ORIGINAL ARTICLE

Chronic-active antibody-mediated rejection with or without donor-specific antibodies has similar histomorphology and clinical outcome – a retrospective study

Kasia A. Sablik[1], Marian C. Clahsen-van Groningen[2], Caspar W. N. Looman[3], Jeffrey Damman[2], Dave L. Roelen[4], Madelon van Agteren[1] & Michiel G. H. Betjes[1]

1 Department of Nephrology and Transplantation, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
2 Department of Pathology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
3 Department of Biostatistics, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
4 Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands

Correspondence
Kasia A. Sablik MD, Department of Nephrology & Transplantation, Erasmus Medical Center, Room Na508, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands.
Tel.: +31 10 7030840;
fax: +31 10 703 40 94;
e-mail: k.sablik@erasmusmc.nl

SUMMARY

Chronic-active antibody-mediated rejection (c-aABMR) is defined as histological evidence of chronic endothelial injury (cg), also known as transplant glomerulopathy, and either microvascular inflammation (MVI) or positivity for C4d. Importantly, the presence of donor-specific antibodies (DSA) is currently still mandatory for the diagnosis of c-aABMR. This retrospective study of 41 c-aABMR patients investigates whether cases suspicious for c-aABMR (DSA negative, $n = 24$) differ from cases of c-aABMR (DSA positive, $n = 17$) with respect to renal histology, allograft function and long-term graft survival. All included patients had progressive loss of allograft function and were diagnosed by for cause biopsy and scored according to the Banff '15 criteria. In all DSApos cases, DSA were *de novo* and the majority was directed against HLA-II being mostly anti-HLA-DQ antibodies. There were no statistically significant differences in clinical characteristics, decline in allograft function and renal allograft survival in cases with or without DSAs. All cases showed chronic histomorphological damage and inflammation, irrespective of the presence of DSA. Renal histology and clinical outcome of patients suspicious for c-aABMR (DSAneg) do not significantly differ from patients with a diagnosis of c-aABMR (DSApos). We believe that our study adds to the ongoing debate regarding the need for DSAs to be present for the diagnosis of c-aABMR.

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Introduction

Chronic-active antibody-mediated rejection (c-aABMR) is currently defined by the Banff classification by (i) histologic evidence of chronic tissue injury, (ii) evidence of current or recent antibody interaction with vascular endothelium and (iii) serologic evidence of donor-specific antibodies (DSA) [1–7]. In the clinical setting, however, it is not uncommon for these diagnostic criteria to appear as an incomplete combination. This is largely due to the requirement of DSA positivity as DSA cannot be detected in a substantial number of patients [8–10].

Cases in which renal histology show chronic endothelial activation as evidenced by glomerular basement

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membrane duplication, known as transplant glomerulopathy (TG), and C4d-positive staining and/or significant microvascular inflammation (MVI) with no detectable DSA are considered suspicious for c-aABMR [9,11–13]. This diagnosis leaves clinicians uncertain about the accuracy of the diagnosis and treatment options for these DSA-negative cases, as previously reported by Halloran *et al.* [14]. Furthermore, those cases classified as suspicious for c-aABMR are frequently excluded from clinical trials, resulting in scarce data about the clinical significance of DSA seropositivity [15–18].

In this study, we investigated whether allograft outcome and renal histomorphology differed between cases suspicious for c-aABMR (DSAneg) and cases with c-a-ABMR (DSApos).

Patients and methods

Study population

We retrospectively included all patients with the histomorphological diagnosis of chronic-active antibody-mediated rejection upon for cause biopsy which was performed after a progressive decline in renal allograft function. Patients were identified from the pathology database at our centre between January 2007 and November 2014.

