Contributions to the study of the complement system in IgA nephropathy and dialysis

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CHAPTER 3

Arteriolar C4d in IgA nephropathy: a cohort study

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ABSTRACT

Rationale & objective: Glomerular C4d (C4dG) as an indicator of lectin pathway of complement activation in IgA nephropathy (IgAN) has been associated with more severe kidney damage. Recent studies have suggested that vascular lesions in IgAN biopsies with complement deposition are also associated with disease progression. We aimed to study the clinical significance of arteriolar C4d (C4dA) in IgAN kidney biopsy tissue.

Study Design: Retrospective Cohort Study.

Setting & participants: Kidney biopsies from 126 adults diagnosed with IgAN under the Oxford Classification were stained using immunohistochemistry and classified according to glomerular and arteriolar C4d deposition. Additionally, vascular lesions including acute and chronic microangiopathy, arteriolar hyalinosis, and arterial intima fibrosis, were characterized.

Predictor: C4dA.

Outcome: Progressive kidney disease defined as the decline in eGFR by ≥50% or occurrence of kidney failure with replacement therapy

Analytical Approach: The association of C4dA and C4dG with baseline clinical and histological characteristics, as well as progressive kidney disease, were assessed with survival analysis using multivariable Cox regression analysis.

Results: C4dA was identified in 21 (17%) patients and was associated with mean arterial pressure, arterial intima fibrosis, and chronic microangiopathy. C4dA was also significantly associated with C4dG and both were associated with progressive kidney disease. In regression analysis, C4dA remained significantly associated with progressive kidney disease after adjusting for other significant predictors, including baseline eGFR, MAP, and the presence of crescents.

Limitations: Findings based on the retrospective evaluation of a single-center's experience, limited number of events, a small number of patients with a broad range of kidney disease stages, and use of immunohistochemistry rather than immunofluorescence to detect C4d.

Conclusions: C4dA is a potential biomarker for disease progression in IgAN. It should be further investigated in larger cohorts to determine the value of C4dA in improving prediction of IgAN disease progression.

INTRODUCTION

Immunoglobulin A (IgA) nephropathy (IgAN) is the most common glomerulonephritis worldwide.[1] Up to 40% of IgAN cases will progress to end-stage kidney disease (ESKD) within 20 years after diagnosis.[2] The use of immunosuppression in IgAN has been subject to controversy and concerns. While the addition of immunosuppressive therapy to supportive care seems to reduce proteinuria, the impact on progressive kidney disease in IgAN patients has been inconsistent.[3,4,5] Moreover, immunosuppression has substantial side effects including the risk of severe infection.[5] Therefore, biomarkers identifying subgroups of IgAN patients with poor prognosis that might benefit from more aggressive forms of treatment are needed.

Although not yet widely used in clinical practice, several studies have shown that glomerular C4d (C4dG) is a valuable biomarker associated with disease progression in IgAN.[6] Moreover, C4dG identifies patients who are at risk for progression at an early stage of the disease,[7] and its predictive value remains after adjustment for other established immunohistological biomarkers.[8] A mechanistic explanation for the mesangial deposition of C4d is the glomerular activation of the lectin pathway of complement.[9] Complement activation has also been documented in the urine and serum of IgAN patients,[10,11] whereas variants in complement genes have been shown to affect IgAN risk and prognosis.[12,13] Consequently, clinical trials have been initiated with different complement inhibitors in IgAN.[14]

The vascular compartment is neglected by the Oxford pathologic classification because its significance in predicting kidney outcomes is uncertain.[15,16,17,18] Nevertheless, the impact of risk factors for vascular disease on the progression of IgAN has been shown in numerous studies.[5,18,19,20] Additionally, microangiopathy lesions in IgAN biopsies have been associated with worse prognosis.[21,22] Interestingly, C4d has been proposed as a marker of thrombotic microangiopathy (TMA) in various kidney diseases, including IgAN.[23,24] We therefore aimed to investigate the significance of arteriolar C4d (C4dA) in a cohort of patients with IgAN and compare it with clinical and histological markers of disease progression.

MATERIALS AND METHODS

Patients

We reviewed the kidney pathology archives from January 2001 to December 2017 at Centro Hospitalar Universitário de São João in Portugal to identify patients who had IgAN diagnosed by initial kidney biopsy. The following information at the time of the kidney biopsy was recorded: age, gender, systolic and diastolic blood pressure, use of anti-hypertensive medication up to one-year prior to the referral to the nephrologist, total cholesterol (TC), triglycerides, high- and low-density lipoprotein cholesterol (HDL-C, LDL-C), use of lipid-lowering medication, smoking status, proteinuria, serum creatinine, history of macroscopic hematuria, comorbid conditions and number of glomeruli in the kidney biopsy specimen. Progression of kidney disease and post-biopsy use of renin-angiotensin system (RAS) blockade and/or immunosuppressive therapy record were retrospectively reviewed. Inclusion criteria were absence of Henoch–Schönlein purpura diagnosis or other conditions, such as diabetes mellitus, liver disease, lupus nephritis, vasculitis or atypical hemolytic uremic syndrome and availability of paraffin-embedded kidney biopsy (with ≥8 glomeruli). The Centro Hospitalar Universitário de São João Ethics Committee reviewed and approved the use of patients' biopsies for the purpose of this study without the need to obtain informed consent. The study was conducted in accordance with the principles originating from the Declaration of Helsinki.

