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An analysis of transplant glomerulopathy and thrombotic microangiopathy in kidney transplant biopsies

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Summary

Glomerular diseases of the transplanted kidney are the most important cause of poor long- term outcome. The estimation of the magnitude of this problem and an elucidation of pathogenic mechanism is essential for improvement of graft survival. This study from the Indian subcontinent aims (i) to determine the incidence of transplant glomerulopathy (TG) and thrombotic microangiopathy (TMA) in a large cohort of indicated renal transplant biopsies, (ii) to evaluate the histological and ultrastructural features of TG and TMA, and (iii) to assess the relationship between the two glomerular lesions. Of a total of 1792 indication renal transplant biopsies received over 5 years (2006–2010), 266 biopsies (of 249 patients) had significant glomerular pathology and were further analyzed along with immunofluorescence, electron microscopy (EM), and C4d immunohistochemistry. TG is the most common glomerular lesion followed by TMA seen in 5.97% and 5.08% of allograft biopsies, respectively, which constitutes 40.23% and 34.2% of biopsies with significant glomerular lesions. Pathologic antibody-mediated rejection (AMR) is associated with both TG and TMA in 71% and 46.5%, respectively. A coexistent TG was found in 18.4% of biopsies with TMA. Endothelial swelling with subendothelial widening, a feature of TMA, is also seen in early TG by EM. Our findings support the concept that TG evolves from a smoldering TMA of various causes.

Introduction

Kidney transplantation is the best therapeutic option for most patients with end- stage kidney disease. In spite of a marked decrease in acute rejection episodes in early post-transplant period, the long-term survival of graft is still poor, and the most important cause for poor long-term outcome is the development of *de novo* or recurrent glomerular disease [1]. Glomerular diseases of transplanted kidneys include those related to transplantation like glomerulitis of antibody-mediated rejection (AMR), transplant glomerulopathy (TG), *de novo* thrombotic microangiopathy (TMA); glomerulopathies acquired from renal donor; and diseases unrelated to the process of transplantation such as *de novo* or recurrent glomerulonephritis.

Glomerular lesions are responsible for upto 36% of graft loss, and TG constitutes the single most common specific histological diagnosis of graft loss [2]. In addition, lesions of TMA in the glomeruli are a significant problem, and their varied morphology can mimic various *de novo* and recurrent glomerular diseases including TG.

Transplant glomerulopathy is defined by the characteristic duplication of glomerular capillary wall observed by light microscopy, as recommended by the Banff working group and is associated with poor allograft survival. TG begins as a focal lesion and involves increasing percentage of capillary loops in an increasing number of glomeruli [3].

It is considered to be a manifestation of chronic antibody antibody-mediated injury, though C4d positivity is seen in 25–50% of cases only in various studies [3–7]. Recent study suggests donor-specific antibody positive, C4d negative (DSA+/C4d−) TG may be classified as chronic AMR, while DSA−/C4d− TG results from T-cell activation [8].

Thrombotic microangiopathy in a renal transplant is often *de novo* rather than recurrent and is limited to the kidneys. It can be due to varied causes including antibody-mediated rejection, calcineurin inhibitor toxicity, cytomegalovirus infection, parvovirus B 19 infection, BK polyoma virus nephritis, anticardiolipin antibodies in hepatitis C virus (HCV)-positive patients or malignancy [9]. It can have varied morphology ranging from fibrin thrombi and endothelial swelling in acute TMA to membranoproliferative lesion or segmental sclerosis of glomerulus or arteriolar hyalinosis, intimal fibrosis and onion-skin hypertrophy of the arteriolar walls [10,11].

Recurrent and *de novo* glomerulonephritides in the renal allograft display the same features as in the native kidneys on light microscopy, immunofluorescence, and electron microscopy (EM). However, the diagnosis is a challenge as it may coexist with glomerular manifestations of acute or chronic rejection as well as those of drug toxicity [12].

This study is from a large tertiary care centre in India, and it analyses the magnitude of the glomerular pathology in renal transplant biopsies with special reference to TG and TMA, as well as various other recurrent or *de novo* glomerular pathologies occurring singly or in combination with transplant-related lesions.

