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ORIGINAL ARTICLE

Renal allograft DARCness in subclinical acute and chronic active ABMR

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SUMMARY

Gene expression profiling of renal allograft biopsies revealed the Duffy antigen receptor for chemokines (DARC) as being strikingly upregulated in antibody-mediated rejection (ABMR). DARC has previously been shown to be associated with endothelial injury. This study aimed at assessing the value of DARC immunohistochemistry as diagnostic marker in ABMR. The study was performed on 82 prospectively collected biopsies of a clinically well-defined population (BORTEJECT trial, NCT01873157) of DSA-positive patients with gene expression data available for all biopsies. Diagnostic histologic assessment of biopsies was performed according to the Banff diagnostic scheme. DARC expression was focally accentuated, on peritubular capillaries (PTC) mostly in areas of interstitial fibrosis and/or inflammation. DARC positivity was associated with diagnosis of ABMR and correlated with DARC gene expression levels detected by microarray analysis. Still, as previously described, a substantial number of biopsies without signs of rejection showed DARC-positive PTC. We did not observe significantly reduced graft survival in cases showing histologic signs of ABMR and being DARC-positive, as compared to DARC-negative ABMR. In summary, the upregulation of DARC, detected by immunohistochemistry, is associated with but not specific for ABMR. We did not observe reduced graft survival in DARC-positive patients.

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Key words
antibody-mediated rejection, Duffy antigen receptor for chemokines, immunohistochemistry gene expression profiling, kidney transplantation

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Introduction

The diagnosis of antibody-mediated rejection (ABMR) is based on a complex set of rules for assessing morphologic and immunohistologic findings in allograft biopsies [1,2]. The Banff classification recently also suggested to incorporate gene expression data for defining rejection in order to overcome the limited specificity of histopathologic lesions for the underlying pathogenic mechanisms [2]. This strategy is mainly supported by the work of the Alberta Transplant Applied Genomics Centre who aimed at defining specific gene expression patterns for established diagnostic categories [3–5]. Gene expression profiling was

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crucial for revealing previously unrecognized conditions like C4d-negative ABMR, by assessing endothelial associated transcripts (ENDAT) [2,3,6]. One of the genes most strikingly upregulated in ABMR was Duffy antigen receptor for chemokines (DARC, also known as atypical chemokine receptor 1, ACKR1) [3]. This promiscuous chemokine binding protein is expressed on red blood cells and on certain vascular endothelial cells [7]. DARC has previously been shown to be associated with sites of endothelial injury [8,9]. In earlier studies of our group, we observed increased expression of DARC on endothelial cells in cases of renal allograft rejection [10] but without specifically addressing its role in patients with ABMR. The present study was designed to investigate whether immunohistochemical staining for DARC is able to refine the diagnosis of ABMR, especially in diagnostically equivocal conditions like C4d-negative ABMR. The study was performed on biopsies of a clinically and pathologically well-defined population of DSA-positive patients who underwent biopsies to analyse morphologic and molecular features of rejection.

Materials and methods

Study population

The biopsies analysed in this study were performed during the screening phase of a randomized controlled trial to assess the impact of proteasome inhibitor bortezomib on the course of late ABMR (BORTEJECT trial; NCT01873157) [11]. The protocol for patient screening has been described in detail elsewhere [11]. Overall, 741 recipients (key inclusion criteria: age >18 years, functioning renal allograft ≥180 days post-transplantation, eGFR >20 ml/min per 1.73 m^2) in outpatient care at the Vienna transplant unit were subjected to HLA antibody monitoring (screening period from October 2013 to February 2015). For 111 patients, out of a total of 1165 patients visiting the nephrology outpatient clinic, HLA class I and/or II DSA were identified with single-antigen flow bead testing [11]. Of these, 86 underwent biopsy (Fig. 1) [11]. The study population consisted of 82 subjects for whom sufficient biopsy material was left for retrospective immunohistochemical DARC staining. Follow-up patient and graft survival data were available for 5 years after transplantation. Patients were followed until their return to dialysis or re-transplantation. All diagnostic and therapeutic measures of the BORTEJECT study were approved by the ethics committee of the Medical University of Vienna [11].

Preparation of slides

All biopsies were formalin-fixed and paraffin-embedded and processed for diagnostic histopathology according to standard procedures [11]. C4d immunohistochemical staining was done on 2 µm paraffin sections according to standard protocol using a polyclonal anti-C4d reagent (BI-RC4D; Biomedica, Vienna, Austria) [11]. DARC immunohistochemical staining was done on new 2 µm paraffin sections using a mouse monoclonal anti-human DARC-Fy6 antibody (kindly provided by Yves Colin, Inserm). The staining procedure in short: after deparaffinizing in xylene and rehydrating in a graded series of alcohol, the slides were heated in Tris-EDTA buffer (pH 9) for 60 min at 98 °C, cooled down to room temperature, incubated in 3% hydrogen peroxide in PBS for 10 min to block endogenous peroxidase, washed in buffer and then incubated in Ultra V Block for 5 min. After washing in PBS, the DARC-Fy6 antibody (dilution 1:100 in 1% BSA/PBS) was added and incubated for 1 h followed by four times washing in PBS, applying primary antibody enhancer, incubated for 10 min at room temperature and washed again four times. Then, HRP polymer was added, incubated for 15 min and washed again 4 times, followed by incubation with ACE (amino ethyl carbazol) chromogen for 5 min and washed again four times in distilled water. Finally, the slides were counterstained with Mayer's Hämalaun for 1 min and a coverslip was mounted with Aquatex® medium (Merck KGaA, Darmstadt, Germany).

