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Original article

Complement activation in patients with diabetic nephropathy

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ABSTRACT

Aim. – Emerging evidence has indicated a role of the complement system in the pathogenesis of diabetic nephropathy (DN), although the pathways of complement activation and their clinicopathological relevance in DN are as yet unclear. The present study aimed to investigate levels of various complement components in plasma and urine of DN patients, and their correlation with clinicopathological parameters.

Methods. – A total of 68 biopsy-proven DN patients with plasma samples were recruited, including 50 patients who also had urine samples available. Seven complement components (C1q, MBL, Bb, C4d, C3a, C5a, soluble C5b-9) were measured by enzyme-linked immunosorbent assay (Elisa), and any associations between their levels and clinicopathological parameters were then investigated.

Results. – In DN patients, plasma levels of C1q, MBL, Bb, C4d, C3a, C5a and sC5b-9 were significantly higher than in diabetes patients without renal involvement, as were also urinary levels except for C1q, which showed no significant differences between the two groups. Also, urinary levels of C3a and C5a were significantly correlated with serum creatinine, urinary protein and estimated glomerular filtration rate, whereas urinary sC5b-9 was significantly correlated with the latter two (and not serum creatinine). In addition, urinary levels of MBL, Bb and C4d were significantly correlated with urinary protein, while C3a, C4d and Bb significantly correlated with the classification of glomerular lesions in DN.

Conclusion. – In DN patients, the complement system is activated and, of the three possible complement pathways, activation of the lectin and alternative pathways is associated with renal damage.

Introduction

Diabetic nephropathy (DN) is among the most common and serious consequences of diabetes mellitus (DM). In 2015, 415 million people were estimated to have DM and this number is expected to rise to 642 million by 2040 [1]. At present, DN is the leading cause of chronic kidney disease and end-stage renal disease (ESRD) around the world, adding a heavy burden to the economy [2,3]. In recent years, immune-mediated inflammation has been recognized as an important factor in the pathogenesis of DN [4,5]. In particular, emerging evidence has indicated a role of the complement system, a major component of innate immunity, in DN development [6,7].

The complement system is typically activated by three different pathways: the classic pathway; the lectin pathway; and the alternative pathway. Activation of all three leads to formation of the membrane attack complex (MAC), which contributes to elimination of foreign cells through lytic and sublytic effects [8]. In addition, the complement system has extensive effects on inflammation, apoptotic cell clearance and T-cell immunity regulation, complementing humoral immunity and interactions with Toll-like receptors or the coagulation system [9]. However, overactivation of complement is detrimental.

Previous studies of the complement system in the development of DN mostly focused on the lectin pathway. In clinical studies, circulating levels of mannose-binding lectin (MBL) were higher in normoalbuminuric patients with DM compared with healthy controls [10,11]. In addition, baseline MBL levels could predict the incidence of albuminuria and progression from macroalbuminuria to ESRD in type 1 DM (T1DM) patients [12,13]. In streptozotocin-induced T1DM mice, knockout of MBL attenuated urinary albumin excretion and collagen IV expression, indicating a causal relationship between MBL and DN [14]. Some studies have highlighted the role of downstream complement components, such as C3, C4 and MAC, in the pathogenesis of DN [15–19]. There are also clues to

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suggest the potential significance of the classic and alternative pathways in the pathogenesis of DN at the transcriptional level [20]. However, these pathways of complement activation and their clinicopathological relevance in DN are as yet still unclear. Therefore, the present study measured levels of various complement components in both plasma and urine samples from DN patients to assess their correlation with clinicopathological parameters.

Methods

Patients and samples

A total of 68 patients with renal-biopsy-confirmed DN from the Department of Nephrology, Peking University First Hospital, were enrolled in our study from February 2004 to May 2017 (Fig. 1). The diagnosis of DM was according to criteria of the American Diabetes Association (ADA) [21], whereas the diagnosis of DN was verified by its characteristic pathological changes, such as glomerular hypertrophy, thickened capillary basement membranes and nodular mesangial sclerosis. Patients with other concurrent renal diseases, such as membranous nephropathy, IgA nephropathy and minimal change disease (MCD), were excluded.