Patients were excluded if the biopsy was within the first-year post-transplantation (inaccurate information about renal allograft function) or the requirement of a minimum of 5 eGFR measurements per year was not met. Patients were divided into two groups based on the presence of detectable DSA in their serum at time of biopsy. Upon diagnosis, all patients, regardless of DSA status (which was unavailable at time of diagnosis), received similar treatment with three doses of 1 g intravenous methylprednisolone (MP) over a 3-day period combined with a single dose of intravenous immunoglobulins (IVIG) (1 g/kg body weight) on the second day of treatment. None of the patients had plasmapheresis. Renal biopsies were re-evaluated and the individual lesions were scored according to the Banff '15 classification by two independent pathologists [7]. In case of differences consensus was reached. Alternative diagnoses for the histomorphological changes compatible with c-aABMR such as hepatitis C virus infection, membranoproliferative glomerulonephritis (MPGN) or (chronic) thrombotic microangiopathy (cTMA) were excluded, by immunofluorescence and clinical analysis [19–23]. C4d was evaluated using immunohistochemical staining of 4 μm slides of formalin fixed paraffin-embedded tissue. Immunofluorescent staining was performed with antibodies against IgG, IgM, IgA, C3c, C1q, kappa and lambda on snap frozen material according to standard diagnostic procedure in our hospital.

Several individual Banff lesions were combined to assess microvascular inflammation and chronicity of the renal parenchyma and vessels. The microvascular inflammation (MVI) score was calculated by combining the glomerulitis and peritubular capillaritis score. A MVI score ≥2 was considered moderate to severe and a diagnostic criterion for c-aABMR. The chronic inflammation score was calculated as (*interstitial fibrosis (ci) + tubular atrophy (ct) + total inflammation (ti)*) as previously described by Patri *et al.* [24].

Renal allograft function

Change in renal allograft function in time was evaluated by including all estimated glomerular filtration rate (eGFR, MDRD) measurements 1 year prior to diagnosis and 1 year after diagnosis for both patient groups [25]. A minimum of five measurements per year at regular intervals was required to give a reliable estimate over time of the decrease in renal allograft function. eGFR measurements influenced by antibiotic treatment, i.v. fluids or other potential factors were excluded from the analysis due to misleading representation of allograft function. Return to dialysis or retransplantation was considered allograft failure.

Characterization of anti-HLA antibodies

All patients were transplanted with a CDC-negative cross-match. To determine the definite diagnosis of each patient, serum samples at the time of biopsy were re-evaluated and tested for the presence of donor-specific antibodies against HLA (DSA). If DSA were found to be present, it was determined whether this concerned *de novo* DSA. If samples were found to be negative, additional screening was performed by analyzing serum samples for DSA in the 2 years prior to diagnosis.

Patient serum samples were screened for the presence or absence of HLA antibodies using the Luminex Screening Assay: Lifecodes Lifescreen Deluxe (LMX) kit, according to the manufacturer's manual (Immunocor Transplant Diagnostics Inc., Stamford, CT, USA). Samples that were considered positive for either HLA class I (HLA-A or HLA-B or HLA-C) or HLA class II (HLA-DQ or HLA-DR) antibodies were further analyzed with a Luminex Single Antigen assay, using LABscreen HLA class I and class II antigen beads (One Lambda, Canoga Park, GA, USA).

Statistical analysis

The characteristics between the DSApos and DSAneg groups were compared using unpaired *t* test for continuous variables, Mann–Whitney *U* test for ordinal variables and chi-square or Fisher exact test for categorical variables. Similarly, a subgroup analysis between DSAneg C4d+ and DSAneg C4d− patients was performed. The histomorphological lesions were analysed as categorical variables with medians and range to provide accurate information on the variety of lesions. Allograft survival was assessed using censored Kaplan–Meier curves and log rank test. Patient follow-up was a minimum of 12 months or until allograft failure. Assessment of changes in renal allograft function over time was performed by multilevel analysis [26].

For the analysis of influence of histological characteristics on graft survival, the Banff classification was divided into two categories. The Banff scores 0 and 1 were considered minimal to mild lesions and the scores 2 and 3 were considered moderate to severe lesion with the exception of v-lesions and C4d staining which were either positive or negative.

