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Peritubular Capillary Basement Membrane Multilayering in Renal Allograft Biopsies of Patients With De Novo Donor-Specific Antibodies

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Background. Severe peritubular capillary basement membrane multilayering (PTCBML) is part of the Banff definition of chronic antibody-mediated rejection. We retrospectively investigated whether assessment of the mean number of layers of basement membrane (BM) around peritubular capillaries (PTC) can be used in a cohort of patients with de novo donor-specific antibodies (dnDSA) as an early marker to predict long-term antibody-mediated injury. **Methods.** This is a retrospective cohort study with 151 electron microscopy samples from 54 patients with dnDSA, assessed at around 1 year after transplantation, for a mean number of BM layers around PTC and in serial biopsies. Graft survival and time to transplant glomerulopathy (TG) development were estimated in survival analyses. **Results.** We found that a mean PTCBML count greater than 2.5 layers assessed in a sample of 25 PTCs around 1 year after transplantation is indicative of the development of TG in patients with dnDSA ($P = 0.001$). In addition, in patients with serial biopsies available for electron microscopy analysis, we could distinguish 2 groups: patients with a mean PTCBML count of 2.5 or less on all biopsies, and patients who developed greater than 2.5 layers at any time after transplantation. The latter group reflected dnDSA patients at risk for TG development ($P < 0.001$). In patients with dnDSA, PTCBML score added significantly to the sensitivity and specificity of prediction of TG compared with microcirculation injury score alone. **Conclusions.** Our results highlight the potential value of assessing the mean number of BM in PTC for early prediction of progression to chronic antibody-mediated injury.

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Chronic antibody-mediated rejection (cAMR) is an important cause of graft loss.[1,2] Transplant glomerulopathy (TG), a defining feature of cAMR, carries a poor prognosis regardless of treatment strategy.[3] Early identification of antibody-mediated injury at a stage where it is potentially reversible is a major focus of current research. Careful examination for ultrastructural endothelial and basement membrane (BM) features in the microcirculation of the graft by electron microscopy (EM) has shown promise in this field.[4-7] Whereas some groups have focused on glomerular and endothelial changes, we are interested in peritubular capillary BM multilayering (PTCBML).

Peritubular capillary BM multilayering was found to be associated with cAMR as early as 1990.[8] Peritubular capillary BM multilayering is a defining histological feature of cAMR in the Banff classification.[9] In the most recent update, PTCBML is diagnostic of cAMR when the worst affected peritubular capillary (PTC) comprises 1 PTC with 7 layers or more and 2 PTC with 5 layers or more.[10]

After observing several cases of TG in our transplant population that were preceded by PTCBML, sometimes by several years, we conducted a small case-control study (16 cases of TG and 16 controls) and found indications that lesser degrees of PTCBML, examined in a sample of 25 capillaries,

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H.dK., M.W., and C.R. identified suitable patients. M.W., P.B., E.S.N., and H.dK. obtained clinical data. J.W.G., A.G.M., and D.T. collected biopsy tissue. H.dK. and C.R. performed data analysis. C.R., H.T.C., and H.dK. assessed histology by light microscopy. H.dK., L.B.M., and J.M. assessed histology by electron microscopy. C.R., H.dK., and H.T.C. designed the study and obtained funding. All authors were involved in writing the article.

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precede and predict TG development.[5] We hypothesized that: (1) a low level of multilayering across a range of PTC might help detect a putative "field-effect" of antibody-mediated injury in the renal microcirculation and provide an early, predictive marker of cAMR and (2) that a mean number of PTCBML may provide as much if not more information than assessing only the worst affected PTC. In the current study, we assess whether, within a cohort of renal transplant patients with de novo donor-specific antibodies (dnDSA), PTCBML predicts TG. To facilitate comparison with other studies, we used several scoring methods. In addition, we assessed whether early PTCBML added any value to other predictive markers associated with outcome in these patients, such as microcirculation inflammation (MI) and complement split product 4d (C4d) staining.[11,12] This in contrast to the current use of PTCBML in the Banff classification, where this feature is used as a diagnostic feature of established cAMR, rather than as a potential early marker predictive of future cAMR.

MATERIALS AND METHODS

Patients and Study Design

All transplant recipients with standard immunological risk (n = 638) receiving a crossmatch-negative kidney transplant at the Imperial College Kidney and Transplant Centre between November 2005 and January 2011 were included in this retrospective cohort study. Seventy-nine (12.4%) patients developed dnDSA as defined previously,[12] and these patients were followed up until April 16, 2013. In addition, 73 DSA-negative control patients with an EM sample available around 1 year after transplantation were randomly selected from our database. Renal transplant tissue was obtained from the Imperial College Healthcare Tissue Bank, which has ethics approval to both collect human tissue and release material to researchers (MREC 07/MRE09/54).