Blood and urine samples were collected on the day of renal biopsy. Blood samples for complement measurement were drawn into ethylenediaminetetraacetic acid (EDTA) tubes, immediately put on ice, and then centrifuged at 3000 × g for 10 min at 4 °C and stored in aliquots at −80 °C until needed. Of our 68 DN patients, urine samples from 50 patients were also available. These were collected and underwent the same procedures as for blood samples. Also, 42 plasma samples and 30 urine samples were collected from DM patients with no renal involvement, as were 31 plasma samples and 19 urine samples from MCD patients, and 30 plasma and urine samples from healthy donors as disease controls. DM patients without renal involvement were identified by urinary albumin/creatinine ratios (uACR) < 30 mg/g and estimated glomerular filtration rates (eGFR) > 60 mL/min/1.73 m^2.

This research was performed in accordance to the Declaration of Helsinki and approved by the ethics committees of Peking University First Hospital. Informed written consent was also obtained from all participants.

Quantification of complement components

To evaluate the degree of complement activation in DN patients, seven complement components – C1q, MBL, Bb, C4d, C3a, C5a and soluble C5b-9 (sC5b-9) – were measured by enzyme-linked immunosorbent assay (Elisa) in plasma and urine. Of these components, Bb, C4d, C3a, C5a and sC5b-9 were measured using commercial Elisa kits from the Quidel Corporation (San Diego, CA, USA) while, to measure levels of MBL, commercial Elisa kits from BioPorto Diagnostics (Hellerup, Denmark) were used. These procedures were all carried out strictly according to manufacturers' instructions. Repeated freeze–thaw cycles were avoided: samples were thawed rapidly at 37 °C, then kept on ice before testing to ensure no additional complement activation.

Detection of C1q was according to our own group's method, as previously described elsewhere [22]. In brief, 50 μL of rabbit anti-human C1q polyclonal antibody (Agilent Technologies, Inc., Santa

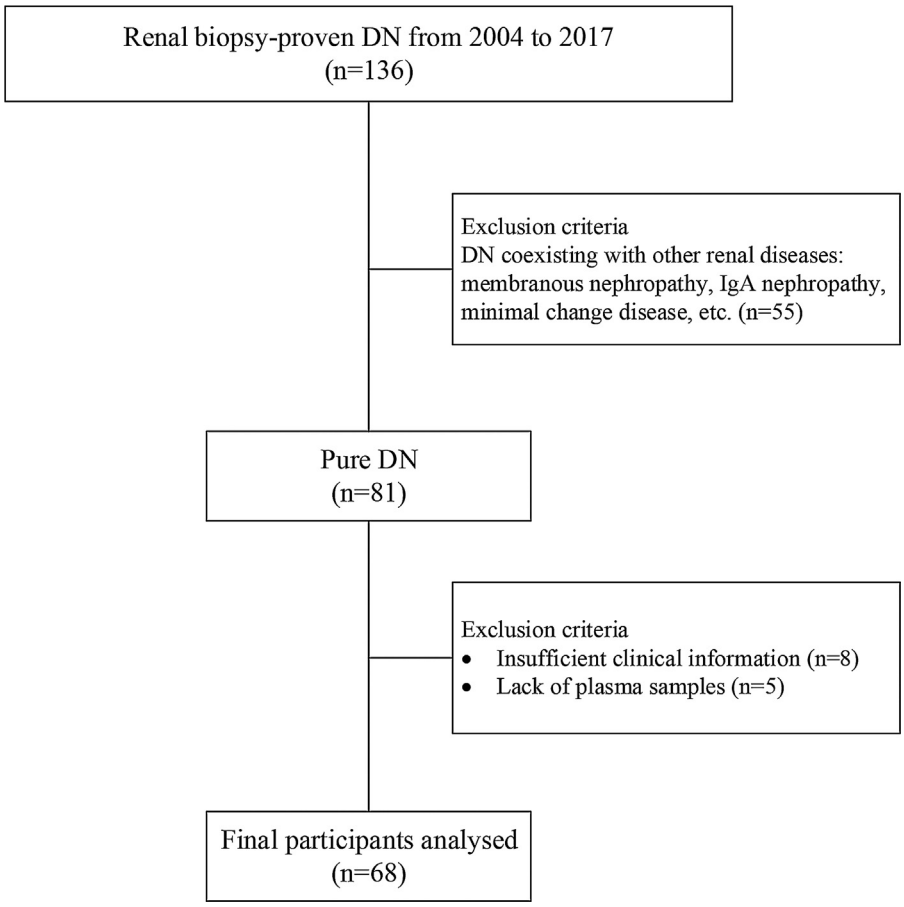

Fig. 1. Flow chart of study participant recruitment.

