Alternative Complement Pathway Activation Products in Urine and Kidneys of Patients with ANCA-Associated GN

Shen-Ju Gou, Jun Yuan, Chen Wang, Ming-Hui Zhao, and Min Chen

Summary

Background and objectives Previous study revealed that complement activation products of the alternative pathway could be detected in renal specimens of human ANCA-associated vasculitis. The current study aimed to investigate the clinical and pathologic significance of complement activation products in the urine and kidneys of patients with ANCA-associated vasculitis.

Design, setting, participants, & measurements Renal biopsy specimens from 29 patients with ANCA-associated vasculitis diagnosed at Peking University First Hospital from January of 2008 to December of 2010 were randomly collected. Urine samples from 27 of 29 patients in active stage and 22 ANCA-associated vasculitis patients in complete remission who were independent of the above-mentioned 29 patients were collected. Urine samples from 28 patients with lupus nephritis and 25 healthy individuals were also collected. The renal deposition of Bb, C3d, and C5b-9 were detected by immunohistochemistry. The urinary levels of Bb, C3a, C5a, and soluble C5b-9 were determined by ELISA.

Results The deposition, measured by the mean optical density of Bb, which is an alternative complement pathway marker, in glomeruli correlated with the proportion of total crescents (r=0.50, P=0.006), the extent of interstitial infiltrate (r=0.59, P=0.001), interstitial fibrosis (r=0.45, P=0.01), and tubular atrophy (r=0.55, P=0.002), whereas it correlated inversely with the proportion of normal glomeruli (r=−0.49, P=0.008). The urinary levels of Bb, C3a, C5a, and soluble C5b-9 were all significantly higher in active compared with remission stage. The urinary levels of Bb in patients with active ANCA-associated vasculitis correlated with the serum creatinine (r=0.56, P=0.002) and correlated inversely with the proportion of normal glomeruli in renal specimens (r=−0.49, P=0.009).

Conclusions The present study provides additional evidence that complement activation through the alternative pathway occurred in the development of ANCA-associated vasculitis. The renal deposition of Bb and urinary Bb levels were associated with the severity of renal injury.

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Due to the number of contributing authors, the affiliations are provided in the Supplemental Material.

Correspondence:
Dr. Min Chen, Renal Division, Department of Medicine, Peking University First Hospital, No.8, Xishiku Street, Xicheng District, Beijing 100034, China. Email: leimeng@public3.bta.net.cn

Introduction

ANCA is associated with systemic small vessel necrotizing vasculitis, including granulomatosis with polyangiitis, microscopic polyangiitis, and Churg–Strauss syndrome, which are collectively termed ANCA-associated vasculitis (AAV). AAV is characterized by pauci-immune necrotizing inflammation of the small blood vessels, and ANCAs are useful serological markers of AAV. The major target antigens of ANCA are the myeloperoxidase (MPO) and proteinase 3 (1,2).

Activation of the complement system mainly follows three different pathways: the classic, lectin, or alternative pathway depending on the activation triggers (3). The complement system is activated in a linear cascade, with activation of one component leading to activation of the next (4). Complement activation products, usually the cleavage products of complement components, are important markers for activation of complement system. Among them, Bb is a marker for activation of the alternative pathway.

Because the histopathological hallmark of ANCA-associated GN is pauci-immune necrotizing crescentic GN and AAV is generally not associated with hypocomplementemia (2,5), it was previously assumed that the complement system was not involved in the development of AAV. Recently, increasing evidences have suggested an important role of complement activation in the pathogenesis of AAV (6–13). In the animal study by Xiao *et al.* (7), it was found that activation of the alternative complement pathway, but not the classic or lectin pathway, was required for induction of GN with anti-MPO IgG. Our previous study in renal histopathology revealed that complement activation products of the alternative pathway could be detected in human ANCA-associated GN (14). Our additional investigation (15) found that circulating Bb level was associated with systemic

disease activity of AAV measured by Birmingham Vasculitis Activity Score (BVAS). However, the clinical and pathologic significance of the complement activation products in kidneys was not clear. Therefore, in the current study, we measured the levels of various complement activation products in both urine and renal specimens of AAV patients, and their correlation with clinical and histopathological parameters was studied.

