see commentary on page 16

# Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody–associated vasculitis

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**Studies in animal models suggest that complement activation is crucial in the pathogenesis of anti-neutrophil cytoplasmic antibody–associated vasculitis (AAV). Here we investigate the circulating complement activation profile of 66 patients with active stage AAV compared to that of 54 patients with AAV in remission. Plasma levels of C3a, C5a, soluble C5b-9, and Bb, all determined by enzyme-linked immunosorbent assay, were significantly higher in active stage than in remission of AAV, while plasma levels of properdin were significantly lower in the former than the latter disease stage. There was no significant difference in the plasma levels of C4d between active stage and remission. The plasma level of Bb in patients with active AAV significantly correlated with the proportion of total and cellular crescents in the renal biopsy, the erythrocyte sedimentation rate, and the Birmingham Vasculitis Activity Scores. Thus, systemic activation of complement by the alternative pathway takes place in human AAV. Circulating Bb might be a useful biomarker in assessing disease activity of AAV.**

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KEYWORDS: alternative pathway; anti-neutrophil cytoplasmic antibody; complement; vasculitis

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Anti-neutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) is characterized by pauci-immune necrotizing inflammation of the small blood vessels. AAV comprises granulomatosis with polyangiitis (GPA, previously named Wegener's granulomatosis), microscopic polyangiitis, and Churg–Strauss syndrome. ANCAs are predominantly IgG autoantibodies directed against neutrophil cytoplasmic constituents, in particular proteinase 3 and myeloperoxidase (MPO),[1,2] and are useful serological markers of AAV.

The complement system has a central role in innate and humoral immunity, and is composed of more than 30 plasma- and membrane-bound proteins. Activation of the complement system mainly follows three different pathways: the classical, the lectin, or the alternative pathway, distinguished by the factors that trigger complement activation. It was previously assumed that the complement system is not involved in the pathogenesis of AAV, as there is little immunoglobulin or complement deposition at lesional sites and AAV is generally not associated with hypocomplementemia. Recent studies in the mouse model of anti-MPO IgG-mediated glomerulonephritis suggest that complement activation, *via* the alternative pathway, is crucial for the disease development.[3–5] Our previous study in human AAV indicated that patients with renal C3c deposition had a higher level of proteinuria and a poorer initial renal function.[6] In a subsequent study, we confirmed complement activation *via* the alternative pathway in kidneys of patients with AAV.[7] However, direct evidence for systemic complement activation in human AAV is lacking. To address this issue, we measured plasma levels of various complement components in patients with AAV in both active and quiescent phases of the diseases, and correlated circulating levels of various complement components with clinical and pathological parameters.

## RESULTS

### Demographic and general data

Of the 66 patients with AAV in active stage, 29 were male and 37 were female, with an age of 55.4 ± 15.7 (range 14–82) years at diagnosis. The median interval from complaints to

diagnosis was 3 (range 0.3–60.8) months. At the time of diagnosis, 7 of the 66 patients had complaints less than 1 month; and 32 of the 66 patients had complaints more than 3 months. The level of initial serum creatinine was 371.3 ± 258.3 (range 57–1168) μmol/l. The level of urinary protein excretion was 2.49 ± 1.67 (range 0–7.35) g per 24 h. The level of Birmingham Vasculitis Activity Scores (BVAS)[8] was 20.3 ± 5.2 (range 11–30). In the renal specimen of these patients, little or no staining for IgG, IgA, or IgM (⩽1+ on a scale of 0 to 4+) was observed by immunofluorescence microscopy. No electron dense deposits were detected by electron microscopy. The clinical and histopathological data were listed in Table 1. In addition, among the 46 patients with active lupus nephritis, the level of systemic lupus erythematosus disease activity index was 21.5 ± 5.7 (range 9–36).

Of the 54 patients in remission stage of AAV, 21 were male and 33 were female, with an age of 59.3 ± 15.2 (range 18–81) years at diagnosis. The level of serum creatinine at sampling was 193.0 ± 147.9 (range 58–642) μmol/l. The BVAS levels of all the 54 patients in remission stage of AAV were zero.

**Table 1 | Clinical and histopathological data of patients with active AAV**

| | Values |
|---|---|
| Total number of patients | 66 |
| Male/female | 29/37 |
| Average age at onset of the disease (years) | 55.36 ± 15.72 |
| *Scr (μmol/l)* | |
| Mean ± s.d. | 371.3 ± 258.3 |
| Range | 57–1168 |
| *eGFR (ml/min per 1.73 m²)* | |
| Mean ± s.d. | 24.43 ± 23.54 |
| Range | 0.7–123.9 |
| ESR (mm per 1 h) | 73.27 ± 41.35 |
| ANCA level (RU/ml) | 115.97 ± 52.48 |
| Renal insufficiency at diagnosis | 60 (89.5%) |
| *Urinary protein (g per 24 h)* | |
| Mean ± s.d. | 2.49 ± 1.67 |
| Range | 0–7.35 |
| Skin rash | 10 (14.9%) |
| Arthralgia | 25 (37.3%) |
| Muscle pain | 32 (47.8%) |
| Pulmonary | 32 (47.8%) |
| ENT | 29 (43.3%) |
| Ophthalmic involvement | 13 (19.4%) |
| Gastrointestinal involvement | 12 (17.9%) |
| Nervous system | 3 (4.5%) |
| BVAS | 20.27 ± 5.18 |
| Average glomeruli per biopsy | 25.62 ± 13.09 |
| *Glomerular lesions (%)* | |
| Total crescents | 59.39 ± 29.00 |
| Cellular crescents | 15.91 ± 20.70 |

Abbreviations: AAV, anti-neutrophil cytoplasmic antibody-associated vasculitis; ANCA, antineutrophil cytoplasmic antibody; BVAS, Birmingham Vasculitis Activity Scores; eGFR, estimated glomerular filtration rate; ENT, ear, nose, and throat; ESR, erythrocyte sedimentation rate; s.d., standard deviation.
Data were collected at presentation.