Immunosuppressive Therapy

Patients received induction therapy of alemtuzumab (Campath-1H; Millennium Pharmaceuticals) 30 mg intravenous or daclizumab (Zenapax; Roche) 2 × 2 mg/kg IV, both with methylprednisolone (500 mg) IV preoperatively, followed by prednisolone 1 mg/kg per day (maximum, 60 mg) for 3 days, then 0.5 mg/kg per day (maximum, 30 mg) for 4 days and then discontinued after day 7. Alemtuzumab-treated patients received maintenance monotherapy consisting of low-dose tacrolimus (mean trough level, 5-8 ng/mL). Daclizumab-treated patients received maintenance therapy consisting of mycophenolate mofetil and low-dose tacrolimus (mean trough level, 8-12 ng/mL).[12,13] First acute rejection episodes either histologically confirmed or clinically suspected as T cell–mediated rejection were mostly (64% of cases) treated with prednisolone + mycophenolate mofetil; in those recognized as AMR, treatment included plasma exchange + IVIg (n = 29).

DSA Assessment

Before transplantation, all donor-recipient pairs had a negative T and B cell complement-dependent cytotoxicity crossmatch and a negative T cell flow cytometric crossmatch. The DSA were assessed using LABScreen mixed beads (One Lambda, Canoga Park, CA). When positive, the specificity of the anti-HLA antibody was identified using LABScreen single-antigen beads. Patients were typed for HLA-A, -B, -Cw, -DR, and -DQ antigens.[12] De novo DSA were defined by (1) pretransplantation either no antibodies, only nondonor-specific HLA antibodies or DSA with an mean fluorescence index (MFI) less than 300 assessed by Luminex single antigen beads; (2) after transplantation, any measurement of a DSA greater than 500 MFI or MFI of 300 to 500 in 2 independent serum samples.

EM and PTCBML Counting

Fresh renal biopsy tissue was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in osmium tetroxide, dehydrated through ascending grades of ethanol, and embedded in Spurr's resin. Ultrathin sections were embedded on ATHENE 400 thin copper 3.05-mm grids and stained with Reynold lead citrate.[14]

For each biopsy on which EM was assessed (151 samples from 54 patients), 25 sequential PTCs were located on low magnification (1900×), and the layers of BM were counted at high magnification (13,500×). Peritubular capillary BM multilayering was counted in the cortex only, including all areas of cortical interstitium (narrow and wide). Only PTC that were mostly visible (>80% not obscured by grid) were assessed; endothelial changes were not recorded. In those cases where the number of layers varied around the circumference, the number of layers recorded corresponded to the maximal number of layers seen avoiding corners and cross-cut sections. In a random sample of the cohort (93 samples from 51 patients), after the first 10 PTCs were counted, the extent of multilayering was noted before completing the full 25 sequential PTCBML examination. The findings were recorded as the number of PTC having one layer (Figure 1A, B), 2 layers, 3 to 4 layers, or 5 or more layers of BM (Figure 1C). In this cohort, no PTC with 7 layers or more of BM was observed. From these recordings, we also derived the mean and median number of PTCBML.

To facilitate comparison of several PTCBML scoring methods,[5,15-20] including the number of PTC with 5 PTCBML or more,[9,17] the number of PTC with 3 PTCBML or more,[5] the 3 most affected PTC,[18,19] and the mean number of BM layers, we used measurements on sets of 10 or 25 PTC, with different cutoff levels, and assessed the predictive value for outcomes. In addition, the mean number of layers in 25 PTC was assessed in 73 DSA-negative patients in EM samples acquired around 1 year after transplantation.

Histopathology

All biopsies (n = 269, indication = 238, surveillance = 31) were graded according to the Banff classification[9] by C.R., with glomerulitis[21] and peritubular capillaritis[9] scored according to the respective Banff classification. Microcirculation inflammation is the sum of glomerulitis + peritubular capillaritis. C4d (BI-RC4D, Biomedica, Austria) was performed on paraffin-embedded sections and classified as positive if greater than 10% of PTC showed circumferential staining.[9] The TG was defined as double contours of the glomerular capillary wall on light microscopy; with no or small amounts of immune-complex deposition on immunofluorescence and/or EM; and absence of hepatitis C infection and of clinical features of thrombotic microangiopathy.[12]

Statistical Analyses

For continuous variables, means with standard deviation (SD) were calculated; for non-Gaussian distributed variable,

