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Original Paper

Is Early Complement Activation in Renal Transplantation Associated with Later Graft Outcome?

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Key Words
Complement • Renal transplantation • MASP-2 • Collectin 11 • CFD • C9 • C1q • C3c • C3d • Renal biopsy

Abstract
Background/Aims: Complement activation is important in post-transplantation renal injury, but data on its role as predictor of transplant outcome/complications when assessed in donor kidneys are lacking. ***Methods:*** In human renal transplant biopsies with delayed graft function (DGF, n=12), antibody mediated rejection (ABMR, n=8), T-cell mediated rejection (TCMR, n=11), 1 year protocol biopsies (control, n=10) and corresponding zero-biopsies we performed immunohistochemical analyses of 6 complement factors using FFPE sections and correlated the findings with kidney function, as assessed by serum creatinine, and morphological changes including interstitial fibrosis and tubular atrophy (IF/TA). ***Results:*** In DGF, TCMR and ABMR significant complement deposition was observed, which was less pronounced in corresponding zero-biopsies. Zero-biopsies with subsequent ABMR showed glomerular complement factor D and C3c expression. Moreover, glomerular C3c and C9 and tubular MASP-2 and Collectin-11 expression in zero-biopsies significantly correlated with serum creatinine at diagnosis of DGF, TCMR or ABMR. Glomerular C1q was significantly increased in ABMR, but not in DGF and TCMR. In contrast, peritubular C1q was significantly enhanced in DGF and TCMR compared to zero-biopsies. Using C3d as a surrogate marker for complement activity we could confirm that stained complement factors are frequently associated with complement activity. ***Conclusion:*** Complement deposition strongly correlated with histopathological changes observed in renal transplants. All 3 complement pathways were operational in biopsies with DGF, TCMR and ABMR albeit with differential abundance

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and localization. Since complement deposition in zero-biopsies correlated with graft function and morphological changes, early specific complement inhibition in renal transplantation may be a new therapeutic option to prevent graft loss.

Introduction

For a long time the complement system was considered exclusively an important part of innate immunity, playing an essential role in defense against bacteria. However, during the last two decades the complement system is recognized to be even more complex and to be also involved in the adaptive immune system [1, 2]. Furthermore, activation of the complement system was found to be involved in the pathogenesis of various renal diseases [2]. After renal transplantation complement activation is involved in renal injury during ischemia/reperfusion [3-6], rejection events [7] and renal dysfunction [6, 8]. The complement system can be activated by three different pathways, (i) the classical pathway, activated by any structure that is recognized by C1q [9], (ii) the lectin pathway, activated when mannan-binding lectin-associated serine protease 2 (MASP-2) complexes with mannose binding lectin 2 (MBL-2), ficolins or Collectin-11 binds to saccharide patterns expressed on bacteria or cells [10, 11] and (iii) the alternative pathway, activated through spontaneous hydrolysis of C3 [12]. An important activator of the alternative complement pathway is complement factor D (CFD) that cleaves factor B. The cleavage product Bb complexes with factor C3b, becoming the C3 convertase [13, 14]. However, in all three activation pathways C3 convertases have the same function: Cleavage of C3 into C3a and C3b fragments and C3b fragment can subsequently unite C3 convertase, producing C5 convertase. Finally, C5 convertases promote the complement cascade by cleaving C5 into C5a and C5b fragments. C5a initiates the building of the membrane attack complex (MAC) consisting of C5b, C6, C7, C8 and multiple C9 molecules [15]. It is known that all activation pathways can be involved in the pathogenesis of renal transplant rejection [1, 16-18], but the precise mechanisms are not well understood albeit this is important in the light of new therapeutical approaches. Furthermore, information on the localization and quantities of different complement components in human renal transplant biopsies are very sparse. Therefore, we analyzed in situ localization and degree of early complement activation in all three pathways using human renal biopsies diagnosed with humoral and cellular rejection as well as delayed graft function. We hypothesize that different transplant complications are mediated by different complement pathways. We further tested if complement activation is already present in zero-biopsies (taken before implantation) and whether this might be a useful predictor for later graft outcome.

Materials and Methods

Renal tissue specimens

In our study we included kidney biopsies collected during and after renal transplantation between 2008 and 2016 from the Department of Nephrology at FAU Erlangen-Nürnberg, Germany. In total 88 formalin-fixed paraffin-embedded (FFPE) specimens of archival kidney biopsies (from the Institute of Pathology, University Hospital, Erlangen, Germany) were used to evaluate the relevance of complement activation in delayed graft function (DGF, n=10), antibody mediated rejection (ABMR, n= 8), T-cell mediated rejection (TCMR, n=12). Biopsies from patients with borderline diagnosis or other co-morbidities like viral infection were excluded from the cohort. Protocol biopsies from kidneys without signs of renal dysfunction or rejection, collected one year after renal transplantation, served as controls (ctr, n=12). Delayed graft function was defined as impaired renal function necessiting dialysis within 10 days post transplantation and lacking signs of rejection. In addition, zero biopsies of all 4 groups were analyzed to detect early changes in transplant biopsies (ctr 0, DGF 0, ABMR 0, TCMR 0). The use of archival material was approved by the

Ethics Committee of the Friedrich-Alexander-University of Erlangen-Nuremberg, waiving the need for retrospective consent for the use of archived rest material (Re.-No.4415). The study groups and patient characteristics are described in Table 1.

Immunohistochemistry

For immunohistochemistry kidneys fixed in formalin, embedded in paraffin were cut into sections of 2 µm. Antigen retrieval was done using pronase E digestion for 40 minutes at 37°C (C1q, C3c) or cooking for 2, 5 min in target retrieval solution pH 6 (DAKO Deutschland). After blocking of endogenous peroxidase using 3% H_2O_2 and Avidin-Biotin block (Vector laboratories, Burlingame, CA, USA), normal goat serum and blotto (1:5) sections were incubated over night at 4°C using the following antibodies diluted in 1%BSA in 50 mM Tris pH 7, 4: C1q, a rabbit polyclonal antibody against human C1q (A0126; DAKO Deutschland, Hamburg, Germany); C3c, a rabbit polyclonal antibody against human C3c (A0062; DAKO Deutschland); C3d, a mouse monoclonal antibody against human C3d (Quidel, Athens, OH, USA); C9, a mouse monoclonal antibody against human C9 (Quidel, Athens, OH, USA); CFD, a mouse monoclonal antibody directed against active human complement factor D (Clone 8E2, provided by F. Hoffmann-La Roche Ltd, Basel, Switzerland [19]); MASP-2, a rabbit polyclonal directed against human Mannan-binding lectin serine peptidase 2 (Sigma Aldrich, Taufkirchen, Germany); Collectin-11, a rabbit polyclonal antibody against Collectin-11 (Sigma Aldrich). After washing with 50 mM Tris pH 7, 4, sections were incubated with biotinylated secondary goat anti-rabbit IgG (BA-1000; Vector laboratories) or horse anti-mouse IgG (BA-2001, Vector laboratories). Bound antibodies were detected using ABC-Kit and DAB-Immpact as a substrate (both from Vector laboratories). Finally, nuclei were stained using hemalaun.

Negative controls for immunostaining included either deletion or substitution of the primary antibody with equivalent concentrations of an irrelevant murine monoclonal antibody or preimmune rabbit IgG.

Semi-quantitative evaluation of complement factors and their localization in renal biopsies

Complement staining in renal biopsies was graded separately in glomeruli, tubuli and the peritubular/interstitial compartment. The semi-quantitative scores (0-4) describe the intensity and distribution of observable complement components in kidney biopsies in a magnification of 200x using light microscopy. Score 0: no deposits. Score 1: weak staining affecting up to 25% of the investigated compartment. Score 2: moderate staining affecting up to 50%. Score 3: substantial staining affecting up to 75% of the investigated compartment. Score 4: highest staining intensity affecting more than 75% of the investigated compartment.

Histopathology and evaluation of retrospective clinical data

Histopathological changes were graded using BANFF classification score for renal transplant biopsies in the process of routine diagnosis [20]. Clinical parameters from patients were retrospectively investigated at the time point of biopsy collection. In addition, transplantation relevant parameters were included for correlation analysis with the compartment specific scores for all investigated complement factors.