Clinical definitions

The estimated glomerular filtration rate (eGFR) was determined using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation.[25] Progressive kidney disease was defined as a decline of at least 50% in the eGFR or progression to ESKD during the follow-up period.[26] Hypertension was defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg and/or use of anti-hypertensive medication, and further classified as hypertension at the time of biopsy (hypertension at biopsy), or hypertension diagnosed ≥1 year prior to the nephrology consultation (previous hypertension). Dyslipidemia was defined as the presence of either TC≥200 mg/dL; LDL-C≥130 mg/dL; HDL-C<40 mg/dL in men, and <50 mg/dL in women; triglycerides≥150 mg/dL; or use of antidyslipidemic medication. Shortly before the kidney biopsy, 24-hour urine was collected to determine proteinuria. End points were recorded until May 2018 or until the he first occurrence of ESKD (eGFR <15 mL/

min/1.73m^2, dialysis, or transplantation). There was no loss to follow-up and no deaths occurred during follow-up.

Kidney pathological evaluation

Paraffin-embedded tissue from kidney biopsies was stained with hematoxylin and eosin, periodic acid-Schiff, silver methenamine and Masson's trichrome. Direct immunofluorescence was used to detect deposition of IgG, IgA, IgM, C3, C1q and fibrinogen. The sections for light microscopy (LM) were graded according to the Oxford MEST-C score of IgAN.[15,27] Further, kidney biopsies were analyzed for vascular lesions and classified as previously described.[22] In brief, "acute" microangiopathic was defined as endothelial cell swelling, subintimal edema, and arteriolar thrombosis and/or fibrinoid necrosis, while lesions with arterial "onion skin" lesion (fibrous intimal thickening with concentric lamination) were considered "chronic". In addition, general microangiopathic lesions was defined as the presence of either or both lesions. Arteries and arterioles were also evaluated for arterial intimal fibrosis (defined as thickening of the arterial wall and narrowing of the vascular lumen produced by fibrotic intimal thickening and replication of the internal elastic lamina)[28] and arteriolar hyalinosis (defined as chronic arteriolar change with amorphous, glassy and eosinophilic material observed in the walls of renal arterioles resulting in thickened arteriolar walls and luminal narrowing)[29] as previously described.[22] Each type of vascular lesion was finally simplified to absent or present to increase statistical power.

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Immunoperoxidase staining was performed on formaldehyde-fixed sections deparaffinized in xylene and rehydrated in graded ethanol. Sections were stained with the polyclonal anti-human C4d (Biomedica, Vienna, Austria), antigen retrieval was performed in a microwave oven using trisethylenediamine tetraacetic acid buffer as antigen retrieval solution. The detection system included the following steps: endogenous peroxidases blockage with H_2O_2 and signal amplification with the horseradish peroxidase (HRP) kit (Universal HRP Multimer kit; Ventana Medical Systems, Tucson, AZ, USA). Diaminobenzidine was used as chromogen and hematoxylin as nuclear stain. The immunohistochemistry (IHC) specificity was controlled by replacing the primary antibodies with irrelevant anti-sera. IHC score analysis was performed as follows: for glomerular C4d as negative (0) or positive (1). Patients were classified as "positive" when >25% of non-sclerotic glomeruli were positive for C4d, as described in Espinosa et al.[30] For arteriolar C4d, the authors followed the same criteria as for the glomeruli, classifying cases as “positive” if C4d IHC was present in more than 25% of the arterioles.

Kidney biopsies from all patients were reviewed independently by three observers (P.C., Q.C., B.F.) who were blinded to the clinical data. All disagreements were finally resolved by the senior pathologist (R.S.).

Statistical analysis

Continuous variables are expressed as mean ± standard deviation (SD) or median with interquartile range (IQR), while categorical variables are expressed as count and percentage.

Associations between categorical variables were tested with the chi-square test or Fisher’s exact test, as appropriate. Numerical variables were tested for normality. The t-test was used to compare groups with respect to numerical variables if the normality assumption was met; otherwise, the Mann–Whitney test was used. Associations were evaluated through the eta-squared statistic, the proportion of variance explained, for numerical outcomes, and through the Cramer V coefficient, the strength of association, for categorical outcomes. Survival from progressive kidney disease was assessed using the Kaplan-Meier method and compared among C4dA and C4dG status using the log-rank test. Associations between baseline variables and progressive kidney disease were estimated using Cox proportional hazards models.

Multivariable models were estimated to evaluate the association of C4dA IHC with progressive kidney disease, while controlling for other significant variables. The reduced sample size and the number of events limited the number of variables to be included in the multivariable model. Thus, initially variables associated with the risk of progressive kidney disease were identified through simple Cox regression models. Second, a multiple regression model was constructed using a stepwise procedure, with variables for inclusion in a multiple regression model selected from identified variables in the first step. The association of C4dA with progressive kidney disease was evaluated using 2 different approaches to build multiple models. The first approach involved selecting from C4dA and the other variables previously identified using a stepwise forward procedure. This approach led to a multiple Cox regression model with five variables, including C4dA. In the second approach, 2 multiple Cox regression models, one for significant clinical variables and the other for significant histological variables, were constructed separately first, and then combined into one. C4dA and C4dG were added separately to the previous models to evaluate the associations between these markers and progressive kidney disease. All nonsignificant variables were removed from the models. Models with C4dA IHC and models with C4dG IHC were compared using the Akaike information criterion (AIC). Hazard ratios (HRs) for C4dA IHC and C4dG IHC and their 95% confidence intervals (CIs) from these models are reported. Harrell concordance index was used to assess and compare discrimination between the 2 models.[31,32]

The association with progressive kidney disease of C4dA IHC, relative to C4dG IHC, was further evaluated using the likelihood ratio test. Two likelihood ratio tests were performed, each to test the multiple regression model with both C4dA IHC and C4dG IHC against the reduced simple model without one of these markers. A rejection of the null hypothesis would suggest that the model with both C4dA IHC and C4dG IHC was preferred compared with the reduced model without one of these.

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In Cox regression models the proportional-hazards assumption was evaluated and if necessary, time-dependent coefficients were considered. Statistical analysis was undertaken using IBM SPSS Statistics (version 24) and R software. $P<0.05$ was considered statistically significant.

