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High mobility group box-1 (hmgb1) in systemic vasculitides

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CHAPTER 6

Are urinary levels of high mobility group box 1 markers of active nephritis in anti-neutrophil cytoplasmic antibody-associated vasculitis?

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Summary

The objective of this study is to evaluate urinary high mobility group box 1 (HMGB1) levels as markers for active nephritis in patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) in comparison with urinary CD4⁺ effector memory T cells and urinary monocyte chemoattractant protein-1 (MCP-1). Twenty-four AAV patients with active nephritis and 12 healthy controls (HC) were evaluated. In nine patients, samples were also obtained during remission. Urinary levels of HMGB1 were measured by Western blot. CD4⁺ T cells and CD4⁺ effector memory T cells (CD4⁺CD45RO⁺CCR7⁺) were determined in urine and whole blood by flow cytometry. Measurement of urinary levels of MCP-1 and serum HMGB1 levels were performed by enzyme-linked immunosorbent assay (ELISA). AAV patients with active nephritis had higher median intensity of HMGB1 in urine than HC [10.3 (7.05–18.50) *versus* 5.8 (4.48–7.01); $p = 0.004$]. Both urinary HMGB1 and MCP-1 levels decreased significantly from active nephritis to remission. The urinary MCP-1/creatinine ratio correlated with Birmingham Vasculitis Activity Score (BVAS) ($p = 0.042$). No correlation was found between the HMGB1/creatinine ratio and 24-h proteinuria, estimated glomerular filtration rate (eGFR), MCP-1/creatinine ratio, BVAS and serum HMGB1. A positive correlation was found between urinary HMGB1/creatinine ratio and CD4⁺ T cells/creatinine ratio ($p = 0.028$) and effector memory T cells/creatinine ratio ($p = 0.039$) in urine. Urinary HMGB1 levels are increased in AAV patients with active nephritis when compared with HC and patients in remission, and urinary HMGB1 levels are associated with CD4⁺ T cells and CD4⁺ effector memory T cells in urine. Measurement of urinary HMGB1 may be of additional value in identifying active glomerulonephritis in AAV patients.

Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAV) are pauci-immune necrotizing systemic vasculitides that affect

predominantly small-sized vessels, and are associated with ANCA against proteinase 3 (PR3) and myeloperoxidase (MPO). AAV include granulomatosis with polyangiitis (GPA) (formerly Wegener's), microscopic polyangiitis (MPA), eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg–Strauss) and renal limited vasculitis (RLV) [1,2]. Kidney involvement is observed in 70–88% of patients with GPA and MPA during follow-up [3–5], and is associated with an increased risk of mortality [5].

High mobility group box 1 (HMGB1) is a nuclear protein that can be released passively by necrotic cells or secreted actively by activated cells. Once in the extracellular milieu, HMGB1 triggers proinflammatory reactions activating both innate and adaptive immunity [6,7]. HMGB1 has been shown to induce the proliferation and survival of T cells [8,9]. However, the influence of HMGB1 on T helper cell polarization is still controversial, as it has been demonstrated that HMGB1 is involved in stimulating a T helper type 1 (Th1) response by dendritic cells [9,10] while directly inducing a Th17 response on CD4⁺ T cells *in vitro* and *in vivo* in experimental autoimmune myocarditis [11,12]. Furthermore, inhibition of regulatory T cell activity with a decreased expression of cytotoxic T lymphocyte antigen-4 (CTLA-4) and forkhead box protein 3 (FoxP3), and a decreased secretion of interleukin (IL)-10 after exposure to HMGB1 have also been reported [13,14].

In systemic lupus erythematosus (SLE), HMGB1 has been shown to be a good biomarker for active lupus nephritis as both serum and urinary HMGB1 levels are increased in patients with active nephritis compared to patients without nephritis and healthy controls (HC). Moreover, both serum and urinary HMGB1 levels were correlated positively with SLE disease activity index (SLEDAI) and negatively with serum complement levels [15,16]. Extracellular HMGB1 expression was increased in renal tissue from patients with active lupus nephritis [16,17].

In patients with GPA, an association between serum HMGB1 levels and active disease has been observed with either granulomatous

manifestations or with active nephritis [18-20]. Furthermore, HMGB1 expression is stronger in kidney tissue from AAV patients with active nephritis than in those with a normal biopsy [20]. However, in 52 AAV patients at disease presentation, no differences could be found in HMGB1 levels when compared to HC [21].