All statistical analysis was performed using the R statistical programming environment and SPSS software version 21. A *P*-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Patient characteristics

We identified 24 patients with the diagnosis of suspicious for c-aABMR (DSAneg) and 17 with c-aABMR (DSApos). The demographic and clinical characteristics for both groups of patients are summarized in Table 1.

Baseline characteristics did not differ significantly between the two groups. The diagnosis was made on average 77 months after transplantation (80 months DSApos vs. 75 months DSAneg, $P = 0.76$). There was, a noteworthy difference of almost 1 g/l more proteinuria in the DSApos patients, although this was not statistically significant ($P = 0.15$). Of interest, no significant differences were found between DSAneg C4d+ and DSAneg C4d− patients (data not shown).

Table 1. Demographic and clinical characteristics.

	DSA positive ($n = 17$)	DSA negative ($n = 24$)	*P*-value
Age, years	49	51	0.64
Gender, *n*			
Female	5	10	
Male	12	14	0.52
Donor type, *n*			
Post mortal	5	7	0.99
Living	12	17	
Donor age, years	45	50	0.27
Previous transplantation, *n*			
Yes	5	12	0.19
No	12	12	
Time to diagnosis, months	80	75	0.76
Total HLA mismatch, mean	3.1	3.4	0.52
PRA current, mean	2.4	4.0	0.55
PRA historical, median	12.1	22.8	0.29
Previous BPAR, *n*			
Yes	4	7	0.74
No	13	17	
Proteinuria at biopsy, g/l	2.00	0.99	0.15
eGFR at baseline, ml/min/1.73 m^2	32	30	0.44
Immunosuppressive regimen ($t = 0$), *n*			
TAC	11	21	
CsA	1	1	
MMF	16	21	
Prednisone	7	6	
Other	1	2	
Renal disease			
Diabetic nephropathy	3	2	
Hypertensive nephropathy	3	4	
IgA nephropathy	1	0	
Hereditary	1	4	
Congenital	3	2	
Other	6	12	

DSA, donor-specific antibody; PRA, panel reactive antibody; BPAR, biopsy-proven acute rejection (incl. borderline changes); TAC, tacrolimus; CsA, cyclosporine A; MMF, mycophenolate mofetil.

Anti-HLA antibodies

Seventeen of the 41 patients were tested positive for circulating DSA at time of biopsy, these cases are included in the c-aABMR (DSApos) group. The vast majority of the circulating DSA detected were directed against HLA-DQ (78%) (Table 2). Except for one patient who had both HLA-DQ and HLA-A antibodies, all other patients only had one specificity of circulating DSA. All circulating DSA were found to be *de novo* DSA. The

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mean fluorescent intensity (MFI) ranged between 2336 and 25 588 with an average MFI of 13 477.

To assess whether DSAneg patients might have had circulating DSA present prior to diagnosis, available serum samples in the 2 years prior to diagnosis of suspicious for c-aABMR were also analyzed for the presence of DSA. A total of 13 of 24 patients had serum samples available in the 2 years prior to diagnosis. For eight of 13 patients, multiple samples were available for testing. One patient tested positive for DSA 1 and 2 years prior to diagnosis, whilst being negative at time of biopsy. The MFI of the DSA (anti-HLA DQ6) decreased from 16 599 2 years prior to biopsy-proven diagnosis to 8331 1 year prior to biopsy.

Histopathological comparison of DSApos and DSAneg cases

All renal biopsies were re-evaluated and the individual lesions were scored according to the Banff 2015 classification [7]. Six patients had an insufficient number of nonsclerotic glomeruli in the biopsy to fulfil the criteria for the scoring of individual lesions according to the Banff classification and were omitted from analysis. A median of 21 glomeruli (18–32) was available per biopsy for the DSApos group of which 13% (5–20%) was globally sclerotic. For the DSAneg group, a median of 13 (10–21) glomeruli was available of which 8% (2–17%) was globally sclerotic. Although the total number of glomeruli was significantly different ($P = 0.02$) between the groups, this was not the case for the percentage of global glomerulosclerosis ($P = 0.22$).

