Glomerular C4d Immunoperoxidase in Chronic Antibody-Mediated Rejection and Transplant Glomerulopathy

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Introduction: The diagnosis of late antibody-mediated rejection (AMR) is compromised by frequent absence of C4d in peritubular capillaries ($C4d_{ptc}$), termed "C4d-negative" AMR. We hypothesized that glomerular capillary C4d ($C4d_{glom}$) reflected endothelial interaction with antibody and could improve immunologic classification of transplant glomerulopathy (TG).

Methods: We evaluated C4d using immunoperoxidase in 3524 consecutive, kidney transplant biopsies from a single center.

Results: $C4d_{glom}$ was detected in 16.5% and $C4d_{ptc}$ in 9.9% of biopsies. $C4d_{glom}$ occurred in 60.3% of TG ($n = 174$) and was absent in normal glomeruli. Epidemiologic risk factors for $C4d_{glom}$ were younger, female, living-donor recipients with early AMR, prior treated rejection, and late presentation using multivariable analysis. Semiquantitative $C4d_{glom}$ score correlated with donor specific antibody (DSA) level, $C4d_{ptc}$, microvascular inflammation (MVI), Banff cg scores, renal dysfunction, and proteinuria. Principal component analysis colocalized $C4d_{glom}$ with histologic AMR. Multivariable analysis of TG found DSA, $C4d_{ptc}$, and post-transplant time associated with $C4d_{glom}$. Addition of $C4d_{glom}$ into Banff chronic AMR schema improved its diagnostic sensitivity for TG (verified by electron microscopy [EM]) from 22.2% to 82.4% and accuracy from 59.6% to 93.9%, compared with Banff 2019 using only $C4d_{ptc}$. Tissue $C4d_{glom}$ and chronic AMR diagnosis incorporating $C4d_{glom}$ were associated with death-censored allograft failure in TG ($P < 0.001$), independent of the severity of glomerulopathy and chronic interstitial fibrosis.

Conclusion: $C4d_{glom}$ is a promising diagnostic biomarker of endothelial interaction with antibody which substantially improved test performance of the Banff schema to correctly classify TG by pathophysiology and prognosticate graft loss. We recommend routine C4d immunoperoxidase to minimize underdiagnosis of late AMR in TG.

Kidney Int Rep (2022) **7**, 1594–1607; https://doi.org/10.1016/j.ekir.2022.04.016

KEYWORDS: antibody-mediated rejection; Banff schema; kidney transplantation

Late-onset AMR is characterized by *de novo* DSA from underimmunosuppression, glomerulitis, and/or peritubular capillary (PTC) inflammation (MVI), C4d deposition, and chronic morphologic changes, including transplant TG and multilamination of PTC basement membranes. Activated glomerular capillary endothelial cells expand the subendothelial space with fibrillary and neomembrane material forming "double contours" on silver staining.[1–4] The pathologic diagnosis of chronic AMR should be accurate and reliable. Underdiagnosis of AMR in the early Banff schema was highlighted by abnormal endothelial transcript expression (indicating molecular AMR) in "C4d-negative" rejection (using diffuse $C4d_{ptc}3$ threshold) and then misclassified as T cell-mediated rejection.[5] Iterative reductions of $C4d_{ptc}$ thresholds to "focal" C4d2 (10%–50% for immunofluorescence) and "minimal" C4d1 (1%–9% immunoperoxidase)[6,7] and incorporation of MVI[8] improved sensitivity and reduced false-negative results; however, histologic AMR without DSA or $C4d_{ptc}$ remains diagnostic challenges.[9–14]

A fundamental weakness for diagnosis of chronic active-AMR (CA-AMR) is over-reliance on $C4d_{ptc}$ and MVI lesions because target PTC are lost from humoral injury. Capillary endothelial cells undergo apoptosis and detachment in acute AMR causing collapse and luminal occlusion of interstitial microcirculation,[15] which progressively disappears with advancing interstitial fibrosis in chronic rejection.[16,17] $C4d_{ptc}$ positivity rates are only 49.4% in TG (weighted

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Received 23 February 2022; revised 24 March 2022; accepted 11 April 2022; published online 21 April 2022

average, 12 studies, $n = 656$ biopsies).[2–4,18–26] $C4d_{ptc}$ most often fluctuates in early subclinical AMR (37.0%) and is insensitive for prediction of parenchymal disease and graft failure.[9,11] One practical solution is evaluation of $C4d_{glom}$, a larger antigenic target for DSA deposition. Cleaved C4b covalently binds to adjacent amino acids and carbohydrate moieties on glomerular endothelial cells and basement membrane collagen, via reactive sulfhydryl groups. Stable C4d remains detectable after proteolytic inactivation as the local "footprint" of classical complement system activation by DSA binding within the glomerular capillaries.[24,27,28]

The 2001 Banff AMR diagnostic schema originally specified linear $C4d_{ptc}$,[29] excluding glomeruli because C4d immunofluorescence of normal glomeruli is variably positive in mesangium, occasional capillary loops, and collagen autofluorescence from sclerosed glomeruli.[30] Chromogenic C4d immunohistochemistry staining of formalin-fixed, paraffin-embedded tissue although less sensitive avoids this problem: background $C4d_{glom}$ is absent in normal glomeruli.[18,30,31] In complement-activating native glomerular diseases (including membranous, lupus, and immune-complex glomerulonephritis [GN]), mesangial and glomerular capillary C4d immunoperoxidase of formalin-fixed, paraffin-embedded is used for salvage when immunofluorescence tissue is absent.[30,31] Several transplant studies reported $C4d_{glom}$ staining in active AMR and chronic TG using immunoperoxidase[1,3,18,25–27,32,33]; however, its use as a diagnostic biomarker is not currently accepted. We hypothesized that: (i) $C4d_{glom}$ represents endothelial interaction with antibody in transplanted kidneys; (ii) the magnitude of $C4d_{glom}$ immunoperoxidase staining correlates with clinical, immunologic, and pathologic humoral activities; and (iii) incorporation into the Banff chronic AMR schema would improve diagnostic sensitivity and improve etiologic classification of late chronic AMR expressed as TG.

We evaluated $C4d_{glom}$ in a well-characterized cohort of 3524 consecutive adequate samples from ABO-compatible kidney transplant recipients (where confounding complement-mediated diseases were excluded) to calculate $C4d_{glom}$ prevalence, epidemiologic risk factors, and correlations with authenticated AMR markers, including circulating DSA, histologic MVI, Banff cg scores, and $C4d_{ptc}$. $C4d_{glom}$ background staining was absent in all preimplantation donor tissues. The suboptimal diagnostic performance of Banff 2019 CA-AMR definition (using only $C4d_{ptc}$)[34] to diagnose confirmed TG was substantially improved by addition of $C4d_{glom}$ and better discriminated graft failure.

METHODS

Specific aims

The specific study aims were to:

1. establish population prevalence rates for $C4d_{glom}$;
2. determine clinical epidemiologic risk factors for $C4d_{glom}$;
3. correlate $C4d_{glom}$ scores against circulating DSA, histologic markers of antibody, renal dysfunction, and proteinuria;
4. evaluate the test performance of $C4d_{glom}$ as a diagnostic biomarker in Banff-defined AMR and confirmed TG;
5. compare the clinical performance of Banff 2019 CA-AMR diagnosis (using $C4d_{ptc}$) to an enhanced Banff definition with $C4d_{glom}$ for TG diagnosis within a common reference standard subset (Banff cg≥1b and normal, verified by EM); and
6. evaluate the clinical impact of $C4d_{glom}$ as a prognostic biomarker for allograft survival in TG.

Study Design

The research design was a retrospective, single-center, observational nested cohort study with prospective data collection. It was investigator-initiated, independent, and undertaken without external funding. Institutional ethics was HREC LNR/12/WMEAD/114. STARD checklist for diagnostic studies is included (Supplementary Table S1). Consecutive, kidney transplant biopsy specimens with sufficient tissue from May 2012 to April 2021 were screened. Indication biopsies for cause, post-treatment verification, and surveillance per protocol (at 0, 1, 3, and 12 months for kidney and additional 3, 5, 7, and 10 years for kidney-pancreas recipients) were included. Nonalloimmune diseases (e.g., diabetic nephropathy, BK virus nephropathy) and conditions that activate the complement (recurrent GN, atypical hemolytic uremic syndrome, thrombotic microangiopathy, and ABO-incompatible transplantation) were excluded.

The principal disease of study interest was TG, defined by light microscopy (LM; Banff cg ≥1) as the archetypical expression of chronic AMR, irrespective of DSA status. A test reference subset of TG (Banff cg ≥1b) and DSA-negative normal controls (both verified by EM) was used for diagnostic test comparison and $C4d_{glom}$ evaluation. Banff cg1a and abnormal endothelial activation or hypertrophy without neomembrane (≥3 capillaries, "cg0e") were independently verified (BN), analyzed separately as diagnostically indeterminate (Figure 1).

