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Abstract

Accurate diagnosis of kidney allograft pathology remains challenging, particularly in borderline biopsies and non-classical antibody-mediated rejection (ABMR), including isolated microvascular inflammation (iMVI). We retrospectively analyzed 219 consecutive kidney allograft biopsies (2019–2024) evaluated by Banff-based histology and parallel Molecular Microscope Diagnostic System (MMDx) testing. Histology and MMDx were concordant in 186/219 biopsies (84.9%). In 20/219 (9.1%), predominantly borderline/equivocal cases, MMDx was pivotal for the final clinicopathological diagnosis. Rejection was identified by both modalities in 45/219 (20.5%), with phenotype agreement in 37/45 (82.2%); rejection versus non-rejection discordance occurred in 17/219 (7.8%). Three-month changes in eGFR and proteinuria did not differ across histology/MMDx rejection concordance groups ($p=0.786$ and $p=0.543$), and graft failure up to 12 months post diagnosis was infrequent ($p=0.324$). Molecular ABMR-related signatures were detected in 12/16 (75.0%) C4d-negative histological ABMR/mixed and in 14/19 (73.7%) DSA-negative iMVI/mixed rejection biopsies. In Banff-relevant strata, molecular rejection was present in 9/12 (75.0%) iMVI (DSA–/C4d–) and 5/6 (83.3%) probable ABMR (DSA+/C4d–). Of 56/219 biopsies (25.6%) deemed histologically limited, MMDx yielded reportable results in all inadequate samples. Overall, integrating MMDx improved diagnostic confidence and refined rejection phenotyping in real-world practice.

Introduction

Accurate diagnosis of kidney allograft pathology is essential for guiding immunosuppressive therapy and optimizing long-term transplant outcomes. Despite substantial advances in histological assessment and the adoption of standardized Banff classification criteria, significant limitations persist in diagnostic sensitivity and specificity-particularly in cases with borderline changes, donor-specific antibody (DSA)-negative/C4d-negative isolated microvascular inflammation (iMVI), and biopsies showing overlapping injury patterns (e.g., viral infection, drug toxicity, chronic scarring).(1-3)

To address these challenges, the most recent Banff classification (2019 update with subsequent 2022 working group addenda)(1, 2) formally incorporated molecular markers of rejection into the diagnostic framework. Assessment of rejection-associated transcripts was recognized as a valuable adjunct to conventional histopathological evaluation-including light microscopy, immunofluorescence, and C4d staining-particularly for clarifying diagnostically ambiguous cases and improving interobserver and intercenter reproducibility.

To implement molecular diagnostics in routine clinical practice, in 2019 we introduced the Molecular Microscope® Diagnostic System (MMDx) at the Centre for Kidney Transplantation, University Medical Centre Ljubljana.(4) This platform utilizes genome-wide microarray-based gene expression profiling coupled with machine learning algorithms to provide an objective, quantitative, transcriptome-based diagnostic readout. MMDx enables the identification of rejection-specific molecular patterns and helps to differentiate rejection from non-immune-mediated injuries such as acute kidney injury/acute tubular necrosis (AKI/ATN), BK polyomavirus-associated nephropathy, or chronic fibrosis. (4-8)

The primary aim of this study was to assess the diagnostic contribution of MMDx in kidney allograft biopsies performed at our center, with particular emphasis on biopsies with equivocal histology and on cases in which molecular results differed from conventional light microscopy, immunopathology and electron microscopy. We further evaluated histology-MMDx concordance and clinically relevant discordance patterns, and examined ABMR classifications in relation to C4d and DSA status in line with the evolving Banff framework.

Materials and Methods

Study design and clinical evaluation

We conducted a retrospective single-center study of adult kidney transplant recipients at the Centre for Kidney Transplantation, Departement of Nephrology, University medical Centre Ljubljana. The study included kidney allograft biopsies with available molecular analysis results performed between 2019 and 2024, following the implementation of the MMDx in routine clinical practice. All consecutive biopsies meeting these criteria were included.

The study cohort included both surveillance biopsies (performed approximately 12 months post-transplant as per our local protocol) and indication biopsies (performed for clinical reasons such as rising serum creatinine, new or worsening proteinuria, detection of DSA, elevated donor derived cell free DNA (dd-cfDNA), or delayed graft function (DGF)).

Clinical data were retrospectively obtained from electronic medical records and included: patient demographics (age, sex), time since transplantation, serum creatinine, and estimated glomerular filtration rate calculated using the CKD-EPI formula (eGFR CKD EPI, ml/min/1.73m²), estimated protein excretion rate (ePER, g/day), presence and specificity of DSA (measured by Luminex single antigen assay), plasma dd-cfDNA levels (where available), and indication for biopsy (if applicable). For each patient, relevant laboratory and immunological work-up at the time of biopsy was documented.

Histopathological evaluation

All kidney allograft biopsies underwent standard Banff-based histological evaluation in parallel with molecular assessment using MMDx. Routine pathology processing included light microscopy, electron microscopy, immunofluorescence, and C4d staining, with lesions scored according to the contemporaneous Banff update used at the time of interpretation.(1, 2, 9, 10) Final histopathological diagnoses were established by multidisciplinary consensus involving nephrologists and nephropathologists.