Reading of the biopsies

The initial reading of the biopsies in the course of the Bortejeect trial was performed on formalin-fixed paraffin-embedded sections applying standard methodology inclusive electron microscopy if necessary. Originally, histologic classification had been performed according to the Banff 2013 scheme by two experienced pathologists [11,12] and was now updated according to current classification rules (Banff 2017), which, however, did not change diagnostic results in this cohort. DARC staining was assessed by two renal pathologists (JK and HR) in a fully blinded manner. Overall signal of DARC-positive staining along peritubular capillaries (PTC) which had to be linear and circumferential was assessed in the total biopsy, and positive percentage was

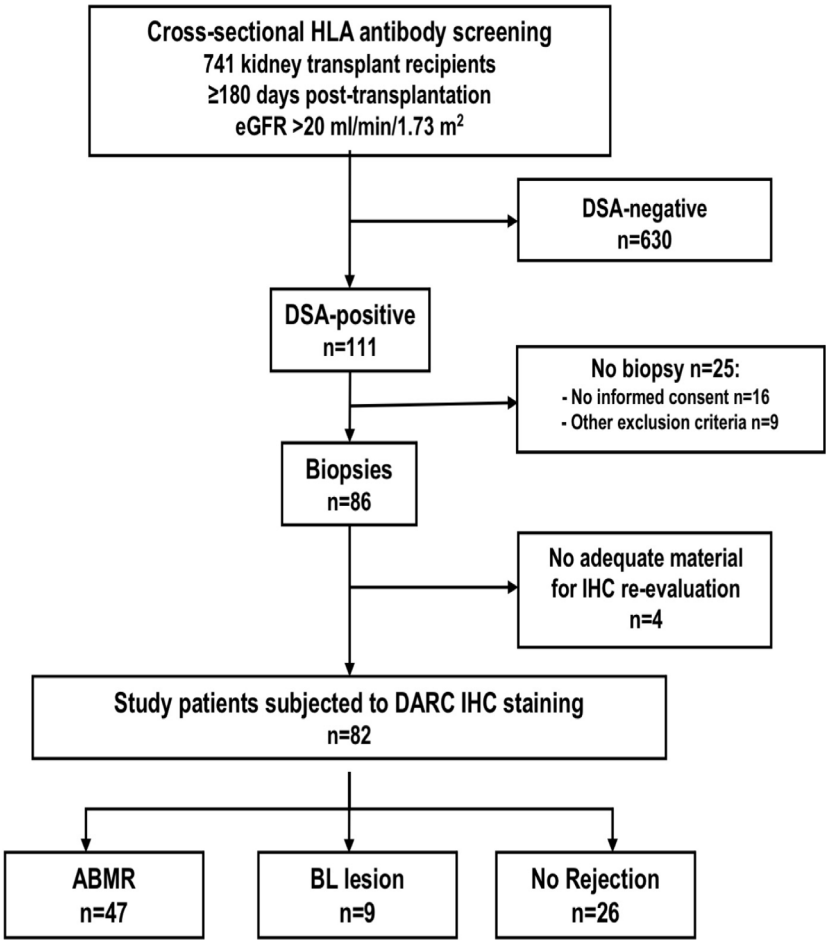

Figure 1 Flow Chart of study population and included biopsies.

estimated in regard to all capillaries – analogous to C4d scoring. The distribution of cortical positively stained PTC was further evaluated and assigned to inflamed, fibrotic and unchanged parenchyma in percentage (10% steps) of total cortical stained PTC. Morphology was evaluated on haematoxylin counterstaining which allowed for sufficient mapping of fibrosis/atrophy or inflammation.

DSA testing

The applied procedure for DSA testing is described in detail elsewhere [11]. In brief, for prescreening, LABScreen Mixed assays (One Lambda, Canoga Park, CA, USA) were applied. Before further analysis, serum samples were heated (56 °C for 30 min) to prevent complement interference. Lab Screen Single Antigen assays (One Lambda) were used for in-depth analysis, setting MFI threshold at >1000. HLA specificity was determined according to results from serologic and/or low- or high-resolution donor/recipient HLA typing, done by the local HLA laboratory or the Eurotransplant database [11]. C1q-binding ability was tested using C1qScreen assays (One Lambda).

Molecular analysis

For molecular analysis, a 3 mm portion of one biopsy core (16-gauge needle) was placed in RNAlater immediately after biopsy and shipped at room temperature or on dry ice to the Alberta Transplant Applied Genomics Centre (ATAGC, University of Alberta, Alberta, Canada) [11]. RNA extraction and gene expression analysis was done as previously described, using PrimeViewGeneChip arrays (Affymetrix, Santa Clara, CA, USA) [11,13]. Gene expression results, including classifiers related to rejection, were analysed using the MMDx platform [11,13,14].

Statistical analysis

The study is a retrospective evaluation of immunohistochemical staining of DARC in kidney transplant biopsies of patients, collected for a prospectively randomized clinical trial [11]. Statistical analysis was performed using IBM SPSS Statistics 24. For descriptive analysis, continuous variables are displayed as median and interquartile range, categorical variables are shown in absolute numbers and percentage, and for correlation, Spearman's correlation coefficient was calculated. For comparative analysis, chi-square test, when necessary Fisher's exact test, and Mann–Whitney *U*-test were applied. Kaplan–Meier event analysis was applied for analysis of graft and patient survival with application of Cox–Mantel log rank test for inter-group comparison. Univariable and multivariable Cox regression analysis was used to test association of DARC with death-censored graft survival. We used a mixed model in order to test the effect of DARC positivity on the trajectory of eGFR. The explanatory variables used as fixed effects were base GFR, time and DARC positivity as well as their interaction. The included random effects of the model were intercept and time. The necessity of the interaction term as well as the random effects was tested with a log-likelihood test. Therefore, the final reported model does not include the interaction term. The covariance structure of the measurements was modelled with an autoregressive covariance of first order. To test for interobserver variability in assessment of DARC positivity, Cohen's kappa was calculated.

