ORIGINAL ARTICLE

Three-month course of intragraft transcriptional changes in kidney allografts with early histological minimal injury – a cohort study

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SUMMARY

The tubulitis with/without interstitial inflammation not meeting criteria for T-cell-mediated rejection (minimal allograft injury) is the most frequent histological findings in early transplant biopsies. The course of transcriptional changes in sequential kidney graft biopsies has not been studied yet. Molecular phenotypes were analyzed using the Molecular Microscope® Diagnostic System (MMDx) in 46 indication biopsies (median 13 postoperative days) diagnosed as minimal allograft injury and in corresponding follow-up biopsies at 3 months. All 46 patients with minimal injury in early biopsy received steroid pulses. MMDx interpreted indication biopsies as no-rejection in 34/46 (74%), T-cell-mediated rejection (TCMR) in 4/46 (9%), antibody-mediated rejection in 6/46 (13%), and mixed rejection in 2/46 (4%) cases. Follow-up biopsies were interpreted by MMDx in 37/46 (80%) cases as no-rejection, in 4/46 (9%) as TCMR, and in 5/46 (11%) as mixed rejection. Follow-up biopsies showed a decrease in MMDx-assessed acute kidney injury ($P = 0.001$) and an increase of atrophy–fibrosis ($P = 0.002$). The most significant predictor of MMDx rejection scores in follow-up biopsies was the tubulitis classifier score in initial biopsies (AUC = 0.84, $P = 0.002$), confirmed in multivariate binary regression (OR = 16, $P = 0.016$). Molecular tubulitis score at initial biopsy has the potential to discriminate patients at risk for molecular rejection score at follow-up biopsy.

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Introduction

The tubulitis with/without interstitial inflammation not meeting criteria for T-cell-mediated rejection (minimal allograft injury) is the most frequent histological finding in early transplant biopsies performed within first two post-transplant weeks [1–3]. A fraction of these cases fulfills criteria for suspicious (borderline) for acute TCMR definition by recent Banff classification [4]. Outcomes of such histological findings remain unclear as sequential biopsies are less available. The molecular assessment of kidney allografts represents a new diagnostic tool capable of overcoming the documented inconsistencies of conventional histology [5]. Our previous microarray study revealed heterogeneity of intragraft molecular pathways in biopsies with borderline

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changes, with higher immune activation in indication biopsies performed within first two post-transplant weeks [6]. It is therefore likely that minimal allograft injury may represent nonhomogenous cohort in respect of transcriptional profiles and theirs outcomes [7–9].

The Molecular Microscope® Diagnostic System (MMDx) has been introduced as a standardized and validated method to improve diagnostics in transplanted organs. Using this platform for assessment, only 26% (12/46) of indication biopsies called suspicious (borderline) for acute TCMR definition by Banff 2005 classification exhibited molecular rejection phenotypes while the majority of biopsies were reclassified as nonrejection [2,3,10]. While biopsy series may improve our understanding of how pathological lesions develop [11–13], the natural history of post-transplant pathologies other than fibrosis is difficult to interpret because of therapy bias [14]. Time courses of molecular events associated with graft pathologies have not been systematically studied to date.

The objective of this retrospective cohort study was to evaluate the development of molecular profiles from early indication biopsies (taken in the first 2 weeks post-transplant) diagnosed by histology as tubulitis with/without interstitial inflammation not meeting TCMR criteria to follow-up protocol biopsies taken at three months.