Statistical analyses

After testing for normal distribution of values using Kolmogorov-Smirnov test, data were analyzed using Kruskal Wallis test and Dunn's multiple comparison test as posthoc test for comparison of different groups. In all tests $p<0.05$ was accepted as statistically significant. Spearman test was used to test correlation of complement deposition with renal injury scores and clinical data.

Table 1. Characteristics of patients. Values were shown as numbers or Mean±SD. Abbreviations: Delayed graft function (DGF); antibody mediated rejection (ABMR); T-cell mediated rejection (TCMR); #at time of diagnosis of complication; *p<0.01 vs. DGF; •p<0.05 vs. control

Variable	Complications in human renal transplantation				SUM/mean
	Control	DGF	ABMR	TCMR	
number of patients	12	12	8	12	44
donor age [years]	47.3±17.3	55.6±15.6	53.1±5.5	59.4±9.9	53.8±14.0
recipient age [years]	44.8±16.3	50.1±15.5	53.1±11.8	45.7±18.7	48.1±15.8
male	8	8	2	5	23
female	4	4	6	7	21
donor serum creatinine [mg/dl]	0.9±0.2	0.9±0.3	0.9±0.3	0.8±0.2	0.9±0.2
recipient serum creatinine [mg/dl]#	1.2±0.3*	7.1±2.9	3.3±1.7*	3.4±2.9*	3.5±1.4
warm ischemia time [min]	35.9±7.1	31.5±16.1	34.1±8.9	30.8±8.8	33.0±10.9
cold ischemia time [min]	525.7±270.6	651.8±268.8	603.9±133.2	431.2±318.4	549.6±275.1
hypertension in donor [%]	58.3	41.6	42.9	41.6	46.1
HLA mismatch	2.6±1.9	3.6±1.1	3.9±0.7	4.1±0.9•	3.5±1.4
living donors [%]	25	8.3	0	25	15.9

Statistical analyses were performed using SPSS for Windows software (version 19.0 SPSS, IBM, Munich, Germany) or GraphPad Prism 5 for Windows software (version 5.02, GraphPad software Inc., San Diego, CA, USA).

Results

Patient characteristics (Table 1)

In our cohort of patients we evaluated zero-biopsies and subsequent transplant biopsies with renal transplants diagnosed for DGF, ABMR and TCMR. Zero-biopsies and 1 year protocol biopsies showing no signs of rejection or renal dysfunction served as controls. Mean donor age, recipient age, percentage of hypertensive kidney donors and living donors, donor serum creatinine, cold ischemia time and warm ischemia time were comparable in all 4 groups (Table 1). In DGF patients serum creatinine levels were about 6 times higher compared to mean levels in controls 1 year post transplantation. Mean serum creatinine levels in ABMR and TCMR were more than 2.5-fold higher than in the control group but did not reach the significance level due to high standard deviation (Table 1). The highest mean HLA-mismatch was detected in the TCMR and ABMR groups being significant higher than in controls (Table 1).

Zero-biopsies from patients that later developed ABMR were already positive for glomerular CFD and C3c (Fig. 1-2)

Activation of the alternative complement pathway by marked CFD staining could be detected in glomeruli from DGF, ABMR and TCMR groups showing five times higher mean score values compared to 1 year protocol biopsies (Fig. 1C, hatched bars). In contrast, glomerular CFD staining in zero-biopsies was much lower and sometimes lacking (Fig. 1A, C). However, in zero-biopsies from patients that later on developed ABMR (ABMR 0) CFD staining was significantly higher compared to the group that later on developed DGF (DGF), when analysis of significance was restricted to zero-biopsies (Fig. 1C, red asterisk). Glomerular CFD was present on parietal epithelial cells (Fig. 2B, PECs) and some podocytes (Fig. 1C, P). In addition, in proximal tubular cells CFD was detected in vesicles (Fig. 1D, E). However, we observed only minor differences in vesicular staining between all investigated groups; significant differences were only found between the lowest CFD staining in control zero-biopsies (ctr 0) and the highest staining score found in the group diagnosed for TCMR (Fig. 1F, TCMR D). Furthermore, CFD was also detected in interstitial cells in groups diagnosed for DGF, ABMR and TCMR (Fig. 1I, suffix D, hatched bars) but was lacking or rarely found in all zero-biopsies and control 1y group (Fig. 1G-I, suffix 0).

C3c, the stable cleavage product of C3, is a marker for complement activation that cannot be assigned to a specific complement activation pathway since all activation pathways lead to C3 cleavage. This marker was already detected on low level in zero-biopsies from transplants that later developed delayed graft function or rejection events (Fig. 2). In most biopsies except controls and ABMR D groups glomeruli showed a moderate staining for C3c (Fig. 2A, C), that was located in mesangial matrix and basement membrane but was not completely restricted to a specific cell type (Fig. 2A). In contrast, in both controls (0-biopsies and 1 year biopsies) we observed only weak glomerular C3c staining being significantly lower than in zero-biopsies from kidneys that later developed TCMR (Fig. 2C). Pronounced glomerular C3c staining was detected in the ABMR D group (Fig. 2B, C). In tubular cells C3c staining was weak, predominantly vesicular located and highest in both ABMR groups but the difference was not significant (Fig. 2E, F). In addition, C3c could be detected at least at low levels in all investigated groups in a peritubular localization including interstitial and peritubular endothelial cells (Fig. 2G-I). Peritubular C3c was most abundant in ABMR D and TCMR D groups being significantly higher compared to both control groups and ABMR 0 group (Fig. 2I). However, peritubular C3c was about 2-times higher in TCMR 0 and DGF D

groups compared to control groups but lacked significance due to high standard deviation (Fig. 2I).

Early complement activation in zero-biopsies is associated with morphological renal changes and reduced kidney function in the post transplant period (Table 2)

Of note, glomerular C3c and C9 in zero-biopsies correlated strongly with serum creatinine at the time point of diagnosis of DGF, TCMR or ABMR (Table 2). Furthermore, the degree of tubular staining of the lectin pathway activators MASP-2 and Collectin-11 in zero-biopsies significantly correlated with renal function as assessed by serum creatinine at the subsequent indication biopsy (Table 2). In addition, tubular MASP-2 and peritubular C9 in zero-biopsies correlated with chronic renal changes as assesses by IF/TA in the biopsies (Table 2).

Complement activation is increased in zero-biopsies from diabetic, hypertensive or smoking donors (Fig. 3)

Since complement activation in zero-biopsies may depend on kidney donor parameters we looked for potential risk factors for renal complement activation. Hereby, we observed higher glomerular CFD and C9 as well as peritubular C9 deposition in hypertensive kidney