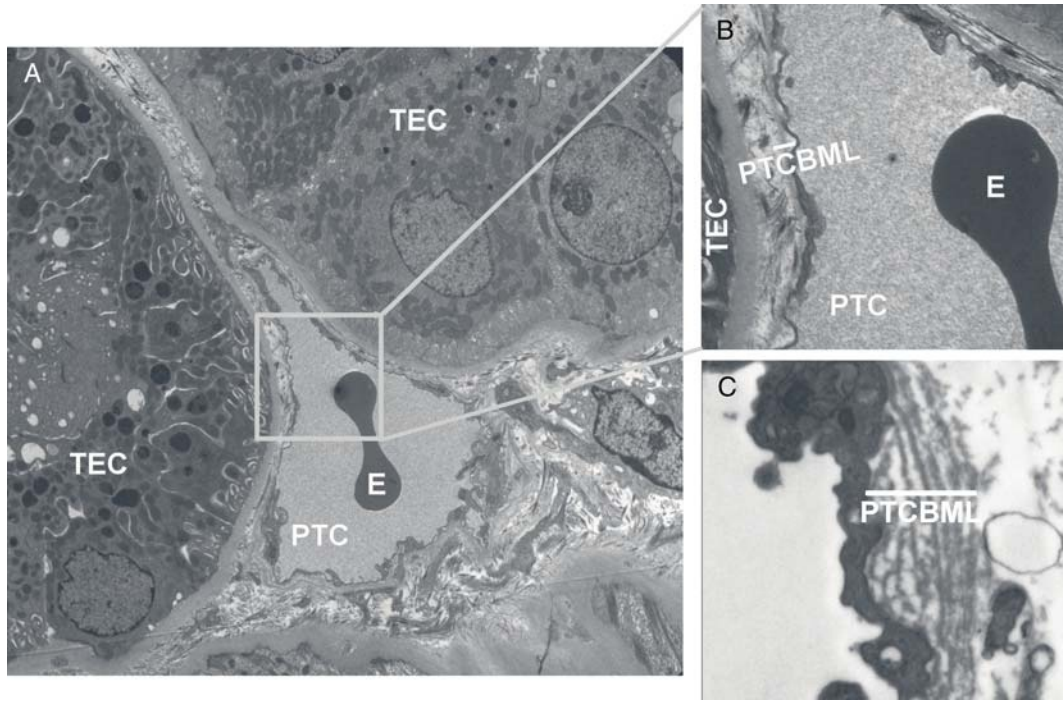


FIGURE 1. Illustration of PTCBML scoring. A, EM picture of an erythrocyte in a peritubular capillary surrounded by tubular epithelial cells (1,900×). B, Enlargement of area, clearly showing the single PTCBM layer (13,000×). C, A peritubular capillary with greater than 5 layers of PTCBM (13,000×). E, erythrocyte; TEC, tubular epithelial cell.

the median and interquartile range (IQR) are expressed. Two groups were defined based on longitudinal PTCBML data: mean PTCBML score in all biopsies of 2.5 or less (n = 25) (nonprogressor) versus at least 1 biopsy with greater than 2.5 mean layers (n = 25) (progressor). Clinical characteristics for the 2 groups were analyzed with Fisher exact test for count data or univariate binary logistic regression analysis for continuous variables. Binary logistic regression was also used for multivariable analysis including univariate variables with P less than 0.2. Graft failure was defined as resuming dialysis and censored for patient death with a functioning graft. The Kaplan-Meier product limit method was used to estimate the kidney allograft survival times and the time to diagnosis of TG. In addition, receiver operator curve (ROC) analysis using a logistic model was performed, defining outcome as TG. Significance was set at $P \leq 0.05$. Statistical calculations were performed using SPSS statistics 20.0 (SPSS, USA).

RESULTS

Characteristics of the Group

In the period 2005 until 2010, a total of 79 of 638 (12.4%) standard immunological risk renal transplant patients developed dnDSA. Information on the clinical demographics can be found in Table 1 (http://onlinelibrary.wiley.com/doi/10.1111/j.1600-6143.2012.04325.x/full#ajt4325-tbl-0001) of our previous article on this cohort.[12] In 7 of 79 (9.7%) dnDSA patients, no biopsy material was available. From the 72 patients with biopsies, we included (1) all patients with an adequate sample for EM analysis around 1 year after transplantation (n = 50); and (2) all patients with 2 EM samples or more for longitudinal analysis (n = 50). This represents a total population of 54 patients because 46 patients from these 2 groups overlap. A total of 151 EM samples were analyzed. A random cohort of 73 DSA-negative patients with an adequate EM sample around 1 year after transplantation was also analyzed. Additional information on distribution of patients, biopsy, and EM samples can be found in Figure 2.

One-Year Posttransplantation Biopsies: Association of PTCBML With Outcome

Our previous case-control study suggested that PTCBML in an early biopsy after transplantation was associated with future progression to TG.[5] To assess which type of PTCBML assessment is most informative as a predictive marker and what its merit is compared to the MI and C4d score, we included 1 EM sample per patient. This sample was the sample closest to 1 year after transplantation (n = 50), with a median of 1.04 years after transplantation (IQR, 0.58-1.40). This time frame was chosen because it corresponds to the posttransplant period where early antibody-mediated damage is likely to develop in patients with dnDSA.[12] Peritubular capillary BM multilayering was considered positive for outcome analysis if the mean number of PTCBML was greater than 2.5 or ≥3 PTC with ≥5 layers (Table 1). For comparison, a control cohort of 73 DSA-negative patients with an EM sample at median 0.91 years after transplantation (IQR, 0.5-1.3) had a mean number of layers of 1.93 (IQR, 1.6-2.1).

Graft survival from time of biopsy did not differ for the mean PTCBML count >2.5 (P = 0.137). Graft survival did differ for ≥3 PTC with ≥5 layers, with 4-year graft survival estimates of 79% in the negative versus 42% in the positive group (P = 0.016). Analysis of the predictive value of MI either dichotomized (comparing MI < 2 with MI ≥ 2) or categorized (comparing MI0, MI1 + 2, and MI ≥ 3) showed significantly worse graft survival for higher MI score (P = 0.011, P = 0.019; respectively). The C4d staining did not associate with graft failure (P = 0.484). Both MI and