MMDx analysis

For MMDx analysis tissue samples were shipped to the Alberta Transplant Applied Genomics Centre, University of Alberta (Edmonton, Canada), and processed according to standard protocols. For the purposes of this study, MMDx results were extracted primarily as the reported diagnostic phenotypes—T cell-mediated rejection (TCMR), ABMR, mixed rejection, or non-rejecting. Transcript patterns suggestive of non-rejection injury (e.g., AKI/ATN or fibrosis-associated signals) were considered descriptively in individual cases but were not the primary focus of the comparative analyses. All MMDx reports were reviewed together with histology and clinical data at weekly multidisciplinary transplant pathology meetings, where an integrated consensus diagnosis was established for each biopsy.

Analysis of concordance and discrepancy

We systematically assessed concordance between conventional histology (Banff-based classification) and MMDx by assigning each biopsy a primary diagnostic category in both modalities. Histology categories included ABMR, TCMR, mixed rejection (ABMR and TCMR), AKI/ATN, calcineurin inhibitor-associated nephropathy (CNI-N), interstitial fibrosis and tubular atrophy (IFTA), recurrence of primary kidney disease, no major abnormalities (normal), and other. MMDx categories included rejection-related classifiers (ABMR, TCMR, mixed rejection, and non-rejecting).

Concordance was evaluated by comparing conventional histology and MMDx with respect to rejection status (rejection vs non-rejection) and, when rejection was present, rejection phenotype (ABMR, TCMR, or mixed). Biopsies were assigned to one of four predefined agreement categories: same phenotype (both methods indicated rejection with the same phenotype), phenotype discordance (both methods indicated rejection but with different phenotype assignments), histology-only rejection (rejection detected by conventional histology only), or molecular-only rejection (rejection detected by MMDx only). These categories were summarized in a bar plot.

All discordant rejection calls and phenotype-discordant cases were re-reviewed in weekly multidisciplinary meetings integrating morphology, C4d/DSA assessment, and clinical context, and were adjudicated with respect to their potential diagnostic and therapeutic implications.

Comparison of kidney function, proteinuria dynamics, and allograft failure across Banff/MMDx rejection concordance groups

To evaluate whether concordant versus discrepant rejection classification was associated with short-term kidney function and graft outcomes, we dichotomized each biopsy as rejection (R) or non-rejection (NR) separately by histology (Banff; any ABMR, TCMR, or mixed) and by MMDx (any ABMR, TCMR, or mixed). Patients were then assigned to one of four concordance groups: Banff NR/MMDx NR, Banff R/MMDx R, Banff R/MMDx NR, and Banff NR/MMDx R. For kidney function and proteinuria dynamics, eGFR was expressed as CKD-EPI (mL/min/1.73m²) and proteinuria as ePER (g/day). Baseline values were taken at the time of kidney biopsy, and follow-up values at 3 months; changes were calculated as $\Delta\text{eGFR} = \text{eGFR}_{3 \text{ months}} - \text{eGFR}_{\text{kidney biopsy}}$ and $\Delta\text{ePER} = \text{ePER}_{3 \text{ months}} - \text{ePER}_{\text{kidney biopsy}}$. Distributions of eGFR, ePER, and their changes were summarized using median and interquartile range and compared across the four groups using the Kruskal–Wallis test.

Categorical outcomes included graft failure (yes/no) and an eGFR decline of at least 30% at 3 months ($\text{eGFR}_{3 \text{ months}} \leq 0.70 \times \text{eGFR}_{\text{kidney biopsy}}$). Graft failure was assessed at 12 months during after the kidney graft biopsy. Categorical outcomes were compared between groups using chi-square testing, with Fisher's exact test used for pairwise comparisons when expected cell counts were small. All tests were two-sided, and p-values <0.05 were considered statistically significant.

Statistical analysis

Continuous variables are presented as median with interquartile range (IQR), and categorical variables as counts and percentages. Comparisons of continuous variables (eGFR, ePER, ΔeGFR , and ΔePER) across Banff/MMDx concordance groups were performed using the Kruskal–Wallis test. Categorical outcomes, including graft failure (yes/no) and an eGFR decline $\geq 30\%$ at 3 months ($\text{eGFR}_{3 \text{ months}} \leq 0.70 \times \text{eGFR}_{\text{kidney biopsy}}$), were compared using the chi-square test, with Fisher's exact test applied when expected cell counts were small. All tests were two-sided, and p-values <0.05 were considered statistically significant. Concordance between histology and MMDx was assessed descriptively by classifying biopsies into predefined agreement categories (same phenotype, phenotype discordance, histology-only rejection, and MMDx-only rejection). Statistical analyses were performed using *GraphPad Prism* (GraphPad Software, San Diego, CA, USA). Graphical representation was generated using a generative AI–assisted tool.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the National Medical Ethics Committee of the Republic of Slovenia (approval number: 0120-87/2021/3). All patients provided written informed consent for surveillance and indication kidney biopsy as part of their clinical care. Owing to the retrospective design and use of routinely collected clinical data, the Committee waived the requirement for additional study-specific informed consent.

Results

Basic characteristics of patients

Between 1 January 2019 and 31 December 2024, 219 kidney allograft biopsies from 186 patients were analyzed with both conventional histology and MMDx. Of these, 73 were surveillance biopsies (median time post-transplant 389 days, range 252 to 497 days) and 146 were indication biopsies (median time post-transplant 1155 days, range 6 to 10.290 days). Indication kidney biopsies were performed due to a rise in serum creatinine (n=106, 72.6%), increased proteinuria (n=11, 7.5%), DGF (n=16, 10.95%), occurrence or increase of DSA/dd-cfDNA (n=9, 6.16%), or other (suspicion of recurrent disease, BK virus-associated nephropathy, and thrombotic microangiopathy (n=4, 2.7%).