RESULTS

Patient, clinical, and pathological data

Isolated hematuria and/or proteinuria were the most common indication for a kidney biopsy (59.5%). Baseline eGFR was lower than 60 ml/min/1.73m^2 in 40.8% of the patients, and baseline proteinuria was higher than 1g/day in 72% of the patients (Table 1). A total of 111 patients were treated with RAS blockade and 29 with immunosuppression. During a median follow-up period of 4 years [IQR 2–6], progressive kidney disease developed in 32 patients (5.46 per 100 patient-years), of whom 31 had a decline in eGFR of ≥50% (5.29 per 100 patient-years) and 28 progressed to ESKD (4.78 per 100 patient-years) (Table 2).

Table 1. Patient Baseline Characteristics and C4dA IHC status

	All (N=126)	C4dA IHC negative (N=105)	C4dA IHC positive (N=21)	*P* value	Association
Clinical Data					
Age (years)	42 ± 15	43 ± 16	39 ± 10	0.2*	
Female	47 (37.3%)	38 (36.2%)	9 (42.9%)	0.6 ¥	
Hypertension					
Hypertensive at biopsy	74 (58.7%)	59 (56.2%)	15 (71.4%)	0.2¥	
Previous Hypertension	44 (34.9%)	35 (33.3%)	9 (42.9%)	0.5¥	
Mean Arterial Pressure	99.4 ± 15.2	97.6 ± 13.2	108.3 ± 21.0	0.03*	0.069(b)
RAS Blockade	68 (54%)	58 (55.2%)	10 (47.6%)	0.5	
Previous macroscopic hematuria	44 (34.9%)	38 (36.2%)	6 (28.6%)	0.6¥	
eGFR (ml/min per $1.73m^2$)	69.1 ± 40.5	74.1 ± 40.1	44.0 ± 33.3	0.002*	0.078(b)
Stage 1	40 (31.7%)	39 (37.5%)	1 (4.8%)	0.003¥	0.354(c)
Stage 2	35 (27.8%)	29 (26.9%)	6 (28.6%)		
Stage 3	26 (20.6%)	22 (21.2%)	4 (19.0%)		
Stage 4	11 (8.7%)	7 (6.7%)	4 (19.0%)		
Stage 5	14 (11.1%)	8 (7.7%)	6 (28.6%)		
Proteinuria (g/day)	1.83 (0.95-3.43)	1.65 (0.9-2.71)	3.48 (1.6-5.2)	0.01**	0.051(a)
Serum IgA (mg/dL)	297 (231-391)	310 (249-404)	249 (225-302)	0.01**	0.064(b)
Serum C3:C4 ratio	4.10 (3.22-4.81)	4.29 (3.45-4.83)	3.23 (2.53-3.64)	0.005**	0.049(a)
Dyslipidemia	45 (35.7%)	34 (32.4%)	11 (52.4%)	0.1¥	
Statin therapy	31 (24.6%)	26 (24.8%)	5 (23.8%)	0.9¥	
Smoker	21 (16.7%)	17(16.2%)	4 (19.0%)	0.9¥	
Histological Data					
IgM positivity	55 (44%)	44 (41%)	11 (52%)	0.4 ¥	
IgG positivity	29 (23%)	20 (19%)	9 (43%)	0.02¥	0.221(a)
C3 positivity	93 (74%)	74 (71%)	19 (91%)	0.04¥	0.189(a)
C1q positivity	18 (14%)	14 (13%)	4 (19%)	0.5 ¥	
M1 score	105 (83%)	87 (83%)	18 (86%)	0.9 ¥	
E1 score	12 (10%)	10 (10%)	2 (10%)	0.9 ¥	
S1 score	62 (49%)	50 (48%)	12 (57%)	0.4 ¥	

Table 1. Continued

	All (N=126)	C4dA IHC negative (N=105)	C4dA IHC positive (N=21)	*P* value	Association
T score				0.006¥	0.289[(b)]
T1 score	34 (27%)	26 (25%)	8 (38%)		
T2 score	5 (4%)	2 (2%)	3 (14%)		
Crescents	11 (9%)	10 (10%)	1 (6%)	0.7¥	
Arterial Intimal Fibrosis	62 (49%)	47 (41%)	15 (71%)	0.03	0.191[(a)]
Arteriolar hyalinosis	48 (38%)	38 (36%)	10 (48%)	0.09	
Microangiopathic lesions	36 (29%)	28 (27%)	8 (38%)	0.1 ¥	
Acute	23 (18%)	18 (17%)	5 (24%)	0.3 ¥	
Chronic	20 (16%)	13 (12%)	7 (33%)	0.01	0.259[(a)]
C4dG IHC positivity	35 (28%)	19 (18%)	16 (76%)	<0.001	0.482[(b)]

Hypertension was defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg or usage of anti-hypertensive medication. Previous Hypertension – Hypertension diagnosed 1 year before Nephrology consultation. RAS=Renin-angiotensin system. Dyslipidemia is defined as the presence of either Total Cholesterol ≥ 200mg/dL, LDL-C ≥ 130 mg/dL, HDL-C < 40mg/dL in men, and < 50mg/dL in women, Triglycerides ≥ 150mg/dL, or current use of antidyslipidemic medication. M1, S1, E1, T1-2 and C1-2 according to Oxford MEST-C Classification. *t-test; ** Mann-whitney; ¥ Chi-square test or Fisher exact test. Values are expressed as number (%), mean±SD or median (interquartile range; non-normally distributed variables). Association is measured with eta-squared or Cramér's V coefficient and interpreted as (a) small, (b) medium and (c) large, as previously described.[34,35]

Table 2. Duration of follow-up according to CKD Stages.