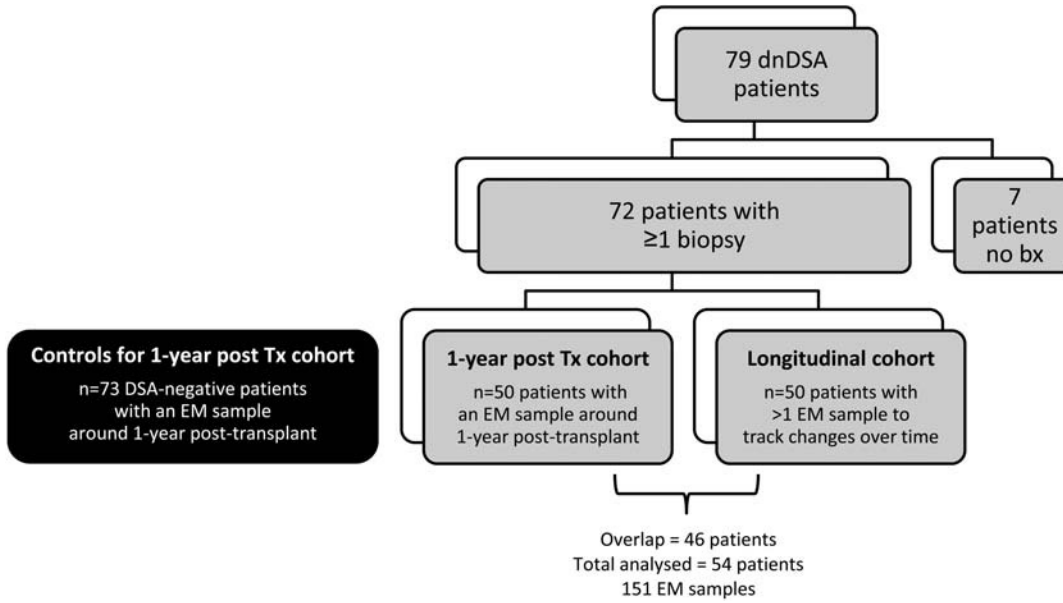


FIGURE 2. Composition of cohort analyzed, based on all renal transplant patients with de novo donor-specific antibodies (n = 79) and electron microscopy tissue (n = 54) analyzed. Grey boxes are samples derived from renal transplant patients with de novo DSA. Black box describes samples derived from renal transplant patients that are DSA negative. Bx, biopsy; 1-year bx, cohort of patients with 1 EM sample closest to 1 year after transplantation; longitudinal, patients with greater than 1 EM sample for study of PTCBML over time; controls, cohort of DSA-negative patients with 1 EM sample closest to 1 year after transplantation.

C4d findings were in keeping with our previously published findings on dnDSA patients.[12]

To estimate TG development from the time of biopsy, 13 patients were excluded because they were lost due to follow-up (n = 6) or had already developed TG before or at index biopsy (n = 7). The remaining 37 patients all had a biopsy for follow-up. A mean PTCBML count greater than 2.5 associated with more TG development from time of index biopsy (P = 0.001). The TG development from the time of biopsy was not associated with ≥3 PTC with ≥5 layers (P = 0.335). However, 5 of 7 patients with this degree of PTCBML had already been excluded from analysis for having established TG. C4d did not associate with TG development, whereas dichotomized MI score did (P = 0.413, P = 0.006; respectively). To establish if AMR treatment had any effect on outcome, we found that AMR treatment at any time after transplantation associated with more TG development from time of index biopsy (P = 0.009, data not shown [DNS]).

In a separate analysis, the area under the ROC for the prediction of TG development was poor for C4d-positive staining (0.62), whereas it was good for PTCBML greater than 2.5 (0.72) and MI score greater than 2 (0.74). Our model showed that adding PTCBML to the MI score results in a significantly better prediction of TG development (area under curve, 0.81; P = 0.006; Figure 3).

Serial Transplant Biopsies; PTCBML Trend Analysis in Relation With Outcome

To do a trend analysis, we included 50 patients who had greater than 1 biopsy with EM; 14 of 50 had 2 biopsies and 36 of 50 had 3 biopsies or greater. The time intervals between the biopsies were variable because these were mostly indication biopsies. Based on previous results,[5] we counted

TABLE 1.

On 1-year posttransplantation biopsies, PTCBML assessment methods and their association with outcome in comparison with other histological parameters

	Graft survival % survival (P)	TG development % TG-free (P)	ROC TG development (AUC)
PTCBML			
Mean: ≤2.5 vs >2.5	73 vs 67 (0.137)	81 vs 31 (0.001)	0.72
≥5 layers: <3 vs ≥3 PTC	79 vs 42 (0.016)	72 vs 53 (0.335)	
MI			
MI: <2 vs ≥2	77 vs 59 (0.011)	79 vs 50 (0.006)	0.74
MI0 vs MI1/2 vs MI ≥ 3	77 vs 76 vs 43 (0.019)	93 vs 41 vs 33 (0.005)	
C4d			
C4d: ≤10% vs >10%	70 vs 72 (0.484)	68 vs 68 (0.413)	0.62

For the outcome parameters graft survival and TG development, P values of Kaplan Meier survival analysis estimates are given per histological parameter. In the third column ROC analyses on TG development are given, which are depicted in Figure 3.
AUC indicates area under the curve.

