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ORIGINAL ARTICLE

# Urinary and serum soluble CD25 complements urinary soluble CD163 to detect active renal anti-neutrophil cytoplasmic autoantibody-associated vasculitis: a cohort study

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## ABSTRACT

**Background.** Early detection of renal involvement in anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is of major clinical importance to allow prompt initiation of treatment and limit renal damage. Urinary soluble cluster of differentiation 163 (usCD163) has recently been identified as a potential biomarker for active renal vasculitis. However, a significant number of patients with active renal vasculitis test negative using usCD163. We therefore studied whether soluble CD25 (sCD25), a T cell activation marker, could improve the detection of renal flares in AAV.

**Methods.** sCD25 and sCD163 levels in serum and urine were measured by enzyme-linked immunosorbent assay in 72 patients with active renal AAV, 20 with active extrarenal disease, 62 patients in remission and 18 healthy controls. Urinary and blood $CD4^+$ T and $CD4^+$ T effector memory (TEM) cell counts were measured in 22 patients with active renal vasculitis. Receiver operating characteristics (ROC) curves were generated and recursive partitioning was used to calculate whether usCD25 and serum soluble CD25 (ssCD25) add utility to usCD163.

**Results.** usCD25, ssCD25 and usCD163 levels were significantly higher during active renal disease and significantly decreased after induction of remission. A combination of usCD25, usCD163 and ssCD25 outperformed all individual markers (sensitivity 84.7%, specificity 95.1%). Patients positive for sCD25 but negative for usCD163 ($n = 10$) had significantly higher C-reactive protein levels and significantly lower serum creatinine and proteinuria levels compared with the usCD163-positive patients. usCD25 correlated positively with urinary $CD4^+$ T and $CD4^+$ TEM cell numbers, whereas ssCD25 correlated negatively with circulating $CD4^+$ T and $CD4^+$ TEM cells.

**Conclusion.** Measurement of usCD25 and ssCD25 complements usCD163 in the detection of active renal vasculitis.

**Keywords:** ANCA vasculitis, glomerulonephritis, renal dysfunction, soluble CD25, soluble CD163

## INTRODUCTION

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) is a group of systemic autoimmune diseases characterized by pauci-immune necrotizing inflammation of small- to medium-sized blood vessels with a predilection for lungs and kidneys. AAV is the leading cause of rapidly progressive glomerulonephritis, especially in the elderly [1, 2].

Although immunosuppressive treatment induces remission in most patients, >30% of patients experience a relapse of disease within the first 3 years after induction of remission [3, 4]. Eventually >70% of all patients develop renal involvement, which is associated with a decline in renal function and increased morbidity and mortality [5, 6]. Therefore, early detection of renal relapses is important to prevent permanent renal dysfunction in AAV.

A potent new marker for active renal vasculitis, soluble cluster of differentiation 163 (sCD163), a macrophage-shed scavenger receptor for haemoglobin/haptoglobin, has recently been identified [7]. Increased levels of urinary soluble CD163 (usCD163) closely reflect active renal vasculitis and correlate with macrophage infiltration into the kidney [7]. Macrophages are key players in renal damage, as they are the most frequent inflammatory cell type present in glomerular crescents [8, 9]. Although high levels of usCD163 reflect active renal vasculitis, between 13% and 27% of the patients with active renal vasculitis

**Table 1. Patient characteristics**

| Characteristics | Inception cohort | Validation cohort | HCs |
|---|---|---|---|
| Number of patients | 41 | 113 | 18 |
| Median age, years (range) | 57.5 (47.4–67.3) | 61.0 (47.9–73.5) | 53.8 (42.8–60.9) |
| Male, *n* (%) | 23 (56.1) | 64 (56.6) | 10 (55.5) |
| ANCA specificity, *n* (%) | | | |
| PR3 | 27 (65.9) | 66 (58.4) | – |
| MPO | 14 (34.1) | 47 (41.6) | – |
| Disease state, *n* (%) | | | |
| Active renal | 24 (58.5) | 48 (42.5) | – |
| Active non-renal | 5 (12.2) | 15 (13.3) | – |
| Remission | 12 (29.3) | 50 (44.2) | – |
| Diagnosis/relapse | | | |
| Active renal | 10/14 | 42/6 | – |
| Active non-renal | 1/4 | 4/11 | – |
| BVAS, active renal | 15 (12–19) | 15 (12–19) | – |
| BVAS, active non-renal | 11 (8–19) | 11 (8–16) | – |
| Serum creatinine (μmol/L) | | | |
| Active renal disease | 175 (94–319) | 266 (120–365) | – |
| Active non-renal disease | 63 (59–128) | 88 (68–129) | – |
| Remission | 86 (68–129) | 114 (86–165) | – |
| Proteinuria (g/L) | | | |
| Active renal disease | 0.8 (0.3–2.2) | 0.7 (0.2–2.0) | – |
| Active non-renal disease | 0.4 (0.0–0.6) | 0.6 (0.0–0.8) | |
| Remission | 0.2 (0.0–0.4) | 0.3 (0.0–0.5) | |
| Immunosuppressive treatment, *n* (%) | 17 (41.4) | 70 (61.9) | 0 (0) |

HCs, healthy controls; MPO, myeloperoxidase; PR3, proteinase 3.

test negative [7]. Thus usCD163 levels alone are insufficient to identify active renal vasculitis in all AAV patients.