There is increasing evidence that T cells play an important role in the pathogenesis of AAV [22]. Infiltrating CD4⁺ T cells are found within granulomatous lesions, and a persistent activation of CD4⁺ T cells from peripheral blood is observed in AAV even during remission [23,24]. The persistent expansion of T cells in AAV patients is associated with a particular subtype of memory CD4⁺ T cells referred to as effector memory T cells (CD3⁺CD4⁺CD45RO⁺CCR7⁻) [25], which are the main cells found in glomerular infiltrates from active AAV patients [26]. The number of CD4⁺ T cells is increased in urine samples from AAV patients with active glomerulonephritis compared to AAV patients in remission and to AAV patients with disease activity in other organs and systems. CD4⁺ effector memory T cells are the main T cell subtype found in urine from AAV patients with renal involvement [27].

Monocyte chemoattractant protein-1 (MCP-1), also designated as CCL2, is a member of the CC chemokine family that acts as a potent monocyte/macrophage attractant to sites of tissue injury and infection [28]. The expression of MCP-1 is increased in renal tissue, and high urinary MCP-1 levels have been observed in different renal diseases [29]. In AAV, urinary MCP-1 levels are significantly higher in patients with active nephritis than in those without renal involvement, a decrease in urinary MCP-1 levels is observed following therapy and a significant correlation is found between urinary MCP-1 and glomerular macrophage infiltration [30]. Moreover, MCP-1 has been shown to be the best urinary marker to discriminate active renal involvement and remission in AAV [31].

This study aims to evaluate whether urinary HMGB1 levels are increased in AAV patients with active renal involvement in comparison to HC and to analyse associations of urinary HMGB1 levels with parameters of renal disease activity, CD4⁺ T cell- and CD4⁺ effector memory T cell counts in urine and urinary MCP-1 levels.

Materials and methods

Patients and controls

Twenty-four patients with AAV and 12 HC were enrolled. Patients and HC had similar mean age (55.63 ± 13.35 years *versus* 49.83 ± 7.46 years; $p = 0.105$) and frequency of females (37.5% *versus* 58.3%, $p = 0.236$). In nine patients samples were also obtained during remission, with a mean interval of 36.2 ± 10.5 months from the time of active disease. A diagnosis of GPA and MPA was established according to the European Medicines Agency algorithm [32], while the diagnosis of RLV was based on the presence of isolated renal involvement, ANCA positivity and/or biopsy-proven pauci-immune necrotizing glomerulonephritis. All AAV patients had active renal involvement and were included either at diagnosis ($n = 10$) or at the time of relapse ($n = 14$). Only seven (29.2%) AAV patients were under immunosuppressive therapy when samples were collected (Table 1). Active nephritis was characterized by active urinary sediment with glomerular erythrocyturia and/or red blood cell casts associated with abnormalities in serum creatinine or decreased estimated glomerular filtration rate (eGFR) and/or with a renal biopsy showing active pauciimmune necrotizing glomerulonephritis. Disease activity was measured by the third version of the Birmingham Vasculitis Activity Score (BVAS) [33]. Remission was defined as a BVAS = 0, including normal urinary sediment, and stable creatinine/eGFR. Table 1 depicts features of AAV patients. None of the AAV patients or HC presented active infection when evaluated. The study was approved by the local Ethical Committee and informed consent (according to the Declaration of Helsinki) was obtained.

Antibodies

The following antibodies were used for flow cytometry analysis: phycoerythrin (PE)-conjugated CCR7, fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO, peridin chlorophyll protein (PerCP)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD3, multiTEST fourcolour antibodies (FITC-conjugated CD3, PE-conjugated CD8, PerCP-conjugated CD45 and APC-conjugated CD4) and isotype-matched control antibodies of irrelevant specificity. All antibodies were purchased from Becton Dickinson (Amsterdam, the Netherlands).

Table 1. Disease features, renal involvement and therapy of AAV patients.

Variables	AAV patients (N=24)
Diagnosis/relapse,	10/14
GPA, <i>n</i> (%)	15 (62.5)
MPA, <i>n</i> (%)	4 (16.7)
RLV, <i>n</i> (%)	5 (20.8)
ANCA positivity, <i>n</i> (%)	23 (95.8)
PR3-ANCA, <i>n</i> (%)	13 (54.2)
MPO-ANCA, <i>n</i> (%)	10 (41.7)
Median BVAS,	13.00 (10.0-18.7)
24-hour proteinuria, g	0.8 (0.5-3.5)
Hematuria (>10RBC/HPF), <i>n</i> (%)	24 (100.0)
Urinary MCP-1/creatinine ratio ng/mmol	18.7 (7.5-63.3)
Serum creatinine, μmol/l	147.5 (91.5-313.0)
Creatinine in spot urine, μmol/l	6.22 ± 2.78
eGFR, ml/minute/1.73m ²	38.0 (18.0-77.0)
Red cell casts, <i>n</i> (%)	10 (41.7)
Current therapy in patients with active disease, <i>n</i> (%)	7 (29.2)
Oral prednisolone	7
Oral cyclophosphamide	3
Azathioprine	2
Mycophenolate mofetil	2
Current therapy in patients in remission, <i>n</i> (%)	5 (55.5)
Oral prednisone	1
Azathioprine	3
Mycophenolate mofetil	1