Results

In this study, 82 DSA-positive kidney recipients subjected to protocol biopsies in the context of the BORTEJECT trial were included. For these 82 biopsies, adequate material for immunohistochemical staining for DARC was available. Baseline characteristics are shown in Table 1. Biopsies were performed at a median of 4.8 years after transplantation. Median eGFR at the time of biopsy was 54 ml/min/1.73 m^2 and protein/creatinine ratio 192 mg/g. The majority of patients was on triple immunosuppression. DSA characteristics and biopsy findings were summarized in Tables 2 and S1. Class II DSA ($n = 55$, 67.1%) was most commonly present. Twenty-three subjects (28%) had C1q-fixing DSA. The majority of the patients had a diagnosis of active ($n = 14$, 17.1%) or chronic/active ($n = 33$, 40.2%) ABMR by Banff 2017 classification. There were no patients with frank T-cell mediated rejection, but 9 (11.0%) specimens showing borderline lesion. Besides donor age and peri-transplant immunoadsorption, there was no difference in baseline demographics and patient characteristics, especially concerning baseline immunosuppression (Table 2). Also, bortezomib administration during BORTEJECT trial did not differ significantly between DARC-negative and DARC-positive cases (Table 2). The patients that were not included in the BORTEJECT trial did not receive any change in management.

DARC staining patterns

As shown in Fig. 2, DARC staining was mainly observed in peritubular capillaries (PTC). The pattern was patchy, and stained PTC were mostly located in the cortex and only rarely also in the medulla. Occasionally, some signal was also present in small venules and arterioles. None of the samples showed staining in glomeruli or arteries. There was a low level of background staining, mainly protein droplets in tubular epithelial cells.

Among 82 study patients, 61 showed positive DARC staining in ≥5% of PTC. We selected these DARC IHC positive biopsies for further analysis. We chose a 5% cut-off to have a high sensitivity for inclusion of DARC-positive cases while avoiding the problem of questionable cases at the border of positivity, especially since we and others observed minimal DARC staining also in normal kidneys or pretransplant biopsies [8,9]. Inter-observer variability for scoring cases as DARC-negative or DARC-positive was quite good with a kappa value of 0.845 ($P < 0.001$). DARC-positive specimens showed in median 5% PTC staining (IQR: 5–20%, range: 5–40%). Subsequently, we compared the amount of DARC-positive vessels in areas of interstitial fibrosis and tubular atrophy (IFTA), within inflammation and in normal parenchyma. Most of the signal was located in areas of interstitial fibrosis (median 90%, IQR: 30–100%, range 0–100%) or inflammation (median 80%, IQR: 20–100%, range 0–100%) with only a minority in unchanged parenchyma (median 0%, IQR: 0–10%, range 0–50%).

DARC expression is associated with signs of rejection

Comparing the DARC-positive and DARC-negative biopsies, we found no differences regarding DSA characteristics, such as HLA class specificity or number of detected DSA (Table 2). There was a significant difference regarding the MFI of the immunodominant DSA [DARC positive: Median MFI 3508 (IQR 1679–9885)

Table 1. Baseline demographics and patient characteristics.

Parameters	Total (n = 82)	DARC-positive (n = 61)	DARC-negative (n = 21)	P value
Variables recorded at the time of transplantation				
Recipient age (years), median (IQR)	47 (35–54)	50 (34–54)	48 (39–54)	0.889
Caucasian	81 (99)			
Donor age (years), median (IQR)	46 (35–58)	50 (39–59)	42 (27–49)	0.031
Female recipient sex, n (%)	37 (45)	31	6	0.126
Live donor, n (%)	14 (17)	11	3	>0.999
ABO-incompatible live donor transplant, n (%)	1 (1)			n.a.
Cold ischaemia time (hours), median (IQR)*	12 (8–17)	12 (9–17)	12 (8–20)	0.784
Recipient of a re-transplant, n (%)	23 (28)	19	4	0.401
HLA mismatch in A, B and DR, median (IQR)	3 (2–4)	3 (2–4)	3 (2–4)	0.850
Current CDC panel reactivity ≥10%, n (%)[†]	14 (18)	12	2	0.327
Preformed anti-HLA DSA, n (%)[‡]	25 (61)	23	2	0.089
Induction with antithymocyte globulin, n (%)	27 (33)	24	3	0.058
Peri-transplant immunoadsorption, n (%)[§]	24 (29)	22	2	0.026
CDC crossmatch conversion before transplantation, n (%)	8 (10)	8	0	0.106
Variables recorded at the time of biopsy				
Time to biopsy (years), median (IQR)	4.8 (2–13)	4.8 (1.8–13)	5.7 (1.9–12)	0.889
Recipient age (years), median (IQR)	55 (45–62)	55 (43–62)	54 (46–62)	0.982
eGFR (ml/min/1.73 m^2), median (IQR)	54 (32–79)	50 (32–79)	57 (49–87)	0.078
Urinary protein/creatinine ratio (mg/g), median (IQR)	192 (78–445)	213 (77–673)	147 (82–279)	0.222
Maintenance immunosuppression, n (%)				
Triple immunosuppression n (%)	64 (78)	47	17	>0.999
Dual immunosuppression, n (%)	18 (22)	14	4	
Immunosuppressive agents				
Tacrolimus, n (%)	50 (61)	38	12	0.769
Cyclosporine A, n (%)	27 (33)	20	7	>0.999
mTOR inhibitor, n (%)	4 (5)	4	1	>0.999
Belatacept, n (%)	1 (1)	0	1	0.256
MPA or azathioprine, n (%)	74 (90)	54	20	0.673
Steroid, n (%)	72 (88)	54	18	0.711
Borteject randomization, n (%)	44 (53.7)	37 (60.7)	7 (33.3)	
Bortezomib administration, n (%)	21 (25.6)	16 (26.2)	4 (19.0)	0.684

ABMR, antibody-mediated rejection; DSA, donor-specific antibody; CDC, complement-dependent cytotoxicity; IQR, interquartile range; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin.