In addition to macrophages, T cells also play a pivotal role in the pathogenesis of AAV. T cells in peripheral blood of both active AAV patients and patients in remission are persistently activated, and various aberrations in T-cell subsets, both numerical and functional, have been identified in AAV patients with active disease [10–16]. Furthermore, increased numbers of $CD4^+$ effector memory T (TEM) cells in the urine have been found to reflect active renal vasculitis disease [17].

Accordingly, T-cell activation markers have been proposed as potential disease activity markers, including CD25, a T-cell interleukin-2 alpha receptor (sIL-2Rα) that has significantly greater expression on $CD4^+$ T cells during active disease compared with healthy controls [16]. Soluble CD25 (sCD25), which is shed from T cells after activation, is increased in the serum of AAV patients with active disease and correlated with vasculitis disease activity and decreased upon induction of remission [18–20]. A recent study identified usCD25 as a potential biomarker for active lupus nephritis, demonstrating increased levels during active disease and in patients in whom induction of disease remission failed [21]. However, in AAV, data on usCD25 levels as a marker for renal disease activity are lacking. Therefore, as both T-cell and macrophage activation play a pivotal role in AAV pathogenesis, we studied whether sCD25 could complement usCD163 in the detection of active renal AAV.

## MATERIALS AND METHODS

### Patients

Patients included in the inception cohort were recruited from the University Medical Center Groningen (UMCG), Groningen, The Netherlands. The inception cohort consisted of 24 patients with active renal disease and five patients with active non-renal AAV. Of 12 patients, paired samples were available, that is, samples collected during active disease and a subsequent sample collected at disease remission.

For the independent validation cohort, samples from the Rare Kidney Disease (RKD) Biobank, Trinity Healthy Kidney Centre, Dublin, Ireland were used. The validation cohort consisted of 48 patients with active renal AAV, 15 patients with active non-renal AAV and 50 patients in remission. Of the 50 patients, 10 patients in remission were also included during active disease. Patient characteristics are listed in Table 1. AAV diagnosis was established according to the Chapel Hill consensus classification criteria [22]. Disease activity was determined using the third version of the Birmingham Vasculitis Activity Score (BVAS) [23]. Active renal vasculitis was defined according to clinical practice as new or increasing haematuria, and/or proteinuria, and/or in serum creatinine. Patients with active disease but without renal involvement were defined using BVAS v3 based on the absence of clinical signs of renal involvement.

Remission was defined as a BVAS of 0, including stable urinary sediment and serum creatinine levels. The study was approved by the local ethics committees and informed consent was obtained from all participants in agreement with the Declaration of Helsinki.

### Sample collection and preparation

For serum, 10 mL of blood was collected and allowed to clot at room temperature for 1 h then centrifuged for 10 min at 1500 *g* and stored at either −20°C (UMCG samples) or −80°C (RKD samples). For patients recruited in the UMCG, spot urine samples were diluted 1:1 in phosphate-buffered saline (PBS)

and centrifuged for 15 min at 1200 *g*, the supernatants were collected and stored at −20°C until use, whereas the cell pellets of the active AAV patients were used for T-cell measurements. Spot urine samples from patients enrolled in the RKD were centrifuged at 2000 *g* for 10 min at 4°C and the supernatants were stored at −80°C until use.

### sCD25 and sCD163 detection

Serum and urine sCD25 and sCD163 levels were detected by commercial sandwich enzyme-linked immunosorbent assay (ELISA) (human sIL-2R DY202, human sCD163 DY1607; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions with minor modifications. For sCD25, serum samples were diluted 1:2 and urine samples were diluted 1:4 and 1:8 in 1% bovine serum albumin (BSA)/PBS.

For sCD163, levels were measured and data were published previously [7]. Data are presented here as well to compare sCD163 with sCD25 and identify patients testing false negative for sCD163. In short, serum samples were diluted 1:200 and 1:400 and urine samples were diluted 1:4 and 1:8 in 1% BSA/PBS. Both the capture antibody and the streptavidin horseradish peroxidase (HRP) conjugate were diluted in 1% BSA/PBS/0.005% Tween to minimize background signal. Levels of sCD25 and sCD163 in urine were corrected for urinary creatinine levels to correct for urinary dilution.

### Phenotyping and quantification of $CD4^+$ T cells in urine and blood samples

$CD4^+$ T and $CD4^+$ TEM cell numbers in urine and blood were previously assessed and published [24]. Here, these data were used to correlate T-cell numbers and migration to sCD25 levels. In short, immediately after voiding, urine was diluted 1:1 with cold PBS and processed as previously described [17]. Isolated mononuclear cells were resuspended in wash buffer (1% BSA/PBS) and stained with anti-CD45RO-FITC, anti-CCR7-PE, anti-CD4-PerCP and anti-CD3-APC for 15 min at room temperature in the dark. In parallel, blood samples were labelled with the aforementioned monoclonal antibodies. Next, cells were treated with 2-mL diluted fluorescence-activated cell sorter (FACS) lysing solution (BD, Amsterdam, The Netherlands) for 10 min and samples were washed twice in wash buffer and immediately analysed on an FACSCalibur system (BD). Data were collected for $10^5$ events for each sample and plotted using WinList software package (Verity Software House Topsham, ME, USA). Positively and negatively stained populations were calculated by quadrant dot-plot analysis, as determined by the isotype controls.