All renal transplant biopsies ($n = 41$) had glomerular basement membrane double contours. The majority of patients in both DSApos and DSAneg cases had severe (cg3) capillary loop involvement. Three patients were diagnosed as a cg1a based on electron microscopic imaging (DSApos $n = 1$; DSAneg $n = 2$). Additionally, all biopsies had a MVI score of more than 2. The DSApos and DSAneg cases showed vast glomerulitis (g3; 86% vs. 95%) in combination with moderate to severe peritubular capillaritis (ptc2-3; 93% vs. 86%). C4d staining was unavailable for one patient (DSApos, $n = 1$). Eight patients were positive upon C4d staining in the DSApos patients (C4d− $n = 8$; unknown $n = 1$), whereas only nine of 24 DSAneg patients were positive upon C4d staining (C4d− $n = 15$). There were four patients (DSApos $n = 1$; DSAneg $n = 3$) with a form of tubulitis ($t \geq 1$) in combination with arteritis. An additional seven patients presented with isolated v-lesions (DSApos $n = 4$; DSAneg $n = 3$).

A comparison between the histomorphological characteristics of the DSApos and DSAneg cases showed no significant differences in the individual Banff lesions score (Table 3). Neither was there a difference in histomorphology between DSAneg C4d+ and DSAneg C4d− patients.

Both the DSApos and DSAneg cases showed a substantial 'chronic inflammation score' of 5 (1–8) and 6 (1–9), respectively. Furthermore, there was considerable presence of MVI. The groups showed no significant differences in the 'chronic inflammation score' or MVI (Table 4) [24].

Table 2. Donor-specific antibodies and mean fluorescent intensity (MFI) values.

	Number of patients	MFI values
DQ1	3	5691
		14 875
		19 464
DQ2	5	17 758
		18 332
		19 016
		20 474
		20 550
DQ3	1*	24 799
DQ4	2	4899
		20 866
DQ7	3	5352
		11 898
		25 588
DP3	1	6443
A2	1	18 996
A9	1*	2336
B60	1	3745

*Patient was positive for both.

Renal allograft function

All patients had a progressive decline in renal allograft function at the time of renal biopsy. The average eGFR at baseline, approx. 31 ml/min/1.73^2, was similar in both patient groups ($P = 0.44$). The DSAneg cases showed a slightly larger average decrease in allograft function of −11.2 (−5.8 to −16.7) ml/min/1.73 m^2/year before diagnosis compared to the DSApos cases who showed a decline of −8.9 (−7.0 to −10.8) ml/min/1.73 m^2/year ($P = 0.06$). After diagnosis and treatment with IVIG/MP both groups showed a more mitigated loss of allograft function than before diagnosis

Table 3. Histomorphological lesions according to the Banff '15 criteria of DSApos and DSAneg cases.

	DSA positive (n = 14)	DSA negative (n = 21)	P-value
Total glomeruli (n, IQR)	21 (18–32)	13 (10–21)	0.02
Global glomerulosclerosis (%, IQR)	13 (5–20)	8 (2–17)	0.22
Interstitial inflammation (range)	2 (0–3)	2 (0–3)	0.80
Tubulitis (range)	0 (0–3)	0 (0–1)	0.78
Arterial inflammation (range)	0 (0–2)	0 (0–2)	0.58
Glomerulitis (range)	3 (1–3)	3 (2–3)	0.63
Peritubular capillaritis (range)	3 (0–3)	2 (0–3)	0.80
Total inflammation (range)	2 (0–3)	3 (0–3)	0.58
Interstitial fibrosis (range)	1 (0–3)	2 (0–3)	0.45
Tubular atrophy (range)	1 (1–3)	1 (0–3)	0.78
Arterial intimal thickening (range)	2 (0–3)	3 (0–3)	0.73
Transplant glomerulopathy (range)	3 (1–3)	3 (1–3)	0.78
Arterial hyalinosis (range)	2 (0–3)	3 (0–3)	0.80
Mesangial matrix (range)	3 (0–3)	3 (0–3)	0.80
C4d peritubular capillaries (range)	0 (0–3)	0 (0–3)	0.39

Table 4. Chronic histomorphological lesions.