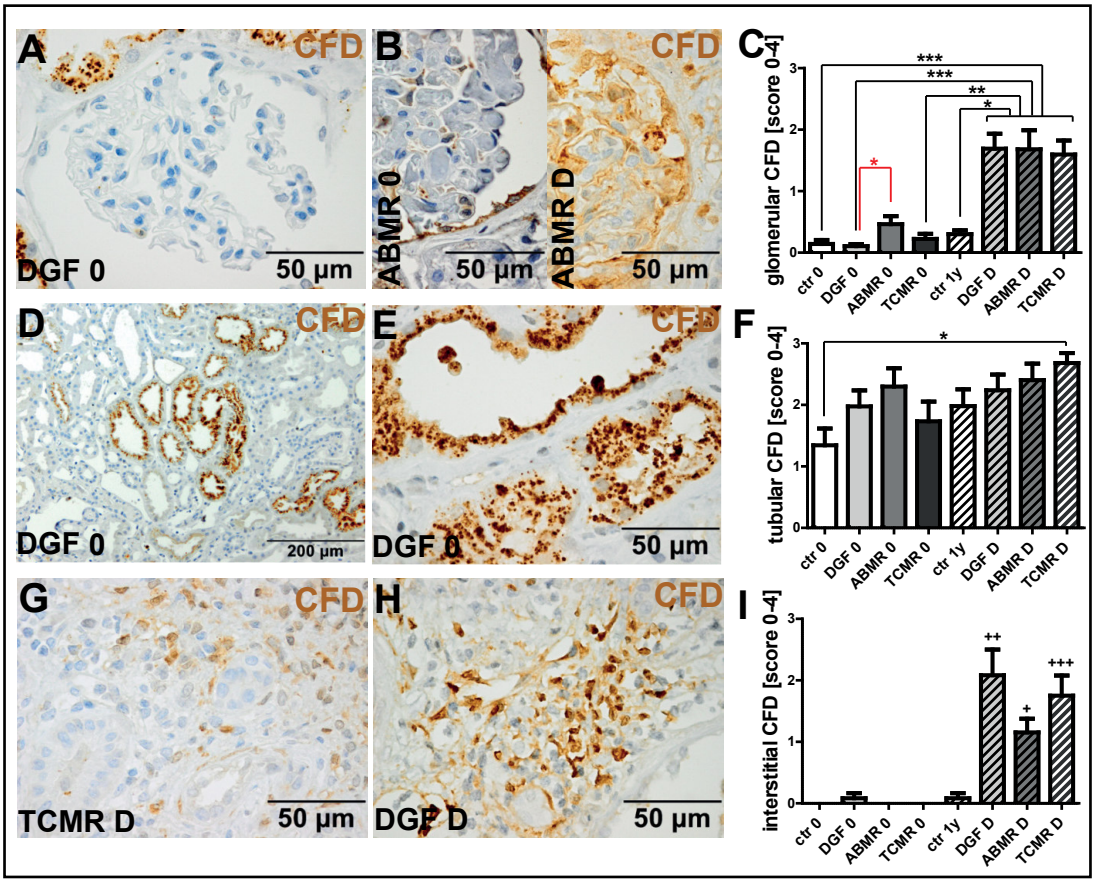

Fig. 1. CFD, an early marker for activation of the alternative complement pathway, in zero-biopsies and transplant biopsies with DGF, ABMR and TCMR. Complement factor D (CFD) was evaluated in the glomerular (A-C), in tubular (D-F) and interstitial (G-I) compartment using immunohistochemistry and semi-quantitative scoring (C, F, I). Renal transplant biopsies diagnosed for delayed graft function (DGF D), antibody mediated rejection (ABMR D) and T-cell mediated rejection (TCMR D) were compared to 1 year protocol biopsies (ctr 1y) as controls and corresponding zero-biopsies (ctr 0, DGF 0, ABMR 0 and TCMR 0). *p<0.05, **p<0.01, ***p<0.001. Red asterisk indicates analysis restricted to zero-biopsies.

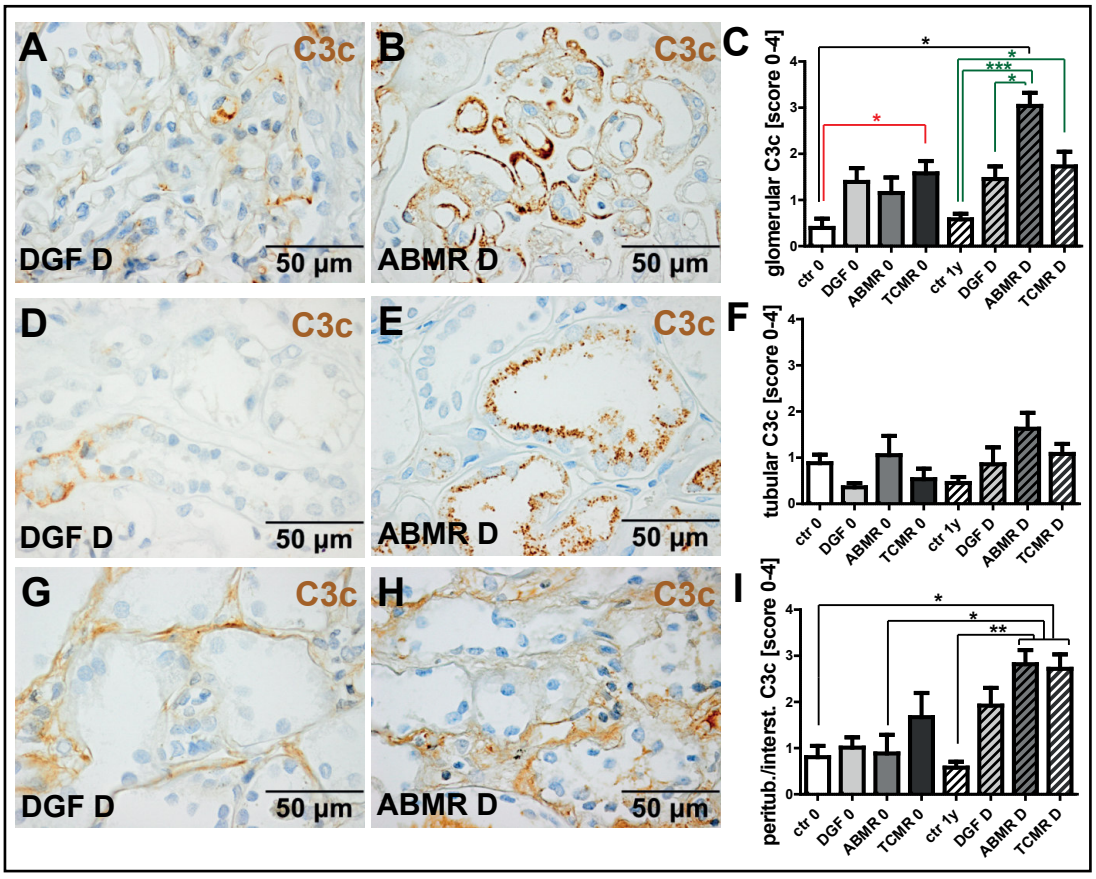

Fig. 2. C3c, a marker for activation of the alternative complement pathway, in zero-biopsies and transplant biopsies with DGF, ABMR and TCMR. Complement factor C3c (C3c) was evaluated in the glomerular (A-C), in tubular (D-F) and peritubular/interstitial (G-I) compartment using immunohistochemistry and semi-quantitative scoring (C, F, I). Renal transplant biopsies diagnosed for delayed graft function (DGF D), antibody mediated rejection (ABMR D) and T-cell mediated rejection (TCMR D) were compared to 1 year protocol biopsies (ctr 1y) as controls and corresponding zero-biopsies (ctr 0, DGF 0, ABMR 0 and TCMR 0). *p<0.05, ***p<0.001. Red asterisk indicates analysis restricted to zero-biopsies. Green asterisk indicates analysis restricted to renal biopsies taken for diagnosis of renal complication.

donors compared to normotensive donors (Fig. 3A-C). Glomerular C9 and CFD staining was increased in diabetic donors compared to non-diabetic patients (Fig. 3D, E). Furthermore, glomerular CFD expression was significantly higher in renal biopsies from donors with high nicotine consumption compared to non-smokers (Fig. 3F).

Compartment specific activation of the classical complement pathway in ABMR (Fig. 4)

C1q, as a marker of the classical complement activation pathway, was only rarely detected in glomeruli of zero-biopsies (Fig. 4A, C) but significantly increased in mesangial and endothelial localization in ABMR D (Fig. 4B, C). Glomerular C1q staining in DGF D and TCMR D biopsies was at background level (Fig. 4C). In contrast, peritubular C1q was significantly higher in DGF D and TCMR D biopsies (Fig. 4E, F) but not in ABMR D and zero-biopsies (Fig. 4D, F). Some arterioles showed a C1q-positive staining, with highest frequency in the TCMR D group, but did not reach the level of significance (Fig. 4G-I).