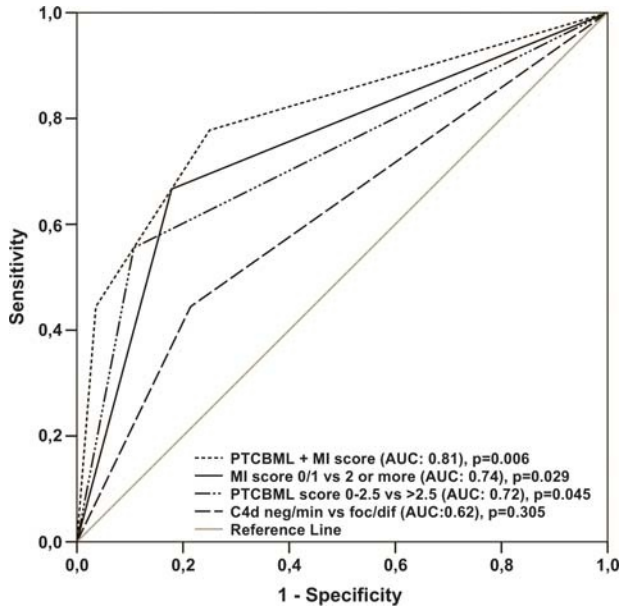


FIGURE 3. ROC analysis comparing PTCBML, MI and C4d score on 1-year posttransplantation biopsies (n = 37). Sensitivity (Y-as) and 1-specificity (X-as) are displayed for dichotomized PTCBML score (mean BM layers out of 25 PTC ≤2.5 vs >2.5), dichotomized MI score (MI score composed of g + ptc <2 vs ≥2) and dichotomized C4d staining (C4d staining of PTC negative/minimal vs focal/diffuse). Reference line is depicted as a grey line. ROC analysis was performed using a logistic model, defining outcome as development of TG within the follow-up. ptc, peritubular capillaritis; g, glomerulitis.

PTCBML, using a mean number of 2.5 BM as a cutoff. Two groups were defined based on PTCBML count during follow-up: patients with PTCBML scores consistently of 2.5 or less (Figure 4 (●), n = 25) (nonprogressors) versus patients with at least 1 biopsy with greater than 2.5 mean layers (Figure 4 (■), n = 25) (progressors). Table 2 shows a comparison of clinical characteristics and immunological risk factors for nonprogressors (≤2.5 BM layers) versus progressors (any biopsy with >2.5 BM layers).

The first time point of EM analysis was used as the index biopsy. Time from transplantation to index biopsy was the same for the nonprogressor and progressor PTCBML groups (P = 0.977), at a median time of 0.56 year (IQR, 0.24-1.18). Comparing time between first and last EM sample, we found no differences for the nonprogressor and progressor PTCBML groups (P = 0.486). In a multivariable analysis comparing nonprogressors with progressors, no significant differences were found (Table 2).

Graft survival at 4 years after first biopsy showed no significant difference between progressors and nonprogressors (P = 0.185; nonprogressors, 73%; progressors, 66% [Figure 5A]). To estimate TG development from index biopsy, 3 progressor group patients with a biopsy greater than 2.5 PTCBML were excluded because they had already developed TG. The TG-free survival at 4 years after index biopsy showed more TG development in the progressor PTCBML group (P < 0.001; nonprogressors, 95%; progressors, 34% [Figure 5B]). Graft survival at 2 years after last biopsy showed a worse trend for patients with a biopsy with greater than 2.5 PTCBML (P = 0.064; nonprogressors, 84%; progressors, 50% [DNS]).

PTCBML Assessment Methods Compared

Review of the literature on PTCBML reveals different methods of assessment with variations in the number of PTC examined, and cutoff levels.[9,15-18,20,22-24] Therefore, on a random subset of our biopsies (93 EM samples from 51 patients), we recorded our counting results after 10 PTCs were analyzed, so that we could carry out a methodological comparison of counts of either 10 or 25 PTC. In this group, median time to biopsy from transplantation was 1.63 years (IQR, 0.5-3.0), and median time to biopsy from dnDSA development was 0.68 years (IQR, 0.0-2.2). We recorded the mean number of BM layers across the set of 10 and the set of 25 PTCs. The count in 25 PTC was set as the "Gold Standard," although this was not validated for outcome. Results were assessed as dichotomized values as well as discrete variables, correlating 10 versus 25 PTC counts.

When assessing mean number of layers of PTCBML, recorded as a dichotomous reading (either less than 2.5 layers or more than 2.5 layers), there is a significant correlation (odds ratio, 0.098; 95% confidence interval, 0.045-0.214) with excellent sensitivity and specificity of the reading in 10 PTCs with the reading in 25 PTCs (sensitivity, 86%; specificity, 92%) (DNS). When considering mean PTCBML as a discrete variable, a simple linear regression was used to predict the PTCBML in 25 PTC based on PTCBML in 10 PTCs (Figure 6). A significant regression equation was found (25PTC = 0.045 + 0.986[10 PTC]), with an r^2 of 0.8731. Thus, counting 10 PTCs for mean PTCBML score is recommended, taking into consideration time and cost effectiveness.