Clara, CA, USA), diluted to 1:5000 in 0.05 M bicarbonate buffer (pH 9.6), was coated onto the wells of Nunc polystyrene microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA) and left overnight at 4 °C. The following was then carried out at 37 °C, taking 1 h per step: blocking with 200 μL of phosphate buffered saline supplemented with Tween 20 (PBST) containing 1% bovine serum albumin (BSA); incubation of 50 μL of each sample with double-diluted standards; and incubation of horseradish peroxidase (HRP)-conjugated goat anti-human IgG monoclonal antibody (Abcam plc, Cambridge, UK) diluted in PBST.

Renal histology

After renal biopsy, specimens were routinely processed for light microscopy, immunofluorescence and electron microscopy. Patients' pathological classifications and histological scores were evaluated according to the 2010 criteria of the Renal Pathology Society[23]. In brief, renal pathological findings were classified as: glomerular lesions (I, IIa, IIb, III, IV); interstitial fibrosis and tubular atrophy (IFTA) (0, 1, 2, 3); interstitial inflammation (0, 1, 2); arteriolar hyalinosis (0, 1, 2); and arteriosclerosis (0, 1, 2).

Statistical analysis

Mean ± standard deviation (SD) was used to describe normally distributed quantitative data, with median and interquartile range (IQR) used for non-normally distributed quantitative data. Multiple groups were compared using one-way analysis of variance (ANOVA) for normally distributed data, and Kruskal-Wallis analysis for non-normally distributed data with Bonferroni correction, which means that corrected *P*-values are presented. The Bonferroni correction was introduced to decrease the chances of making type I errors, caused by multiple comparisons, by adjusting levels of significance. For convenience of demonstration, corrected *P*-values were used to show results calculated by the original *P*-value multiplied by the number of comparisons between groups. Urinary levels of complement components were normalized for urinary creatinine to correct for the influence of dilution. To adjust for the confounding effect of urinary protein, a covariance analysis model was used: urinary levels of complement were taken as a logarithmic transformation based on 2 to normalize the data, and zero was substituted by the minimum value decreased by one order of magnitude. Possible correlation between two numerical parameters was assessed by Pearson's or Spearman's test as appropriate. Correlation between ordinal categorical variables and numerical parameters was analyzed by Spearman's test. The difference was considered significant at $P < 0.05$. All analyses were done using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

DN patients' general clinical data

Of the 68 patients with DN, 53 were male and 15 were female, and aged 47.49 ± 11.27 (range 23–71) years at the time of renal biopsy. Other general clinical data for these patients are summarized in Table 1.

Table 1
General clinical data for patients with diabetic nephropathy.

Parameter	Values
Patients, *n*	68
Gender, male/female	53/15
Age, years	47.49 ± 11.27
Known duration of diabetes, years	10.02 ± 6.05
HbA_{1c}, %	6.76 ± 1.56
Fasting plasma glucose, mmol/L	5.77 (4.86, 7.44)
Serum albumin, g/L	30.85 ± 6.10
Triglycerides, mmol/L	1.80 (1.36, 3.09)
Cholesterol, mmol/L	
Total	5.27 (4.12, 6.57)
Low-density lipoprotein	3.10 ± 1.64
High-density lipoprotein	1.04 ± 0.30
Serum creatinine, μmol/L	143.85 (112.00, 229.90)
Urinary protein, g/24 h	6.33 ± 4.35
eGFR, mL/min/1.73 m^2	44.28 ± 26.79
Classification of glomerular lesions, *n* (%)	
I	6 (8.82)
IIa	11 (16.18)
IIb	18 (26.47)
III	26 (38.24)
IV	7 (10.29)

Data are presented as means ± SD or as medians (IQR); eGFR: estimated glomerular filtration rate.