**Plasma levels of soluble C5b-9 (Sc5b-9), C5a, and C3a**

The level of plasma Sc5b-9 was significantly higher in patients with AAV with active disease compared with patients in remission and normal controls (955.81 ± 321.39 vs. 345.99 ± 106.84 ng/ml, $P<0.01$; 955.81 ± 321.39 vs. 360.82 ± 164.51 ng/ml, $P<0.01$), but significantly lower than that in patients with lupus nephritis (955.81 ± 321.39 vs. 1477.68 ± 733.80 ng/ml, $P<0.01$). There was no significant difference in plasma concentration of Sc5b-9 between patients with AAV in remission and normal controls (345.99 ± 106.84 vs. 360.82 ± 164.51 ng/ml, $P>0.05$) (Figure 1a).

Plasma C5a levels were significantly higher in patients with active AAV compared with patients in remission, patients with lupus nephritis, and normal controls (51.65 ± 34.75 vs. 9.78 ± 5.81 ng/ml, $P<0.01$; 51.65 ± 34.75 vs. 23.29 ± 18.37 ng/ml, $P<0.01$; 51.65 ± 34.75 vs. 8.19 ± 5.44 ng/ml, $P<0.01$, respectively). There was no significant difference in plasma levels of C5a between patients with AAV in remission and normal controls (9.78 ± 5.81 vs. 8.19 ± 5.44 ng/ml, $P>0.05$) (Figure 1b).

The plasma level of C3a in patients with active AAV was significantly higher compared with patients in remission, patients with lupus nephritis, and normal controls (2178.67 ± 668.91 vs. 268.50 ± 211.53 ng/ml, $P<0.01$; 2178.67 ± 668.91 vs. 1164.83 ± 1063.07 ng/ml, $P<0.01$; 2178.67 ± 668.91 vs. 100.87 ± 70.55 ng/ml, $P<0.01$, respectively). However, the level of plasma C3a in patients with AAV in remission was still significantly higher compared with normal controls (268.50 ± 211.53 vs. 100.87 ± 70.55 ng/ml, $P<0.01$) (Figure 1c).

**The plasma levels of Bb and properdin**

As data from animal models suggest that complement activation *via* the alternative pathway has a crucial role in the development of AAV,[3] we further investigated plasma levels of two important factors in the alternative pathway, properdin and Bb. Measurement of properdin and Bb in plasma provides evidence for the involvement of the alternative complement pathway.[9–11]

The plasma levels of Bb were significantly higher in patients with AAV in active stage compared with patients in remission and normal controls (1.30 ± 0.68 vs. 0.62 ± 0.34 μg/ml, $P<0.01$; 1.30 ± 0.68 vs. 0.63 ± 0.26 μg/ml, $P<0.01$, respectively). No significant difference was found in plasma level of Bb between AAV patients in remission and normal controls (0.62 ± 0.34 vs. 0.63 ± 0.26 μg/ml, $P>0.05$). The plasma levels of properdin were significantly lower in AAV patients with active disease compared with patients in remission and normal controls (10.35 ± 5.64 vs. 35.96 ± 13.87 μg/ml, $P<0.01$; 10.35 ± 5.64 vs. 22.57 ± 9.67 μg/ml, $P<0.01$, respectively). The plasma levels of properdin in AAV patients in remission were significantly higher than that in normal controls (35.96 ± 13.87 vs. 22.57 ± 9.67 μg/ml, $P<0.01$). However, there were no significant differences in Bb and properdin levels between patients with active AAV and patients with lupus nephritis (1.30 ± 0.68 vs. 1.47 ± 0.87 μg/ml, $P>0.05$; 10.35 ± 5.64 vs. 11.61 ± 6.92 μg/ml, $P>0.05$, respectively) (Figure 1d and e).