Subjects and methods

This study material comprises all indication renal transplant biopsies submitted to the department of Histopathology, PGIMER, Chandigarh, India for the diagnosis of graft dysfunction in the form of raised creatinine, proteinuria, or hematuria over a period of 5 years from January 2006 to December 2010. Protocol biopsies were excluded from the study as they are infrequently and irregulary done in our institute.

Histopathological examination

Screening

All the renal transplant biopsies were screened for any glomerular changes by going through the routine H & E and PAS-stained slides. A minimum of one H&E and one PAS-stained slide was used to screen and, whenever needed, special stains like Jones silver methanamine, Masson trichrome, Marshal scarlet blue, Phosphotungstic acid haematoxylin stains were used. The biopsies were screened and analyzed by four pathologists (SR, KJ, RD, RN) independently and later simultaneously in a multiheader microscope and good consensus of opinion was reached in every case.

Immunohistochemistry for C4d

C4d staining was performed by immunoperoxidase method using rabbit antihuman C4d polyclonal antibody from AbD serotec (catalogue no. 03000230) at dilution of 1:20 as the primary antibody, antigen extraction by pressure cooker method and horse radish peroxidase conjugated antibody (HRP REAL Envision from DAKO catalogue no: K 5007) as the secondary antibody and diaminobenzidine as the substrate. Immunostaining was done using protocol described by Regele *et al.* [13], which is modified and standardized in the department. A known case of antibody-mediated rejection was used as external control.

Direct immunofluorescence (DIF)

Direct immunofluorescence (DIF) was performed for IgG, IgM, IgA, fibrinogen, kappa, Lambda, C3, and C1q using FITC-targeted antibody (DAKO) at a dilution of 1:20. DIF was also performed in selected cases on paraffin sections using the protocol standardized in the department modified from the method described by Nasr *et al.* [14]. The antigen extraction was performed by digesting deparaffinized tissue using 50–100 µl of Proteinase K diluted with phosphate buffer saline at pH 7.2, and further DIF was performed as in frozen sections.

Electron microscopy (EM)

Tissues were fixed in glutaraldehyde, postfixed in osmium, embedded in Epon 812. Thin sections were cut and stained with lead citrate and uranyl acetate. Grids were visualized by Zeiss 906 transmission electron microscopy and photographed by digital photography. Electron microscopy was performed on paraffin-embedded tissue by taking the tissue out of the paraffin block, deparaffinized with repeated changes of xylene over a period of 3 h (six changes). Subsequently, tissue was rehydrated with graded alcohol and later fixed with glutaraldehyde 2%. The tissue was then processed as done routinely [15].

Clinical data

The clinical data of the cases were retrieved from the original requisition forms and patient files.

Analysis of histopathological, immunohistochemical, immunofluorescence, and electron microscopy findings

The biopsies were assessed in detail, and scoring of various parameters was done as recommended in the Banff schema 2009 updates [16]. Criteria for assessment of acute antibody-mediated rejection comprised (i) Acute tubular necrosis (ATN)-like changes in the tubules, and (ii)

peritubular capillaritis with or without glomerulitis or capillary thrombosis [16]. C4d positivity in peritubular capillaries (PTCs) was also recorded. However, data on the presence or absence of circulating donor-specific antibodies was invariably not available. Hence, the diagnosis made was of "Pathologic AMR" rather than "AMR". TG was defined as glomerular capillary loop thickening with double contours identified in methanamine silver stain or PAS-stained sections affecting at least 10% of the peripheral capillary loops in a nonsclerotic glomerulus (Banff schema 2009) with/without circumferential multilamination of glomerular basement membrane in electron microscopy due to formation of new layer(s) of basal lamina. Subendothelial electron-lucent widening is seen in early stages of TG by electron microscopy. However, the expansion of subendothelial space without new layer(s) of basal lamina cannot be taken as specific diagnostic feature of TG as it is seen in TMA also [17]. TMA is identified by light microscopy as glomerular or vascular fibrin thrombi, fibrinoid necrosis of arterioles, prominent mucoid to concentric intimal thickening with luminal obliteration in small arteries and arterioles, presence of red blood cells in the thickened vascular walls. Glomeruli, in addition, may show various other changes in subacute or chronic phase which includes endothelial swelling or vacuolation, subendothelial fibrin deposition demonstrable by special stains like Marshal scarlet blue, mesangial hypercellularity, mesangiolysis, bloodless appearance, podocyte prominence, segmental or gobal collapse and small crescents (Fig. 1). As the subacute and chronic stages of TMA resembles TG [10] a diagnosis of TG was made in the presence of glomerular capillary loop thickening with double contours affecting at least 10% of the peripheral capillary loops (Banff 2009); and in same glomerulus, a coexistent TMA was considered only in the presence of definite fibrin thrombi or other features as defined above. A combination of TG and TMA was also diagnosed in situations where different glomeruli in the renal biopsy showed features of both.