Levels of complement components in plasma

Compared with healthy controls, plasma levels of C1q, MBL, Bb, C4d, C3a, C5a and sC5b-9 in DM patients without renal involvement were not significantly different. In contrast, in patients with DN, plasma levels of these seven complement components were significantly higher than in DM patients with no renal involvement (86.54 ± 24.62 vs 72.64 ± 17.74 μg/mL, corrected $P = 0.01$; 4382.5 ± 3361.79 vs 2276.05 ± 1640.94 ng/mL, corrected $P = 0.04$; 1.00 ± 0.52 vs 0.59 ± 0.13 μg/mL, corrected $P < 0.001$; 12.97 (8.93, 21.67) vs 1.93 (1.08, 2.75) μg/mL, corrected $P < 0.001$; 163.01 (88.16, 644.85) vs 55.07 (44.52, 85.37) ng/mL, corrected $P < 0.001$; 9.27 (3.49, 15.63) vs 5.11 (2.97, 7.13) ng/mL, corrected $P = 0.006$; and 238.73 ± 112.34 vs 132.45 ± 32.09 ng/mL, corrected $P < 0.001$, respectively).

Compared with MCD patients, the C4d level was significantly higher in DN patients [12.97 (8.93, 21.67) vs 0.96 (0.50, 1.57) μg/mL, corrected $P < 0.001$], whereas there were no significant differences in levels of C1q, MBL, Bb, C3a, C5a and sC5b-9 between these two groups of patients (Fig. 2). The main results were summarized in Table 2.

Levels of complement components in urine

Urinary levels of complement components were normalized for urinary creatinine to correct for the influence of dilution. Unlike plasma levels, urinary levels of MBL, Bb, C3a, C5a and sC5b-9 in DM patients without renal involvement were significantly higher than in healthy controls [0.12 (0.05, 0.21) vs 0 (0, 0) ng/mg, corrected $P < 0.001$; 0.05 (0.03, 0.07) vs 0 (0, 0) μg/mg, corrected $P < 0.001$; 0.02 (0.01, 0.03) vs 0 (0, 0) ng/mg, corrected $P < 0.001$; 0.05 (0.03, 0.08) vs 0 (0, 0.01) ng/mg, corrected $P < 0.001$; and 6.15 (4.97, 11.58) vs 0 (0, 0.07) ng/mg, corrected $P < 0.001$, respectively]. However, there were no significant differences in urinary levels of C1q or C4d between DM patients with no renal involvement and healthy controls.

In patients with DN, urinary levels of MBL, Bb, C4d, C3a, C5a and sC5b-9 were all significantly higher than in DM patients without renal involvement [1.7 (0.49, 5.97) vs 0.12 (0.05, 0.21) ng/mg, corrected $P < 0.001$; 0.30 (0.14, 0.98) vs 0.05 (0.03, 0.07) μg/mg, corrected $P < 0.001$; 0.17 (0.03, 1.11) vs 0 (0, 0) μg/mg, corrected $P < 0.001$; 10.14 (1.98, 45.5) vs 0.02 (0.01, 0.03) ng/mg, corrected $P < 0.001$; 10.22 (4.66, 27.59) vs 0.05 (0.03, 0.08) ng/mg, corrected $P < 0.001$; and 79.56 (22.84, 288.07) vs 6.15 (4.97, 11.58) ng/mg, corrected $P < 0.001$, respectively]. However, there was no significant difference in urinary C1q levels between DN patients and DM patients with no renal involvement.