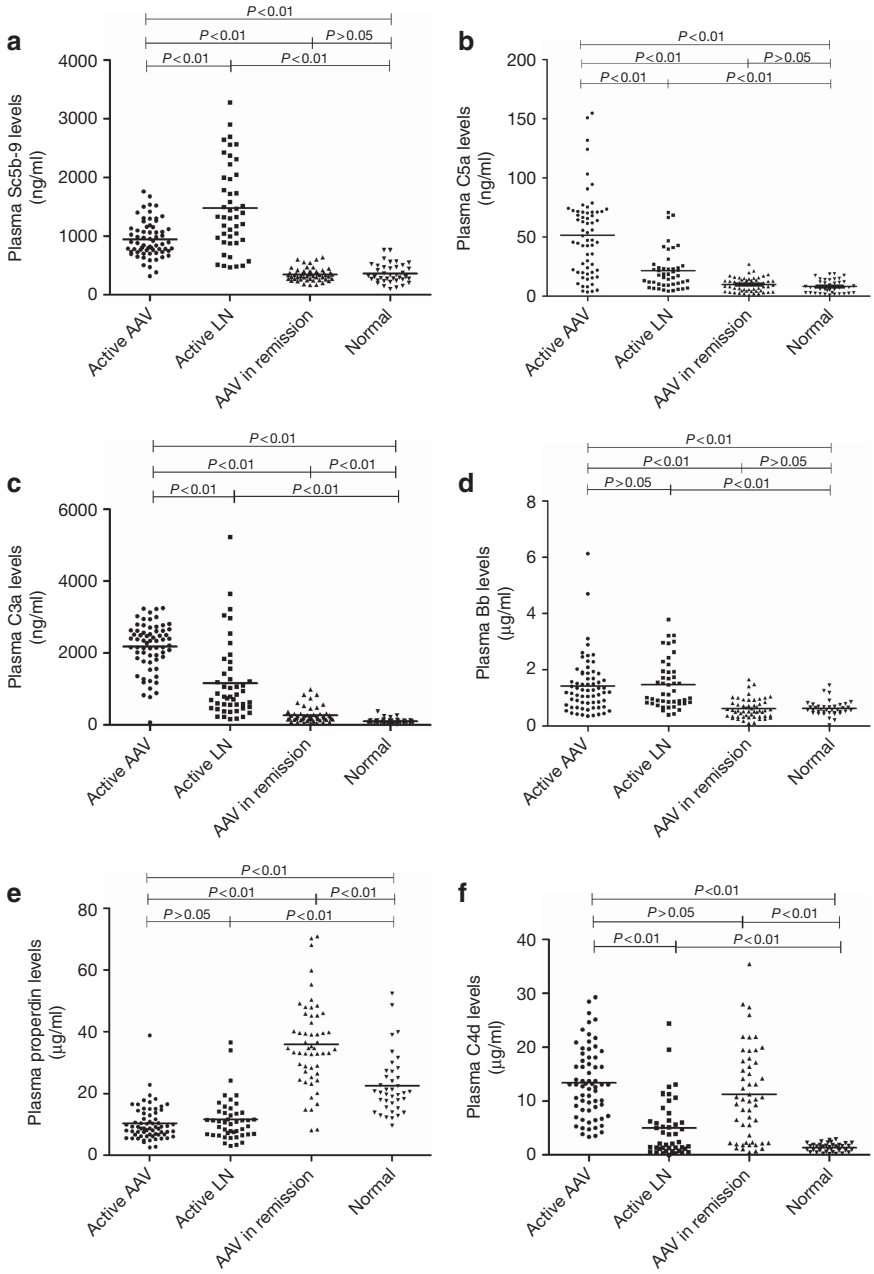


**Figure 1 | Plasma levels of complement components in patients with active anti-neutrophil cytoplasmic antibody–associated vasculitis (AAV), active lupus nephritis (LN), remission of AAV, and in normal controls.** Horizontal solid lines indicated the mean level. (**a**) Plasma levels of Sc5b-9. (**b**) Plasma levels of C5a. (**c**) Plasma levels of C3a. (**d**) Plasma levels of Bb. (**e**) Plasma levels of properdin. (**f**) Plasma levels of C4d.

### Plasma levels of C4d

The complement fragment C4d is a product derived from activation of C4, which is needed in both the classic pathway and the lectin pathway.

The plasma levels of C4d were significantly higher in patients with AAV with active disease compared with patients with lupus nephritis and normal controls ($13.43 \pm 6.56$ vs. $4.59 \pm 4.97$ μg/ml, $P < 0.01$; $13.43 \pm 6.56$ vs. $1.36 \pm 0.82$ μg/ml, $P < 0.01$, respectively). The plasma level of C4d in AAV patients in remission was significantly higher than that in normal controls ($11.24 \pm 8.49$ vs. $1.36 \pm 0.82$ μg/ml, $P < 0.01$). However, there was no significant difference in the plasma levels of C4d between AAV patients with active disease and AAV patients in remission (Figure 1f).

### Plasma levels of complement components in sequential samples from AAV patients

To further confirm the changes of circulating complement levels between active and quiescent disease, sequential plasma samples of both active stage and remission stage, from 20 patients with AAV, were measured. The plasma levels of Sc5b-9, C5a, C3a, and Bb were significantly higher in active stage