Statistical analysis

A descriptive analysis of the data was performed. Appropriate correlation and tests of significance were derived using SPSS software 17.0 (SPSS Inc., Chicago, IL, USA) wherever needed.

Results

The department of Histopathology received a total of 1792 indication transplant biopsies over a 5-year period. After screening, 362 of 1792 biopsies were found to have glomerular changes. The biopsies with only glomerulitis of AMR (n = 50; 2.79%) and glomerular sclerosis related to arteriosclerosis or of unknown significance like ischemic changes and glomerular enlargement (n = 46) were separated out. Hence, 266 sof 1792 indication biopsies had significant glomerular lesion (14.84%) defined as those lesions with features diagnostic of TG or TMA as defined above in light microscopy; those having well-defined morphological abnormalities like thickening of glomerular capillary loops, diffuse or focal endocapillary proliferation, mesangial proliferation, membranoproliferative patterns, crescents, and segmental sclerosis (Table 1). They were further analyzed by special stains, immunohistochemistry, DIF, and EM. These 266 biopsies with significant glomerular lesions were from 249 patients. Of 266 biopsies selected for detailed analyses, EM was done in 54 biopsies and DIF in 148 biopsies.

Demographic and clinical profile of patients

The age of the patients (n = 249) ranged from 17 to 66 years (mean 37 years). Male: female ratio was 7:1. The basic disease was largely unknown, and most patients had a diagnosis of ESRD or chronic glomerulonephritis. Symptomatic or asymptomatic rise in creatinine was the most common indication of renal biopsy (n = 195) followed by proteinuria (n = 87). In nine patients, the first kidney transplant was lost and the biopsy under study was from the second transplant. Twenty-six patients were positive for HCV, 12 for HBsAg, two each for CMV, parvovirus and BKV. Two patients had serological positivity for both HCV and HBsAg and one patient for HBsAg and CMV. The donor-specific antibody status was not determined. Various clinical parameters are summarized in Table 2.

On the basis of light microscopy, DIF, and EM, the prevalence of various glomerular lesions as diagnosed in our study is summarized in the Table 1. In addition, the biopsies also revealed pathologic AMR (as defined above) in 41.35%, morphological features of calcineurin inhibitor (CNI) toxicity in 11.65%, acute cellular rejection (ACR) in 6.02%, and granulomatous inflammation in one case. ACR cases included borderline, grade I and grade II ACR. There were no biopsies with grade III ACR.

Transplant glomerulopathy

There were 107 biopsies with TG in a total of 1792 renal allograft biopsies over a period of 5 years (5.97%). It constituted 40.23% of all significant glomerular lesions (107/266 in biopsies or 102/249 patients). The duration of transplantation at the time of biopsy ranged from 1 month to 180 months with a mean of 52.5 months. There was only a single case of less than 3-month duration. The duration was 3 months to less than 1 year, 1–5 years and more

