay. Abcam’s Quantikine ELISA is composed of antibodies are directed against amino acids: 41 – 1045 and should theoretically detect all the isoforms. Five of the six available assays are monoclonal (all commercial assays) and the in house assay from the University of Aarhus is polyclonal. Urine analyte measurement has more physicochemical challenges than blood analyte measurement due to the wide variation in normal range of urinary pH, urine concentration, timing of urine collection, storage conditions, and the presence of additional proteins and potential bacterial contamination.

2.1.2. Derivation of a robust diagnostic cut off range

Standardised reference and diagnostic cut off ranges are needed to translate these findings to useful clinically meaningful and useful tests. A validated diagnostic range allows assessment of false positives, false negatives and allow assessment of the true diagnostic utility of the test. Diagnostic cut off calculations are used to maximise both the sensitivity and specificity of the test [149, 150].

2.1.3. Decision tree analysis

Decision tree analysis will be used to integrate clinical and laboratory data in addition to novel biomarkers. Statistical methodologies used will include recursive partitioning and conditional inference. These tools will allow us to then estimate the additional benefit our biomarker of interest adds and whether or not use of additional current clinical biomarkers improves the diagnostic capabilities[151].
2.1.4 Alternative methods of prediction modelling

Prediction models are used to assisting in clinical decision making and prognostications. Prediction model selection requires knowledge of the outcome of interest (i.e. renal vasculitis flare), parameters included, model selection, model evaluation and model validation[152]. Prediction models can use either traditional methodologies such as logistic, linear regression or survival analysis or machine learning methodologies including artificial neural networks or tree models.

Coxs proportionate hazard model is frequently used for survival analysis as it allows for time censoring and multivariate analysis. It is frequently used to predict outcomes such as cancer survival from a baseline timepoint[153]. Limitations include the collinear variables, lack of handling of missing variables requiring imputation and requirement to transform data to a linear form[154, 155].

Decision trees use classification, regression and random forest to predict prespecified outcomes. Machine learning algorithm approaches have advantages over traditional regression modelling[156]. The lack of a predefined hypothesis is of particular use in disease modelling when there are abundant potential predictors and interactions between predictors, which are common in biological processes. This allows potential discovery of unexpected predictors. Recursive partitioning is used to build classification or regression models via a two-step process with a resultant binary tree. Rpart trees are constructed by selecting the first single variable which best splits the data into two groups. The data is separated, and this repeated in each subgroup recursively until the subgroups reach a minimum size or no further improvement can be made. Cross validation is then used to trim back the tree[157].
2.2. Aims

2.2.1. Hypothesis

1. Different sCD163 assays detect different sCD163 isoforms or amino acid sequences. We hypothesise that comparing all available assays will allow identification of the optimal sCD163 assay for detection of active renal vasculitis.

2. We hypothesise that an accurate diagnostic cut off range can be derived and validated.

3. We hypothesise that decision tree analysis will assist in diagnostic accuracy by combining clinical and urinary biomarkers for the detection of active renal vasculitis.

2.3 Sample Processing

2.3.1. Rare Kidney Disease Biobank, Trinity College Dublin - Serum and Urine Processing

Bio samples were collected according to pre-determined protocolized procedures. Serum and urine were spun at 2000g for 10 minutes then frozen at -80°C until shipping on dry ice to central Trinity College Dublin repository [127].

2.3.2. Central Pathology Laboratory, St. James’s Hospital, Dublin – Urine Processing

Urine samples were collected according to pre-determined protocolized procedures. Upon arrival at CPL urine was assessed for red blood cell casts if received in less than 4 hours, spun at 2000g for 10 minutes then frozen at -80°C until experimental assay.
2.4. usCD163 Assay Details

usCD163 was measured by five sandwich ELISA assays:

1. R&D Systems Duoset (DY1607) at a 1:4 dilution. All assays were performed in duplicate. This is a solid phase sandwich ELISA with quoted range of 156-10,000 pg/ml. This assay was selected as it has been previously validated in urine in the clinical settings of renal vasculitis at diagnosis and lupus nephritis[97, 127, 147, 148]. As this assay involves a manufacturing step of coating the plates with capture antibody it does not meet accreditation standards as a diagnostic clinical grade test. See protocol in figure 2.1.

2. Abcam Quantikine (ab155428) undiluted. All assays were performed in duplicate. This is a solid phase sandwich ELISA with quoted range of 0.032 ng/ml - 8 ng/ml and sensitivity <30 pg/ml. This assay was selected due to their pre-coated plates, hence lack of a manufacturing step. Additionally, Abcam quotes a high level of sensitivity with proposed detected of sCD163 concentrations as low as 0.032ng/ml. This assay was performed at the Central Pathology Lab in St. James’s Hospital, Dublin by Medical Laboratory Scientists. See protocol in figure 2.2.

3. R&D Systems Quantikine (DC1630) undiluted. All assays were performed in duplicate. This is a solid phase sandwich ELISA with a quoted range of 1.6 - 100 ng/ml and sensitivity 0.613. This assay was selected due to their pre-coated plates, hence lack of a manufacturing step. The manufacturer was unable to provide information on the antibodies used in this assay and their own Duoset assay. See protocol in figure 2.3.

4. Aarhus ELISA was performed at the University of Aarhus as per their protocol.[113] This is the first developed sCD163 assay and was performed in-house by Professor Moller’s team. It was selected as it is a polyclonal sandwich ELISA and hypothetically should detect all potential CD163 isoforms.

5. Euroimmun sCD163 ELISA was developed specifically for measurement of urine (rather than serum or cell culture supernatant) sCD163. This assay is a solid phase sandwich ELISA with capture antibody pre-coated on each plate. This assay lacks the manufacturing step of the aforementioned Duoset R&D ELISA and will bear the CE marking of approval. CE Marking on a product is a manufacturer’s declaration that the product complies with the
essential requirements of the relevant European health, safety and environmental protection legislation. This assay was available to our group but at time of thesis submission was not yet available for commercial purchase. This assay was performed at the Central Pathology Lab in St. James’s Hospital, Dublin by Medical Laboratory Scientists.

6. IQ Systems ELISA was developed in collaboration with Aarhus University (see ELISA No. 4) and given similarities better these two assays, sample volume and cost restrictions we did not include this assay in our experimental design.

As per prior studies of usCD163 we normalized the urine sCD163 level to the creatinine level as determined by a modified Jaffe technique. Urine creatinine and protein were measured by Roche Cobas Creatinine plus (05 6612 7) and Total Protein (11877801) modules respectively by Medical Scientists at the Central Pathology Laboratory, St. James’s Hospital, Dublin.
2.5. usCD163 Assay Protocols

2.5.1. R&D Systems Duoset sCD163 ELISA

Kit: R&D Systems DY1607

Day 1:

1. Determine amount of capture antibody (aliquots in -80C research freezer) solution required (50ul per well) and prepare a 1:120 dilution in PBS. Add capture antibody overnight at room temperature. Ensure plate is covered.

Day 2:

2. Wash plate 3X with PBS + Tween 20 (add 0.5 ml of Tween 20 to 1 litre of PBS). Dry plate on tissue paper.

3. Block wells with 1% BSA + PBS (prepare by adding 1g of BSA to 100 ml of PBS) for 1 hr at room temperature (RT). Add 150ul per well so determine how much required.

4. Wash plate 3X with PBS + Tween 20. Dry plate. Ensure plate is covered with either clingfilm or paraffin.

5. Organise samples for each plate.

6. Prepare standards for each plate by adding 18 ul of standard to 1 ml of 1% BSA + PBS. Mix and dilute into 500ul of 1% BSA + PBS. Repeat 6 times, leave last vial with just 1% BSA + PBS (= background).

7. Wash plate 3X with PBS + Tween 20. Dry plate on tissue paper.

8. Add 50ul of standards and 50ul of samples to appropriate wells for 2 hours at RT. Ensure plate is covered with either clingfilm or paraffin.

9. Wash plate 3X with PBS + Tween 20. Dry plate with tissue paper.

10. Add detection antibody (make up in 1% BSA + PBS ) at 1:60 for 1 hour at RT. Add 50ul per well.

11. Wash plate 3X with PBS + Tween 20. Dry plate on tissue paper.

12. Add streptavidin-HRP (kept in fridge in Lab 1.06) at 1:40. Add 50ul per well for 20 minutes at RT.

13. Wash plate 3X with PBS + Tween 20. Dry plate on tissue paper.

14. Add 50ul of TMB (kept in coldroom next to Room 2.13) to each well. Incubated for 20 minutes at RT (avoid placing the plate in direct light, colour will be BLUE)

15. Add 25ul of stop solution for each well (colour will be YELLOW)

16. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.

Figure 2.1. R&D Systems Duoset sCD163 ELISA Protocol
2.5.2. Abcam Quantikine sCD163 ELISA

CD163:

1. Prepare standards using Assay Diluent B.
2. When preparing Standards, it is critical to briefly spin down the vial first to ensure all powder is removed from cap – DO NOT Vortex.
3. After adding 400μl to CD163 Std vial, invert and flick tube – DO NOT VORTEX. Spin down vial.
4. Label 8 tubes 1-8.
5. Prepare Std 1 by adding 80 μl CD163 stock std to 420 μl of 1X Assay Diluent B (Dilute 5X Assay Diluent B 5-fold with dH2O prior to use)
6. Pipette 300 μl of 1X Assay Diluent in to tubes 2-8
7. Transfer 200 μl from tube 1 to tube 2 etc until tube 7. Tube 8 is just Assay diluent – serving as the zero std.
8. Pipette 100 μl of std/neat urine/1QC into appropriate wells.

9. Prepare wash solution – 20ml of 20X Wash Buffer Concentrate into 380ml dH2O to yield 1X Wash Buffer.
10. Prepare Biotinylated Ab - Briefly spin the Biotinylated anti-Human CD163 vial before use. Add 100μl Assay Diluent B into vial – The detection Ab concentrate should be diluted 80-fold with 1X Diluent B - 80μl Biotinylated Ab added to 6400μl Diluent B for half-plate.
11. Discard the solution. Wash by filling each well with 300μl of wash buffer using multichannel pipette. Complete removal of liquid at each step is essential. Invert plate and blot against clean paper towels.
12. Add 100μl of 1X prepared Biotinylated Ab and Incubate for 1hour
13. Prepare Streptavidin solution – Briefly spin the vial and pipette up and down to mix gently. Add 50μl of Streptavidin concentrate into 10ml 1X Diluent B to prepare 200-fold diluted HRP-Streptavidin solution. Mix well.
14. Repeat step 11.
15. Add 100μl of prepared Streptavidin solution to each well and incubate for 45mins.
16. Repeat step 11.
17. Add 100μl of TMB One-step Substrate reagent to each well. Incubate for 30mins.
18. Add 50μl of Stop solution to each well. Read at 450nm immediately.

Figure 2.2. Abcam Quantikine sCD163 ELISA Protocol
2.5.3. R&D Systems Quantikine sCD163 ELISA

**ASSAY PROCEDURE**

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100 μL of Assay Diluent RD1-34 to each well.

4. Add 50 μL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.

5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 μL of Human CD163 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Figure 2.3. R&D Systems Quantikine sCD163 ELISA Protocol*
2.5.4. Euroimmun sCD163 ELISA Protocol

**Inkubation: sCD163-ELISA**

**Antikörper-beschichtete Mikrowells**

**Pipettieren:**
- 100 µl je Reagenzgefäß (100 µl per well)

**Pipette:**
- 50 µl je Reagenzgefäß (50 µl per well)

**Inkubieren:**
1h bei Raumtemperatur (18°C - 25°C) bei mittlerer Drehzahl (ca. 400 U/min) auf einem Orbital-Shüttler inkubieren

**Incubate:**
Cover the wells
1h at room temperature (18°C - 25°C) at medium speed (approx. 400 rpm) on an orbital shaker

**Waschen:**
3x

**Wash:**
- 300 µl (man.)/450 µl (aut.) je Reagenzgefäß
  - reaction time: 30-60 s per washing cycle

**Waschen:**
3x

**Wash:**
- 300 µl (man.)/450 µl (aut.) per well
  - reaction time: 30-60 s per washing cycle

**Pipettieren:**
- 100 µl je Reagenzgefäß (100 µl per well)

**Pipette:**
- 100 µl per well

**Inkubieren:**
1h bei Raumtemperatur (18°C - 25°C) bei mittlerer Drehzahl (ca. 400 U/min) auf einem Orbital-Shüttler inkubieren

**Incubate:**
1h at room temperature (18°C - 25°C) at medium speed (approx. 400 rpm) on an orbital shaker

**Waschen:**
3x

**Wash:**
- 300 µl (man.)/450 µl (aut.) je Reagenzgefäß
  - reaction time: 30-60 s per washing cycle

**Pipettieren:**
- 100 µl je Reagenzgefäß (100 µl per well)

**Pipette:**
- 100 µl per well

**Incubieren:**
30 min bei Raumtemperatur (18°C - 25°C) bei mittlerer Drehzahl (ca. 400 U/min) auf einem Orbital-Shüttler inkubieren

**Incubate:**
30 min at room temperature (18°C - 25°C) at medium speed (approx. 400 rpm) on an orbital shaker

**Waschen:**
3x

**Wash:**
- 300 µl (man.)/450 µl (aut.) je Reagenzgefäß
  - reaction time: 30-60 s per washing cycle

**Pipettieren:**
- 100 µl je Reagenzgefäß (100 µl per well)

**Pipette:**
- 100 µl per well

**Inkubieren:**
15 min bei Raumtemperatur (18°C - 25°C)

**Incubate:**
15 min at room temperature (18°C - 25°C)

**Waschen:**
3x

**Wash:**
- 100 µl je Reagenzgefäß (100 µl per well)

**Stoplösung:**
- stop solution

**Auswerfen:**
- Photoanalytische Messung (450 nm)

**Evaluate:**
- photometric measurement (450 nm)

Figure 2.4. Euroimmun sCD163 ELISA Protocol
2.6. Statistical methodology

2.6.1. Urine normalisation

Urine values were normalised to urine creatinine to correct for potential confounding effects of urine dilution. This is performed by dividing the concentration of the urinary analyte of interest by the urine creatinine concentration. This technique has been extensively validated clinically with the widespread use of albumin and protein to creatinine ratios[158]. Spot urine albumin to creatinine ratio and spot protein to creatinine ratio are widely used in the monitoring of chronic kidney disease and diabetes[159]. Day to day variation in ACR and PCR occurs but this is less significant with greater degrees of proteinuria[158, 160]. Urine analyte value normalisation to urine creatinine is also widely used in urinary analyte measurement and all existing sCD163 literature to date [127, 148, 161].

Creatinine is a by-product of muscle metabolism and is excreted in urine at a constant rate via glomerular filtration[162]. Potential caveats of urine creatinine measurement include its variability with dietary protein load, muscle bulk and sex[163-166].

Urine is an ideal biospecimen as it is be easily and non-invasively obtained with general abundant supply. Spot urine samples are traditionally used in urinary analyte experiments as 24-hour urine collections for analytes are limited by collection technique, incomplete collection, bacterial contamination and sample degradation. Up to 50% of 24-hour urine collections are incomplete, limiting their diagnostic utility[167]. Spot urine sample use is limited by the differing effects of urinary concentration with varying dilution effect and sample volume.

2.6.2. Cut off Derivation

To generate receiver-operator characteristic (ROC) curves and determine the most appropriate diagnostic cut off levels I used the OptimalCutpoints package using R Studio version 0.99.902. This requires data to transformed into a data frame prior to analysis. [149]

2.6.2.1. Technique Selection
The Youden index was selected to maximize the sum of sensitivity and specificity; other methods include MaxSp (to maximise specificity), MaxSe (to maximise sensitivity), MaxProdSpSe (to maximise the product of specificity and sensitivity) and SpEqualSe (using criterion of equality of sensitivity and specificity). Despite investigating the diagnostic utility of these alternate calculations the Youden index yielded the most clinically relevant cut off values. [150]

2.6.2.2. R Script Used

X: Defines the biomarker of interest
Status: Defines the outcome of interest
Methods: Cut off technique selected i.e., Youden, MaxSe
Data: name of your data frame file (must be .txt)
Other components of code can be altered as required.

library("OptimalCutpoints")
install.packages("OptimalCutpoints")

# Youden calculation
optimal.cutpoint.Youden<-optimal.cutpoints(X = "Duoset_CD163_Normalised_Creatinine_ng_mmol", status = "ModSelectionGroup", tag.healthy = "6", methods = "Youden", data = df, pop.prev = NULL, categorical.cov = NULL, control = control.cutpoints(), ci.fit = TRUE, conf.level = 0.95, trace = FALSE)

#plot your work
plot(optimal.cutpoint.Youden, legend = FALSE)

#summary
summary(optimal.cutpoint.Youden)

2.6.2.3. R Script Output

Below is typical output received from the above coding:
Area under the ROC curve (AUC): 0.938 (0.874, 1.002)

CRITERION: Youden
Number of optimal cut-offs: 1

<table>
<thead>
<tr>
<th>cut-off</th>
<th>Estimate</th>
<th>95% CI lower limit</th>
<th>95% CI upper limit</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>270.7500000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Se</td>
<td>0.9130435</td>
<td>0.71962066</td>
<td></td>
</tr>
<tr>
<td>0.9892900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp</td>
<td>0.8947368</td>
<td>0.75195062</td>
<td></td>
</tr>
<tr>
<td>0.9705655</td>
<td></td>
<td></td>
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<tr>
<td>PPV</td>
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<td>0.65185537</td>
<td></td>
</tr>
<tr>
<td>0.9788070</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPV</td>
<td>0.9444444</td>
<td>0.80603022</td>
<td></td>
</tr>
<tr>
<td>0.9850629</td>
<td></td>
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</tr>
<tr>
<td>DLRPositive</td>
<td>8.6739130</td>
<td>3.40351690</td>
<td></td>
</tr>
<tr>
<td>22.1055954</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLRNegative</td>
<td>0.0971867</td>
<td>0.02573557</td>
<td></td>
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<tr>
<td>0.3670117</td>
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<tr>
<td>FP</td>
<td>4.0000000</td>
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<td></td>
</tr>
<tr>
<td>FN</td>
<td>2.0000000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal criterion</td>
<td>0.8077803</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.6.3. Decision Tree

Decision tree analyses were performed using R Studio version 0.99.902. rpart, ctree and party packages were used.[151]

Clinical variables of new/worse proteinuria, new/worse hematuria, serum creatinine, c-reactive protein, age, and sex, in addition to the biomarkers of interest, usCD163 and uMCP-1. These clinical variables were included in the decision tree irrespective of whether or not they were recorded as an active BVAS/WG item.

2.6.3.1. Technique Selection

Recursive partitioning was selected as it creates a decision tree that attempts to classify subjects by splitting into sub-populations of subjects based on dichotomous independent variables. It is termed recursive as each sub-population can be split indefinitely until a stopping criterion is achieved.

2.6.3.2. R Script Used

#load your packages
packages(party)
install.packages("party")
library(party)
install.packages("rpart")
library(rpart)
install.packages("rattle")
install.packages("rpart.plot")
install.packages("RColorBrewer")
library("rattle")
library("rpart.plot")
library("RColorBrewer")

#load your dataset
data <- read.table('VCRC_Final_Masterfile_October_2016.txt', header=T)
input.dat <- data[,c(1:320),]

#needs to be a data frame
df=data.frame(data)
#classify

#input.dat$Class <- NA
#input.dat$Class[which(input.dat$Classification=="Remission_Renal ")]
  <- 0
#input.dat$Class[which(input.dat$Classification=="Active_Renal")]
  <- 1

data$Class <- NA
data$Class[which(data$SelectionGroup=="2")]
  <- 0
data$Class[which(data$SelectionGroup=="3")]
  <- 0
data$Class[which(data$SelectionGroup=="5")]
  <- 0
data$Class[which(data$SelectionGroup=="1")]
  <- 1
data$Class[which(data$SelectionGroup=="4")]
  <- 2

#names

names(data)

#rpart model all variables included

fit <- rpart(ModSelectionGroup ~ MH_PGA_Today 
  + RBCCastsToday 
  + VCRC_Diagnosis 
  + ElevatedSerumCreatinineToday 
  + Creatinine_Current_Serum Derived_mg_dl 
  + Protein 
  + Blood 
  + Gender 
  + Age 
  + aRedBloodCellCasts 
  + ANCA_ELISA 
  + Duoset_CD163_Normalised_Creatinine_ng_mmol 
  + MCP1_normalised_creatinine_pg_mmol 
  + PCRRatio_mg_mmol 
  + Haematuria 
  + New_Haematuria 
  + New_Proteinuria 
  + Proteinuria 
  + X._INCREASE_CREAT, 
  method="class", data=df)

plot(fit, uniform=TRUE, compress=TRUE, main="Classification Tree")

summary(fit)
fancyRpartPlot(fit)
#load your packages

packages(party)
install.packages("party")
library(party)

install.packages("rpart")
library(rpart)

install.packages("rattle")
install.packages("rpart.plot")
install.packages("RColorBrewer")

library("rattle")
library("rpart.plot")
library("RColorBrewer")

install.packages("randomForest")
library("randomForest")

#load your dataset

data <- read.table('VCRC_Final_Masterfile_October_2016.txt',
                   header=T)
input.dat <- data[c(1:320),]

#needs to be a data frame

df=data.frame(data)

#classify

#input.dat$Class <- NA
#input.dat$Class[which(input.dat$Classification=="Remission_Renal "] <- 0
#input.dat$Class[which(input.dat$Classification=="Active_Renal")]
<- 1

data$Class <- NA
data$Class[which(data$SelectionGroup=="2")]
<- 0
data$Class[which(data$SelectionGroup=="3")]
<- 0
data$Class[which(data$SelectionGroup=="5")]
<- 0
data$Class[which(data$SelectionGroup=="1")]
<- 1
data$Class[which(data$SelectionGroup=="4")]<-2

#names
names(data)

#rpart model all in
fit<-rpart(ModSelectionGroup~MH_PGA_Today+
  +RBCCastsToday
  +VCRC_Diagnosis
  +ElevatedSerumCreatinineToday
  +Creatinine_Current_Serum_derived_mg_dl
  +Protein
  +Blood
  +Gender
  +Age
  +aRedBloodCellCasts
  +ANCA_ELISA
  +Duoset_CD163_Normalised_Creatinine_ng_mmol
  +MCP1_normalised_creatinine_pg_mmol
  +PCRRatio_mg_mmol
  +Haematuria
  +New_Haematuria
  +New_Proteinuria
  +Proteinuria
  +X._INCREASE_CREAT,
method="class",data=df)
plot(fit,uniform=TRUE,compress=TRUE,main="Classification Tree")
text(fit,use.n=TRUE,all=TRUE,cex=.8)
summary(fit)
fancyRpartPlot(fit)
2.7. Conclusions

usCD163 can be reliably measured by a variety of research grade commercial assays. One of the challenges in translating results from a research grade assay into clinical practice are the lack of a standardised reference range and the inability of treating Physicians to obtain a diagnostic grade result that they can reliably act upon in their clinical practice. Collaboration between Professor Little, Trinity College Dublin, St. James’s Hospital and Euroimmun has led to the delivery of a diagnostic grade usCD163 assay. This assay is compared to existing assays in chapter 4.

Advanced statistical methodologies beyond absolute concentrations increases are required to both accurately compare a new biomarker to existing tools and to integrate its use amongst existing clinical tools. Decision trees are used in chapter 3 as well a collaboration with the University of Groningen. [97]

A robust diagnostic cut off point is required for translation into clinical practice. The Youden index in addition to other techniques are utilised in chapters 3,4 and 5. The use of clear cut off points allows accurate phenotyping of false positive and false negative cases. This step is vital in outlining clinical caveats and informing real world utility of the usCD163 assay.
Chapter 3:

Urinary Soluble CD163 and Monocyte Chemoattractant Protein-1 in the Identification of Renal Flare in ANCA-Associated Vasculitis
3.1 Abstract

Objectives: Prior work has shown that urinary soluble CD163 (usCD163) displays excellent biomarker characteristics for detection of active renal vasculitis using samples that included new diagnoses with highly active renal disease. This study focused on use of usCD163 in detection of the more clinically relevant state of mild renal flare and compared results of usCD163 testing directly to testing of urinary monocyte chemoattractant protein 1 (uMCP1).