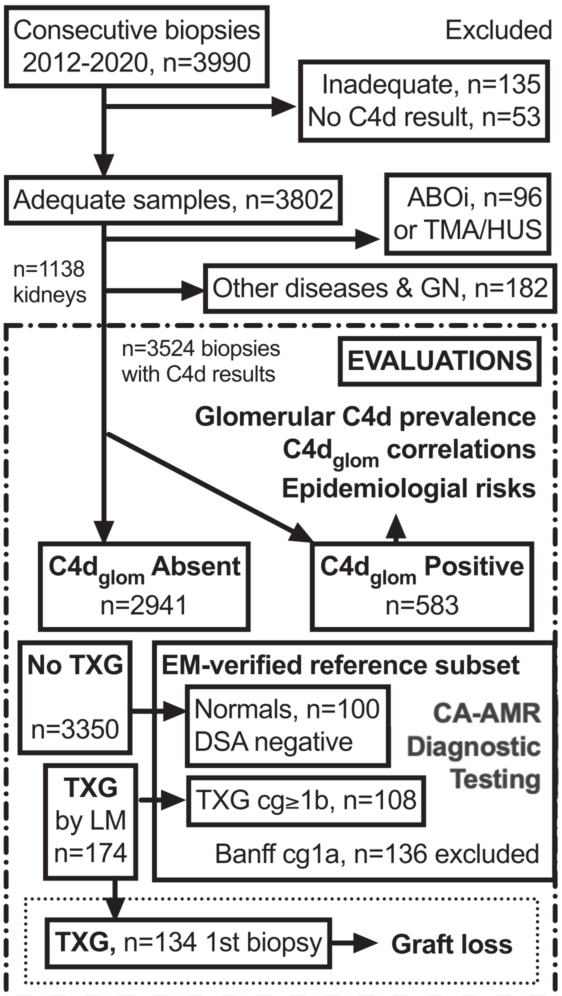

Figure 1. Study flow diagram. Numbers are biopsy samples, except for graft outcome which used the first biopsy in a unique kidney for actuarial survival (i.e., not total kidney number). Absent C4d was treated as indeterminant. ABOi, ABO-incompatible; aHUS, (atypical) haemolytic uremic syndrome; TMA, thrombotic microangiopathy; TG, transplant glomerulopathy.

Assessment of $C4d_{glom}$ Immunopathology and Antibody

Histology was contemporaneously scored by 6 specialized nephropathologists and classified using Banff 2019 AMR schema from original lesion scores.[34] All samples were tested for C4d using immunoperoxidase in formalin-fixed, paraffin-embedded tissue. Epitope retrieval of unstained sections used mild cell conditioning medium (Ultra CC1, Ventana systems, Tucson, AZ, incubated 95 °C for 36 minutes), specific C4d antihuman primary antibody (rabbit polyclonal anti-C4d antibody, Cell Marque, CA, 37 °C for 40 minutes), and visualized using indirect, biotin-free detection (Ultra DAB, Ventana Benchmark ULTRA), as best practice technical recommendations.[35]

$C4d_{glom}$ was defined by linear staining of ≥3 glomerular capillary loops. Mesangial staining was disregarded (present in 3.6%, Figure 2b). The dichotomized $C4d_{glom}$ interobserver kappa score was excellent at 0.950 (CHP and MS). $C4d_{glom}$ was semiquantitatively scored as: $C4d_{glom}1$, faint and/or segmental pattern of any single glomerulus (≤9% glomeruli, Figure 2d); $C4d_{glom}2$, mild-to-strong intensity in 10% to 50% glomeruli (segmental or global pattern, Figure 2c and e); and $C4d_{glom}3$, mild-to-strong diffuse staining in most (>50%) glomeruli (Figure 2f). Reference native histology (n = 21) and $C4d_{glom}$ donor samples (n = 140) were retrospectively verified by a single, blinded pathologist (CHP; Supplementary Table S2a).

Tissue for EM was immediately fixed in modified Karnovsky's solution at biopsy procurement, postfixed with osmium tetroxide, dehydrated in ascending ethanol series, polymerized in Epon resin, ultrathin sectioned, with digital images of 2 or more glomeruli obtained by transmission EM. Anti-HLA specific IgG DSA used specific class I and/or class II assays (LABScreen Single Antigen Bead, Luminex, One Lambda, CA), with positivity defined as median fluorescence intensity (MFI) ≥ 500. HLA class I (A, B, C) and class II (DRB1/3/4/5, DQ α/β) alleles were defined by two-field sequence-based typing (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) after 2017, replacing single-field molecular HLA typing by sequence-specific oligonucleotides (LABType SSO, One Lambda).

Statistical Analysis

Unpaired Student *t* test or Wilcoxon rank sum tested parametric and nonparametric nominal data, respectively, and Pearson's or Spearman's tests for correlations. Analysis of repeated samples used univariable and multivariable generalized estimating equations (GEEs). Multivariable models were constructed following backward elimination and adjusted for confounding factors. Collinearity diagnostics verified final models. $C4d_{glom}$ score results were confirmed by ordinal logistic regression analysis as ordered categorical values. Survival analyses used first occurrence of $C4d_{glom}$ or TG from a single unique kidney (avoiding double-counting repeat samples). Kaplan–Meier actuarial survival (log-rank test) was used for binary predictors and Cox regression for multivariable factors. Time-to-event was calculated from index cases. P values were 2-sided, and probability < 0.05 was considered significant. Data are expressed as mean ± SD, unless stated.

RESULTS

Population Screening and Study Exclusions

From 3990 consecutive biopsies screened, exclusions were as follows: unsatisfactory tissue and/or absent glomeruli ($n = 135$); ABO-incompatible kidneys ($n = 62$), (atypical) haemolytic uremic syndrome ($n = 31$) or thrombotic microangiopathy ($n = 3$); nonalloimmune

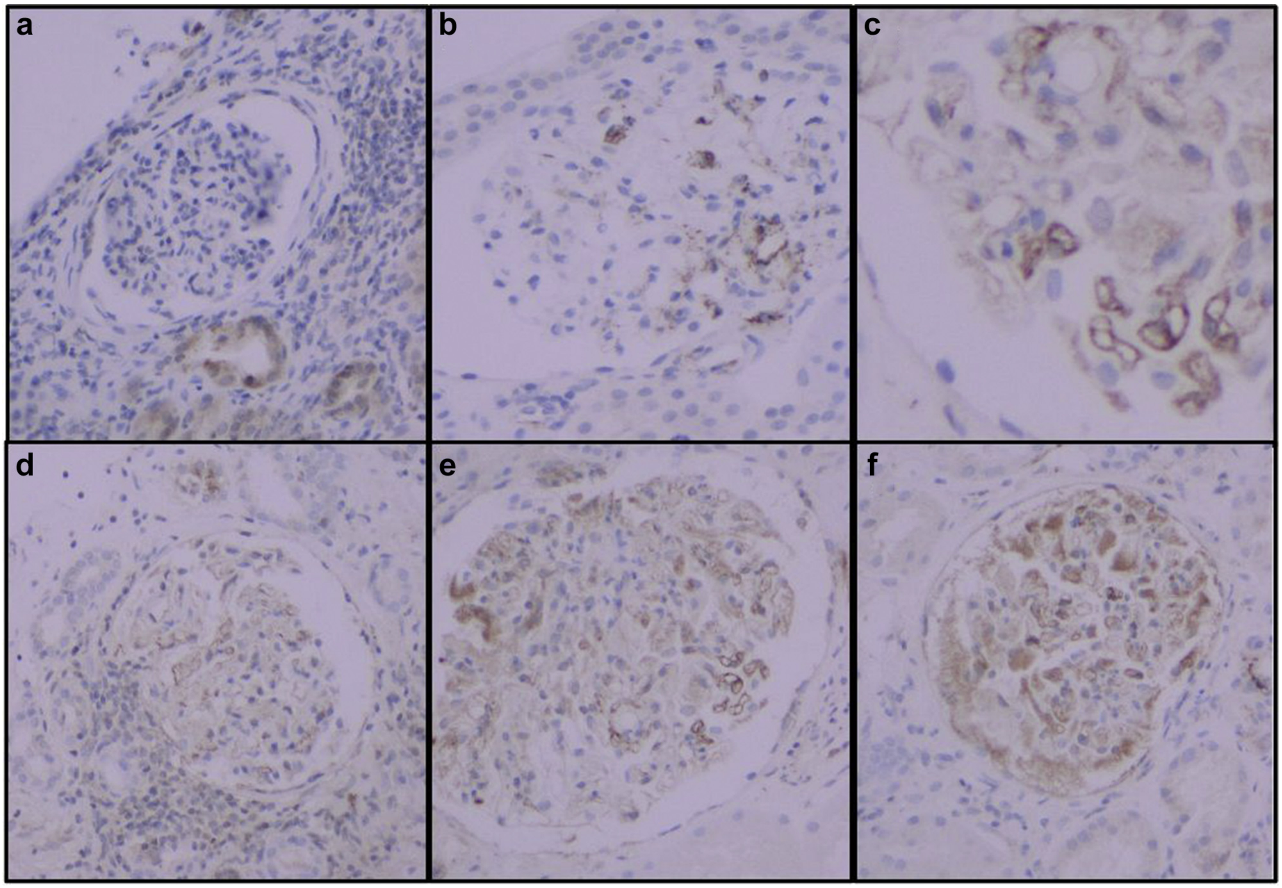

Figure 2. Spectrum of $C4d_{glom}$ immunohistochemistry. (a). Normal glomerulus without C4d staining ($C4d_{glom}0$). (b). Nondiagnostic mesangial C4d staining (not counted). (c). Detail of glomerular loop C4d staining. (d). $C4d_{glom}1$, faint, and/or segmental pattern of any single glomerulus. (e). $C4d_{glom}2$, mild-to-moderate intensity staining of 10% to 50% glomeruli (segmental or global pattern). (f). $C4d_{glom}3$, mild-to-strong diffuse staining in most glomeruli (>50%). Immunoperoxidase from formalin-fixed paraffin-embedded tissue. $C4D_{glom}$, glomerular capillary C4d.

disease and GN ($n = 182$); and unavailable $C4d_{glom}$ results ($n = 53$); leaving 3524 included samples (mean 3.1 ± 1.8 per patient, range 1–13) from 1138 recipients (Figure 1 and Supplementary Table S2b).