Materials and Methods

Patients and Samples

Renal biopsy specimens from 29 patients with active AAV diagnosed in Peking University First Hospital from January of 2008 to December of 2010 were randomly collected in this study. Here, active AAV referred to the initial onset of AAV before commencing immunosuppressive treatment with active clinical manifestations of AAV, particularly active GN (with dysmorphic erythrocytes or red cell casts in the urine). The plasma levels of complement activation products of these patients were measured as described previously (15). Sera samples of these patients with active AAV were also collected for detection of ANCA-IgG subclasses. Urine samples from 27 of 29 patients were collected on the day of renal biopsy before commencing immunosuppressive treatment. Urine samples of 22 patients who achieved complete remission after immunosuppressive therapy were also collected at their regular ambulatory visits between January of 2011 and April of 2012. Complete remission was defined as the "absence of disease activity attributable to active disease qualified by the need for ongoing stable maintenance immunosuppressive therapy" as described previously (16). All 51 patients were positive for perinuclear ANCA and MPO-ANCA at diagnosis. All the patients met the Chapel Hill Consensus Conference definition of AAV (17) and had complete clinical and pathologic data. Patients with secondary vasculitis or coexistence of other renal disease were excluded.

Urine samples from 25 age- and sex-matched healthy individuals were collected as the normal control; samples from 28 patients with renal biopsy-proven diffuse lupus nephritis (class IV according to the abbreviated version of the International Society of Nephrology/Renal Pathology Society classification) (18) in active stage diagnosed in the same period at our center were collected as the disease control. All the patients with lupus nephritis fulfilled the 1997 American College of Rheumatology revised criteria for systemic lupus erythematosus (19).

The urine samples of patients and controls were centrifuged at 2000 $\times$ *g* for 20 minutes at 4°C, and the supernatant 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 hour. Repeated freeze/thaw cycles were avoided. The research was in compliance with the Declaration of Helsinki and approved by the ethics committee of our hospital. Written informed consent was obtained from each participant.

Renal Histology

Renal histology of patients with AAV was evaluated according to the previous standardized protocol (20–22). The presence of glomerular lesions, including fibrinoid necrosis, crescents, and glomerulosclerosis, was calculated as the percentage of the total number of glomeruli in a biopsy. Interstitial and tubular lesions were scored semiquantitatively on the basis of the percentage of the tubulointerstitial compartment affected: interstitial infiltrate (− for 0%, + for 0%–20%, ++ for 20%–50%, and +++ for >50%), interstitial fibrosis (− for 0%, + for 0%–50%, and ++for >50%), and tubular atrophy (− for 0%, + for 0%–50%, and ++ for >50%).

Detection of Deposition of Complement Activation Products in Renal Specimens by Immunohistochemistry

To study the deposition of complement activation products in kidneys of patients with AAV, complement activation products Bb, C3d, and C5b-9 were detected by immunohistochemistry on renal biopsy specimens of patients with ANCA-associated GN (Supplemental Material) as previously described (14).

Since our previous study in renal histopathology revealed that activation of the complement system by the alternative pathway and not the classic or lectin pathway occurred in the development of human ANCA-associated GN (14), we detected the deposition of Bb, which is unique in activation of the alternative pathway, in renal specimens in the current study. The three known complement pathways converge at the level of the C3 molecule, and C3d was one of the products derived from C3 activation (23). In the present study, we detected C3d and C5b-9, which are indicators of activation of the common pathway, in renal specimens.

The renal staining of Bb, C3d, and C5b-9 in glomeruli was evaluated by the Image Pro Plus analysis software 6.0 (Media Cybernetics, Silver Spring, MD). The positive signals were quantified as the mean optical density (integrated option density/area).