Methods: Patients with ANCA-associated vasculitis (AAV, n=88, primarily GPA n= 73) were identified within a serially sampled, longitudinal, multi-center cohort. Creatinine-normalised usCD163 and uMCP-1 levels were measured by ELISA and, both alone and in combination, were compared between times of active renal AAV and levels during remission and/or active non-renal AAV. Renal flare diagnosis was based on physician assessment using standardised tools which did not include renal biopsy.

Results: Samples from 320 study visits included times of active renal vasculitis (n=39), remission (233), and active extra-renal vasculitis (48). Median creatinine levels in were 0.9mg/dl (interquartile range, IQR 0.8-1.2) in remission and 1.4mg/dl (1.0-1.8) during renal flare. usCD163 levels were higher in patients with active renal vasculitis compared with patients in remission and those with active extra-renal vasculitis, with median values of 162ng/mmol (IQR 79-337), 44ng/mmol (17-104), and 38ng/mmol (7-76), respectively, p<0.001. uMCP-1 levels were also higher in patients with active renal vasculitis compared with patients in remission and those with active extra-renal vasculitis, with median values of 10.6pg/mmol (IQR 4.6-23.5), 4.1pg/mmol (2.5-8.4) and 4.1pg/mmol (1.9-6.8), respectively, p<0.001). The proposed diagnostic cut-points for usCD163 and uMCP-1 were 72.9ng/mmol and 10.0pg/mmol, respectively. usCD163 and uMCP-1 levels were marginally correlated ($r^2=0.11$, p<0.001). Combining novel and existing biomarkers using recursive tree partitioning indicated that elevated usCD163 plus either elevated uMCP1 or new/worse proteinuria improved positive likelihood ratio of active renal vasculitis to 19.2.

Conclusion: A combination of usCD163 and uMCP1 measurements appear to be useful in identifying the diagnosis of subtle renal vasculitis flare.
Urinary Soluble CD163 & MCP-1 in the Identification of Subtle Renal Flare in ANCA-Associated Vasculitis

Results

Methods
Retrospective, longitudinal study
88 patients, 320 encounters

Conclusions
usCD163 and uMCP-1 are elevated in subtle renal vasculitis flare. Using recursive tree partitioning usCD163, uMCP1 and new onset proteinuria lead to improved diagnostic fidelity with PLR of 19.2 and NLR of 0.6.
3.2. Introduction

To date, usCD163 has been assessed as a biomarker of crescentic glomerulonephritis in AAV at time of diagnosis and in class III and IV lupus nephritis [127[148]]. These cohorts included patients with severe kidney disease with little doubt about the presence of active glomerulonephritis based on existing clinical parameters. Little doubt about the presence of active glomerulonephritis based on existing clinical parameters. There is an unmet need for clinical biomarkers in AAV to identify patients with active renal vasculitis flares. In the absence of overt rapidly progressive glomerulonephritis with marked loss of kidney function, this diagnosis is often difficult, with a broad differential diagnosis and frequent requirement for kidney biopsy. Dipstick positive haematuria and proteinuria, while useful in patients at the onset of disease, remain elevated for a median of 448 and 346 days respectively after diagnosis of AAV [77], thereby reducing their utility as discriminators of active glomerular inflammation.

Tam et al previously showed potential utility for uMCP1 in active renal vasculitis with some indication that the levels then fall in remission[98]. This biomarker is currently being used as a secondary outcome measure in clinical trials although it has not entered routine clinical practice.[74]

To address this clinical unmet need, we sought to test the utility of usCD163 and uMCP1, alone or in combination, as a diagnostic tool in subtle renal vasculitis flare. The Vasculitis Clinical Research Consortium has access to a rich longitudinal clinical and biological sample set, including patients suffering from a wide range of levels of renal disease in AAV. We used this unique resource to determine usCD163 and uMCP1 biomarker characteristics in this setting, and to explore ways in which they could be integrated with existing clinical biomarkers to maximise utility for the identification of patients with mild flares of renal vasculitis.
3.3. Aims

sCD163 is produced following inflammatory cleavage from the surface of glomerular macrophages, whereas MCP1 is derived from intrinsic renal cells in response to inflammatory stimuli and results in recruitment of monocytes. As these are discrete elements in the process of renal macrophage accumulation, we were interested in determining whether the pattern of urinary excretion was different, and whether usCD163 could identify cases mis-characterised by uMCP1 and vice versa.

As this retrospective cohort is derived from a largely Rheumatology based practice we specifically chose this cohort to assess the role of usCD163 in this phenotypically milder setting than our planned prospective study of renal vasculitis flare (chapter 4) which is largely Nephrology practice based with an increased severity of kidney disease.

We addressed the following hypotheses:

(1) usCD163 concentrations are increased in clinically subtle renal vasculitis flare.

(2) uMCP-1 aids in the diagnosis of clinically subtle renal vasculitis flare, both in isolation and in combination with usCD163.

As any new biomarker will not be likely be used in isolation from existing best practice, we tested both usCD163 and uMCP1 in combination with new haematuria, creatinine rise, CRP, and new proteinuria. To identify the optimal combination of tests we used unbiased recursive partitioning to generate a combination of markers that maximised specificity, i.e., to “rule out” the presence of active renal vasculitis.
3.4. Methods

3.4.1. Patients and clinical data collection

Patients were enrolled in the Vasculitis Clinical Research Consortium (VCRC) Longitudinal Study of patients with granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), or eosinophilic granulomatosis with polyangiitis (eGPA), from 8 referral centers in the United States and Canada. All patients were enrolled using protocols approved by the institutional review boards or ethics committees of all participating sites and written informed consent documents in keeping with The Declaration of Helsinki.

All enrolled patients met the American College of Rheumatology (ACR) criteria for GPA modified to include ANCA, or the Chapel Hill Consensus Conference definition of MPA, or ACR Classification Criteria for EGPA which was adapted so that biopsy proof of small-vessel vasculitis was not required.

Clinical data, including measures of disease activity and immunosuppression, were collected on a quarterly or annual basis. We identified patients with a visit associated with a renal or non-renal AAV flare and targeted for analysis up to two encounters during remission both before and after the flare visit. We also included patients initially presenting with active renal vasculitis but with only remission encounters thereafter.
3.4.2. Measures of vasculitis disease activity

Information on specific manifestations of vasculitis was recorded using the Birmingham Vasculitis Activity for Wegener’s Granulomatosis Score (BVAS/WG), physician global assessment scores as recorded using a visual analogue scale, urinalysis for blood and protein, red blood cells casts, c-reactive protein, and serum creatinine [99, 168].

Active renal disease was determined by the physician-investigator and was informed by the presence of new or worse haematuria, new or worse proteinuria, urinary red blood cell casts, and/or rise in serum creatinine >30% as interpreted by the clinician as being due to active renal vasculitis.

Additional clinical variables were created using available clinical data including new/worse hematuria, new/worse proteinuria, percentage increase in serum creatinine to mirror real life clinical decision making.

3.4.3. Collection and storage of urine samples

Urine was collected by the patients in sterile cups and aliquoted without further manipulation, frozen at −80°C at each participating clinical site, shipped on dry ice to the VCRC specimen repository, and stored at -80°C until used for this study. Samples were received in Trinity College Dublin on dry ice and frozen at -80°C until sample processing.

3.4.4. Clinical laboratory tests

Serum creatinine and urinalyses (dipstick and microscopy) were performed in CLIA-approved laboratories at the clinical sites per standard practice for collection and processing of outpatient specimens. Results of dipstick and microscopic urinalyses were recorded in the VCRC database as positive or negative (without further quantification) for blood, protein, red blood cells (RBC), and RBC casts. RBCs and RBC casts could also be noted as positive based on examination by the investigator or a nephrologist colleague at the time of the patient visit, but such an examination was not required. It was not recorded whether assessment for RBCs or casts was made by the clinical laboratory or the investigator. The presence or absence of dysmorphic RBCs was not
recorded. Glomerular filtration rate (GFR, ml/min per 1.73 m² body surface area) was calculated from serum creatinine using the modified diet in renal disease (MDRD) formula [169].

3.4.5. Urinary biomarker assays

usCD163 was measured by three commercially-available ELISA kits (1-3) and one in house polyclonal usCD163 kit. usCD163 assay methodology is discussed in detail in chapter 2 Methods.

1. R&D Systems Duoset (DY1607) at a 1:4 dilution [127].
2. Abcam Quantikine (ab155428) undiluted.
3. R&D Systems Quantikine (DC1630) undiluted.
4. Aarhus ELISA was performed at the University of Aarhus as per their protocol [170].

uMCP-1 was measured by commercially-available ELISA (R&D Systems Duoset DY279) at a 1:5 dilution [98]. All assays were performed in duplicate. See protocol in figure 3.1

Urine creatinine and protein were measured by Roche Cobas Creatinine plus (05 6612 7) and Total Protein (11877801) modules respectively. Urine biomarker values were normalised to urine creatinine, as discussed in detail in chapter 2.

R&D Systems Duoset sCD163 (DY1607) and R&D Systems Duoset MCP-1 (DY279) were selected due their prior use in publications as a biomarker in ANCA associated vasculitis [101, 127, 148]. sCD163 Quantikine kits from Abcam (ab155428) and R&D Systems (DC1630) were selected due to their pre-coated plates, hence lack of a manufacturing step. In order for an accredited clinical laboratory to perform and gain NEQAS validation for sCD163 testing the kit must not possess a manufacturing step. Aarhus sCD163 ELISA was selected as it was the original sCD163 ELISA and due to its polyclonality.
1. **Plate preparation (Day 1)**
   a. Dilute capture Ab (1/120)
   b. 83.3ul of capture Ab into 10mls PBS per plate (1 plate=10mls; 10,000/120= 83.3ul)
   c. Pipette 100ul into each well of 96 well plate
   d. Incubate overnight wrapped in clingfilm
2. **Plate preparation (Day 2)**
   a. Wash plate in wash buffer (PBS with tween 20) x 3 washes
   b. Block plates using 300ul of 1% BSA (reagent diluent)
   c. Incubate at room temperature x 1 hour
   d. Wash plate in wash buffer (PBS with tween 20) x 3 washes
3. **Assay procedure (day 2)**
   a. Add 100ul of sample per well in duplicate as per plate plan
   b. Take 7.15 ul (1000 pg) from frozen stock, thaw and add to 1 ml of reagent diluent = 1000 pg/ml (tube 1). Add 500ul of reagent diluent to remaining 6 tubes (tubes 2 to 7) and serially transfer 500 ul from tube 1 to 2, tube 2 to 3, tube 3 to 4, tube 4 to 5, tube 5 to 6, tube 6 to 7 (should end up with 1 ml in tube 7). Mix each tube with pipettor as you go along. [An extra tube, tube 8 (not shown) will just have reagent diluent.
   c. Add 100ul of standard per well from A to H.
   d. Cover with clingfilm and incubate x 2 hours at room temperature
4. Wash plate in wash buffer (PBS with tween 20) x 3 washes
5. Add 100ul of detection Ab
   a. Aliquot 166.6 ul of detection Ab into 10mls of 1% BSA for each plate
   b. Then add 100ul Dab to each well
6. Cover and incubate x 2 hours
7. Wash plate in wash buffer (PBS with tween 20) x 3 washes
8. Streptavidin
   a. Dilute streptavidin 1:40 in 1% BSA
   b. 100ul streptavidin to 10mls BSA
   c. Add 100ul streptavidin to each well
9. Cover and incubate x 20mins at room temp, not in direct light
10. Wash plate in wash buffer (PBS with tween 20) x 3 washes
11. Substrate solution
    a. Add 100ul of TMB to each well (Do not wash!)
    b. Incubate x 20 mins, not in direct light
12. Stop solution
    a. Add 50ul of stop solution to each well
13. Microplate reader at 450nm

*Figure 3.1: uMCP-1 ELISA experimental protocol*
3.4.6. Statistical methodology

3.4.6.1. Descriptive Statistics
Clinical, laboratory data and ELISA results were analysed using GraphPad Prism version 6. Biomarker values were non-normally distributed and are thus reported as median and interquartile ranges. Kruskal Wallis and Mann Whitney U tests were used to analyse determine the significance of associations for non-paired samples. Wilcoxon rank testing was used to analyse determine the significance of associations for paired samples. Correlations were measured using spearman and Pearson correlation coefficients.

3.4.6.2. Optimal Cut Point
To generate receiver-operator characteristic (ROC) curves and determine the most appropriate diagnostic cut off levels I used the OptimalCutpoints package using R Studio version 0.99.902. This analysis is described in detail in chapter 2 Methods.

3.4.6.3. Decision Tree Analysis
Decision tree analyses were performed using R Studio version 0.99.902. rpart, ctree and party packages were used. [17, 18]. Clinical variables of new/worse proteinuria, new/worse hematuria, serum creatinine, c-reactive protein, age, and sex, in addition to the biomarkers of interest, usCD163 and uMCP-1. This analysis is described in detail in chapter 2 Methods.

3.4.6.4. Mixed Effect Modelling
This analysis was performed by Statistician collaborators (Jason Wyse). To confirm that the association of usCD163 and uMCP1 with active renal vasculitis remained robust after taking into account the intra-individual repeated measures employed in this study, the transformed trajectories of CD163 were modelled using mixed effects models. For this purpose, the group with active renal disease was compared to all other groups combined. Within-patient errors were modelled as longitudinal using an autoregressive correlation structure (order 1). The autocorrelation parameter was estimated. This parameter can be thought of as a measure of memory, with a value of zero corresponding to the more usual assumption of independent errors. Model fitting was carried out using the nlme package in R. To quantify the performance of the regression tree outputs in three different prevalence scenarios we used the formula $P_1 = (LR^*P_0)/(1-P_0+LR^*P_0)$, where $P_0$ is pre-test probability and $P_1$ is post-test probability. The three scenarios were: 1. High probability of stable remission ($P_0=5\%$); 2. Clear extra-renal flare with some urinary abnormalities ($P_0=40\%$); 3. High probability renal flare ($P_0=70\%$).
3.5. Results

3.5.1. Demographics

Urine and clinical data were obtained at 320 clinical encounters from 88 patients, with active renal disease present in 39 (12.2%) of the encounters (Tables 3.1 and 3.2).

These encounters were characterised as follows:

1. Patients with renal flare (n=39) and remission encounters selected from either before or after the renal flare visit (n=116 in 39 patients).
2. Patients with non-renal flare (n=48) and remission encounters (n=106 in 48 patients) selected from before or after this flare encounter.
3. Patients with remission encounters only, having presented with renal vasculitis at diagnosis (n=10).

The study population included many patients with GPA (82.9% of total study population) with minimal kidney impairment. Renal flares were often subtle, as indicated by median creatinine levels in remission and during renal flare of 0.9mg/dl (interquartile range, IQR 0.8-1.2) and 1.4mg/dl (1.0-1.8), respectively.
Table 3.1 Demographic and Clinical Characteristics of Study Subjects. The active renal and active non-renal patients contributed sample and clinical data at
the time of flare, as well as 1-3 samples at visits both before and after the flare event. IQR=Interquartile range, GPA=Granulomatosis with polyangiitis,
MPA=Microscopic polyangiitis, EGPA=Eosinophilic granulomatosis with polyangiitis. ANCA=anti-neutrophil cytoplasmic antibody, PR3=proteinase 3,
MPO=myeloperoxidase.
<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th>ACTIVE RENAL</th>
<th>ACTIVE NON-RENAL</th>
<th>REMISSION</th>
<th>P-VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Reactive Protein, mg/dL (IQR)</td>
<td>1.4 (1-1.8)</td>
<td>0.8 (0.7-0.9)</td>
<td>0.9 (0.8-1.2)</td>
<td>0.0028</td>
</tr>
<tr>
<td>Physician global Assessment, 0-10 (SD)</td>
<td>5.7 (1.9)</td>
<td>4.4 (1.3)</td>
<td>0.1 (0.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current IS? N (%)</td>
<td>28 (71.8)</td>
<td>36 (85.1)</td>
<td>169 (72.8)</td>
<td>ns</td>
</tr>
<tr>
<td>Renal Parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR baseline*, median (ml/min), (IQR)</td>
<td>54 (37.2-90.5)</td>
<td>101 (83.1-121)</td>
<td>79 (54.4-98.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Serum Creatinine, median (mg/dL), (IQR)</td>
<td>1.4</td>
<td>0.8</td>
<td>0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine level change, % (IQR)</td>
<td>6.5 (-1.2 to 13.3%)</td>
<td>-2.8 (-13.3 to 7.5%)</td>
<td>0 (-7.2 to 8.3%)</td>
<td>ns</td>
</tr>
<tr>
<td>Dipstick Haematuria, n (%)</td>
<td>26 (66)</td>
<td>12 (25)</td>
<td>65 (27)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dipstick Proteinuria, n (%)</td>
<td>29 (74.5)</td>
<td>6 (12.5)</td>
<td>79 (33.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RBC Casts Present, n (%)</td>
<td>33.3% (13)</td>
<td>2.1% (1)</td>
<td>7 (2.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>New Proteinuria, n (%)</td>
<td>14 (35.9)</td>
<td>2 (4.2)</td>
<td>23 (8.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>New Haematuria, n (%)</td>
<td>12 (30.8)</td>
<td>12 (25)</td>
<td>24 (8.9)</td>
<td>0.0011</td>
</tr>
<tr>
<td>Acute Kidney Injury (%)</td>
<td>10.3</td>
<td>4.2</td>
<td>3.4</td>
<td>0.0813</td>
</tr>
</tbody>
</table>

Table 3.2: Clinical characteristics according to disease activity. Differences between groups measured by Kruskal Wallis test. IQR=Interquartile range, eGFR=estimated glomerular filtration rate (MDRD equation[169]), current IS= currently prescribed immunosuppression. Acute kidney injury determined by AKIN Criteria. *refers to the eGFR at the earliest time point.
3.5.2. Correlation between usCD163, uMCP-1 and Proteinuria

usCD163 and uMCP-1 levels from each ELISA kit were compared. All assays correlated significantly with each other, but correlation coefficients were variable (p<0.0001). See table 3.3 and figure 3.2 and 3.3.

The correlation between usCD163 (R&D Systems Duoset) and uMCP1 was weakly positive ($r^2=0.217$, p<0.001, Figure 3.2 A). There was also a weak correlation of each parameter with urine protein excretion rate ($r^2=0.02$, p<0.001 for usCD163 and $r^2=0.14$, p<0.001 for uMCP1, Figure 3.2.B/C).

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>usCD163 R&amp;D QUANTIKINE</th>
<th>usCD163 AARHUS</th>
<th>usCD163 R&amp;D DUOSET</th>
<th>USCD163 ABCAM QUANTIKINE</th>
<th>UMCP-1 R&amp;D DUOSET</th>
</tr>
</thead>
<tbody>
<tr>
<td>USCD163 R&amp;D QUANTIKINE</td>
<td>-</td>
<td>$r^2=0.256$</td>
<td>$r^2=0.732$</td>
<td>$r^2=0.184$</td>
<td>$r^2=0.342$</td>
</tr>
<tr>
<td>USCD163 AARHUS</td>
<td>$r^2=0.256$</td>
<td>-</td>
<td>$r^2=0.181$</td>
<td>$r^2=0.145$</td>
<td>$r^2=0.204$</td>
</tr>
<tr>
<td>USCD163 R&amp;D DUOSET</td>
<td>$r^2=0.732$</td>
<td>$r^2=0.181$</td>
<td>-</td>
<td>$r^2=0.499$</td>
<td>$r^2=0.217$</td>
</tr>
<tr>
<td>USCD163 ABCAM QUANTIKINE</td>
<td>$r^2=0.184$</td>
<td>$r^2=0.145$</td>
<td>$r^2=0.499$</td>
<td>-</td>
<td>$r^2=0.148$</td>
</tr>
<tr>
<td>UMCP-1 R&amp;D DUOSET</td>
<td>$r^2=0.342$</td>
<td>$r^2=0.204$</td>
<td>$r^2=0.217$</td>
<td>$r^2=0.148$</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.3: Correlation coefficients between measures of usCD163 as measured by four assays and uMCP-1 as measured by Spearman correlation. All associations reached significance with p<0.0001.
Figure 3.2: Correlation between usCD163, uMCP-1 and Proteinuria (A): Correlation between usCD163* and uMCP-1 ($r^2=0.11$). (B): Correlation between urine protein: creatinine ratio (PCR) and uMCP-1 ($r^2=0.14$). (C): Correlation between urine PCR and usCD163* ($r^2=0.12$). Logarithmic scales (log10) are used to compare each assay. *= usCD163 measured by R&D Systems Duoset assay, $p<0.0001$. n=320
Figure 3.3. Graphic representation of correlations between each usCD163 assay and uMCP-1 as measured by Pearson correlation. All correlations reached statistical significance (p<0.0001). Logarithmic scales (log10) are used to compare each assay. n=320
3.5.3. usCD163 and uMCP1 are elevated in the presence of active renal vasculitis flare

Median usCD163 concentrations (R&D Systems Duoset) were higher in patients with active renal vasculitis at 162ng/mmol (IQR 79-337) compared with patients in remission at 44ng/mmol (17-104), and those with active extra-renal vasculitis at 38ng/mmol (7-76), p<0.001, (Figure 3.4A). The area under the ROC curve for usCD163 in distinguishing patients with active renal flare from those without active renal vasculitis was 0.794 (Figure 3.4.B). Median uMCP-1 concentrations were also higher in patients with active renal vasculitis at 10.6pg/mmol (IQR 4.6-23.5) compared with patients in remission at 4.1pg/mmol (2.5-8.4) and those with active extra-renal vasculitis at 4.1pg/mmol (1.9-6.8), p<0.001 (Figure 3.4.C). The area under the ROC curve for uMCP-1 was 0.687 (Figure 3.4D).

usCD163 concentrations as measured by all assays were higher in patients with active renal vasculitis compared with patients in remission and those with active extra-renal vasculitis. See Figure 3.5 and Table 3.4.
Figure 3.4: usCD163 (R&D Systems Duoset) and uMCP-1 levels across the entire cohort (n=320). (A): usCD163 levels in patients with active [A] renal vasculitis (n=39) compared to those in remission [R] (n=233) and those with active non-renal vasculitis (n=48). Upper line denotes cut-off of 143 ng/mmol (optimising sensitivity derived from the Rule Out decision tree (figure 3.8)) and the lower line denotes the Youden index cut-off of 72.9ng/mmol. The boxes in panels (A) and (C) indicate the fraction of positive samples in each group. Kruskal Wallis testing performed to determine significance between groups (B): Receiver operating characteristic (ROC) curve of usCD163 comparing active renal vasculitis to remission and active non-renal vasculitis. (C): uMCP-1 levels in patients with active [A] renal vasculitis compared to those in remission [R] and those with active non-renal vasculitis. Upper line denotes cut-off of 20 ng/mmol (optimising sensitivity derived from the Rule Out decision tree (figure 4)) and the lower line denotes the Youden index cut-off of 10ng/mmol. (D): ROC curve of uMCP-1 comparing active renal vasculitis to remission and active non-renal vasculitis. ****p<0.0001, ***p<0.001
Figure 3.5: usCD163 levels from each usCD163 across the cohort (n=320) active renal vasculitis (n=39) compared to remission (n=48) and active non-renal vasculitis (n=233). (A): usCD163 levels as measured by Abcam Quantikine ELISA. (B): usCD163 levels as measured by R&D Systems Duoset ELISA. Upper line denotes cut-off of 143 ng/mmol (optimising sensitivity derived from the Rule Out decision tree (figure 3.4.9)) and the lower line denotes the Youden index cut-off of 72.9ng/mmol. (C): usCD163 levels as measured by Aarhus ELISA. (D): usCD163 levels as measured by R&D Systems Quantikine ELISA. Bar and Whiskers 10-90th centiles. Line represents median. Kruskal Wallis testing performed to determine significance between groups ****p<0.0001, ***p<0.001
<table>
<thead>
<tr>
<th>Test</th>
<th>Median (IQR)</th>
<th>Median (IQR)</th>
<th>AUC</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>USCD163 R&amp;D DUOSET NG/MMOL</td>
<td>167 (IQR 81.6-359.6)</td>
<td>42.73 (IQR 16.29-101.4)</td>
<td>0.794</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>USCD163 R&amp;D QUANTIKINE PG/MMOL</td>
<td>280.2 (IQR 140.7-508.3)</td>
<td>98.6 (IQR 52.6-218.7)</td>
<td>0.744</td>
<td>0.0004</td>
</tr>
<tr>
<td>USCD163 ABCAM QUANTIKINE PG/MMOL</td>
<td>6.35 (IQR 2.43-14.85pg/mmol)</td>
<td>1.6 (IQR 0.6-3.8)</td>
<td>0.803</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>USCD163 AARHUS UG/MMOL</td>
<td>4.94 (IQR 3.56-7.25)</td>
<td>3.35 (IQR 2.63-4.71)</td>
<td>0.737</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 3.4. usCD163 levels in active and remission renal vasculitis (both remission and active non-renal vasculitis) based on each experimental assay. Values are reported as median and interquartile ranges. Groups compared using Mann Whitney U test. IQR=Interquartile range, AUC = area under the curve.
3.5.4. usCD163 and uMCP levels do not differ based on ANCA disease subtype

There was no difference in either usCD163 (R&D Systems Duoset) or uMCP-1 levels in active renal vasculitis when stratified by disease subtype of GPA compared to MPA, with median usCD163 values of 144.9ng/mmol (IQR 62.4-307.2), 450ng/mmol (249.9-475.3), respectively, p=0.4 and median uMCP-1 values of 7.8pg/mmol (3.7-21.8) and 10.8pg/mmol (4.9-39.6), respectively, p=0.8 (figure 3.6 A, 3.6 B).