Lectin-mediated complement pathway activators Collectin-11 and MASP-2 were predominantly increased in ABMR and TCMR (Fig. 5, 6)

In zero-biopsies from controls glomerular MASP-2 was almost lacking (Fig. 5C) but was significantly increased in 1 year control biopsies (Fig. 5C, ctr 1y). Glomerular MASP-2 staining was predominantly localized in capillaries being highest in biopsies from kidneys diagnosed for ABMR and TCMR (about 10x higher compared ctr 0; Fig. 5B, C). MASP-2 staining was minimal in tubular cells of both control groups and 2-3.5 times increased in 0- and diagnostic biopsies from patients with DGF, ABMR and TCMR reaching significance for comparison of control 1 year protocol biopsies (ctr 1y) with ABMR D (Fig. 5F). In addition, TCMR D group showed significantly higher tubular MASP-2 levels compared to controls, when analysis was restricted to diagnostic biopsies (Fig. 5F, green). MASP-2 was also detected in interstitial cells including neutrophil granulocytes (Fig. 5G). Interstitial MASP-2 was nearly absent in all 0-biopsy groups and ctr 1y group (Fig. 4I). Significant amounts of interstitial MASP-2 were detected in all 3 diagnosis groups with renal complications with highest levels in TCMR D group (Fig. 5H, I). Collectin-11, the second lectin complement pathway activator, showed a comparable staining pattern to MASP-2 (Fig. 6). Glomerular Collectin-11 was again highest in biopsies from ABMR D and TCMR D groups and localized predominantly in glomerular capillaries (Fig. 6A-C). Interestingly, mean values for tubular Collectin-11 were slightly higher in zero-biopsies than in corresponding diagnostic biopsies

Table 2. Complement activation in 0-biopsies is associated with subsequent functional and morphological changes in renal transplants at later time points. *marks significant results

No.	Correlation of serum creatinine at time point of post-transplant pathology diagnosis with	Correlation-coefficient	p-value
1	tubular Collectin 11 in zero-biopsy	0.406*	0.017
2	tubular MASP2 in zero-biopsy	0.419*	0.014
3	glomerular C3c in zero-biopsy	0.534*	0.002
4	glomerular C9 in zero-biopsy	0.514*	0.001
No.	Correlation of IF/TA at time point of post-transplant pathology diagnosis with	Correlation-coefficient	p-value
1	tubular MASP2 in zero-biopsy	0.345*	0.027
2	peritubular C9 in zero-biopsy	0.311*	0.036

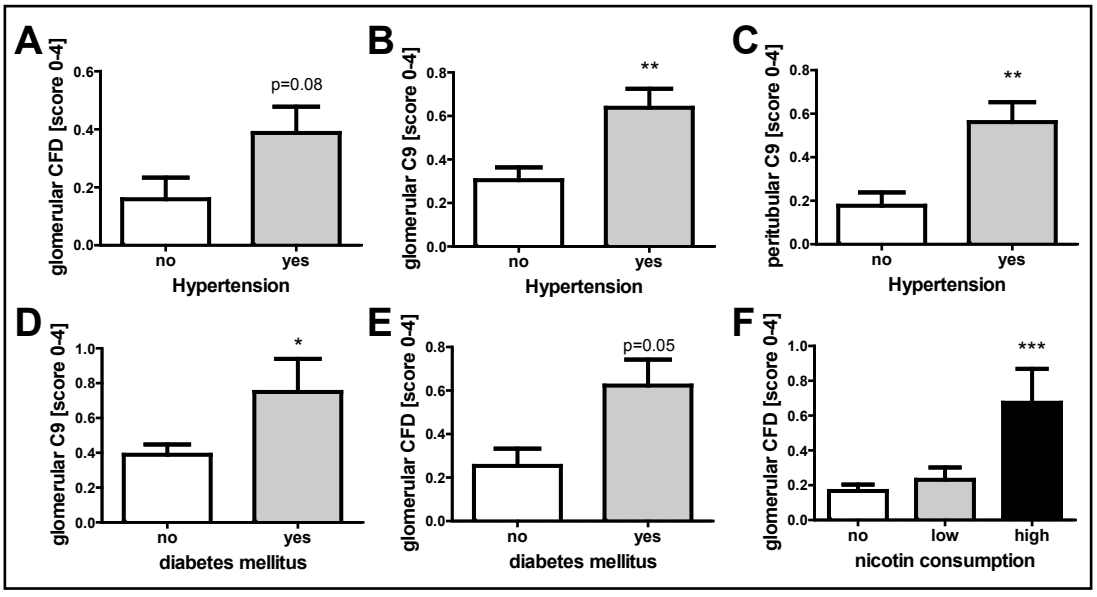

Fig. 3. Risk factors for complement activation in donor biopsies. Semi-quantitative evaluation of glomerular CFD (A), glomerular C9 (B) and peritubular C9 (C) from non-hypertensive and hypertensive donors were compared using zero-biopsies. Furthermore, glomerular C9 (D) and glomerular CFD (E) was compared between healthy and diabetic donors. Glomerular CFD deposition in zero-biopsies was dependent on nicotine consumption (F). *p<0.05, **p<0.01, ***p<0.001.

(Fig. 6F). In addition, Collectin-11 was detected in peritubular capillaries and interstitial cells including inflammatory cells with quite similar distribution as MASP-2 (Fig. 6G-I and 5I).

MAC complex component C9 is upregulated in ABMR and TCMR (Fig. 7)

In transplant renal biopsies the MAC complex component C9 was detected predominantly in glomeruli and peritubular space (Fig. 7) and only rarely found in an intracellular tubular localization (Fig. 7D-F). Glomerular C9 staining was frequently detected near the vascular pole (Fig. 7A) and in highly damaged glomeruli showing no restriction to a special glomerular cell type (Fig. 7B). The highest abundance for glomerular C9 was detected in biopsies from patients with ABMR (score 1.7±0.4) and the lowest in both control groups showing mean values below 0.5 (Fig. 7C). In zero-biopsies from patients that later developed renal rejection or dysfunction mean C9 scores were higher compared to controls, but did not reach significance (Fig. 7C). In ABMR D and TCMR D groups marked C9 staining of tubular basement membrane (Fig. 7H) reaching 3 times higher scores compared to controls and TCMR 0 group (Fig. 7I) was seen. In biopsies from patients with DGF peritubular C9 staining was marginal increased compared to controls (Fig. 7G, I).