CKD Stages*	Duration of follow-up (years)
All	4 (2 - 6)
Stage 1	*5 (3 – 9)*
Stage 2	*5 (4 – 7.2)*
Stage 3	*4 (2 – 5.8)*
Stage 4	*1 (1 – 4)*
Stage 5	*0.3 (0.1 – 0.6)*

*CKD – Chronic Kidney Disease. CKD Stages according to Kidney Disease: Improving Global Outcomes (KDIGO) classification. Values are expressed as median (interquartile range).

Regarding baseline characteristics, older age, hypertension, higher mean arterial pressure (MAP), low serum C3:C4 ratio, lower eGFR, higher proteinuria, dyslipidemia, and smoking were all associated with progressive kidney disease (Table 3).

In histologic examination according to the Oxford classification, 83.3%, 49.2%, and 9.5% of patients' biopsy specimens were scored M1, S1, and E1, respectively; 27% of the biopsies were scored as having T1, and 4% as T2. C1 and C2 were present in 11 patients (8.7%), but only one case was classified as C2. Therefore, we aggregated the variable to the presence or absence of crescents, as previously described.[22,33] Immunofluorescence data of kidney biopsies showed positivity for IgM in 43.7%, IgG in 23.0%, C3 in 73.8%, and C1q in 14.3%. IgM positivity, E1, T1, T2, and crescents were positively associated with progressive kidney disease (Table 3).

Among vascular lesions, acute, chronic and general microangiopathic lesions were present in 18.3%, 15.9%, and 28.6% of cases, respectively. Furthermore, arterial intima fibrosis and arteriolar hyalinosis were found in 49% and 38% of patients, respectively. All vascular lesions were associated with progressive kidney disease (Table 3).

Table 3. Associations of baseline characteristics with progressive kidney disease (decline of at least 50% in the eGFR or progression to ESKD during the follow-up period).

Clinical and Histological data	HR to Progressive kidney disease (95%CI)	*P* value
Clinical Data		
Age (per year)	1.027 (1.004 – 1.050)	0.02
Female gender (n, %)	1.375 (0.651 – 2.904)	0.4
Hypertension*		
Hypertensive at biopsy	7.722 (2.350 – 25.372)	0.001
Mean Arterial Pressure** (per 1mmHg)	1.053 (1.033 – 1.072)	<0.001
Previous Hypertension	2.429 (1.211 – 4.872)	0.01
RAS blockade	1.703 (0.772 – 3.330)	0.1
Previous macroscopic hematuria	0.721 (0.328 – 1.583)	0.4
eGFR** (per 1ml/min per 1.73m^2)	0.942 (0.926 – 0.958)	<0.001
Proteinuria** (per 1g/day)	1.296 (1.169 – 1.436)	<0.001
Serum IgA** (per 1mg/dL)	0.997 (0.995 – 1.002)	0.2
Serum C3:C4** (per unit of ratio)	0.632 (0.437 – 0.915)	0.02
Dyslipidemia	2.069 (1.032 – 4.149)	0.04
Statin therapy	1.481 (0.701 – 3.135)	0.3
Smoker	3.574 (1.732 – 7.375)	0.001
Histological Data		
IgM positivity	2.137 (1.044-4.372)	0.04
IgG positivity	0.935 (0.402 – 2.172)	0.9
C3 positivity	1.345 (0.549 – 3.294)	0.5

Table 3. Continued

Clinical and Histological data	HR to Progressive kidney disease (95%CI)	*P* value
C1q positivity	0.931 (0.326 – 2.664)	0.9
M1 score	1.249 (0.436 – 3.576)	0.7
S1 score	0.822 (0.410 – 1.649)	0.6
E1 score	3.189 (1.307 – 7.781)	0.01
T0 score	*Reference category*	
T1 score	15.449 (5.860 – 40.725)	<0.001
T2 score	26.252 (7.007 – 98.358)	<0.001
Crescents	4.857 (2.079 – 11.349)	<0.001
Arterial Intima Fibrosis	3.418 (1.532 – 7.629)	0.003
Arteriolar hyalinosis	4.355 (1.681 – 11.282)	0.002
Microangiopathic lesions	3.930 (1.777 – 8.691)	0.001
Acute microangiopathy	2.889 (1.307 – 6.388)	0.009
Chronic microangiopathy	2.983 (1.353 – 6.576)	0.007
C4dG IHC positivity	2.627 (1.311– 5.265)	0.006
C4dA IHC positivity	4.024 (1.962 – 8.250)	<0.001

* Defined as defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg or usage of anti-hypertensive medication. **Quantitative variables. Previous Hypertension – Hypertension diagnosed 1 year before Nephrology consultation. RAS=Renin-angiotensin system. Dyslipidemia was defined as the presence of either TC ≥ 200 mg/dL, LDL-C ≥ 130 mg/dL, HDL-C < 40 mg/dL in men, and < 50 mg/dL in women, Triglycerides ≥ 150 mg/dL, or current use of antidyslipidemic medication. M1, S1, E1, T1, T2, according to Oxford MEST-C Classification. HR – Hazards Ratio.

Associations of C4dA and C4dG with baseline characteristics

Arteriolar C4d positivity (C4dA IHC) was present in 21 patients (17%; Fig 1). Furthermore, C4dA IHC was significantly associated with lower eGFR, serum IgA, serum C3:C4 ratio and higher proteinuria and MAP, as well as with the presence of IgG, C3, T1 and T2, arterial intima fibrosis, chronic microangiopathy and C4dG IHC in the biopsy (Table 1). Based on previously proposed criteria,[34,35] the strength of association of C4dA IHC with eGFR, MAP, IgA and T1 and T2 is medium, whereas for the other variables it is considered small.

Regarding microangiopathic lesions, four cases presented with C4dA as well as acute and chronic microangiopathic lesions (CKD stages 2, 3, 4 and 5). Three C4dA positive cases had chronic, but not acute, microangiopathic arteriolar lesions (CKD stages 2, 4, and 5), while only one case presented with C4dA positivity and acute, but not chronic, microangiopathic lesions (CKD stage 4).