![Box and whiskers plot](image)

**Figure 3.6:** (A): Box and whiskers plot (10-90\textsuperscript{th} centiles) of levels of usCD163 (R&D Systems Duoset) in renal vasculitis flare (n=39) and remission (n=281) as stratified by diagnostic subgroup. MPA= microscopic polyangiitis (clear boxes), GPA=granulomatosis with polyangiitis (shaded grey boxes). (B): Box and whiskers plot (10-90\textsuperscript{th} centiles) of levels of uMCP-1 in renal vasculitis flare and remission as stratified by diagnostic subgroup. Shaded grey boxes = granulomatosis with polyangiitis (GPA), Clear boxes=microscopic polyangiitis (MPA). GPA flare n=33, MPA flare n=3, GPA Non-Flare n=169, MPA Non-flare n=16. Groups compared using Kruskal Wallis testing. NS= non-significant.
3.5.5. usCD163 and uMCP concentrations are not elevated pre-flare

usCD163 (R&D Systems Duoset) levels were not increased prior to renal flare with median pre-flare levels of 1.9ng/mmol (IQR 0.4-5.3). See Figure 3.7 (A).

3.5.6. usCD163 and uMCP levels are not altered by urinalysis findings

When those with active renal vasculitis were stratified by urinary findings there were no significant differences in usCD163 levels (R&D Systems Duoset). usCD163 concentrations were 184.6ng/mmol (IQR 61.3-293.2) in those with new hematuria, 132.6ng/mmol (47.3-352.5) in those with no new hematuria, 190.5ng/mmol (86.9-421) in those with new proteinuria and 112.9ng/mmol (42-294.1) in those with no new proteinuria, p value of 0.531 (Figure 3.7 (B)). sCD163 values reported as measured by R&D Systems Duoset ELISA.

Figure 3.7: (A): Paired levels of usCD163 (R&D Systems Duoset) at pre-flare visit and at renal vasculitis flare visit (n=39). (B): Levels of usCD163 in those with active renal vasculitis stratified by those with new haematuria (n=12), no new haematuria (n=20), new proteinuria (n=14) and no new proteinuria (n=20), (respectively, p=0.5 and 0.2) as measured by Kruskal Wallis testing.
3.5.7. Derivation of Optimal Diagnostic Ranges

We derived a series of potential diagnostic ranges based on calculations using the Youden index which maximising both sensitivity and specificity, maximising sensitivity and maximising specificity,

The proposed diagnostic cut points in this setting for usCD163 (R&D Systems Duoset) and uMCP-1 were 72.9ng/mmol and 10.0pg/mmol, respectively. Given that the R&D Systems Duoset assay diagnostic ranges provided the most optimal diagnostic cut off level (based on optimal criterion) we selected that assay for use in further studies and in the primary data presented in this chapter. See Table 3.5.
Table 3.5: Biomarker characteristics. In each case, the ability of the biomarker to correctly classify patients with active renal vasculitis was tested within a heterogenous cohort also including patients in remission and those with active extra-renal disease. *The decision tree cut-points were determined by recursive partitioning. Sens= sensitivity, Spec= specificity, PPV=positive predictive value, NPV=negative predictive value, PLR=positive likelihood ratio, NLR=negative likelihood ratio, AUC=area under the receiver operator characteristic curve. usCD163= urine soluble CD163, uMCP-1= urine monocyte chemoattractant protein 1.

<table>
<thead>
<tr>
<th>CUT POINT METHOD</th>
<th>DIAGNOSTIC LEVEL</th>
<th>SENS</th>
<th>SPEC</th>
<th>PPV</th>
<th>NPV</th>
<th>PLR</th>
<th>NLR</th>
<th>AUC</th>
<th>OPTIMAL CRITERION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>USCD163</strong> R&amp;D Systems DUOSET</td>
<td>Youden</td>
<td>72.9 ng/mmol</td>
<td>79.5%</td>
<td>67.3%</td>
<td>25.2</td>
<td>95.9</td>
<td>2.4</td>
<td>0.3</td>
<td><strong>0.79</strong></td>
</tr>
<tr>
<td><strong>USCD163</strong> R&amp;D Systems QUANTIKINE</td>
<td>Youden</td>
<td>135.3pg/mmol</td>
<td>84.2%</td>
<td>58.6%</td>
<td>21.6</td>
<td>96.4</td>
<td>2.0</td>
<td>0.3</td>
<td><strong>0.74</strong></td>
</tr>
<tr>
<td><strong>USCD163</strong> Abcam QUANTIKINE</td>
<td>Youden</td>
<td>1724.8pg/mmol</td>
<td><strong>94.4%</strong></td>
<td>54.2%</td>
<td>20.7</td>
<td><strong>98.7</strong></td>
<td>2.1</td>
<td>0.1</td>
<td><strong>0.79</strong></td>
</tr>
<tr>
<td><strong>USCD163</strong> AARHUS</td>
<td>Youden</td>
<td>4.28ug/mmol</td>
<td>66.7%</td>
<td><strong>70.8%</strong></td>
<td>22.4</td>
<td>94.4</td>
<td>2.3</td>
<td><strong>0.5</strong></td>
<td><strong>0.73</strong></td>
</tr>
<tr>
<td><strong>UMCP-1 R&amp;D Systems DUOSET</strong></td>
<td>Youden</td>
<td>10.0 pg/mmol</td>
<td>53.9%</td>
<td>82.2%</td>
<td>29.6</td>
<td>92.8</td>
<td>3.0</td>
<td>0.6</td>
<td><strong>0.68</strong></td>
</tr>
<tr>
<td><strong>DECISION TREE</strong></td>
<td>Recursive Partitioning</td>
<td>CD163 &gt;143 ng/mmol*</td>
<td>41.0%</td>
<td>97.9%</td>
<td>72.7</td>
<td>92.3</td>
<td>19.2</td>
<td>0.6</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCP-1 &gt;20 pg/mmol*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>New/Worse Proteinuria</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
3.5.8. Longitudinal Modelling of usCD163 and uMCP1

To allow visualisation of usCD163 trends over time with reference to renal and extra-renal vasculitis flares, serial measurements of usCD163 and uMCP1 are depicted in Figure 3.8. usCD163 as measured by R&D Systems Duoset assay.
Figure 3.8: Levels of usCD163 in serial patient samples plotted over time from date of diagnosis (depicted as time 0). In each case samples taken at the time of active vasculitis are marked in black. (A): usCD163, black circles depicting active renal vasculitis (n=39) with clear circles denoting remission vasculitis (n=113) (B): usCD163, black circles depicting active non-renal vasculitis (n=48) with clear circles denoting remission vasculitis (n=114). (C): uMCP1, black circles depicting active renal vasculitis (n=39) with clear circles denoting remission vasculitis (n=113). (D): uMCP1, black circles depicting active non-renal vasculitis (n=48) with clear circles denoting remission vasculitis (n=114).
3.5.9. Modelling of longitudinal usCD163 and uMCP1 values against disease activity

To validate the association between usCD163 and uMCP1 levels and active renal vasculitis, while taking into account the intra-individual repeated measures in the study dataset, we generated and fitted a mixed-effects model that incorporated both clinical co-variates and accounted for the serial nature of the measurements. This demonstrated that the only parameters significantly associated with the presence of active renal flare were usCD163 and uMCP1 (Table 3.6). This confirmed that the presence of active renal flare was a significant determining factor in observing elevated usCD163 and uMCP1 levels (Table 3.7).

<table>
<thead>
<tr>
<th></th>
<th>USCD163 Coefficient value</th>
<th>USCD163 p-value</th>
<th>UMCP1 Coefficient value</th>
<th>UMCP1 p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RENAL FLARE</td>
<td>4.362</td>
<td>&lt;0.0001</td>
<td>1.850</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NO RENAL FLARE</td>
<td>-0.881</td>
<td>0.002</td>
<td>-0.332</td>
<td>0.016</td>
</tr>
<tr>
<td>GENDER (MALE)</td>
<td>0.105</td>
<td>0.747</td>
<td>-0.010</td>
<td>0.949</td>
</tr>
<tr>
<td>AGE</td>
<td>-0.001</td>
<td>0.916</td>
<td>0.005</td>
<td>0.295</td>
</tr>
<tr>
<td>ANCA +</td>
<td>-0.100</td>
<td>0.798</td>
<td>0.074</td>
<td>0.692</td>
</tr>
<tr>
<td>NEW PROTEINURIA</td>
<td>0.085</td>
<td>0.777</td>
<td>0.141</td>
<td>0.328</td>
</tr>
<tr>
<td>NEW HAEMATURIA</td>
<td>0.131</td>
<td>0.630</td>
<td>-0.136</td>
<td>0.319</td>
</tr>
</tbody>
</table>

Table 3.6: Mixed effects modelling of novel biomarkers in longitudinal samples. The Coefficient value for renal flare gives the typical level of transformed biomarker for a female, ANCA-negative renal patient in a renal flare episode. This also represents the baseline level for the other explanatory variables i.e., it represents the typical level for females, ANCA-negative, no new proteinuria and no new haematuria. The remaining Coefficient values represent differences from this baseline for a patient with the given characteristic. For example, a male patient in remission will typically have a transformed sCD163 level of 3.586 (= 4.362 - 0.881 + 0.105). The p-values confirm that renal flare episodes are the only significant characteristic in explaining the transformed biomarker values through mixed effects modelling. ANCA= anti-neutrophil cytoplasmic antibody. usCD163 measured by R&D Systems Duoset Assay. N=320
3.5.10. Combining usCD163 and uMCP1 with existing biomarkers improves diagnostic fidelity

To determine whether combining the two biomarkers enhances diagnostic fidelity we generated a recursive partitioning tree that sought to maximise distinction between “active renal vasculitis” and “no active renal vasculitis” by sequentially adding parameters that improved prediction. In addition to usCD163 and uMCP1, we included new/worse onset proteinuria, new/worse haematuria, CRP and rise in creatinine level in the model. This was an attempt to place usCD163 and uMCP-1 within the context of existing clinical approaches to identifying renal flare. In all models tested, usCD163 was identified as the first node in the tree (Table 3.7). A subsequent testing strategy in usCD163 positive individuals that incorporated uMCP1 and then new onset proteinuria maximised diagnostic fidelity and increased specificity to 97.9% and positive likelihood ratio to 19.2.

As these biomarker statistics are intimately dependent on pre-test probability (Figure 3.9B), and to provide an estimate of real world diagnostic accuracy, we estimated post-test probability of confirming or excluding renal flare in three scenarios: (i) some degree of proteinuria or haematuria but nothing else to suggest active disease (pre-test probability of active renal vasculitis 5%), (ii) clear extra-renal flare with some degree of proteinuria or haematuria (40%) and (iii) high clinical probability of renal flare (70%). In the latter two scenarios, the post-test probability of a positive result following the recursive tree algorithm was 93% and 98% respectively (Table 3.7), potentially obviating the need for kidney biopsy in these settings.

<table>
<thead>
<tr>
<th>PRE-TEST PROBABILITY (ODDS)</th>
<th>POST-TEST PROBABILITY OF HAVING RENAL FLARE IF TEST POSITIVE</th>
<th>POST-TEST PROBABILITY OF NOT HAVING RENAL FLARE IF TEST NEGATIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 (0.052)</td>
<td>0.51</td>
<td>0.03</td>
</tr>
<tr>
<td>0.40 (0.67)</td>
<td>0.93</td>
<td>0.22</td>
</tr>
<tr>
<td>0.70 (2.33)</td>
<td>0.98</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 3.7 Post-decision tree probability in a series of hypothetical scenarios. Using the calculated positive likelihood ratios and negative likelihood ratios, post-test probability was compiled for situations where the pre-test probability varied between 5%, 40% and 70%. usCD163 as measured by R&D Systems Duoset assay.
Figure 3.9: Recursive partitioning was applied to the dataset to identify variables that maximised correct classification of patients. In the resulting decision tree (A), variables are depicted in ovals. Black cells indicate tree termination with classification of the patient as having active renal disease (n=22), whereas grey cells indicate tree termination with the patient classified as not having active renal disease (n=298). CC=correctly classified, AR=active renal vasculitis, RR=remission renal vasculitis / active non-renal vasculitis. (B) Likelihood ratio plot depicting the change in post-test probability (Y-axis) following application of the algorithm in a range of pre-test probability scenarios (X-axis). N=320
3.6. Discussion

It remains difficult to distinguish active vasculitis in the kidney from other causes of renal injury, such as infection, acute tubular necrosis, allergic interstitial nephritis, and paraprotein-mediated kidney disease. We examined the ability of testing for usCD163 and uMCP1 to identify subtle active renal vasculitis in a large multi-centre cohort. We found that both biomarkers were elevated in the presence of active renal vasculitis, with usCD163 displaying a slightly larger area under the ROC than uMCP1. The low degree of correlation between usCD163 and uMCP1 highlights the fact that each reflects a different component of the glomerular macrophage recruitment and activation pathway. In this setting of subtle clinical evidence of active renal vasculitis, the moderate clinical utility of each biomarker in isolation was enhanced by using usCD163 to exclude active vasculitis, and then grouping the “usCD163+ / uMCP1+” and “usCD163 / new proteinuria” as the two “Yes” nodes, giving a positive LR of 19. This decision tree approach reflects more accurately the use of novel biomarkers in clinical practice.

We have previously identified usCD163 as a promising biomarker in glomerulonephritis [127], and it is similarly emerging as a potentially useful test in lupus nephritis [148]. In our prior study, we observed excellent biomarker characteristics, with positive LR for usCD163 alone of 20.7, negative LR of 0.17 and area under the ROC curve of 0.93. However, most patients with active disease in the prior study were recruited at the time of diagnosis, had severe disease (often mandating dialysis), and included patients with anti-glomerular basement antibody disease. A subset of the current sample set has been tested previously for uMCP1 (among other biomarkers) by Lieberthal et al [99]. Although a similar degree of elevation in uMCP1 was observed in active renal disease, this study differed from the current one in identifying positive uMCP1 results in active non-renal disease. A slightly different assay was used (R&D pre-coated Quantikine DCP00), versus the R&D Duoset DY279 in the current study, which may have contributed to the divergent results. Of note, Tam et al reported low uMCP1 levels in patients with active non-renal disease using the same Duoset assay used in the current study, although there were only 6 patients in this group [98].

To assess the true clinical utility of these biomarkers, we chose to test them in a more clinically challenging and relevant environment: seemingly mild (or early) renal flare. In this setting, positive/negative likelihood ratio values of 2.4/0.3 and 3.0/0.6 for usCD163 and uMCP1 respectively suggest borderline clinical utility and reflect the fact that these patients may require other methods of definitive diagnosis such as renal biopsy. However, when unbiased recursive tree partitioning was
applied to the biomarkers alongside conventional markers, usCD163 and uMCP1 emerged as the first and second nodes respectively, with new onset proteinuria adding additional fidelity. The positive and negative likelihood ratios of the algorithm were 19.2 and 0.6 respectively, indicating high potential clinical value. In the setting of intermediate or high pre-test clinical probability, this algorithm provided an estimated post-test probability in excess of 90%. It is not uncommon currently for such patients to undergo kidney biopsy; our results suggest that, in many cases, it may not be necessary to perform a biopsy, and to embark on appropriate treatment based on the algorithm results.

When considering the benefits and caveats of biomarker interpretation we must take into account comparison with renal biopsy. usCD163 and uMCP-1 measurement is non-invasive, inexpensive (estimated cost per sample $50), serial testing may be used to monitor response to treatment, samples can be sent from remote locations who do not have facilities to perform renal biopsy. Caveats include potential for false positive or negative results. This risk is mitigated by use of two biomarkers, inclusion in a diagnostic algorithm and clinical follow up. Biopsy provides the gold standard for diagnosis of renal disease and allows assessment of not only current disease activity but also of chronicity. The Berden classification of renal vasculitis provides prognostic information regarding long term renal outcomes[33]. This has been validated in biopsies at the time of diagnosis and is not informative regarding the risk of future renal vasculitis flare. Repeat biopsy for monitoring of potential flare activity is impractical and is not currently standard of care. There is also significant risk and cost associated with renal biopsy with an overall estimated risk of major bleeding of 2.2-2.6% and estimated outpatient biopsy cost of $4000 with additional costs incurred if inpatient biopsy or admission for management of complications is required. [171]

Although both usCD163 and uMCP1 are linked to the recruitment of activated macrophages to the injured glomerulus, these markers reflect different stages of this process. sCD163 is actively shed from the surface of glomerular macrophages in the presence of pro-inflammatory peptides, particularly ADAM17/TACE [130]. The level of usCD163 is presumed to reflect the burden of activated macrophages in situ. On the other hand, MCP-1 (CCL2) is a chemokine that specifically attracts blood monocytes and tissue macrophages to its source, via interaction with its cell surface receptor, CCR2. Renal cells produce MCP-1 in response to various pro-inflammatory stimuli. Indirectly, MCP-1 has the potential to drive renal fibrosis by macrophage recruitment, and via direct induction of a fibrotic response in glomerular mesangial cells. Therefore, it is conceivable that MCP-1 appears in the urine earlier in the process that sCD163, providing a rationale for testing for both proteins when clinical uncertainty remains after use of traditional biomarkers. This premise was supported by our finding of
a low degree of correlation between the two biomarkers in urine. The moderate correlation between urine PCR and the biomarkers suggests that, in cases of heavy proteinuria, there may be leakage of the sCD163 or MCP1 from serum into urine, but that this is a minor consideration overall, with most of the measured protein coming from the inflamed nephron.

The principal limitation of our study is the lack of gold standard kidney biopsy to diagnose active renal vasculitis. This is likely to have led to misclassification of some cases and may account for some of the high values observed in remission and active extra-renal disease. For example, in our prior published work examining usCD163, using a Youden cut-point, the test was positive in 3.7%, <1%, and 1.8% of patients in remission, those with active extra-renal vasculitis, and healthy controls respectively. However, we observed a positive test in 25% and 34.7% of patients in remission and with active extra-renal disease using a similar cut-point method in the current cohort. Even using a cut-point that maximised specificity, much higher false positive rates were observed in the current study (18.0-18.7%). In some of these cases, the patient was classified by the treating physician as having active extra-renal vasculitis, and satisfied BVAS/WG criteria for same, but had new-onset haematuria by dipstick and/or slight elevation in creatinine level on the visits after treatment. One may infer that these cases may also have had subtle renal vasculitis. The effect of this diagnostic uncertainty on biomarkers’ performance is not known. In addition, the use of ROC curve analysis has variable utility in datasets with repeated measures, but it does represent the best option for describing biomarker performance. An important additional limitation is that we have not included a validation cohort for the classification algorithm, and it should be noted that the high likelihood ratio is derived from a relatively small number of cases (22).

The presence of active BVAS/WG items informed the definition of patient groups with active renal and extra-renal vasculitis. These were recorded by the investigator physician only if they were considered, in their clinical judgement, to be due to active vasculitis. This clinical judgement was disregarded for the purpose of the decision tree, so that changes in serum creatinine, proteinuria and hematuria were included at face value. When treated in this manner, usCD163 and uMCP1 were selected in an unbiased fashion as the first and second nodes in the model, and that proteinuria was the only clinical marker that added utility, while hematuria, creatinine level, and CRP did not. This analysis does not include the physician assessment of whether observed changes were due to active vasculitis; however, in clinical practice, such an assessment would be added on top of such a decision tree, as it would be added on top of the individual parameters.
In summary, an algorithm that combines usCD163 with uMCP-1 and new-onset proteinuria may aid diagnosis of subtle renal flare in AAV. The biomarkers of the future will be incorporated into machine learning algorithms that incorporate existing clinical parameters and additional variables, such as environmental changes. We have taken the first steps in the vasculitis field to realize this novel approach. The ability of this approach to reduce the need for kidney biopsy will need to be tested in a prospective clinical study.
Chapter 4:

Urinary Soluble CD163 is Diagnostic of Renal Vasculitis Flare
4.1 Abstract

Background: ANCA-associated vasculitis (AAV) is a rare, chronic autoimmune disease. 70% of patients ultimately develop kidney involvement with glomerulonephritis and 26% of patients with AAV develop ESKD within 5 years of diagnosis [1, 2]. There is an urgent need for non-invasive tools to detect active renal inflammation to prevent irreversible end organ damage including end stage kidney disease. usCD163 has been shown to be elevated in active renal vasculitis at diagnosis, in subtle renal vasculitis flare and in lupus nephritis. To date, however, no prospective studies on the diagnostic utility of usCD163 have been performed.

Methods: Patients with a known diagnosis of AAV were prospectively recruited at the time of potential renal vasculitis flare from a multicentre longitudinal cohort. Physician initial clinical impression was recorded as high probability of flare or possible flare at the time of sampling. An independent adjudication committee that was blinded to usCD163 results, made a final adjudication on renal vasculitis flare using renal biopsy if available or BVAS major renal criteria. usCD163 levels were measured by clinical and research grade ELISA with results normalised to urine creatinine.

Results: 84 patients were included in final analysis, 31 (36.9%) were adjudicated as having a renal vasculitis flare. Of those with RV flare, the median usCD163 concentration was 805.8ng/mmol creatinine (IQR 439 to 1705ng/mmol) as measured by clinical grade ELISA. In non-RV flare the median usCD163 concentration was 100.0ng/mmol creatinine (IQR 52-174ng.mmol, p<0.0001). The area under the curve for detection of active renal vasculitis was 0.947 (95% CI 0.90-0.99, p<0.0001). A diagnostic cut-off that optimized the biomarker’s differentiating ability when equal weight is given to sensitivity and specificity matched our previously defined optimal cut-off (derived using Youden index at 253ng/mmol), giving 96.8% sensitivity and 86.8% specificity, likelihood ratio 6.14.