Continuous data are presented as median and interquartile range (IQR) or as mean and standard deviation. GPA: granulomatosis with polyangiitis; HPF = high power field; MCP-1 = monocyte chemoattractant protein-1; MPA = microscopic polyangiitis; MPO = myeloperoxidase; PR3 = proteinase 3; RBC = red blood cells; RLV = renal limited vasculitis.

Sample preparation and flow cytometry

Ethylenediamine tetraacetic acid (EDTA) blood and urine samples were collected from AAV patients and analysed immediately by flow cytometry for total CD4⁺ T cells and CD4⁺ effector memory T cells (CD3⁺CD4⁺CD45RO⁺CCR7⁻). After voiding, 100 ml of urine was diluted 1:1 with cold phosphate-buffered saline (PBS) and centrifuged at 558g for 15 minutes. The supernatant was collected for HMGB1 measurement, whereas the sediment was resuspended with 10 ml of PBS. We took 50 µl from the cell suspension for quantitative measurement using a TrueCount tube and mononuclear cells were isolated from the remaining cell suspension with densitygradient centrifugation by Lymphoprep (Axis-Shield, Oslo, Norway). Washing buffer (1% bovine serum albumin in PBS) was added to mononuclear cells isolated from urine and mixed with appropriate concentrations of anti-CD45RO-FITC, anti-CCR7-PE, anti-CD4-PerCP and anti-CD3-APC, and incubated for 15 min at room temperature in the dark. Whole blood samples were labelled using the same protocol. All samples were treated with 2 ml diluted fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson) for 10 min and samples were washed twice in washing buffer and analysed by flow cytometry. Four-colour staining was analysed by FACS Calibur (Becton Dickinson), and data were collected for 10⁵ events in every sample and plotted with Win-List software (Verity Software House, Topsham, ME, USA). Positively and negatively stained populations were calculated by quadrant dot-plot analysis, based on isotype controls (Fig. 1).

Quantification of effector memory T cells

TrueCount tubes (Becton Dickinson) were used to quantify CD4⁺ T cells in peripheral blood and urine. Briefly, 20 µl of MultiTEST four-colour antibodies (CD3-FITC, CCR7-PE, CD45RO-PerCP and CD4-APC) and 50 µl of sample (urine or blood) were put into bead-containing TrueCount tubes.

The cell suspension was processed and analysed and the absolute number of CD4⁺ T cells and CD4⁺ effector memory T cells was determined using a previously described protocol [27]. Results were displayed as cells/ml in urine and as x10⁶ cells/ml in peripheral blood. In order to correct for variations in urine dilution, urine CD4⁺ T cell and CD4⁺ effector memory T cell counts were also expressed as ratios to urinary creatinine.

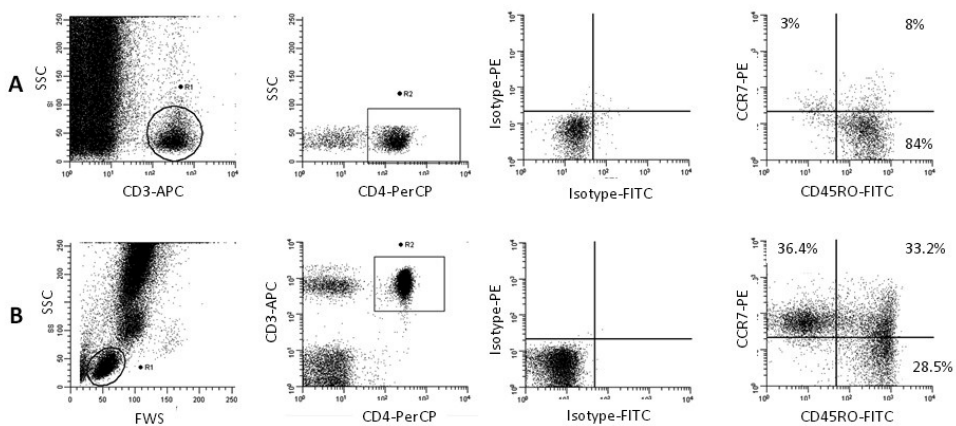


Fig. 1. Representative flow cytometry analysis of CD4⁺ effector memory T cells in urine and whole blood. Samples were stained with allophycocyanin (APC)-conjugated anti-CD3, peridin chlorophyll protein (PerCP)-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CCR7 and fluorescein isothiocyanate (FITC)-conjugated anti-CD45RO. The encircled areas show lymphocytes and the quantification of T cells was performed by quadrant dot-plot analysis. CD4⁺ effector memory T cells in urine (a) and in peripheral blood (b).