	DSA positive (n = 14)	DSA negative (n = 21)	P-value
Microvascular inflammation score (g + ptc) (range)	5 (3–6)	5 (3–6)	0.80
Chronic inflammation score (ci + ct + ti) (range)	5 (1–8)	6 (1–9)	0.58

($P < 0.001$). The response to treatment was similar regardless of DSA status (change in renal allograft loss: DSAneg: −5.5 ml/min/1.73 m^2/year vs. DSApos: −5.4 ml/min/1.73 m^2/year ($P = 0.93$) (Fig. 1). Similarly, there was no significant difference in response to therapy upon comparison of DSAneg C4d+ and DSAneg C4d− patients.

There was no significant difference (log rank; $P = 0.93$) in overall graft survival between DSApos and DSAneg cases (12.2 vs. 13 years, respectively) (Fig. 2). In addition, there was no significant difference in survival after diagnosis with an average survival of 4.6 years in the DSApos cases vs. 3.7 years in the DSAneg cases (log rank; $P = 0.58$) (Fig. 3).

A decrease in graft survival was seen in all cases with high tubular atrophy, interstitial fibrosis and total inflammation (log rank; $P = 0.033$ for ct 2-3 vs. 0-1; $P = 0.002$ for ci 2-3 vs. 0-1; $P = 0.025$ for ti 2-3 vs. 0-1) (Fig. 4a–c). It is not possible to show a correlation between cg score and graft survival. As stated previously, only three out 41 patients were diagnosed as cg1a based on electron microscopic imaging. The remaining patients had a cg score of at least 2. Finally, the presence or absence of C4d did not associate with a difference in graft survival (log rank; $P = 0.44$) (Fig. 4d).

Discussion

The contribution of donor-specific antibodies to the diagnosis of c-aABMR has become an important topic of discussion [10,27]. The Banff '15 criteria do not allow for the classifying diagnosis of c-aABMR if there are no DSA present. Instead, these cases are diagnosed as suspicious for c-aABMR. Therefore, the significance of DSA and their contribution to the loss of allograft function and relation to renal histomorphology have not yet been fully explored. We found that there are no histological or clinical differences between cases with c-aABMR and cases suspicious for c-aABMR.

Most studies have used transplant glomerulopathy (with or without DSA) as the histopathological diagnosis and have not described the level of microvascular inflammation and chronic tissue damage between cases with or without DSAs. However, to comprehend the possible effect of DSAs in the setting of c-aABMR, DSAs must be evaluated in the context of the Banff '15 classification.

In this study, we report of 17 cases of c-aABMR and 24 cases suspicious for c-aABMR. In the c-aABMR group, all DSA were *de novo* and the vast majority of the circulating DSA detected were directed against HLA-DQ (78%). These findings are in line with

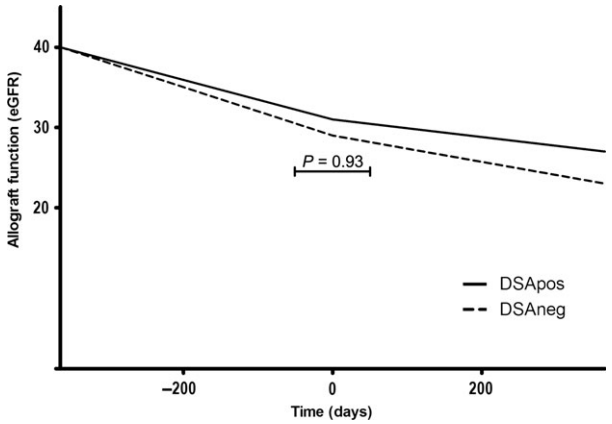

Figure 1 Allograft function of both DSApos and DSAneg patients in the year prior to and the year after c-aABMR diagnosis. Change in renal allograft loss: DSAneg: −5.5 ml/min/1.73 m^2/year vs. DSApos: −5.4 ml/min/1.73 m^2/year ($P = 0.93$).