Conclusion: usCD163 is elevated in renal vasculitis flare and remains low in flare mimics in a prospectively sampled cohort. usCD163 has been validated as a diagnostic test for active renal vasculitis.
Urinary Soluble CD163 is Diagnostic of Renal Vasculitis Flare

**Methods**

**Results**

<table>
<thead>
<tr>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>PPV</th>
<th>NPV</th>
<th>FP/FPN</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>USCD163 &gt;253</td>
<td>NG/MMOL</td>
<td>96.8%</td>
<td>86.8%</td>
<td>81%</td>
<td>97.8%</td>
</tr>
<tr>
<td>KIDNEY BIOPSY</td>
<td>88.2%</td>
<td>(63.6-98.6%)</td>
<td>100%</td>
<td>(15.8-100%)</td>
<td>100%</td>
</tr>
<tr>
<td>RBC CASTS</td>
<td>30%</td>
<td>(6.7-65.2%)</td>
<td>78.3%</td>
<td>(56.2-92.5%)</td>
<td>37.5%</td>
</tr>
</tbody>
</table>

**Conclusions:** usCD163 is **elevated in renal vasculitis flare** and remains **normal in flare mimics**. usCD163 has similar biomarker characteristics to renal biopsy and is superior to current non-invasive clinical tools.
**4.2. Introduction**

ANCA-associated vasculitis (AAV) is a rare, chronic autoimmune disease characterised by periods of remission and relapse. AAV is frequently associated with kidney involvement characterised by focal necrotising glomerulonephritis. There is an urgent need for non-invasive methods of detection of active renal inflammation to prevent irreversible end organ damage including end stage kidney disease.

Up to 40% of patients experience a relapse within the first five years after initial diagnosis[15]. Overall, up to 70% of patients ultimately develop kidney involvement with glomerulonephritis[32, 33]. Collectively, glomerulonephritis (including renal AAV) is the second commonest cause of end stage kidney disease (ESKD) [172-174]. Up to 26% of patients with AAV develop ESKD within 3-5 years of diagnosis[175, 176]. ESKD outcomes are poor with 5-year survival on dialysis of 55% and annual costs of $36 billion[177]. Clinical presentation with renal vasculitis can range from asymptomatic microscopic haematuria with preserved glomerular filtration rate to dialysis requiring acute kidney injury. It is estimated that 43% patients with renal AAV progress to ESKD without clinically detected renal vasculitis activity[175].

The gold standard for diagnosis of renal involvement in AAV is kidney biopsy. However, kidney biopsy is invasive with hemorrhage in up to 11% and major bleeding requiring blood transfusion or embolization in up to 0.9% and estimated per procedure healthcare costs of $1394-1800 USD [88, 89, 91, 171, 178]. Sequential procedures are uncommon, in routine clinical practice. Of those recruited to a randomised controlled trial of AAV induction therapy only 44% of those with renal flare underwent diagnostic confirmation with a kidney biopsy[46, 47]. Factors that are associated with increased biopsy risk include advanced age, reduced glomerular filtration rate (GFR), hypertension and those receiving plasma exchange, all of which are common in AAV[179]. Kidney biopsy is not required for diagnosis of renal AAV flare in clinical guidelines or trial protocols[50, 93].

Alternate clinical tools such as Birmingham vasculitis activity score (BVAS) are not diagnostic criteria, and were designed as standardised scoring systems based on expert opinion [40]. Non-invasive clinical tools for detection of active renal inflammation include serum creatinine, hematuria, proteinuria and red blood cell (RBC) casts. However, these biomarkers lack sensitivity for detection of early renal structural and functional loss and do not reliably differentiate active vasculitis from flare mimics such as sepsis, urinary tract infections, and adverse drug reactions. Changes in serum creatinine do not distinguish active renal vasculitis from other aetiologies, and there may be substantial loss of function
prior to an observed rise[81]. Persistent hematuria is common, portends an increased risk of renal relapse but in isolation lacks specificity for detection of active renal vasculitis[79]. RBC cast assessment is limited by inter-operator variability, time sensitivity and is not generally available outside centres of expertise [80, 180-183]. Inflammatory markers such as c-reactive protein are limited by lack of specificity, as they are non-specific and elevated in potential vasculitis mimics including infection[184, 185]. ANCA measurement has limited value in identifying relapse as rising titers can occur in up to 40% of patients without new or worsening disease activity, and relapse can occur without an ANCA rise[24, 84].

Urine is an ideal biospecimen as it is readily available, levels of biomarkers in urine may reflect local inflammation and retrospective studies raise the possibility that they may remove the need for kidney biopsy. [130, 186, 187]. CD163 functions as a monocyte/macrophage-specific scavenger receptor for haemoglobin–haptoglobin complexes[109]. The soluble form (sCD163) is present in high levels in serum, with proposed function in innate defense by reversibly binding bacteria and free hemoglobin [110, 188]. Urine soluble CD163 possesses many ideal biomarker properties: very low levels in health, stability at room temperature (for up to 7 days) and ease of measurement by ELISA[113, 127]. Prior work in retrospective cohorts has shown usCD163 to be a biomarker of active renal vasculitis at diagnosis, subtle renal vasculitis flare and lupus nephritis. [97, 127, 147, 148, 161]. Elevated levels in the urine are due to local production from macrophages within crescents in crescentic glomerulonephritis. To date, no study has prospectively examined the diagnostic utility of usCD163 in the diagnosis of renal vasculitis flare. We therefore conducted a multi-centre prospective observational study of the utility of urine sCD163 in the diagnosis of renal AAV flare using a clinical grade assay.
4.3 Aims

4.3.1 Hypothesis

We hypothesise that usCD163 concentrations are elevated in the renal vasculitis flare and remain low in renal vasculitis flare mimics.

4.3.2 Research Questions

Does urine sCD163 level distinguish active renal vasculitis from other causes of clinical deterioration in patients with a prior diagnosis of ANCA vasculitis?

A secondary objective is to build a model that compares the precision of urine sCD163 alone with sequential addition of existing urine biomarkers including hematuria and proteinuria, mirroring what would happen in clinical practice.

a. usCD163 is elevated in renal vasculitis flare
b. usCD163 is not elevated in renal vasculitis flare mimics
c. usCD163 is superior to current non-invasive diagnostic tools
4.4. Methods

4.4.1 Patients and clinical data collection

Patients were enrolled in this prospective observational study via the Rare Kidney Disease (RKD) Biobank based at Trinity College Dublin from seven referral centres in Ireland. All enrolled patients met the American College of Rheumatology (ACR) criteria for GPA modified to include ANCA, or the Chapel Hill Consensus Conference definition of MPA, or ACR Classification Criteria for EGPA (adapted so that biopsy proof of small-vessel vasculitis was not required).

All patients were enrolled using protocols approved by ethics committees of the participating sites and written informed consent was obtained and documented in keeping with the Declaration of Helsinki. These patients have consented to providing up to 5 sample sets over a period of 5 years and therefore a repeat consent process will not be required.

Treating Physicians notified the study team (Professor Little, Dr. Moran) of potential recruits. Prior recruitment to the RKD Registry was confirmed. If not previously recruited then formal recruitment including informed consent and biospecimens was obtained. Clinical information was obtained regarding current and previous measures of disease activity (see 4.4.2), urine samples were collected (see 2.3.1). At one month treating Physician was contacted and information regarding subsequent clinical diagnosis and treatment was obtained.

Final diagnostic category (renal vasculitis flare or renal vasculitis remission) was adjudicated by an expert committee. The expert committee was composed of three Nephrologists (Professor Mark Little, Dr. Sarah Moran, Dr. Jennifer Scott) and one Immunologist (Dr. Niall Conlon), who reviewed the clinical information in detail including chart reviews and requests for clarification from treating Physicians. The committee was blinded to usCD163 results and adjudicated as to the cause of the potential flare encounter. Active vasculitis was considered to be present if kidney biopsy was revealed active renal vasculitis as defined by pauci-immune crescentic glomerulonephritis or BVAS score >0 with one or more major renal items, including the presence of haematuria by urine dipstick or a rise of serum creatinine of greater than 30%.
4.4.2 Measures of vasculitis disease activity

At the time of potential AAV renal flare treating Physicians were asked to provide the following information clinical impression in addition routine clinical measurements including urine microscopy, dipstick urinalysis, urine protein: creatinine ratio, serum ANCA, creatinine and c-reactive protein. See figure 4.1. Renal biopsy was performed for clinical indication as per treating Physician discretion. Physicians were asked whether they at the time of clinical review they felt that renal vasculitis flare was not possible, possible or highly probable. At one month Physicians were asked their clinical impression of that encounter (i.e. renal flare, systemic flare, other aetiology). Clinical information was retrospectively collected from the last clinical encounter prior to the potential flare incident and prospectively from the next visit following it. Vasculitis disease activity was recorded using the Birmingham Vasculitis Activity Score (BVAS)[168].
Figure 4.1: Clinical information proforma distributed to treating Physicians to obtain relevant clinical information at time of clinical encounter.
4.4.3 Collection and storage of urine samples

As per Methods chapter 2, section 2.3.1.

4.4.4 usCD163 Assay Methodology

To maximise translation into clinical practice we utilised a pre-coated diagnostic grade sandwich sCD163 ELISA (Euroimmun GMBH). This ELISA was developed and optimised for the measurement of urinary soluble CD163. In our prior work we used a capture ELISA (R&D Systems, human sCD163 Duoset, DY1607 ELISA). This assay involves a manufacturing step with capture antibody coating and therefore does not meet the required standards for a diagnostic clinical grade test[97, 127, 147, 148]. Euroimmun developed a prototypic diagnostic grade usCD163 assay which lacks the manufacturing step of the aforementioned Duoset R&D ELISA and bears the CE marking of approval. CE Marking on a product is a manufacturer's declaration that the product complies with the essential requirements of the relevant European health, safety and environmental protection legislation. To assess inter-assay performance, we measured usCD163 measured using both ELISA methods in a subset of patients.

We normalized the urine sCD163 level to the creatinine level as determined by a modified Jaffe technique. See Methods chapter 2, section 2.5.1 and 2.5.4.
4.4.5 Statistical Methodology

4.4.5.1 Target Recruitment:

Target Recruitment: Of those that present with a clinical decline potential renal vasculitis flare, we estimated that 25% will have an actual flare and 75% will have an alternative diagnosis (i.e., 1:3 ratio). Based on our previous work, we expected an effect size \((d = (m_1 - m_2)/\text{common error variance})\) of 0.8. Therefore, to detect a difference in urine sCD163 value at a significance level of 0.05 with a power of 80% between those with and without flare, we calculated that we needed to recruit 104 patients (to include 26 with true flare). Within the ten recruiting centres over 400 patients with ANCA vasculitis have previously been recruited to the RKD registry and biobank. We expected 75 of this cohort to meet the inclusion criteria each year, which equates to 113 patients over 1.5 years, of whom 28 (25%) will have a renal relapse with active renal vasculitis. Interim analysis was performed at 9 months when 44 subjects were recruited patients. In the interim analysis 32% (14) were adjudicated as renal flare, and 68% (31) were adjudicated as no renal flare. usCD163 concentration (as measured by R&D Duoset ELISA) was higher in renal flare than remission with median concentrations of 469.6 ng/mmol (IQR 363.8-2974 ng/mmol) and 25.4ng/mmol (IQR 3.9-80.8 ng/mmol, \(p<0.0001\)). Based on these results showing a clear difference recruitment was halted based on those results despite less than 113 patients recruited. Actual Recruitment: Screening targets were achieved with a total of 121 recruited over 23 months with 85 patients. (See figure 4.2).

![Screening Targets and Achievements](image)

*Figure 4.2: Screening: Blue line represents actual subjects screened and orange lines represents target screening.*
4.4.5.2 Descriptive Statistics

Clinical, laboratory data and ELISA results were analysed using GraphPad Prism version 6. Biomarker values were non-normally distributed and are thus reported as median and interquartile ranges. Kruskal Wallis, Mann Whitney U tests, chi squared, unpaired t tests were used to determine the significance of associations for non-normally and normally distributed data. Correlations were measured using Spearman correlation coefficient.

4.4.5.3. Diagnostic Cut-Off Derivation

Optimal cut off ranges were performed using R Studio version 0.99.902. To generate receiver-operator characteristic (ROC) curves and determine the most clinically relevant diagnostic cut-offs we used the OptimalCutpoints package. The Youden calculation was selected to maximize the sum of sensitivity and specificity[150]. See Methods chapter 2, section 2.6.1.

4.4.5.4. Net reclassification index calculation

Commonly used statistical methods to estimate prediction of risk and relative improvements in risk estimation with area under the curve (or c-statistic) one of the most commonly used methods. The net reclassification index (NRI) is an attractive concept as it reports reclassification[142]. I calculated NRI based on Pencina’s original formula[142].

4.4.5.5. Decision Tree Analysis

Decision tree analyses were performed using R Studio version 0.99.902. rpart, ctree and party packages were used. [17, 18]. Clinical variables of new/worse proteinuria, new/worse hematuria, serum creatinine, percentage creatinine change from baseline, >30% increase in serum creatinine, c-reactive protein, current ANCA, percentage change in ANCA titre, RBC casts, BVAS haematuria, age, and sex, in addition to usCD163. See Methods chapter 2, section 2.6.2.
4.5 Results

4.5.1. Baseline Characteristics

121 encounters were screened for inclusion (See Figure 4.3). Of these, 37 encounters were excluded from analysis, as 18 had no urine sample available for biomarker analysis, 14 were within 6 months of diagnosis, in three cases it was not possible to adjudicate on the flare and two did not meet diagnostic criteria for ANCA-associated vasculitis. Of the 84 encounters included in the final analysis, 31 (36.9%) were adjudicated as being a renal flare. Of the 53 (63.1%) adjudicated as non-renal flare the final diagnoses were: systemic flare with no renal involvement in 13 (24.5%), sepsis in 11 (20.8%), isolated haematuria without clear cause in 11 (20.8%), other in 8 (15.1%), AKI of non-vasculitic origin in 7 (13.2%) and non-vasculitic CKD progression in 3 (5.6%).

Baseline demographics and renal function prior to study enrollment with potential renal vasculitis flare were similar between those subsequently diagnosed with renal flare and not, with the exception of higher levels of proteinuria (urine PCR) in those with subsequent renal flare (See table 4.1).
Figure 4.3: Flow diagram of recruitment from screening to enrollment and subsequent diagnosis. Inception and validation cohorts usCD163 values were measured by different assays. High Probability and Possibly denote Physician impression at the time of study enrollment. Renal Flare and No Renal Flare denote blinded adjudication committee diagnosis.
Table 4.1: Baseline characteristic derived from clinical parameters from last review prior to study visit. MPA= microscopic polyangiitis, GPA = granulomatosis with polyangiitis, EGPA= eosinophilic granulomatosis with polyangiitis, AAV/aGBM denotes overlap syndrome of dual positive ANCA and aGBM antibodies, MTX = methotrexate, MMF = mycophenolate mofetil, SD= standard deviation, IQR = interquartile range. Differences between flare and remission groups measured by Mann Whitney U and T tests.

<table>
<thead>
<tr>
<th>BASELINE CHARACTERISTICS</th>
<th>RENAL FLARE (N=31)</th>
<th>REMISSION (N=53)</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENDER</strong></td>
<td>Female 48.4% (15)</td>
<td>Female 35.9% (19)</td>
<td>-</td>
</tr>
<tr>
<td><strong>AGE</strong></td>
<td>63.1 yrs. (±16.2 yrs.)</td>
<td>60.3 yrs. (±13.5)</td>
<td>-</td>
</tr>
<tr>
<td><strong>DIAGNOSIS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPA, %, (N)</td>
<td>54.8% (17)</td>
<td>52.9% (28)</td>
<td>-</td>
</tr>
<tr>
<td>GPA, %, (N)</td>
<td>38.7% (12)</td>
<td>36.5% (19)</td>
<td>-</td>
</tr>
<tr>
<td>EGPA, %, (N)</td>
<td>3.2% (1)</td>
<td>3.9% (2)</td>
<td>-</td>
</tr>
<tr>
<td>DOUBLE + AAV/AGBP, %, (N)</td>
<td>3.2% (1)</td>
<td>5.8% (3)</td>
<td>-</td>
</tr>
<tr>
<td><strong>DISEASE DURATION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEDIAN (IQR)</td>
<td>3.9 years (IQR 1.7-6.5)</td>
<td>4.1 years (IQR 1.7-6.5)</td>
<td>-</td>
</tr>
<tr>
<td><strong>PRIOR RENAL INVOLVEMENT</strong></td>
<td>86.7% (26)</td>
<td>83.0% (44)</td>
<td>-</td>
</tr>
<tr>
<td>BASELINE GFR</td>
<td>48.8 (±27.8)</td>
<td>53 (±19.1)</td>
<td>-</td>
</tr>
<tr>
<td>BASELINE CREATININE</td>
<td>117 (IQR 79-249)</td>
<td>118 (IQR 99-146)</td>
<td>-</td>
</tr>
<tr>
<td>BASELINE HAEMATURIA</td>
<td>2+ (IQR 0-3+)</td>
<td>1+ (0-2+)</td>
<td>-</td>
</tr>
<tr>
<td>BASELINE PROTEINURIA</td>
<td>1+ (IQR 0-3+)</td>
<td>0+ (IQR 0-2+)</td>
<td>-</td>
</tr>
<tr>
<td>BASELINE PROTEIN: CREATININE RATIO</td>
<td>96 (IQR 36-329)</td>
<td>34 (IQR 16-59)</td>
<td>0.0088</td>
</tr>
<tr>
<td>CURRENT IMMUNOSUPPRESSION</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NONE</td>
<td>56% (14)</td>
<td>39.2% (20)</td>
<td>-</td>
</tr>
<tr>
<td>AZATHIOPRINE</td>
<td>24% (6)</td>
<td>33.3% (17)</td>
<td>-</td>
</tr>
<tr>
<td>MMF</td>
<td>8% (2)</td>
<td>17.7% (9)</td>
<td>-</td>
</tr>
<tr>
<td>MTX</td>
<td>4% (1)</td>
<td>1.9% (1)</td>
<td>-</td>
</tr>
<tr>
<td>RITUXIMAB</td>
<td>8% (2)</td>
<td>0% (0)</td>
<td>-</td>
</tr>
<tr>
<td>OTHER</td>
<td>0% (0)</td>
<td>7.8% (4)</td>
<td>-</td>
</tr>
<tr>
<td>CURRENT CORTICOSTEROIDS</td>
<td>34.8% (8)</td>
<td>39.6% (19)</td>
<td>-</td>
</tr>
</tbody>
</table>
4.5.2. Study Visit Clinical Characteristics

Clinical characteristics from the time of potential renal vasculitis flare are outlined in table 4.2. Notably those subsequently adjudicated as having a renal flare had higher serum creatinine, higher percentage increase in serum creatinine, greater proportion meeting BVAS criteria of increased in creatinine >30%, higher urinalysis protein, urinalysis blood as well as protein: creatinine ratio. Physician impression of high probability was greater in those with RV flare as well as those who underwent renal biopsy.
Table 4.2: Clinical characteristics at time of study visit (flare or flare mimic) in both cohorts combined. eGFR=estimated glomerular filtration rate, CRP=c-reactive protein, ANCA=anti-neutrophil cytoplasmic antibody, PCR=protein: creatinine ratio, UA= dipstick urinalysis, RBC=red blood cell, BVAS=Birmingham Vasculitis Activity Score, clinical impression was as per treating Physicians opinion at time of study visit, SD= standard deviation, IQR = interquartile range. Differences between flare and remission groups measured by Mann Whitney U and T tests.
4.5.3. Optimisation of clinical grade sCD163 ELISA

4.5.3.1. usCD163 cut off ranges

Optimal diagnostic cut off levels for diagnosis of renal vasculitis flare were derived using the Youden index as >253ng/mmol (Euroimmun assay n=84).

4.5.3.2. usCD163 Assay Correlation

usCD163 was measured in a subgroup (n= 42) using two usCD163 assays to determine assay performance. usCD163 measurements using Euroimmun and R&D DuoSet values were highly correlated with p<0.0001 and r of 0.773. Synchronous urine protein: creatinine ratio and eGFR correlated with usCD163 with r of 0.7987 and 0.5914, p<0.0001. See Figure 4.4.
Figure 4.4: (A): XY plot of usCD163 values (n=42) as measured by R&D Systems Duoset (Assay A) and Euroimmun (Assay B) ELISAs. (B): XY plot of combined assays for usCD163 and estimated GFR (n=80). (C): XY plot of combined assays for usCD163 and urine protein: creatinine ratio (n=58). A-C are significantly correlated with p<0.0001 (spearman testing).
4.5.4. usCD163 is elevated in renal vasculitis flare

31 (36.9%) of participants had a confirmed diagnoses of renal vasculitis (RV) flare adjudicated. Of those with RV flare, the median usCD163 concentration was 805.8ng/mmol creatinine (IQR 439 to 1705) as measured by clinical grade ELISA. In non-RV flare the median usCD163 concentration was 100.0ng/mmol creatinine (IQR 52-174, p<0.0001, figure 4.5A). The area under the curve for detection of active renal vasculitis was 0.95 (95% CI 0.90-1.0, p<0.0001, figure 4.5B).

Figure 4.5 (A): usCD163 concentrations as per adjudicated diagnosis of renal vasculitis flare or no renal vasculitis flare. Dotted lines represent diagnostic cut off value of >253ng/mmol. ****= p<0.0001 as calculated by Mann Whitney testing. (B): Receiver operator of usCD163 ng/MMol comparing renal vasculitis flare to non-flare (p<0.0001). AUC=area under the curve. N=84. usCD163 as measured by Euroimmun ELISA.
4.5.5. usCD163 levels do not differ by BVAS major renal criteria

usCD163 levels do not differ in those adjudicated as remission renal vasculitis when groups are stratified by BVAS major criterion indication for potential flare as measured in 84 patients by diagnostic grade Euroimmun assay. Median values for hematuria as defined by ≥10 red blood cells per high power field, of moderate on urinalysis were 240ng/mmol (IQR 91.3 to 475), rise in serum creatinine >30% were 426ng/mmol (IQR 88.4-889), both hematuria & rise in serum creatinine >30% were 852ng/mmol (IQR 468-1938) or no renal BVAS criteria were 88.2ng/mmol (IQR 39.2-149), p<0.0001. See figure 4.6.

Figure 4.6: usCD163 values in adjudicated non-flare as per Birmingham Vasculitis Activity Score (BVAS) major renal criterion. + denotes criterion present, - denotes criterion absent. Dotted lines represent diagnostic cut off value of >253ng/mmol. Scatter dot plot with bar and lines represent median and interquartile ranges. Medians compared across groups using Kruskal Wallis testing ****p<0.0001, n=84. usCD163 as measured by Euroimmun ELISA.
4.5.6. **usCD163 is not elevated in renal vasculitis flare mimics**

usCD163 remains low across non-flare diagnostic groups as measured in 84 patients by diagnostic grade Euroimmun assay. Diagnosis category was confirmed by adjudication committee (see section 4.4.1). Values did not significantly differ across groups as measured by Kruskal Wallis testing. See Table 4.3 and Figure 4.7.