*Cold ischaemia time (documented for both deceased and living donor transplants) was not recorded for 4 recipients.

[†]CDC panel reactivity was not recorded for 5 recipients.

[‡]Pretransplant DSA data were available for 41 recipients (solid-phase HLA antibody screening on the wait list was implemented at the Vienna transplant unit in July 2009).

[§]According to our local standard, sensitized patients (until 2009: ≥40% CDC-PRA; since 2009: preformed DSA) were subjected to an earlier detailed protocol of peri-transplant immunoadsorption.

Table 2. Baseline DSA characteristics and biopsy results.

Parameters	Total (*n* = 82)	DARC-positive (*n* = 61)	DARC-negative (*n* = 21)	*P* value
DSA characteristics				
HLA class I DSA, *n* (%)	42 (51.2)	32 (52.5)	10 (47.6)	0.702
HLA class II DSA, *n* (%)	55 (67.1)	40 (65.6)	15 (71.4)	0.242
Number of DSA, median (IQR)	1 (1–2)	1 (1–2)	1 (1–2)	0.501
DSA MFI_max, median (IQR)	3009 (1476–8740)	3508 (1679–9885)	1802 (1240–4020)	0.030
C1q-binding DSA, *n* (%)	23 (28.0)	20 (32.8)	3 (14.3)	0.104
Biopsy results*				
Banff categories, *n* (%)				
Banff 2017 ABMR, *n* (%)	47 (57.3)	40 (65.6)	7 (33.3)	0.010
Acute/active ABMR, *n* (%)	14 (17.1)	12 (19.7)	2 (9.5)	0.502
Chronic/active ABMR, *n* (%)	33 (40.2)	28 (45.9)	5 (15.2)	0.075
Banff borderline lesion, *n* (%)	9 (11.0)	7 (11.5)	2 (9.5)	>0.99
Single lesions (Banff score)†				
ptc score, median (IQR)	0 (0–2)	1 (0–2)	0 (0–0.5)	0.006
g score, median (IQR)	1 (0–2)	1 (0–2)	0 (0–1)	0.049
t score, median (IQR)	0 (0–0)	0 (0–0)	0 (0–0)	0.481
i score, median (IQR)	0 (0–0)	0 (0–0)	0 (0–0)	0.956
ti score, median (IQR)	1 (0–1)	1 (0–1)	0 (0–1)	0.078
cg score, median (IQR)	0 (0–1)	0 (0–2)	0 (0–0.5)	0.086
ci score, median (IQR)	2 (1–3)	2 (1–3)	1 (0–2)	0.001
ct score, median (IQR)	1 (0–2)	1 (1–2)	0 (0–1)	0.001
cv score, median (IQR)	1 (0–2)	1 (0.75–2)	1 (0–1)	0.069
mm score, median (IQR)	0 (0–1)	0 (0–1)	0 (0–0.75)	0.332
C4d score, median (IQR)	0 (0–2)	0 (0–2)	0 (0–1)	0.498
High-grade MLPTC, *n* (%)	16 (20.3)	14 (24.1)	2 (9.5)	0.153
C4d in peritubular capillaries, *n* (%)	26 (31.7)	21 (34.4)	5 (23.8)	0.365
Molecular results‡				
Molecular ABMR score, median (IQR)	0.26 (0.06–0.67)	0.40 (0.14–0.78)	0.06 (0.02–0.28)	<0.001
Molecular TCMR score, median (IQR)	0.01 (0.01–0.02)	0.01 (0.01–0.02)	0.01 (0.00–0.01)	0.034
Molecular all-rejection score, median (IQR)	0.39 (0.09–0.78)	0.55 (0.15–0.81)	0.06 (0.02–0.39)	0.001
Molecular atrophy/fibrosis score, median (IQR)	0.28 (0.17–0.60)	0.38 (0.20–0.63)	0.19 (0.09–0.48)	0.011
Molecular acute kidney injury score, median (IQR)	−0.01 (−0.32 to 0.28)	0.055 (−0.247 to 0.327)	−0.21 (−0.49 to 0.035)	0.006
DARC gene expression, median (IQR)	8.24 (7.52–9.12)	8.68 (7.91–9.32)	7.52 (6.99–8.14)	<0.001

ABMR, antibody-mediated rejection; DSA, donor-specific antibody; IQR, interquartile range; MFI_max, mean fluorescence intensity of the immunodominant DSA; MLPTC, multilayering of peritubular capillary basement membranes.

*Morphologic lesions were scored according to the Banff 2017 classification of renal pathology[2].

†For the following lesions biopsy material was not sufficient for a subset of patients: ptc (*n* = 2), g (*n* = 3), t (*n* = 2), I (*n* = 2), ti (*n* = 1), cg (*n* = 2), ci (*n* = 1), ct (*n* = 1), cv (*n* = 12), and high-grade MLPTC (*n* = 3), respectively.

‡Gene expression analysis was performed in 79 of the 82 patients.