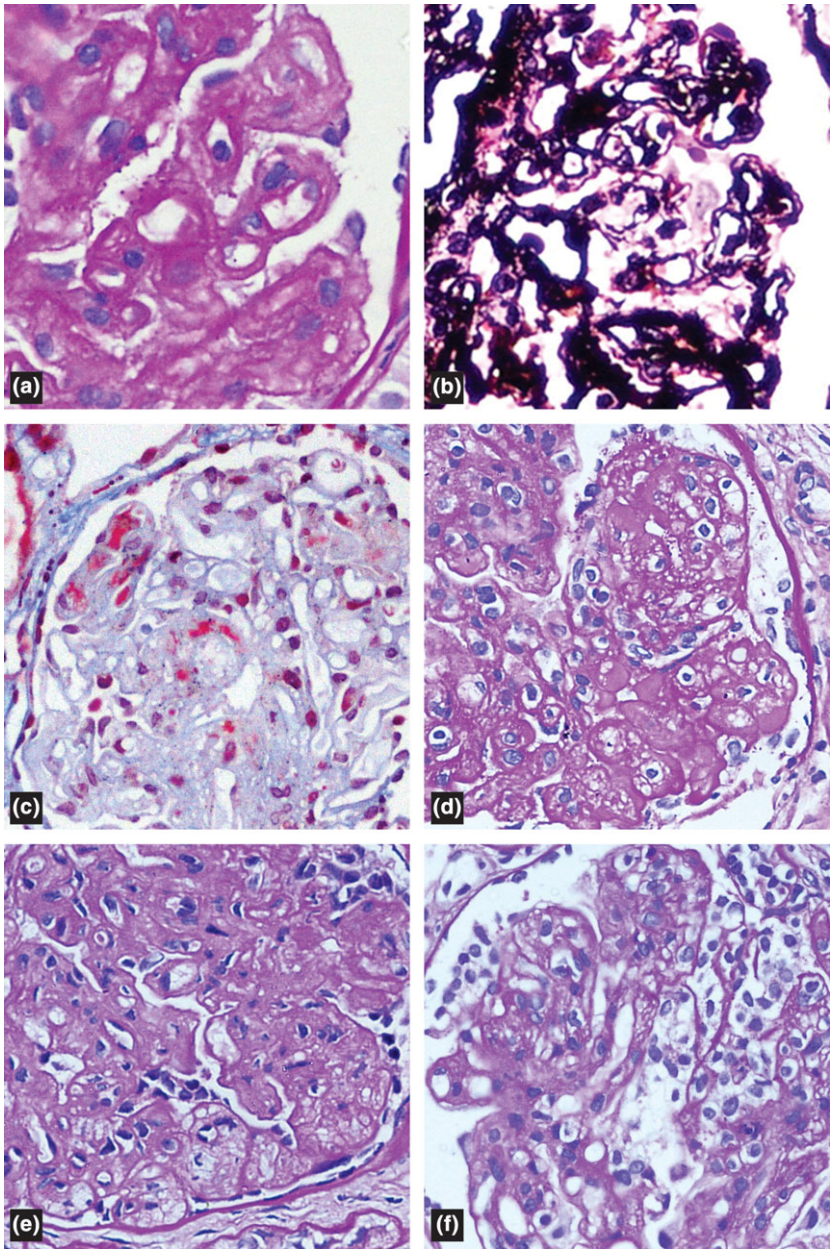

Figure 1 Panel of microphotographs showing glomeruli with different morphological changes. (a, b) A case of transplant glomerulopathy (TG) with duplicated appearance of peripheral capillary loops (×400, PAS (a) and Jones silver stain (b)). (c) A case of thrombotic microangiopathy (TMA) with subendothelial and luminal fibrin deposition highlighted by Marshal scarlet blue stain (×400). (d) Glomerular loop thickening with endothelial swelling (×400, PAS). (e). Marked endothelial swelling and vacoulation in a case of TMA (×400, PAS). (f) A case of TMA with podocyte prominence (×400, PAS).

than 5 years in 9%, 60%, and 30% cases, respectively. At the time of biopsy, creatinine was elevated in 87% and proteinuria was present in 97% of patients. The clinical data is summarized in Table 2.

Pathological features in biopsies with transplant glomerulopathy were graded as per the Banff schema and are summarized in Table 3. C4d immunostaining positivity along peritubular capillaries was graded by Banff criteria; it was absent or <10% in 26% cases, focal positive (10–50%) in 5.6% cases, and diffuse positive (>50%) in 68.2%. Glomerular C4d positivity was seen in 78.7% cases. There was morphological evidence of AMR in the form of peritubular capillaritis with or without glomerulitis or capillary thrombosis or ATN in 68% of biopsies which could be confirmed by C4d immunostaining. In another three biopsies, there was morphological evidence of AMR though the C4d was

Table 1. Prevalence of various glomerular lesions in allograft biopsies.