Absolute numbers of $CD4^+$ T cells were quantified in urine and blood using TruCOUNT Tubes (BD). In brief, 20 µl of MultiTEST four-colour antibodies (CD3-FITC, CD8-PE, CD45-PerCP and CD4-APC) and 50 µl of sample (urine or blood) were added to bead-containing TruCOUNT tubes. The cell suspension was processed and analysed and absolute counts for $CD4^+$ T and TEM cells were calculated as described earlier [17].

### Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, CA, USA). As sCD25 and sCD163 levels in serum and urine were non-normally distributed, differences between the groups were analysed using the Kruskal–Wallis test with *post hoc* Dunn's test. Wilcoxon matched pairs testing was used for paired analysis. Correlations were tested using Spearman's rank correlation. For usCD25, usCD163 and serum soluble CD25 (ssCD25), receiver operating characteristics (ROC) curves were generated between patients with active renal vasculitis and patients in remission. In the inception cohort, optimal cut-off values were calculated using the Youden index [25]. The optimal cut-off values were then applied to the validation cohort. Recursive partitioning was used to calculate optimal combinations of usCD25 and usCD163 and ssCD25 and ssCD163 for detection of active renal vasculitis (R version 3.3.3, R Project for Statistical Computing, Vienna, Austria). Cut-off values derived from the inception cohort were used for this analysis and the optimal combination of markers was applied on the combination of the inception and validation cohort. P-values $<0.05$ were considered significant.

## RESULTS

### sCD25 levels in urine and serum reflect vasculitis activity

Patients with active renal vasculitis had significantly higher ssCD25 levels compared with patients in remission and healthy controls in the inception cohort and patients with active non-renal disease in the validation cohort (Figure 1A). A significant decrease in ssCD25 levels was found in paired samples of patients after achieving remission compared with the levels during active disease (median 937 versus 494 pg/mL) (Figure 1C).

In urine, usCD25 levels were significantly higher in patients with active renal vasculitis compared with healthy controls. Moreover, in the validation cohort, usCD25 levels also differed significantly between patients with active renal disease and patients in remission (Figure 1B). usCD25 levels decreased significantly upon remission (median 210 versus 90 ng/mmol) (Figure 1D).

Patients with active renal disease who received induction treatment before sampling had significantly lower ssCD25 levels (median 1369 versus 798 pg/mL) and lower usCD25 levels (median 190 versus 59 ng/mmol). Maintenance treatment did not affect ssCD25 or usCD25 levels significantly (Supplementary data, Table S1). ANCA specificity did not influence usCD25 or ssCD25 levels during active disease or in remission (data not shown).

### usCD25 correlate with urine $CD4^+$ T cells

As sCD25 is shed from activated T cells, correlations between ssCD25 and $CD4^+$ T or $CD4^+$ TEM cell counts in blood and correlations between usCD25 and $CD4^+$ T or $CD4^+$ TEM cells in urine were tested. A negative trend between ssCD25 levels and circulating $CD4^+$ T ($\rho = -0.431$, $P = 0.050$) or $CD4^+$ TEM cells ($\rho = -0.405$, $P = 0.068$) was observed (Figure 2A and B). In contrast, usCD25 levels correlated positively with