Compared with MCD, urinary levels of C1q, MBL, Bb, C4d, C3a, C5a and sC5b-9 were all significantly higher in patients with DN

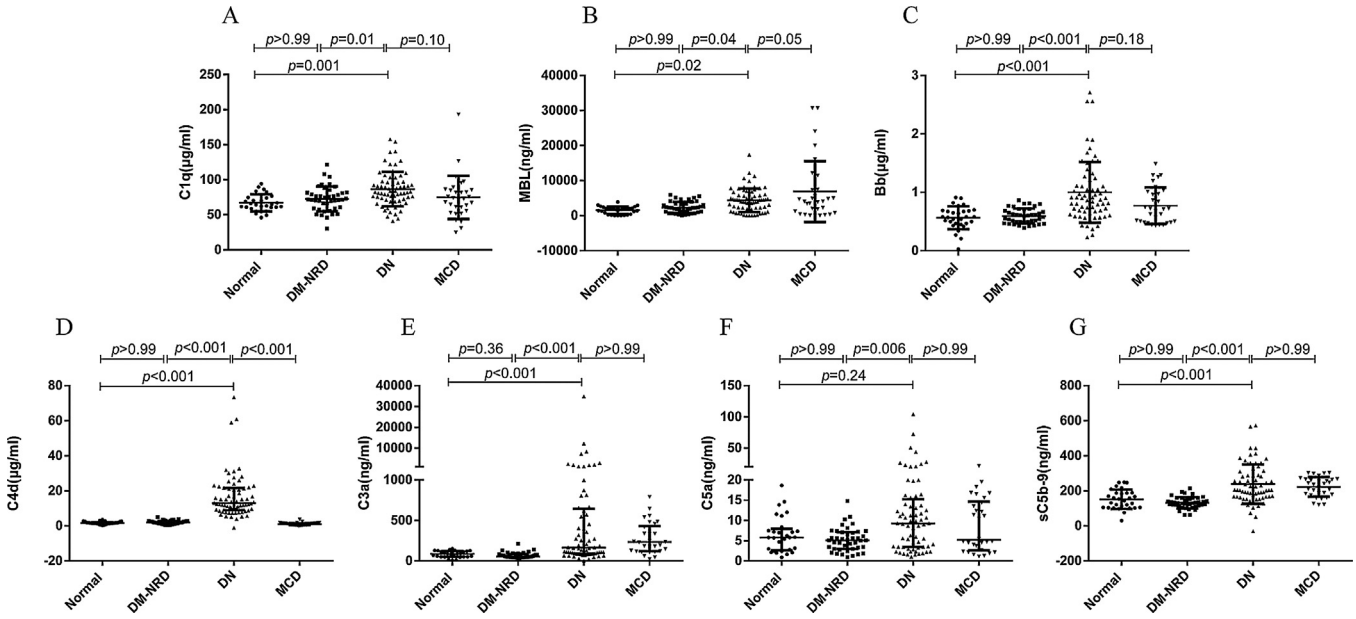

Fig. 2. Plasma levels of complement components in patients with diabetic nephropathy (DN): (A) C1q; (B) MBL (mannose-binding lectin); (C) Bb; (D) C4d; (E) C3a; (F) C5a; and (G) sC5b-9 (soluble C5b-9). *P*-values with Bonferroni correction. DM: diabetes mellitus; MCD: minimal change disease; NRD: non-renal disease.

Table 2
Levels of complement components in plasma.

	Normal	DM-NRD	DN	MCD
C1q (μg/mL)	67.1 ± 12.1	72.64 ± 17.74	86.54 ± 24.62[a,b]	74.65 ± 30.85
MBL (ng/mL)	1542.83 ± 1029.89	2276.05 ± 1640.94	4382.5 ± 3361.79[a,b]	6889.56 ± 8666.72
Bb (μg/mL)	0.56 ± 0.2	0.59 ± 0.13	1.00 ± 0.52[a,b]	0.77 ± 0.31
C4d (μg/mL)	1.6 (1.24, 2.12)	1.93 (1.08, 2.75)	12.97 (8.93, 21.67)[a,b,c]	0.96 (0.50, 1.57)
C3a (ng/mL)	86.92 (51.23, 121.58)	55.07 (44.52, 85.37)	163.01 (88.16, 644.85)[a,b]	233.16 (120.71, 429.07)
C5a (ng/mL)	5.86 (2.44, 11.11)	5.11 (2.97, 7.13)	9.27 (3.49, 15.63)[b]	5.16 (2.6, 14.65)
sC5b-9 (ng/mL)	154.89 ± 55.6	132.45 ± 32.09	238.73 ± 112.34[a,b]	223.05 ± 55.72

Data are presented as means ± SD or as medians (IQR); DM: diabetes mellitus; DN: diabetic nephropathy; MBL: mannose-binding lectin; MCD: minimal change disease; NRD: non-renal disease; sC5b-9: soluble C5b-9.
[a] $P < 0.05$ vs normal control after Bonferroni correction.
[b] $P < 0.05$ vs DM-NRD after Bonferroni correction.
[c] $P < 0.05$ vs MCD after Bonferroni correction.