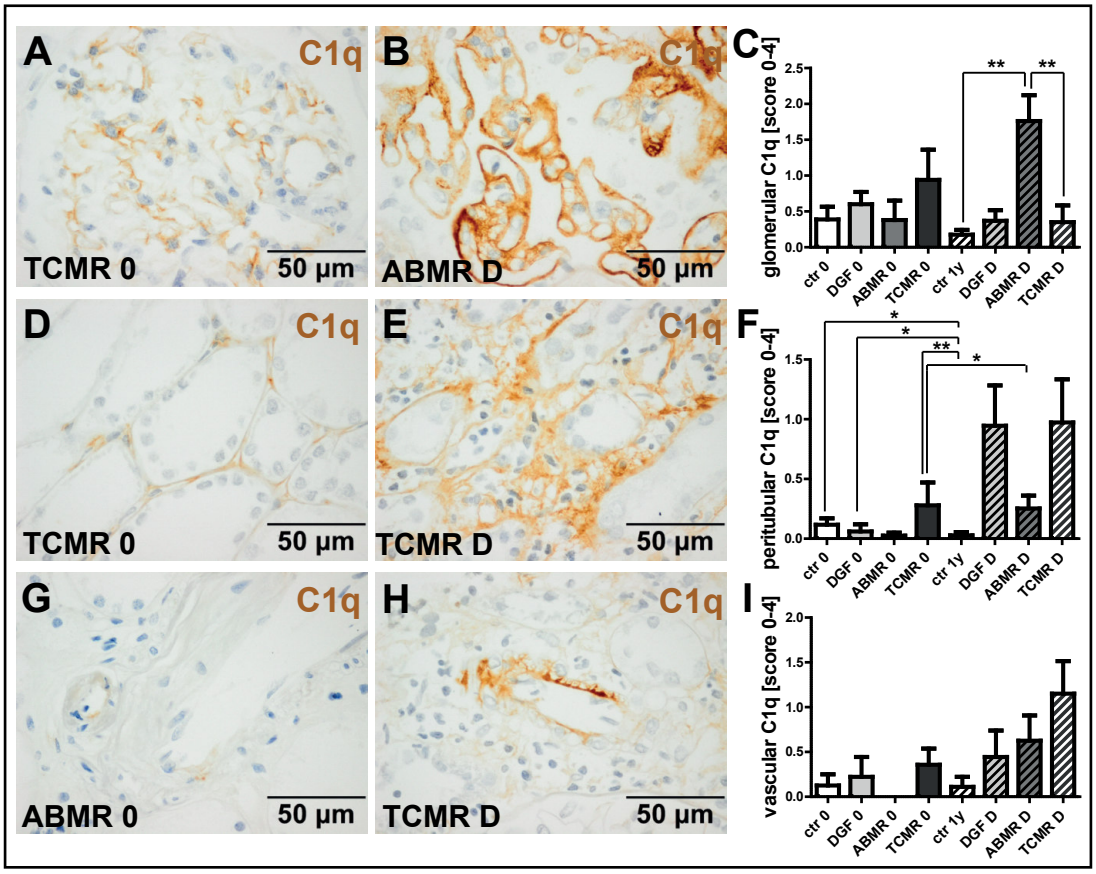

Fig. 4. C1q, a marker for the classical complement activation pathway, in zero-biopsies and transplant biopsies with DGF, ABMR and TCMR. Complement factor C1q (C1q) was evaluated in the glomerular (A-C), in peritubular (D-F) and vascular (G-I) compartment using immunohistochemistry and semi-quantitative scoring (C, F, I). Renal transplant biopsies diagnosed for delayed graft function (DGF D), antibody mediated rejection (ABMR D) and T-cell mediated rejection (TCMR D) were compared to 1 year protocol biopsies (ctr 1y) as controls and corresponding zero-biopsies (ctr 0, DGF 0, ABMR 0 and TCMR 0). *p<0.05, **p<0.01.

Complement deposition in renal biopsies is frequently associated with complement activation (Fig. 8, 9)

It is unclear if detection of complement pathway initiators, factors and cleavage products is associated with local complement activation. To address this question, we used C3d as a surrogate marker for activated complement. Tubular staining of MASP-2 and Collectin-11, the initiators of the lectin activated complement pathway, co-localizes with the activity marker C3d (Fig. 8A-F, green arrows). In contrast, staining of MASP-2 and Collectin-11-positive interstitial cells did not correlate with C3d, indicating intracellular localization. Furthermore, C3d staining was weak but in part co-localized with C1q- and CFD-positivity in renal transplant biopsies (Fig. 8G, H; Fig. 9A, B, green arrows), while only some interstitial cells weakly positive for CFD were negative for C3d (Fig. 9A, B, red arrows). Interestingly, in C3c-positive areas C3d staining was absent in the majority of cases (Fig. 9C, D). Furthermore, we did not observe exact co-localization of C9 as a part of the membrane attack complex and C3d (Fig. 9E-H). We rather found C3d-positive renal structures lacking C9 (Fig. 9E, F) and C9-positive staining lining the tubular basement membrane that were lacking in C3d

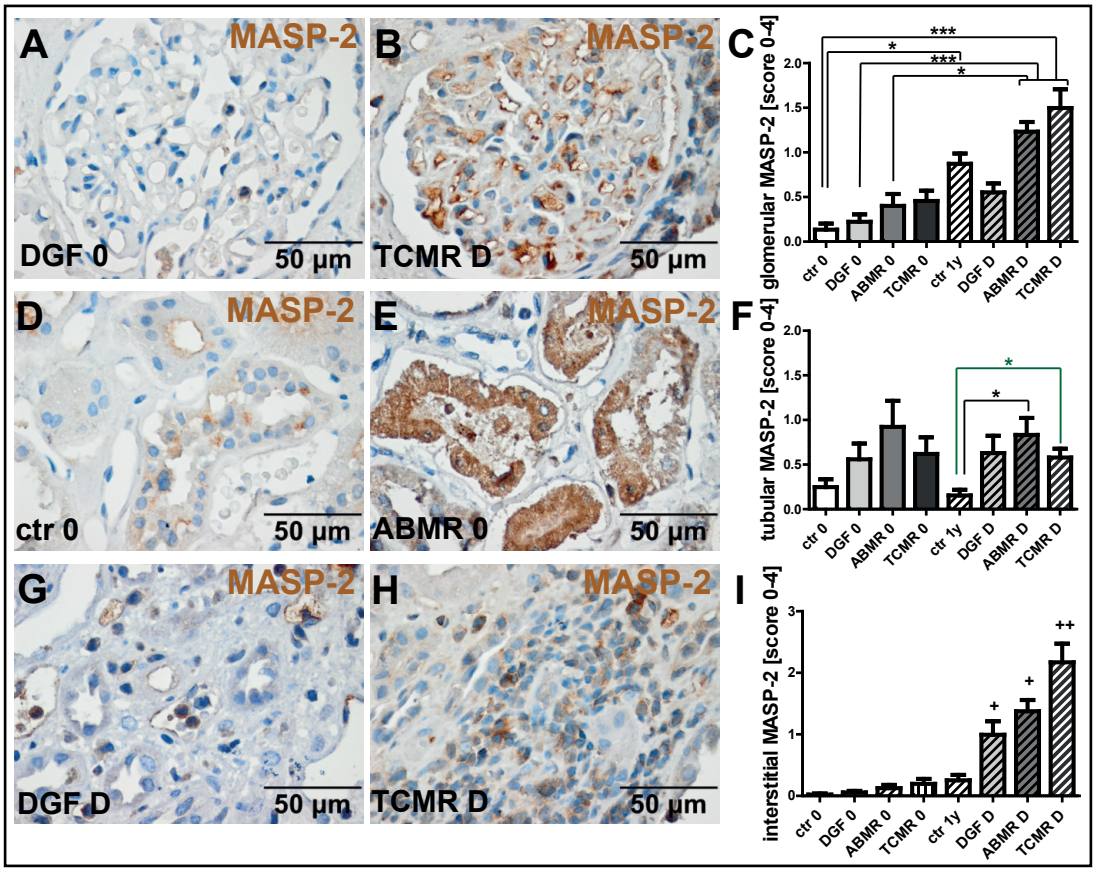

Fig. 5. MASP-2, an early marker for activation of the lectin complement pathway, in zero-biopsies and transplant biopsies with DGF, ABMR and TCMR. Mannan-binding lectin serine protease 2 (MASP-2) was evaluated in the glomerular (A-C), in tubular (D-F) and interstitial (G-I) compartment using immunohistochemistry and semi-quantitative scoring (C, F, I). Renal transplant biopsies diagnosed for delayed graft function (DGF D), antibody mediated rejection (ABMR D) and T-cell mediated rejection (TCMR D) were compared to 1 year protocol biopsies (ctr 1y) as controls and corresponding zero-biopsies (ctr 0, DGF 0, ABMR 0 and TCMR 0). *p<0.05, **p<0.01, ***p<0.001. +p<0.05 vs. all zero-biopsies and ctr 1y. ++p<0.01 vs. all zero-biopsies and ctr 1y. Green asterisk indicates analysis restricted to renal biopsies taken for diagnosis of renal complication.

staining (Fig. 9G, H). However, some tubules with C9-positive basolateral lining showed weak cytosolic C3d staining (Fig. 9G, H, asterisks).

Complement activation in follow-up biopsies at diagnosis is highly associated with functional and morphological changes in renal transplants (Table 3)

In our analyses we observed numerous associations of different complement activation pathway components with changes in renal histology and function in biopsies from DGF, TCMR and ABMR kidneys. Positive correlations with serum creatinine levels could be demonstrated for all investigated complement components, showing the best correlation with glomerular and interstitial CFD and intracellular tubular C9 (Table 3). In addition, we observed strong correlations of most complement components with renal histopathological changes with focus on inflammatory events (Table 3). However, we focused on associations with high correlation coefficients > 0.500 and high p-values (Table 3).