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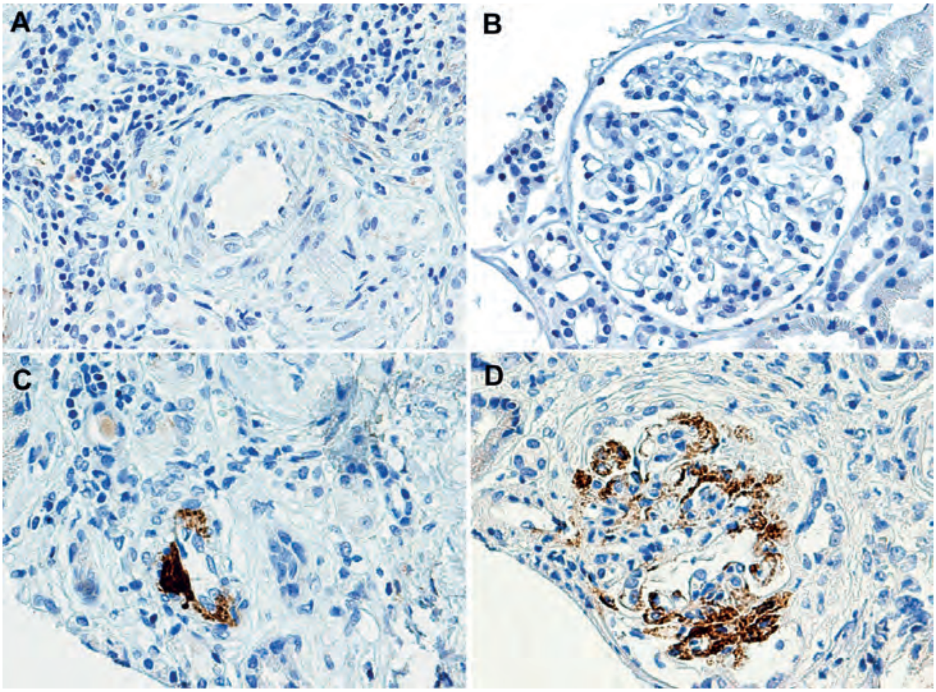
Patients with positive C4dG IHC had significantly higher baseline proteinuria compared with patients without (Table 4). Furthermore, C4dG IHC was associated with the presence of IgM, IgG, T1, and T2 in the biopsy (Table 4).

Figure 1



Kidney tissue from patients with Immunoglobulin A nephropathy (IgAN) was stained for the presence of C4d through immunohistochemistry (IHC). Both glomerular (C4dG) and arteriolar (C4dA) staining was evaluated. (A) Negative C4dA IHC, (B) Negative C4dG IHC, (C) Positive C4dA IHC, and (D) Positive C4dG IHC.

Table 4. Patient Baseline Characteristics and C4dG IHC status

	C4dG IHC negative N=91	**C4dG IHC positive N=35**	***P*-value**	**Association**
Clinical Data				
Age (years)	42±17	39±10	0.1*	
Female	31(34.1)	16(45.7)	0.2 ¥	
Hypertension				
Hypertensive at biopsy	53(58.2)	20(57.1)	0.9¥	
Previous Hypertension	32(35.2)	11(31.4)	0.7¥	
Mean Arterial Pressure	98.36±14.49	102.13±17.02	0.2 *	

Table 4. Continued

	C4dG IHC negative N=91	C4dG IHC positive N=35	*P*-value	Association
RAS Blockade	49(53.8)	18(51.4)	0.8¥	
Previous macroscopic hematuria	35(38.5)	9(28.1)	0.1¥	
eGFR (ml/min per 1.73m^2)	73.61 ± 41.30	58.01 ± 37.16	0.05*	
Stage 1	32(35.2)	8(22.9)	0.1 ¥	
Stage 2	27(29.7)	8(22.9)		
Stage 3	19(20.9)	7(20.0)		
Stage 4	5(5.5)	6(17.1)		
Stage 5	8(8.8)	6(17.1)		
Proteinuria (g/day)	1.54(0.75-2.75)	2.7(1.44-4)	0.01**	0.178(a)
Serum IgA (mg/dL)	302(234-389)	288(220-401)	0.6**	
Serum C3:C4 ratio	4.25(3.44-4.91)	3.66(2.6-4.68)	0.07**	
Dyslipidemia	30(33.0)	14(40.0)	0.5¥	
Statin therapy	22(24.2)	5(14.3)	0.9¥	
Smoker	15(16.5)	7(20)	0.6¥	
Histological Data				
IgM positivity	32(35.2)	23(25.3)	0.003 ¥	0.266 (a)
IgG positivity	15(16.5)	14(40.0)	0.008¥	0.240 (a)
C3 positivity	65(71.4)	27(77.1)	0.4¥	
C1q positivity	10(11.0)	8(22.9)	0.1 ¥	
M1 score	74(81.3)	30(85.7)	0.7¥	
E1 score	8(8.8)	4(11.4)	0.7 ¥	
S1 score	43(47.2)	18(51.4)	0.7 ¥	
T score			0.002¥	0.338(b)
T1 score	20(22.0)	14(40.0)		
T2 score	1(1.1)	4(11.4)		
Crescents	8(8.8)	3(8.6)	0.9¥	
Arterial Intimal Fibrosis	43(47.3)	18(51.4)	0.8¥	
Arteriolar hyalinosis	32(35.2)	15(42.9)	0.3¥	
Microangiopathic lesions	25(27.5)	11(31.4)	0.6 ¥	
Acute microangiopathy	16(17.6)	7(20.0)	0.7 ¥	
Chronic microangiopathy	13(14.3)	7(20.0)	0.4¥	
C4dA IHC positivity	5(5.5)	16(76.2)	<0.001¥	0.482(b)