[1.07 (0.28, 4.78) vs 0 (0, 0.34) ng/mg, corrected $P = 0.006$; 1.7 (0.49, 5.97) vs 0 (0, 0) ng/mg, corrected $P < 0.001$; 0.30 (0.144, 0.98) vs 0.01 (0.01, 0.03) μg/mg, corrected $P < 0.001$; 0.17 (0.03, 1.11) vs 0.01 (0.01, 0.03) μg/mg, corrected $P < 0.001$; 10.14 (1.98, 45.5) vs 0 (0, 0.04) ng/mg, corrected $P < 0.001$; 10.22 (4.66, 27.59) vs 0.05 (0.01, 0.17) ng/mg, corrected $P < 0.001$; and 79.56 (22.84, 288.07) vs 0.07 (0, 3.62) ng/mg, corrected $P < 0.001$, respectively] (Fig. 3). After adjusting for urinary protein, differences between the two groups remained significant except for urinary levels of C1q and C4d. The main results were summarized in Table 3.

Associations between complement components and clinicopathological parameters

In patients with DN, urinary levels of C3a significantly correlated with serum creatinine (sCr), urinary protein and eGFR ($r = 0.369$, $P = 0.009$; $r = 0.562$, $P < 0.001$; and $r = -0.448$, $P < 0.001$, respectively). Likewise, urinary C5a levels also correlated significantly with sCr, urinary protein and eGFR ($r = 0.387$, $P = 0.005$; $r = 0.600$, $P < 0.001$; and $r = -0.437$, $P = 0.002$, respectively), while urinary sC5b-9 significantly correlated with urinary protein and eGFR ($r = 0.619$, $P < 0.001$; and $r = -0.310$, $P = 0.03$, respectively). In addition, urinary levels of C4d, MBL and Bb significantly correlated with urinary protein levels ($r = 0.487$, $P = 0.001$; $r = 0.518$, $P < 0.001$; and $r = 0.565$, $P < 0.001$, respectively).

Regarding pathological parameters, urinary levels of C3a, C4d and Bb significantly correlated with classifications of glomerular DN lesions ($r = 0.322$, $P = 0.027$; $r = 0.305$, $P = 0.037$; and $r = 0.431$, $P = 0.002$, respectively). Of the 50 DN patients with urine samples, six were classified as I, seven as IIa, 14 as IIb, 18 as III and five as IV. However, there was no significant correlation between urinary levels of complement components and tubulointerstitial or vascular lesions.

It was observed that, in some DN cases, complement concentrations in urine were below the limit of detection. More specifically, of our DN patients, 10 had undetectable urinary concentrations of C1q, three of urinary MBL, five of urinary C4d, three of urinary C3a, five of urinary C5a, three of urinary sC5b-9 and none of urinary Bb. DN patients were then divided into two groups based on whether or not they had such detectable urinary concentrations (except Bb), and their clinical parameters were compared (Table S1; see supplementary materials associated with this article online). To further elucidate this issue, correlation analyses were performed for the subgroup of DN patients with detectable urinary levels of complement components (Table S2; see supplementary materials associated with this article online). The results were generally in line with those for the entire cohort of