HMGB1 measurement by Western blot in urine

Three ml of urine supernatants, removed after centrifugation, were concentrated between 30 and 300 times with Vivaspin 6[®] tubes (Sartorius Stedim Biotech, Gottingen, Germany). Sodium dodecyl sulphate (SDS) buffer

was added to concentrated urine, and the volume of urine loaded to the gel was corrected for concentration factor. Western blot was performed as described previously [20]. In brief, proteins were resolved by 12.5% SDS-polyacrylamide gel electrophoresis (Criterion gel Bio-Rad, Veenendaal, the Netherlands) and transferred to polyvinylidene fluoride membrane (Millipore, Amsterdam, the Netherlands) followed by blocking with Odyssey buffer (LI-COR Biotechnology, Lincoln, NE, USA). Membranes were then incubated with anti-HMGB1 mouse monoclonal antibody 1:1000 (R&D Systems, Abingdon, UK) overnight at 4°C and with goat anti-mouse IgG antibodies labelled with IRDye800 (1:10000; LI-COR Biotechnology). Blots were scanned with Odyssey infrared Imaging System (LI-COR Biotechnology). A lysate of Jurkat cells was prepared and this lysate was included twice in each blot as standard. HMGB1 levels were presented as the fluorescence intensity against the standard. To perform correlation studies, creatinine levels were measured in unconcentrated urine and urinary levels of HMGB1 were expressed as HMGB1 intensity/creatinine ratio in intensity/mmol.

ELISA for serum HMGB1

Serum HMGB1 levels in AAV patients were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Shino Test, Sagamihara, Kanagawa, Japan). Levels were expressed in ng/ml.

Urinary MCP-1

Urinary levels of MCP-1 in patients and controls were measured by ELISA. Briefly, the ELISA plate was coated with monoclonal anti-MCP-1 antibodies diluted 1:250 (R&D Systems). After blocking the plate for 1 h, urine samples and standards were incubated for 2 h. The standard curve was built with sequential dilutions of recombinant human MCP-1 (R&D Systems) and biotinylated polyclonal goat anti-human MCP-1 antibodies (R&D Systems)

were used as detection antibodies. Results were corrected for urinary dilution with spot urine creatinine and expressed as MCP-1/creatinine ratio in pg/mmol.

Statistical analysis

Statistical analysis was carried out with spss software version 20.0 and graphs were built using Graph Pad Prism version 3.02. Categorical variables were presented as total number and percentage while continuous variables were presented as mean \pm standard deviation (s.d.) or as median and interquartile range (IQR) as appropriate. Comparison between groups was performed using χ^2 test or Fisher's exact test for categorical variables and Student's *t*-test or Mann–Whitney *U*-test for continuous variables. Correlations between continuous variables were performed with the Spearman's rank correlation coefficient. Wilcoxon's signed-rank test was used to analyse paired urinary HMGB1 and MCP-1 samples. The significant level accepted was 5% (p -value < 0.05).

Results

Serum HMGB1 levels

No significant differences were found in serum HMGB1 levels among AAV subsets [GPA: 2.86 (0.56–4.08) ng/ml *versus* MPA: 1.25 (0.56–7.80) ng/ml *versus* RLV: 2.36 (1.34–10.61) ng/ml, $p = 0.760$] (Fig. 2a) or onset/relapse patients [2.36 (0.56–3.42) ng/ml *versus* 2.48 (0.88–5.20) ng/ml, $p = 0.502$] (Fig. 2b). No significant correlations were found between serum HMGB1 levels and BVAS ($\rho = 0.073$; $p = 0.741$), proteinuria ($\rho = 0.102$; $p = 0.669$), urinary MCP1/creatinine ratio ($\rho = 0.086$; $p = 0.728$) or eGFR ($\rho = 0.303$; $p = 0.195$).