When assessing 3 PTCs or greater with 5 layers or greater, recorded as a dichotomous reading (either yes or no), there is a significant correlation (odds ratio, 1.330; 95% confidence interval, 1.005-1.769) with unacceptable sensitivity and perfect specificity of the reading in 10 PTC for the reading in 25 PTCs (sensitivity, 25%; specificity, 100%) (DNS). Simple linear regression used to predict the number of PTC with 3 layers or

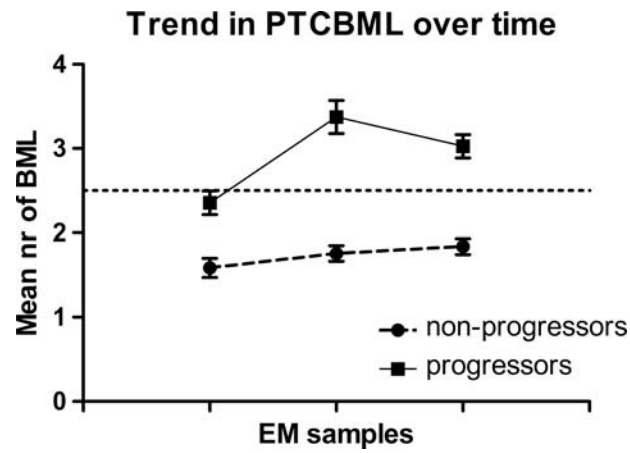


FIGURE 4. Number of PTCBML over time as a mean of 25 PTC counts for nonprogressors and progressors. The stippled line denominated by circles (●, n = 25) are the nonprogressors and the continuous line denominated by squares (■, n = 25) are the progressors. Per sample mean ± SEM is depicted. The mean number of BML of 25 counted PTC is depicted on the Y-axis. On the X-axis three time points are depicted where all (n = 50) have a sample for the first and the last point and for the intermediate point some samples are missing. The X-axis is depicted without a relation to time.

TABLE 2.

Comparison of PTCBML nonprogressors and progressors for clinical characteristics and immunological risk factors

	Nonprogressors (n = 25)	Progressors (n = 25)	Univariate *P* value	Multivariable *P* value
Sex donor (% male)	57	39	0.365	
Age donor (yr; median [IQR])	46 (36-54)	53 (44-60)	0.056	0.220
Type donor (% DDRT)	48	40	0.776	
Sex recipient (% male)	52	76	0.140	0.486
Age recipient (yr; median [IQR])	42 (26-52)	43 (35-56)	0.127	0.397
Ethnicity (%) white/Asian/Afrocar/other	40/40/20/0	36/44/12/8	0.630	
Total HLA mismatches	4 (3-4)	4 (3-5)	0.656	
Retransplanted (% yes)	8	8	1.000	
Preemptive (% yes)	8	8	1.000	
Induction therapy (% campath)	92	84	0.667	
Total follow-up (y; median[IQR])	4.4 (3.7-5.8)	5.4 (4.0-5.9)	0.161	0.194
Time to DSA (y; median [IQR])	0.24 (0.03-0.47)	0.5 (0.27-0.94)	0.201	
Anti-HLA class I DSA (% yes)	56	60	1.000	
Anti-HLA class II DSA (% yes)	60	84	0.114	0.567
C4d staining (% focal/diffuse)	28	28	1.000	
MI (% ≥2)	20	44	0.128	0.170

Analysis of the 2 PTCBML groups as determined on their first index biopsy using Fisher exact test and binary logistic regression for univariable and multivariable analysis. For data not normally distributed, the median and IQR is expressed. In the multivariable analysis, no variables held any significance.
Afrocar, Afro-Caribbean; DDRT, deceased donor renal transplant; FU, follow–up; HLA = human leukocyte antigen; IQR = interquartile range; MI = microcirculation inflammation; yr = year.

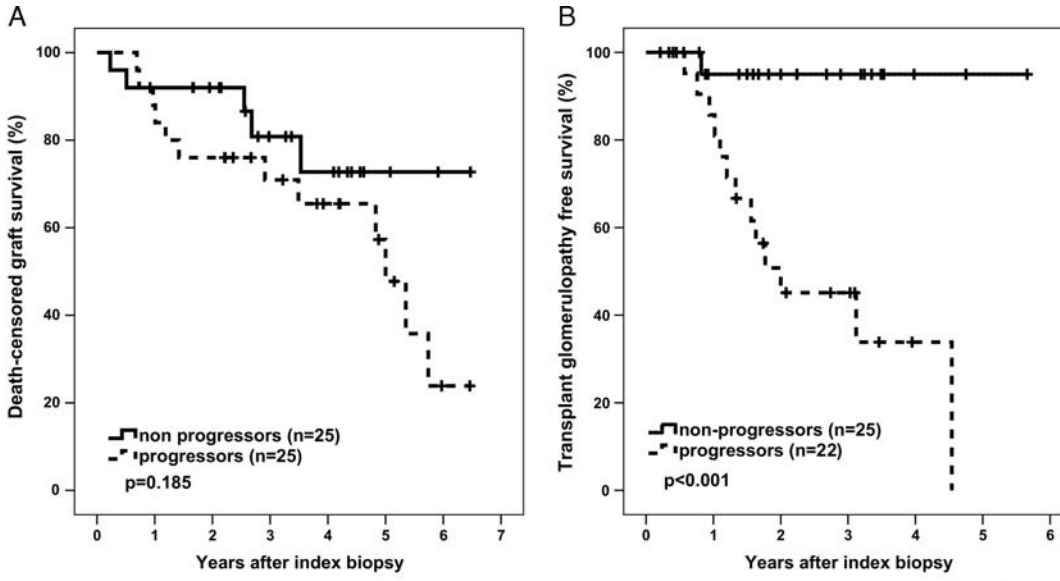
greater or 5 layers or greater in 25 PTC based on PTCBML in 10 PTC shows still significant, but inferior, correlation; r^2 0.8094 (25PTC = 0.7158 + 2.292[10PTC]) and r^2 0.8079 (25PTC = 0.2464 + 2.476[10PTC]), respectively (DNS).