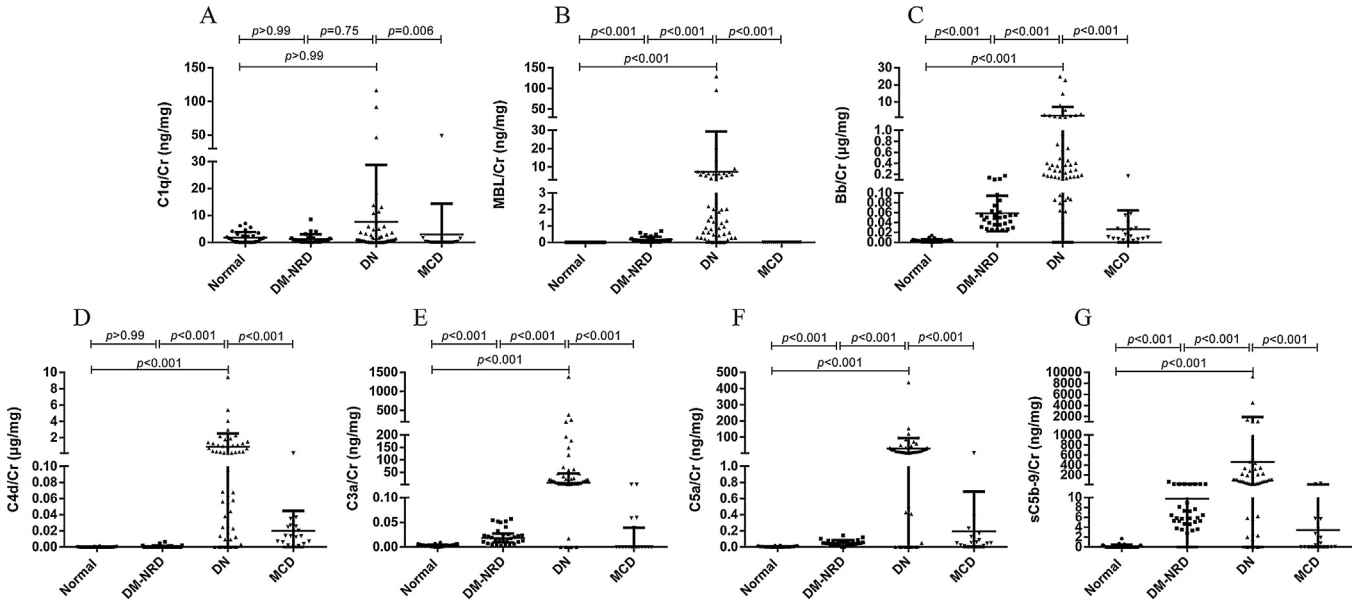

Fig. 3. Urinary levels of complement components in patients with diabetic nephropathy (DN): (A) C1q; (B) MBL (mannose-binding lectin); (C) Bb; (D) C4d; (E) C3a; (F) C5a; and (G) sC5b-9 (soluble C5b-9). *P*-values with Bonferroni correction. DM: diabetes mellitus; MCD: minimal change disease; NRD: non-renal disease.

Table 3
Levels of complement components in urine.

	Normal	DM-NRD	DN	MCD
C1q (ng/mg)	1.11 (0.04, 2.79)	0.63 (0.4, 1.57)	1.07 (0.28, 4.78)	0 (0, 0.34)
MBL (ng/mg)	0 (0, 0)	0.12 (0.05, 0.21)[a]	1.7 (0.49, 5.97)[a,b,c]	0 (0, 0)
Bb (μg/mg)	0 (0, 0)	0.05 (0.03, 0.07)[a]	0.30 (0.14, 0.98)[a,b,c]	0.01 (0.01, 0.03)
C4d (μg/mg)	0 (0, 0)	0 (0, 0)	0.17 (0.03, 1.11)[a,b,c]	0.01 (0.01, 0.03)
C3a (ng/mg)	0 (0, 0)	0.02 (0.01, 0.03)[a]	10.14 (1.98, 45.5)[a,b,c]	0 (0, 0.04)
C5a (ng/mg)	0 (0, 0.01)	0.05 (0.03, 0.08)[a]	10.22 (4.66, 27.59)[a,b,c]	0.05 (0.01, 0.17)
sC5b-9 (ng/mg)	0 (0, 0.07)	6.15 (4.97, 11.58)[a]	79.56 (22.84, 288.07)[a,b,c]	0.07 (0, 3.62)

Data are presented as means ± SD or as medians (IQR); DM: diabetes mellitus; DN: diabetic nephropathy; MBL: mannose-binding lectin; MCD: minimal change disease; NRD: non-renal disease; sC5b-9: soluble C5b-9.
[a] $P < 0.05$ vs normal control after Bonferroni correction.
[b] $P < 0.05$ vs DM-NRD after Bonferroni correction.
[c] $P < 0.05$ vs MCD after Bonferroni correction.