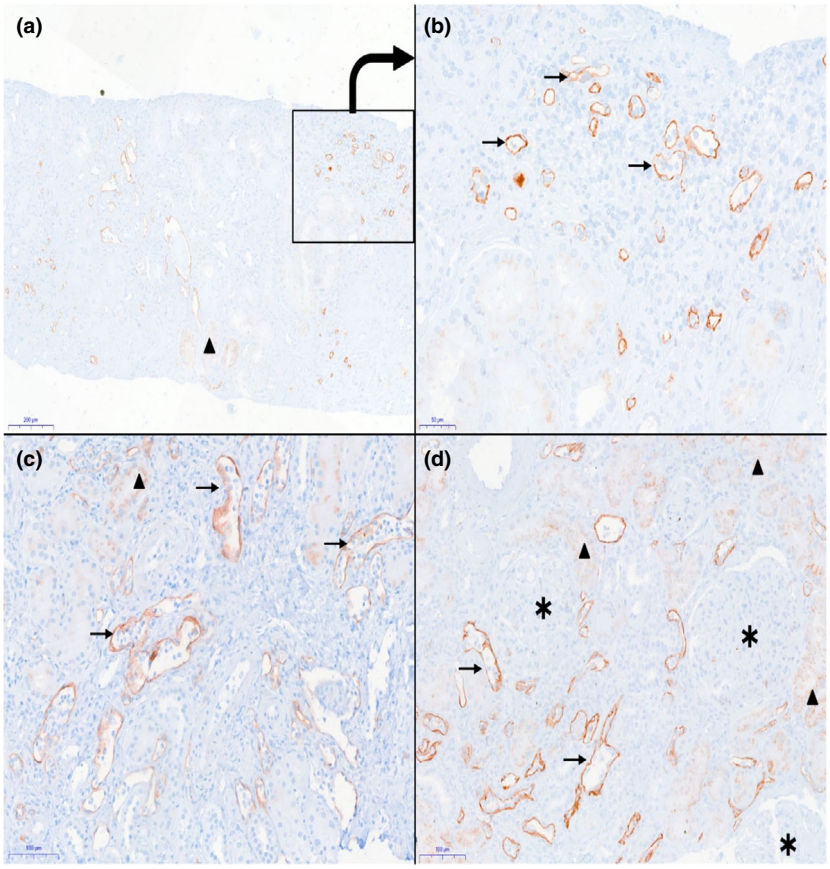

Figure 2 DARC IHC: DARC staining is always patchy (a), and mostly associated with areas of inflammation (a, b) and/or fibrosis (c). Staining is almost exclusively confined to endothelial cells of PTC while glomeruli are completely spared (d) (asterisks). Weak background staining in tubular epithelial cells is not uncommon (triangles).

vs. DARC negative: 1802 (1240–4020), $P = 0.030$] but not in regard to C1q-fixing DSA [DARC positive: 20 (32.8) vs. DARC negative 3 (14.3), $P = 0.104$].

Most of the ABMR cases were DARC positive ($n = 40$ vs. $n = 7$, $P = 0.01$). Still, a substantial amount of biopsies without any signs of rejection and the majority of borderline lesions showed DARC-positive PTC (Fig. 3). There were significant differences with respect to ptc score ($P = 0.012$) and g score ($P = 0.049$), but not C4d positivity ($P = 0.365$). In lesions reflecting chronic damage, we found significant differences in ci score ($P = 0.001$) and ct score ($P = 0.001$) and a trend towards higher cg ($P = 0.086$), ti ($P = 0.078$) and cv scores ($P = 0.069$).

DARC IHC correlation with gene expression data from MMDx

On a molecular level, DARC gene expression confirmed results of IHC, meaning higher gene expression scores being linked to more DARC-positive PTC in biopsies ($r_s = 0.564$, $P < 0.001$; Fig. 4) and a significant difference in median DARC gene expression in DARC-positive vs. DARC-negative biopsies [Median DARC expression: 8.68 (IQR 7.91–9.32) vs. 7.52 (6.99–8.14), $P < 0.001$]. Looking at the molecular scores, we also found a significant difference in the molecular ABMR score ($P < 0.001$), rejection score ($P = 0.001$), atrophy/fibrosis score ($P = 0.011$) and to a lesser extent a difference in molecular TCMR score ($P = 0.034$; Table 2). At the same time, percentage of DARC-positive PTC showed positive, albeit moderate correlation with ABMR score ($r_s = 0.488$, $P < 0.001$), atrophy/fibrosis score ($r_s = 0.383$, $P < 0.001$), all-rejection score ($r_s = 0.465$, $P < 0.001$) and acute injury score ($r_s = 0.447$, $P < 0.001$) but not with TCMR score ($r_s = 0.197$, $P = 0.081$; all Fig. 5).

DARC IHC and graft survival

Five-year overall and death-censored graft survival rates from index biopsy were 67.1% and 78%, respectively (Fig. 6a,b). Using Kaplan-Meier analysis and log rank

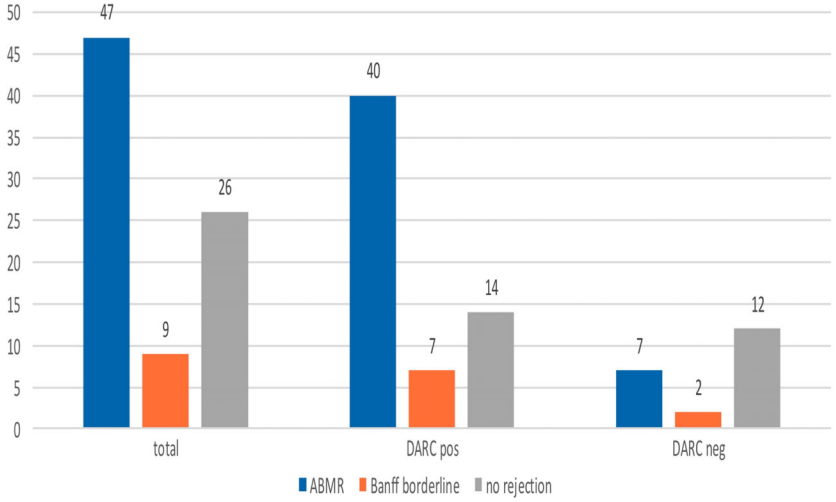

Figure 3 Distribution of Banff diagnosis according to DARC IHC positivity.