<table>
<thead>
<tr>
<th>FINAL ADJUDICATED DIAGNOSIS</th>
<th>USCD163 (NG/MMOL, MEDIAN, IQR)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RENAL FLARE</td>
<td>805.8 (439.1 -1705)</td>
<td>31 (36.9)</td>
</tr>
<tr>
<td>SYSTEMIC FLARE</td>
<td>88.2 (71.6 – 197.8)</td>
<td>13 (15.5)</td>
</tr>
<tr>
<td>ACUTE KIDNEY INJURY*</td>
<td>105.4 (48.4 – 191.0)</td>
<td>7 (8.3)</td>
</tr>
<tr>
<td>SEPSIS</td>
<td>141.0 (75.5 – 232.7)</td>
<td>11 (13.1)</td>
</tr>
<tr>
<td>CKD PROGRESSION</td>
<td>103.8 (54.7 – 524.9)</td>
<td>3 (3.6)</td>
</tr>
<tr>
<td>ISOLATED HEMATURIA</td>
<td>53.0 (24.4 – 95.8)</td>
<td>11 (13.1)</td>
</tr>
<tr>
<td>OTHER</td>
<td>122.9 (42.1 – 201.3)</td>
<td>8 (9.5)</td>
</tr>
</tbody>
</table>

Table 4.3. Final adjudicated diagnosis and usCD163 values in renal vasculitis flare and renal vasculitis flare mimics as measured by Euroimmun assay. N=84. CKD=Chronic kidney disease. *Not due to renal vasculitis

![Figure 4.7: usCD163 in adjudicated non-flare as per diagnostic category (n=84). Dotted lines represent diagnostic cut off value of >253ng/mmol. AKI= acute kidney injury, CKD= chronic kidney disease. Bar and lines represent median and interquartile ranges. usCD163 as measured by Euroimmun ELISA.](image)
**4.5.7. Kidney Biopsy Findings**

22.6% (19/84) patients underwent kidney biopsy at per treating Physician discretion. Of this group, 89.5% (17) were adjudicated as active renal vasculitis and 10.5% (2) as remission renal vasculitis. In those with active renal vasculitis, 88.2% (15) patients had kidney biopsy findings of crescentic glomerulonephritis. Berden subclasses were focal 41.6% (5), crescentic 25% (3), sclerotic 16.7% (2) and mixed 16.7% (2). Within the adjudicated active renal vasculitis subgroup, 14.7% (2) did not have crescentic glomerulonephritis identified on kidney biopsy. One of the biopsies was deemed to be inadequate with insufficient glomeruli for diagnosis (diagnosis of renal vasculitis flare was made on clinical grounds by treating Physicians), the other biopsy revealed age related vascular changes and due to high clinical suspicion of active renal vasculitis repeat kidney biopsy was performed one month later which revealed crescentic glomerulonephritis of Berden focal class. Descriptive features of biopsy reports as described in table 4.4.

<table>
<thead>
<tr>
<th>Kidney Biopsy Findings</th>
<th>FLARE N=17</th>
<th>NO FLARE N=2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Crescentic GN</strong></td>
<td>88.2% (15)</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Berden Class</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focal</td>
<td>33.3% (5)</td>
<td>-</td>
</tr>
<tr>
<td>Crescentic</td>
<td>26.6% (4)</td>
<td>-</td>
</tr>
<tr>
<td>Sclerotic</td>
<td>20% (3)</td>
<td>-</td>
</tr>
<tr>
<td>Mixed</td>
<td>20% (3)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Number of Glomeruli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Crescents</td>
<td>20 (IQR13-35)</td>
<td></td>
</tr>
<tr>
<td>% Normal Glomeruli</td>
<td>28.6 (IQR 6.9-66.7)</td>
<td>0</td>
</tr>
<tr>
<td>% Globally Sclerosed Glomeruli</td>
<td>28.6% (IQR 14.3-48.3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>32% (IQR 6.3-50)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 4.4. Kidney biopsy findings. Results reported as median and interquartile ranges. GN= glomerulonephritis, IFTA = interstitial fibrosis and tubular atrophy, IQR = interquartile range.*
4.5.8. usCD163 Biomarker characteristics

Optimal diagnostic cut off levels for diagnosis of renal vasculitis flare were derived using the Youden index as >253ng/mmol. Using these ranges usCD163 has favorable biomarker characteristics with AUC of 0.947, PPV of 86.2% and NPV of 96%. Diagnosis of renal vasculitis was determined by the adjudication committee (see section 4.4.1). When compared to adjudication committee diagnosis usCD163 was superior to red blood cell casts, major renal BVAS score (>12) and Physician clinical impression of high probability of flare. See table 4.5.

<table>
<thead>
<tr>
<th></th>
<th>SENSITIVITY</th>
<th>SPECIFICITY</th>
<th>PPV</th>
<th>NPV</th>
<th>FP/FN</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>USCD163 &gt;253 NG/MMOL</td>
<td>96.8% (83.3-99.9%)</td>
<td>86.8% (74.7-94.5%)</td>
<td>81% (65.8-99.4%)</td>
<td>97.8% (88.4-99.1%)</td>
<td>7/1</td>
<td>0.95 (0.904,0.996)</td>
</tr>
<tr>
<td>BVAS RENAL &gt;12</td>
<td>66.7% (47.2-82.7%)</td>
<td>94.3% (84.4-98.8%)</td>
<td>86.7% (68.3-94.1%)</td>
<td>83.3% (69.1-96.1%)</td>
<td>3/10</td>
<td>0.874 (0.796,0.951)</td>
</tr>
<tr>
<td>PHYSICIAN IMPRESSION: HIGH PROBABILITY</td>
<td>90.3% (74.2-97.9%)</td>
<td>79.2% (65.9-89.2%)</td>
<td>71.8% (56.3-92.3%)</td>
<td>93.3% (81.2-96.7%)</td>
<td>11/3</td>
<td>0.848 (0.771,0.924)</td>
</tr>
<tr>
<td>KIDNEY BIOPSY</td>
<td>88.2% (63.6-98.6%)</td>
<td>100% (15.8-Na)</td>
<td>100% (58.5-Na)</td>
<td>50% (18.9-Na)</td>
<td>0/2</td>
<td>0.941 (0.862,1.02)</td>
</tr>
<tr>
<td>RBC CASTS</td>
<td>30% (6.7-65.2)</td>
<td>78.3% (56.2-92.5%)</td>
<td>37.5% (17.7-72.4%)</td>
<td>72% (30.89.9%)</td>
<td>5/7</td>
<td>0.541</td>
</tr>
</tbody>
</table>

Table 4.5: Biomarker characteristics of each variable compared to adjudication committee diagnosis of renal vasculitis flare or non-renal vasculitis flare. usCD163 (n=84), BVAS major renal criteria (n=83), Physician Impression (high probability or possible renal vasculitis flare, n=84), kidney biopsy findings of crescentic glomerulonephritis (n=19) and RBC casts (n=33). BVAS = Birmingham Vasculitis Activity Score. PPV = positive predictive value. NPV= negative predictive value. FP= false positive. FN= false negative. AUC = area under the curve.

4.5.9. Characteristics of usCD163 mis-classified cases

9.5% (8) of cases were misclassified using usCD163 diagnostic cut off of >253ng/mmol and 10.5% (2) with renal biopsy. Clinical characteristics of those misclassified by usCD163 measurement are detailed in table 4.6.
<table>
<thead>
<tr>
<th>CLASSIFICATION</th>
<th>USCD163</th>
<th>CLINICAL COURSE</th>
<th>RENAL BIOPSY BVAS RENAL</th>
<th>RBC CASTS</th>
<th>SERUM CREATININE (% CHANGE)</th>
<th>ANCA TITRE (% INCREASE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CASE 1 (2015)</td>
<td>False Negative</td>
<td>112.9 ng/mmol</td>
<td>AKI with RBC casts, treated empirically for flare.</td>
<td>N/A</td>
<td>10</td>
<td>Yes</td>
</tr>
<tr>
<td>CASE 2 (53)</td>
<td>False Positive</td>
<td>270.9ng/mmol</td>
<td>Sepsis (pulmonary &amp; UTI)</td>
<td>N/A</td>
<td>10</td>
<td>No</td>
</tr>
<tr>
<td>CASE 3 (22)</td>
<td>False Positive</td>
<td>2126ng/mmol</td>
<td>AKI, stroke, lung mass, then sudden death.</td>
<td>N/A</td>
<td>12</td>
<td>NA</td>
</tr>
<tr>
<td>CASE 4 (2510)</td>
<td>False Positive</td>
<td>575.5ng/mmol</td>
<td>AKI, Renal Thrombus</td>
<td>No CGN. *insufficient size</td>
<td>12</td>
<td>NA</td>
</tr>
<tr>
<td>CASE 5 (8094)</td>
<td>False Positive</td>
<td>371.9ng/mmol</td>
<td>Systemic Flare (MSK, cutaneous)</td>
<td>N/A</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td>CASE 6 (8119)</td>
<td>False Positive</td>
<td>271.7ng/mmol</td>
<td>Systemic Flare (MSK, cutaneous)</td>
<td>N/A</td>
<td>6</td>
<td>No</td>
</tr>
<tr>
<td>CASE 7 (12069)</td>
<td>False Positive</td>
<td>524.9ng/mmol</td>
<td>AKI with haematuria &amp; proteinuria</td>
<td>N/A</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>CASE 8 (12084)</td>
<td>False Positive</td>
<td>293.6ng/mmol</td>
<td>Systemic Flare (MSK, ENT)</td>
<td>N/A</td>
<td>10</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 4.6: Clinical characteristics of misclassified subjects based on usCD163 optimal diagnostic cut off range >253ng/mmol. usCD163 as measured by clinical grade Euroimmun ELISA. AKI = acute kidney injury, UTI= urinary tract infection, MSK= musculoskeletal, ENT = ear, nose and throat.
4.5.9. Effect of proteinuria on usCD163 false positive rate cases

Urine sCD163 concentration was strongly correlated with urine protein: creatinine ratio (PCR) in this prospective cohort (figure 4.4 C). Despite this correlation, the fraction of usCD163 false positives for the detection of renal vasculitis flare did not increase with increased proteinuria (Figure 4.8). This is likely because increasing proteinuria in this restricted setting is usually due to active renal vasculitis.

![Figure 4.8](image)

*Figure 4.8. Diagnostic classifications using usCD163 >253ng/mmol for the diagnosis of renal vasculitis flare stratified by urine protein: creatinine ratio. Percentage of diagnostic classification expressed as FP=false positive, TP= true positive, FN= false negative, TN= true negative. N =82*

<table>
<thead>
<tr>
<th></th>
<th>&lt;30 mg/mmol</th>
<th>30-100 mg/mmol</th>
<th>100-300 mg/mmol</th>
<th>&gt;300 mg/mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>False Positive</td>
<td>3.6% (3)</td>
<td>2.4% (2)</td>
<td>1.2% (1)</td>
<td>1.2% (1)</td>
</tr>
<tr>
<td>True Positive</td>
<td>1.2% (1)</td>
<td>10.9% (9)</td>
<td>13.4% (11)</td>
<td>9.8% (8)</td>
</tr>
<tr>
<td>False Negative</td>
<td>1.2% (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>True Negative</td>
<td>37.8% (31)</td>
<td>17.1% (14)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>43.9% (36)</td>
<td>30.4% (25)</td>
<td>14.6% (12)</td>
<td>10.9% (9)</td>
</tr>
</tbody>
</table>

*Table 4.7: Diagnostic classifications using usCD163 >253ng/mmol for the diagnosis of renal vasculitis flare stratified by urine protein: creatinine ratio. FP=false positive, TP= true positive, FN= false negative, TN= true negative, PCR = protein: creatinine ratio. N=82*
4.6 Discussion

One of the greatest challenges in caring for patients with AAV is the early detection of active disease prior to accrual of irreversible end organ damage with up to 26% of patients progressing to ESKD within 3-5 years of diagnosis. The chronic relapsing remitting course of vasculitis renders immunosuppression challenging with conflicting risks of excess immunosuppression and clinical relapse. Renal vasculitis flare is likely under-diagnosed with 13% of dialysis independent patients developing end stage kidney disease in the absence of a diagnosed renal vasculitis relapse[39]. usCD163 is a promising biomarker of crescentic glomerulonephritis with elevated concentrations in renal vasculitis at diagnosis, subtle flare and lupus nephritis[97, 127, 147, 148].

This multicentre prospective study of patients with known ANCA-associated vasculitis presenting with a potential renal vasculitis flare shows that usCD163 concentrations are elevated in renal vasculitis flare and remain low in clinically relevant mimics. usCD163 has favorable biomarker characteristics for the detection of renal vasculitis flare with cut offs of >253ng/mmol with sensitivity of 96.8%, specificity of 96.8%, and AUC of 0.95. usCD163 was superior to currently used non-invasive clinical tools including red blood cell cast assessment, renal Birmingham Vasculitis Activity Score >12 and physician impression. usCD163 has similar biomarker characteristics to the current gold standard of kidney biopsy. Kidney biopsy detection of crescentic glomerulonephritis in this population had sensitivity of 88.2%, specificity of 100% and AUC of 0.941.

In clinical practice it is often difficult to distinguish active renal vasculitis from clinical mimics such as infection, non-glomerular haematuria, non-vasculitic acute kidney injury and chronic kidney disease progression. We have demonstrated that that usCD163 levels remain low in these clinically relevant mimics. The prospective nature and inclusion of flare mimics provides a real-world estimate of the utility of usCD163 and contextualises its use in conjunction with traditional biomarkers.

This is the first study of usCD163 to use a clinical grade assay which allows evidence-based translation into clinical practice. We validated the use of a diagnostic grade usCD163 assay (Euroimmun) by comparing it to the previously validated research grade usCD163 assay (R&D Systems, Duoset) in a subset of 42 patients. The two assays were highly correlated with $r^2$ of 0.773 ($p<0.0001$). The reported usCD163 concentrations with this diagnostic grade assay in active renal vasculitis compared favorably with our prior
work at time of diagnosis and subtle flare with median values of 805.8ng/mmol, 560ng/mmol and 162ng/mmol, respectively. Derived cut-off values were similar to prior published work with longitudinal cohort, time of diagnosis and subtle flare groups values of >253ng/mmol, >300ng/mmol and >143ng/mmol (in combination with uMCP-1), respectively [39, 41]. The validation of this clinical grade ELISA allows clinical translation of usCD163 from a research test to a clinically available diagnostic tool.

A challenge we faced in our study design was in accurately determining which participants had a renal vasculitis flare. Renal biopsy was not mandated as this was a prospective observational study. We defined renal vasculitis flare using a blinded final adjudication by expert committee of disease activity. This committee reviewed renal major and minor BVAS criteria, trends in serum creatinine, urinary protein, red blood cell casts, subsequent clinical management (immunosuppression), and renal biopsy (if available)). This pragmatic approach has also been taken in clinical trials, RITAZAREM enrolled patients with relapsing AAV with a diagnosis of relapse based on one major or three minor disease activity items on the Birmingham Vasculitis Activity Score (BVAS/WG). RITUXVAS required renal biopsy with necrotizing glomerulonephritis or red cell casts on urine microscopy or ≥ ++ haematuria. Only 39.3% (33) of patients in this study underwent kidney biopsy, in RITAZAREM 44% of participants underwent kidney biopsy.

Strengths of our study include its multi-centre prospective nature, inclusion of Physician clinical impression, currently used clinical tools and blinded final adjudication by expert committee of disease activity. The prospective nature of this study allowed for inclusion of real-world potential flare mimics including hematuria, sepsis and CKD progression. Potential weakness includes a relatively sample size of 84 (121 patients screened) with kidney biopsy only performed in a subset of 19 as per treating Physician discretion.

This study has validated usCD163 as a diagnostic marker of renal vasculitis flare using a clinical grade assay. This will allow translation of usCD163 from research assay to a clinical grade test that could be used at the bedside. Next steps to further validate the role of usCD163 as a diagnostic marker of renal vasculitis flare would include a prospective trial mandating both biopsy and usCD163 measurement.
Chapter 5:

Urinary Soluble CD163 in the Setting of Proteinuria
Abstract

Background: Prior work has demonstrated that urinary soluble CD163 (usCD163) displays excellent biomarker characteristics for detection of active renal vasculitis in patients with newly diagnosed ANCA-associated vasculitis (AAV). We sought to assess the levels of usCD163 in high grade proteinuria as we were concerned that the given the 2 log higher levels of sCD163 in serum that in the setting of high-grade proteinuria leakage of serum sCD163 could occur across the glomerular basement membrane (GBM) leading to detection of non-locally produced sCD163 in urine.

Methods: Patients with biopsy proven nephrotic syndrome with paired urine samples from time of remission (urine protein <0.5g/day) and of active nephrotic syndrome (urine protein >3.5g/day) were selected from a multicentre longitudinal cohort. Patients with active renal vasculitis (RV), remission RV with proteinuria, remission RV without proteinuria and healthy controls were identified from a multicentre longitudinal cohort with available paired urine and serum samples. usCD163 levels were measured in urine by sandwich ELISA. Values were normalised to urine creatinine, protein and albumin.

Results: In the nephrotic syndrome (NS) cohort: 65 patients were identified (MN n=22, MCD n=20, FSGS n=23). In the renal vasculitis cohort: 39 patients were enrolled: ten with active renal vasculitis (RV), 10 in remission RV with residual proteinuria, 9 in remission RV with no proteinuria and 10 healthy controls.

In the NS cohort: Median usCD163 concentrations (normalised to urine creatinine) were elevated in active primary nephrotic syndrome compared to remission primary nephrotic syndrome with median values of 465ng/mmol and 5.1ng/mmol, p<0.0001, respectively. Correction for proteinuria by normalising usCD163 values to urine protein attenuates the elevated signal in active nephrotic syndrome with median usCD163 concentrations in active nephrotic syndrome and remission primary nephrotic syndrome of 0.82ng/mg and 0.92ng/mg, p=0.3926, respectively.

In those with remission vasculitis with residual proteinuria median usCD163 (creatinine normalised) concentrations are elevated at 167ng/mmol compared to healthy controls at 1ng/mmol and remission vasculitis without proteinuria at 1ng/mmol but concentrations are significantly less than active renal vasculitis at 510ng/mmol, respectively (p<0.00001). Without correction for urinary protein remission vasculitis with residual proteinuria values are within the remission renal vasculitis range from our
previously validated diagnostic thresholds of <300ng/mmol at diagnosis, <253ng/mmol at renal vasculitis flare. In those with remission vasculitis with residual proteinuria usCD163 (protein normalised) concentrations are elevated at 1.3ng/mg compared to healthy controls at 0ng/mg and remission vasculitis without proteinuria at 0.1ng/mg but concentrations are significantly less than active renal vasculitis with median concentrations of 7.8ng/mg, respectively (p=0.0001).

**Conclusion:** usCD163 levels are increased in high-grade proteinuria. Correction for urinary protein attenuates this signal in primary nephrotic syndrome but not in active renal vasculitis.
5.1.1. Visual Abstract

Results

usCD163 is elevated in high grade proteinuria
usCD163 levels are normalized by urine protein in active nephrotic syndrome but not in active renal vasculitis

Methods

Urinary Soluble CD163 Interpretation in the Setting of Proteinuria
5.2. Introduction

We have previously validated usCD163 in the setting of renal vasculitis at diagnosis, renal flare and throughout induction therapy [97, 127, 147]. A clinical concern remains that usCD163 detection may be a sophisticated measure of proteinuria. In this chapter I sought to assess the impact on usCD163 levels in clinical settings with minimal renal sCD163 production and high-grade proteinuria. In this setting, the detection of sCD163 in urine may reflect passage of serum sCD163 across the glomerular filtration barrier. Primary nephrotic syndrome is characterised by extensive foot process effacement with no local macrophages present. usCD163 detection in primary nephrotic syndrome is therefore likely to reflect serum “spill over” into urine rather than local production. In order to investigate the effect of proteinuria on the interpretation of usCD163 concentrations in the setting of ANCA-associated vasculitis, I investigated usCD163 levels in patients in clinical remission with persistent proteinuria, clinical remission without proteinuria, active renal vasculitis and healthy controls. Patients with primary nephrotic syndrome were identified through a collaboration with the Nephrotic Syndrome Study Network (NEPTUNE). Serial samples were obtained from periods of active nephrosis (urine protein >3.5g/day) and remission (urine protein <0.5g/day)[189]. We identified patients with ANCA-associated vasculitis with paired urine and serum samples from the Rare Kidney Disease Biobank and Registry.

5.2.1. Nephrotic Syndrome Study Network

The Nephrotic Syndrome Study Network (NEPTUNE) was established to investigate the underlying disease mechanisms of primary nephrotic syndrome, elucidate pathogenesis, and identify therapeutic targets for clinical trials. It is a National Institute of Health (NIH) funded initiative with 23 sites throughout North America. General inclusion criteria are a diagnosis of focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD), or membranous glomerulonephritis (MN) with proteinuria ≥ 500 mg/day and GFR > 30mls/minute. To date NEPTUNE has recruited more than 650 rigorously phenotyped participants. At baseline, NEPTUNE collected information on demographics, clinical history, physical examination, as well as synchronous tissue samples from renal biopsy, blood and urine samples. Once baseline assessment is completed, participants are followed over 30 months to collect data concerning health, quality of life, outcomes (partial remission, complete remission, end stage kidney disease), urine and blood samples. The primary outcome is a composite measure of change in urinary protein excretion and change in renal function.
5.2.2. Minimal Change Disease

Minimal change disease (MCD) is the commonest cause of idiopathic nephrotic syndrome in children and young adults, accounting for 70%–90% of nephrotic syndrome[190]. Its clinical course is characterized by abrupt onset of peripheral edema and frothy urine with investigations revealing high grade proteinuria and profound hypoalbuminemia[191]. In adults, MCD accounts for approximately 15% of idiopathic nephrotic syndrome. Renal biopsy typically reveals normal light microscopy, no immunofluorescence positivity and diffuse foot effacement on electron microscopy[192].

The aetiology of MCD remains elusive but clinical activity is frequently associated with allergies, infections and medications. Treatment primarily consists of corticosteroids with additional immunosuppression such as rituximab, cyclophosphamide, calcineurin inhibitors and mycophenolate mofetil reserved for those with steroid resistant, steroid dependent or frequently relapsing disease[193].

Long term prognosis is usually excellent with steroid responsive subtypes rarely leading to end stage kidney disease. Declining renal function and steroid resistance are atypical and re-biopsy often reveals focal and segmental glomerulosclerosis lesions leading to reclassification.

Given the lack of inflammatory changes on light microscopy and absence of macrophages we estimate that usCD163 concentration would be within the normal range as there is no local source of sCD163, unless there is leak of serum sCD163 across the glomerular filtration barrier due to the extensive foot process effacement.
5.2.3. Focal and Segmental Glomerulosclerosis

Focal and segmental glomerulosclerosis (FSGS) is a pattern of histologic injury on renal biopsy. In its primary form, termed immunologically mediated focal segmental glomerulosclerosis it is the leading cause of adult-onset nephrotic syndrome. Prognosis is variable with a significant proportion progressing to end stage kidney disease.

The aetiology of FSGS remains elusive with supporting evidence for a circulating factor leading to nephrosis supported by rapid recurrence post-transplantation as well as treatment responsiveness to immunosuppression and plasma exchange. Non-immunologically mediated FSGS is a secondary adaptive form with a wide range of potential aetiologies including low nephron load, excessive nephron workload and reduced nephron capacity. Kidney biopsy has characteristic features with the Columbia Classification of five histological subtypes[194]. Light microscopy reveals capillary lumen obliteration, include adhesion to Bowman’s capsule at the tip, hyper cellularity, foam cells, segmental sclerosis[195]. Immunofluorescence is classical negative, but IgM positivity can occur[196]. Electron microscopy reveals diffuse foot process effacement.

Initial treatment is with corticosteroids[197, 198]. In cases of steroid resistance calcineurin inhibitors, mycophenolate mofetil, rituximab and plasma exchange are trialled. Long term prognosis is variable with steroid resistance and certain histologic variants portending a worse outcome.

Traditionally immunologically mediated FSGS has not traditionally been viewed as a macrophage driven disease however foam cells are thought to originate from monocytes and macrophages with foam cell accumulation characteristic of FSGS[199]. One study of childhood nephrotic syndrome showed that there were CD8 + infiltrates in those with FSGS with a more aggressive clinical course[200].

We estimate that usCD163 concentration would be within the either within normal range or minimally elevated as there is minimal local sCD163 production, unless there is leak of serum sCD163 across the glomerular filtration barrier due to foot process effacement.
5.2.4. Membranous Nephropathy

Membranous nephropathy (MN) is a form of glomerulonephritis characterised by immune complex deposition on the glomerular basement membrane with associated glomerular basement membrane thickening[201, 202].

80% of cases are a primary autoimmune form associated with circulating IgG4 autoantibody to the podocyte membrane antigen PLA2R or anti-THSD7A[203]. In its secondary form it is associated with malignancy, infections such as hepatitis B and systemic autoimmune conditions such as lupus. Clinical presentation varies from sub-nephrotic range proteinuria with impaired GFR to fulminant nephrosis. Untreated, an estimated one third of patients undergo spontaneous remission, one-third progress to ESKD over 10 years, and the remaining third have non-progressive chronic kidney disease.