Diagnosis	Number of biopsies	% of significant glomerular lesions $n = 266$	% of total allograft biopsies $n = 1792$
Transplant glomerulopathy	107	40.23	5.97
Thrombotic microangiopathy	91	34.20	5.08
IgA nephropathy	41	15.41	2.29
Membranous GN	16	6.02	0.89
FSGS	13	4.89	0.73
Membrano proliferative GN type I	5	1.88	0.28
Diffuse proliferative GN	3	1.13	0.17
Pauci immune crescentic GN	2	0.75	0.11
AntiGBM disease	2	0.75	0.11
Diabetic nephropathy	2	0.75	0.11
Dense deposit disease	2	0.75	0.11
Light chain deposit disease	1	0.38	0.06
CMV glomerulopathy	1	0.38	0.06

negative. Thus, of 107 biopsies, there was morphologic evidence of AMR in 71% biopsies. Associated features of CNI toxicity was evident in eight biopsies (7.5%) while ACR was seen in six biopsies (5.6%). There was no statistical difference between the grades of TG (cg1, cg2, cg3) in terms of glomerulitis(g), tubulitis(t), tubular atrophy(ct), interstitial inflammation (i), interstitial fibrosis (ci), PTC dilatation, and capillaritis.

Table 3. Pathological features in biopsies with transplant glomerulopathy.

	Feature	Score	No. (%)
Banff cg score (cg)	cg0	<10%	0
	cg1	10–25%	28 (26.2)
	cg2	26–50%	31 (29.0)
	cg3	>50%	48 (44.9)
Banff glomerulitis score (g)	g0	0	47 (43.93)
	g1	<25%	21 (19.63)
	g2	25–75%	14 (13.08)
	g3	>75%	25 (23.36)
Banff tubulitis score (t)	t0	0	86 (80.4)
	t1	1–4	9 (8.4)
	t2	5–10	10 (9.3)
	t3	>10	2 (1.9)
Banff tubular atrophy score (ct)	ct0	0	18 (16.8)
	ct1	1–25%	47 (43.9)
	ct2	26–50%	30 (28.0)
	ct3	>50%	12 (11.2)
Peritubular capillary dilatation		Absent	14 (13.1)
		Focal	18 (16.8)
		Diffuse	75 (70.1)
Peritubular capillaritis		0	19 (17.76)
		3–4	55 (51.4)
		5–10	28 (26.17)
		>10	5 (4.67)
Interstitial inflammation score (i)	i0	0–10%	22 (20.6)
	i1	11–25%	63 (58.9)
	i2	26–50%	20 (18.7)
	i3	>50%	2 (1.9)
Interstitial fibrosis score (ci)	ci0	0–5%	47 (43.9)
	ci1	5–25%	32 (29.9)
	ci2	26–50%	18 (16.8)
	ci3	>50%	10 (9.3)

Thrombotic microangiopathy

Thrombotic microangiopathy was morphologically diagnosed as defined above (Fig. 1). Glomerular and or vascular TMA was present in 114/1792 (6.4%) of renal allograft biopsies.

91/1792 (5.08%) biopsies had glomerular TMA with or without vascular TMA and constituted 34.20% of significant glomerular lesions (91/266). In another 23 biopsies, TMA was found only in the blood vessels. At the time of biopsy, serum creatinine was elevated in 74.7%, and proteinuria was present in 26.3% of patients. The clinical data is summarized in Table 2. TMA was associated with AMR confirmed by C4d staining in 50 biopsies (43.9%) while 3 (2.6%) of them had morphological evidence of AMR; however, C4d was negative. Thus, TMA was associated with

Table 2. Summary of clinical data.