Study Population Demographics

The mean (±SD) age was 47.2 ± 12.7 years, 61.8% male, 7.2% retransplanted, 79.0% received deceased donor kidney, and 31.1% kidney-pancreas transplants. HLA mismatch was 3.9 ± 1.8. Induction was basiliximab in 83.9%, antithymocyte globulin in 8.9%, desensitization in 0.6%, nil in 5.6%, and unknown in 1.0%. Early (≤3 months) acute interstitial, vascular, and C4d-positive antibody rejection occurred in 19.6%, 4.6%, and 5.4%, respectively; and 16.9% received dialysis for delayed function. Prior rejection episodes before biopsy diagnosis were treated with methylprednisolone in 37.4% and antithymocyte globulin in 8.9% of the cases.

Immunosuppression at biopsy included the following: tacrolimus (92.5%) or cyclosporine (6.6%); azathioprine (6.3%), mycophenolate (87.6%), or leflunomide (3.4%); sirolimus/everolimus (1.3%); and prednisolone in 99.6%. Daily doses were: 7.4 ± 6.4 mg for tacrolimus (trough 8.9 ± 3.7 ng/ml); 207 ± 110 mg for cyclosporine (172 ± 141 ng/ml); 1.81 ± 0.38 g for mycophenolate mofetil; and 14.1 ± 6.1 mg for prednisolone.

Prevalence of Glomerular C4d by Time and Diagnosis

The population prevalence of C4d was 16.5% (583/3524) for $C4d_{glom}$ and 9.9% for $C4d_{ptc}$ (350/3524). When classified by dominant clinicopathologic diagnoses, $C4d_{glom}$ occurred in 25.0% of subclinical rejection, 24.8% acute rejection, 21.2% chronic-active T cell mediated rejection, 74.0% chronic AMR, 15.2% interstitial fibrosis and tubular atrophy, 31.9% for calcineurin inhibitor nephrotoxicity, and 11.3% in normal/minor abnormalities (Figure 3 and detailed Supplementary Table S2b). $C4d_{glom}$ occurred in 54.8% for excluded ABO-incompatible, 32.3% (atypical) haemolytic uremic syndrome, 33.3% thrombotic microangiopathy, 9.1% BK virus allograft nephropathy (including inflammation/rejection), and 34.6% for GN cases. TG occurred in 4.9% (174 of 3524) and positive for $C4d_{glom}$ in 60.3% (105 of 174).

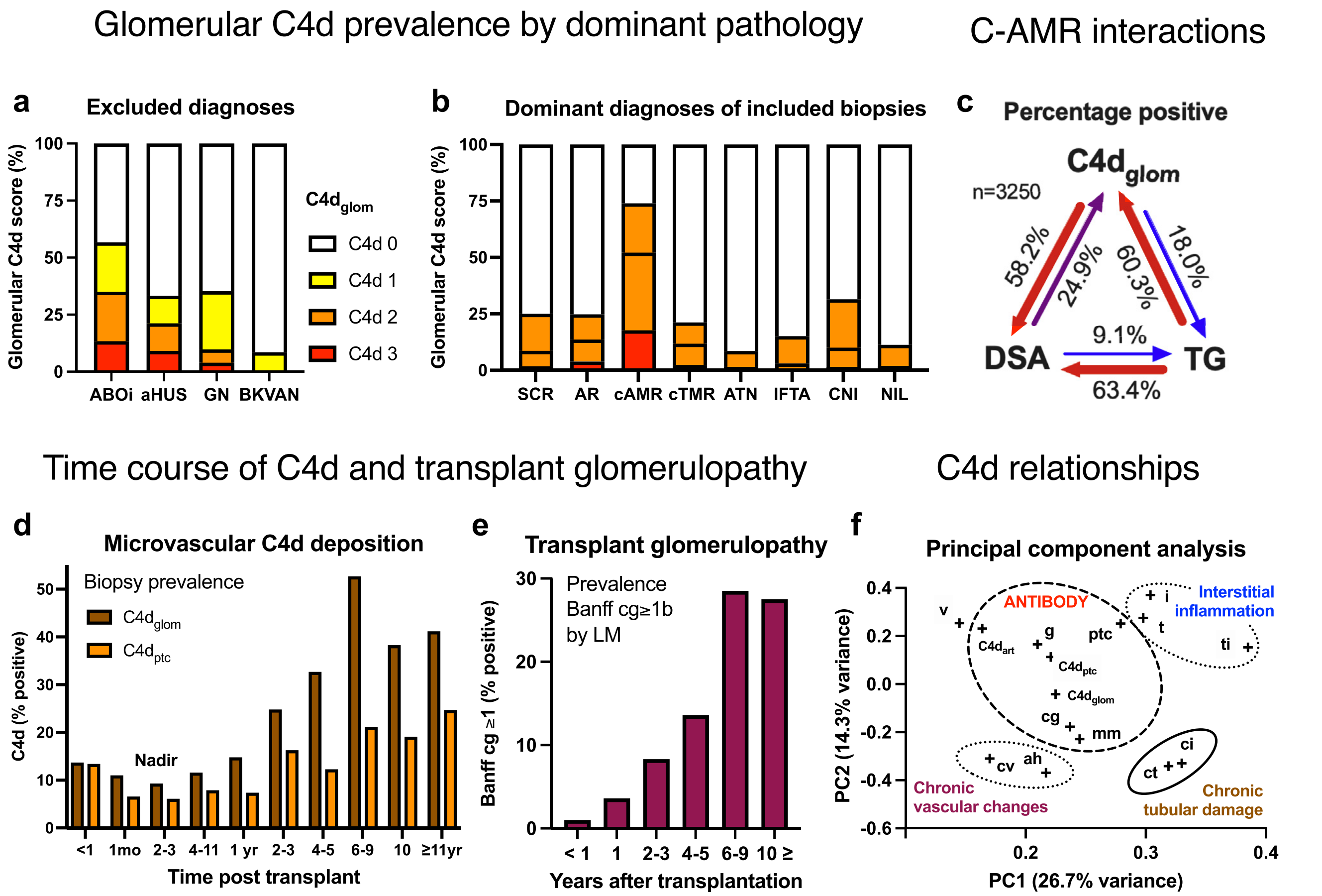

Figure 3. $C4d_{glom}$ prevalence and clinical relationships. (a) The prevalence of $C4d_{glom}$ immunoperoxidase staining in: a. Excluded adequate samples ($n = 278$); and (b) Included study samples ($n = 3524$) classified by dominant clinicopathologic diagnosis including acute clinical and subclinical rejection, but greatest $C4d_{glom}$ in chronic AMR. Panel key and biopsy numbers: SCR, subclinical rejection ($n = 220$); AR, acute rejection ($n = 286$); cAMR, chronic AMR ($n = 96$); cTCMR, chronic TCMR ($n = 85$); ATN, acute tubular necrosis ($n = 286$); IFTA ($n = 1147$); CNI, calcineurin inhibitor nephrotoxicity, (n = 69); NIL, ($n = 1223$). (c). Proportional interactions between $C4d_{glom}$, DSA, and TG ($n = 3250$ with complete data). Arrowheads indicate feature numerator proportional to sample positive (e.g., DSA detected in 58.5% of $C4d_{glom}$ positive). (d). Time course of C4d ($C4d_{glom}$ and $C4d_{ptc}$) as percentage of biopsies obtained from each time period. (e). Corresponding time-dependent prevalence of TG (Banff cg≥1) diagnosed by light microscopy. (f) Principal component analysis found $C4d_{glom}$ colocalized with acute histologic AMR markers (Banff g, ptc, $C4d_{ptc}$) and chronic glomerular features (Banff cg and mm). AMR, antibody mediated rejection; $C4D_{glom}$, glomerular capillary C4d; DSA, donor-specific antibody; TCMR, T cell mediated rejection; TG, transplant glomerulopathy.

A modest early (<1 month) peak and nadir at 2 to 3 months was followed by progressive and sustained increase in $C4d_{glom}$, paralleling TG and exceeding $C4d_{ptc}$ prevalence (Figure 3d and e). $C4d_{glom}$ positive cases demonstrated DSA (in 58.2%) and TG (in 18.0%), whereas TG had DSA (in 63.4%) and $C4d_{glom}$ (60.3%; Figure 3c). Principal component analysis found $C4d_{glom}$ colocalized with AMR histology including Banff cg, mm, ptc, g, and $C4d_{ptc}$ (Figure 3f).

Epidemiologic and Histologic Predictors of Glomerular C4d

Tabulated comparisons (Table 1 and Supplementary Tables S2 and S3a, S3b) and univariable binomial GEE ($n = 3524$ biopsies from 1132 patients) of clinical and demographic factors found that $C4d_{glom}$ was predicted by younger, female, living donor recipients who experienced early T cell-mediated rejection (OR 1.705, 95% CI 1.349–2.154) or AMR (OR 2.598, 95% CI 1.698–3.974) and prior rejection treatment (pulse corticosteroids or antithymocyte globulin, antithymocyte globulin, Supplementary Table S4a). Multivariable binomial GEE confirmed younger, female, living-donor recipients with early AMR, rejection treatment, and late presentation as independent epidemiologic risks for $C4d_{glom}$ (Supplementary Table S4c and S5).

Independent histologic determinates of $C4d_{glom}$ included chronic glomerulopathy (Banff cg and mm scores) and AMR indicators including DSA, Banff $C4d_{ptc}$ and ptc scores, when adjusted for time using multivariable binomial GEE (Table 2 and Supplementary Table S4d and S5). Glomerulitis lost significance. Multivariable ordinal regression confirmed $C4d_{glom}$ scores increased with Banff cg, mm, ptc, and $C4d_{ptc}$ scores, (log_eMFI) DSA, and later post-transplant time ($P < 0.001$; Supplementary Table S5).