Those with persistent proteinuria >4g/day after six months of supportive therapy or who develop complications of nephrotic syndrome, are treated with immunosuppressive therapy. Commonly used regimens include corticosteroids and cyclophosphamide, calcineurin inhibitors, and rituximab[201, 204-209].

Traditionally primary MN has not traditionally been viewed as a macrophage driven disease however increased inflammatory cells including macrophages within the interstitium are associated with lower renal survival.

We estimate that usCD163 concentration would be within normal range unless there is leak of serum sCD163 across the glomerular filtration barrier due to foot process effacement.
5.2.5. usCD163 in Lupus Nephritis

Lupus nephritis is classified by the International Society of Nephrology/Renal Pathology Society classification based on degree of proliferation and histological pattern[210]. Classes III and IV are characterised by crescentic glomerulonephritis. Class V lupus nephritis has a membranous pattern with no associated proliferation or crescents and clinical features of nephrotic syndrome including high grade proteinuria. Endo et al have demonstrated that usCD163 concentrations are elevated in lupus nephritis. They further investigated the effect of normalisation of usCD163 levels by urine protein concentration, they demonstrated persistent usCD163 elevation in class III and IV but normalisation of usCD163 levels in class V [148]. In figure 5.1. Endo et al demonstrate a trend of increased usCD163 in class V lupus nephritis and that is attenuated when corrected for proteinuria. These findings have been replicated in cohorts from the University of Ohio and Mexico City with increased usCD163 concentrations in active lupus nephritis[161].

![Figure 5.1: Urinary sCD163 concentrations in various kidney diseases and LN. Urinary protein excretion of sCD163 (A and B) corrected by urinary Cr or urinary total protein (u-Cr and u-TP) in renal diseases (left) and LN classified according to ISN/RPS criteria (right). Each patient is represented by a dot and the mean of each group is shown as a horizontal bar. *P < 0.05. In (B) and (D), data from normal subjects were excluded from the analysis because of the absence of proteinuria. IgAN = IgA nephropathy, AAV= ANCA associated vasculitis, MCNS = minimal change nephrotic syndrome, MN = membranous nephropathy, DMN = diabetic nephropathy[148].](image-url)
5.2.6. Urinary analytes interpretation and value normalisation

Urine is an ideal biospecimen as it is easily and non-invasively obtained with general abundant supply. Spot urine samples are traditionally used in urinary analyte experiments as 24-hour urine collections for analytes are limited by collection technique, incomplete collection, bacterial contamination and sample degradation. Up to 50% of 24-hour urine collections are incomplete, limiting their diagnostic utility[167]. Spot urine sample use is limited by the differing effects of urinary concentration with varying dilution effect and sample volume.

Accurate assessment of urinary biomarker concentrations is complex, in particular in the setting of kidney diseases. Urinary analyte detection may reflect local production or serum passage across the glomerular filtration. Measurement of analytes in urine compared to serum poses many challenges including but not limited to urinary concentration, proteinuria, presence of bacteria, pH and the integrity of the glomerular filtration barrier.

The glomerular filtration barrier is composed of fenestrated glomerular capillaries endothelium, glomerular basement membrane and podocytes foot processes[211]. Passage across the glomerular filtration barrier is dependent on electrical charge, molecule size and capillary pressure. Disruption of any of the physical components of the glomerular filtration barrier can lead to passage of plasma proteins into urine. This is manifest in primary nephrotic syndrome with diffuse podocytopathy leading to high grade proteinuria and leakage of other plasma proteins into urine. Crescentic glomerulonephritis can affect the glomerular filtration barrier with focal glomerular basement rupture. Systemic factors can also affect urinary analyte concentrations, including increased albuminuria with intense exercise and fever [212-215]. It is estimated that 70% of the urinary proteome is of local urogenital origin and 30% from plasma proteome passage through the glomerular filtration membrane[216].

A major challenge in the interpretation of urinary proteomics is determining the optimal method of data normalisation. Demonstration of local expression of a renal origin for a protein of interest, for example mRNA expression of sCD163 in glomeruli of patients with ANCA-associated vasculitis and lupus nephritis is a vital step in linking the structural phenotype and functional measures using urinary biomarkers[127]. Detection in urine does however confirm that the analyte concentration accurately reflects intra-renal molecular events in the presence of a disrupted glomerular filtration barrier.
5.2.6.1 Protein normalisation of urinary analytes

Proteinuria, in particular albuminuria has been strongly and consistently associated with adverse renal outcomes including progression to end stage kidney disease[217, 218]. A variety of strategies have been proposed to account for urinary analyte interpretation in the context of proteinuria as there are no guidelines for urinary biomarker standardisation. A distinguishing feature of usCD163 compared to other urinary biomarkers of interest is the strength of supporting evidence of its relationship to crescentic glomerulonephritis as evidenced by mRNA microdissection and our efforts to define the clinically relevant caveats to allow accurate translation of usCD163 into clinical practice[127, 161]. Some authors have accounted for proteinuria by reporting a correlation with subsequent discussion regarding the relationship between analytes and proteinuria without reporting of attempted correction strategies. This approach has been commonly used and limits the potential for clinical translation[219]. Other authors have proposed including albuminuria in predictive models in addition to the urinary analyte of interest [220]. Another strategy has been the demonstration that despite a strong correlation between an analyte of interest and proteinuria in a particular population, that there is no increased signal in other proteinuria kidney diseases[221]. The healthy urinary proteome study reported proteome values based on relative abundance using intensity-based absolute quantification (iBAQ). The iBAQ of specific peptide reports its intensities divided by the number of theoretically observable peptides of the proteins. This methodology ranked CD163 as the 961st most abundant urinary protein in health [222]. This chapter proposes the use of the same methodology as Endo et al to account for the potential confounding effect of proteinuria in this population with reporting of normalisation of usCD163 to proteinuria in the setting of nephrotic syndrome[148]. Urine analytes protein correction methodology has also been reported in bladder cancer and endemic nephropathy, [223-226].

5.2.6.2 Creatinine normalisation of urinary analytes

Normalisation for urinary creatinine concentration is frequently used as a technique to normalise urinary analyte concentrations[127, 148, 161]. This technique has been used in this chapter and throughout this thesis correct for potential confounding effects of urine dilution [161, 220]. This is performed by dividing the concentration of the urinary analyte of interest by the urine creatinine concentration. This is proposed to improve diagnostic accuracy by accounting for urinary concentration and has been extensively validated including the use of albumin and protein to creatinine ratios[158]. Spot urine albumin to creatinine ratio and spot protein to creatinine ratio are widely used in the monitoring of chronic kidney
disease and diabetes[159]. Day to day variation in ACR and PCR occurs but this is less significant with greater degrees of proteinuria[158, 160]. Creatinine is a by-product of muscle metabolism and is excreted in urine at a constant rate via glomerular filtration[162]. Potential limitations of this include alterations in creatinine excretion based on age, sex, race, protein intake and muscle mass[163-166, 169].

5.2.6.3 Fractional excretion of urinary analytes
Fractional excretion of electrolytes such as sodium is an established method of assisting in the diagnosis of pre-renal acute kidney injury[227, 228]. The authors proposed a FENa cut off of <1% based on physiological estimates of sodium filtration in the setting of pre-renal azotemia. This is in widespread clinical use, in particular for the diagnosis of hepatorenal syndrome[229]. To account for alterations in sodium excretion with concomitant diuretic use, the fractional excretion of urea was proposed with FEUrea of <35% in pre renal AKI[230]. Fractional excretion of NGAL, a biomarker of acute kidney injury has been reported with similar results to urinary NGAL concentration alone[231].

5.3. Aims

5.3.1. Hypothesis

1. We hypothesize that in primary nephrotic syndrome:
   a. The absolute concentrations of usCD163 are elevated due to passage of serum sCD163 across the glomerular filtration barrier into urine.
   b. The potential observed increase in usCD163 concentration in active nephrotic syndrome is attenuated when usCD163 concentrations are normalised to urine protein concentration.

2. We hypothesize that in remission AAV with persistent proteinuria usCD163 concentrations are elevated compared to remission AAV without persistent proteinuria, but these concentrations remain below the diagnostic cut off thresholds for active renal vasculitis.
5.3.2. Research questions
To address these hypotheses, we conducted two studies in the distinct groups of primary nephrotic syndrome and ANCA-associated vasculitis and present these results in parallel.

a. Is urine sCD163 elevated in active primary nephrotic syndrome?
b. Is urine sCD163 elevated in remission AAV with persistent proteinuria?
c. Does correction for urine protein concentration normalise usCD163 levels in primary NS?
d. Does calculation of fractional excretion of sCD163 relative to protein and albumin improve diagnostic fidelity?
5.4. Methods

Two cohorts were used address our hypotheses, primary nephrotic syndrome and ANCA-associated vasculitis.

5.4.1. Inclusion criteria - Primary Nephrotic Syndrome

1. Primary nephrotic syndrome
   a) Focal and segmental glomerulosclerosis (FSGS)
   b) Membranous glomerulonephritis (MN)
   c) Minimal change disease (MCD)

2. Availability of paired urine samples from both
   a) Urine protein < 0.5g/day
   b) Urine protein > 3.5g/day

5.4.2. Inclusion criteria - ANCA-associated Vasculitis

1. ANCA-associated Vasculitis
   a) Active renal vasculitis (haematuria and proteinuria on urinalysis)
   b) Remission vasculitis with persistent proteinuria (proteinuria > 2+ on urinalysis)
   c) Remission vasculitis without proteinuria (proteinuria 0 on urinalysis)
   d) Healthy controls

2. Availability of synchronous paired
   a) Urine sample
   b) Serum sample
5.4.3. Techniques used to account for effect of proteinuria

As we hypothesised that a proportion of the detected usCD163 in the setting of proteinuria is due to passage of serum sCD163 across the glomerular filtration barrier we used the following strategies.

1. Urine protein correction

\[
\frac{\text{usCD163 pg/ml}}{\text{uProtein mg/dL}}
\]

2. Fractional Excretion of sCD163 to Protein

\[
\frac{\text{urine sCD163 ng/L} \times \text{serum Protein g/L}}{\text{urine Protein g/L} \times \text{serum sCD163 ng/L}}
\]

3. Urine albumin correction

\[
\frac{\text{usCD163 pg/ml}}{\text{uAlbumin mg/L}}
\]

4. Fractional Excretion of sCD163 to Albumin

\[
\frac{\text{usCD163 ng/L} \times \text{sAlbumin g/L}}{\text{uAlbumin g/L} \times \text{ssCD163 ng/L}}
\]
5.4.4. Patients and clinical data collection

5.4.4.1. Primary Nephrotic Syndrome

At diagnosis of nephrotic syndrome subjects were enrolled with synchronous biopsy, blood, urine, molecular, histopathological samples obtained along with detailed clinical phenotypic information. Biopsy diagnosis was confirmed by two independent nephropathologists blinded to the original diagnosis[189]. After enrollment, NEPTUNE participants undergo clinical evaluation and urine and blood sampling at 4-month intervals for the first year then every 6 months.

5.4.4.2. ANCA-associated Vasculitis

At enrollment to the Rare Kidney Disease Biobank synchronous blood and urine samples in addition to detailed clinical phenotypic information was obtained. After enrollment, participants underwent clinical evaluation and urine and blood sampling at regular intervals with additional visits at times of disease activity.

5.4.5. Collection and storage of urine samples

5.4.5.1. Primary Nephrotic Syndrome

Bio samples were collected according to pre-determined protocolized procedures. Urine was spun at 1000g for 12 minutes then frozen at -80°C until shipping on dry ice[189].

5.4.5.2. ANCA-associated Vasculitis

Samples were collected according to pre-determined protocolized procedures as per Methods chapter 2, section 2.3.1.

5.4.6. usCD163 Assay Methodology

usCD163 assay was performed using commercial usCD163 sandwich ELISA (R&D Systems Duoset) as per Methods chapter 2, section 2.5.1.
5.4.7. Statistical Methodology

5.4.7.1. Descriptive Statistics – Primary Nephrotic Syndrome and AAV

Clinical, laboratory data and ELISA results were analysed using GraphPad Prism version 6 and 8. Biomarker values were non-normally distributed and are thus reported as median and interquartile ranges. Kruskal Wallis, Wilcoxon matched pairs signal test, Mann Whitney U tests, chi squared, unpaired t tests were used to determine the significance of associations for non-normally and normally distributed data. Correlations were measured using Spearman correlation coefficient.

5.4.7.2. Fractional Excretion - AAV

1. In order to calculate the fractional excretion of albumin to sCD163 we used the following formula:

\[
\frac{\text{usCD163 ng/L x sAlbumin g/L}}{\text{uAlbumin g/L x ssCD163 ng/L}}
\]

2. In order to calculate the fractional excretion of creatinine to sCD163 we used the following formula:

\[
\frac{\text{urine creatinine (mmol/L) x serum sCD163 (ng/ml)}}{\text{urine sCD163 (ng/ml) x serum creatinine (mmol/L)}}
\]

3. In order to calculate the fractional excretion of protein to sCD163 ratio we used the following formula:

\[
\frac{\text{urine sCD163 ng/L x serum Protein g/L}}{\text{urine Protein g/L x serum sCD163 ng/L}}
\]

5.4.7.3. Derivation of diagnostic cut offs.

Optimal cut off ranges were derived using the Youden index to maximise sensitivity and specificity using R Studio version 1.1.456. Optimal cutpoints packages was used. See method chapter 2 section 2.6.2.
5.5. Results – Primary Nephrotic Syndrome

5.5.1. Baseline characteristics

65 patients were studied (MN n=22, MCD n=20, FSGS n=23). Median age at onset of NS was 34.5 years (IQR 13.5-59.8 years), median eGFR was 77.3 mls/min/1.73m² (SD ±30.3 mls/min/1.73m²). Between groups those with MN were older at diagnosis with mean age of 58 years and had lower eGFR with mean value of 68.3 mls/min. Those with MCD had the highest median proteinuria during active disease at 10.1 g/L. (See table 5.1)

<table>
<thead>
<tr>
<th>N=65</th>
<th>FSGS (n=23)</th>
<th>MCD (n=20)</th>
<th>MN (n=22)</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE AT DIAGNOSIS (YEARS)</td>
<td>15 (IQR 2-35)</td>
<td>16 (IQR 5-29)</td>
<td>58 (36.9-65.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EGFR (MLS/MIN)</td>
<td>77.3 (SD ±30.3)</td>
<td>96.5 (SD ±29.6)</td>
<td>68.3 (SD ±19.7)</td>
<td>0.0161</td>
</tr>
<tr>
<td>BODY MASS INDEX</td>
<td>20.8 (IQR 19.7-2.4)</td>
<td>24.5 (IQR 20.7-28.3)</td>
<td>28.2 (IQR 24.7-30.5)</td>
<td>0.1043</td>
</tr>
<tr>
<td>SYSTOLIC BLOOD PRESSURE (MMHG)</td>
<td>109.7 (SD ±4.8)</td>
<td>117.3 (SD ±18.5)</td>
<td>115.6 (SD ±18.2)</td>
<td>0.8754</td>
</tr>
<tr>
<td>DIASTOLIC BLOOD PRESSURE (MMHG)</td>
<td>59 (IQR 48-72)</td>
<td>70 (IQR 65-78)</td>
<td>77 (69-79)</td>
<td>0.1062</td>
</tr>
<tr>
<td>PROTEINURIA (DURING NEPHROSIS) (G/L)</td>
<td>5.6 (IQR 3.8-6.1)</td>
<td>10.1 (IQR 7.0-11.7)</td>
<td>4.8 (IQR 3.5-5.6)</td>
<td>0.0005</td>
</tr>
<tr>
<td>PROTEINURIA (DURING REMISSION) (G/L)</td>
<td>0.05 (IQR 0.03-0.1)</td>
<td>0.04 (IQR 0.02-0.07)</td>
<td>0.06 (IQR 0.03-0.09)</td>
<td>0.3926</td>
</tr>
</tbody>
</table>

Table 5.1: Baseline characteristics of subjects with primary nephrotic syndrome. Differences across groups measures by Kruskal Wallis testing. FSGS= focal and segmental glomerulosclerosis, MCD= minimal change disease, MN = membranous nephropathy, eGFR= estimated glomerular filtration rate, IQR = interquartile range, SD=standard deviation, ns= non-significant.
5.5.2. usCD163 concentration is elevated in active primary nephrotic syndrome

Median usCD163 concentration (normalised to urine creatinine) is elevated in active primary nephrotic syndrome with median concentrations of 465ng/mmol (IQR 188-884 ng/mmol) compared to remission primary nephrotic syndrome with median concentrations of 5.1ng/mmol (IQR 0-24.7 ng/mmol, p<0.0001), respectively. usCD163 concentration is elevated in all subgroups of nephrotic syndrome. (See figure 5.2A, 5.3A & B and tables 5.2, 5.5).

5.5.3. usCD163 concentrations are attenuated when corrected for urine protein in active primary nephrotic syndrome

When usCD163 levels are corrected for urinary protein, there is attenuation of the elevated signal in active nephrotic syndrome compared to remission nephrotic syndrome which was observed with urinary creatinine corrected values. Median protein corrected usCD163 concentrations in active nephrotic syndrome are 0.82ng/mg (IQR 0.3-1.65) and in remission nephrotic syndrome are 0.92ng/mg (IQR 0-5.52, p=0.0773), respectively. (See figure 5.2 B, 5.3 C, 5.7 and tables 5.2, 5.5).

5.5.4. usCD163 concentrations are decreased when corrected for urine albumin in active primary nephrotic syndrome

When usCD163 levels are corrected for urinary albumin, there is a decrease in usCD163 concentrations in active nephrotic syndrome compared to remission nephrotic syndrome with median usCD163 concentrations in active nephrotic syndrome of 1.53ng/mg albumin (IQR 0.74-3.1) and remission nephrotic syndrome 2.59 ng/mg albumin (IQR 0-36)), p=0.0021, respectively. (See figure 5.2 C, 5.3 D and table 5.2).
Figure 5.2: Levels of usCD163 in each disease subtype in both remission (protein <0.5g/day) and active nephrosis (protein >3.5g/day). (A) urine creatinine corrected, N=65 paired samples, (B) urine protein corrected, N=61 paired samples. (C) urine albumin corrected, N=60 paired samples.

Figure 5.3: Levels of usCD163 in each disease subtype in both remission (protein <0.5g/day) and active nephrosis (protein >3.5g/day). (A) uncorrected, raw usCD163, (B) urine creatinine corrected, dotted red line represents diagnostic cut off for active vasculitis. (C) urine protein corrected. (C) urine albumin corrected. FSGS = focal and segmental glomerulosclerosis. MN = membranous glomerulonephritis. MCD = minimal change disease. N=65 paired samples.
<table>
<thead>
<tr>
<th>USCD163</th>
<th>CLINICAL STATUS</th>
<th>FOCAL AND SEGMENTAL GLOMERULOSCLEROSIS</th>
<th>MINIMAL CHANGE DISEASE</th>
<th>MEMBRANOUS GLOMERULONEPHRITIS</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw pg/ml</td>
<td>Nephrotic</td>
<td>2935 (IQR 1705-6418)</td>
<td>6414 (IQR 1609-14491)</td>
<td>6414 (IQR 3735-10045)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Nephrotic</td>
<td>0 (IQR 0-239.8)</td>
<td>114 (0-332.8)</td>
<td>180.5 (0-423.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Creatinine corrected ng/mmol</td>
<td>Nephrotic</td>
<td>287.8 (IQR 145.4- 587.8)</td>
<td>539.2 (IQR 114.3-1511)</td>
<td>604.2 (IQR 305.8- 1423)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Remission</td>
<td>0 (IQR 0-13.8)</td>
<td>0 (IQR 0-28.8)</td>
<td>7.7 (IQR 0-33.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Protein corrected ng/mg</td>
<td>Nephrotic</td>
<td>0.82 (IQR 0.5-1.44)</td>
<td>0.43 (IQR 0.23-1.61)</td>
<td>1.01 (IQR 0.49-1.86)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Remission</td>
<td>3.94 (IQR 0.01-9.63)</td>
<td>0 (IQR 0-4.16)</td>
<td>0.82 (IQR 0-5.61)</td>
<td>ns</td>
</tr>
<tr>
<td>Albumin corrected ng/mg</td>
<td>Nephrotic</td>
<td>1.11 (IQR 0.77-2.0)</td>
<td>0.97 (IQR 0.28-2.77)</td>
<td>2.22 (IQR 1.49-4.25)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Remission</td>
<td>3.24 (IQR 0-32.2)</td>
<td>0 (IQR 0-65.5)</td>
<td>3.35 (IQR 0-36.1)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 5.2: usCD163 levels in each disease subcategory as normalised to each variable (in bold) and each clinical setting of nephrosis (proteinuria >3.5g/day) and remission (proteinuria <0.5g/day). IQR = interquartile range. Differences in median values across groups assessed by Kruskal Wallis testing. N=65 paired samples.
5.5.5. *usCD163* levels correlate poorly with urine protein and albumin concentrations

In the setting of active nephrosis *usCD163* levels correlate poorly but significantly with spot protein: creatinine ratio ($r^2 = 0.121, 0.0005$). In the setting of remission nephrotic syndrome *usCD163* levels do not correlate with spot protein: creatinine ratio ($r^2 = 0.008, p=0.9470$). See figure 5.4 A & B.

In the setting of active nephrosis *usCD163* levels correlate poorly but significantly with spot albumin: creatinine ratio ($r^2 = 0.032, 0.00087$). In the setting of remission nephrotic syndrome *usCD163* levels do not correlate with spot albumin: creatinine ratio ($r^2 = 0.009, p=0.9470$). See figure 5.4 C & D.

Figure 5.4: Correlation between *usCD163* (normalised to urine creatinine) and spot protein: creatinine ratio (mg/mmol) in (A) active nephrosis (proteinuria >3.5g/day), $n=65$ and (B) remission (proteinuria <0.5g/day), $n=65$. Correlation between *usCD163* (normalised to urine creatinine) and spot albumin: creatinine ratio (mg/mmol) in (A) active nephrosis (proteinuria >3.5g/day), $n=65$ and (B) remission (proteinuria <0.5g/day), $n=64$. Axes formatted in log10 scale.
5.5.6. usCD163 levels normalised by urine creatinine do not predict renal function decline

Median usCD163 concentrations (normalised to urine creatinine) in renal function decline defined as end stage kidney disease or 40% reduction in GFR and stable renal function were 435.3ng/mmol (IQR 276.5-906.1ng/mmol) and 418.9ng/mmol (IQR 171.4-418.9ng/mmol, p=0.8650), respectively.
5.6. Results – ANCA-associated Vasculitis

5.6.1. Demographics

39 patients met inclusion criteria: ten with active renal vasculitis, ten in remission with residual proteinuria, nine in remission with no proteinuria and ten healthy controls. Those with active vasculitis and remission vasculitis with residual proteinuria had lower eGFR and higher proteinuria than remission vasculitis without proteinuria. See table 5.3.
Table 5.3: Baseline characteristics of those with active renal vasculitis, remission vasculitis with proteinuria, remission vasculitis without proteinuria. Healthy controls are not included as they have no recorded laboratory values (of note they are screened with urinalysis). Proteinuria as measured on scale of 0 to 3+. IQR = interquartile range. SD=standard deviation, PCR = protein: creatinine ratio, UA= urinalysis. P values derived from Kruskal Wallis testing for gender, urinalysis, PCR and serum creatinine. P values derived from Kruskal Wallis testing.