	Biopsies with significant glomerular lesions	Transplant glomerulopathy	Thrombotic microangiopathy
No: of biopsies	266	107	114
Age range (Mean)	17–66 years (37 years)	17–66 years (38.4 years)	18–62 years (38.2 years)
Duration of transplantation at the time of biopsy (Mean)	3 days to 204 months (46.8 months)	1–180 months (52.5 months)	3 days to 204 months (46.9 months)
Mean serum creatinine at the time of biopsy	3.7 mg/dl	4.18 mg/dl	3.85 mg/dl
Mean urine protein at the time of biopsy	2.62 g/24 h	2.38 g/24 h	3.36 g/24 h

pathologic AMR in a total of 53 (46.5%) biopsies. Evidence of CNI toxicity was seen in 28 (24.6%) biopsies and in one case there was evidence of cytomegalovirus infection confirmed by immunohistochemistry. Both AMR as well as CNI toxicity would have contributed to the development of TMA in 6 (5.3%) biopsies. In 38 (33%) biopsies, the etiology is not clear. A coexistent TG.was found in 21/114 biopsies with TMA (18.4%).

The combination of TG or TMA associated with other glomerulonephritis was diagnosed with the help of DIF and EM. TG was associated with dense deposit disease and thin glomerular basement membrane disease in one biopsy each. Similarly, TMA was associated with IgA nephropathy in three cases. There was a single biopsy with TG, TMA, and IgA nephropathy.

Analysis of serial biopsies

Of 249 patients with significant glomerular lesions, 16 patients had more than one biopsy. 15 patients had two and one had three biopsies. Two patients had TMA in the initial biopsy and TG in subsequent biopsies. One had TG in the initial biopsy but had TMA in the subsequent one. Five patients had TG in both the biopsies. One patient had TMA in the initial biopsy and focal segmental glomerulosclerosis (FSGS) subsequently.

Ultrastructural findings in TG and TMA

Electron microscopy was done in 54 biopsies with a diagnosis of TG (14 cases), TMA (14 cases), IgA nephropathy (16 cases), membranous glomerulonephritis (five cases), FSGS (two cases), diabetic nephropathy, light chain deposit disease, anti glomerular basement membrane disease (GBM) disease (one case each). Of 14 cases with TG, glomeruli and PTCs were present for evaluation in 12 cases of which only three biopsies had multilamination of GBM, a diagnostic feature of TG, while four showed subendothelial expansion by fluffy material. Nine biopsies showed moderate (5–6 layers) to severe (≥7 layers) multilamination of basement membrane of PTCs. The rest of them did not show any specific ultrastructural changes. Of 14 cases with TMA, subendothelial expansion by electron luscent material was found in eight biopsies; none of them showed multilamination of GBM. Five biopsies had moderate (5–6 layers) to severe (≥7 layers) multilamination of basement membrane of PTCs (Fig. 2).

Discussion

The present study which is the largest study on glomerular pathology in renal allograft in India (ref: PUBMED SEARCH) shows that *de novo* or recurrent glomerular lesions excluding glomerulitis and arterionephrosclerosis are seen in 14.84% of indicated renal allograft biopsies. A similar study was performed by Chan *et al.* [18], who found the prevalence of significant glomerulopathy to be 33%. In our study, TG is seen in 5.97% of biopsies, which is similar to 5.1% reported by Sis *et al.* [7], but is less frequent compared with the 9.5% reported by Gloor *et al.* [3] in a study which included protocol biopsies also. Thus, TG is the most common glomerular pathology in renal allografts. The prevalence of TG was 1.6% (18/1111) by Sijpkens *et al.* [6], 5.36% (62/1156) by Horita *et al.* [19] and 19.1% (28/146) of allografts with significant glomerular lesions by Chan *et al.* [18].

The incidence of peritubular capillary C4d staining in TG is various studies was reported to be 53% by Regele *et al.* [5], 40% by Sijpkens *et al.* [6], 26% by Sis *et al.* [7], 25% by Gloor *et al.* [3], and 68.2% in the present study. Regele *et al.* [13] first reported the use of C4d staining on paraffin sections by using a novel anti-C4d polyclonal antibody. In this study, the C4d positive group had less of tubular atrophy and interstitial fibrosis as compared with C4d negative group, which can be explained by the fact that PTCs gradually disappears in chronic cases [20].