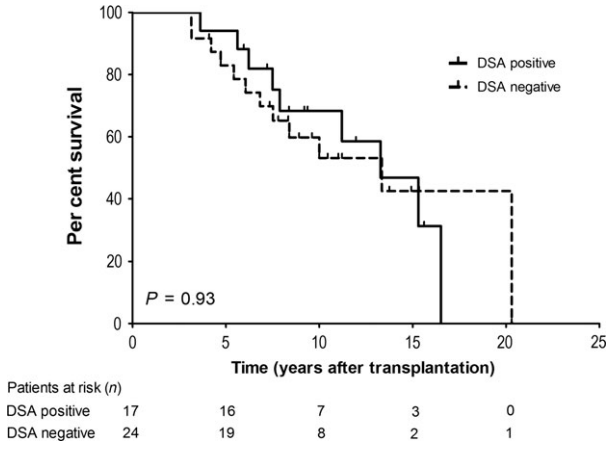

Figure 2 Overall graft survival for DSApos versus DSAneg cases.

previous publications showing a range of 20–80% DSA positivity in TG cases with *de novo* anti-HLA-DQ antibodies most frequently found [13,24,28,29].

The presence of DSA was not associated with a difference in response to therapy or graft survival. The latter finding is in accordance with previous studies that did not show a significant impact of DSA on renal allograft survival in transplant patients with evidence for antibody-mediated rejection either acute or chronic [14,15,18,24]. In addition, remarkably similar clinical characteristics and no differences in the renal allograft histomorphology were observed between the DSApos and DSAneg cases. Irrespective of DSA status, there was substantial chronic histomorphological damage as manifested by glomerular basement membrane double contours, chronic inflammation in addition to microvascular inflammation.

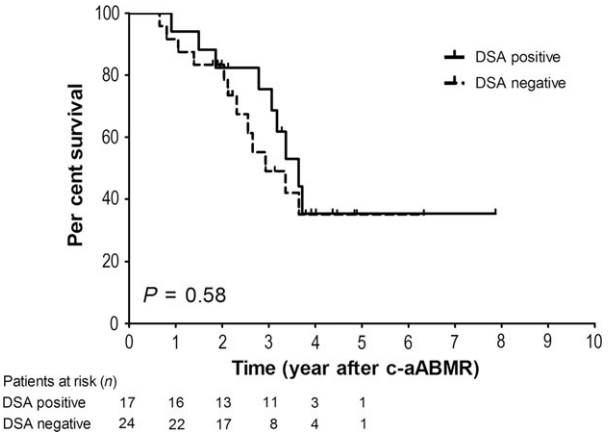

Figure 3 Graft survival after c-aABMR diagnosis for DSApos versus DSAneg cases.

Chronic histological damage has previously been identified as one of the most important attributing factors to kidney allograft loss irrespective of diagnosis [28,30]. Patri *et al.* [24] have previously shown that a chronic inflammation score, combining *interstitial fibrosis*, *tubular atrophy* and *total inflammation* is associated with long-term graft survival in patients with transplant glomerulopathy. We too have found that, regardless of DSA status, cases with moderate to severe interstitial fibrosis, tubular atrophy and total inflammation showed significantly worse allograft survival.

The absence of detectable DSA should not discard the involvement of an antibody-mediated process [8,13]. The evolving knowledge regarding DSA in combination with the complexity of HLA antibody assessment should prompt cautious interpretation and diagnostic decisions based on DSA in a chronic-active antibody-mediated rejection process [27,31,32]. Both the current (in-)ability to detect non-HLA antibodies and the possible fixing of DSA to the kidney transplant should be taken into consideration when testing negative for DSA. The relative importance of the latter explanation is not unequivocal as a study showed that a similar percentage of DSA positivity was found in both eluates of renal allografts and serum samples of renal transplant patients [33]. Furthermore, the moment of DSA assessment is of significant importance due to the possible transient nature of (detectable) DSA [11,27]. With the clinical manifestation often lagging behind the histomorphological changes and the fluctuation in DSA positivity, DSA testing might lead to a false-negative result when tested at a single moment in time. We searched for this possibility; however, only one patient was found to be DSA negative at time of biopsy but DSA positive 1 and 2 years prior to biopsy.