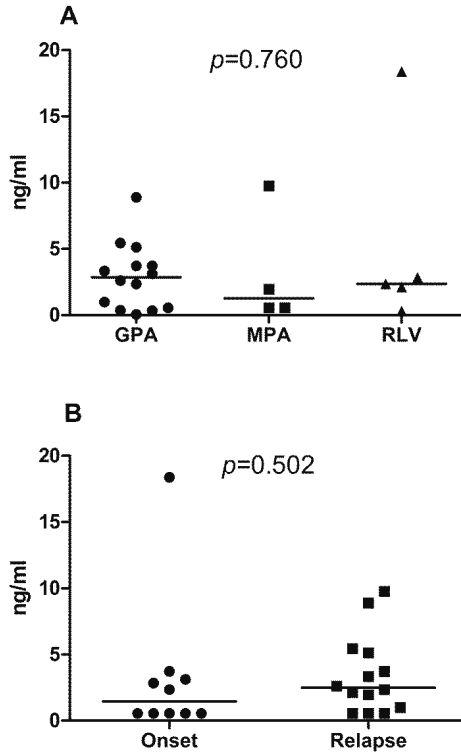


Fig. 2. Serum high mobility group box 1 (HMGB1) levels in subgroups of anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients. Serum HMGB1 levels are similar in different AAV subsets (a) and in patients at onset or with relapsing disease (b).

Urinary HMGB1 levels

AAV patients with active nephritis presented significantly higher median HMGB1 levels in urine in comparison to HC [10.3 (7.05–18.50) *versus* 5.8 (4.48–7.01); $p = 0.004$] (Fig. 3a). A representative blot used for urinary measurement of HMGB1 is shown in Fig. 3b with samples from patients with active nephritis and HC. In AAV patients, in order to correct for differences in urinary concentrations, analyses were performed with urinary HMGB1/urinary creatinine ratios. No difference could be found in median HMGB1/creatinine ratio in urine between AAV patients under immunosuppressive therapy and

those without [1.80 (1.43–2.24) *versus* 1.94 (0.67–3.62); $p = 0.688$] or between AAV patients with and without use of prednisolone [1.84 (1.43–2.24) *versus* 1.94 (0.67–3.62); $p = 0.738$]. In nine AAV patients urinary samples were collected again during remission (36.2 ± 10.5 months later). Urinary HMGB1/creatinine ratio decreased significantly when these samples were compared with those obtained during active nephritis [0.031 (0.017–0.135) *versus* 0.740 (0.360–2.110) $p = 0.0078$] (Fig. 4).

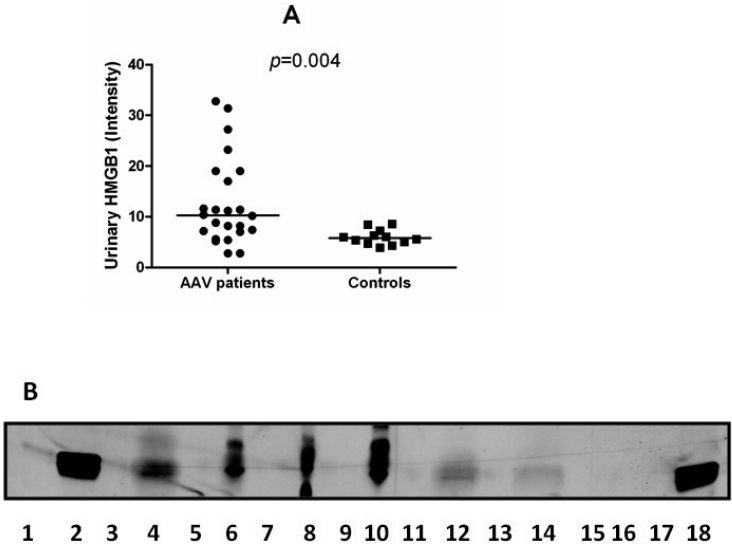


Fig. 3. Detection of urinary high mobility group box 1 (HMGB1) in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active glomerulonephritis and in healthy controls (HC). (a) AAV patients with active nephritis present significantly higher urinary HMGB1 levels than HC ($P = 0.004$). (b) A representative blot for measuring urinary HMGB1 levels. Lane 1: molecular weight marker; lanes 2 and 18: positive control; lanes 4, 6, 8, 10 and 12: urine samples from AAV patients; lanes 14 and 16: urine sample from HC.