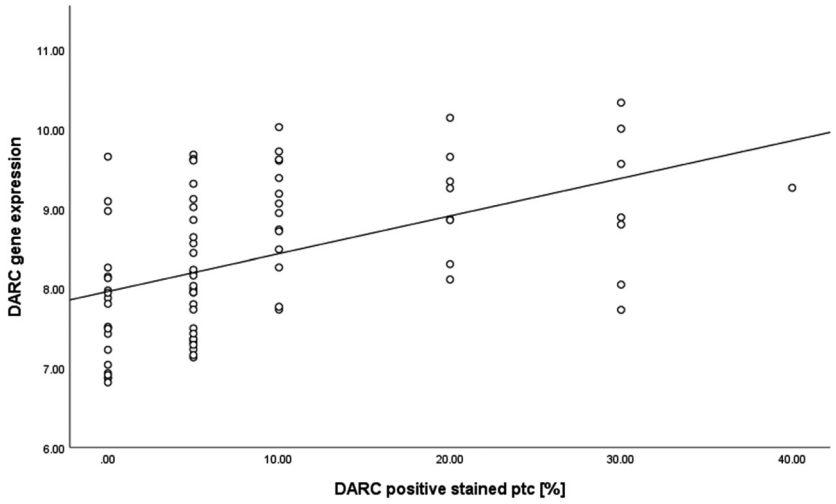

Figure 4 Correlation of DARC gene expression with DARC IHC: dot plots depicting DARC gene expression and percentage of DARC-positive stained PTC.

test, overall graft survival was worse in DARC-positive biopsies (DARCneg 85.7% vs. DARCpos 60.7%; $P = 0.046$) but this effect was lost in death-censored graft survival analysis (DARCneg 90.5% vs. DARCpos 73.8%; $P = 0.106$; Fig. 6c,d). Neither uni- nor multivariable regression analysis comparing DARC and other histologic markers of ABMR (g, ptc, C4d, high-grade-MLPTC, cg) showed a significant effect of DARC positivity on death-censored graft survival (Table S2). Additionally, and as expected, diagnosis of ABMR (ABMRneg 94.3% vs. ABMRpos 66.0%; $P = 0.004$) had a strong effect on death-censored graft survival (Fig. 6e). Also in uni- and multivariable regression analysis, diagnosis of ABMR remained statistically significant for reduced death-censored graft survival in a model including diagnosis of ABMR, DARC positivity and clinical features with a $P < 0.25$ in univariable analysis [donor age, prior transplantation, recipient sex, eGFR (Mayo-formula)], whereas in this model DARC also had no significant additional effect (Table S3).

Because of the fact that DARC positivity does not perfectly match with ABMR diagnosis, we wanted to evaluate whether DARC positivity had an additional effect on death-censored graft survival. Therefore, we defined four subgroups (group1 = DARC−/ABMR−, group2 = DARC+/ABMR−, group3 = DARC−/ABMR+, group4 = DARC+/ABMR+) and performed another group comparison on death-censored graft survival (Fig. 6f). Remarkably, biopsies being DARC positive and showing signs of ABMR performed worst (graft

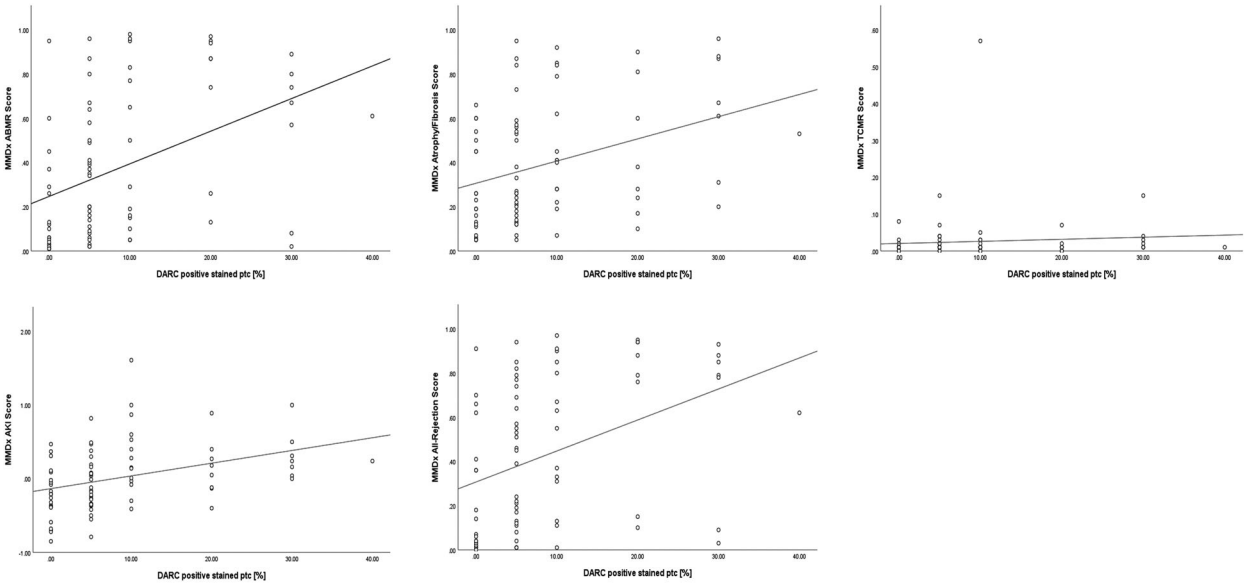

Figure 5 Correlation of percentage of DARC stained PTC with different MMDx scores: dot plots depicting different molecular scores obtained with MMDx and percentage of DARC-positive stained PTC.