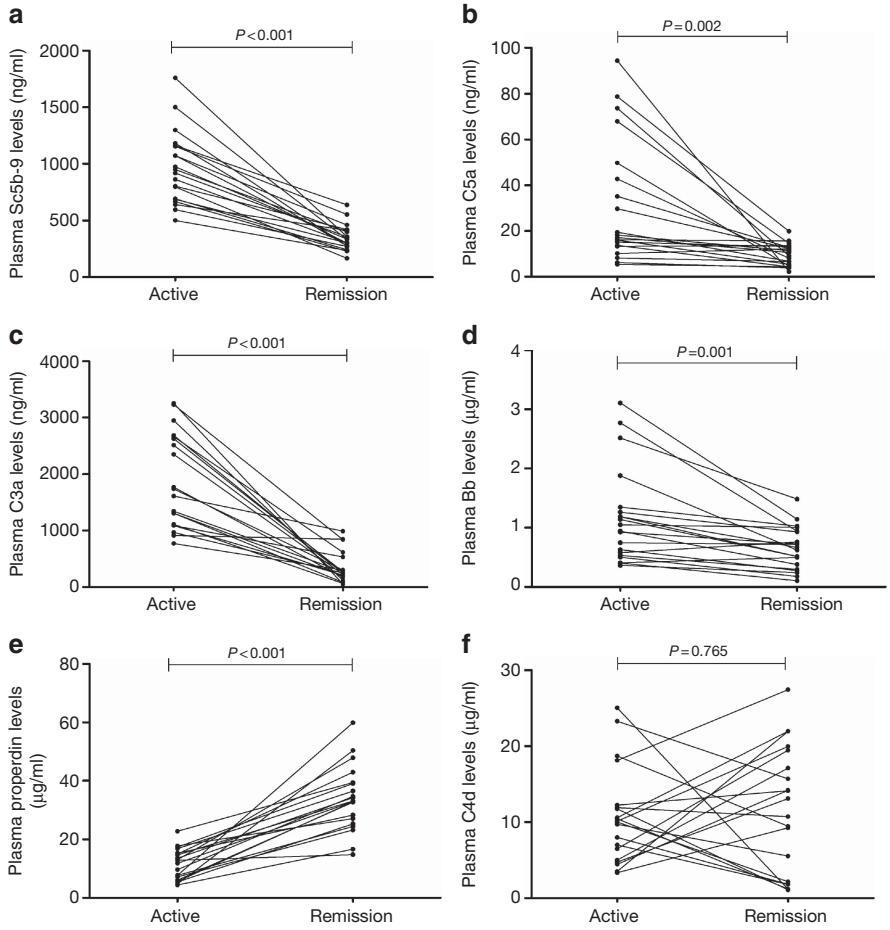


**Figure 2 | Changes of circulating complement levels in 20 anti-neutrophil cytoplasmic antibody–associated vasculitis (AAV) patients with sequential plasma samples.** (**a**) Plasma levels of Sc5b-9. (**b**) Plasma levels of C5a. (**c**) Plasma levels of C3a. (**d**) Plasma levels of Bb. (**e**) Plasma levels of properdin. (**f**) Plasma levels of C4d.

than that in remission ($964.98 \pm 319.51$ vs. $343.25 \pm 113.98$ ng/ml, $P < 0.001$; $31.63 \pm 27.14$ vs. $9.64 \pm 4.75$ ng/ml, $P = 0.002$; $1863.54 \pm 832.90$ vs. $340.66 \pm 274.46$ ng/ml, $P < 0.001$; $1.17 \pm 0.80$ vs. $0.65 \pm 0.36$ μg/ml, $P = 0.001$, respectively). For every patient, plasma levels of Sc5b-9 and C3a in remission were much lower than those in active stage. Eighteen of these 20 patients had a decrease in plasma level of C5a in remission compared with that in active stage, whereas only two patients had a very slight increase in plasma level of C5a in remission compared with that in active stage. Similarly, 18 of these 20 patients had a decrease in plasma level of Bb in remission compared with that in active stage, whereas two patients had a very slight increase in plasma level of Bb in remission compared with that in active stage. The plasma level of properdin was significantly lower in active stage than that in remission ($11.17 \pm 5.39$ vs. $34.13 \pm 10.60$ μg/ml, $P < 0.001$). For every patient, the plasma level of properdin in remission stage was much higher than that in active stage. However, no significant difference was found in plasma level of C4d between the active and the remission stage ($10.73 \pm 6.25$ vs. $11.47 \pm 8.32$ μg/ml, $P = 0.765$) (Figure 2).

### Plasma levels of intact complement components C4, C2, and factor B

The plasma levels of C4, C2, and factor B were significantly higher in patients with AAV in active stage than those in normal controls ($0.22 \pm 0.08$ vs. $0.18 \pm 0.03$ mg/ml, $P = 0.01$; $60.69 \pm 18.51$ vs. $47.63 \pm 8.75$ μg/ml, $P < 0.01$; $418.18 \pm 160.40$ vs. $295.99 \pm 91.94$ μg/ml, $P < 0.01$, respectively). The plasma levels of C4, C2, and factor B were significantly lower in patients with lupus nephritis than those in normal controls ($0.10 \pm 0.07$ vs. $0.18 \pm 0.03$ mg/ml, $P < 0.01$; $29.00 \pm 21.25$ vs. $47.63 \pm 8.75$ μg/ml, $P < 0.01$; $213.83 \pm 239.33$ vs. $295.99 \pm 91.94$ μg/ml, $P < 0.01$, respectively). The plasma levels of C4, C2, and factor B were still significantly higher in patients with AAV in remission than those in normal controls ($0.20 \pm 0.04$ vs. $0.18 \pm 0.03$ mg/ml, $P = 0.02$; $55.32 \pm 17.21$ vs. $47.63 \pm 8.75$ μg/ml, $P = 0.01$; $399.11 \pm 94.46$ vs. $295.99 \pm 91.94$ μg/ml, $P < 0.01$, respectively). However, there were no significant differences in plasma levels of C4, C2, and factor B between patients with AAV in active stage and those in remission ($0.22 \pm 0.08$ vs. $0.20 \pm 0.04$ mg/ml, $P > 0.05$; $60.69 \pm 18.51$ vs. $55.32 \pm 17.21$ μg/ml, $P > 0.05$;

418.18 ± 160.40 vs. 399.11 ± 94.46 μg/ml, $P > 0.05$, respectively) (Figure 3).