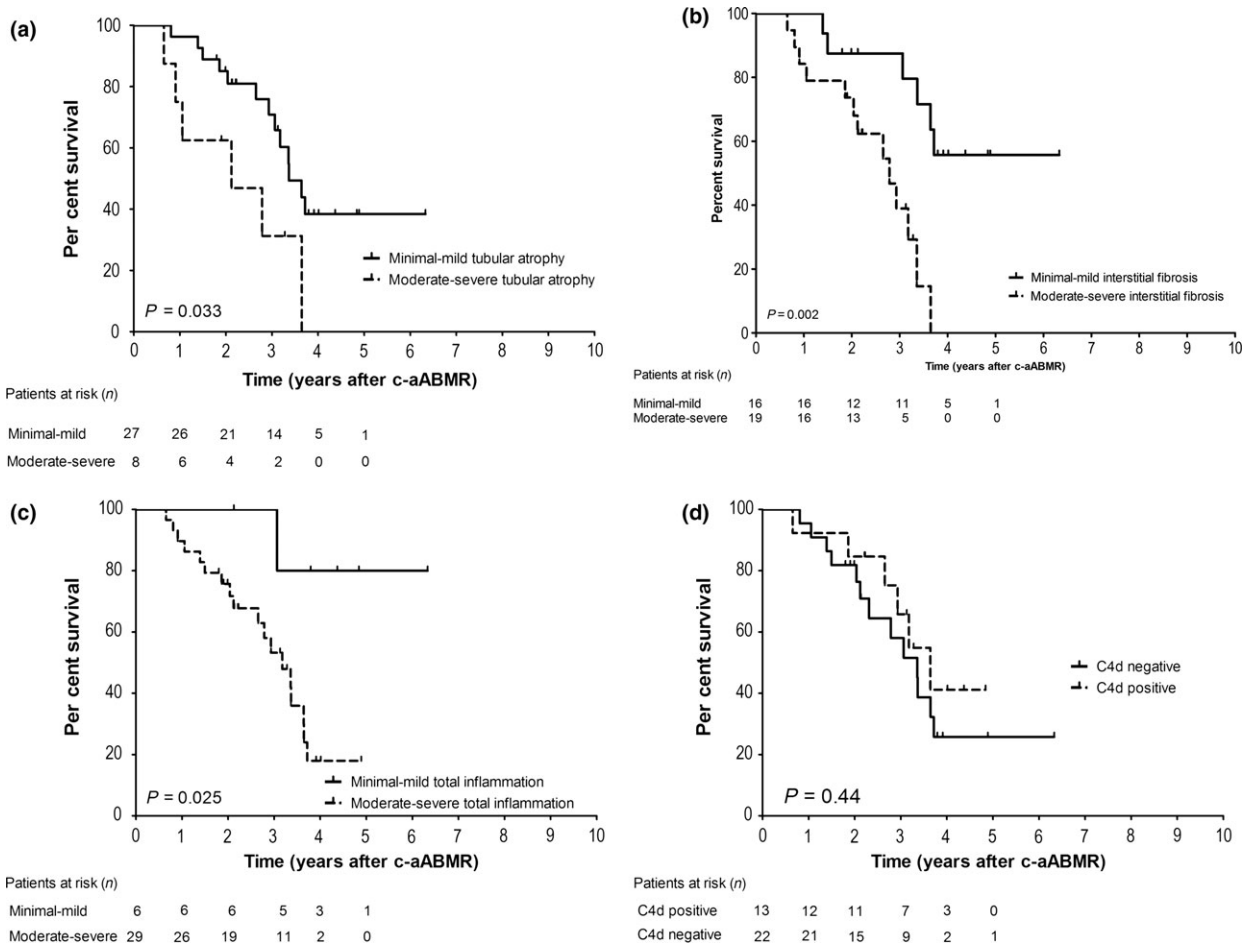

Figure 4 Histomorphological lesions (a) tubular atrophy, (b) interstitial fibrosis, (c) total inflammation and (d) C4d in the peritubular capillaries associated with graft survival.