Table 1. Clinical and demographic differences by $C4d_{glom}$

Category	$C4d_{glom}$	Absent	*P* value
Biopsies (*n*)	583	2941	
Post-transplant (mo)	38.1 ± 62.5	14.3 ± 33.0	<0.001
Indication biopsy, *n* (%)	224 (38.4)	771 (26.2)	<0.001
Pretransplant factors:			
Recipient age (yr)	44.0 ± 12.9	47.9 ± 12.6	<0.001
Recipient female	252 (43.2)	1094 (31.2)	0.006
Living donor	150 (25.7)	592 (21.1)	0.002
Early clinical events before biopsy time:			
Delayed function	71 (12.3)	523 (17.8)	<0.001
Early acute cellular rejection	160 (27.7)	529 (18.0)	<0.001
Early vascular rejection	34 (5.9)	129 (4.4)	0.004
Early humoral rejection	70 (12.1)	117 (4.0)	<0.001
Prior i.v. corticosteroids	309 (53.2)	1075 (36.6)	<0.001
Prior antithymocyte globulin	168 (28.9)	508 (17.3)	<0.001
Selected histopathology and antibody results:			
DSA (MFI)	3989 ± 6655	948 ± 2504	<0.001
Banff g score	0.2 ± 0.5	0.06 ± 0.3	<0.001
Banff ptc score	0.4 ± 0.7	0.08 ± 0.4	<0.001
Banff cg score	0.3 ± 0.7	0.03 ± 0.2	<0.001
Banff mm score	0.4 ± 0.7	0.08 ± 0.3	<0.001
$C4D_{glom}$ score	1.4 ± 0.6	0 ± 0.0	<0.001
$C4D_{ptc}$ score	0.4 ± 0.8	0.07 ± 0.3	<0.001

AMR, antibody mediated rejection; $C4D_{glom}$, glomerular capillary C4d; $C4D_{ptc}$, C4d in peritubular capillary; DSA, donor specific antibody; MFI, median fluorescence intensity. Comparison of biopsy samples ($n = 3524$) stratified by any capillary loop glomerular C4d immunoperoxidase staining ($C4d_{glom} \geq 1$). Mean ± SD for continuous data, *n* (%) for countable results (detailed in Supplementary Table S3).

Table 2. Predictors of $C4D_{glom}$

Model 1: Epidemiologic risk factors for $C4D_{glom}$			
	OR	95% CI	*P* value
Months post-transplant	1.010	1.008–1.013	<0.001
Recipient age (yr)	0.978	0.970–0.987	<0.001
Living donor	1.346	1.037–1.748	0.026
Early (≤3 mo) AMR	1.700	1.092–2.646	0.019
Pulse corticosteroid treatment	1.402	1.111–1.767	0.004
Prior antithymocyte globulin	1.460	1.126–1.895	0.004
Model 2: Antibody-mediated histologic determinants of $C4D_{glom}$			
	OR	95% CI	*P* value
$C4D_{ptc}$ score	2.959	2.292–3.819	<0.001
Banff mm score	2.147	1.633–2.824	<0.001
Banff cg score	1.697	1.199–2.402	0.003
Banff ptc score	1.477	1.181–1.847	<0.001
Any DSA (MFI >500)	1.582	1.244–2.012	<0.001
Months post-transplant	1.006	1.003–1.009	<0.001

AMR, antibody mediated rejection; $C4D_{glom}$, glomerular capillary C4d; $C4D_{ptc}$, C4d in peritubular capillary; DSA, donor specific antibody; MFI, median fluorescence intensity; OR, odds ratio.
Model 1 describes epidemiologic risk factors of $C4D_{glom}$ with independent variables limited to "causal" clinical and demographic predictors ($n = 3524$ biopsies from 1129 patients, constant = 0.632) controlled for time after transplantation using multivariable binomial generalized estimating equation. Model 2 illustrates determinant of $C4D_{glom}$ by histologic AMR indicators, circulating DSA, and presentation time (constant −2.408, detailed Supplementary Table S4).

Glomerular C4d Correlations With Antibody and Histology

Within the study population ($n = 3524$), $C4d_{glom}$ score correlated with the following: microvascular antibody markers including Banff ptc (rho = 0.216, $P < 0.001$), g (rho = 0.180, $P < 0.001$), and MVI scores (rho = 0.231, $P < 0.001$); chronic glomerular morphologic changes such as Banff cg (rho = 0.290, $P < 0.001$) and mm (rho = 0.281, $P < 0.001$) scores; $C4d_{ptc}$ scores (rho = 0.291, $P < 0.001$); and immunodominant DSA MFI (r = 0.411, $P < 0.001$; Figure 4 and Supplementary Table S6). Stronger $C4d_{glom}$ categories paralleled Banff acute microvascular and total inflammation, chronic antibody and fibrosis scores (Figure 4). Moderate $C4d_{glom} \geq 2$ associated with renal dysfunction and proteinuria ($P < 0.001$).

The test performance of $C4d_{glom}$ as an individual biomarker to diagnose AMR was evaluated using multiple acute and chronic Banff definitions ($n = 3524$ samples). For "definitive" Banff 2019 CA-AMR (all 3 criteria needed), the diagnostic sensitivity of $C4d_{glom}$ was 69.2% (84.7% specificity) and 72.2% (84.6% specificity) for active AMR. For "suspicious" AMR (at least 2 criteria present), sensitivities were 45.7% to 50.5% (specificity 87.2%–88.5%; Supplementary Table S7). In the EM-verified reference subset, the sensitivity of $C4d_{glom}$ to detect TG (all Banff cg≥1b) was 66.7% (specificity 99.0%), 42.4% for DSA-negative TG, and 79.0% for DSA-positive TG (Table 3 and Supplementary Table S8).

TG and Glomerular C4d

The population prevalence of TG was 4.9% (diagnosed by LM, 174 of 3524) comprising 109 Banff cg1 (62.7%), 36 cg2 (20.7%), and 29 (16.6%) cg3 cases. TG occurred at 79.9 ± 90.7 months with higher serum creatinine (207 ± 132 μmol/l vs. 168 ± 153 for Banff cg0, $P < 0.001$) and urinary albumin/creatinine (98.5 ± 167 mg/mmol vs. 17 ± 47, $P < 0.001$; Supplementary Table S9). Independent epidemiologic predictors of TG were female ($P = 0.007$), living donor recipients ($P < 0.001$), with prior pulse corticosteroid treatment ($P = 0.010$), late presentation and higher DSA (MFI, $P < 0.001$; Supplementary Table S10) using multivariable binomial GEE (2371 biopsies from 1038 patients).

In TG ($n = 174$), $C4d_{glom}$ occurred in 48.6%, 83.3%, and 75.9% of Banff cg1, cg2, and cg3 cases, respectively. $C4d_{glom}$ scores correlated with Banff cg (rho = 0.367, $P < 0.001$), MVI (rho = 0.230, $P = 0.002$), ptc (rho = 0.200, $P = 0.008$), g (rho = 0.164, $P = 0.031$); mm (rho = 0.243, $P < 0.001$); $C4d_{ptc}$ scores (rho = 0.232, $P = 0.002$); and DSA ($\log_e$MFI, rho = 0.351, $P < 0.001$). $C4d_{glom}$ in TG was independently predicted by DSA ($P = 0.007$) and $C4d_{ptc}$ ($P = 0.047$), when controlled for time using multivariable binomial GEE (Table 4). Banff cg and mm lost