DISCUSSION

There is currently a renewed interest in ultrastructural examination of endothelial and BM features in the microcirculation of the renal allograft, as markers for early antibody-mediated injury. The current Banff classification for renal allograft pathology includes PTCBML as a feature of established cAMR.[9] Based on our previous findings, we assessed the value of lesser degrees of PTCBML as an early predictor of a chronic antibody-mediated damage. From this retrospective study, we conclude that, in patients with dnDSA, a mean PTCBML count greater than 2.5 assessed in 25 PTC around 1 year after transplantation is indicative of development of TG. In a longitudinal assessment, PTCBML score of, or progressing to, a mean number greater than 2.5 PTCBML identified dnDSA patients at risk for TG development. Furthermore, we found that when the read-out for PTCBML is a mean number of BM layers, a count of 10 PTC is as good as a count of 25. Therefore, when assessing a biopsy around 1 year after transplantation in patients with dnDSA, we recommend counting the number of BM layers in 10 PTC and calculating the mean; a mean count of PTCBML above 2.5 predicts TG development. This does not mean that all 10 PTC have 2 to 3 layers, but indicates that, in line with our postulate that DSA has a "field effect" on the microcirculation, on average, the 10 PTCs have more than the expected 1 or 2 layers.

Peritubular capillary BM multilayering was first incorporated in to the Banff definition of cAMR following detailed publications by Ivanyi et al.[17,18,25-27] Although the initial cutoff for cAMR was 3 PTCs or greater with 5 layers or greater, the current Banff definition requires 1 PTC with 7 layers or greater and 5 layers or greater in 2 additional PTCs.[10] There are no widely accepted guidelines as to how many PTC should be assessed, and a survey of the literature shows that methods vary,[24] with some authors only assessing the worst-affected PTC (without mention of numbers of PTC examined),[19] and others counting anything between 4[6] and 25[5] PTCs for this feature.[9,15-18,20,22,23,28] We noticed an increase in number of PTC with greater than 5 BML when a larger sample of PTC was assessed (25 vs 10). However, the effect of sample size was less pronounced when looking at the mean number of PTCBML. This suggests the need for future studies to address PTCBML counting methodology. Liapis et al, in a recent publication, recommend to scan at least 15 to 20 PTCs for the 3 worst affected and find that presence of severe changes (1 PTC with ≥7 layers and one other with ≥5 layers at least) is helpful in establishing a diagnosis of cAMR, whereas absence of such severe PTCBML is a strong indicator excluding cAMR. In contrast, using a "within disease" approach, specifically looking in patients with a dnDSA and in a biopsy around 1 year after transplantation, we find that 3 PTCs with 5 layers or greater of BM is predictive of graft loss, probably indicating established cAMR. However, by using this approach, we did not address the issue of specificity of this feature.

In addition to its role in the diagnosis of established cAMR, EM may help detect early antibody-mediated injury, at levels not diagnostic for cAMR. This is the main question we have addressed in the current study. Significant PTCBML can develop as early as 3 months after transplantation.[6] In a small vintage-matched case-control study which included sensitized and ABOi cases, and nonsensitized controls, we previously found that if 10 PTCs or greater had 3 layers or greater of BM, this was associated with TG development.[5] Patients who went on to develop TG had a mean of 2.58 BM layers, as opposed to 1.67 in the control group.[5] Based on this, we chose a cutoff of 2.5 mean layers of BM as a potential marker for future TG. In the current study, a "within disease" cohort comprising only patients with dnDSA was analyzed. We found that a mean greater than 2.5 BM layers in a biopsy



Number of patients at risk for							
A. death-censored graft failure							
Years after index biopsy	0	1	2	3	4	5	6
non-progressor (n=25)	25	22	19	12	9	3	1
progressor (n=25)	25	22	19	14	10	5	1
B. transplant glomerulopathy development							
Years after index biopsy	0	1	2	3	4	5	6
non-progressor (n=25)	25	17	11	8	2	1	0
progressor (n=22)	25	18	8	6	1	0	0

FIGURE 5. Death-censored graft survival (A) and transplant glomerulopathy free survival (B) stratified by PTCBML. Kaplan-Meier curve and descriptive table of death-censored graft survival (A) and transplant glomerulopathy-free survival (B) comparing the nonprogressor group (solid line, n = 25) with the progressors (stippled line, n = 25 for graft survival analysis, n = 22 for TG development analysis).