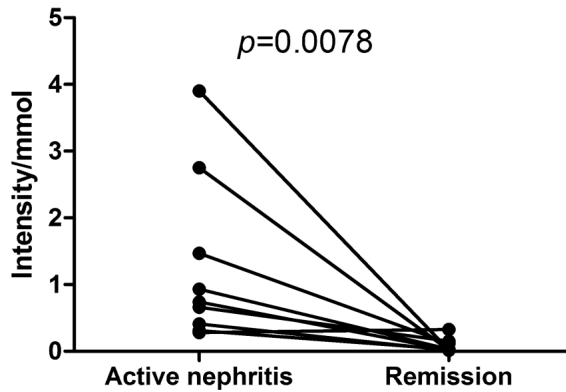


Fig. 4. Urinary high mobility group box 1 (HMGB1)/creatinine ratio in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active nephritis *versus* remission. A significant decrease in urinary HMGB1 levels is observed from active renal disease to remission at a mean 36.2 ± 10.5 months later.

Associations of urinary HMGB1 levels with clinical and laboratory parameters in AAV

No differences could be found in urinary HMGB1/creatinine ratios among different AAV subsets [GPA: 1.86 (1.32–3.12) *versus* MPA: 2.58 (1.91–3.57) *versus* RLV: 1.08 (0.30–2.35); $p = 0.186$], onset/relapse patients [1.96 (1.21–2.86) *versus* 1.84 (1.07–3.02); $p = 0.852$] or MPO-ANCA positivity *versus* PR3-ANCA positivity [2.24 (1.08–3.36) *versus* 1.86 (1.12–2.59); $p = 0.841$]. In order to evaluate whether urinary HMGB1/creatinine ratio was associated with parameters of renal involvement, systemic disease activity or with serum HMGB1 levels in AAV patients, we calculated the correlation coefficient between urinary HMGB1/creatinine ratio and 24-h proteinuria ($\rho = -0.151$; $p = 0.515$), eGFR ($\rho = -0.178$; $p = 0.452$), BVAS ($\rho = 0.018$; $p = 0.934$) and serum HMGB1 ($\rho = -0.241$; $p = 0.279$). None of these comparisons led to significant correlations.

Urinary HMGB1 levels and CD4⁺ T cells

As the presence of CD4⁺ effector memory T cells in urine has been found to reflect renal disease activity in AAV [26], we evaluated whether urinary HMGB1 could be associated with CD4⁺ T cell- and CD4⁺ effector memory T cell counts in urine. A positive correlation was found between urinary HMGB1/creatinine ratio and CD4⁺ T cells/creatinine ratio ($\rho = 0.431$; $p = 0.028$) and effector memory T cells/creatinine ratio ($\rho = 0.403$; $p = 0.039$) (Fig. 5a,b). The urinary HMGB1/creatinine ratio did not correlate with CD4⁺ T cells ($\rho = -0.153$; $p = 0.498$) or with CD4⁺ effector memory T cells in peripheral blood ($\rho = -0.222$; $p = 0.320$). Furthermore, urinary CD4⁺ T cell- and effector memory T cell counts were not correlated with BVAS ($\rho = -0.108$; $p = 0.652$ and $\rho = -0.180$; $p = 0.449$, respectively), proteinuria ($\rho = 0.105$; $p = 0.680$ and $\rho = 0.091$; $p = 0.791$, respectively) and eGFR ($\rho = 0.154$; $p = 0.542$ and $\rho = 0.152$; $p = 0.548$, respectively).

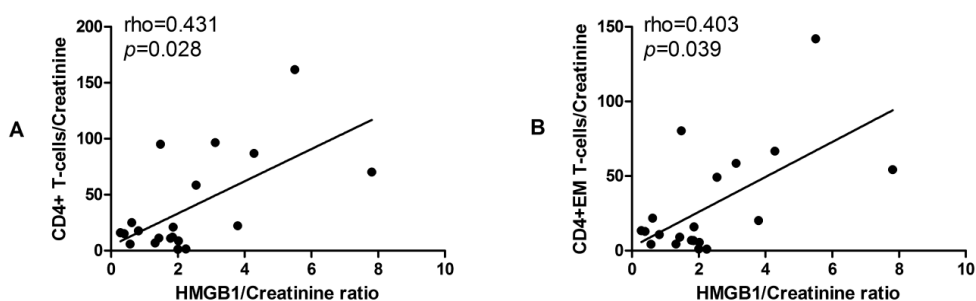


Fig. 5. Urinary high mobility group box 1 (HMGB1), CD4⁺T cell and CD4⁺ effector memory T cell counts in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active glomerulonephritis. A significant positive correlation was found between urinary HMGB1/creatinine ratio (a) and CD4⁺ T cells and CD4⁺ effector memory T cell counts in urine (b).