Following clinicopathological correlation and multidisciplinary consensus integrating laboratory data, conventional histology and MMDx findings, the distribution of leading diagnoses (final report) among 219 biopsies was: 19 with no major abnormalities (8.7%), 68 with CNI-N or IFTA as the leading finding (31.1%), 26 with ATN/AKI (11.9%), 55 with rejection (25.1%), 8 with BK virus-associated nephropathy (3.7%), 9 with recurrent glomerulonephritis (4.1%), and 34 with other pathology (15.5%), including diabetic nephropathy, nephrocalcinosis, thrombotic microangiopathy, ischemic changes, pyelonephritis, light chain deposition disease, de novo glomerulonephritis, and nonspecific inflammatory infiltrates.

Baseline clinical, laboratory, and immunological characteristics at the time of kidney allograft biopsy are summarized in Table 1.

Table 1. The basic characteristics of patients with surveillance and indication kidney biopsy, including laboratory and immunological work-up at the time of kidney biopsy.

Variable	Surveillance biopsy (n=73)	Indication biopsy (n=146)
Age, years	50.5 (39.3–63.0)	57.0 (45.8–65.3)
Time post-KTx, days	388 (375–404)	1155 (93–3566)
Serum creatinine, $\mu\text{mol/L}$	108 (94–137)	173 (134–216)
eGFR, mL/min/1.73 m^2	59.0 (49.0–71.5)	35.0 (26.0–46.0)
ePER, g/day^*	0.11 (0.00–0.21)	0.43 (0.20–1.36)
dd-cfDNA, %*	0.46 (0.18–0.75)	0.68 (0.38–1.68)
DSA present, n (%)	1 (1.4%)	29 (19.9%)

Continuous variables are presented as median (IQR), and donor-specific antibodies are presented as n (%). Abbreviations: KTx, kidney transplantation; eGFR, estimated glomerular filtration rate; ePER, estimated protein excretion rate; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibodies. *ePER data were available for 211 (96.3%) patients, and dd-cfDNA for 52 (23.7%) patients.

Concordance between histology and MMDx findings

A Sankey plot summarizing transitions between the preliminary histology-based diagnostic categories assigned before MMDx results were available and the rejection-focused molecular classifications is shown in Figure 1, illustrating the expected asymmetry between a comprehensive histological taxonomy and a rejection-centered molecular framework.

Among 219 biopsies, 186 (84.9%) were ultimately considered concordant after multidisciplinary clinicopathological correlation. Within this concordant group, 20 cases (9.1% of the total cohort) with borderline or otherwise equivocal findings required MMDx input to establish the final consensus diagnosis because histology alone was not sufficiently decisive.

Most discordance reflected framework mismatch or phenotype-level differences rather than true disagreement on the presence of rejection. This distinction is particularly important in frequent non-rejection histological diagnoses—such as recurrence of primary disease, CNI-N, IFTA, or ATN—where MMDx provides added value by objectively determining whether a concomitant rejection-associated molecular signal is present. Furthermore, 52 biopsies were provisionally classified into a separate group of other pathologies (“H-Other”) based on the preliminary histologic assessment before MMDx results were available. This category included a heterogeneous range of mixed-injury patterns, such as combined chronic injury with acute tubular injury, infection-related pathology including BK virus-associated nephropathy, thrombotic microangiopathy, and other less frequent entities, as well as biopsies in which histology was not sufficiently conclusive for definitive categorization. In these cases, MMDx proved clinically useful both by excluding molecular evidence of rejection—thus helping avoid unnecessary intensification of immunosuppression—and by supporting rejection phenotypes when present, thereby increasing diagnostic confidence in multidisciplinary evaluation. For example, in six out of eight biopsies with BK virus-associated nephropathy and prominent inflammatory infiltrates, MMDx showed no molecular evidence of rejection, helping to resolve the key diagnostic dilemma of whether the inflammation reflected viral injury alone or a concomitant alloimmune process with opposite therapeutic implications. Similarly, in four out of six biopsies with histological features of thrombotic microangiopathy, MMDx showed no molecular evidence of rejection, helping to distinguish non-alloimmune endothelial injury from concomitant rejection in a diagnostically challenging setting.

Focusing on rejection, 45/219 biopsies (20.5%) were classified as rejection by both histology and MMDx (Figure 2). Among these, 37/45 (82.2%) showed complete concordance in rejection phenotype (ABMR n=29, TCMR n=3, mixed n=5). In the remaining 8/45 (17.8%), both methods identified rejection but differed in phenotypic assignment (e.g., isolated TCMR on histology versus mixed rejection by MMDx); all of these were ultimately classified as rejection in the final clinicopathological consensus report.

True rejection versus non-rejection discordance was observed in 17/219 biopsies (7.8%). Histology identified rejection in 9 cases (ABMR n=6; TCMR n=3) without corresponding molecular evidence of rejection; all were classified as rejection in the final report. Conversely, MMDx indicated rejection in 8 cases without histological evidence of rejection; final integrated diagnoses in these biopsies were non-rejection entities, including IFTA/CNI-N (n=3), BK virus-associated nephropathy (n=1), chronic pyelonephritis (n=1), and nonspecific inflammatory changes (n=3).