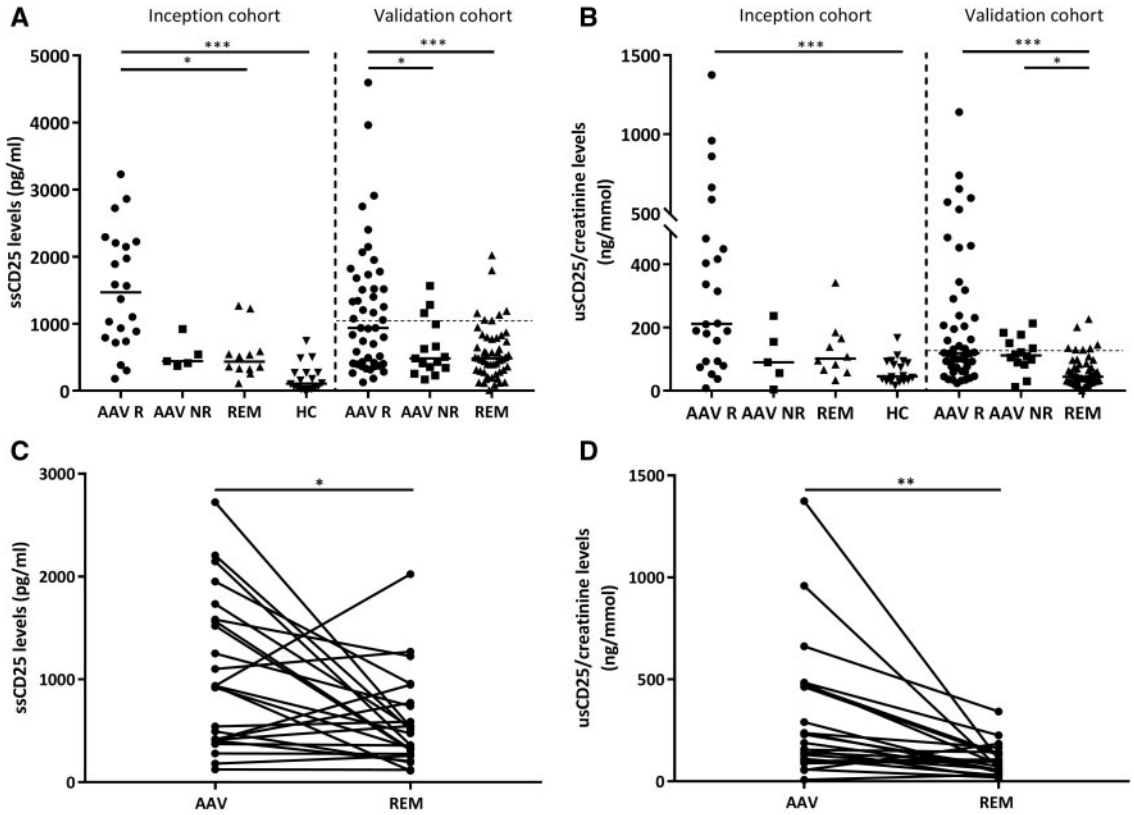


**FIGURE 1:** sCD25 levels in **(A)** serum and **(B)** urine. Paired sCD25 levels were measured during active disease and remission states in **(C)** serum and **(D)** urine. AAV R, patients with active renal disease; AAV NR, patients with active disease without renal involvement; REM, patients in remission; HC, healthy controls. Dotted line represents the cut-off value (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

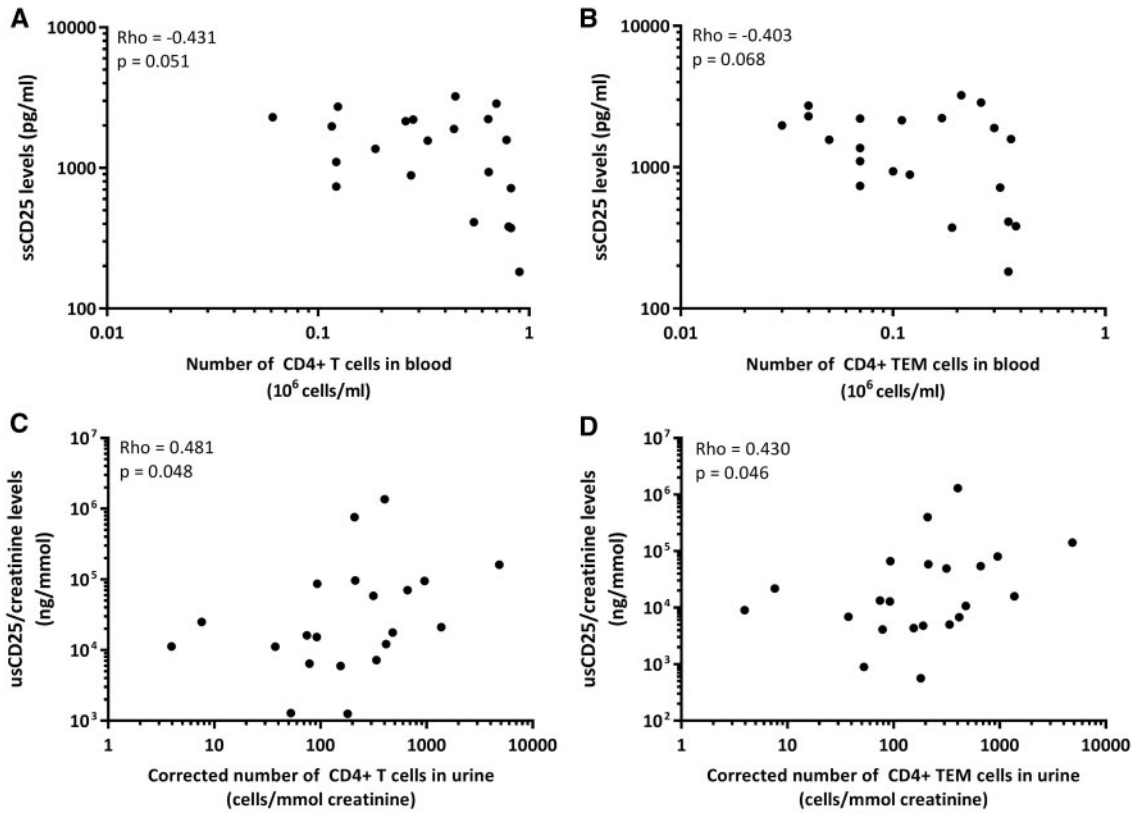


**FIGURE 2:** Correlations between ssCD25 levels and circulating **(A)** $CD4^+$ T and **(B)** $CD4^+$ TEM cells and correlations between usCD25 levels and **(C)** urinary $CD4^+$ T and **(D)** $CD4^+$ TEM cells.