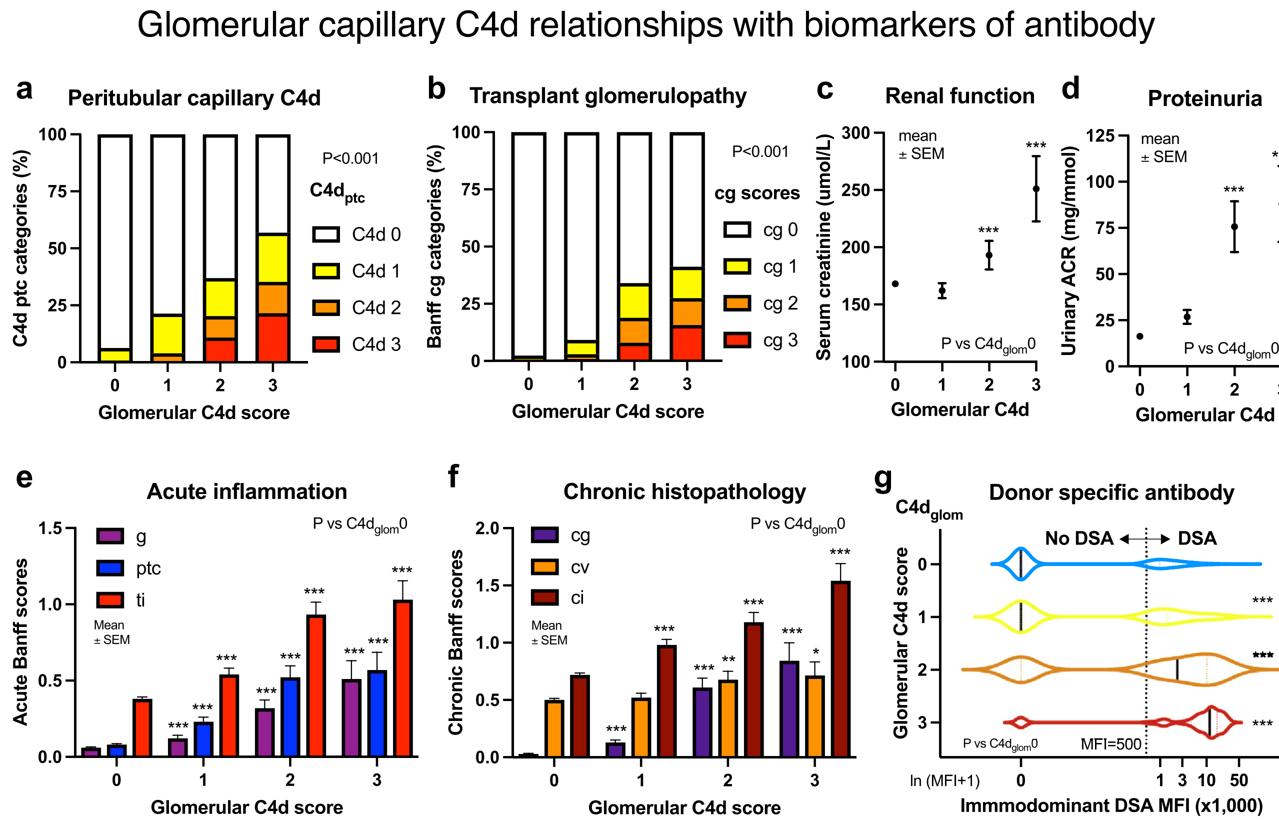

Figure 4. $C4d_{glom}$ relationships with antibody markers. The positive relationship of $C4d_{glom}$ semiquantitative scores categories with: (a) C4d in peritubular capillaries ($C4d_{ptc}$, $P < 0.001$); (b) Transplant glomerulopathy severity (Banff cg score, $P < 0.001$); (c) Renal function; (d) Proteinuria by spot ACR; (e) Microvascular inflammation as glomerulitis (Banff g) and peritubular capillaritis (ptc), and total cortical inflammation (ti); (f) Chronic glomerulopathy (Banff cg), fibrointimal hyperplasia (Banff cv), and chronic interstitial fibrosis (Banff ci) scores; (g) MFI values of the immunodominant DSA (log_{10}scale, violin plots, Bar median, MFI >500 broken line). Mean ± SEM. Key: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus $C4d_{glom}0$. ACR, albumin/creatinine ratio; $C4D_{glom}$, glomerular capillary C4d; $C4D_{ptc}$, C4d in peritubular capillary; DSA, donor specific antibody; MFI, median fluorescence intensity.

Table 3. Diagnostic performance of $C4D_{glom}$ biomarker in AMR

1. Overall study population using Banff 2019 AMR criteria			
	Prevalence (%, n/N)	Sensitivity, %	Specificity, %
Suspicious active AMR	11.32 (339/3524)	46.1	87.2
Definitive active AMR	2.04 (72/3524)	72.2	84.6
Suspicious CA-AMR	12.43 (438/3524)	50.5	88.3
Definitive CA-AMR	2.21 (78/3524)	69.2	84.7
Total suspicious active and CA-AMR	14.67 (517/3524)	45.7	88.5
Total definitive active and CA-AMR	3.43 (121/3524)	65.3	85.2
2. Ultrastructure verified TG in reference subset by DSA			
DSA +ve TG (cg≥1b)	43.18 (76/176)	79.0	99.0
All TG (cg≥1b) ± DSA	53.37 (111/208)	66.7	99.0
DSA-negative TG	24.81 (33/133)	42.4	99.0

AMR, antibody mediated rejection; $C4D_{glom}$, glomerular capillary C4d; CA-AMR, chronic-active antibody mediated rejection; DSA, donor-specific antibody; TG, transplant glomerulopathy.
Summary diagnostic test performances of the $C4d_{glom}$ component to diagnose[1] active and chronic Banff 2019 AMR as "definitive" (all 3 criteria present) or "suspicious" (≥2 diagnostic criteria) defined by light microscopy from the study population ($n = 3524$); and[2] TG ($n = 108$) in the reference subset and DSA-negative normal cases ($n = 100$) all verified by electron microscopy. Key: CA-AMR. AMR prevalence percentage by definition used (disease/subtotal tested, detailed Supplementary Table S7).

significance. Sensitivity analysis demonstrated Banff cg failed to independently predict $C4d_{glom}$ when DSA and $C4d_{ptc}$ were included into the multivariable model (Supplementary Table S11a and S11b)

Determinants of Transplant Glomerular Morphology

Analyses restricted to histologic variables found TG was independently determined by mesangial matrix expansion, glomerulitis, and $C4d_{glom}$, when chronic fibrosis and vascular changes were controlled by

Table 4. Predictors of $C4D_{glom}$ in TG

Predictor	OR	95% CI	P value
Any DSA (MFI >500)	2.809	1.328–5.943	0.007
Any $C4D_{ptc}$ ($C4d_{ptc}$ ≥1)	2.542	1.039–6.219	0.047
Months post-transplant	1.007	1.000–1.013	0.003

$C4D_{glom}$, glomerular capillary C4d; $C4D_{ptc}$, C4d in peritubular capillary; DSA, donor specific antibody; MFI, median fluorescence intensity; OR, odds ratio; TG, transplant glomerulopathy.
Predictors of glomerular capillary loop C4d immunoperoxidase staining ($C4d_{glom}$ ≥1) in TG defined by light microscopy (Banff cg ≥1, $n = 124$ biopsies with contemporaneous DSA results) in the reference subset using multivariable binomial generalized estimating equation. Constant −0.937, estimate of common correlation 0.30. $C4d_{glom}$ was not predicted by Banff cg using multivariable analysis (detailed Supplementary Table S11).

Table 5. Histologic determinants of TG

Determinant	OR	95% CI	*P* value
Banff g score	2.929	2.219–3.866	<0.001
$C4D_{glom}$ score	2.235	1.744–2.866	<0.001
Banff ci score[a]	1.417	1.125–1.785	0.003
Banff cv score	1.400	1.077–1.820	0.012

$C4D_{glom}$, glomerular capillary C4d; OR, odds ratio; TG, transplant glomerulopathy.
[a]Post-transplant time could substitute for Banff ci score (detailed Supplementary Table S10).
Multivariable predictors of TG defined by light microscopy (Banff cg ≥1, $n = 3524$ biopsies from 1106 patients) using binomial generalized estimating equation restricted to pathologic independent variables.

multivariable binomial GEE ($n = 3524$ biopsies, 1106 patients, Table 5 and Supplementary Table S10). $C4d_{ptc}$ and ptc lost significance. TG was independently predicted by $C4d_{glom}$ ($P < 0.001$) in all multivariable histologic models, irrespective of post-transplant time, DSA, glomerulitis, Banff ci and cv. $C4d_{ptc}$ lost significance being superseded by $C4d_{glom}$ (Supplementary Table S10). Ordinal regression confirmed Banff cg score increased by $C4d_{glom}$, Banff mm and ci scores, DSA, and late TG presentation ($P = 0.003$ to $P < 0.001$, data not found).

Diagnostic Utility of Banff 2019 AMR Criteria for TG

A reference subgroup of proven TG was defined and verified by EM ($n = 359$ including Banff 1a, DSA prevalence 59.3%) for comparative diagnostic testing. The principal study group of interest was LM-defined, EM-verified TG (Banff cg≥1b, $n = 108$), irrespective of DSA status. A normal control group was formed by exclusion of 936 indication biopsies (leaving 2588 protocol), with contemporaneous DSA results ($n = 1295$), which tested negative ($n = 781$), with available EM (excluding Banff cg1a, cg0e, and multilamination of PTC≥4, $n = 373$), with nil acute Banff scores ($n = 267$), and absent/minimal chronic pathology (allowing only Banff ci≤1), leaving 100 verified normal controls without AMR or DSA.

In EM-confirmed pathology, $C4d_{glom}$ occurred in 1% of NIL, 20.6% cg0e, 29.3% DSA-negative TG, and 57.5% of DSA-positive TG (Figure 5). In DSA-positive TG, $C4d_{glom}$ occurred in 47.1% Banff cg1a and 77.5% for Banff cg≥1b, and unrelated to cg score (Figure 5b), and MVI in 26.2% Banff cg1a and 54.7% for Banff cg≥1b. Cross comparison showed lower $C4d_{ptc}$ prevalence relative to $C4d_{glom}$ in all TG subcategories (Figure 5d and e). The test performances of Banff 2019 CA-AMR schema (using $C4d_{ptc}$ for criterion 2, endothelial interaction with antibody) to diagnose TG (Banff cg≥1b, $n = 108$) irrespective of DSA (detected in 65.7%) were evaluated against normal controls without DSA ($n = 100$) in the EM-verified reference subset. Mild Banff cg1a (invisible by LM, $n = 136$) and cg0e minor endothelial changes ($n = 388$) were excluded as indeterminant diagnoses.

The Banff 2019 CA-AMR criteria demonstrated suboptimal sensitivity of 22.2% to detect TG (accuracy 59.6%, specificity 100%), which primarily failed from infrequent expression of diagnostic lesions including $C4d_{ptc}$ (23.1%), glomerulitis (39.8%), PTCs (14.8%), MVI≥2 (29.6%, and noncountable with Banff i≥1, 23.1%), and/or DSA (65.7%). In contrast to 23.1% $C4d_{ptc}$ prevalence, $C4d_{glom}$ was present in 66.7% of TG, and either biomarker occurred in 70.4%. Expansion of Banff CA-AMR definition to include $C4d_{glom}$ and/or $C4d_{ptc}$, the sensitivity for TG diagnosis increased to 82.4% and accuracy improved to 90.9% (Table 6 and Supplementary Table S12).