<table>
<thead>
<tr>
<th></th>
<th>ACTIVE RENAL (N=10)</th>
<th>REMISSION PROTEINURIC (N=10)</th>
<th>REMISSION NON-PROTEINURIC (N=9)</th>
<th>HEALTHY CONTROL (N=10)</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AGE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEAN, SD YEARS</td>
<td>64.8 (SD 12.1)</td>
<td>52.1 (SD 14.4)</td>
<td>55.7 (SD 17.4)</td>
<td>68.2 (SD 14)</td>
<td>0.0609</td>
</tr>
<tr>
<td><strong>SEX %, (N)</strong></td>
<td>Male 90% (9)</td>
<td>Male 100% (10)</td>
<td>Male 44.4% (4)</td>
<td>Male 50% (5)</td>
<td>0.0105</td>
</tr>
<tr>
<td><strong>UA PROTEIN MEDIAN, IQR</strong></td>
<td>2.5+ (IQR 1.8-3+)</td>
<td>3+ (IQR 2-3+)</td>
<td>0+ (IQR 0-0+)</td>
<td>N/A</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>UA BLOOD MEDIAN, IQR</strong></td>
<td>3+ (IQR 2.8-3+)</td>
<td>1+ (IQR 0-2.3+)</td>
<td>0+ (IQR 0-1+)</td>
<td>N/A</td>
<td>0.0003</td>
</tr>
<tr>
<td><strong>URINE PCR MEDIAN, IQR</strong></td>
<td>58 (IQR 47-167)</td>
<td>168 (IQR 98-212)</td>
<td>19 (IQR 9-27)</td>
<td>9.7 (IQR 5.7-9.9)</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>SERUM CREATININE MEDIAN, IQR</strong></td>
<td>220 (IQR 128-291)</td>
<td>198 (IQR 128-312)</td>
<td>100 (IQR 80-141)</td>
<td>76 (IQR 62-79)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
5.6.2. usCD163 concentrations are elevated in remission vasculitis with residual proteinuria compared to remission vasculitis without proteinuria

In those with remission vasculitis with residual proteinuria creatinine normalised usCD163 concentrations are elevated at 167ng/mmol compared to healthy controls (1ng/mmol) and remission vasculitis without proteinuria at 1ng/mmol but concentrations are significantly less than active renal vasculitis at 510ng/mmol, p<0.00001 with medians compared across groups using Kruskal Wallis testing. usCD163 concentrations in remission vasculitis with residual proteinuria values are less than our previously validated diagnostic thresholds of <300ng/mmol at diagnosis and <253ng/mmol at renal vasculitis flare[127]. See table 5.4 and figure 5.5A & B. Serum sCD163 values did not differ between groups in keeping with our prior work[127]. See table 5.4 and figure 5.5 C.

![Figure 5.5: (A) urine sCD163 values uncorrected. (B) urine sCD163 values corrected for urine creatinine concentration. (C) serum sCD163 values uncorrected. Line represents diagnostic cut for active renal vasculitis of 253ng/mmol. AR= active renal vasculitis (n=10), RP = remission proteinuric vasculitis (n=9), RNP = remission non-proteinuric vasculitis (n=10, HC = healthy controls (n=10). Bar and lines represent median and interquartile ranges. Groups compared using Kruskal Wallis testing. ns=non-significant, ****p≤0.0001. sCD163 as measured by Duoset ELISA.](image-url)
5.6.3. Correction of usCD163 concentration for urine protein and albumin attenuates increased usCD163 concentration in those with remission vasculitis with residual proteinuria.

In those with remission vasculitis with residual proteinuria median protein normalised usCD163 concentrations are elevated at 1.3ng/mg when compared to healthy controls at 0ng/mg and remission vasculitis without proteinuria 0.1ng/mg but are significantly less than active renal vasculitis 7.8ng/mg, p=0.0001 with medians compared across groups using Kruskal Wallis testing. Utilising a serum: urine protein: sCD163 ratio yielded similar values to proteinuria correction alone. See table 5.4 and figure 5.6.A &C.

In those with remission vasculitis with residual proteinuria median albumin normalised usCD163 concentrations are elevated at 2.1ng/mg when compared to healthy controls at 0ng/mg and remission vasculitis without proteinuria at 1.1ng/mg but concentrations are significantly less than active renal vasculitis at 22.4ng/mg, p<0.0001 with medians compared across groups using Kruskal Wallis testing. Utilising a serum: urine albumin: sCD163 ratio yielded similar values to albuminuria correction alone. See table 5.4 and figure 5.6.B &D.
Figure 5.6: usCD163 values as corrected by various measures. (A) urine sCD163 values uncorrected. (B) serum sCD163 values uncorrected. (C) urine sCD163 values corrected for urine creatinine concentration. Line represents diagnostic cut for active renal vasculitis of 300ng/mmol. (D) urine: serum usCD163: protein ratio. (E) usCD163 values corrected for urine protein concentration. (F) usCD163 values corrected for urine albumin concentration. AR= active renal vasculitis (n=10), RP = remission proteinuric vasculitis (n=9), RNP = remission non-proteinuric vasculitis (n=10), HC = healthy controls (n=10). Bar and lines represent median and interquartile ranges. Groups compared using Kruskal Wallis testing. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 sCD163 as measured by Duoset ELISA.
### Table 5.4: Levels of soluble CD163 values in active renal vasculitis, remission vasculitis with proteinuria, remission vasculitis without proteinuria.

Differences between groups measured by Kruskal Wallis testing. AUC=area under the curve (derived using optimal cutpoints). IQR = interquartile range, sCD163 as measured by Duoset ELISA.
5.6.4. Defining a diagnostic cut off point for those with urine protein or albumin normalised values.

Diagnostic cut off ranges were derived for: urine albumin with cut off of 5.3ng/mg with AUC of 0.815, fractional excretion of albumin with cut off of 1.16 with AUC of 0.864, urine protein cut off of 6.3 ng/mg with AUC of 0.932, fractional excretion of protein cut off of 1.6 with AUC of 0.916, and creatinine normalised with cut off of 362ng/mmol with AUC of 0.916. These values were derived using remission renal vasculitis with and without persistent proteinuria as the comparator group. See table 5.4.
5.7. Results – Combined cohorts

Creatinine normalised usCD163 concentrations are elevated in nephrotic syndrome with median levels up to 2-3x previously reported diagnostic limits for active renal vasculitis. However, use of urine protein correction instead of urinary creatinine sCD163 correction attenuates this signal in active nephrotic syndrome but does not attenuate the signal in those with active renal vasculitis. See figure 5.7 and table 5.5.

![Figure 5.7](image)

Figure 5.7: usCD163 values corrected for urinary (A) creatinine and (B) protein. AAV=ANCA=associated vasculitis. NS=nephrotic syndrome. Bar and lines represent median and interquartile ranges. ns=not significant, **p≤0.01, ****p≤0.0001. Dotted line in (A) represents diagnostic cut off range of 253ng/mmol and (B) represents diagnostic cut off range of 6.3ng/mg for the detection of active renal vasculitis. Significances are reported using Kruskal Wallis testing across all groups (wider bar) and using Mann Whitney testing when comparing active renal vasculitis and active nephrotic syndrome (narrower bar).
Table 5.5: Concentrations of (A) creatinine normalised usCD163 and (B) protein normalised
usCD163. Concentrations reported as median and interquartile ranges (IQR). Significance derived from
Kruskal Wallis testing across groups. sCD163 measured using Duoset ELISA.
5.8 Discussion

This thesis has focused on usCD163 as a biomarker of active renal vasculitis. To safely inform its clinical translation it is vital to understand the clinical caveats of usCD163 interpretation. We therefore studied usCD163 in the setting of proteinuric kidney diseases. We were concerned that despite the glomerular origin of sCD163 in the setting of crescentic glomerulonephritis, in the setting of glomerular filtration barrier injury passage of serum sCD163 could occur leading to detection of non-renal produced sCD163 in urine [113]. In health, usCD163 does not cross the glomerular basement membrane due to its high molecular weight (130kDa).

The ideal scenario in which to assess the potential detected of non-glomerular origin sCD163 in urine is the clinical setting of primary nephrotic syndrome, with glomerular filtration barrier injury due to widespread diffuse foot process effacement. Primary nephrotic syndrome generally lacks macrophages on light microscopy; however, extensive diffuse foot process effacement leads to passage of serum proteins across the glomerular filtration barrier. This phenomenon is clinically manifest with loss high molecular weight proteins such as albumin, immunoglobulins and clotting factors including protein S and antithrombin III [232, 233]. Creatinine normalised usCD163 concentrations were elevated in active nephrotic syndrome compared to remission nephrotic syndrome. There were no significant differences between creatinine normalised usCD163 amongst diagnoses of minimal change disease, FSGS or membranous nephropathy.

Creatinine normalised usCD163 concentrations were elevated in the clinical setting of active primary nephrotic syndrome compared to active renal vasculitis with median levels of 465ng/mmol which is similar to medial levels of 510ng/mmol in our active vasculitis population and twice the diagnostic threshold for active vasculitis (at diagnosis >300ng/mmol and in flare >253ng/mmol). In paired samples from the same patients in remission we found usCD163 levels (normalised to urinary creatinine) were within the normal range.

When usCD163 levels are normalised to urinary protein, there is attenuation of the elevated signal in active nephrotic syndrome compared to remission nephrotic syndrome which was observed with urinary creatinine corrected values. This supports the hypothesis that in the setting of nephrotic range proteinuria urinary creatinine normalised sCD163 concentrations may be elevated in the absence of crescentic glomerulonephritis.
The effect of proteinuria on usCD163 concentrations in renal vasculitis, both active and remission also required further study. Leveraging the RKD biobank subjects were identified with prior renal vasculitis in clinical remission with persistent proteinuria and compared to subjects with prior renal vasculitis in clinical remission without proteinuria, subjects with active renal vasculitis and healthy controls. Healthy controls were of similar age and sex distribution but had lower serum creatinine values and less proteinuria compared to vasculitis patients.

Urine creatinine normalised usCD163 concentrations were elevated in remission renal vasculitis with proteinuria compared to remission renal vasculitis without proteinuria and healthy controls. However, urine creatinine normalised usCD163 concentrations in remission renal vasculitis were lower than active renal vasculitis. A proposed urine CD163 protein normalised cut off range for the detection of active renal vasculitis was proposed with AUC of 0.932.

An additional goal of this chapter was to assess the utility of different methods of correcting for potential passage of sCD163 across the glomerular filtration barrier. Both urine protein and urine albumin normalisation of usCD163 concentrations were attempted as, in health neither protein nor albumin should cross the glomerular filtration barrier. Fractional excretion of sCD163 relative to albumin and protein was attempted as an additional method to assess the relative serum contribution to urine detected sCD163 concentrations. Thus replicating formulae used to assess fractional excretion of sodium [234]. Use of a fractional excretion ratio of paired serum and urine CD163 relative to protein and albumin produced similar results with preservation of signal in active renal vasculitis.

The use of total urine protein rather than urine albumin holds more biological plausibility for glomerular filtration barrier leakage as albumin has a lower molecular weight than total protein and is more freely filtered. Additionally, the use of total protein account for all urinary protein therefore including any usCD163 that is filtered.

Given the favorable biomarker characteristics, ease of translation into clinical practice we favour urine total protein correction. Based on the experimental work contained within this chapter, I would recommend in clinical practice that samples sent for usCD163 assay have urine creatinine and total protein measured in addition to urine sCD163. In the setting of high-grade proteinuria usCD163 concentrations would be reported as both urine creatinine and protein normalised with reference ranges to allow accurate clinical interpretation.
Our urine protein correction findings were similar to those reported by Endo et al when they explored the role of usCD163 in lupus nephritis. In this setting distinguishing between lupus nephritis classes III/IV and V is vital as they have differing treatments and prognoses. Urine protein correction successfully attenuated the increased signal in class V but not in classes III and IV.

Future directions from this work include the validation of the derived diagnostic cut off points for active renal vasculitis and remission renal vasculitis with usCD163 normalisation with urine protein in addition to urine creatinine.
Chapter 6:

Discussion
6.1. Introduction

In this thesis I have identified a highly sensitive and specific association between urinary sCD163 and active renal vasculitis in the clinical setting of flare in both prospective and retrospective studies. I have validated the clinical utility of usCD163 in the setting of high-grade proteinuria. There is a strong biologic rationale for usCD163 in the monitoring of renal vasculitis activity as it is a macrophage marker that is strongly expressed in glomerular crescents with direct shedding of soluble CD163 protein from the glomerular crescent cell surface directly into the urinary space leads to elevated levels. usCD163 is easily measured by commercial ELISA with this thesis validating a diagnostic grade assay. See figure 6.1.

Figure 6.1. Pathway from research protein of interest to validated diagnostic grade assay.
6.2. Key findings of this thesis:

6.2.1. usCD163 is elevated in subtle renal vasculitis flare and is superior to uMCP-1.

In collaboration with the Vasculitis Clinical Research Consortium usCD163 and uMCP-1 were measured in a serially sampled longitudinal multicentre cohort with clinically mild disease.

Both biomarkers were elevated in the presence of active renal vasculitis, with usCD163 displaying a slightly larger area under the ROC than uMCP1. The low degree of correlation between usCD163 and uMCP1 highlighted their differing origin is the fact that each reflects a different component of the glomerular macrophage recruitment and activation pathway. In this setting of subtle clinical evidence of active renal vasculitis, the moderate clinical utility of each biomarker in isolation was enhanced by using usCD163 to exclude active vasculitis, and then grouping the “usCD163+ / uMCP1+” and “usCD163 / new proteinuria” as the two “Yes” nodes, giving a positive LR of 19. This decision tree approach is more accurately the use of novel biomarkers in clinical practice.

usCD163 and uMCP-1 correlated poorly confirming alternate biological characteristics (monocyte and macrophage recruitment). Strengths include the large sample size, longitudinal nature. Weaknesses include the lack of biopsy data, minimal information on putative flare.

6.2.2. usCD163 is diagnostic of renal vasculitis flare

A national prospective multi-centre study was performed to assess the diagnostic utility of usCD163 in those with suspected renal vasculitis flare.

I found that usCD163 is elevated in renal vasculitis flare with concentrations remaining normal in renal vasculitis flare mimics such as sepsis, isolated hematuria and extra renal vasculitis. usCD163 displayed exceptional biomarker characteristics in this setting with an AUC of 0.95 with superiority to RBC casts, BVAS criteria, change in serum creatinine. It had similar biomarker characteristics to renal biopsy with detection of renal inflammation in those with negative biopsies but clinical treatment for flare. The use of a diagnostic grade assay in this chapter will enhance the potential clinical translation of sCD163 into clinical use.

Strengths of this chapter include the multicentre, prospective study design and inclusion of real-world flare mimics as denominator, large sample size and longitudinal nature. The primary weakness of this study was the lack of biopsy confirmation of renal and non-renal flare diagnoses.
6.2.4. usCD163 is elevated in the clinical high-grade proteinuria but correction for urinary protein and/or albumin normalises sCD163 level in those without active renal vasculitis

In this retrospective multi-centre international collaboration, we showed that usCD163 concentration is elevated in the setting of primary nephrotic syndrome with nephrotic range proteinuria with levels up to twice the diagnostic cut off for active vasculitis. In this cohort usCD163 should be undetectable as there is no local source of sCD163 production, however in this cohort there is extensive diffuse foot process effacement suggesting leak of serum sCD163 across the glomerular filtration barrier. When sCD163 levels were corrected for either urine protein or albumin levels were attenuated in nephrotic syndrome.

In this retrospective multi-centre study, we showed that usCD163 concentration is increased in remission AAV with persistent proteinuria compared to remission, but levels remained significantly less than those with active vasculitis. When results were normalised to urine protein, urine albumin and ratios of urine to serum sCD163, albumin and protein there was no significant difference between remission proteinuric and remission non-proteinuric subjects, but sCD163 concentrations remained elevated in active renal vasculitis. We proposed the use of a urine protein normalised CD163 concentration in the setting of high-grade proteinuria.
6.3. Clinical caveats of usCD163

6.3.1. Safe translation into clinical practice

Translation of any biomarker safely into clinical practice requires clear definition of false positive and negative results to enable treating Physicians to accurately and safely interpret assay results in a clinical context. Caveats identified to date include diabetes mellitus, high-grade proteinuria, other forms of glomerulonephritis, renal necrosis and renal vasculitis flare prediction.

6.3.2. Diabetic Nephropathy

Macrophages are implicated in the pathogenesis of progressive diabetic nephropathy[235, 236]. Given the general increasing prevalence of type II diabetes worldwide and occurrence of steroid related diabetes in this disease population defining the usCD163 signature in diabetic nephropathy is of great clinical importance[237-239]. To answer this pertinent question, we have collaborated with the National University of Ireland Galway to measure usCD163 in their longitudinal cohort of patients with type II diabetes mellitus. These assays will be performed on 600 samples using the clinical grade usCD163 assay in Trinity College Dublin.

6.3.3. Proteinuria

High-grade proteinuria as a clinical caveat is addressed in chapter 5 of this thesis. usCD163 concentrations are increased with nephrotic range proteinuria but are normalised by correction for urine protein. We recommend that in the setting of high-grade proteinuria and renal vasculitis that correction for urine total protein is performed to assess the level of locally produced usCD163.

6.3.4. Renal Infarction/Necrosis

Drilling into the clinical phenotypes of our false positive cases is vital to understand the potential clinical scenarios in which usCD163 may have lower specificity for renal AAV. One of these areas is of renal necrosis. Two patients in the course of our prospective studies were clinically diagnosed with renal infarction and papillary necrosis, both of which had elevated usCD163 levels. This is an area that will require further monitoring and study. We plan to address this query by prospectively recruiting all patients with renal infarction as disease controls to the Rare Kidney Diseases Biobank to assess the expected range of usCD163 in this population.
6.3.5. Future flare prediction

In our multicentre longitudinal retrospective study of usCD163 in subtle renal vasculitis flare we compared usCD163 levels at pre-flare visits to remission and active renal vasculitis with no increase compared to baseline levels noted. In the prospective study of renal vasculitis flare two patients were enrolled with possible flare one year prior to actual flare developing. There was no signal of increased usCD163 in these subject’s pre-flare either. This is in keeping with our understanding of usCD163 pathophysiology as macrophages are only present within glomeruli during disease activity not prior to activity.

6.3.6. Standardised diagnostic ranges

Prior to the advent of a diagnostic grade assay determination of a robust diagnostic range was a major clinical challenge. As we gain further understanding of the assay characteristics it cut off ranges may be further defined. What is encouraging however are the similarities in diagnostic cut off ranges using the clinical grade assay and our prior work with values of greater than 253ng/mmol in renal flare (both R&D Systems, Duoset and Euroimmun) and greater than 300ng/mmol at time of diagnosis (R&D Systems, Duoset). We have collaborated with Professor Moller at the University of Aarhus, who developed the first sCD163 assay regarding the development of an international set of standard concentrations.

Additionally, further work is required to assess optimal diagnostic ranges in other disease subtype such as lupus nephritis and anti-glomerular basement membrane disease as in these disease states a higher percentage of glomeruli are involved and median values appear higher. Further study in these clinical conditions using a diagnostic grade assay will define whether or not the same diagnostic thresholds apply to all forms of crescentic glomerulonephritis or not.

Selecting a diagnostic cut off range is highly nuanced, balancing both statistical possibilities with clinically acceptable risk. Diagnostic ranges can be set to maximise sensitivity, specificity or a combination of both. I performed extensive assay and diagnostic cut off point validation with the most clinically relevant cut off derived using the Youden index to maximise both sensitivity and specificity.
6.3.7. Limitations of inclusion of Eosinophilia with Granulomatosis with Polyangiitis

Eosinophilic granulomatosis with polyangiitis (EGPA) is characterised by eosinophil-rich and necrotizing granulomatous inflammation. In contrast to MPA and GPA, only 30-40% of cases are ANCA positive[20]. ANCA positivity is more frequent when glomerular disease is present. Renal involvement is less common than MPA and GPA with estimated of 7.7-25% of patients. Inclusion of patients with EGPA in chapters 3 and 4 was performed as we had pre-selected inclusion criteria of all AAV meeting Chapel Hill Consensus criteria. Given the lower prevalence of renal involvement in EGPA there is a lower pre-test probability than including patients with MPA and GPA[240].
6.4. Translation from promising research assay to clinical test

The conversion of research laboratory assay to a clinical grade diagnostic test is a great challenge. Potential characteristics that could limit the clinical translation of a biomarker include to protein physicochemical characteristics such as instability at room temperature, specific storage conditions required, assay characteristics such as manufacturing steps, clinical laboratory accreditation, assay reproducibility, correlation with other previously validated assays and a lack of robust clinical phenotyping.

Through my PhD with Professor Little we have transitioned from identifying a novel protein in urine of patients with AAV, demonstrating biological plausibility using animal model, immunohistochemical staining of human kidney with renal vasculitis which provided a solid foundation for my thesis and goal of personalising care of patients with AAV. Through three retrospective cohort studies and one prospective observational study we have clearly identified the clinical utility of usCD163 in the detection of active renal vasculitis at time of disease flare and throughout induction therapy. Importantly, we have identified the potential clinical caveats of the assay with notable concerns in the setting of high-grade proteinuria. We have addressed this potential caveat and found correction using urine protein concentration improves the diagnostic fidelity of this usCD163. Potential other areas of clinical caveat include usCD163 interpretation in those with co-existent diabetes mellitus given the presence of macrophages in diabetic nephropathy. Our group are currently investigating the role of usCD163 as a marker of diabetic nephropathy progression and this will further inform our knowledge. In clinical practice in patient with AAV and coexisting diabetes mellitus the role of usCD163 may be more as a “rule out” test rather than empirically treating based on a positive result.

A key step in the translation of usCD163 from research to diagnostic grade assay has been collaboration with industry. Having rigorously validated commercially available research grade and in-house research grade ELISAs we were then able to partner with industry to develop a diagnostic grade commercially available usCD163 ELISAs. We identified the R&D Systems Duoset sCD163 assay as the most accurate research grade usCD163 ELISA in chapter 2 and then used this assay to validate the newly developed diagnostic grade ELISA (Euroimmun) in chapter 4. This assay has received CE marking, ISO 15189 and NEQAS accreditation is planned to allow for widespread adoption by clinical laboratories at reasonable cost with minimal training and no additional equipment required.
6.5. Impact

6.5.1. Patient impact

The use of a non-invasive tool to monitor renal inflammation is greatly beneficial to individual patients as it may enable earlier detection of renal inflammation with resultant reduction in glomerulosclerosis and chronic kidney disease progression. Renal biopsies may be avoided and their resultant potential complications such as bleeding limited. An additional disruptive factor for patients undergoing biopsy is the need to avoid heavy lifting for at least two weeks post procedure to avoid haemorrhage. Chronic health conditions are associated with increased anxiety and stress, reassurance from a normal usCD163 result may be of benefit to patients living with this relapsing chronic condition.

This work and the RKD biobank have been supported by the Vasculitis Ireland Association (VIA), the Irish patient advocacy group. Provisional results of this work were presented at the VIA annual meeting. The positive findings of this work we hope will continue to encourage patients with vasculitis to engage with and participate in clinical research going forwards.

6.5.2. Healthcare Impact

The implications for healthcare are broad. A reduction in renal biopsies would lead to significant direct and indirect cost savings. Outpatient renal biopsy cost is estimated at €800, however inpatient biopsy and admission for management of complications is significantly higher. There are safety implications with potential morbidity and mortality associated with biopsy. This patient population frequently possesses many of the risk factors for haemorrhage including advanced CKD, anticoagulant use and hypertension.

Additional implications include potential increased detection of active renal vasculitis, there is a subpopulation of renal vasculitis patients progress to end-stage kidney disease without clinically evident vasculitis flare. These patients may have ongoing undetected renal inflammation that is ascribed clinically to CKD progression. CKD and ESKD have increased morbidity and mortality with cardiovascular disease as a primary cause of death. Undetected untreated inflammation risks both local organ damage but also venous thromboembolic and cardiovascular risk. usCD163 may be more sensitive than renal biopsy given potential sampling error with examination of relatively few glomeruli of millions possibly affected.
Another concern is the safety implication of a potentially missed diagnosis of flare. The overall risk of this appears low with excellent negative predictive values but it must be borne in mind when considering implications of widespread usCD163 usage.