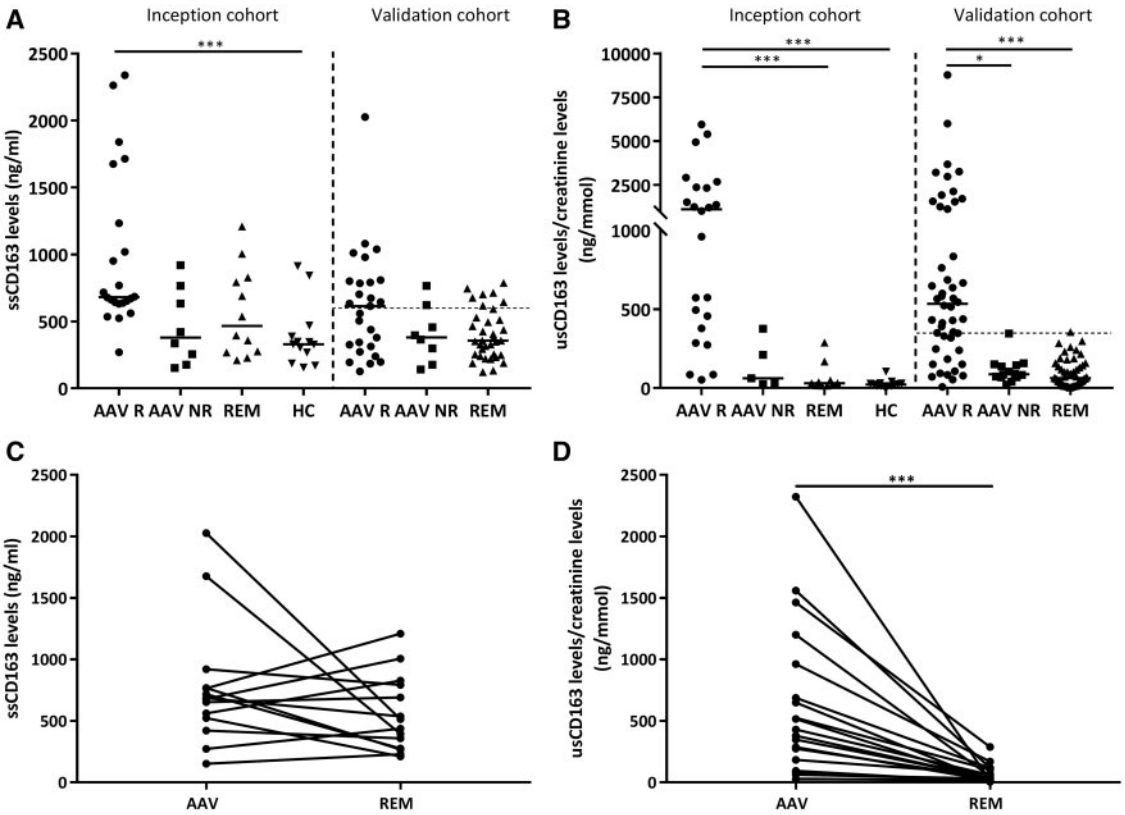


**FIGURE 3:** sCD163 levels in **(A)** serum and **(B)** urine. Paired sCD163 levels were measured during active disease and remission states in **(C)** serum and **(D)** urine. AAV R, patients with active renal disease; AAV NR, patients with active disease without renal involvement; REM, patients in remission; HC, healthy controls. Dotted line represents the cut-off value (*$P < 0.05$; ***$P < 0.001$).

urinary $CD4^+$ T ($\rho = 0.481$, $P = 0.048$) and $CD4^+$ TEM ($\rho = 0.430$, $P = 0.046$) cell numbers in active renal AAV patients (Figure 2C and D).

### usCD163 levels are highly increased during active renal AAV

Patients with active disease had significantly higher ssCD163 levels compared with healthy controls, but not patients in remission, in the inception cohort (Figure 3A). Levels did not decrease upon induction of remission (median 681 versus 495 ng/mL) (Figure 3C). usCD163 was significantly higher in patients with active renal disease than in patients with active non-renal disease, those in remission and healthy controls. This was confirmed in the validation cohort (Figure 3B) and levels decreased significantly upon induction of remission (median 404 versus 35 ng/mmol) (Figure 3D). Moreover, the presence of induction therapy at the time of sampling was associated with lower ssCD163 levels (median 678 versus 255 ng/mL) but did not influence usCD163 levels (median 678 versus 574 ng/mmol) in patients with active renal disease. Maintenance treatment did not influence usCD163 or ssCD163 levels significantly (Supplementary data, Table S1).

### Utility of usCD163 as a biomarker of active renal vasculitis

To test whether the soluble markers reflected active renal vasculitis, ROC curves were generated for patients with active renal disease, active non-renal disease and those in remission. Using the inception cohort data, optimal cut-offs for usCD25 (>125 ng/mmol), ssCD25 (>1050 ng/mL), usCD163 (>350 ng/mmol) and ssCD163 (>630 pg/mL) were calculated (Table 2).

The cut-off values were applied to the validation cohort. In the validation cohort, usCD25 and usCD163 were comparable in the detection of active renal vasculitis (65.7% versus 70.8%), whereas ssCD25 and ssCD163 were less sensitive (60.0% and 62.5%, respectively). In total, 19.6% and 2.5% of those that tested positive using usCD25 and usCD163, respectively, did not have active renal disease. ssCD25 and ssCD163 performed less well compared with usCD163 (Table 2).

In the total cohort, results were similar to the validation cohort and these results indicate that usCD163 acts as the most promising single marker in the detection of active renal disease; however, 27.8% of the patients with active renal disease still tested false negative.

The utility of traditional markers for active renal vasculitis [proteinuria, haematuria, serum creatinine and C-reactive protein (CRP)] was also tested. Of these, CRP tested the most accurate, with a sensitivity of 77.9% and a specificity of 71.6%. However, CRP alone was not able to detect active disease, as 55% of the patients without renal disease also tested positive (Supplementary data, Table S2).