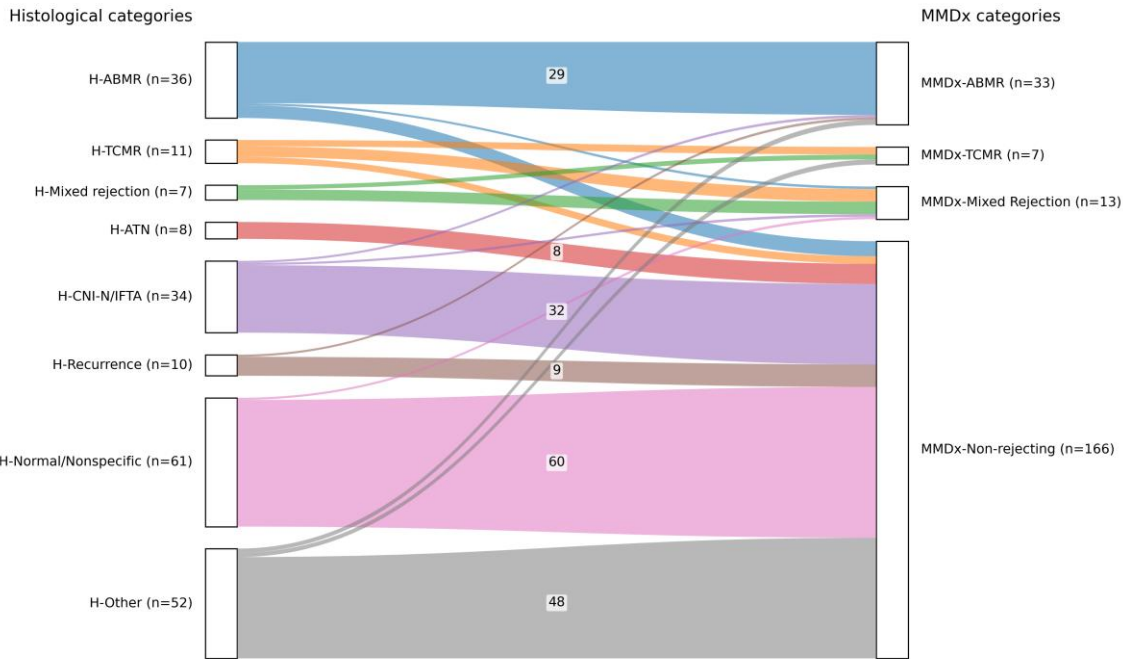


Figure 1. Sankey (alluvial) plot of agreement between preliminary histology-based and molecular (MMDx) diagnoses (n = 219).

The Sankey diagram visualizes patient-level concordance and discordance between the primary diagnostic category assigned by conventional histopathology (left; H) and the corresponding Molecular Microscope Diagnostic System (MMDx) classification (right). The width of each flow is proportional to the number of biopsies in that transition. Histology categories include ABMR, TCMR, mixed ABMR+TCMR, ATN, IFTA, recurrence, other/unclassified, and normal/nonspecific. MMDx categories include ABMR, TCMR, mixed ABMR+TCMR, and no rejection. Numbers displayed on selected flows indicate case counts for larger transitions (labels shown for $n \geq 7$).

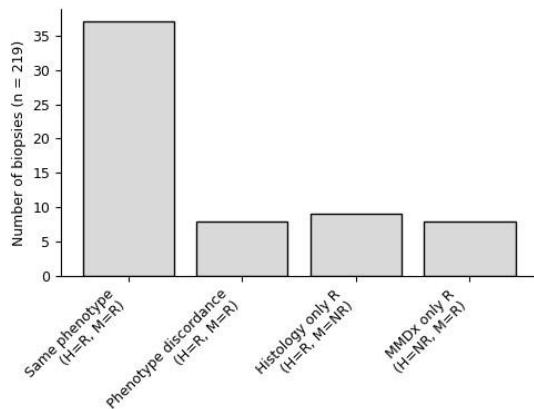


Figure 2. Rejection concordance between histology and MMDx (n = 219).

Bar plot showing agreement between conventional histology (H) and MMDx (M) for rejection status and phenotype. “Same phenotype” indicates concordant ABMR, TCMR, or mixed rejection. “Phenotype discordance” indicates rejection by both methods but different phenotype. “Histology only R” and “MMDx only R” denote rejection detected by one method only. R=rejection, NR=no rejection.

Comparison of clinical data and graft failure rate in rejection and non-rejection categories by histology and MMDx findings