Graft failure and Glomerular C4d and CA-AMR Diagnosis

Death-censored graft survival from first TG index biopsy in 134 kidneys (56.7% with $C4D_{glom}$, 76/134) was followed for 45.5 months (median, interquartile range 22–74). Kaplan–Meier survival was reduced by $C4d_{glom}$ ≥1 (log rank 10.973, $P < 0.001$, Figure 6a), $C4d_{ptc}$ ≥1 (log rank 6.317, $P = 0.012$), and DSA (log rank 6.826, $P = 0.009$). TG graft loss also increased with $C4d_{ptc}$ (Figure 6d). TG from indication biopsies ($n = 73$, 105.1 months, $C4D_{glom}$ 65.8%, $C4d_{ptc}$ 28.8%) demonstrated accelerated failure and 5-year graft survival of 29.6% with $C4D_{glom}$ (log rank 9.600, $P = 0.002$; Figure 6e). Sensitivity analysis (all first biopsies with rejection of all phenotypes and normal) from the study population ($n = 695$ unique kidneys) confirmed $C4D_{glom}$ (17.3% prevalence) was associated with graft loss (log rank 8.160, $P = 0.004$, Figure 6f).

Univariable predictors of graft loss included younger, living donor recipients, HLA mismatch, later presentation time, and serum creatinine. DSA (hazard ratio [HR] 2.778, 95% CI 1.248–6.181), $C4d_{glom}$ (HR 3.412, 95% CI 1.581–7.366, $P = 0.002$), $C4d_{ptc}$ (HR 2.352, 95% CI 1.182–4.679), Banff ti, ci, ct, cv, cg, and mm scores also predicted graft failure using univariable Cox regression (Supplementary Table S13). Banff PTC was marginal ($P = 0.051$) and glomerulitis unrelated.

$C4d_{glom}$ was an independent pathologic predictor of graft failure in TG (P = 0.003), along with Banff cg and ci scores using multivariable Cox regression. Presentation time and fibrosis were interchangeable indicators of time-dependent scarring (Table 7 and Supplementary Table S13). DSA and MVI lost significance to $C4d_{ptc}$ and/or $C4d_{glom}$ in multivariable models. A mixed clinicopathologic model found independent predictors of graft loss were late presentation in younger recipients with renal dysfunction, severe

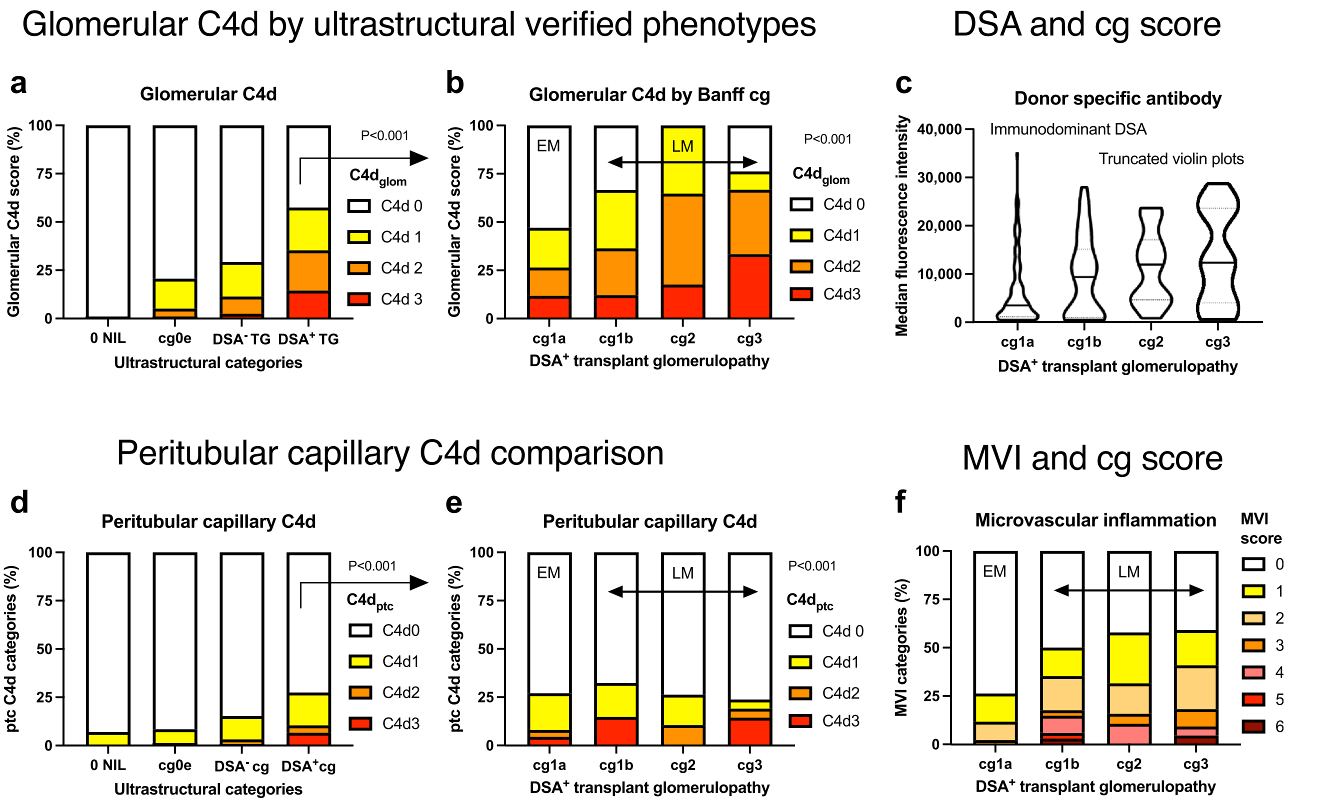

Figure 5. C4d$_{glom}$ by ultrastructural phenotypes. A reference subgroup comprised TG (n = 359) defined and verified by EM, DSA-negative normal controls (NIL, n = 100), and indeterminant samples with minor glomerular capillary endothelial abnormalities on EM (cg0e). (a) C4d$_{glom}$ score categories in NIL, cg0e, and TG diagnosed by electron microscopy (EM, Banff cg≥1a) subclassified as DSA-negative or -positive (MFI > 500). (b) C4d$_{glom}$ in DSA-positive TG according to Banff cg scores were 47.1% (Banff cg1a) and 77.5% for Banff cg≥1b (LM diagnosed), and unrelated to cg scores. (c) Violin plots of immunodominant DSA MFI mirrored C4d$_{glom}$ (panel b). Panels d and e are direct comparisons (a and b) of C4d$_{ptc}$ in NIL, cg0e, TG diagnosed by LM, and DSA-positive Banff cg1a and cg≥1b. C4d$_{ptc}$ was less intense than C4d$_{glom}$ immunoperoxidase in all TG groups. (f) Microvascular inflammation (MVI, Banff g+ptc) occurred in 26.2% of EM-defined DSA-positive cg1a, and 54.7% of LM-diagnosed TG (Banff cg≥1b). Key: Stack bars of ordinal Banff scores, P versus NIL group. C4D$_{glom}$, glomerular capillary C4d; DSA, donor specific antibody; EM, electron microscopy; LM, light microscopy; MFI, median fluorescence intensity; MVI, microvascular inflammation; TG, transplant glomerulopathy.

glomerulopathy, and interstitial fibrosis (Banff cg and ci scores), with marginal significance for C4d$_{glom}$ and C4d$_{ptc}$ (P = 0.093 and 0.097, respectively).

Table 6. Test performance of conventional and expanded Banff 2019 CA-AMR criteria to diagnose transplant glomerulopathy

Sensitivity	Specificity	PPV	NPV	Accuracy
Conventional Banff 2019 CA-AMR test criteria: Using C4d$_{ptc}$ only.				
22.2%	100.0%	100.0%	54.3%	59.6%
(14.8–31.2)	(96.4–100)	(NA)	(51.8–56.8)	(59.6–66.3)
Expanded Banff 2019 CA-AMR criteria: Incorporating C4d$_{glom}$ and C4d$_{ptc}$.				
Sensitivity	**Specificity**	**PPV**	**NPV**	**Accuracy**
82.4%	100.0%	100.0%	84.0%	90.9%
(73.9–89.0)	(96.4–100)	(NA)	(77.8–88.8)	(86.1–94.4)

AMR, antibody mediated rejection; C4D$_{glom}$, glomerular capillary C4d; C4D$_{ptc}$, C4d in peritubular capillary; CA-AMR, chronic-active antibody mediated rejection; DSA, donor specific antibody; NA, not applicable; NPV, negative predictive value; PPV, positive predictive value.
Direct comparison of test performances of (i) Banff 2019 CA-AMR (C4d$_{ptc}$ used for endothelial interaction with antibody, AMR criterion 2) and (ii) an expanded Banff 2019 schema (incorporating both C4d$_{ptc}$ and/or C4d$_{glom}$) to diagnose transplant glomerulopathy (Banff cg^{3}1b), defined by light microscopy (and verified by electron microscopy, n = 108, DSA in 65.7%) with ultrastructural-verified normal controls without DSA (n = 100). All 3 criteria are required for chronic AMR diagnosis ("definitive", identical disease prevalence of 51.92% (108 of 208) in this head-to-head comparison (detailed, Supplementary Table S12). Results are percentages (95% CI).