Patients and methods

Study design and patients

We identified 46 early indication biopsies with minimal kidney allograft injury defined as tubulitis with or without interstitial inflammation not meeting Banff criteria for TCMR or mixed rejection performed between 2007 and 2019, with follow-up biopsies at 3 months (3 M) in which a 2–4 mm section of each biopsy core was stored in RNA*later*™ for future molecular assessment. The second part of the core was examined by histology per standard of care. Patients with examined indication biopsies had no previous biopsy-confirmed pathology. All patients with minimal kidney allograft injury defined by Banff 2005 [15] to 2017 [16] as borderline suspicious for acute TCMR (including isolated tubulitis) in early indication biopsies were treated by steroid pulses according to center protocol (Fig. 1). Molecular phenotypes assigned by MMDx were compared between indication and 3 M follow-up biopsies. Majority of 3-month biopsies (39/46, 85%) were scheduled as protocol biopsies, while in seven cases the biopsies were performed for cause. All patients provided informed consent in advance of the biopsy procedure, allowing us to perform molecular analysis. The study was approved by Ethics Committee of the Institute for Clinical and Experimental Medicine (G-16-06-09).

Transplant demographics of the study cohort are given in Table 1. All were first kidney grafts: 34/46 (74%) were from deceased donors (of which 22 were ECDs), and 12/46 (26%) from living donors. Eight patients (8/46, 17%) received T-cell-depletive rATG induction because they were considered high immunological risk. Maintenance immunosuppression consisted of CNI inhibitors (mainly tacrolimus), mycophenolate mofetil (MMF), and steroids.

Renal graft biopsies and MMDx

Biopsies were performed using a semiautomatic biopsy gun 16G needle under ultrasound guidance. The larger

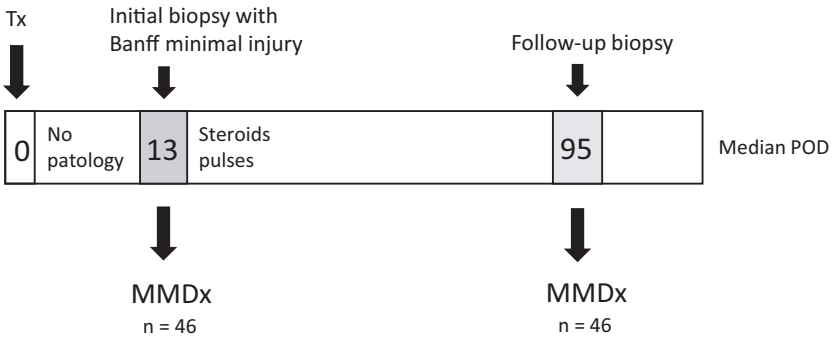

Figure 1 Study design. Early initial biopsies (performed at median 13 postoperative day, POD) with minimal kidney allograft injury defined as tubulitis with or without interstitial inflammation not meeting Banff criteria for TCMR or mixed rejection were identified. All patients in this study were treated by steroid pulses after initial biopsy showing borderline changes according to previous Banff classification valid in time when patients were treated. Follow-up biopsies were performed at median 95 POD. All biopsies were analyzed besides conventional histology also by MMDx, the Molecular Microscope® Diagnostic System.

Table 1. Basic transplant demographics of studied cohort.

n	46
Recipient age, years	51 [25; 81]
Recipient gender, male, *n* (%)	32 (69.6%)
Retransplantation, *n* (%)	0 (0%)
Type of donor, deceased, *n* (%)	34 (73.9%)
Donor age, years	53 [24; 80]
Donor gender, male, *n* (%)	22 (47.8%)
ECD donor, *n* (%)	22 (47.8%)
Dialysis vintage, months	17 [0; 144]
HLA mismatch	3 [0; 6]
Peak PRA	4 [0; 73]
DGF, *n* (%)	29 (63%)
Cold ischemia, h	15 [0; 24]
Original disease, *n* (%)	
Diabetes	5 (10.9%)
Hypertension	8 (17.4%)
Glomerulonephritis	17 (37%)
Polycystic kidney disease	6 (13%)
Other	10 (21.7%)
Creatinine at biopsy (μmol/l)	249 [134; 820]
Immunosuppression	
Tacrolimus	43
Cyclosporine	3
MMF/azathioprine	46
Steroids	43

DGF, delayed graft function; ECD, expanded criteria donor; MMF, mycophenolate mofetil; PRA, panel reactive antibodies.