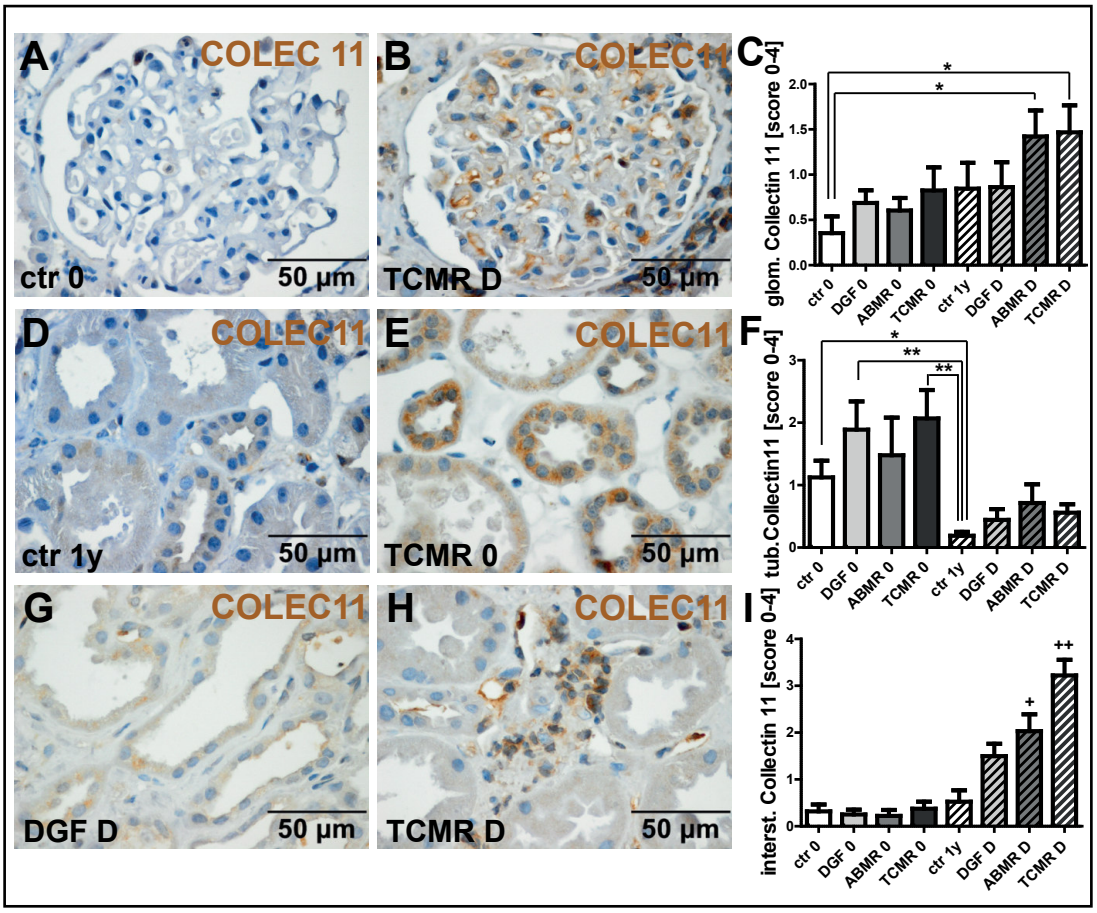

Fig. 6. Collectin-11, an early marker for activation of the lectin complement pathway, in zero-biopsies and transplant biopsies with DGF, ABMR and TCMR. Collectin-11 (COLEC11) was evaluated in the glomerular (A-C), in tubular (D-F) and interstitial (G-I) compartment using immunohistochemistry and semi-quantitative scoring (C, F, I). Renal transplant biopsies diagnosed for delayed graft function (DGF D), antibody mediated rejection (ABMR D) and T-cell mediated rejection (TCMR D) were compared to 1 year protocol biopsies (ctr 1y) as controls and corresponding zero-biopsies (ctr 0, DGF 0, ABMR 0 and TCMR 0). *p<0.05, **p<0.01. +p<0.05 vs. all zero-biopsies and ctr 1y. ++p<0.01 vs. all zero-biopsies and ctr 1y. Green asterisk indicates analysis restricted to renal biopsies taken for diagnosis of renal complication.

Discussion

Predictive value of complement activation in donor biopsies for later outcome

This study, to our knowledge, is the first to investigate different local complement activation pathways in zero-biopsies and to evaluate its predictive value for later graft outcome. Overall, complement activation in zero-biopsies was generally weaker compared to the degree of activation observed in DGF and rejection biopsies except for tubular Collectin-11 which was markedly expressed already in zero-biopsies. Recent studies identified Collectin-11 as an important mediator of renal ischemia/reperfusion injury transiently upregulated in a mouse model early after injury induction [21]. Since Collectin-11 is one of the initiators of the lectin mediated complement pathway [10, 16], this may reflect early tubular complement activation due to ischemic injury which is present in all zero-biopsies. Using Mannose binding lectin (MBL)-deficient mice [22] or MASP-2-deficient mice [23], the pattern recognition molecule MBL and the Mannan-binding lectin-associated serine protease-2 (MASP-2) were identified as an important initiators of complement activation during ischemia/reperfusion injury underlining the important role of the lectin activated pathway in renal transplantation. However, in human biopsies we detected Collectin-11 in both zero-biopsies and later biopsies at moderate levels.

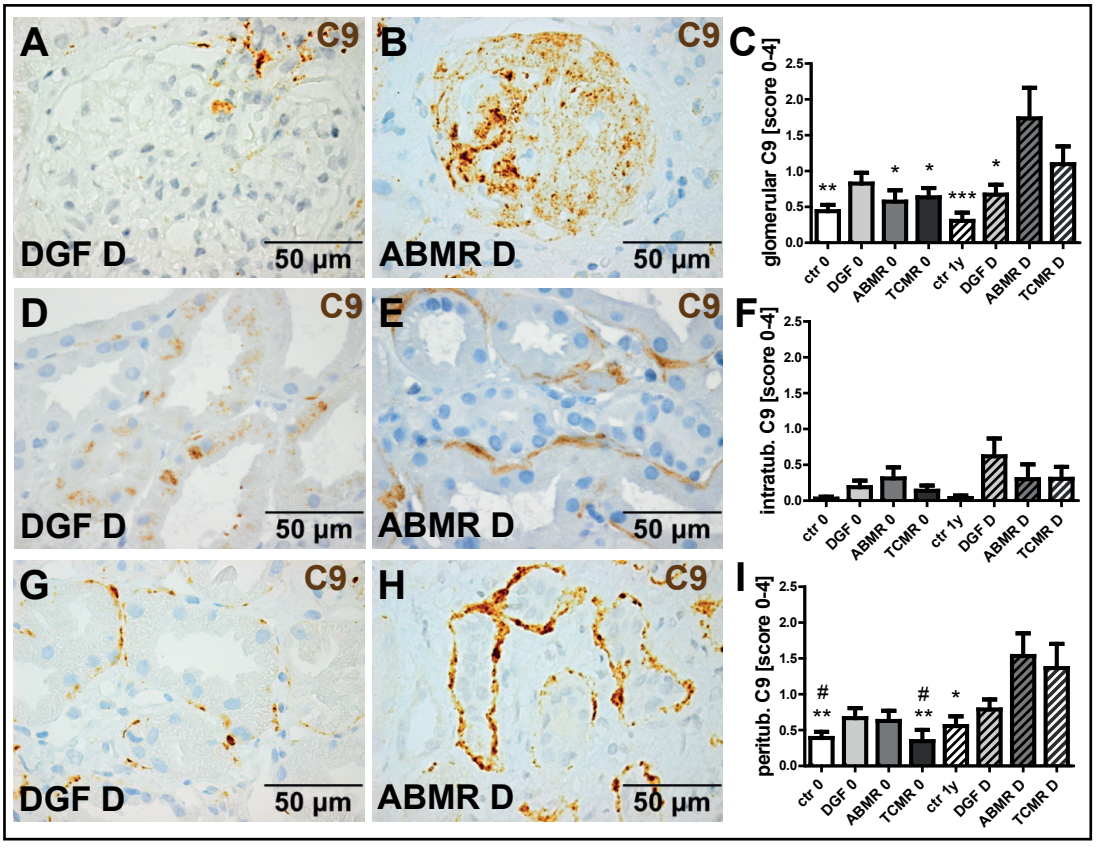

Fig. 7. C9, an essential component of the MAC, in zero-biopsies and transplant biopsies with DGF, ABMR and TCMR. Complement factor 9 (C9) was evaluated in the glomerular (A-C), in intratubular (D-F) and peritubular (G-I) compartment using immunohistochemistry and semi-quantitative scoring (C, F, I). Renal transplant biopsies diagnosed for delayed graft function (DGF D), antibody mediated rejection (ABMR D) and T-cell mediated rejection (TCMR D) were compared to 1 year protocol biopsies (ctr 1y) as controls and corresponding zero-biopsies (ctr 0, DGF 0, ABMR 0 and TCMR 0). *p<0.05 vs. ABMR D, **p<0.01 vs. ABMR D, ***p<0.001 vs. ABMR D. #p<0.05 vs. TCMR D.