### usCD25 and ssCD25 complements usCD163 for the detection of active renal AAV

To study whether the sensitivity of detecting active renal vasculitis could be further increased compared to usCD163 alone,

**Table 2. Statistical analysis of the different markers**

| Marker | AUC | Sensitivity (%) | Specificity (%) | PPV | NPV | +LR | −LR | Cut-off |
|---|---|---|---|---|---|---|---|---|
| Inception cohort (AAV R = 24, AAV NR/REM = 17) | | | | | | | | |
| usCD25 | 0.80 | 70.8 | 76.5 | 80.9 | 65.0 | 3.0 | 0.38 | >125 ng/mmol |
| ssCD25 | 0.82 | 65.7 | 80.4 | 84.2 | 63.6 | 3.8 | 0.40 | >1050 pg/mL |
| usCD163 | 0.94 | 75.0 | 94.1 | 94.7 | 72.3 | 13.0 | 0.27 | >350 ng/mmol |
| ssCD163 | 0.70 | 67.3 | 56.2 | 73.1 | 66.7 | 1.9 | 0.35 | >630 ng/mL |
| Validation cohort (AAV R = 48, AAV NR/REM = 65) | | | | | | | | |
| usCD25 | 0.81 | 65.7 | 80.4 | 67.4 | 74.6 | 2.8 | 0.46 | – |
| ssCD25 | 0.76 | 60.0 | 72.7 | 61.7 | 71.2 | 2.2 | 0.55 | – |
| usCD163 | 0.87 | 70.8 | 98.5 | 97.1 | 82.1 | 36 | 0.30 | – |
| ssCD163 | 0.66 | 62.5 | 75.4 | 65.2 | 73.1 | 2.5 | 0.50 | – |
| Total cohort (AAV R = 72, AAV NR/REM = 82) | | | | | | | | |
| usCD25 | 0.80 | 66.7 | 78.6 | 71.6 | 72.3 | 2.9 | 0.43 | – |
| ssCD25 | 0.78 | 61.8 | 73.7 | 68.2 | 69.3 | 2.4 | 0.50 | – |
| usCD163 | 0.91 | 72.2 | 97.5 | 96.3 | 80.0 | 30.0 | 0.28 | – |
| ssCD163 | 0.66 | 66.1 | 71.9 | 68.1 | 71.9 | 2.4 | 0.44 | – |
| Decision tree | – | 84.7 | 95.1 | 93.8 | 87.6 | 16.0 | 0.16 | – |

+LR, positive likelihood ratio; −LR, negative likelihood ratio; AAV R, patients with active renal disease; AAV NR, patients with active disease without renal involvement; NPV, negative predictive value; PPV, positive predictive value; REM, patients in remission.

we tested various combinations of markers using recursive partitioning. The optimal combination comprised usCD163, usCD25 and ssCD25. In this analysis, patients were tested first for usCD163 and patients with usCD163 levels above the cut-off value were considered to have active renal disease (true positives 51, false positives 1; Figure 4B). Next, patients who did not reach the cut-off for usCD163 were further evaluated using usCD25 and ssCD25. Patients who reached the cut-off for both usCD25 and ssCD25 were classified in the tree as having active renal disease (true positives 10, false positives 2; Figure 4B). Patients who tested negative for all three markers were classified as patients without active renal disease (true negatives 79, false negatives = 11; Figure 4B). This combination led to an increase in sensitivity compared to usCD163 alone (84.7% versus 72.2%; Table 2).

Additional analysis between the three groups revealed that patients who did not reach the cut-off for usCD163 but did test positive for usCD25 and ssCD25 ($n = 10$) had significantly higher levels of CRP and significantly lower levels of serum creatinine and proteinuria compared with patients who tested positive for usCD163 ($n = 51$). Moreover, the number of proteinase 3–ANCA-positive patients tended to be higher in the us/ssCD25-positive group compared with the usCD163-positive group (P = 0.07) (Table 3).

Patients who tested false negative ($n = 11$) had significantly lower serum creatinine compared with the usCD163-positive group and significantly lower CRP compared with the us/ssCD25-positive group (Table 3). Nine of the 11 (73%) false-negative patients received immunosuppressive treatment before sampling, which was higher than in the usCD163- and us/ssCD25-positive groups (41% and 40%, respectively) (Table 3).

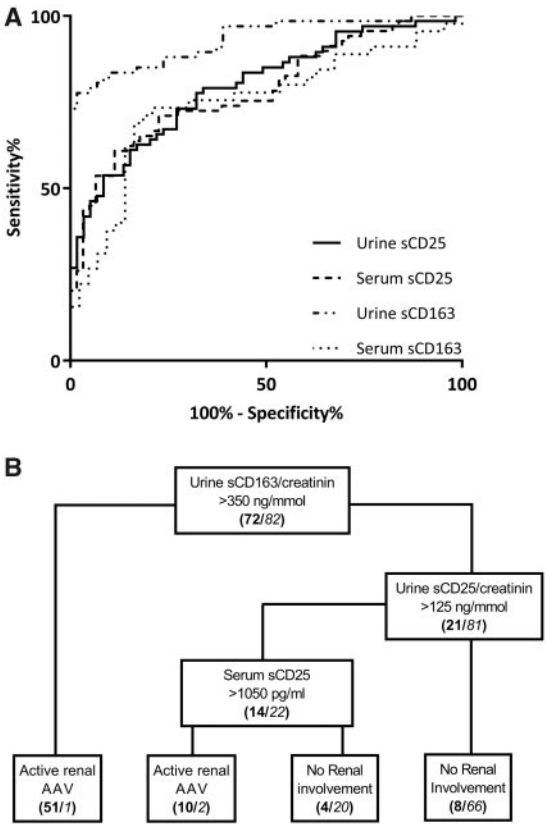


**FIGURE 4:** (**A**) ROC curve for ssCD25, usCD25 and usCD163. (**B**) Decision tree as generated by recursive partitioning. The first number represents patients with active renal disease (bold, $n = 72$) and the second number represents patients with active disease without renal involvement and remission patients (italic, $n = 82$).