For this analysis, we dichotomized each biopsy as rejection (R) or non-rejection (NR) based on histology (Banff; any ABMR, TCMR, or mixed ABMR+TCMR) and, separately, based on MMDx (any ABMR, TCMR, or mixed ABMR+TCMR). This yielded four Banff/MMDx concordance groups: Banff NR/MMDx NR (n=159), Banff R/MMDx R (n=45), Banff R/MMDx NR (n=9), and Banff NR/MMDx R (n=7). Baseline eGFR (CKD-EPI, mL/min/1.73m²) differed across groups (Kruskal–Wallis p=0.024), with median baseline eGFR of 45.0 [IQR 32.0–62.0] in NR/NR, 35.0 [13.0–54.0] in R/R, 40.0 [27.0–46.0] in R/NR, and 40.0 [34.0–54.5] in NR/R. At 3 months, eGFR differences were not statistically significant (p=0.080): NR/NR 49.5 [36.0–65.5], R/R 41.0 [27.0–56.0], R/NR 52.0 [25.0–60.0], and NR/R 47.0 [32.0–65.0]. Short-term eGFR dynamics were comparable between groups: median Δ eGFR (3 months – baseline) was 2.0 [–3.0 to 7.0] in NR/NR, 0.0 [–4.0 to 6.5] in R/R, 9.0 [–3.0 to 12.0] in R/NR, and 3.0 [–5.5 to 5.0] in NR/R (p=0.786); percent change also did not differ (p=0.859). An eGFR decline $\geq$ 30% at 3 months was uncommon and similar across groups (NR/NR 5/154 [3.2%], R/R 3/43 [7.0%], R/NR 0/9, NR/R 0/7; chi-square p=0.579) (Figure 3a).

Proteinuria dynamics were evaluated as the change in ePER from kidney biopsy to 3 months (Δ ePER = ePER_3 months – ePER_kidney biopsy) across the four Banff/MMDx concordance groups. Data were available for 174/219 patients (Banff NR/MMDx NR n=124; Banff R/MMDx R n=36; Banff R/MMDx NR n=7; Banff NR/MMDx R n=7). The distribution of Δ ePER did not differ significantly between groups (Kruskal–Wallis p=0.543), indicating broadly similar short-term proteinuria trajectories regardless of concordance/discordance between histology and MMDx rejection status (Figure 3b).

Allograft failure rates 12 months after the biopsy did not differ significantly across the four groups (chi-square p=0.324): NR/NR 3/158 (1.9%), R/R 3/45 (6.7%), R/NR 0/9, and NR/R 0/7. Relative to the NR/NR reference group, the R/R group showed a numerically higher allograft failure risk (Fisher’s exact OR 3.69; p=0.124), but this did not reach statistical significance, consistent with the low number of events.

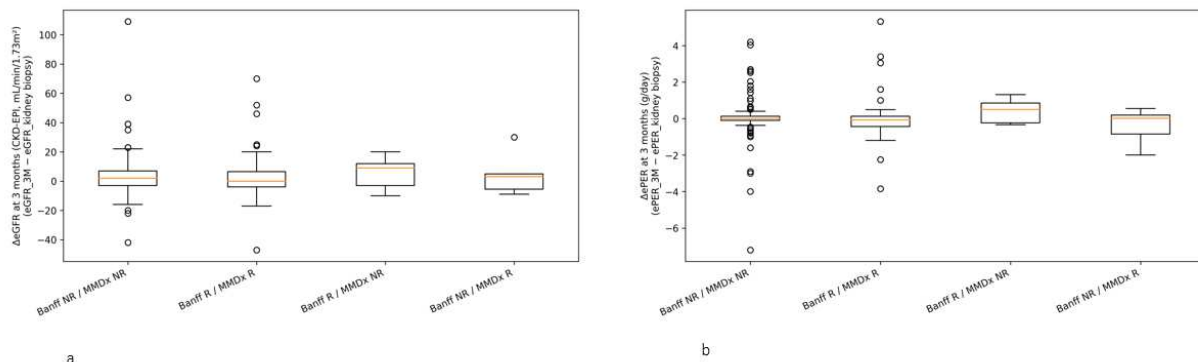


Figure 3. a. Change in eGFR at 3 months by Banff 2022/MMDx concordance groups. Boxplots show $\Delta eGFR$ at 3 months (CKD-EPI, mL/min/1.73m²), calculated as $eGFR_{3 \text{ months}} - eGFR_{\text{kidney biopsy}}$, across four groups defined by rejection (R) vs non-rejection (NR) on histology (Banff) and MMDx (Banff NR/MMDx NR; Banff R/MMDx R; Banff R/MMDx NR; Banff NR/MMDx R). Circles denote outliers. Between-group differences in $\Delta eGFR$ were not statistically significant (Kruskal–Wallis $p=0.786$). **b. Change in proteinuria at 3 months by Banff/MMDx concordance groups.** Boxplots show $\Delta ePER$ (g/day), calculated as $ePER_{3 \text{ months}} - ePER_{\text{kidney biopsy}}$, across the same four Banff/MMDx concordance groups. Circles denote outliers. Between-group differences in $\Delta ePER$ were not statistically significant (Kruskal–Wallis $p=0.543$).

ABMR in the context of C4d and DSA: histology and MMDx concordance

In line with recent Banff updates, we analyzed histological ABMR/mixed rejection across C4d and DSA strata. C4d status was available for all 219 biopsies. Among C4d-positive cases ($n = 28$), histology identified ABMR/mixed in 24/28 (85.7%) and MMDx in 22/28 (78.6%); concordance within histological ABMR/mixed C4d-positive cases was 22/24 (91.7%). In C4d-negative histological ABMR/mixed biopsies ($n = 16$), MMDx supported ABMR/mixed in 12/16 (75.0%). Baseline DSA was positive in 30 and negative/unknown in 189 biopsies. Histological ABMR/mixed was present in 24/30 (80.0%) DSA-positive and 19/189 (10.1%) DSA-negative/unknown cases. Molecular ABMR-related phenotypes were identified in 21/24 (87.5%) DSA-positive and 14/19 (73.7%) DSA-negative/unknown histological ABMR/mixed biopsies.