Quantification of Urinary Levels of Complement Components

In accordance with the immunohistochemical staining in renal specimens, complement activation product levels of Bb, C3a, C5a, and soluble C5b-9 (sC5b-9) in urine samples were also measured. Urinary levels of the above human complement activation products were determined by ELISA. In addition, the urinary levels of C1q and mannose-binding lectin (MBL) were also detected by ELISA. Commercial kits of human complement components Bb, C3a, C5a, sC5b-9 (Quidel Corporation, San Diego, CA), C1q, and MBL (Uscn Life Science, Wuhan, China) were used. The assays were conducted according to the manufacturer's instructions.

To analyze the association between the levels of complement activation products and ANCA IgG subclasses, MPO-ANCA IgG subclasses (IgG1, IgG2, IgG3, and IgG4) were measured using ELISA (Supplemental Material).

Statistical Analyses

Descriptive statistics for normally or non-normally distributed data were presented as mean ± SD or median and interquartile range, respectively. To adjust for the influence of urinary protein excretion on urinary complements

concentration, an analysis of covariance model was used. Details of the statistical analyses are described in the Supplemental Material.

Results

Demographic and General Data

Among 29 patients with AAV in active stage, 12 patients were men, and 17 patients were women. The level of BVAS (24) was 20.1±4.9 (range=11–30). In the renal specimens of these patients, little or no staining for IgG, IgA, or IgM (≤1+ on a scale of 0–4+) was observed by immunofluorescence microscopy. No electron-dense deposits were detected by electron microscopy. The clinical and histopathological data are listed in Table 1. In addition, among 28 patients with active lupus nephritis, the level of Systemic Lupus Erythematosus Disease Activity Index was 20.4±6.1 (range=9–36).

Among 22 patients in remission stage of AAV, 8 patients were men, and 14 patients were women, with an age of 62.5±14.9 (range=23–89) years at diagnosis. The clinical and histopathological data at initial onset of these 22 patients are listed in Table 2. The level of serum creatinine at sampling was 1.3 (1.0–1.6) mg/dl. The BVAS levels at sampling of all 22 patients in remission stage of AAV were zero.

Table 1. Clinical and histopathological data of 29 patients with active ANCA-associated vasculitis

Parameters	Number
Number	29
Men/women	12/17
Average age at onset of the disease (yr)	58.3±15.4
Scr (mg/dl)	
Median (IQR)	2.4 (1.2–4.4)
Range	0.7–11.4
eGFR (ml/min per 1.73 m^2)	
Median (IQR)	20.2 (11.1–51.8)
Range	0.7–118.9
ESR (mm/h)	63.8±43.2
ANCA level (RU/ml)	138.9±51.3
eGFR<60 ml/min per 1.73 m^2 at diagnosis	23 (79.3%)
Urinary protein (g/24 h)	
Median (IQR)	1.9 (0.8–3.1)
Range	0–5.2
Skin rash	3 (10.3%)
Arthralgia	7 (24.1%)
Muscle pain	9 (31.0%)
Pulmonary	18 (62.1%)
ENT	12 (41.4%)
Ophthalmic involvement	6 (20.7%)
Gastrointestinal involvement	3 (10.3%)
Nervous system	4 (13.8%)
BVAS	20.1±4.9
Average glomeruli per biopsy	26.0±11.0
Glomerular lesions (%)	
Total crescents	57.8±30.5
Cellular crescents	46.5±27.6
Fibrous crescents median (IQR)	4.5 (0–14.0)
Tubulointerstitial lesions	
Interstitial infiltration (−/+/++/+++)	1/5/8/15
Interstitial fibrosis (−/+/++)	3/8/18
Tubular atrophy (−/+/++)	1/7/21

Scr, serum creatinine; IQR, interquartile range; eGFR, estimated GFR; ESR, erythrocyte sedimentation rate; ENT, ear, nose and throat; BVAS, Birmingham Vasculitis Activity Scores.

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 1, A–F). Moreover, they could also be detected in some small vessels (Figure 1, G–L), which is consistent with our previous observation (14).

The numbers of glomeruli analyzed for Bb, C3d, and C5b-9 per biopsy of patients with AAV were 14.3±8.3 (range=4–35), 15.5±9.1 (range=4–34), and 12.1±7.1 (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, as described previously (15).