Graft failure in TG was better determined when diagnosis incorporated C4d$_{glom}$. TG (Banff cg≥1b) classified using Banff 2019 CA-AMR diagnostic criteria (n = 134, 12.7% with all criteria) had no impact on outcome (log rank HR 1.354, 95% CI 0.0469–3.904, P = 0.739; Figure 6b, Supplementary Table S14). In comparison, diagnosis of CA-AMR definition using C4d$_{ptc}$ and/or C4d$_{glom}$ more accurately predicted graft failure in TG (log rank HR 3.081, 95% CI 1.603–5.922, P = 0.002; Figure 6c).

DISCUSSION

The current histologic diagnostic algorithm for Banff chronic AMR is neither accurate nor reliable, with a poor sensitivity to detect TG due to infrequent expression of key diagnostic criteria (C4d$_{ptc}$, MVI, and DSA). Failure to accurately classify "C4d-negative" AMR (lacking C4d$_{ptc}$ and/or DSA) compromises clinical management. Inclusion of C4d$_{glom}$ (with C4d$_{ptc}$) into the Banff schema CA-AMR (as criterion 2, endothelial interaction with antibody) dramatically increased the diagnostic sensitivity and accuracy for EM-verified TG

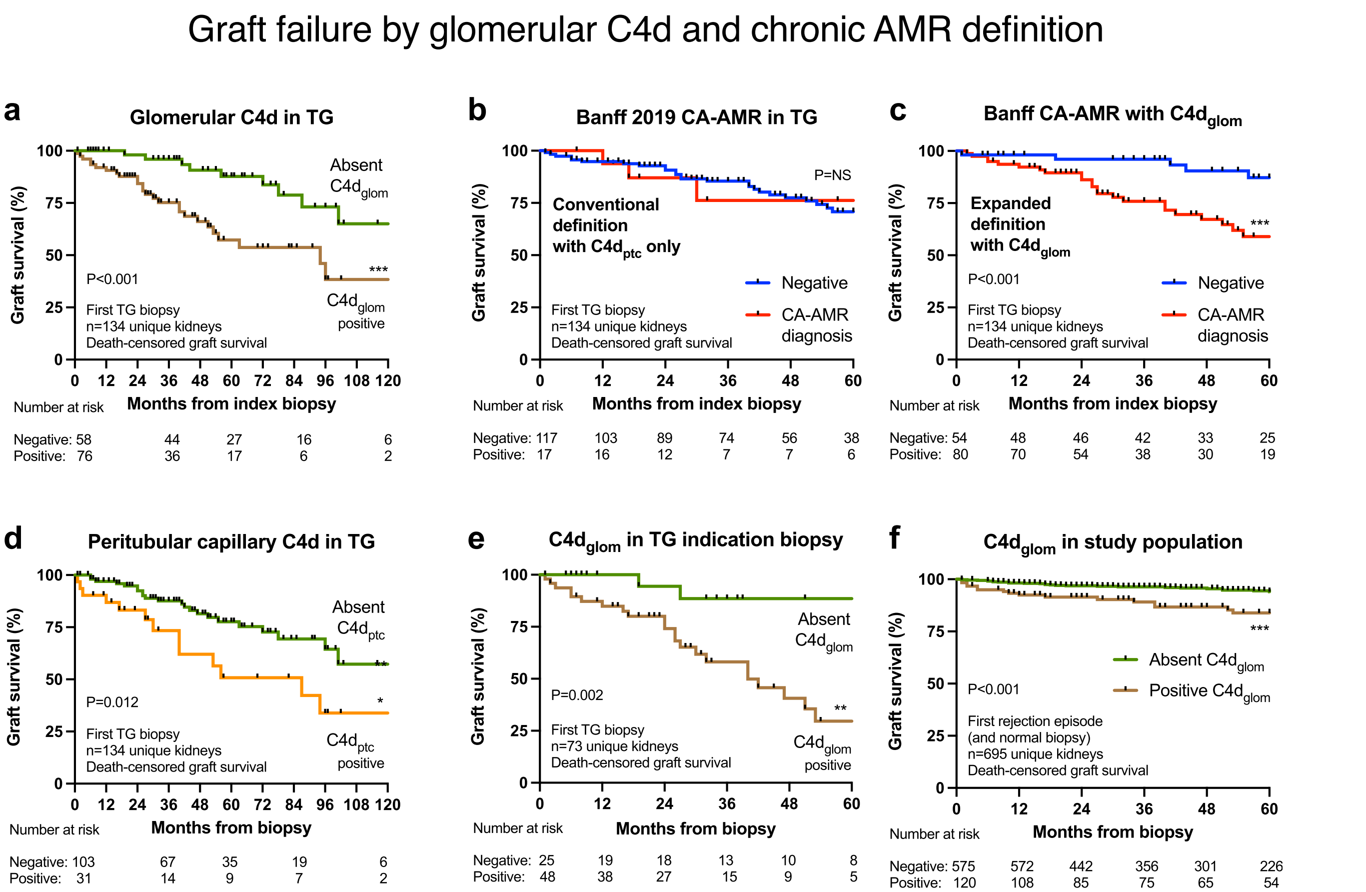

Figure 6. Allograft survival by $C4d_{glom}$ and Banff CA-AMR classifiers. (a) Kaplan–Meier death censored graft survival of TG (from first index biopsy) of 134 unique kidney transplants dichotomized by $C4d_{glom}$ immunoperoxidase ($P < 0.001$, vs. $C4d_{glom}0$ TG). (b) Actuarial graft loss of TG indistinguishable by Banff 2019 CA-AMR criteria (using only $C4d_{ptc}$). (c) Actuarial graft loss curves separated using expanded Banff CA-AMR criteria ($C4d_{ptc}$ and $C4d_{glom}$) with greater graft loss in TG kidneys ($P = 0.002$). (d) Graft survival of TG dichotomized by $C4d_{ptc}$ ($n = 134$, $P < 0.012$ vs. negative $C4d_{ptc}$). (e) Graft survival of TG diagnosed by indication biopsy (73 unique kidney transplants) dichotomized by $C4d_{glom}$ (P = 0.002 vs. negative $C4d_{glom}$). (f) Graft survival of the study population (from first index biopsy) of 575 unique kidney transplants dichotomized by $C4d_{glom}$ ($P < 0.001$ vs. negative $C4d_{glom}$). AMR, antibody mediated rejection; $C4D_{glom}$, glomerular capillary C4d; $C4D_{ptc}$, C4d in peritubular capillary; CA-AMR, chronic-active antibody mediated rejection; TG, transplant glomerulopathy.

compared with Banff 2019 definition using only $C4d_{ptc}$, and was associated with allograft failure. We consider TG expressing $C4d_{glom}$ as *prima facie* evidence of chronic AMR, irrespective of DSA status.

Our hypothesis that glomerular C4d is a tissue biomarker reflecting circulating antibody was supported by the following: correlations with DSA detection and MFI level; associations with all histologic AMR diagnostic lesions ($C4d_{ptc}$, glomerulitis, MVI, and Banff cg); colocalization of $C4d_{glom}$ with AMR pathologic features using principal component analysis; strong independent association of Banff cg score with $C4d_{glom}$ using multivariable analyses controlling for confounding variables; biologically plausible epidemiologic predictors (prior AMR and treated rejection); and prognostication of graft failure. Normal glomerular capillaries constitutively express high-level class II HLA-DP and HLA-DQ proteins, which are co-expressed with HLA-DR, in addition to class I MHC; which present a microvascular targets for DSA.[36] After endothelial binding and complement activation by DSA, stable C4d covalently binds adjacent cells and underlying glomerular basement membrane as visible $C4d_{glom}$.[24] Ultrastructural immunogold localized $C4d_{glom}$ deposition to subendothelial areas and podocytes.[21] The graded and proportional relationship of $C4d_{glom}$ scores with DSA and histologic AMR signifies a semiquantitative biomarker reflecting humoral "activity" in tissue.

Table 7. Histologic determinants of graft failure in transplant glomerulopathy

Determinant	HR	95% CI	*P* value
Banff cg score	2.932	1.777–4.840	<0.001
$C4D_{glom}$ score	1.593	1.171–2.168	0.003
Banff ci score[a]	1.993	1.282–3.099	0.002

$C4D_{glom}$, glomerular capillary C4d; $C4D_{ptc}$, C4d in peritubular capillary; DSA, donor specific antibody; HR, hazard ratio; MVI, microvascular inflammation.
[a]Banff ci score could substitute for post-transplant time (detailed Supplementary Table S13).
Parsimonious histologic predictors of death-censored allograft failure in chronic transplant glomerulopathy (first biopsy occurrence in a single unique kidney, $n = 134$) using multivariable Cox regression. DSA and MVI lost significance when either $C4D_{ptc}$ or $C4D_{glom}$ were incorporated into the multivariable model.