Urinary MCP-1 levels

AAV patients with active nephritis presented higher urinary MCP-1 levels than HC [99.19 (20.31–247.20) pg/ml *versus* 26.96 (5.74–59.58) pg/ml;

$p = 0.035$]. With regard to urinary MCP-1/creatinine ratios in AAV patients, levels decreased significantly when AAV patients with active nephritis achieved remission [6.79 (3.50–48.89) pg/mmol *versus* 0.11 (0.06–2.72) pg/mmol; $p = 0.0039$] (Fig. 6a). A positive correlation was found between urinary MCP-1/creatinine ratio with BVAS ($\rho = 0.447$; $p = 0.042$) (Fig. 6b) but not with 24-h proteinuria ($\rho = 0.426$; $p = 0.069$) or with eGFR ($\rho = -0.152$; $p = 0.545$). In addition, no correlation could be found between urinary HMGB1/creatinine and urinary MCP-1/creatinine levels ($\rho = -0.164$; $p = 0.478$) or between urinary MCP-1/creatinine ratio and CD4⁺ T cells/creatinine ratio in urine ($\rho = 0.300$; $p = 0.226$), CD4⁺ effector memory T cells/creatinine ratio in urine ($\rho = 0.243$; $p = 0.332$), CD4⁺ T cell counts in peripheral blood ($\rho = -0.060$; $p = 0.801$) and with CD4⁺ effector memory T cell counts in peripheral blood ($\rho = -0.147$; $p = 0.537$).

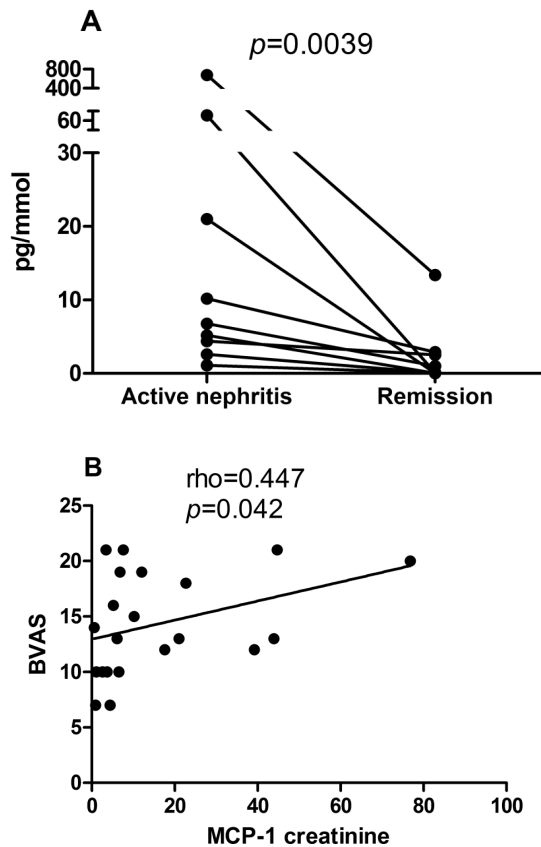


Fig. 6. Urinary monocyte chemoattractant protein-1 (MCP-1)/creatinine ratio in anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients with active disease and remission and correlation with Birmingham Vasculitis Activity Score (BVAS). Urinary MCP-1/creatinine ratio decreased significantly when patients with active nephritis achieved remission (a) and urinary MCP-1 creatinine ratio was correlated positively with BVAS (b).

Discussion

In this study, we observed that AAV patients with active glomerulonephritis present higher urinary HMGB1 levels than HC and patients in remission, and urinary HMGB1 correlates with the number of CD4⁺ T cells and CD4⁺ effector memory T cells in urine. However, urinary HMGB1 levels, CD4⁺ T cells and CD4⁺ effector memory T cells were not associated with

systemic disease activity (i.e. BVAS) or other parameters of renal involvement in AAV (i.e. 24-h proteinuria, eGFR and urinary MCP-1). In contrast, urinary MCP-1 levels were correlated positively with BVAS in AAV patients with active nephritis.

Non-invasive parameters are generally used to monitor disease activity and response to therapy in patients with renal involvement in AAV such as haematuria with dysmorphic red cells, proteinuria and renal function [33]. However, these parameters may not be sufficiently sensitive and specific to differentiate active glomerular vasculitis from permanent damage. Thus, investigation of novel urinary biomarkers to assess active glomerulonephritis in AAV is worthwhile. Levels of urinary MCP-1 have been associated with active renal vasculitis, response to therapy and prognosis in AAV [30,31]. Higher urinary MCP-1 levels in active renal vasculitis in AAV rather than changes in circulating MCP-1 seem to reflect increased renal production [30]. Therefore, we compared urinary MCP-1 as a biomarker for active renal involvement with urinary HMGB1. Indeed, in accordance with a previous study [30], we found a positive correlation between urinary MCP-1 levels and BVAS. However, urinary HMGB1 levels were not correlated with urinary MCP-1 or BVAS. Thus, increased urinary HMGB1 levels in AAV active nephritis seem to be a reflection of the underlying pathological inflammatory process in the kidney rather than a biomarker for systemic disease activity of AAV in clinical practice.