Hypertension was defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg or usage of anti-hypertensive medication. Previous Hypertension diagnosed 1 year before Nephrology consultation. RAS=Renin-angiotensin system. Dyslipidemia was defined as the presence of either Total Cholesterol ≥ 200mg/dL, LDL-C ≥ 130 mg/dL, HDL-C < 40mg/dL in men, and < 50mg/dL in women, Triglycerides ≥ 150mg/dL, or current use of antidyslipidemic medication. M1, S1, E1, T1-2 and C1-2 according to Oxford MEST-C Classification. *t-test; ** Mann-whitney; ¥ Chi-square test or Fisher exact test. Values are expressed as number (percent), mean±SD or median (interquartile range; non-normally distributed variables). Association is measured with eta-squared or Cramér's V coefficient and interpreted as (a) small, (b) medium and (c) large. [34,35]

Associations of C4dA and C4dG with outcomes.

C4dA IHC was associated with progressive kidney disease (Table 3). This effect remained significant after adjusting for microangiopathic lesions, arterial intima fibrosis, arteriolar hyalinosis and proteinuria, separately (Table 5). Kaplan–Meier plots (Figure 2) show the longer survival for patients negative for both C4dA and C4dG IHC, compared with patients positive for 1 of these markers, or for both (Fig 2, P<0.001 by log-rank test).

Table 5. Multivariable Cox regression analysis – association of C4dA IHC, as compared to C4dG IHC, with progressive state of kidney disease (decline of at least 50% in the eGFR or progression to ESKD during the follow-up period).

	C4dA IHC			**C4dG IHC**		
	HR (95%CI)	***P* value**	**AIC**	**HR (95%CI)**	***P* value**	**AIC**
Model 1	4.024 (1.962-8.250)	<0.001	281.19	2.627 (1.311-5.265)	0.006	286.36
Model 2	3.531 (1.608-7.752)	0.002	230.05	1.382 (0.639-2.989)	0.4	238.17
Model 3	2.381 (1.006-5.634)	0.05	194.03	1.798 (0.807 – 4.008)	0.2	195.70
Model 4	3.179 (1.331-7.592)	0.009	194.83	2.135 (0.988-4.613)	0.05	197.51

Data are presented as hazard ratio (HR) plus 95% confidence interval and Akaike Information Criterion (AIC)

Model 1: crude (C4dA IHC or C4dG IHC)
Model 2: adjusted for histological biomarkers: T1, T2 and Crescents
Model 3: adjusted for clinical variables: smoking, eGFR and MAP at the time of biopsy
Model 4: adjusted for baseline variables: Crescents, MAP and eGFR

Table 3 displays all variables that were significantly associated with progressive kidney disease in Cox regression analyses. Multivariable models were constructed to evaluate and compare the associations of C4dA and C4dG IHC with progressive kidney disease, while controlling for other significant variables. Following the first approach described in the “Materials and Methods” section for multivariable analysis, the final model included the variables eGFR, IgM positivity, MAP, crescents, and C4dA IHC, but not C4dG IHC (Table 6). The second approach described in the “Materials and Methods” section for multivariable analysis resulted in the models presented in Table 5. Both C4dA and C4dG IHC were associated with progressive kidney disease (crude model—Model 1). In the final model (Model 4), C4dA IHC remained significant, while C4dG IHC did not (p=0.054), and the AIC was slightly lower for the C4dA IHC model. Harrell’s concordance indexes were calculated for both final models, and their values were validated using bootstrapping. Slightly higher values were obtained for both the C4dA IHC model (0.950; 95% CI: 0.923–0.974) than for the C4G IHC model (0.943; 95% CI: 0.926–0.970), but without reaching a statistically significant difference. Models 4 for C4dA IHC and C4dG IHC in Table 5 were re-estimated with patients with a follow-up of longer than 1 year. In these models the significance of C4dA (HR=3.99; 95% CI: 1.25–12.85, p=0.02), but not C4dG IHC (HR=2.30; 95% CI: 0.91–5.83, p=0.08), was maintained.

Furthermore, likelihood ratio tests were used to compare crude models (simple Cox models with C4dA or C4dG) with the model that included both C4dA and C4dG as covariates. The rejection of H_0 when comparing the simple C4dG model with the model that included both markers (P=0.01) indicates that C4dA offers a statistically significant improvement in the survival model with C4dG alone. However, when comparing the C4dA-only model with the 2-marker model, the test indicates that given the information provided by C4dA IHC, the added value of C4dG IHC is not statistically significant (P=0.3).