### Association between circulating levels of various complement components and clinicopathological parameters of patients with active AAV

Of the 66 patients with AAV in active stage, correlation analysis showed that plasma levels of C5a correlated with that of Sc5b-9 and C3a ($r = 0.382$, $P < 0.01$; $r = 0.424$, $P < 0.01$; respectively). Plasma properdin levels inversely correlated with the proportion of crescents in the renal specimen ($r = -0.292$, $P < 0.05$). Plasma Bb levels correlated with plasma levels of C5a, C3a, and Sc5b-9 ($r = 0.285$, $P < 0.05$; $r = 0.257$, $P < 0.05$; $r = 0.384$, $P < 0.01$, respectively). Plasma Bb levels also correlated with BVAS, the level of erythrocyte sedimentation rate, the proportion of total crescents, and the proportion of cellular crescents ($r = 0.286$, $P < 0.05$; $r = 0.311$, $P < 0.05$; $r = 0.266$, $P < 0.05$; $r = 0.358$, $P < 0.01$, respectively) (Figure 4). In addition, plasma Bb levels marginally inversely correlated with the proportion of normal glomeruli ($r = -0.241$, $P = 0.05$).

### Immunohistochemistry for Bb, C3d, and C5b-9

In renal specimens of patients with AAV, immunohistochemical examination revealed that Bb, C3d, and C5b-9 could be detected along the glomerular capillary wall and mesangial area of glomeruli of patients with AAV in a granular pattern (Figure 5). In addition, they could also be detected in some small arteries, which is consistent with our previous observations.[7]

The number of glomeruli analyzed for Bb, C3d, and C5b-9 per biopsy of patients with AAV was 14.34 ± 8.26 (range 4–35), 15.48 ± 9.13 (range 4–34), and 12.07 ± 7.06 (range 4–27), respectively. The mean optical density of Bb, C3d, and C5b-9 in glomeruli was 0.34 ± 0.23, 0.62 ± 0.30, and 0.05(0.02–0.13), respectively. Correlation analysis showed that the mean optical density of Bb in renal specimens correlated with the mean optical density of C3d and C5b-9 ($r = 0.733$, $P < 0.001$; $r = 0.611$, $P < 0.001$, respectively). The mean optical density of C3d correlated with that of C5b-9 ($r = 0.436$, $P = 0.018$). The mean optical density of C5b-9 in renal specimens inversely correlated with plasma level of C5a and Sc5b-9 ($r = -0.390$, $P = 0.037$ and $r = -0.448$, $P = 0.015$, respectively). No correlation was found between the mean optical density of Bb in renal specimens and the plasma level of Bb, and no correlation was found between the mean optical density of C3d in renal specimens and the plasma level of C3a.

## DISCUSSION

Recently, increasing evidence of complement activation in the pathogenesis of AAV was provided by studies in animal models.[3–5] Our previous study suggested that, in human renal histopathology, complement activation *via* the alternative pathway participated in the development of ANCA-associated glomerulonephritis.[7] However, the evidence for