Restricting analysis to cases with both C4d and DSA available ($n = 42$), probable ABMR (DSA+/C4d-) accounted for 6/42 (14.3%) and iMVI (DSA-/C4d-) for 12/42 (28.6%). In probable ABMR, MMDx classified 4/6 (66.7%) as ABMR/mixed, 1/6 (16.7%) as TCMR, and 1/6 (16.7%) as non-rejecting. In iMVI, MMDx identified ABMR in 8/12 (66.7%), TCMR in 1/12 (8.3%), and non-rejecting profiles in 3/12 (25.0%).

Across the combined histological ABMR/mixed spectrum ($n = 43$), 3-month graft function was overall stable (median $\Delta oGF -0.5$ mL/min/1.73 m² [IQR -4.8 to 4.8]) with graft failure in 2/43 (4.7%). All patients were managed according to the histological diagnosis of rejection; treatment intensity and specific regimens were individualized and not formally analyzed. When stratified by molecular phenotype, ABMR-related molecular profiles (molecular ABMR/mixed; $n = 35$) showed stable function (median $\Delta oGF 0.0$ [-5.8 to 4.0]) with graft failure in 2/35 (5.7%), accompanied by a median $\Delta creatinine$ of -1.5 $\mu\text{mol/L}$ (-45.0 to 9.8) and a modest decrease in proteinuria (median $\Delta -0.14$ g/day [-0.83 to 0.13]). In contrast, molecular non-rejecting cases ($n = 6$) had no graft failures (0/6) and a median ΔoGF of 4.5 (-3.5 to 11.8), but demonstrated an increase in proteinuria (median $\Delta +0.80$ g/day [0.50 to 0.90]) despite a median $\Delta creatinine$ of -14.5 $\mu\text{mol/L}$ (-37.8 to 8.0). Within Banff-relevant C4d/DSA strata, probable ABMR (DSA+/C4d-; $n = 6$) showed a more pronounced short-term decline in function (median $\Delta oGF -9.0$ [-12.2 to -3.5]) with graft failure in 1/6 (16.7%), whereas iMVI (DSA-/C4d-; $n = 12$) had a median ΔoGF of -2.0 (-5.0 to 0.5) with graft failure in 1/12 (8.3%). Molecular sub-stratification within these phenotypes, including creatinine and proteinuria dynamics, is summarized in Table 2.

Table 2. 3-month functional evolution and 12-month graft failure in the ABMR spectrum by molecular phenotype.

Group	n	Graft failure n (%)	Δ oGF median (IQR)	Δ Creatinine median (IQR)	Δ Proteinuria median (IQR)
ABMR spectrum (histological ABMR/mixed)	43	2 (4.7)	-0.5 (-4.8 to 4.8)	-1.0 (-39.0 to 11.5)	-0.04 (-0.44 to 0.42)
Molecular ABMR-related	35	2 (5.7)	0.0 (-5.8 to 4.0)	-1.5 (-45.0 to 9.8)	-0.14 (-0.83 to 0.13)
Molecular non-rejecting	6	0 (0.0)	4.5 (-3.5 to 11.8)	-14.5 (-37.8 to 8.0)	0.80 (0.50 to 0.90)
Molecular TCMR-only	2	0 (0.0)	-1.5 (-1.8 to -1.3)	35.0 (32.5 to 37.5)	0.16 (-0.01 to 0.33)
Probable ABMR (DSA+/C4d-)	6	1 (16.7)	-9.0 (-12.2 to -3.5)	31.0 (4.5 to 74.8)	0.20 (-1.10 to 0.49)
Molecular ABMR-related	4	1 (25.0)	-11.5 (-21.5 to -9.5)	60.5 (23.0 to 191.2)	-1.10 (-1.68 to -0.45)
Molecular non-rejecting	1	0 (0.0)	20.0	-39.0	0.80
Molecular TCMR-only	1	0 (0.0)	-2.0	30.0	0.49
Isolated MVI	12	1 (8.3)	-2.0 (-5.0 to 0.5)	5.0 (0.5 to 18.0)	0.03 (-0.17 to 0.18)
Molecular ABMR-related	8	1 (12.5)	-1.0 (-5.0 to 3.0)	2.0 (-4.0 to 7.0)	-0.04 (-0.17 to 0.10)
Molecular non-rejecting	3	0 (0.0)	-4.0 (-7.0 to -3.0)	9.0 (7.0 to 25.5)	1.10 (1.00 to 1.21)
Molecular TCMR-only	1	0 (0.0)	-1.0	40.0	-0.17

Abbreviations: ABMR, antibody-mediated rejection; TCMR, T cell-mediated rejection; DSA, donor-specific antibodies; C4d, complement split product C4d; MVI, microvascular inflammation; Δ , 3-month change from biopsy baseline; eGFR (CKD-EPI, mL/min/1.73 m²), estimated glomerular filtration rate according to CKD EPI; IQR, interquartile range. "Probable ABMR" denotes DSA+/C4d- and "isolated MVI" denotes DSA-/C4d-. Molecular ABMR-related indicates an ABMR or mixed molecular phenotype; molecular non-rejecting indicates no molecular evidence of rejection; molecular TCMR-only indicates an isolated TCMR molecular phenotype. Values are median (IQR). Δ denotes 3-month value minus baseline, graft failure refers to period up to 12 months post kidney graft biopsy/diagnosis. Raw values are shown for strata with n=1.