section of the biopsy core was histologically examined, and the results were interpreted or reinterpreted according to the 2019 Banff criteria [4,16]. A small piece of the biopsy specimen (2–4 mm) cut from the middle of biopsy core was immediately placed in RNA*later*™ (Qiagen, Hilden, Germany) and stored at −80 °C for transcriptomic analysis by MMDx. Retrospectively selected samples with minimal allograft injury changes in early indication biopsies and their follow-up biopsies at 3 M were sent on dry ice to the Alberta Transplant Applied Genomics Centre (ATAGC, University of Alberta, Edmonton, Canada). RNA extraction and gene expression analysis used PrimeView GeneChip arrays (Affymetrix, Santa Clara, CA, USA) and were performed as previously described [2]. Classifiers related to rejection (ABMR, TCMR, and all rejection) or acute kidney injury (AKI), inflammation, and chronic injury (atrophy/fibrosis score) were generated using a published reference set of 1208 biopsy specimens [2,17]. To distinguish between early-stage, fully developed, and late-stage ABMR, archetype analysis group assignment was used in combination with time post-transplant, and classifier scores [17]. Severity of MMDx rejection was determined according to respective TCMR and/or ABMR classifier scores, plus archetype scores and MMDx classifier scores predicting relevant histological lesions [5,18].

Statistical analyses

Continuous data presented as median with minimum/maximum were compared by the Mann–Whitney *U*-test. Paired comparisons were performed using the Wilcoxon signed paired test. Categorical variables were presented as absolute and relative frequencies and compared by Fisher's exact test.

We used receiver operating characteristic (ROC) curves and the calculation of the area under the curve (AUC) to evaluate whether a particular molecular score in the first biopsy predicted the molecular rejection score for the 3 M follow-up biopsy. To determine which variable(s) remained important risk factor(s) after adjustment for other variables, binary logistic regression was performed. Selected molecular scores were re-categorized into binary variables using optimized cut-offs from ROC analysis with highest specificity and sensitivity (Table 2). Because of the limited biopsy numbers, only the four most significant molecular scores from univariate regression analysis (with $P \leq 0.05$) were entered into the multivariate binary regression model. The backward Wald method of binary logistic regression was used to select the variables with the highest contribution to the model. A test of the full model against a constant-only model was statistically significant, indicating that the model reliably identified biopsies with positive rejection scores at the 3 M follow-up biopsy ($\chi^2 = 14.21$, $P = 0.001$ with df = 2). The robustness of the model was validated using 46-fold leave-one-out cross-validation.

For statistical analysis, IBM SPSS STATISTICS 24 (SPSS, Inc., Chicago, IL, USA) and the GRAPHPAD PRISM5 (GraphPad, San Diego, CA, USA) were used.

Results

The 3-month course of histological changes

All indication biopsies performed early after transplantation (median 13 postoperative days) were diagnosed as tubulitis with ($n = 15$) or without ($n = 31$) interstitial inflammation not meeting the criteria for TCMR (Fig. 2) [16]. There were 28/46 biopsies with

Table 2. Prediction of MMDx rejection at 3 months based on MMDx scores in the initial biopsy with minimal allograft injury.

MMDx score in indication BL biopsy	AUC	*P* value	95% Confidence interval	Cut-off	Sensitivity	Specificity
t > 1 probability	0.839	0.002	0.69–0.99	0.075	88.9	70.3
i > 1 probability	0.830	0.002	0.66–1.00	0.095	77.8	59.5
ptc > 0 probability	0.806	0.005	0.68–0.94	0.205	88.9	64.9
TCMR score	0.775	0.011	0.59–0.96	0.015	77.8	59.5
Inflammation score	0.763	0.015	0.59–0.93	2.635	77.8	64.9
ABMR score	0.722	0.040	0.57–0.88	0.095	66.7	70.3
ct > 1 probability	0.718	0.045	0.51–0.93	0.135	66.7	54.1
Rejection score	0.713	0.049	0.53–0.9	0.085	77.8	59.5
AKI score	0.706	0.058	0.51–0.91			
Atrophy–fibrosis score	0.700	0.066	0.48–0.92			
g > 0 probability	0.626	0.245	0.42–0.84			
DSA propability	0.563	0.561	0.35–0.78			
cg > 0 probability	0.389	0.306	0.11–0.67			