This is the first report demonstrating increased urinary HMGB1 levels in AAV patients with active renal involvement. To date, only serum HMGB1 levels have been evaluated in AAV patients presenting active glomerulonephritis, but associations with parameters of renal involvement have not been analysed [19,20]. Serum HMGB1 levels are lower in AAV patients with renal involvement at presentation in comparison to non-renal patients [21] and in GPA patients with predominantly vasculitic manifestations than in GPA patients with granulomatous manifestations [19]. Bruchfeld *et al.*,

however, observed higher serum HMGB1 levels in AAV patients with active glomerulonephritis than in AAV patients with inactive renal disease [20]. Serum HMGB1 levels decreased 6–9 months after baseline when a new biopsy showed improvement in renal histopathology and the expression of HMGB1 in renal tissue decreased from active disease to remission [20]. Similarly to serum levels and tissue expression of HMGB1, urinary HMGB1/creatinine ratio decreased significantly when patients with active nephritis achieved remission in the current study.

Differently from AAV, the association between HMGB1 and renal involvement is well established in SLE. Both serum and urinary HMGB1 levels are higher in SLE patients with active lupus nephritis in comparison to patients without renal involvement and HC [15,16]. Furthermore, both serum and urinary levels of HMGB1 correlated with SLEDAI and complement levels while serum HMGB1 was also associated significantly with proteinuria and anti-dsDNA levels [15,16]. Of note, urinary HMGB1 levels have been measured by Western blot, as HMGB1 ELISAs are not validated for measurement in urine. Also serum levels of HMGB1 in SLE patients are detected by Western blot, because of the presence of anti-HMGB1 antibodies in lupus serum [15,16]. These antibodies are hardly present in AAV patients, so HMGB1 levels can be measured by ELISA in these sera [21]. In lupus nephritis, the cytoplasmic and extracellular expression of HMGB1 in renal tissue was higher than in control renal tissue and did not decrease with follow-up [16,17].

It remains speculative whether urinary HMGB1 in renal AAV results from release by local renal inflammation and infiltrating cells or necrosis in the kidney. The association between urinary HMGB1 and urinary T cells as well as the lack of correlation between urinary HMGB1 and urinary MCP-1 levels, a well-known inflammatory urinary marker [34], suggests passive release of HMGB1 by necrotic cells from the kidney. HMGB1 released from necrotic cells is in a reduced form and has chemotactic properties, whereas HMGB1

released from activated cells has a disulphide bond between C23 and C45 and C106 in the thiol form. This form of HMGB1 acts differently, as it can induce cytokine production by signalling through Toll-like receptor (TLR)-4 [35]. Clearly, the source of urinary HMGB1 in renal AAV needs further evaluation.

The presence of CD4⁺ T cells, mainly effector memory T cells, in urine reflects renal involvement in AAV and a reduction in the number of these cells in urine is observed following treatment [27]. Indeed, the number of CD4⁺ effector memory T cells was increased in urine in AAV patients with active nephritis, but no correlation could be found with BVAS, 24-hour proteinuria and eGFR in this study. However, both CD4⁺ T cell⁻ and CD4⁺ effector memory T cell counts in urine were associated with urinary HMGB1. These correlations could indicate a possible interplay between HMGB1 and the adaptive immune response in active nephritis in AAV [8–12]. Otherwise, the lack of association between urinary MCP-1 levels and CD4⁺ T cell⁻ and CD4⁺ effector memory T cell counts in urine could be explained by the fact that the chemoattractant effect of MCP-1 is predominantly exerted on monocytes and macrophages rather than on T cells [28].

Limitations of this study include the relatively low number of AAV patients evaluated and the lack of comparison with AAV patients with active disease but without renal involvement.

Urinary HMGB1 levels are increased in AAV patients with active nephritis in comparison to HC and decrease when remission is achieved. Urinary HMGB1 levels correlate with urinary CD4⁺ T cell⁻ and urinary CD4⁺ effector memory T cell counts. However, this analysis suggests that urinary HMGB1 reflect renal involvement in AAV less well than urinary MCP-1.

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