Urinary Levels of Bb, C3a, C5a, sC5b-9, C1q, and MBL

Urinary levels of complement components were normalized for urinary creatinine levels to correct for differences in dilution.

The urinary Bb levels were significantly higher in AAV patients in active stage compared with AAV patients in remission, patients with lupus nephritis, and normal controls (0.11 [0.04–0.21] versus 0.01 [0–0.03] μg/mg creatinine (Cr), $P<0.001$; 0.11 [0.04–0.21] versus 0.02 [0–0.04] μg/mg Cr, $P=0.001$; and 0.11 [0.04–0.21] versus 0.01 [0–0.02] μg/mg Cr, $P<0.001$, respectively). No significant difference was found in urinary levels of Bb between AAV patients in remission and normal controls (0.01 [0–0.03] versus 0.01 [0–0.02] μg/mg Cr, $P=0.19$) (Figure 2A).

The urinary C3a levels were significantly higher in AAV patients in active stage compared with AAV patients in remission, patients with lupus nephritis, and normal controls (3.25 [0.99–13.30] versus 0.02 [0.01–0.05] ng/mg Cr, $P<0.001$; 3.25 [0.99–13.30] versus 0.20 [0.01–2.11] ng/mg Cr, $P<0.001$; 3.25 [0.99–13.30] versus 0.01 [0–0.01] ng/mg Cr, $P<0.001$, respectively). However, the levels of urinary C3a in patients with AAV in remission were still significantly higher than levels of normal controls (0.02 [0.01–0.05] versus 0.01 [0–0.01] ng/mg Cr, $P<0.001$) (Figure 2B).

The urinary C5a levels were significantly higher in patients with AAV in active stage compared with AAV patients in remission and normal controls (1.93 [0.67–9.01] versus 0.09 [0.01–0.20] ng/mg Cr, $P<0.001$ and 1.93 [0.67–9.01] versus 0.03 [0.01–0.05] ng/mg Cr, $P<0.001$, respectively). There was no significant difference in urinary C5a levels between patients with active AAV and patients with lupus nephritis. No significant difference was found in urinary levels of C5a between AAV patients in remission

Table 2. Clinical and histopathological data at initial onset of 22 patients with ANCA-associated vasculitis whose urine samples were collected in remission stage

Parameters	Number
Number	22
Men/women	8/14
Average age (yr)	62.5±14.9
Scr (mg/dl)	
Median (IQR)	1.8 (1.0–2.6)
Range	0.6–9.8
eGFR (ml/min per 1.73 m^2)	
Median (IQR)	41.9 (24.3–77.1)
Range	5.7–120.3
ESR (mm/h)	61.2±40.4
ANCA level (RU/ml)	121.9±72.3
eGFR<60 ml/min per 1.73 m^2 at diagnosis	13 (59.1%)
Urinary protein (g/24 h)	
Median (IQR)	0.8 (0.3–1.6)
Range	0–3.2
BVAS	17.0±4.8
Average glomeruli per biopsy	26.7±12.8
Glomerular lesions (%)	
Total crescents	45.0±30.2
Cellular crescents	38.8±28.0
Fibrous crescents median (IQR)	0 (0–3.6)
Tubulointerstitial lesions	
Interstitial infiltration (−/+/++/+++)	1/3/9/9
Interstitial fibrosis (−/+/++)	3/8/11
Tubular atrophy (−/+/++)	1/9/12

Scr, serum creatinine; IQR, interquartile range; eGFR, estimated GFR; ESR, erythrocyte sedimentation rate; BVAS, Birmingham Vasculitis Activity Scores.

and normal controls (0.09 [0.01–0.20] versus 0.03 [0.01–0.05] ng/mg Cr, P=0.06) (Figure 2C).