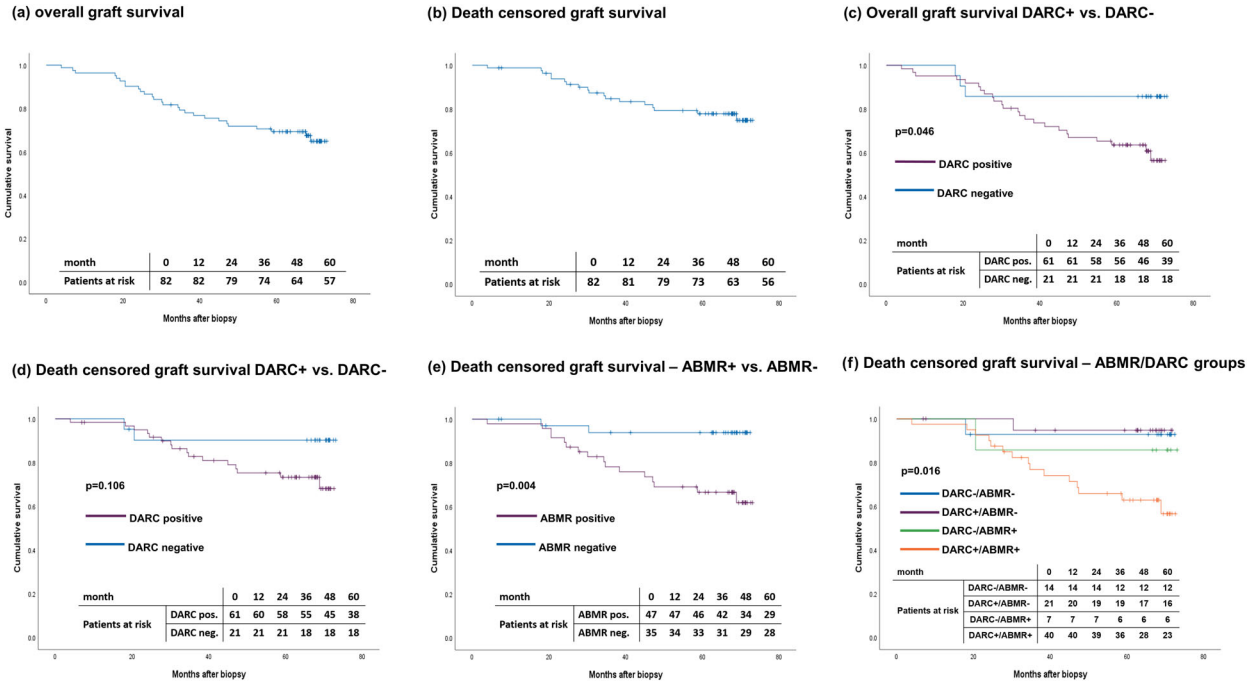

Figure 6 (a–f) graft survival analysis in total population and in regard to ABMR and DARC positivity.

survival 62.5%, $P = 0.016$) whereas the other three groups performed roughly the same with a somewhat worse outcome in group 3 (graft survival 92.9%, 95.2 and 85.7% respectively). However, in pairwise comparison, the difference between group 3 and 4 did not reach statistical significance (Table S4). As we set the threshold for DARC IHC positivity rather low at 5%, we analysed other cut-offs for DARC positivity on graft survival in which we could not identify a significant difference compared to the applied threshold of 5% (Figs S1 and S2). As DARC IHC positivity is associated with ci and ct lesion score, we substituted DARC+/− with ci+/− and ct+/− in the group analysis described above and observed similar results for ci and ct (Fig. S3a,b).

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DARC positivity and eGFR course

Finally, we aimed to assess the impact of DARC positivity on eGFR trajectories using a mixed model with intercept and time as random effects as described in the methods section. DARC positivity showed no statistically significant association with eGFR in our model (Effect estimate on eGFR for DARC positivity: −0.11 ml/min/1.73 m^2, $P = 0.958$, CI −4.23 to 4.05).

Discussion

DARC is of interest for transplant immunology for several reasons: It is expressed on endothelial cells at the site of inflammatory cell recruitment, the number of DARC-positive vessels increases during injury where it has multifaceted immunomodulatory effects [15] and it was found to be strikingly upregulated in ABMR by gene expression analysis in renal allograft biopsies [16]. In a very comprehensive transcriptional profiling analysis of 703 biopsies, Halloran *et al.* [3] compared expression patterns between several disease states in transplanted as well as in nontransplanted kidneys. DARC was found to be one of the most upregulated genes in ABMR [3]. This is particularly surprising because of its expression in only a small proportion of cells, that is endothelial cells of PTCs.

In previous studies, we investigated the expression of DARC in various forms of native kidney and renal allograft injury [10]. There, we documented the increase of DARC-positive peritubular capillaries in C4d positive ABMR and at sites of interstitial fibrosis. These studies were, according to the diagnostic definition of ABMR at the time, restricted to C4d positive cases and likely underestimated the true incidence of ABMR. However, this association was recently confirmed in the abovementioned gene expression profiling that revealed DARC among the most strikingly upregulated transcripts in ABMR. In this study, we took advantage of a very well characterized cohort of prospectively collected renal allograft biopsies from DSA-positive patients. This allowed us to comprehensively assess whether DARC localization by IHC adds to the standard evaluation of allograft biopsies.

A strength of this study is the validation of IHC results by gene expression data from microarray analyses that closely matched the amount of DARC expression detected by IHC and thus confirmed the feasibility of quantifying DARC expression by IHC. Our observation that DARC-positive vessels are predominantly located within areas of inflammation or IF/TA was also confirmed by the correlation of DARC IHC with gene expression scores that are indicative of acute kidney injury, overall rejection and atrophy/fibrosis (Table 2, Fig. 5) [17]. In addition, we could indeed show that immunohistochemically detected DARC expression, that is exclusively confined to PTC, is associated with the histologic and molecular diagnosis of ABMR. It is important to mention that this association is not perfect and a substantial number of biopsies without signs of rejection show DARC positivity and vice versa (Fig. 3). It was also surprising to us that we did not find an association of DARC expression with transplant glomerulopathy, which is the hallmark of chronic ABMR. But one has to keep in mind that the group sizes are different, and the group comprising ABMR without DARC staining is only made up of seven cases.