Similar evidence has previously been provided for the fluctuating nature of C4d-positive staining which has led to the development of the C4d-negative category of ABMR [4,27]. Now, in addition to C4d-positive staining, a MVI score ≥2 is regarded as evidence of current or recent antibody interaction with the vascular endothelium. Gupta *et al.* [34] demonstrated a strong association between pathogenesis-based transcript sets related to ABMR and a MVI score ≥2. These changes in the Banff diagnostic criteria gave newfound importance to microvascular inflammation in the diagnosis of ABMR. Halloran *et al.* recently provided supporting evidence for the importance of microcirculation lesions, rather than DSA, in the classification of ABMR cases. The progression to kidney failure in the patients was independent of DSA status and TG lesions in combination with MVI were molecularly confirmed as ABMR cases even when DSA was not detected via current methods [14].

This study has some obvious limitations. As it is of retrospective nature, it allows for unknown bias and includes a selection of patients with (suspicious for) c-aABMR related to progressive loss of allograft function. Our data describe a patient group that has had a for cause biopsy due to a progressive decline in renal allograft function over a period of at least 12 months. The severity in capillary loop alterations might differ from a population of c-aABMR patients diagnosed based on a protocol biopsy without clinical observable graft deterioration. This difference may explain why the vast majority of our patients has severe glomerular basement membrane double contours (cg3). Notably, this bias is not related to the presence of DSA as this information was unavailable for both clinician and pathologist at time of diagnosis.

Secondly, it includes a relatively small sample size. The study does, however, in comparison with previous studies, contain complete and detailed information regarding the presence of circulating DSA presence at time of biopsy. Unfortunately, this information does not include data on non-HLA antibodies. Furthermore,

it includes a histologically well-defined, homogenous group of patients with (suspicious for) c-aABMR. Additionally, all patients have undergone a standardized treatment regimen irrespective of DSA status allowing for accurate comparison of both clinical and histological data.

For the current study, we have chosen to use the previous Banff classification (2015), as this classification allows for the use of the Banff category 2 'suspicious for' c-aABMR. The use of the most updated version of the Banff classification (2017) would have led to the exclusion of a total of 15 patients as they would not meet the updated criteria for c-aABMR [35]. Due to the current alterations, a significant group of our patients (n = 15), both DSA− and C4d−, is left undiagnosed and would have been excluded from further analysis. Interestingly, our findings suggest that patients with a biopsy classified as 'suspicious' for c-aABMR according to the Banff 2015 classification also represent c-aABMR. We believe that including these cases in the current study illustrates that C4d and DSA are not necessary in the diagnosis of c-aABMR and subsequent graft outcome when cg and MVI are present.

In conclusion, we show that renal histology and clinical outcome of patients with c-aABMR do not significantly differ from patients suspicious for c-aABMR. We believe that the data we presented adds to the growing body of evidence that the presence of DSA is not a *conditio sine qua non* to establish the diagnosis of c-aABMR when other criteria indicative of chronic tissue injury and current/recent antibody interaction with vascular endothelium are met.

Authorship

KAS: participated in research design, data collection, data analysis and writing of the article. MCCG: participated in research design, data collection, data analysis and writing of the article. CWNL: participated in research design, data analysis and writing of the article. JD: participated in research design, data collection, data analysis and writing of the article. DLR: participated in research design, data analysis and writing of the article. MA: participated in research design, data collection and writing of the article. MGHB: participated in research design, data collection, data analysis and writing of the article.

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Conflicts of interest

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