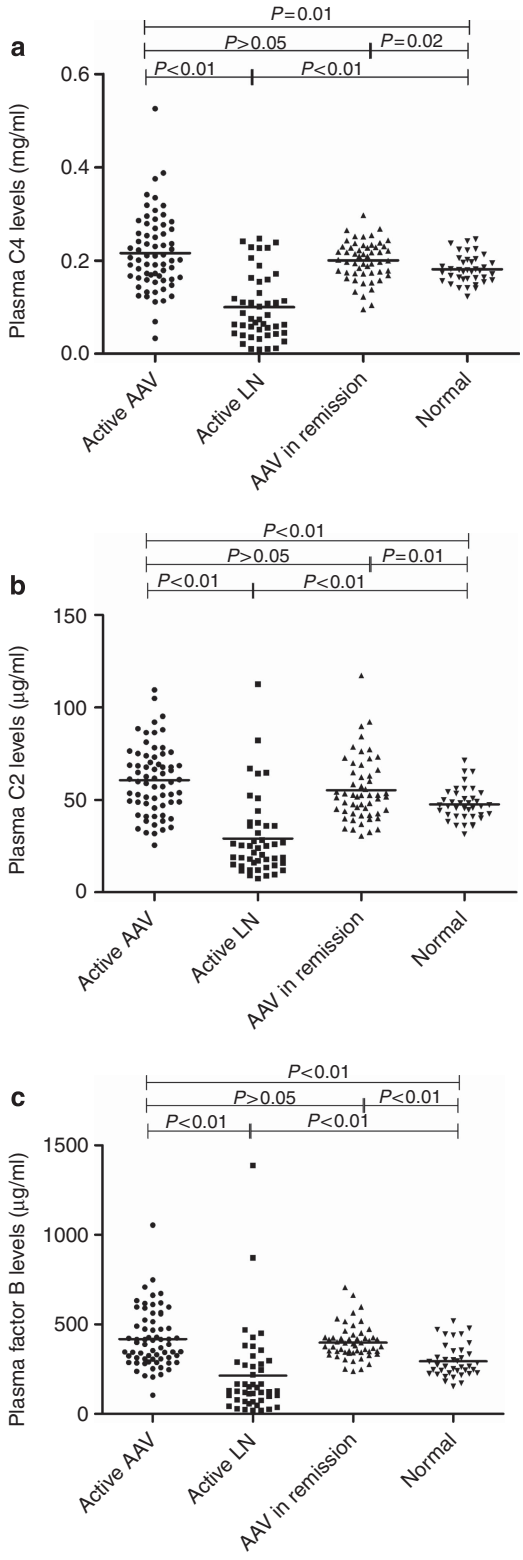


**Figure 3 | Plasma levels of C4, C2, and factor B in patients with active anti-neutrophil cytoplasmic antibody–associated vasculitis (AAV), active lupus nephritis (LN), remission of AAV, and in normal controls.** Horizontal solid lines indicated the mean level. (**a**) Plasma levels of C4. (**b**) Plasma levels of C2. (**c**) Plasma levels of factor B.

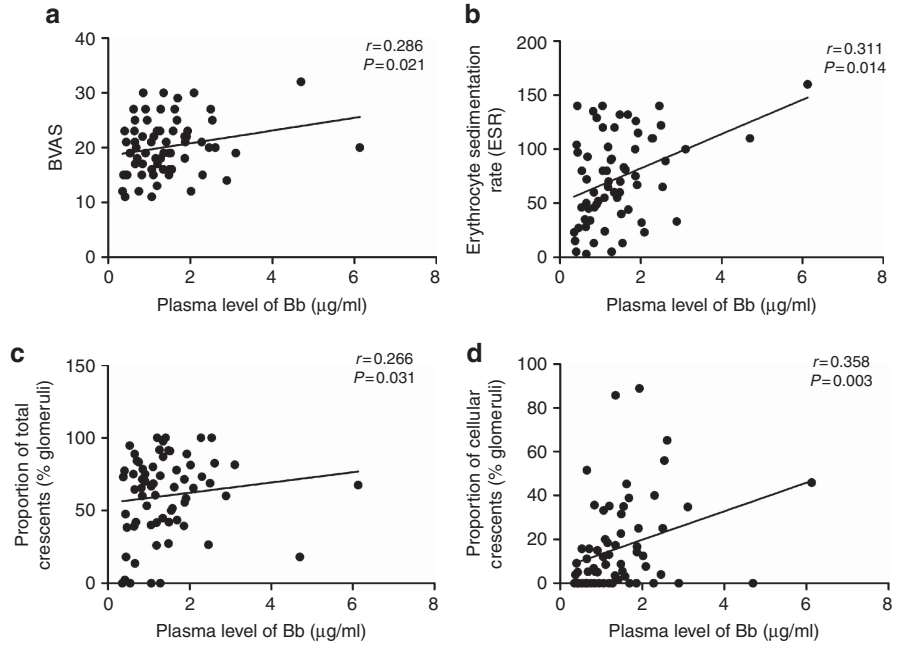


**Figure 4 | Association between circulating levels of Bb and clinicopathological parameters of patients with active anti-neutrophil cytoplasmic antibody–associated vasculitis (AAV).** (**a**) Association between plasma levels of Bb and Birmingham Vasculitis Activity Scores (BVAS). (**b**) Association between plasma levels of Bb and erythrocyte sedimentation rate. (**c**) Association between plasma levels of Bb and the proportion of total crescents in renal specimens. (**d**) Association between plasma levels of Bb and the proportion of cellular crescents.

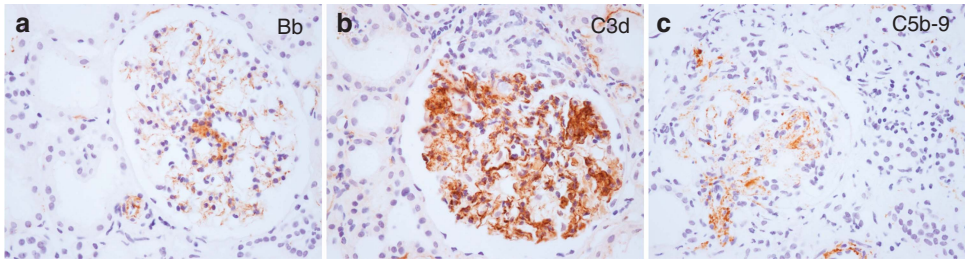


**Figure 5 | Immunohistochemistry staining for complement components Bb, C3d, and C5b-9 in renal specimens of patients with anti-neutrophil cytoplasmic antibody (ANCA)–associated vasculitis.** (**a**) Immunohistochemical staining of Bb in glomerulus of ANCA-associated vasculitis. (**b**) Immunohistochemical staining of C3d in glomerulus of ANCA-associated vasculitis. (**c**) Immunohistochemical staining of C5b-9 in glomerulus of ANCA-associated vasculitis.

circulating activation of complement system in AAV, a systemic autoimmune disease, was lacking. Therefore, in the current study, we measured various circulating complement components to investigate systemic complement activation profile of patients with AAV.