MMDx in the context of inadequate or insufficient kidney allograft biopsy samples

In our cohort of 219 kidney allograft biopsies, 56 (25.6%) were rated diagnostically limited, including 35 (16.0%) suboptimal and 21 (9.6%) inadequate for not meeting one or more Banff adequacy criteria. Limitations most commonly reflected low glomerular yield, absence of arteries, poor sample integrity (fragmented/sclerotic tissue), or predominantly medullary sampling. Inadequate biopsies had fewer glomeruli (median 4, IQR 2–5) than adequate samples (median 12, IQR 8–16); 20/21 (95.2%) contained <10 glomeruli. Cortical representation was lower and more variable (median 80% cortex, IQR 48–94), and 5/21 (23.8%) had $\leq$ 10% cortex. Regardless of histological adequacy, samples submitted for MMDx were sufficient in all biopsies, including the required 3–5 mm of tissue. MMDx yielded reportable results in all inadequate biopsies, indicating rejection-associated signatures in 8/21 and no molecular evidence of rejection in 11/21. Even among samples with $\leq$ 10% cortex, MMDx suggested rejection in 4/5 cases, supporting confirmation or exclusion of rejection when histological interpretation was limited.

Discussion

In this real-world, single-center cohort, incorporating MMDx into routine kidney allograft biopsy assessment provided clinically meaningful diagnostic support in scenarios where conventional histology is most vulnerable—namely borderline or overlapping injury patterns, non-classical ABMR presentations, and limited tissue adequacy. Our experience supports the current Banff direction toward integrated

diagnostics whereby molecular information does not replace microscopy, but adds a complementary layer that can increase confidence, refine phenotyping, and reduce uncertainty in borderline or confounded biopsies.

A key practical aspect of MMDx implementation at our centre was the progressive integration of molecular readouts into routine reporting. In a subset of biopsies with borderline or equivocal histological features, definitive categorization was intentionally deferred until the MMDx result was available, and the final diagnosis was established during multidisciplinary review incorporating both modalities. Accordingly, 20/219 biopsies (9.1%) required MMDx input to reach a final clinicopathological diagnosis. This workflow explains the relative absence of borderline rejection in the final diagnostic distribution of our cohort: cases that might otherwise have remained “borderline” were reassigned to a more specific consensus category once molecular data were considered, pointing to a pragmatic decision-support effect during a multi-year period of progressive integration into routine practice.

When interpreting histology–MMDx discrepancies, it is essential to recognize the different diagnostic scopes of the two approaches.(11, 12) Conventional histology captures the full range of alloimmune and non-alloimmune pathology, whereas the current MMDx framework is primarily designed to distinguish rejection from non-rejection and, if present, to phenotype rejection as ABMR, TCMR, or mixed.(12) Consequently, a proportion of discordance is anticipated and does not necessarily indicate methodological inaccuracy. Processes such as recurrent disease, infection-related injury, drug toxicity, or chronic scarring lack dedicated molecular classifiers and therefore, by design, map to the “non-rejecting” category unless accompanied by rejection-associated transcripts. In our cohort, this scope-related mismatch accounted for the most frequent discordance pattern. Nonetheless, molecular testing remained clinically informative by objectively indicating whether rejection-associated activity coexisted with a non-rejection histologic process, supporting risk–benefit decisions regarding immunosuppression escalation.

Beyond scope-related differences, true rejection versus non-rejection discordance was uncommon (17/219, 7.8%) and occurred predominantly in biopsies in which histology favored a non-alloimmune primary diagnosis, most often AKI/ATN, CNI-N, advanced chronic scarring, recurrence of primary kidney disease, infection-related injury, and thrombotic microangiopathy. MMDx may be particularly valuable in BK virus-associated nephropathy, where concomitant TCMR is often difficult to distinguish from virus-driven tubulointerstitial inflammation on histology alone. A similar diagnostic advantage applies to biopsies with thrombotic microangiopathy, where the central question is often whether the lesion reflects isolated non-alloimmune endothelial injury or an overlapping alloimmune process, particularly ABMR. In these settings, MMDx may help both by identifying rejection-related molecular activity when present and by excluding ABMR when such activity is absent, thereby improving therapeutic confidence in situations with directly opposing treatment implications. More broadly, an MMDx “rejection” call in these contexts may plausibly reflect concomitant alloimmune activity superimposed on the dominant histologic process, sampling differences between tissue allocated to histology versus transcriptomics (especially in cortex-poor specimens), or overlap between injury-associated inflammatory programs and rejection-associated transcript patterns.

When both modalities identified rejection, discrepancies involved phenotype assignment (8/45, 17.8% phenotype discordance within the rejection/rejection group), consistent with the difficulty of resolving mixed or transitional ABMR/TCMR patterns by morphology alone—particularly when MVI is subtle or tissue is limited. In these cases, molecular phenotyping provided an additional, independent source of evidence that supported multidisciplinary adjudication of the dominant process. Importantly, short-term functional trajectories did not differ significantly across Banff/MMDx rejection concordance groups. This likely reflects the clinical context in which MMDx is applied and the limited discriminatory power of short-term endpoints in a heterogeneous, low-event cohort, where early graft function is shaped by multiple downstream determinants. Consequently, separation of outcomes may require larger event numbers and longer follow-up.