around 1 year after transplantation correlates with future TG development. Our control group of DSA-negative patients had a mean PTCBML count of 1.91. Although the cutoff of 2.5 BM layers may appear low, comparable numbers were noted in other publications. Looking at PTCBML concurrent with cAMR, Ivanyi et al[17] found a mean number of circumferential layers of 2.87 ± 1.83 (range, 0-7.36). Looking at PTCBML before cAMR development, Wavamunno et al[6] found that around 1 year after transplantation, the mean number of layers of PTCBML in cases that went on to develop TG was greater than 2, whereas the controls had mostly 2 layers or less. When assessed in native kidney biopsies, mean number of BM layers was 0.53 ± 0.65 (range, 0-2.78).[17] We were not able to show a relationship between mean PTCBML greater than 2.5 around 1 year after transplantation and graft loss, although it does predict later TG development. The relative short follow-up after TG development might have hidden an association with graft survival, but only a longer follow-up of this cohort can tell.

We also hypothesized that progression of PTCBML over time could be a marker of ongoing antibody-mediated injury. We performed a longitudinal analysis, comparing PTCBML

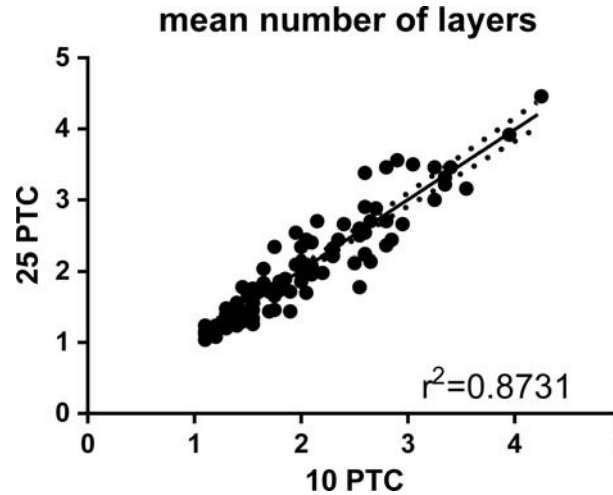


FIGURE 6. Correlation of 10 versus 25 PTCs counts for basement membrane multilayering in 93 samples of 51 patients. Discrete data of 10 and 25 PTC counts for basement multilayering were assessed with linear regression, with counts in 25 PTC set as the "gold standard."

nonprogressors (all samples ≤2.5 mean BML) with progressors (all patients having at some point a sample with >2.5 mean number of BML). There were no significant differences in clinical characteristics or immunological risk factors between the 2 groups. However, TG development was significantly more likely to occur in the PTCBML progressors group. In the longitudinal cohort of dnDSA patients, all trends in PTCBML were seen: low to low, low to high, high to high, and even high to low. Of note is that time intervals between biopsies were variable as most were taken on clinical indication, although this was not significantly different for the nonprogressor and progressor group. Sampling effect may explain some of the observations, but it is interesting to note that not all PTCBML progresses, as far as we can assess in a routine setting. Loss of multilayering over time could be a reflection of loss of PTC.[29]

Peritubular capillary BM multilayering is not specific for cAMR.[15,17,19] Problems with specificity arise with each potential new marker for early AMR, including transcript analysis and MI.[12,30] The C4d staining of PTCs was thought to be the most specific and sensitive marker of AMR, but may be less informative than MI.[12,31] Combining all these features may be more informative than considering each in isolation.[32] In the case of PTCBML, as pointed out by Ivanyi et al,[18] obstructive uropathy, chronic tubulointerstitial nephritis, thrombotic microangiopathy, radiation nephritis, analgesic nephropathy, and Balkan nephropathy should be excluded as causes of multilayering. Hence, knowing that PTCBML can be caused by other factors should not lead to disregarding PTCBML as a valid marker. The cores of transplanted tissue derived through biopsy procedure are routinely split for light and EM. Sometimes, the light microscopy sample is insufficient to yield a firm diagnosis, and additional data from ultrastructural examination have the potential to consolidate a diagnosis. Here, we found that PTCBML was associated with MI and, based on ROC analyses, PTCBML was of additive value to the MI score for the prediction of TG development increasing the area under curve to 0.81.

Limitations of our study are mainly associated with the retrospective nature of our cohort as was mentioned in our previous article.[12] Biopsy and DSA data were not standardized for this cohort. The biopsies included were mostly indication biopsies, whereas a prospective, 1-year surveillance biopsy cohort of patients with dnDSA might better suit the questions addressed. In addition, some patients had no biopsy samples available at all, although patient outcomes did not significantly differ between patients with and without biopsy samples. Our focus was on PTCBML assessment and prediction of outcome within a cohort of dnDSA patients.

Our conclusion is that, in patients with dnDSA, a mean number greater than 2.5 PTCBML in a biopsy around 1 year after transplantation or developing over a series of biopsies is associated with TG development. The PTCBML score is of additive value to the MI score for prediction of TG development, and when EM is not available, we recommend using the MI score alone in patients with dnDSA for outcome prediction. We suggest that a mean greater than 2.5 PTCBML could be used to stratify patients with dnDSA (where possible in conjunction with MI and transcript analysis) so that those at increased risk of antibody-mediated damage could be closely monitored and/or included in new clinical trials.

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