$C4d_{glom}$ immunoperoxidase is not a new diagnostic lesion but previously reported in acute and chronic rejection involving antibody. Feucht observed abundant diffuse $C4d_{glom}$ staining in 46.2% (42/93) and focal/segmental staining in 18.6% (8/43) in early (≤1 months) indication biopsies (n = 93) from sensitized, re-transplanted recipients with high panel-reactive antibodies, followed by accelerated graft loss.[33] A subsequent study of high immunologic risk (regrafted, sensitized, DSA) recipients demonstrated $C4d_{glom}$ in 100% of early and late acute cellular rejection (27/27 and 25/25) and all late "chronic rejection" samples (12/12), versus $C4d_{ptc}$ in 84.5%, 84.6%, and 83.3%, respectively.[32] Shimizu *et al.*[3] reported that $C4d_{glom}$ in 92% (81% diffuse, 11% focal) of TG (n = 50, mean Banff cg = 1.9, glomerulitis 76%, PTCs 86%) was diagnostically superior to $C4d_{ptc}$ (occurring in 57%). Gloor *et al.*[1] reported that $C4d_{glom}$ in 33.3% (9/28) of TG (n = 55, 49% subclinical, cg = 1.8) was more sensitive than $C4d_{ptc}$ (17.9%). Sijpkens *et al.*[25] found $C4d_{glom}$ in 90.9% of TG (10/11, cg = 2.3) and 15.3% (2/13) of "chronic allograft nephropathy" cases versus 36.4% for $C4d_{ptc}$ (4/11). Batal *et al.*[18] correlated $C4d_{glom}$ (25.3% TG prevalence) with Banff cg score. For acute AMR, Kikic *et al.*[37] and Valente *et al.*[38] correlated $C4d_{glom}$ with DSA, glomerulitis, $C4d_{ptc}$, and univariable graft loss. Gasim *et al.*[21] reported $C4d_{glom}$ in 66.7% of TG (vs. 26.7% $C4d_{ptc}$, n = 30, cg = 2.6) which correlated with cg score, DSA, and $C4d_{ptc}$. Hence, multiple studies consistently found superior sensitivity for $C4d_{glom}$ in TG and chronic (active) AMR compared with $C4d_{ptc}$.

Our study indicated that $C4d_{glom}$ is an ideal diagnostic biomarker of antibody in TG, with excellent test sensitivity and accuracy, high prevalence, and superior prognostication of graft failure compared with $C4d_{ptc}$. C4d immunoperoxidase from automated PPFE tissue processing and indirect, biotin-free detection (Ultra DAB, Ventana Benchmark), produced clean and consistent sections without background staining. $C4d_{glom}$ was absent in preimplantation donor kidneys and DSA-negative verified normal transplants (0%–1%), with high negative predictive values. Nondiagnostic mesangial staining (containing C3) in very few glomeruli (3.6% of kidneys) was easily distinguished from the capillary loop pattern. Minor nonspecific $C4d_{glom}$ occurred in 15.4% of native diabetic glomerulopathy and occasional sclerosed glomeruli. The 2009 multicenter study of $C4d_{ptc}$ immunoperoxidase ("BIFQUIT") reported moderate agreement for dichotomized Cohen's kappa between institutions, laboratories, and observers of 0.63, 0.77, and 0.77 (Banff 2019 AMR $C4d_{ptc}$≥1 vs. $C4d_{ptc}$0),[35] where results varied by epitope recovery protocol, antibody incubation, avidin-biotin detection, and cutoff values. Our dichotomized kappa for $C4d_{glom}$ was 0.95. TG was independently predicted by $C4d_{glom}$ in all multivariable histologic models (irrespective of time, DSA status, glomerulitis, Banff ci and cv).

C4d immunoperoxidase easily visualizes glomerular capillaries against well-defined anatomical landmarks within the whole PPFE section, rather than the small separate immunofluorescence core of unfixed frozen cortex containing few glomeruli with endogenous mesangial fluorescence, and nonspecific binding and autofluorescence from collagen comprising capillary loops and sclerosed glomeruli.[21,33,39,40] Gasim correlated $C4d_{glom}$ immunofluorescence with glomerular basement membrane duplication and neomembrane formation in TG and postulated glomerular remodeling and potential chronic AMR as explanations. Staining within thickened glomerular basement membrane segments was often less intense or absent with areas of lamina rara interna expansion ($C4d_{glom}$ was 20.6% in cg0e). Our immunoperoxidase study of TG found $C4d_{glom}$ was not independently predicted by Banff cg when circulating DSA and $C4d_{ptc}$ were controlled by multivariable analysis, indicating $C4d_{glom}$ represents a tissue-bound biomarker of antibody "activity" rather than nonspecific glomerular thickening.

The current Banff chronic AMR schema uses 3-tier diagnostic criteria comprising the following: (1) DSA (or $C4d_{ptc}$ surrogate); (2) C4d ($C4d_{ptc}$), conditional MVI, and/or expression of validated AMR transcripts/classifiers reflecting endothelial interaction with DSA; and (3) chronic tissue injury as TG and/or multilamination of PTC.[34] Noninclusion of $C4d_{glom}$ compromised the diagnostic sensitivity for CA-AMR. Addition of $C4d_{glom}$ immunoperoxidase (to $C4d_{ptc}$ in criterion 2) improved sensitivity (from 22.2% to 82.4%) and accuracy (from 59.6% to 93.9%) for the diagnosis of TG in a head-to-head comparison within a EM-verified reference set. Specificity was 100%. Gasim reported 54% sensitivity and 84% specificity for $C4d_{glom}$ immunoperoxidase for TG (n = 82, including Banff 1a), with strong staining being 100% specific. In comparison, $C4d_{glom}$ immunofluorescence was 89% sensitive but less specific at 48%.[21] We recommend reporting "positive" $C4d_{glom}$ immunoperoxidase and not mislabelling "C4d negative" AMR simply by absence of $C4d_{ptc}$. Incorporation of $C4d_{glom}$ for diagnosis of antibody was supported by good sensitivity and specificity results in acute and chronic AMR Banff diagnoses (by LM) and EM-confirmed TG. $C4d_{glom}$ strongly associated with graft failure in all scenarios. The 5-year graft survival of TG in indication biopsies was only 29.6%. We advocate inclusion of $C4d_{glom}$ in Banff schema for CA-AMR diagnosis (with caveats for GN, aHUS/thrombotic microangiopathy, and ABOi as differential diagnoses).[21,30,40]

Study strengths include our large, well-phenotyped cohort with adequate tissue samples, universal C4d testing, Banff lesion scoring using current thresholds, DSA detected by solid phase Luminex SAB, case verification by election microscopy within a reference subset, and complete longitudinal follow-up. Conclusions were corroborated by 39 exploratory, secondary, and sensitivity analyses with verification in independent cohorts. Our retrospective, single center study used prospectively collected data. We lacked AMR transcriptomics which may have further may aid etiologic diagnosis.[41] C4d immunoperoxidase results of formalin-fixed paraffin-embedded tissue cannot be extrapolated to immunofluorescence of unfixed tissue which display endogenous C4d in mesangium, occasional capillary loops, and nonspecific sclerosed glomeruli.[21,33,39,40] Further collaborative research using normal controls for background staining, archetypal DSA-positive TG to assess individual laboratory sensitivity, specificity, and reproducibility using modern automated technologies utilizing current protocols (including biotin-free visualization methods), $C4d_{glom}$ score standardization, and gene signature are unmet clinical needs.[35]

In summary, $C4d_{glom}$ immunoperoxidase is a promising diagnostic and prognostic biomarker of endothelial interaction with antibody, which correlated with circulating DSA, severity of glomerulopathy, diagnostic histologic markers of AMR, and was associated with graft failure. Incorporation of $C4d_{glom}$ into Banff 2019 chronic AMR schema substantially improved diagnostic sensitivity and test accuracy, reduced immunologic and misclassification of TG cases. We recommend scoring $C4d_{glom}$ in TG and advocate its inclusion into the Banff schema to minimize underdiagnosis of late chronic AMR.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This study was investigator-initiated, independent, and undertaken without external funding.

Data Sharing Statement

Extensive summary data and analysis are presented within the supplemental material which contains 14 highly detailed tables of de-identified summated clinical data with their univariable and multivariable statistical analyses to allow for open scientific scrutiny. Federal privacy laws and local institutional ethics forbid the placement of confidential individual patient information onto any public data-sharing website nor allow for its unauthorized sharing. Specific questions of clinical science may be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors participated in manuscript review and histologic definitions. CHP was responsible for blinded reference histology and for C4d background, MS for C4d comparisons, and BJN for research design and data analysis.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Table S1. STARD guidelines for diagnostic studies: checklist.

Table S2a. Background normal capillary loop glomerular staining.

Table S2b. $C4d_{glom}$ prevalence in transplanted kidneys by dominant diagnosis.

Table S3a. Clinical and demographic differences by glomerular C4d.

Table S3b. Detailed descriptive pathology by glomerular C4d staining.

Table S4a. Univariable clinical predictors of glomerular C4d using GEE.

Table S4b. Univariable histologic determinants of glomerular C4d.

Table S4c. Multivariable epidemiologic risk factors for glomerular C4d.

Table S4d. Multivariable histologic determinants of glomerular C4d.

Table S5. Confirmatory analysis: Antibody-related determinants of $C4d_{glom}$ using ordinal regression against clinical and histologic risk factors.

Table S6. Correlations with glomerular and peritubular capillary C4d.

Table S7. Diagnostic performance of $C4d_{glom}$ against antibody-mediated rejection diagnosed using Banff 2019 criteria in population and TG subset.

Table S8. Diagnostic performance of $C4d_{glom}$ in reference test subset of transplant glomerulopathy verified by electron microscopy ($n = 359$). Transplant glomerulopathy and relation of Baff cg cases using binomial GEE.

Table S9. Detailed descriptive summary data of transplant glomerulopathy.

Table S10. Predictors of transplant glomerulopathy using GEE (3 models).

Table S11a. Sensitivity analysis I: Univariable predictors of glomerular C4d in transplant glomerulopathy using binomial GEE.

Table S11b. Sensitivity analysis II: Multivariable predictors of $C4d_{glom}$ in transplant glomerulopathy.

Table S12. Ultrastructural verified reference test performance of Banff 2019 AMR diagnostic criteria in transplant glomerulopathy: head-to-head comparison to expanded criteria incorporating $C4d_{glom}$.

Table S13a. Univariable determinants of graft failure using Cox regression.

Table S13b. Multivariable predictors of graft failure using Cox regression.

Table S14. Kaplan Meier graft failure in TG by Banff AMR definitions.

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