### usCD25, ssCD25 and usCD163 correlate with serum creatinine, CRP and proteinuria

To study if ssCD25, usCD25 or usCD163 correlated to other disease markers, correlations between ssCD25, usCD25 or usCD163 and serum creatinine, serum creatinine increase and decrease, CRP, proteinuria and BVAS were tested. usCD25 correlated positively with CRP ($\rho = 0.370$, P = 0.002) and negatively with serum creatinine ($\rho = -0.275$, P = 0.024) and proteinuria ($\rho = -0.427$, P = 0.005). ssCD25 correlated positively with CRP (P = 0.002, $\rho = 0.382$) and serum creatinine ($\rho = 0.240$, P = 0.047).

**Table 3. Patient characteristics based on the decision tree (active patients only)**

| | usCD163$^{+}$ | ssCD25 and usCD25$^{+}$ usCD163$^{-}$ | ssCD25 and usCD25$^{-}$ usCD163$^{-}$ |
|---|---|---|---|
| *N* | 51 | 10 | 11 |
| Diagnosis/relapse, *n* | 36/15 | 6/4 | 7/4 |
| Anti-PR3-positive, *n* (%) | 21 (40) | 8 (80) | 5 (45) |
| Anti-MPO-positive, *n* (%) | 30 (60) | 2 (20) | 6 (55) |
| BVAS | 15 (13–20) | 15 (11–19) | 14 (12–17) |
| Started induction therapy before sampling, *n* (%) | 21 (41) | 4 (40) | 8 (73)[a,b] |
| CRP (mg/L) | 23 (7–65) | 99 (59–190)[b] | 19 (5–49)[a] |
| Leucocytes ($\times 10^9$/L) | 10.6 (8.6–12.9) | 11.1 (8.7–17.0) | 8.3 (6.6–12.0) |
| Serum creatinine (μmol/L) | 277 (147–459) | 90 (64–189)[b] | 135 (108–158)[b] |
| Proteinuria (g/L) | 1.5 (0.4–2.6) | 0.3 (0.1–0.3)[b] | 0.3 (0.1–0.6) |

Values are presented as median (range) unless stated otherwise.
[a]Significant difference ($p < 0.05$) compared to ss/usCD25$^{+}$.
[b]bSignificant difference ($p < 0.05$) compared to usCD163$^{+}$.
MPO, myeloperoxidase; PR3, proteinase 3.

Opposite to usCD25, usCD163 levels correlated positively with serum creatinine ($\rho = 0.546$, $P = 0.001$) and proteinuria ($\rho = 0.430$, $P = 0.005$). No correlation with disease activity (BVAS) or ANCA titre was observed for any of the three markers. Moreover, none of the markers correlated with an increase in serum creatinine 6 months before or a decrease 6 months after active disease.

usCD25 and ssCD25 ($\rho = 0.438$, $P = 0.0002$) as well as usCD163 and ssCD163 ($\rho = 0.421$, $P = 0.004$) correlated positively. ssCD25 and ssCD163 also correlated positively ($\rho = 0.421$, $P = 0.004$), but usCD25 and usCD163 did not ($P = 0.76$).

## DISCUSSION

Early detection of renal involvement in AAV is of great clinical importance to allow early initiation of treatment to limit renal damage. Recently usCD163 has been identified as a promising urinary marker for the detection of active renal vasculitis in the setting of a known diagnosis of AAV [7]. However, some patients with active renal vasculitis still test false negative using usCD163 alone. In this study, we demonstrate that measuring usCD25 and ssCD25 complements usCD163 in the detection of active renal vasculitis, reducing the number of patients testing false negative. Moreover, high sCD25 levels, an indication for T-cell activation and migration, might reflect an earlier disease stage characterized by high CRP levels but still limited renal damage compared with patients displaying high usCD163 levels.

There is a strong biological rationale to measure both sCD25 and sCD163 as potential markers for renal vasculitis since sCD25 reflects activation of T cells and sCD163 reflects activation of M2 macrophages, both of which are pivotal cellular players in AAV pathogenesis. Macrophages play an important role in the development of renal damage and scarring. Moreover, macrophages are the most abundant cell type present in glomerular crescents [8, 9] and levels of usCD163 correlate to the number of macrophages present in the kidney [7].

In addition to macrophages, T cells also play a pivotal role in the induction of renal damage [26]. Here we found that usCD25 levels correlated positively to urinary T and TEM cell numbers. Not only were CD4$^{+}$ TEM cells in the urine previously found to reflect renal involvement in AAV [17], these cells have also been suggested to play a key role in inducing kidney injury in AAV [2, 26]. Depletion of CD4$^{+}$ T cells in a mouse study significantly attenuated the development of crescentic glomerulonephritis in a model of myeloperoxidase autoimmunity [26]. The observed correlation between usCD25 with both CD4$^{+}$ T and CD4$^{+}$ TEM cells may reflect renal T-cell accumulation in those patients. This contention is further supported by the negative correlation between circulating CD4$^{+}$ T or CD4$^{+}$ TEM cells and ssCD25, which suggests that after activation and CD25 shedding, T-cells migrate towards the kidney.