While linear C4d positivity on PTCs is well known to be indicative of AMR, the significance of glomerular capillary wall staining by C4d in paraffin section is not clear. In our study, glomerular C4d staining was seen in 78.7% of the biopsies with TG; as compared with 12% by Regele *et al.* [5], 90% by Sijpkens *et al.* [6], 32% by Gloor *et al.* [3]. Sijpkens *et al.* [6] have considered glomerular C4d positivity as a manifestation of TG. A recent study by Kikic *et al.* [21] has studied the significance of C4d positivity in glomeruli, PTCs and arterioles. In univariate analysis they have found that glomerular C4d positivity is associated with inferior graft survival, however the significance was lost in multivariate analysis.

In this study, the prevalence of TMA was 6.4% of renal allograft biopsies. In the study by Satoskar *et al.* [22], there were a total of 59 patients of 960 with glomerular and or vascular TMA (6.1%). Of these 59 patients, 25 had glomerular TMA alone (2.6%), while in our study it was noted in 71 patients (3.96%). The incidence of TMA in literature varies from 0.8% to 14% [11,22–25]. Our study shows that the prevalence of glomerular TMA is high and, as mentioned in the review by Ponticelli [26], this is an underrated complication.

The diagnosis of vascular TMA and acute glomerular TMA is straight forward as defined above; however, it is met with difficulty and difference of opinion in case of subacute or chronic glomerular TMA as it can be confused with TG and other glomerular diseases with MPGN pattern. In the absence of a definite diagnostic criteria to distinguish chronic TMA from TG, a diagnosis of TMA

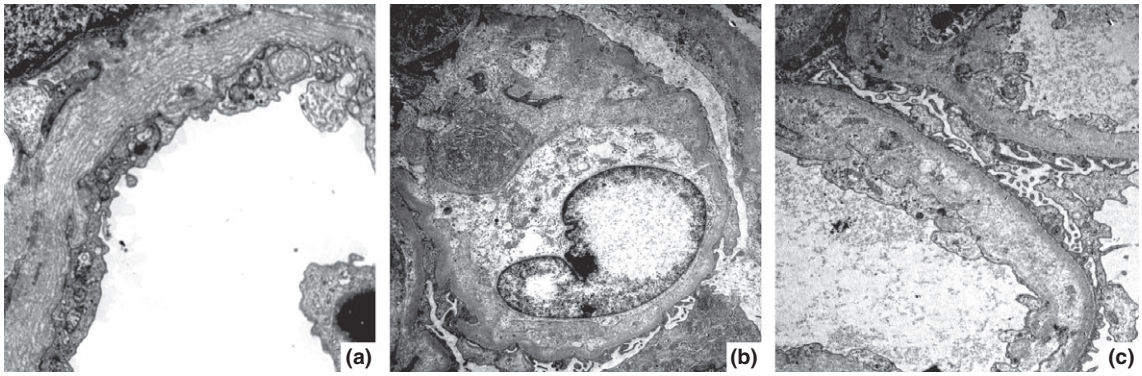

Figure 2 Electron microscopic features; (a) Peritubular capillary basement membrane multilamination upto 10 layers in a case of transplant glomerulopathy (TG; ×17100). (b) Endothelial swelling seen in glomerular capillaries in a case of thrombotic microangiopathy (TMA; ×10300). (c) Sub endothelial widening by fluffy electron luscent material in a case of TMA(×17100).