Activation of the complement system leads the cleavage of C3 to C3a and C3b. The three pathways converge at the formation of C5 convertases capable of cleaving C5 to C5a and C5b, and then membrane attack complex C5b-9 is generated by the assembly of C5b through C9. In the current study, the significant difference in plasma levels of C5a and Sc5b-9 between AAV patients in active stage and in remission as well as healthy controls indicates that complement activation occurs in the development of AAV. Interestingly, the plasma level of Sc5b-9 in patients with active AAV was significantly lower than that in patients with active SLE, whereas the plasma levels of C3a and C5a in patients with active AAV were significantly higher than that in patients with active SLE. CD59 is an important complement membrane inhibitor, which could prevent the formation of the membrane attack complex by blocking the interaction of the C5b-8 complex with C9. It was found that the proportions of several kinds of circulating blood cells with CD59 expression were significantly reduced in SLE patients.[12] Decreased CD59 level might contribute to a significant increase in plasma Sc5b-9 level in SLE patients. However, the CD59 expression in patients with AAV needs further investigation.

In this study, the plasma level of fragment Bb, which indicates activation of the alternative pathway,[9–11] was significantly higher in AAV patients of active stage than that in remission and normal controls. It was also found in the

current study that plasma Bb concentration correlated with plasma levels of C3a, C5a, and Sc5b-9, which reflect the systemic activation of the complement system. These suggested the complement activation *via* the alternative complement pathway occurs in the development of AAV. More importantly, plasma Bb concentration correlated with erythrocyte sedimentation rate, BVAS, and the proportion of crescents in renal histopathology of patients with AAV, which indicated that circulating Bb level could reflect both systemic and renal disease activity. In the anti-MPO-induced AAV mouse model,[13] mice deficient for factor B, which is unique in activation of the alternative pathway, were completely protected from disease induction,[3] indicating that activation of factor B is indispensable in the pathogenesis of AAV. The close association between circulating Bb level and disease activity suggests it to be a potentially useful biomarker for evaluating disease activity in AAV. In the present study, we also found that the plasma level of Bb in patients with lupus nephritis was significantly higher than that in normal controls, which has been consistent with previous studies.[9,14,15] There is increasing evidence from animal and human studies that the alternative pathway is involved in the pathogenesis of lupus nephritis,[16,17] which is consistent with the results presented here. Compared with lupus nephritis, similar plasma levels of Bb were observed in patients with AAV, providing additional evidence for activation of the alternative pathway in AAV.

The current study found that the plasma level of properdin in patients with AAV in active stage was significantly lower than that in normal controls. The level of properdin inversely correlated with the proportion of crescents in renal specimens of AAV. These observations provide additional evidence that activation of the alternative complement pathway occurs in the development of AAV.

The plasma level of C4d in patients with AAV in active stage was significantly higher than that in normal controls, but there was no significant difference in plasma levels of C4d between patients in active stage and in remission. This indicated that the classic and/or lectin pathway were also activated in patients with active AAV, but they were probably not pathogenic. The complement components, especially those in classic and lectin pathways, have functions for clearing apoptotic and necrotic cells. Ogden *et al.*[18] demonstrated that both MBL and C1q, the first components of the lectin pathway and classical pathway, could bind to apoptotic cells and facilitate their clearance by phagocytes, such as macrophage. Gullstrand *et al.*[19] demonstrated that classical pathway components C1q, C4, C2, and C3 are involved in phagocytosis of apoptotic cells by phagocytes. In the present study, we cannot rule out the possibility that the activation of lectin pathway and/or classical pathway of the complement system was involved in clearing apoptotic or necrotic cells in the chronic inflammation process of AAV. In addition, the increase of C4d in patients with lupus nephritis in the present study was consistent with previous reports.[15] Interestingly, the plasma level of C4d in patients with active AAV was much higher than that of patients with lupus nephritis. This might be attributed to a somewhat more fulminant disease process of ANCA-associated glomerulonephritis than lupus nephritis, e.g., rapid loss of renal function and crescentic formation is more common in AAV than in SLE. Therefore, AAV might have higher level of inflammation and lead to more apoptotic or necrotic cells, and then more C4d might be produced during the process of clearing apoptotic or necrotic cells.

In the current study, another interesting finding was that in the remission stage of AAV, in contrast to plasma C5a and Sc5b-9 levels that decreased to normal levels, plasma C3a levels were still significantly higher than that in normal controls. It seemed that the terminal part of the complement cascade became quiescent in the remission stage, and C3 activation was not followed by C5 activation or membrane attack complex formation. The reasons remain speculative, but may also be associated with the clearance of apoptotic bodies because C3b is an important opsonin. C3b fragments are under strict control by a complex system of serum and membrane proteins. CR1 is a receptor for the complement fragments C3b and C4b and is expressed on many different cell types. It allows phagocytic cells to ingest particles that have activated complement and promotes the cleavage of C3b and C4b by the serum enzyme C3b/C4b inactivator.[20] Many other complement regulators, such as CD55, CD46, and factor H, could also inhibit complement amplification *via* targeting on different components.[21] All of the above may contribute to the regulating mechanism preventing C3b from activating C5 in the remission stage of AAV. However, the exact mechanism still needs further investigation.