The analysis of ABMR across C4d and DSA strata further illustrates the added value of integrated diagnostics. Non-classical ABMR phenotypes, including C4d-negative and/or DSA-negative presentations, remain diagnostically challenging, particularly when immunologic markers are absent or borderline.(1, 6) In our cohort, a substantial proportion of histologically defined iMVI cases still demonstrated an ABMR-consistent molecular signature. This is in line with prior observations that transcriptomic evidence of alloimmune endothelial injury may be detectable even in the absence of complement deposition or circulating DSA at the time of biopsy.(13-15) At the same time, a minority of biopsies with iMVI lacked a dominant molecular rejection signal, pointing to the biological heterogeneity of this subgroup and cautioning against uniform therapeutic escalation based solely on morphology. A similar pattern was observed in probable ABMR (DSA+/C4d-), where molecular phenotypes were heterogeneous but frequently mapped to ABMR or mixed rejection. Together, these findings support the evolving Banff perspective that complement deposition is not obligatory for biologically active antibody mediated injury and that integration of MVI, serology, and molecular profiling may more accurately reflect ongoing alloimmune activity than any single marker alone.(1)

Importantly, all patients in the ABMR spectrum were managed according to the histological diagnosis of rejection, with individualized treatment intensity. However, no treatment-adjusted analysis was performed in this study and therapeutic conclusions based on molecular stratification cannot be drawn. MMDx showed high concordance with histology in C4d-positive ABMR, whereas C4d-negative phenotypes, including iMVI and probable ABMR, exhibited greater molecular dispersion. In descriptive short-term follow-up, iMVI cases did not show uniformly adverse outcomes, while probable ABMR (DSA+/C4d-) demonstrated a more pronounced median decline in eGFR and a numerically higher graft-failure rate. Although limited by small subgroup sizes and non-standardized treatment exposure, these findings are consistent with biological variability within C4d-negative ABMR and illustrate how transcriptomic profiling may further delineate this spectrum beyond morphology alone.(14, 16)

A particularly pragmatic strength of molecular testing in our cohort was its usefulness in tissue-limited biopsies. Inadequate or suboptimal samples are common in routine practice and may not satisfy Banff adequacy criteria, yet clinical decisions still must be made. In such circumstances, MMDx provided reportable phenotypes across all inadequate specimens and offered actionable information regarding the presence or absence of rejection-associated molecular activity. Importantly, this observation is consistent with prior multicenter experience demonstrating that molecular classifiers of rejection remain

informative even in biopsies with a high medullary component. Madill-Thomsen and colleagues (17) showed that molecular assessment of rejection and injury can be reliably performed in medulla-predominant samples, with preservation of diagnostic discrimination for ABMR and TCMR despite reduced cortical representation. Similarly, large discrepancy analyses from the INTERCOMEX and related cohorts have reported that transcriptome-based diagnostics maintain performance across variable tissue composition, supporting their robustness to sampling heterogeneity.(4, 7) These data, together with our results, suggest that although cortex-poor sampling remains suboptimal and should be minimized whenever feasible, transcriptomic profiling can partially mitigate the diagnostic uncertainty imposed by limited histological material. However, molecular testing does not eliminate the need for adequate sampling: both morphology and transcriptomics remain dependent on specimen quality, and optimal biopsy technique remains essential.

Our study has several strengths: consecutive biopsies reflecting routine clinical case-mix, systematic multidisciplinary clinicopathological integration, inclusion of both surveillance and indication biopsies across a broad post-transplant range, and explicit consideration of tissue adequacy. Limitations include the retrospective, single-center design and incorporation bias, as MMDx contributed to the consensus process (i.e., the final diagnosis was not independent of the molecular readout). In addition, many non-alloimmune processes remain outside the current MMDx phenotype classifier set; therefore, clinicopathological correlation remains indispensable for recurrence, infection-related injury, drug toxicity and other entities in which a molecular “non-rejecting” call does not imply benign pathology. Finally, outcome analyses were constrained by low event rates and limited statistical power, although graft failure status was ascertained at 12 months in all patients.

In summary, our five-year experience indicates that MMDx is most impactful as a complementary modality within an integrated diagnostic workflow. Its greatest clinical value is the objective assessment and phenotyping of rejection in biopsies with limited tissue, complex mixed injury patterns, and non-classical ABMR presentations, while multidisciplinary interpretation remains essential to correctly contextualize molecular “non-rejecting” results in the presence of clinically relevant non-alloimmune pathology.

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Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the National Medical Ethics Committee of the Republic of Slovenia (approval number: 0120-87/2021/3). All patients provided written informed consent for surveillance and indication kidney biopsy as part of their clinical care. Owing to the retrospective design and use of routinely collected clinical data, the Committee waived the requirement for additional study-specific informed consent.

Data availability statement

The datasets generated and/or analyzed during the current study are not publicly available because they contain pseudonymized patient-level clinical data and are subject to institutional and ethical restrictions, but are available from the corresponding author on reasonable request and with appropriate institutional approval.

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Author contributions

N.B. collected the data and contributed to the initial drafting of the manuscript. Ž.V.H. conceived the study, led the interpretation of the data, and wrote the manuscript. M.A. contributed to the study design, interpretation of clinical data and critical revision of the manuscript. N.K. reviewed the histopathological components and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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