was entertained after exclusion of TG in light microscopy. Though the presence of thrombus in the glomerular capillary loops is the defining feature of acute glomerular TMA, they cannot be seen in later stages. Similarly, endothelial swelling with subendothelial widening is also a manifestation of glomerular TMA. Segmental and global collapse of the glomerular tuft is part of TMA in the setting of kidney transplants. Nadasdy *et al.* [27] have suggested that the morphologic pattern of collapsing glomerulopathy in renal allografts may not represent the same disease process as collapsing glomerulopathy in native kidneys, but rather represent a pattern of renal injury, which may occur due to hemodynamic disturbance. It may occur by direct visceral epithelial cell injury by diverse causes including ischemia and immunosuppression, which causes dysregulated cycling podocyte phenotype [28]. Cellular crescents were seen in 0.36% of all the glomeruli and 1.1% of the biopsies with TMA. Crescents are described in TMA and can be seen in 5.6% cases of TMA with 0.9% cases having more than 50% crescents [29]. In the study by Zarifian *et al.* [25], crescents were seen in association with cyclosporin-related TMA in 24% (9/38 with TMA) of the biopsies, involving less than 10% of the glomeruli in most biopsies. Unlike acute or subacute TMA, chronic TMA especially with mesangiocapillary pattern is difficult to differentiate from TG and other glomerular diseases with MPGN pattern. In late healed stages, TMA can lead to segmental or global sclerosis. In our study, segmentally sclerosed lesions were found in 2.6% of the glomeruli. An attempt was made to identify the cause of TMA in every biopsy. AMR is the most common (46.5%) cause of TMA in this study. Satoskar *et al.* [22] have also shown that 55% of *de novo* TMA patients had evidence of AMR. The second common cause of TMA in this study is CNI toxicity and the cause of TMA could not be delineated in 33% of our cases. TMA in transplant setting can be the result of a number of factors including viral infections, drugs, rejection, and complement abnormalities. CNI toxicity can also occur without any morphological evidence. Hence, the identification of causes in such cases needs more extensive workup.

The existence of C4d negative AMR has been reported by Sis *et al.* [30] as well as Haas [31]. In our study also, 3% of biopsies with morphological features of AMR were C4d negative. Nine of 107 TG cases (8.4%) and eight of 91 patients with TMA (8.8%) were positive for HCV, but a causal relationship between HCV and TG or TMA cannot be determined from our study; as shown by Baid-Agrawal *et al.* [32].

Wavamunno *et al.* [33] have documented that the early ultra structural changes in TG include endothelial cell swelling and vacuolation, and widening of the subendothelial space due to expansion of lamina rara interna filled with floccular electron-lucent material. Formation of lamina densa inner to expanded lamina rara interna occurs in later stages. These early changes are similar to the EM appearance of TMA, as seen in few of our cases suggesting an overlap between TG and TMA. Cosio *et al.* [34] mentioned that although activation of coagulation plays a role in the pathogenesis of TG, microthrombi are not often seen in TG. In our study, 21/114 biopsies with TMA had coexisting TG. The Banff working group has considered TG as an equivalent to chronic antibody-mediated rejection provided C4d is diffusely positive in PTCs [16]. However, C4d positivity is seen only in 25–61% of the cases of TG. One of the attractive hypotheses which can explain the pathogenesis of TG is a chronic smoldering TMA as described by Satoskar *et al.* [22]. Whether repeated episodes of TMA either due to AMR or CNI toxicity or any other cause such as HCV infection [35] is a precursor lesion of TG has been speculated but not proven. Our observations support this hypothesis for the following reasons: (i) various studies including ours have shown the association of TMA with

AMR, CNI toxicity and various infections like CMV, HCV, BK polyoma virus and Parvo virus B19 [9], (ii) endothelial injury and remodeling plays an important role in the pathogenesis of both the conditions. Recent papers highlight the central role played by endothelial injury and subsequent endothelial changes in the development of varied spectrum of microvasular changes including TG and TMA [36,37], (iii) TMA and TG can coexist in same biopsies, (iv) occasionally patients (two patients in this study) have glomerular TMA in first biopsy and TG in subsequent biopsies, and (v) the morphology of chronic TMA overlaps with TG in light microscopy as well as in EM [17,33]. Baid-Agrawal *et al.* [32] have shown that there are three overlapping pathways involving AMR, HCV, and TMA leading to the development of TG. Though it is a fact that such observational studies may not be sufficient to prove a hypothesis, the current study highlights the burden of TMA in allograft biopsies and its possible role in the evolution of TG.

Authorship

SS: collection and analysis of data, helped design the study, and wrote the paper. KJ: designed the study, performed and supervised the conduction of the study, analysis of data, and helped in writing of the paper. RD and RN: one of the four members who performed the study by examining the biopsy material. MM and VS: contributed patient data and biopsy material for the study.

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