The urinary sC5b-9 levels were significantly higher in AAV patients in active stage compared with AAV patients in remission and normal controls (52.73 [3.46–142.44] versus 0.20 [0–2.89] ng/mg Cr, P<0.001 and 52.73 [3.46–142.44] versus 0.56 [0–1.14] ng/mg Cr, P<0.001, respectively). There was no significant difference in urinary sC5b-9 levels between patients with active AAV and patients with lupus nephritis. No significant difference was found in urinary levels of sC5b-9 between AAV patients in remission and normal controls (0.20 [0–2.89] versus 0.56 [0–1.14] ng/mg Cr, P=0.82) (Figure 2D).

The results of the urinary C1q levels and MBL levels are detailed in Figure 2, E and F and Supplemental Material.

After adjusting for urinary protein excretion, all the results of the comparison above were consistent with the results before adjusting for urinary protein excretion, except for the urinary C1q and MBL levels, which were significantly higher in patients with active AAV compared with patients with lupus nephritis (P=0.002 and P<0.001, respectively).

Association between Renal Deposition of Complement Activation Products and Clinicopathologic Parameters of Patients with Active AAV

The mean optical density of Bb in glomeruli correlated with the proportion of total crescents, extent of interstitial infiltrate, interstitial fibrosis, and tubular atrophy in renal specimens (r=0.50, P=0.006; r=0.59, P=0.001; r=0.45, P=0.01; r=0.55, P=0.002, respectively). Moreover, the mean optical density of Bb in glomeruli correlated inversely with the proportion of normal glomeruli (r=−0.49, P=0.008) (Figure 3). The mean optical density of C5b-9 in glomeruli correlated with the proteinuria level of 24 hours (r=0.63, P<0.001).

Association between Urinary Levels of Complement Activation Products and Clinicopathologic Parameters of Patients with Active AAV

The levels of urinary complement activation products for correlation analysis were normalized for urinary creatinine. The urinary levels of Bb correlated with the urinary levels of C3a (r=0.72, P<0.001). The urinary levels of Bb correlated with serum creatinine and the proportion of total crescents in renal specimens (r=0.56, P=0.002; r=0.40, P=0.04, respectively). The urinary levels of Bb correlated inversely with the proportion of normal glomeruli in renal specimens (r=−0.49, P=0.009). However, no correlation was found between the levels of urinary complement activation products and the mean optical density of complement activation products in glomeruli. No significant correlation was found between the levels of complement activation products in urine and plasma (data not show).

Discussion

Recent studies in animal models provided strong evidence that the complement system activation, particularly through the alternative pathway, is involved in the pathogenesis of AAV (6–8). Our previous study in renal histology revealed that complement activation products of the alternative pathway could be detected in human ANCA-associated GN (14). To further investigate the importance of the complement system activation through the alternative pathway in the development of human ANCA-associated GN, we analyzed the association between levels of various complement activation products in both urine and renal specimens of AAV patients and clinicopathologic parameters of AAV patients.

In the present study, the evidence of Bb, C3d, and C5b-9 depositions in glomeruli further confirmed that activation of the alternative complement pathway occurred in patients with AAV. The mean optical density of Bb in glomeruli correlated with the proportion of total crescents and correlated inversely with the proportion of normal glomeruli, which was found to be the histologic parameter with the best predictive value for the renal function (21). In addition, the mean optical density of Bb in glomeruli correlated closely with the extent of interstitial infiltrate, interstitial fibrosis, and tubular atrophy. All of these results indicated that the glomerular deposition of Bb, which is unique in activation of the alternative pathway, could reflect the severity of renal histopathological lesion in AAV.