3

Table 6. Univariable and multivariable analyses with progressive kidney disease

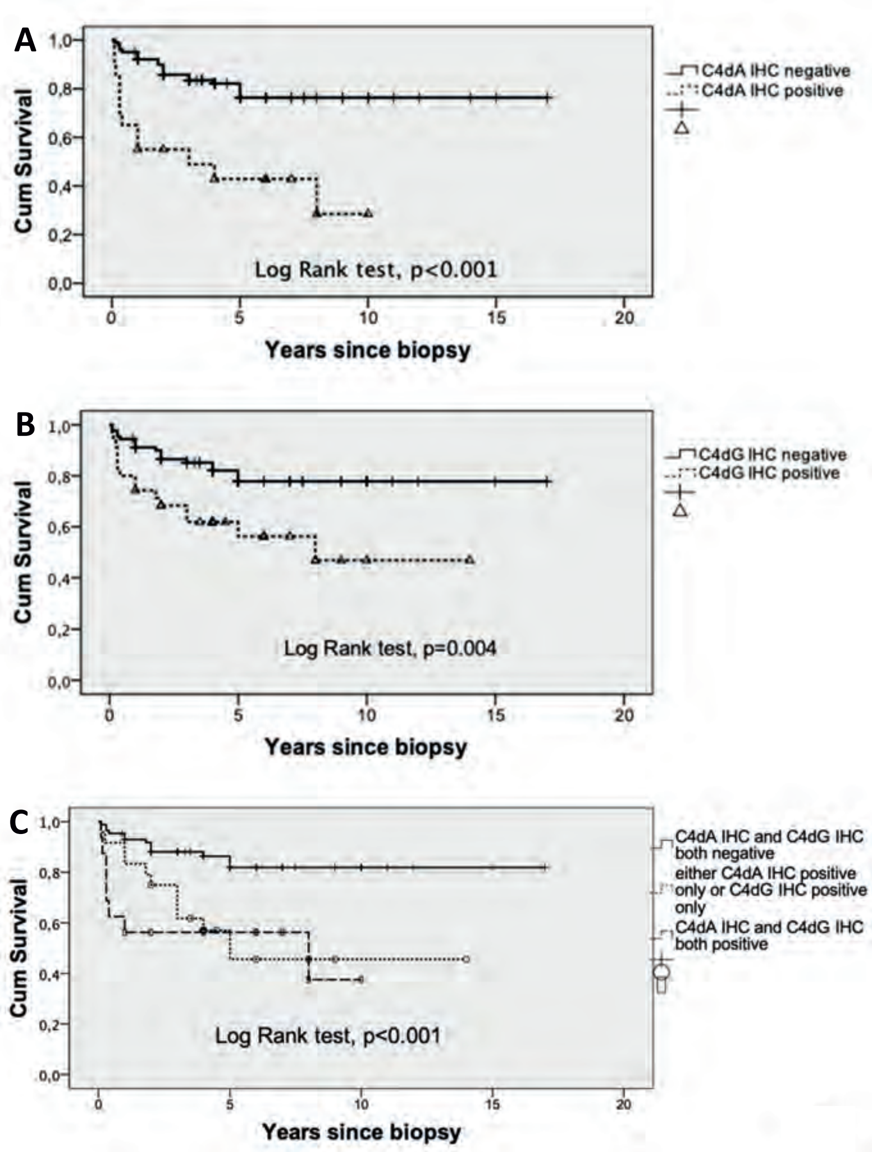
	HR to Progressive kidney disease (95%CI)	***P* value**
Univariable Age (per year)	1.027 (1.004 – 1.050)	0.02
Hypertension*		
Hypertensive at biopsy	7.722 (2.350 – 25.372)	0.001
Mean Arterial Pressure (per 1mmHg)	1.053 (1.033 – 1.072)	<0.001
Previous Hypertension	2.429 (1.211 – 4.872)	0.01
eGFR (per 1ml/min per 1.73m^2)	0.942 (0.926 – 0.958)	<0.001
Proteinuria (per 1g/day)	1.296 (1.169 – 1.436)	<0.001
Serum C3:C4 (per unit of ratio)	0.632(0.438 – 0.913)	0.02
Dyslipidemia	2.069 (1.032 – 4.149)	0.04
Smoker	3.574(1.732 – 7.375)	0.001
IgM positivity	2.137(1.044-4.372)	0.04
E1 score	3.189 (1.307 – 7.781)	0.01
T0 score	*Reference category*	
T1 score T2 score	15.449 (5.860 – 40.725) 26.252 (7.007 – 98.358)	<0.001 <0.001
Crescents Arterial Intimal Fibrosis	4.857 (2.079 – 11.349) 3.418 (1.532 – 7.629)	<0.001 0.003
Arteriolar hyalinosis	4.355 (1.681 – 11.282)	0.002
Microangiopathic lesions	3.930 (1.777 – 8.691)	0.001
Acute microangiopathy	2.889(1.307 – 6.388)	0.009

Table 6. Continued

	HR to Progressive kidney disease (95%CI)	*P* value
Chronic microangiopathy	2.983 (1.353 – 6.576)	0.007
C4dG IHC positivity	2.627 (1.311– 5.265)	0.006
C4dA IHC positivity	4.024 (1.962 – 8.250)	<0.001
Multivariable		
eGFR (ml/min per 1.73m^2)	0.932 (0.910 – 0.954)	<0.001
IgM positivity	2.812 (1.242 – 6.368)	0.01
MAP	1.030 (1.007 – 1.053)	0.01
Crescents	5.098 (1.823 – 14.257)	0.002
C4dA IHC positivity	3.294 (1.369 – 7.924)	0.008

Univariable and multivariable Cox regression analyses with progressive kidney disease in IgAN patients. Progressive kidney disease was defined as a decline of at least 50% in the eGFR or progression to end-stage renal disease during the follow-up period. *Defined as defined as systolic blood pressure greater than 140 mmHg and/or diastolic blood pressure greater than 90 mmHg or usage of anti-hypertensive medication. Previous Hypertension – Hypertension diagnosed 1 year before Nephrology consultation. RAS=Renin-angiotensin system. Dyslipidemia was defined as the presence of either TC ≥ 200mg/dL, LDL-C ≥ 130 mg/dL, HDL-C < 40mg/dL in men, and < 50mg/dL in women, triglycerides ≥ 150mg/dL, or current use of antidyslipidemic medication. M1, S1, E1, T1, T2, according to Oxford MEST-C Classification.

Figure 2



Kaplan–Meier analysis of kidney survival according to arteriolar C4d (C4dA) immunohistochemistry (IHC) and glomerular C4d (C4dG) IHC. (A) C4dA-negative versus C4dA-positive. (B) C4dG-negative versus C4dG-positive. (C) Both C4dA- and C4dG-negative versus either C4dA-positive only or C4dG-positive only versus both C4dA- and C4dG-positive. Kidney survival defined as not reaching the kidney disease progression end point (decline of at least 50% in glomerular filtration rate or progression to kidney failure).