The findings in this study are in line with observations in our previous studies and indicate that DARC expression on endothelial cells (EC) is not simply another diagnostic marker of ABMR but might be associated with, or result from different mechanisms of tissue injury like TCMR or crescentic glomerulonephritis in native kidneys [9,10]. Although DARC expression on EC obviously lacks specificity for ABMR, it might still be a marker for active, ongoing immune reactions and thus might be of biological and potentially also of diagnostic relevance. Experimental studies do indeed suggest a pro-inflammatory effect of DARC expressed on blood endothelial cells by facilitating transcytosis and immobilization of chemokines on the luminal surface of endothelial cells [18]. Apically exposed chemokines are thought to enhance leukocyte migration and mediate inflammatory response [15,19,20]. This concept nicely fits with our observation that microvascular inflammation like glomerulitis and peritubular capillaritis is significantly more pronounced in DARC-positive biopsies. If DARC-bound chemokines do indeed enhance microvascular inflammation, it might contribute to a more severe course of the disease with accelerated graft loss. This prompted us to test whether DARC positivity in our cohort was associated with more aggressive rejection resulting in faster deterioration of kidney function and subsequent graft loss.

Overall, we did not observe a significantly reduced death-censored graft survival for DARC-positive (>5% DARC-positive PTC) vs. DARC-negative biopsies in uni- or multivariable analysis. In these models, only baseline eGFR and cg score remained statistically significant predictors for graft survival. This observation is in line with Waiser *et al.* who studied 54 patients with

biopsy proven ABMR for 24 months of follow-up and with Viglietti *et al.* who aimed at defining a dynamic prognostic score to predict allograft survival [21,22]. Within the group of patients with diagnosis of ABMR, however, DARC expression was associated with reduced graft survival (Fig. 6) as compared to patients with DARC-negative ABMR and DARC-positive patients without ABMR. However, this effect was not statistically significant in pairwise comparison and similar results are shown if DARC is substituted with ci or ct lesion. Even though this is not totally surprising as DARC staining was also associated with ct and ci, this of course limits the relevance of worse graft survival in DARC-positive ABMR cases. In line with this observation, Viglietti *et al.* [22] also identified interstitial fibrosis/tubular atrophy as predictive for graft survival in their model of graft loss in ABMR. Still, biologically, DARC is a marker for endothelial injury and much about its detailed function and acting during the course of inflammation is still unknown. But one can speculate that its upregulation might occur before tissue remodelling and fibrosis and upregulation of DARC in the context of, or driven by an inflammatory environment might indeed aggravate rejection, or at least represent a marker of a more aggressive alloimmune response. DARC expression because of other reasons does not seem to have the same putatively deleterious consequences. The design of our study does unfortunately not allow to further address this issue since our study cohort is defined by DSA positivity, and we therefore did neither investigate native kidneys nor transplant biopsies with TCMR or protocol biopsies. We also did not systematically collect sequential biopsies that would be helpful to follow the level of DARC expression and its consequences over time. Another limitation of the study is the relatively small number of DARC-negative patients (especially within the ABMR group), which might affect the reliability of statistical analyses. Results have therefore to be interpreted with caution. In addition, during the long follow-up period, difference in subsequent management of immunosuppression might influence our results. 44 of our patients were included in the BORTEJECT trial, receiving bortezomid or placebo. DARC positivity was not associated with bortezomib administration ($P = 0.648$). As described by Farsad *et al.* [11], there is no effect of bortezomib on patient survival or graft survival nor on graft function in 24-month analysis. Five of these patients were subsequently included in a phase 1 trial with Anti-C1s monoclonal antibody BIVV009 [23], one of them having been DARC negative. One other patient received additional tocilizumab (DARC−), and for one patient, immunosuppression was adjusted because of BK viraemia (DARC+). For the other patients, no additional treatment is documented, besides initiation of mycophenolic acid for one patient. In conclusion, we infer no substantial effect of bortezomib or systematic relevance of other differences in management to our analysis.

In summary, we conclude that DARC expression in renal allograft biopsies is confined to peritubular capillaries and can reliably be detected by immunohistochemistry. We found that upregulation of DARC is neither specific for ABMR nor generally associated with an adverse outcome. We also did not observe significantly reduced graft survival in cases showing histologic signs of ABMR and being DARC-positive, as compared to DARC-negative ABMR. Moreover, we found a similar impact on graft survival for ci and ct lesion score, further limiting the diagnostic value of DARC expression in cases with established tissue lesions. But, since it is conceivable that DARC staining might precede interstitial fibrosis and atrophy, studies investigating DARC staining in early rejection without fibrosis are required to conclusively determine the prognostic value of DARC.

Authorship

JK: involved in data collection, analysis, manuscript writing and editing. FE and GAB: involved in data collection, analysis and manuscript editing. KN and AK: involved in data analysis, manuscript writing and editing. YCA, J-PC and SS: involved in generation of DARC antibody and manuscript editing. HR: involved in data collection, analysis, manuscript writing, editing and supervision.

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Conflicts of interest

The authors of this manuscript have no conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Death-censored graft survival in regard to per cent DARC-positive PTC – category '5%' vs. '≥5%'.

Figure S2. Death-censored graft survival in regard to per cent DARC-positive PTC – category '≥5 <20%' vs. '≥20%'.

Figure S3. (a and b) Graft survival analysis in regard to ABMR and ci, respectively ct positivity.

Table S1. Occurrence of rejection and selected Banff lesion scores at baseline biopsy.

Table S2. Uni- and multivariable regression analysis, testing effects of DARC on death-censored graft survival in comparison with histologic variables.

Table S3. Uni- and multivariable regression analysis, testing effects of DARC on death-censored graft survival in comparison with diagnosis of ABMR and clinical variables.

Table S4. Pairwise comparison of ABMR/DARC groups on death-censored graft survival.

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