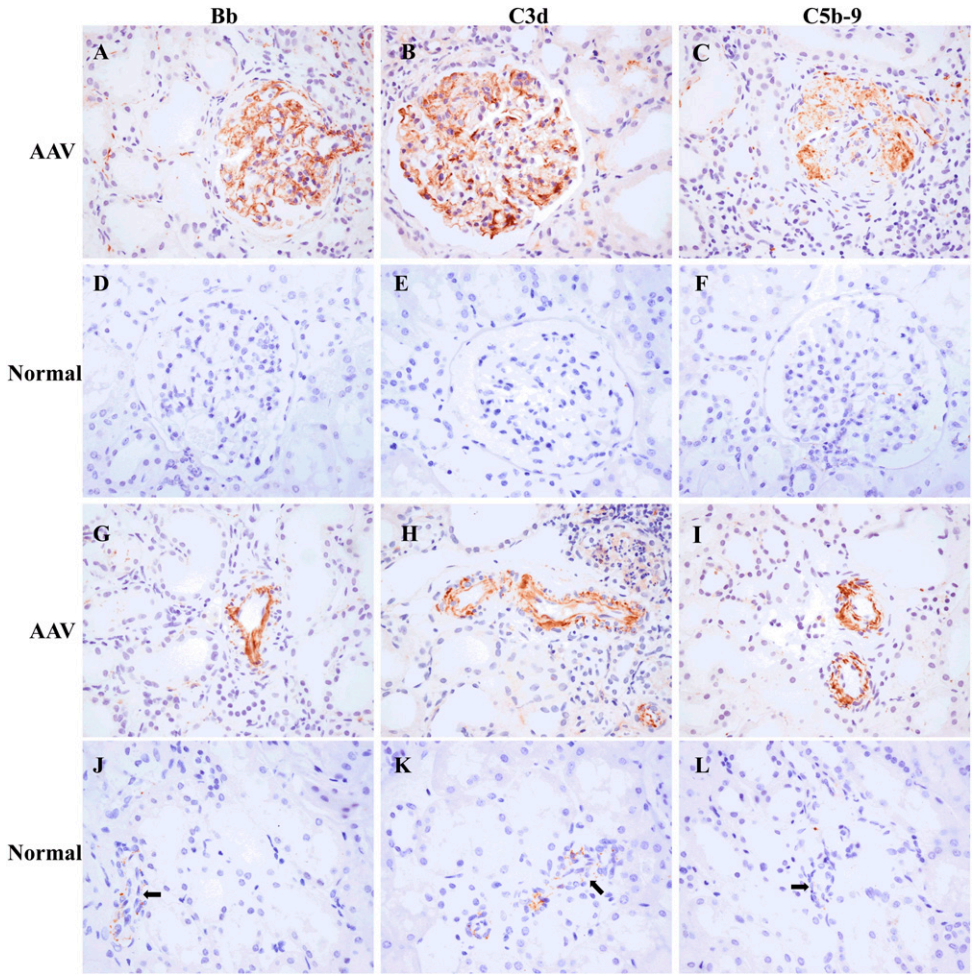

Figure 1. | Immunohistochemistry staining for complement activation products Bb, C3d, and C5b-9 in renal specimens of patients with ANCA-associated vasculitis (AAV) and normal controls. (A–C) Immunohistochemical staining of Bb, C3d, and C5b-9 in glomerulus of AAV. (D–F) Immunohistochemical staining of Bb, C3d, and C5b-9 in glomerulus of normal control. (G–I) Immunohistochemical staining of Bb, C3d, and C5b-9 in small vessel wall of AAV. (J–L) Immunohistochemical staining of Bb, C3d, and C5b-9 in small vessel wall of normal control (arrows indicating the small vessel).

In the present study, urinary Bb levels were significantly higher in patients with active AAV compared with patients in remission and normal controls. Furthermore, urinary Bb levels correlated with serum creatinine and correlated inversely with the proportion of normal glomeruli, which indicated that urinary Bb levels could also reflect the severity of renal injury. These results, together with the results of Bb deposition in renal histology described above, were consistent and further extend our previous finding that circulating levels of Bb closely correlated with the systemic disease activity of AAV (15). They also highlight the importance of complement activation through the alternative pathway in the pathogenesis of AAV.

In the present study, the urinary levels of Bb, C3a, C5a, and sC5b-9, after adjustment for urinary creatinine, were all significantly higher in AAV patients with active AAV than patients in remission and normal controls. There were no differences in urinary levels of Bb, C5a, and sC5b-9 between patients with AAV in remission and normal controls, but the urinary levels of C3a were still significantly higher in patients with AAV in remission than normal controls. The differences in these complement levels in urine between active stage and remission were quite similar to the differences of the circulating complement levels, which were described in our previous study (15). This finding indicated that the complement activation products excretion from the kidney, one of the most vulnerable organs in AAV, was, in part, a reflection of systemic activation of complements.