In the present study, no positive correlation between the plasma levels and the renal deposits of complement components was observed. As a systemic disease, the circulating complement activation status might not necessarily correlate with the local renal complement activation status.

In conclusion, the present study provided direct evidence for that systemic activation of complement *via* the alternative pathway occurred in human AAV. Circulating level of fragment Bb might be a useful biomarker in monitoring systemic disease activity and renal disease activity in AAV.

## MATERIALS AND METHODS

### Patients and blood samples

Sixty-six patients with active AAV, diagnosed at the Peking University First Hospital from January 2007 to December 2010, were enrolled in this study. All the patients had renal involvement and received renal biopsy. Plasma samples of these patients were collected on the day of renal biopsy and before commencing the immunosuppressive treatment. Plasma samples of 54 patients with AAV, who achieved complete remission, as described previously,[22] after immunosuppressive therapy, were also collected at their regular ambulatory visits between January 2011 and April 2012. There were 20 patients who had plasma samples both in active stage and remission. All these 100 patients were positive for perinuclear ANCA (P-ANCA) and MPO-ANCA at diagnosis. All the patients met the Chapel Hill Consensus Conference definition of AAV[23] and had complete clinical and pathological

data. Patients with secondary vasculitis or with coexistence of other renal disease were excluded.

Plasma samples of 39 age- and gender-matched healthy blood donors and 46 patients with renal biopsy-proven diffuse lupus nephritis (class IV, according to the abbreviated version of the ISN/RPS classification[24]) in active stage, diagnosed in the same period in our center, were collected as normal control and disease control, respectively. All the patients with lupus nephritis fulfilled the 1997 American College of Rheumatology revised criteria for SLE.[25]

The blood samples of patients and controls were drawn into EDTA tubes and put on ice immediately. The blood samples were centrifuged at 2000 *g* for 15 min at 4 °C within 30 min after collection and the plasma was stored in aliquot at −80 °C until use. When testing, after rapid thawing at 37 °C, the frozen specimens were transferred immediately onto ice before use within 1 h. Repeated freeze/thaw cycles were avoided. The research was in compliance with the Declaration of Helsinki Principles and approved by the ethics committee of our hospital. Written informed consent was obtained from each participant.

### Quantification of plasma complement components levels

Plasma concentrations of human complement components were determined by enzyme-linked immunosorbent assay, including complement fragments C5a (Quidel, San Diego, CA), C3a (Quidel), Bb (Quidel), soluble C5b-9 (Sc5b-9, Quidel), properdin (Uscnk Life Science, Wuhan, China), C4d (Quidel), C4 (Abcam, Cambridge, UK), C2 (Uscnk Life Science), and factor B (Uscnk Life Science). All the complement components were assayed according to the manufacturer's instructions.

### Renal histology

Renal histology of patients with AAV was evaluated according to the previous standardized protocol.[26–28] The presence of glomerular lesions, including fibrinoid necrosis, crescents, and glomerulosclerosis, were calculated as the percentage of the total number of glomeruli in a biopsy.

### Detection of renal deposition of complement components by immunohistochemistry

Renal specimens from 29 patients in active stage of AAV, who were randomly selected from the 66 patients described above, were included for the immunohistochemistry study.

C3d, one of the products derived from C3 activation, could bind covalently via thiolester bond to its acceptor molecule at the activated site.[29] Therefore, we detected the C3 activation in renal specimen by C3d staining. In the present study, staining of complement components was performed by immunohistochemistry as previously described.[7] As our previous study already proved that staining of C4d, the common component of the classic and lectin pathway, was negative in the renal specimen of ANCA-associated glomerulonephritis,[7] we stained Bb, C3d, and C5b-9 in renal specimens.

Staining of complement components was performed by immunohistochemistry as previously described.[7] Immunohistochemical staining for Bb, C3d, and C5b-9 was performed on 4-μm deparaffinized sections of formaldehyde-fixed renal tissue using mouse anti-human Bb monoclonal antibodies (Quidel), rabbit anti-human C3d polyclonal antibodies (Dako A/S, Copenhagen, Denmark) and mouse anti-human C5b-9 monoclonal antibodies (Abcam) as primary antibodies. Antibodies against Bb, C3d, and C5b-9 were used in dilutions of 1:50, 1:500, and 1:50, respectively. The sections for C3d staining were treated with 0.4% pepsin (Zhongshan Golden Bridge Biotechnology, Beijing, China) 45 min for antigen retrieval. The sections for staining Bb and C5b-9 were treated with 0.5 mg/ml proteinase K for antigen retrieval.

The sections were examined by light microscopy. The renal staining of Bb, C3d, and C5b-9 in glomeruli was evaluated by the Image Pro Plus analysis software 6.05 (Media Cybernetics, Silver Spring, MD). The positive signals were quantified as the mean optical density (integrated option density/area).

### Statistical analysis

Differences of quantitative parameters between groups were assessed using the *t*-test (for normally distributed data) or nonparametric test (for non-normally distributed data). The relationships between two continuous variables were analyzed using Spearman's rank correlation. The difference was considered significant if a *P*-value was $<0.05$. Analysis was performed with SPSS statistical software package (version 13.0, Chicago, IL).

## DISCLOSURE

All the authors declared no competing interests.

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