Of note, zero-biopsies from patients without DGF or rejection episodes showed the lowest complement activation for most complement components. However, differences in complement activation in 0-biopsy groups were weak and rarely reached significance. Despite relatively low complement activation in donor biopsies the levels of tubular Collectin-11 and MASP-2 as well as glomerular C3c and C9 significantly correlated with serum creatinine levels at the time point of later indication biopsies. In addition, tubular MASP-2 and peritubular C9 expression in zero-biopsies also correlated with IF/TA pointing to a possible role of complement activation in donor biopsies for fibrogenesis. A role of Collectin-11 in mediation of renal tubulointerstitial fibrosis was recently confirmed in an ischemia/reperfusion model using Collectin-11 deficient mice [24]. Currently, comparable studies using MASP-2, Collectin-11, C1q, CFD, C3c and C9 in zero-biopsies for investigation of renal complement activation are lacking. Furthermore, in this study we could demonstrate that deposition of complement factors to some extend is associated with complement activation, as shown by C3d-staining as surrogate marker of complement activation. Intracellular staining of complement factors lacking co-localization with the activation marker C3d might be involved in the pathogenesis of transplant nephropathy since interacellular complement factors can rapidly initiate the complement cascade after release from complement expressing cells. The relevance of local complement mRNA expression was also shown in earlier studies that reported significant upregulation of different complement components using microarrays in zero-biopsies from deceased versus living donors also indicating a role of complement activation mediating transplant injury especially in renal transplant with

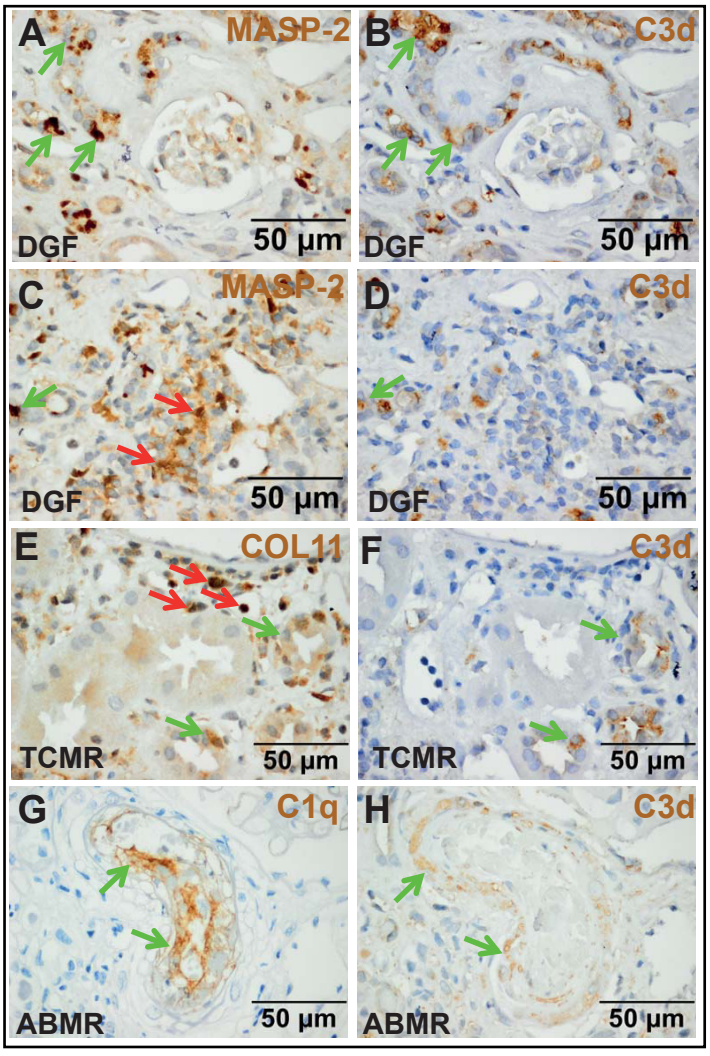

Fig. 8. Co-localization of lectin- and classical complement pathway initiators with C3d in renal transplant biopsies. Consecutive paraffin-sections were used for co-localization studies by immunohistochemistry. MASP-2 (A) co-localized with tubular C3d (B; green arrows in A and B) but C3d (D) was almost lacking in MASP-2-positive interstitial cells (C, red arrows). While C3d (F) was also absent in Collectin 11-positive interstitial cells (E, red arrows), Collectin 11-positive tubuli (E, green arrows) also stained for C3d (F, green arrows). C1q-positive vessels (G, green arrows) were weakly positive for C3d (H, green arrows).

longer cold ischemia time [25]. In addition, there is also a systemic complement activation in deceased donors, as described for serum levels of C5b-9, C4d, factor Bb and MBL in deceased donors compared to living donors or controls [26]. In an earlier study investigating zero-biopsies using immunohistochemistry, the complement cleavage product C4d was detected in more than 40% of the glomeruli and 6% of arterioles without association with a higher risk to develop ABMR or TCMR [27]. However, none of the investigated zero-biopsies showed C4d-positive peritubular capillaries which are typically used for diagnosis of the most common ABMR type I [27].

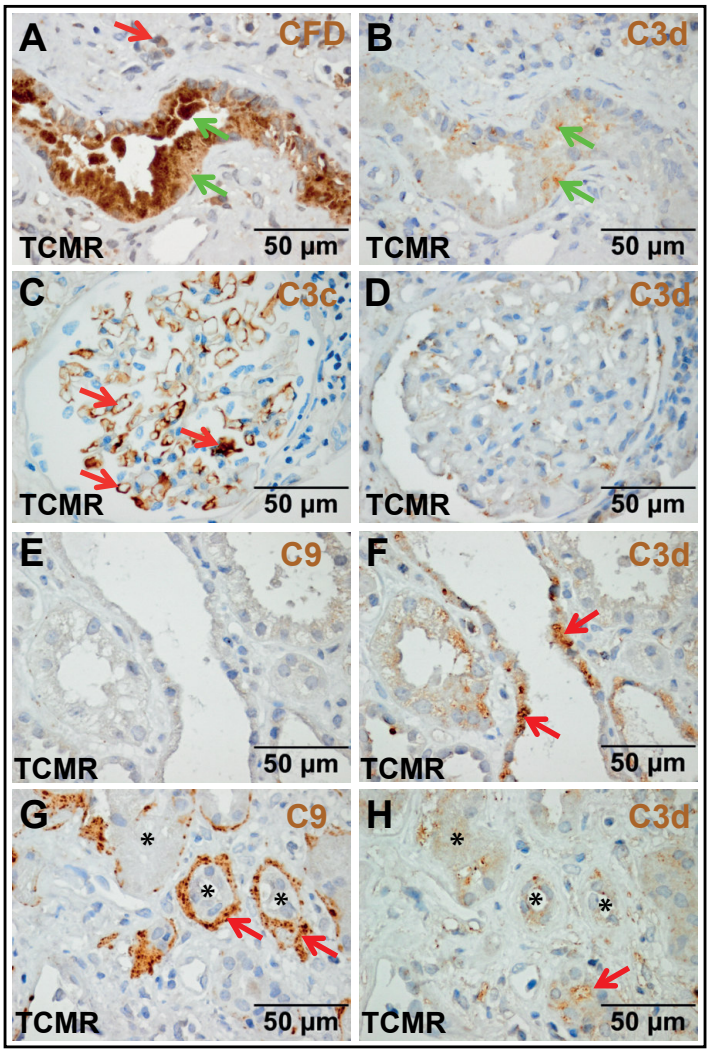

Fig. 9. Co-localization of alternative complement pathway initiator CFD, C3c and C9 with C3d in renal transplant biopsies. Consecutive paraffin-sections were used for co-localization studies by immunohistochemistry. CFD (A) co-localized with weakly stained tubular C3d (B; green arrows in A and B) but C3d (B) was almost absent in CFD-positive interstitial cells (A; B, red arrows). Glomerular C3c (C, red arrows) did not co-localize with C3d (D). C3d-positive tubuli (F, red arrows) did not stain for C9 (E). C9-staining lining the tubular basolateral membrane (G, red arrows) frequently showed weak cytosolic C3d staining of the same tubules (H, asterisks in G and H).