In the present study, the urinary levels of C1q and MBL in patients with active AAV were significantly higher than urinary levels in normal controls. This result indicated that the classic and lectin pathways might be also activated in AAV. However, the urinary levels of C1q and MBL in AAV patients in remission stage were still significantly higher than those levels in normal controls, which suggested that activation of the classic and lectin pathways might be not pathogenic in AAV. The complement components in the classic and lectin pathways were found to have functions for clearing apoptotic and necrotic cells (25,26). Both MBL

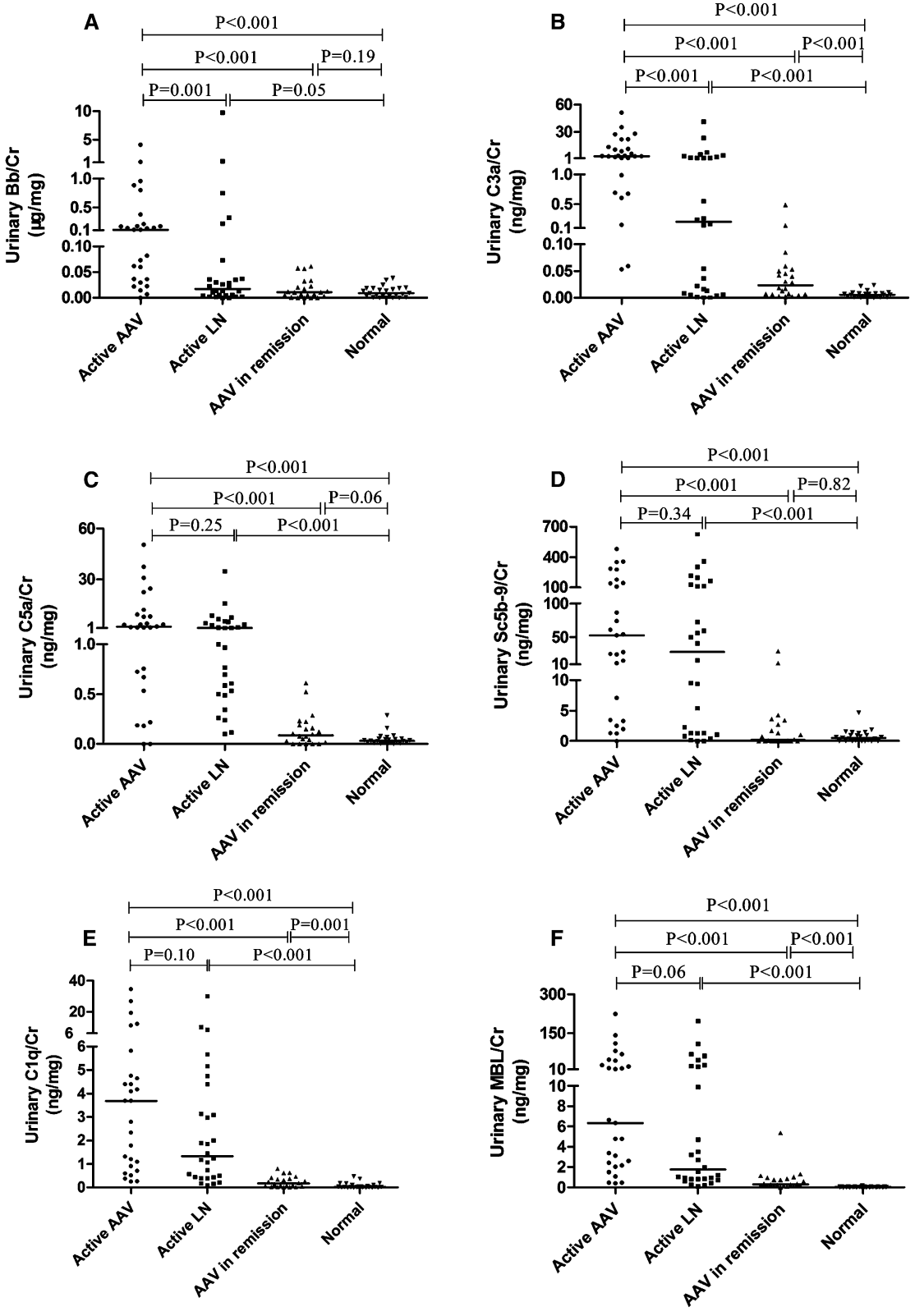

Figure 2. | Urinary levels of complement components in patients with active AAV, active lupus nephritis (LN), and remission of AAV and normal controls. Horizontal solid lines indicated the median level. (A) Urinary levels of Bb. (B) Urinary levels of C3a. (C) Urinary levels of C5a. (D) Urinary levels of soluble C5b-9 (sC5b-9). (E) Urinary levels of C1q. (F) Urinary levels of mannose-binding lectin (MBL). Cr, creatinine.

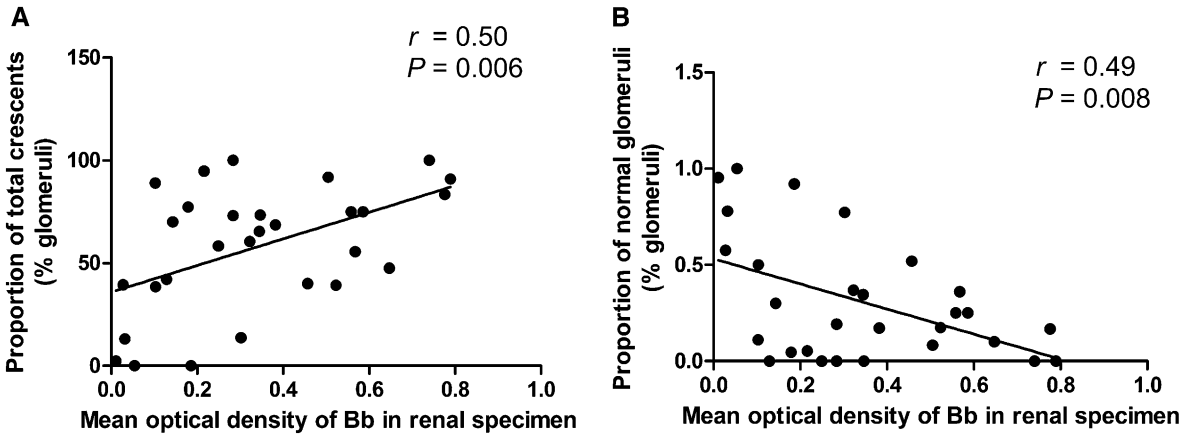

Figure 3. | Association between glomerular deposition of Bb and the severity of renal injury of patients with AAV. (A) Association between the mean optical density of Bb in glomeruli and the proportion of total crescents in renal specimens. (B) Association between the mean optical density of Bb in glomeruli and the proportion of normal glomeruli in renal specimens.

and C1q could bind to apoptotic cells and initiate their uptake into macrophages. We speculated that the elevated urinary levels of C1q and MBL in both active stage and remission might account for clearing apoptotic or necrotic cells in the chronic disease process of AAV, but this result remains additional confirmation.

The urinary complement activation products might come from two sources (27,28). First, they might derive from the circulation because of damaged glomerular basement membrane. Second, complement cascades might be activated locally in kidneys, and then, the complement activation products are, therefore, released into the urine. In addition, the activation of complement cascades in kidney might occur in not only glomeruli but also the lumen of tubules. Our results could not clarify the origin of complement activation products in urine, which was a limitation of the current study.

In conclusion, the present study provides additional evidence that complement activation through the alternative pathway occurred in the development of AAV. The renal deposition of alternative complement pathway activation product Bb and urinary Bb levels were associated with the severity of ANCA-associated GN.

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Disclosures

None.

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S.-J.G. and J.Y. contributed equally to this work.

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