Use of usCD163 outside of the field of vasculitis could have even broader implications as discussed above as lupus, IgA nephropathy or have higher prevalence and present earlier in life. Another potential use that would greatly impact on healthcare service provision is as screening test for crescentic glomerulonephritis in undifferentiated acute kidney injury in situations where access to urgent kidney biopsy is limited.

6.5.3. Societal Impact for Irish population

The Irish research prioritization exercises have articulated the requirement for translation of clinical research into commercial sable innovation. This novel biomarker has potential for generation of new employment opportunities, licensing agreements, export income or a spin-off company, any of which would provide benefit to the Irish public in excess of the initial project investments.

6.5.4. Personal impact

This work was funding by the Health Research Board (HRB) of Ireland whose motto is health, research, action. I was awarded the National Specialist Registrar Academic Fellowship in 2013 which allowed me to combine my clinical and academic training with the ultimate goal of a career as a Clinician Scientist.

The NSAFP was an innovative training programme which selected two individuals from all fields of medicine biennially and offered the combined clinical higher specialist training and the opportunity to complete a PhD. I estimate the overall cost of my training in this programme at €400,000. An additional benefit I derived from the programme was to obtain sub-speciality training in vasculitis and glomerulonephritis. I am therefore on completion of this programme ready to progress academically to intermediate development grants such as the Wellcome Trust Career Development Grant.

Over the course of this fellowship, I have had one first author original paper with three further planned imminently (chapters 4,5,6) as well as seven co-author publications, three invited reviews, multiple oral and poster presentations[35, 66, 77, 97, 127, 147, 241-243].
6.6. Preliminary Data on usCD163 throughout induction therapy

6.6.1 An unmet need
An unmet need exists in the personalised guidance of immunosuppressive therapy intensity. Urine sCD163 is a marker of glomerular crescent macrophage activation with prior work demonstrating increased usCD163 levels in renal ANCA-associated vasculitis (AAV) at diagnosis and flare.

6.6.2. Methods
We prospectively enrolled patients with crescentic glomerulonephritis (CGN) undergoing cytotoxic induction therapy and obtained serial detailed clinical phenotypic information and urine samples. Those who did not undergo renal biopsy had met ACR or Chapel Hill Consensus Conference classification criteria for AAV with clinical evidence of renal activity (increase in serum creatinine >30%, new/worse hematuria or proteinuria). See figures 6.2 and 6.3. usCD163 was measured undiluted by commercial ELISA prototype (Euroimmun). See methods chapter 2 section 2.4.4. Urine biomarker values were normalised to urine creatinine. Refractory disease was defined as per EULAR guidelines with lack of treatment response despite adequate immunosuppression and persistent positive BVAS score.

Figure 6.2: Graphic demonstration of research sampling from diagnosis to switch visit. Urine containers represent usCD163 samples, blood tubes represent serum research samples.
Figure 6.3: Flow diagram of recruitment from screening to study conclusion

- Screened (47)
  - Ineligible (5):
    - Did not receive induction therapy (4)
    - No CGN on Biopsy (1)
  - Consented (42)
    - Excluded (9):
      - Insufficient urine samples (9)
  - Completed Follow-up (33)

- ANCA Vasculitis (25)
  - Microscopic Polyangiitis (16)
  - Granulomatosis with Polyangiitis (9)
  - aGBM Disease (6) (Dual + 4)
  - IgA Vasculitis (1)
  - Class IV Lupus Nephritis (1)

- Other Vasculitis (8)
  - Treatment Responsive (24)
  - Treatment Refractory (9)
6.6.3. Baseline Characteristics

Urine samples and clinical data were obtained at 202 clinical encounters from 33 patients, of whom 26 (78.7%) had AAV, 5 (15.2%) had anti-glomerular basement membrane disease and 2 (6%) had other CGN. CGN was a new diagnosis in 68.7% (23) of patients. 78.7% (26) of patients underwent renal biopsy confirmation of CGN. Of those a diagnosis of renal AAV the Berden classification was crescentic in 25% (6), mixed in 25% (6), focal in 20.8% (5), sclerotic in 4% and biopsy was non-diagnostic in 8.3% (2)[33]. Mean eGFR at enrollment was 26.5mls/min (SD ±18.2). Induction therapy comprised corticosteroids plus cyclophosphamide alone in 15 (45%), rituximab alone in 9 (27%) and both cyclophosphamide and rituximab in 9 (27%). Median usCD163 level was 1091ng/mmol (IQR 347-1705ng/mmol), 589ng/mmol (IQR 266-589), 226ng/mmol (IQR 154-401) and 200ng/mmol (IQR 126-283ng/mmol) at diagnosis, one, three and six months respectively. See table 6.1.
<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th>AAV</th>
<th>NON-AAV</th>
<th>SIGNIFICANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=33</td>
<td>N=26</td>
<td>N=7</td>
<td></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female %, (N)</td>
<td>42.3% (11)</td>
<td>42.9% (3)</td>
<td>ns</td>
</tr>
<tr>
<td>Age mean, (SD)</td>
<td>62.9 years (11.8)</td>
<td>49.8 years (SD 23.8)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPA, %, (N)</td>
<td>65.4% (17)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GPA, %, (N)</td>
<td>34.6% (9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>aGBM, %, (N)</td>
<td>-</td>
<td>71.4% (5)</td>
<td>-</td>
</tr>
<tr>
<td>Other*, %, (N)</td>
<td>-</td>
<td>28.6% (2)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Treatment Refractory</strong></td>
<td>15.3% (4)</td>
<td>71.4% (5)</td>
<td>0.0086</td>
</tr>
<tr>
<td>Disease Duration median, (IQR), months</td>
<td>0 (0 – 3.1)</td>
<td>0(0 – 2.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Biopsy Berden Classification, %, (N)</td>
<td>Crescentic 24% (6), Mixed 24% (6)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>%, (N)</td>
<td>Focal 20% (5),</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Sclerotic 4% (1),</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>No Biopsy 20% (5),</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Non-Diagnostic 8% (2)</td>
<td>-</td>
<td>-</td>
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<td><strong>Induction therapy</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Ever Cyclophosphamide %, (N)</td>
<td>76.9% (20)</td>
<td>100% (7)</td>
<td>ns</td>
</tr>
<tr>
<td>Current Cyclophosphamide</td>
<td>65.4% (17)</td>
<td>100% (7)</td>
<td>ns</td>
</tr>
<tr>
<td>Current IV/ PO/ IV + PO</td>
<td>12/3/2</td>
<td>5/1/1</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma Exchange %, (N)</td>
<td>23.1% (6)</td>
<td>71.4% (5)</td>
<td>ns</td>
</tr>
<tr>
<td>Ever Rituximab % (N)</td>
<td>58.3% (14)</td>
<td>44.4% (4)</td>
<td>ns</td>
</tr>
<tr>
<td>Current Combined Rituximab &amp; Cyclophosphamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Renal Parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diagnosis eGFR mean, (SD), mls/min</td>
<td>26.6 (± 17.4)</td>
<td>26.1 (± 22.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Diagnosis Creatinine median, (IQR), umol/L</td>
<td>248 (136-413)</td>
<td>269 (146-386)</td>
<td>ns</td>
</tr>
<tr>
<td>Diagnosis CRP Median, (IQR), mg/L</td>
<td>41 (4-109)</td>
<td>61 (17-161mg/L)</td>
<td>ns</td>
</tr>
<tr>
<td>Diagnosis Haematuria Mean, (± SD)</td>
<td>2.6+ (± 0.6+)</td>
<td>2.6+ (±0.8+)</td>
<td>ns</td>
</tr>
<tr>
<td>Diagnosis Proteinuria Mean, (± SD)</td>
<td>2.3+ (± 0.6)</td>
<td>2.1+ (± 0.9)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Table 6.1 Demographic and Clinical Characteristics of Study Subjects. The treatment responsive and refractory contributed samples throughout induction therapy. IQR=Interquartile range, GPA = Granulomatosis with polyangiitis, MPA = Microscopic polyangiitis, aGBM= anti-glomerular basement membrane disease, Other* = IgA vasculitis, class IV lupus nephritis. Berden Biopsy Classification only validated in AAV, therefore only subjects with AAV included (n=26), **Ever and Current Rituximab figures are identical, eGFR= estimated glomerular filtration rate, CRP = C-reactive protein, ANCA= anti-neutrophil cytoplasmic antibody. Range for urinalysis haematuria and proteinuria is 0-4+. Diagnosis = visit 1. Differences between groups tested using Mann Whitney U test.
6.6.4. usCD163 levels fall throughout induction therapy

usCD163 levels fall from the time of diagnosis with time of diagnosis median usCD163 level of 1091ng/mmol (IQR 347-1705ng/mmol), with rapid fall by 1-2 months to 589ng/mmol (IQR 266-589). (p <0.0001). See table figure 6.2 and figure 6.4 and 6.5. However, in treatment refractory CGN levels remain elevated compared to those with treatment responsive CGN.

In those who are refractory median levels at diagnosis, one month and three months are 1616ng/mmol (IQR 825-2167ng/mmol), 2021ng/mmol (IQR 1116-2768ng/mmol) and 686ng/mmol (116-1218ng/mmol), respectively. In those who are responsive median levels at diagnosis, one month and three months are 889.3ng/mmol (IQR 330-1586ng/mmol), 589ng/mmol (IQR 266-1043ng/mmol) and 208.2ng/mmol (IQR 161 – 291ng/mmol), respectively. Levels are significantly different between the cohorts at one and three months with p values of 0.0016 and 0.0093, respectively. See table figure 6.2 and figure 6.4 and 6.5

Figure 6.4: usCD163 levels from time of diagnosis (month 0) to 12 months. Clear circles represent treatment responsive cases, black shapes represent treatment refractory cases. Circle= AAV diagnosis, Triangle= non-AAV diagnosis. Differences between groups calculated by Kruskal Wallis testing comparing usCD163 over time. sCD163 measured using Euroimmun ELISA.
6.6.5. Preliminary Discussion of usCD163 concentrations throughout induction therapy

A responsive marker of active renal inflammation throughout induction therapy is lacking. Despite significant progress designing and validating effective induction and maintenance treatment strategies personalised individual patient care is limited by lack of sensitive and specific tools to identify the presence of ongoing renal inflammation or, conversely the absence of inflammation. This prospective multicentre observational study of usCD163 throughout induction therapy for crescentic glomerulonephritis demonstrates that usCD163 levels are elevated at diagnosis, fall throughout induction therapy in those who are treatment responsive and identify those with resistant disease by one month.

Adverse events related to AAV therapy are highly prevalent and are the leading cause of mortality in the first year following diagnosis. One-year mortality is 11.1% with 59% of deaths caused by therapy associated adverse events and 14% of deaths caused by active vasculitis[13]. Additionally, 53% of patients develop infections requiring antimicrobial therapy and 28% were hospitalized for treatment of infectious complications within the first year following diagnosis[59]. The high rate of infections and their associated mortality risk imply that there is a significant population we are potentially over-treating but this risk is balanced by the persistent risk of vasculitis related end organ complications including end stage kidney disease.

Our current tools to monitor renal inflammation include serum creatinine, urinalysis and BVAS criteria but lack sensitivity and specificity with over 40% of patients having persistent haematuria at time of switch to maintenance therapy[77]. A tool for identifying refractory disease at an early stage is lacking.
6.7. Implications beyond ANCA-associated vasculitis

usCD163 is now a well validated biomarker in the setting of active renal vasculitis, but this work has broader implications for other forms of crescentic glomerulonephritis in particular lupus nephritis. Robust work is required to validate or even potentially alter the diagnostic ranges in lupus nephritis due to the extent of crescents which is traditionally greater than in ANCA associated vasculitis.

6.7.1. Lupus Nephritis

As discussed in introductory chapters sCD163 has been shown to be elevated in urine class III and IV lupus nephritis using a research grade assay in addition to immunohistochemical staining of renal biopsy specimens. What is lacking however is validation of reference ranges for active lupus nephritis, prospective validation of its role for diagnosis of lupus nephritis flares in those with synchronous biopsy specimens. usCD163 could potentially have a role in identifying those refractory to LN induction therapy at an earlier time point, allowing alteration in immunosuppressive regimen prescribed. Potentially, retrospective use of trial data with accompanying urine samples could answer this clinically relevant question and address an area of great need.

6.7.2. IgA Nephropathy

An additional area of potential use for usCD163 is in the clinical setting of IgA nephropathy. IgAN is the world’s commonest primary glomerular disease[244]. It has a broad clinical phenotype with variable clinical presentations and progression rates. Overall, 10-50% of patients progress to end stage kidney disease (ESKD) over 20 years[245-247]. Treatment decisions are currently driven by clinical, laboratory and histologic findings. A critical feature of IgAN management is the detection of disease activity prior to accrual of significant damage. Our current limited ability to predict outcome in individual patients highlights the great clinical need for a non-invasive tool to assess disease activity in IgAN and tailor individual treatments[248].

There is increasing recognition of the role of macrophages in IgAN with a recent University of Toronto collaboration showing that CD163 has a 6.48-fold increased signal in E1 endocapillary proliferation compared to E0 proliferation, based on the Oxford classification in renal biopsy transcriptomic data[249, 250]. Prior pathological studies have shown that macrophages, which were identified as CD68 positive cells are increased in IgAN in particular in the presence of proliferative lesions such as endocapillary cellularity or mesangial hypercellularity and their presence associates with a worse
Additionally, crescents occur in up to 38% of IgA nephropathy biopsies and their presence associated with a higher risk of a >50% decline in eGFR or ESRD[255]. Urine monocyte chemoattractant protein-1 levels have been shown to be increased in IgAN with elevated levels associated with higher grades of proteinuria and lower GFR. MCP-1 is produced primarily by resident renal cells, such as mesangial and tubular epithelial cells, and results in chemoattraction of monocytes, which presumably differentiate to become the plentiful glomerular macrophages. The utility of MCP-1 as a urine biomarker is limited as it is relatively instable ex vivo thus limiting its translation into clinical practice[130].

6.7.3. Undifferentiated acute kidney injury

Rapid, accurate identification of those with a clinical suspicion of inflammatory kidney disease using usCD163 could be used to triage AKI cases requiring specialist input. A clinical trial could enable assessment of the utility of usCD163 in the detection of inflammatory kidney disease in remote or rural populations. Potential challenges could include an increased rate of renal biopsy but equally may allow rapid identification of those with inflammatory renal disease requiring urgent treatment. Initial steps would include retrospective study in collaboration with an AKI consortium, utilising their closely phenotyped samples to allow safe design of an interventional trial for the assessment of the utility of usCD163 in the diagnosis of AKI aetiology.

6.7.4. Dialysis dependent acute kidney injury

In those with AAV and dialysis dependence an estimated 50% of patients will recover independent renal function. I hypothesis that if usCD163 concentrations remain elevated there is likely ongoing crescentic glomerulonephritis which may be amenable to ongoing immunosuppression. Conversely, if usCD163 concentrations remain low in the setting of dialysis dependence, then there is low probability of regaining independent renal function. There is a great clinical need to futility and limit immunosuppressive exposure in renal limited AAV.

6.7.5. usCD163 in those undergoing for cause renal biopsy

This would involve measurement of usCD163 at time of synchronous renal biopsy in all comers to further define the structure function relationship between usCD163 and renal histological findings.
6.7.6. Does usCD163 at time of AAV diagnosis predict renal survival

Using the RKD biobank we will assess all usCD163 at time of diagnosis (with subgroup treatment naïve) and see if this is predictive of one, two, five-year composite renal endpoint of ESKD, failure to recover GFR x% from diagnosis. Secondary end points would include proteinuria at timepoints, KFRE at timepoints. Comparison will be made between Berden classification prediction of outcome and usCD163 prediction of outcome.

6.8. Summary

In summary, usCD163 is strongly associated with active renal vasculitis in the clinical setting of flare (both prospectively and retrospectively) and during induction therapy including switch to maintenance immunotherapy. There is robust background biologic rationale for usCD163 as a biomarker of crescentic glomerulonephritis as it is a macrophage marker that is strongly expressed in glomerular crescents with direct shedding of soluble CD163 protein from the glomerular crescent cell surface directly into the urinary space leads to elevated levels. usCD163 is easily measured and a diagnostic grade assay is now available for clinical use. High-grade proteinuria can lead to an elevated urinary level, but this is easily attenuated using urine protein correction. This work has widespread clinical application with potential reduction in requirements for renal biopsy and translation to other forms of renal disease including lupus nephritis, IgA nephropathy and diabetic nephropathy.
7.0 Bibliography


8.0 Appendix I: List of Tables

**Chapter 1**

Table 1.1. 2x2 factorial design table

**Chapter 2**

No tables

**Chapter 3**

Table 3.1 Demographic and Clinical Characteristics of Study Subjects. The active renal and active non-renal patients contributed sample and clinical data at the time of flare, as well as 1-3 samples at visits both before and after the flare event. IQR=Interquartile range, GPA=Granulomatosis with polyangiitis,
MPA=Microscopic polyangiitis, EGPA=Eosinophilic granulomatosis with polyangiitis. ANCA=anti-neutrophil cytoplasmic antibody, PR3=proteinase 3, MPO=myeloperoxidase.

Table 3.2. Clinical characteristics according to disease activity. Differences between groups measured by Kruskal Wallis test. IQR=Interquartile range, eGFR=estimated glomerular filtration rate (MDRD equation[169]), current IS= currently prescribed immunosuppression. Acute kidney injury determined by AKIN Criteria. *refers to the eGFR at the earliest time point.

Table 3.3. Correlation coefficients between measures of usCD163 as measured by four assays and uMCP-1 as measured by Spearman correlation. All associations reached significance with p<0.0001.

Table 3.4. usCD163 levels in active and remission renal vasculitis (both remission and active non-renal vasculitis) based on each experimental assay. Values are reported as median and interquartile ranges. Groups compared using Mann Whitney U test. IQR=Interquartile range, AUC = area under the curve.

Table 3.5. Biomarker characteristics. In each case, the ability of the biomarker to correctly classify patients with active renal vasculitis was tested within a heterogenous cohort also including patients in remission and those with active extra-renal disease. *The decision tree cut-points were determined by recursive partitioning. PPV=positive predictive value, NPV=negative predictive value, PLR=positive likelihood ratio, NLR=negative likelihood ratio, AUC=area under the receiver operator characteristic curve. usCD163= urine soluble CD163, uMCP-1= urine monocyte chemoattractant protein 1.

Table 3.6. Mixed effects modelling of novel biomarkers in longitudinal samples. The Coefficient value for renal flare gives the typical level of transformed biomarker for a female, ANCA-negative renal patient in a renal flare episode. This also represents the baseline level for the other explanatory variables i.e., it represents the typical level for females, ANCA-negative, no new proteinuria and no new haematuria. The remaining Coefficient values represent differences from this baseline for a patient with the given characteristic. For example, a male patient in remission will typically have a transformed sCD163 level of 3.586 (= 4.362 - 0.881 + 0.105). The p-values confirm that renal flare episodes are the only significant characteristic in explaining the transformed biomarker values through mixed effects modelling. ANCA= anti-neutrophil cytoplasmic antibody. usCD163 measured by R&D Systems Duoset Assay. N=320

Table 3.7 Post-decision tree probability in a series of hypothetical scenarios. Using the calculated positive likelihood ratios and negative likelihood ratios, post-test probability was compiled for
situations where the pre-test probability varied between 5%, 40% and 70%. usCD163 as measured by R&D Systems Duoset assay

Chapter 4

Table 4.1. Baseline characteristic derived from clinical parameters from last review prior to study visit. MPA= microscopic polyangiitis, GPA = granulomatosis with polyangiitis, EGPA= eosinophilic granulomatosis with polyangiitis, AAV/aGBM denotes overlap syndrome of dual positive ANCA and aGBM antibodies, MTX = methotrexate, MMF = mycophenylate mofetil, SD= standard deviation, IQR = interquartile range. Differences between flare and remission groups measured by Mann Whitney U and T tests.

Table 4.2. Clinical characteristics at time of study visit (flare or flare mimic) in both cohorts combined. eGFR=estimated glomerular filtration rate, CRP=c-reactive protein, ANCA=anti-neutrophil cytoplasmic antibody, PCR=protein: creatinine ratio, UA= dipstick urinalysis, RBC=red blood cell, BVAS=Birmingham Vasculitis Activity Score, clinical impression was as per treating Physicians opinion at time of study visit, SD= standard deviation, IQR = interquartile range. Differences between flare and remission groups measured by Mann Whitney U and T tests.

Table 4.3. Final adjudicated diagnosis and usCD163 values in renal vasculitis flare and renal vasculitis flare mimics as measured by Euroimmun assay. N=84. CKD=Chronic kidney disease. *Not due to renal vasculitis

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Chapter 5

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9.0 Appendix II: List of Figures

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glomeruli, (3) in the periglomerular region, (4) within tubules, and (5) in the interstitial compartment. (C) CD163 scores in each of the respective five regions stratified by clinical diagnosis (upper graphs) and antibody specificity (lower graphs), (P<0.05; **P<0.01; ***P<0.001). (D–I) Images depict representative low power (×40 magnification) views of healthy control (D) and vasculitic (E) kidney, alongside high power (×400) views of healthy control kidney (F), a glomerulus with mild vasculitic injury (G, arrow), a severely affected glomerulus with established crescent formation (H, arrow), and a glomerulus with a fibrous crescent from previous vasculitic injury (I, arrow, ×200). MPA, microscopic polyangiitis; GPA, granulomatosis with polyangiitis.[127]

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**Chapter 2**

**Figure 2.1.** R&D Systems Duoset sCD163 ELISA Protocol

**Figure 2.2.** Abcam Quantikine sCD163 ELISA Protocol

**Figure 2.3.** R&D Systems Quantikine sCD163 ELISA Protocol

**Figure 2.4.** Euroimmun sCD163 ELISA Protocol

**Chapter 3**

**Figure 3.1.** uMCP-1 ELISA experimental protocol
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Figure 3.3. Graphic representation of correlations between each usCD163 assay and uMCP-1 as measured by Pearson correlation. All correlations reached statistical significance (p<0.0001). Logarithmic scales (log10) are used to compare each assay. n=320

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**Chapter 4**

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**Figure 4.3.** Flow diagram of recruitment from screening to enrollment and subsequent diagnosis. Inception and validation cohorts usCD163 values were measured by different assays. High Probability and Possibly denote Physician impression at the time of study enrollment. Renal Flare and No Renal Flare denote blinded adjudication committee diagnosis.

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Chapter 5
**Figure 5.1:** Urinary sCD163 concentrations in various kidney diseases and LN. Urinary protein excretion of sCD163 (A and B) corrected by urinary Cr or urinary total protein (u-Cr and u-TP) in renal diseases (left) and LN classified according to ISN/RPS criteria (right). Each patient is represented by a dot and the mean of each group is shown as a horizontal bar. *P < 0.05. In (B) and (D), data from normal subjects were excluded from the analysis because of the absence of proteinuria. IgAN = IgA nephropathy, AAV= ANCA associated vasculitis, MCNS = minimal change nephrotic syndrome, MN = membranous nephropathy, DMN = diabetic nephropathy [148].

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remission proteinuric vasculitis (n=9), RNP = remission non-proteinuric vasculitis (n=10), HC = healthy controls (n=10). Bar and lines represent median and interquartile ranges. Groups compared using Kruskal Wallis testing. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001 sCD163 as measured by Duoset ELISA.

**Figure 5.7:** usCD163 values corrected for urinary (A) creatinine and (B) protein. AAV=ANCA-associated vasculitis. NS=nephrotic syndrome. Bar and lines represent median and interquartile ranges. ns=not significant, ****p≤0.0001. Dotted line in (A) represents diagnostic cut off range of 253ng/mmol (see chapter 4) and (B) represents diagnostic cut off range of 6.3ng/mg. Significances are reported using Kruskal Wallis testing across all groups (wider bar) and using man Whitney testing when comparing active renal vasculitis and active nephrotic syndrome (narrower bar).

**Chapter 6**

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**Figure 6.2:** Graphic demonstration of research sampling from diagnosis to switch visit. Urine containers represent usCD163 samples, blood tubes represent serum research samples.

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