DISCUSSION

In IgAN, accurate risk stratification for kidney disease progression at diagnosis remains a challenge. The major finding of the current study is that arteriolar C4d deposition is associated with disease progression in IgAN. Moreover, the association of C4dA with progressive kidney disease was observed in both unadjusted and adjusted models using two different approaches. Extending these findings, C4dA had a mildly stronger association with disease progression than did C4dG. These results provide evidence that C4dA in the kidney biopsy specimen is a novel biomarker for IgAN progression.

Deposition of C4d is a widely used biomarker for complement activation because C4d has a long half-life given that it remains covalently bound to surfaces.[36] Although recently challenged, [37,38] the paradigm of C4d as a split product without any described physiological activity or specific receptor still remains. In IgAN, C4dG is the result of activation of the lectin pathway; however, this has not been proven for C4dA. The lectin pathway has recently been shown to be activated by the cholesterol crystals in atherosclerosis, providing a possible explanation for C4dA.[39] Other potential triggers for vascular C4d deposition include microvascular injury due to hypertension,[40] and intravascular cellular debris or injured endothelium.[23] However, a role for the classical pathway has recently been suggested because C1q and C4d co-localized in glomerular capillaries and arterioles of IgAN biopsies.[24] We did not find this co-localization in glomeruli or arterioles. Nevertheless, C4dG IHC positivity, but not C4dA IHC, was associated with IgM deposition, suggesting complement activation by immunocomplexes in glomeruli but not in vessels. Further studies should focus on whether C4dA is a marker of immune complex-mediated injury in IgAN, or if it is the consequence of nonspecific pro-atherosclerotic stimuli and/or vascular endothelial injury due to progression of CKD.

The strength of C4dA IHC was tested against the most potent clinical and histological prognostic variables in this cohort. A previously large cohort study established a prediction model for disease progression in IgAN at the time of kidney biopsy.[33] In line with these findings, the current study used most of the risk factors previously described for the regression analysis and C4dA remained significantly associated with outcome after adjustment. This association was also maintained in a subgroup analysis of patients with at least 1-year follow-up, thereby excluding very advanced cases to mitigate the possibility that C4dA is a marker of advanced damage. After multivariable modeling, eGFR, MAP, and crescents also remained in both final models, but not proteinuria. In IgAN, proteinuria is a well-studied risk factor for progression to ESKD.[33,41] However, the STOP-IgAN trial underscores the finding that progression is not solely dependent on proteinuria.[5]

Besides confirming the association between C4dG IHC and progressive disease,[7,8,30] the current study also compared the use of C4dA IHC with C4dG IHC as biomarkers in IgAN. C4dG IHC and C4dA IHC were both associated with kidney disease outcomes, but C4dA IHC had a stronger association with progressive kidney disease than did C4dG IHC. Importantly, because C4dG is widely accepted as a prognostic marker in IgAN, C4dA analysis could be easily added without the need for another procedure while still improving its predictive power.

The vascular compartment is not included in the Oxford Classification for IgAN because it was shown to not predict kidney disease outcomes in the original and validation cohorts.[16,27] However, vascular lesions, such as arterial intima fibrosis, arteriolar hyalinosis and microangiopathy, have recently been shown to be associated with worse outcome.[17,22,24] In particular, microangiopathic lesions have been proposed to be incorporated in the classification.[21,22] Interestingly, C4d has been shown to be a reliable general marker for TMA in various kidney diseases,[23] including IgAN.[24] Our results regarding the prevalence and significance of microangiopathy in IgAN are in line with those of previous studies.[22,24] Although C4d showed a correlation with microangiopathic lesions in this study, the concept of C4d as a common denominator in TMA could not be demonstrated. However, these differences might also be explained by the relatively lower number of acute lesions in our study and the fact that our evaluation for microangiopathic lesions focused only on arterioles and not on the combination with glomeruli as in these previous studies.[23,24] In accordance with others,[17] our data show that arteriolar hyalinosis and arterial intima fibrosis predict progressive disease in IgAN. Notably, C4dA remained associated with outcome even after adjusting for the presence of these vascular lesions. Altogether, the current study demonstrates the importance of vascular disease in IgAN and underscores the rationale that the clinical approach should go beyond proteinuria (as well as eGFR and hypertension) analysis to manage this disease.[42]

The current study has both limitations and strengths. This is a single-center retrospective cohort study; furthermore, very advanced IgAN cases were included, limiting the long-term predictive value of C4dA IHC. Nevertheless, in a subanalysis without these cases, C4dA remained significantly associated with kidney disease outcome. In addition, we did not determine the incremental value of C4dA over validated risk-prediction models for disease progression due to the limited sample size and patient heterogeneity.[33] Moreover, the underlying mechanism for C4d deposition in vessels remains to be established. We acknowledge that the current findings are limited to detection of C4d by IHC and therefore cannot be fully extrapolated to the presence of the molecule per se. In fact, immunofluorescence for C4d detection remains the current gold standard. However, although differences may exist between IHC and immunofluorescence, these might not be as significant as generally claimed.[30,43,44] Last, due to the focal nature of C4d deposition, potential misclassification cannot be excluded. However, the strengths of the current study include histological assessment by 3 masked independent experts, use of a hard and clinically relevant outcome (disease progression), and the extensive statistical analysis that involved 2 different approaches for C4d analysis in IgAN.

In conclusion, our study shows that C4dA IHC deposition can be used as a biomarker for IgAN progression. These findings offer new options for the inclusion of C4d staining in biomarker panels in IgAN and provide new data for the importance of the vascular compartment in this disease. We propose that C4d analysis in IgAN not be restricted to glomeruli but extended to vessels because this is a powerful, inexpensive, and easy-to-perform biomarker of disease progression.

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PART B

The Complement System in Dialysis