Risk factors for complement activation in donor kidneys

In our study we identified hypertension, diabetes mellitus and high nicotine consumption as risk factor for complement activation. Significant differences were detected for glomerular CFD and peritubular C9 indicating that deleterious effects of donor hypertension, diabetes mellitus or high nicotine on renal grafts are potentially mediated by activation of the alternative pathway.

Negative effects of donor nicotine consumption are known and include impaired kidney function [28]. An association of donor smoking with DGF was only seen using bivariate analysis [29] but this relationship became insignificant when adjusted for correlated outcome data [30]. Complement activation is known in both diabetes mellitus [31] and hypertension [32]. In addition, both factors are included in calculation of risk quantification for selection of deceased kidney donors [33]. Here, we suggest that these negative effects are possibly mediated by complement activation since there is a large body of evidence indicating that renal injury in transplantation is mediated by complement [1].

Table 3. Complement activation in renal biopsies is highly associated with subsequent functional and morphological changes in renal transplants. *marks significant results.[1] Only correlations with p-values below 0.01 are shown. [2]Only correlations with correlation coefficient above 0.500 and a p-value below 0.001 are listed

No.	Correlation of serum creatinine at time point of renal biopsy with[1]	Correlation-coefficient	p-value
1	tubular Collectin 11 in renal biopsy	0.450*	0.003
2	interstitial MASP2 in renal biopsy	0.408*	0.008
3	tubular C1q in renal biopsy	0.556*	<0.001
4	glomerular CFD in renal biopsy	0.624*	<0.001
5	interstitial CFD in renal biopsy	0.615*	<0.001
6	interstitial C3c in renal biopsy	0.439*	0.005
7	glomerular C9 in renal biopsy	0.456*	0.003
8	intratubular C9 in renal biopsy	0.618*	<0.001
No.	Correlation of renal changes at time point of renal biopsy with complement component[2]	Correlation-coefficient	p-value
1	glomerulitis in renal biopsy with glomerular C1q in renal biopsy	0.611*	<0.001
2	glomerulopathy in renal biopsy with glomerular C1q in renal biopsy	0.626*	<0.001
3	gnterstitial inflammation in renal biopsy with interstitial Collectin 11 in renal biopsy	0.655*	<0.001
4	parench. interstitial inflam. in renal biopsy with interstitial Collectin 11 in renal biopsy	0.511*	<0.001
5	parench. interstitial inflam. in renal biopsy with interstitial CDF in renal biopsy	0.511*	<0.001
6	peritubular capillaritis in renal biopsy with glomerular C3c in renal biopsy	0.537*	<0.001

Differences in level and site of complement activation in ABMR, TCMR and DGF

Earlier studies on complement activation in human DGF or transplant rejection focused on serum complement or gene expression as mentioned above [25-27]. There are limited studies using immunohistochemistry or immunofluorescence staining for evaluation of complement mediated injury in human biopsies indicating a relevance of C3c in the pathogenesis of allograft rejection. Glomerular C3c deposition in early humoral renal allograft rejection was associated with a poor short-term outcome [34, 35]. In contrast, the role of C3d on graft outcome is unclear. While in one study peritubular C3d deposition was found to be an indicator of a variant of acute renal rejection with worse clinical outcome [36] there was no association of C3d deposition with graft outcome in a second study [37].

In this study we assessed whether different renal transplant complications including DGF, ABMR and TCMR are associated to different complement activation pathways or activation in different renal compartments. The relatively low numbers of patients per group are a limitation of the study, but his is due to the fact that patients with well defined diagnosis and related zero-biopsies are not very frequent even in our large cohort of transplant biopsies. As expected, we could not identify complement activation pathways exclusively activated in a specific transplant complication. However, there are clear differences between transplant complication groups regarding the pattern of complement deposition: In biopsies from ABMR we detected complement activation by all three activation pathways. However, in ABMR biopsies C1q was clearly detected in glomeruli and lacking in peritubular localization. In addition, C1q was found in DGF and TCMR in peritubular localization but lacking in glomeruli. In contrast, in a small study with 24 patients, mesangial C1q detection by immunofluorescence staining was not restricted to ABMR [38] and information on peritubular C1q localization is lacking. Furthermore, C3c was highest in ABMR biopsies in all investigated compartments, reaching significance in glomeruli. Despite C4d positivity is more specific for ABMR, C3c deposition was shown to be associated with graft loss in an earlier study [35].

Interestingly, in TCMR components of the lectin-mediated complement pathway like MASP-2 and Collectin-11 were present in high quantities compared to ABMR and DGF

particularly in the interstitial compartment. Recently, the lectin-mediated complement pathway was suggested as the primary mode of complement activation and subsequent renal injury after ischemic insult [21-23]. Of note, both MASP-2 and Collectin-11 were positive in a subpopulation of interstitial cells. These cells include neutrophil granulocytes and presumably additional inflammatory cells. In animal models MASP-2 was detected in similar peritubular localization [23], but Collectin-11 was detected predominantly in tubular cells [21]. However, our results support that lectin pathway might be involved in mediating renal injury during kidney transplantation as demonstrated in animal models using wild type versus MBL, MASP-2 or Collectin-11 deficient mice [21-23].

DGF is also clearly associated with complement activation of all three investigated pathways. However, we failed to detect membrane attack complex (MAC) formation, as assessed by C9, indicating that DGF is mediated by earlier complement factors e.g. binding of C3a and C5a to their receptors. A role of the lectin pathway in pathogenesis of DGF in human kidney transplantation has been shown by single nucleotide polymorphism (SNP) analysis. Gene polymorphism in the Ficolin 2 gene, another activator of the lectin-mediated complement pathway, increased the affinity to carbohydrates and is associated with a higher risk of DGF and acute rejection [39]. In a current study complement receptor 1 blocking is tested for prevention of DGF targeting complement effects mediated by C3d and C4b [40]. Inhibition of terminal complement activation using a C5 inhibitor resulted in reduced DGF in animal models [6]. However, studies of C5 inhibition for preventing DGF in humans are currently not completed.

Conclusion

In this study we observed different patterns of intensity and localization of complement activation dedicated to different activation pathways in renal transplant biopsies with DGF, TCMR and ABMR. Despite little complement detection in zero-biopsies, its positive correlation with later outcome indicated that complement activation can still be present in donor kidneys or is initiated early after explantation. Deposition of complement factors is in part associated with local complement activation. Complement deposition in renal biopsies with DGF, TCMR and ABMR is strongly associated with histopathological changes. Therefore, early inhibition of complement activation in renal transplantation might be effective in the prevention of transplant complications and graft loss.

Abbreviations

1y (protocol biopsy collected 1 year after transplantation); ABMR (antibody mediated rejection); C1q (Complement factor C1q); C3c (stable Complement factor C3c cleavage product); C3d (cleavage product of C3b); C9 (Complement factor 9); CFD (Complement factor D); Ctr (control); DGF (Delayed graft function); MASP-2 (Mannan-binding lectin-associated serine protease 2); TCMR (T-cell mediated rejection).

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Disclosure Statement

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