In the patient cohorts tested here, usCD25 levels were increased in active disease, decreased upon remission and were found to be a reasonable marker for active vasculitis [area under the curve (AUC) 0.80]. However, usCD25 alone was not specific for renal involvement, as 30% of patients without renal involvement also tested positive for usCD25. This might be due to active filtration or passive leakage of sCD25 by the kidney. However, as proteinuria was found to be negatively correlated with usCD25, passive leakage of sCD25 is unlikely.

For ssCD25, our results are in line with those from earlier studies reporting elevated ssCD25 levels during active disease that decrease upon remission [18–20]. With an AUC of 0.78, the marker potential was similar to that of usCD25, but ssCD25 was found to be more specific for renal involvement, as only 10% of patients without renal disease tested positive. Previously, O'Reilly *et al.* [7] showed that usCD163 alone is a potent marker for active renal disease, and with sensitivity between 73 and 96%, it outperforms ssCD25 and usCD25. In contrast to O'Reilly *et al*, we found in our validation cohort significantly higher ssCD163 levels in patients with active renal disease compared with patients in remission. These results, however, could not be validated in a larger validation cohort and could be partly explained by differences in the number of patients receiving immunosuppressive treatment at the time of sampling. Moreover, the discrepancy could further be explained by the distinction of patients with active renal and non-renal active disease that was applied in this study but not by O'Reilly *et al.*

Interestingly, in our cohort, 10 patients with active renal vasculitis who tested negative for usCD163 had high ssCD25 and usCD25 levels. Clinical parameters differed between these two groups. Patients negative for usCD163 but with high usCD25 and ssCD25 levels had higher CRP levels and lower serum creatinine and proteinuria. These results suggest that levels of sCD25 may increase at an earlier stage of the disease process when renal injury is still limited. In contrast, usCD163 correlated positively with proteinuria and serum creatinine, which suggests that usCD163 might be associated with more established renal damage. These results indicate that usCD25, ssCD25 and usCD163 have different kinetics during the development of renal injury in AAV, which may reflect different stages of the inflammatory process. This is emphasized by the lack of correlation between usCD25 and usCD163.

Based on our results, measuring usCD25 and ssCD25 in combination with usCD163 could improve the detection of renal flares in AAV patients before extensive renal damage has occurred. However, 15% of the patients with active renal disease still tested false negative when this combination was used. This observation can be partly explained by the higher number of patients who received induction therapy before sampling in the false-negative group compared with the true-positive group (73% versus 41%). In patients who received induction therapy days before sampling, levels of ssCD25 were significantly lower and usCD25 levels tended to be lower, which might explain these false-negative results.

A limitation of using usCD25 and ssCD25 as well as usCD163 as indicators of disease activity is that these markers are not disease specific. Previous studies have demonstrated elevated usCD25 and ssCD25 levels in septic patients with or without renal involvement [27–29], and increased usCD25 and usCD163 levels have been reported in lupus nephritis [21, 30]. Although these markers are, thus, not specific for active renal vasculitis in AAV, and therefore cannot be used to diagnose AAV, their utility as markers of active renal vasculitis in patients with an established diagnosis of AAV is promising. An additional limitation of our study is its retrospective design. Therefore, additional prospective studies are necessary to substantiate the value of combined measurement of sCD163 and sCD25 in the detection of active renal AAV.

In conclusion, our results indicate that usCD25 and ssCD25 complements usCD163 in the detection of active renal vasculitis in AAV patients. Our results also suggest that elevated usCD25 and ssCD25 levels reflect an earlier stage of development of renal vasculitis in AAV patients, which could be of clinical importance as an early sign of active disease. Further studies should be carried out to confirm and extend our findings in a prospective manner.

## SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

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## CONFLICT OF INTEREST STATEMENT

None declared.

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# pNaKtide ameliorates renal interstitial fibrosis through inhibition of sodium-potassium adenosine triphosphatase-mediated signaling pathways in unilateral ureteral obstruction mice

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## ABSTRACT

**Background.** Sodium-potassium adenosine triphosphatase (Na/K-ATPase) has been shown to regulate Src activity by combining with Src to keep it in an inactive form. We previously reported that Na/K-ATPase was downregulated in unilateral ureteral obstruction (UUO) animals. In this study, we examined whether inhibition of Na/K-ATPase-mediated Src signaling pathways ameliorated renal interstitial fibrosis induced by UUO.

**Methods.** UUO was performed on male C57BL/6J mice. pNaKtide, a mimic of Na/K-ATPase, was administered by intraperitoneal injection on Day 0 and Day 4 after ureteral ligation. Markers of interstitial fibrosis, inflammation and oxidative stress and transforming growth factor-β1 (TGF-β1) expression were examined after the mice were sacrificed on Day 7. Activation of Src and its downstream signaling effectors, including extracellular regulated protein kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase B (AKT), were evaluated.

**Results.** pNaKtide administration markedly attenuated myofibroblast accumulation and extracellular matrix deposition in obstructed kidneys. Also, pNaKtide significantly reduced the increased expression of 8-iso-prostaglandin F2α, TGF-β1, interleukin-6 and monocyte chemoattractant protein-1 (MCP-1), as well as reduced macrophage infiltration, in UUO animals. All these changes were obtained, along with inhibition of Src and its downstream effector activity.