DN patients. However, there was no significant correlation between plasma levels of complement components and clinicopathological parameters.

Discussion

The present study measured seven complement components (C1q, MBL, Bb, C4d, C3a, C5a, sC5b-9) in plasma and urine to determine the status of complement activation in DN patients. Plasma levels of all of them in DN patients were significantly higher than in DM patients without renal involvement, indicating that all three-complement pathways were activated in the circulation of DN patients. Urinary levels of complement components showed similar results, with levels of MBL, Bb, C4d, C3a, C5a and sC5b-9 all significantly higher than in DM patients without renal involvement, whereas levels of C1q did not differ between these two patient groups.

To further investigate the potential role of complement activation in DN, the association of complement component levels and clinicopathological parameters were also investigated. In previous studies, C1q was reported to bind to autologous protein that had undergone abnormal changes, for instance, advanced glycation end-products [24] and oxidized low-density lipoproteins [25], both of which are relevant to DN [26,27]. However, there was no significant correlation between C1q levels and clinicopathological parameters, indicating that the classic pathway may not be playing a pathogenic role in DN. Except for C1q, urinary levels of MBL, Bb, C4d, C3a, C5a and sC5b-9 correlated well with urinary protein levels. Proteinuria is a well-known marker of disease severity and predictor of ESRD in DN [28–30], thereby suggesting that urinary levels of MBL, Bb, C4d, C3a, C5a and sC5b-9 could reflect severity of renal damage in DN. Moreover, urinary levels of C3a, C4d and Bb significantly correlated with classification of glomerular DN lesions. Taken together, this might indicate the pathogenic role of the lectin and alternative pathways in DN. It is also noteworthy that, among the urinary levels of MBL, Bb, C4d, C3a, C5a and sC5b-9, C3a and C5a also correlated with eGFR and sCr, while sC5b-9 correlated with eGFR. This might be highlighting the relatively more important role of C3a, C5a and sC5b-9 in the development of DN. In fact, Li et al. [31,32] found that both C3a and C5a receptor antagonists improved renal function in T2DM rats.

Complement proteins in urine may be derived in two ways: (i) injured glomeruli could leak complement proteins together with other types of serum proteins into the urinary space; and (ii) complement proteins could pass through the glomerular barrier along with other serum proteins and further activate tubular epithelial cells to overexpress complement components [33]. Therefore, proteinuria nephropathy is often accompanied by complement activation. Thus, to eliminate the influence of proteinuria on complement activation as much as possible, a

covariance analysis model was used to compare urinary levels between DN and MCD patients: urinary levels of all seven complement components were significantly higher in DN than in MCD patients. After adjusting for urinary protein, differences between these two groups remained significant except for urinary levels of C1q and C4d, indicating a role of complement activation in the development of DN.

However, it was totally unexpected that levels of complement components in plasma were not significantly correlated with clinicopathological parameters, whereas urinary levels were well correlated. To some extent, this may reflect the importance of locally produced complement components in DN. Except for circulatory complement components in glomerular capillaries, renal resident cells, such as glomerular mesangial, glomerular epithelial and tubular epithelial cells, have long been known to synthesize complement components locally [34–36], and cytokines such as interferon (IFN)-γ and interleukin (IL)-2 are known mediators of locally synthesized complement. In DN, inflammatory cytokines, mainly IL-1, IL-6 and IL-18, as well as tumor necrosis factor (TNF)-α, are involved in its development and progression [37]. Thus, it is reasonable to speculate that these cytokines might also mediate local biosynthesis of complements in the disease.

In conclusion, the complement system is activated in DN patients and, of the three complement pathways, activation of the lectin and alternative pathways is associated with renal damage.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.diabet.2018.04.001.

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