ABMR, antibody-mediated rejection; AUC, area under curve; i, interstitial inflammation; ptc, peritubular capillaries; t, tubulitis; TCMR, T-cell-mediated rejection.

For the purpose of this study, the MMDx rejection was defined as both TCMR (cut-off >0.1) and/or ABMR (cut-off >0.2) positive scores.

i0t1 (61%), 12/46 with i1t1 (26%), 1/46 with i1t2 (2%), 3/46 with i0t3 (6%), and 2/46 with i1t3 (4%, Fig. 3).

Histological diagnosis of 3 M follow-up biopsies showed 22/46 no-rejection findings (48%), 4/46 (9%) with borderline suspicious for acute TCMR, and 9/46 (20%) with isolated tubulitis. In 7/46 patients at 3 M (15%), rejection was diagnosed; 3/7 TCMR IB, 1/7 DSA-negative suspicious for chronic active ABMR, and 3/7 chronic TCMR grade II. Three cases at 3 M follow-up were diagnosed with BKV nephropathy and one with pyelonephritis (Fig. 2, Table S1).

In the subset of 28 patients with i0t1 category in the initial biopsy, the 3 M follow-up biopsies showed four borderline suspicious for acute TCMR (14.3%), six isolated tubulitis (21.4%), two TCMR IB (7.1%), one chronic TCMR (3.5%), and one BKVN. In this subset, tubulitis resolved in 15 (54%) patients. Three cases of severe tubulitis without interstitial inflammation (i0t3) were observed in the initial biopsy. In the follow-up 3 M biopsy, chronic ABMR, BKVN, and persistent isolated tubulitis were diagnosed.

In 12 kidneys with Banff i1t1 in the initial biopsy, two (17%) progressed to acute TCMR IB and chronic TCMR at 3 M, respectively, isolated tubulitis (33%) was confirmed in four cases, and BKVN was found in one patient. In three cases of i1t2-3 category in early initial biopsies, acute pyelonephritis, chronic TCMR, and normal findings were found at 3 M.

Individual histology lesions in early initial biopsies with minimal injury did not predict rejection (ABMR and TCMR) in 3 M follow-up biopsies (Table S2).

Molecular phenotypes of minimal allograft injury in early indication biopsies

Early indication biopsies with minimal allograft injury ($N = 46$) were interpreted by MMDx in 34/46 (74%) cases as no-rejection (NR), in 4/46 cases as T-cell-mediated rejection (TCMR, 9%), in 6/46 cases as antibody-mediated rejection (ABMR, 13%), and in 2/46 (4%) cases as mixed rejection (Fig. 2).

All four patients with TCMR by MMDx also displayed moderate/extensive MMDx scores reflecting inflammation and AKI, minimal/moderate scores predicting atrophy–fibrosis, high signals of probability of tubulitis and interstitial inflammation.

All six MMDx ABMR patients had molecular ABMR classifier scores slightly above the threshold for ABMR positivity (median 0.27 with threshold >0.2), and all six displayed elevated MMDx scores reflecting glomerulitis (g > 0, median 0.42 with threshold 0.2), peritubular capillaritis (ptc > 0, median 0.56 with threshold >0.23), and DSA (median 0.53 with threshold >0.35), indicating MMDx mild early ABMR. One MMDx ABMR patient additionally demonstrated a higher MMDx score reflecting interstitial inflammation (i > 1), tubulitis (t > 1), and tubular atrophy (ct > 1; Table S1).

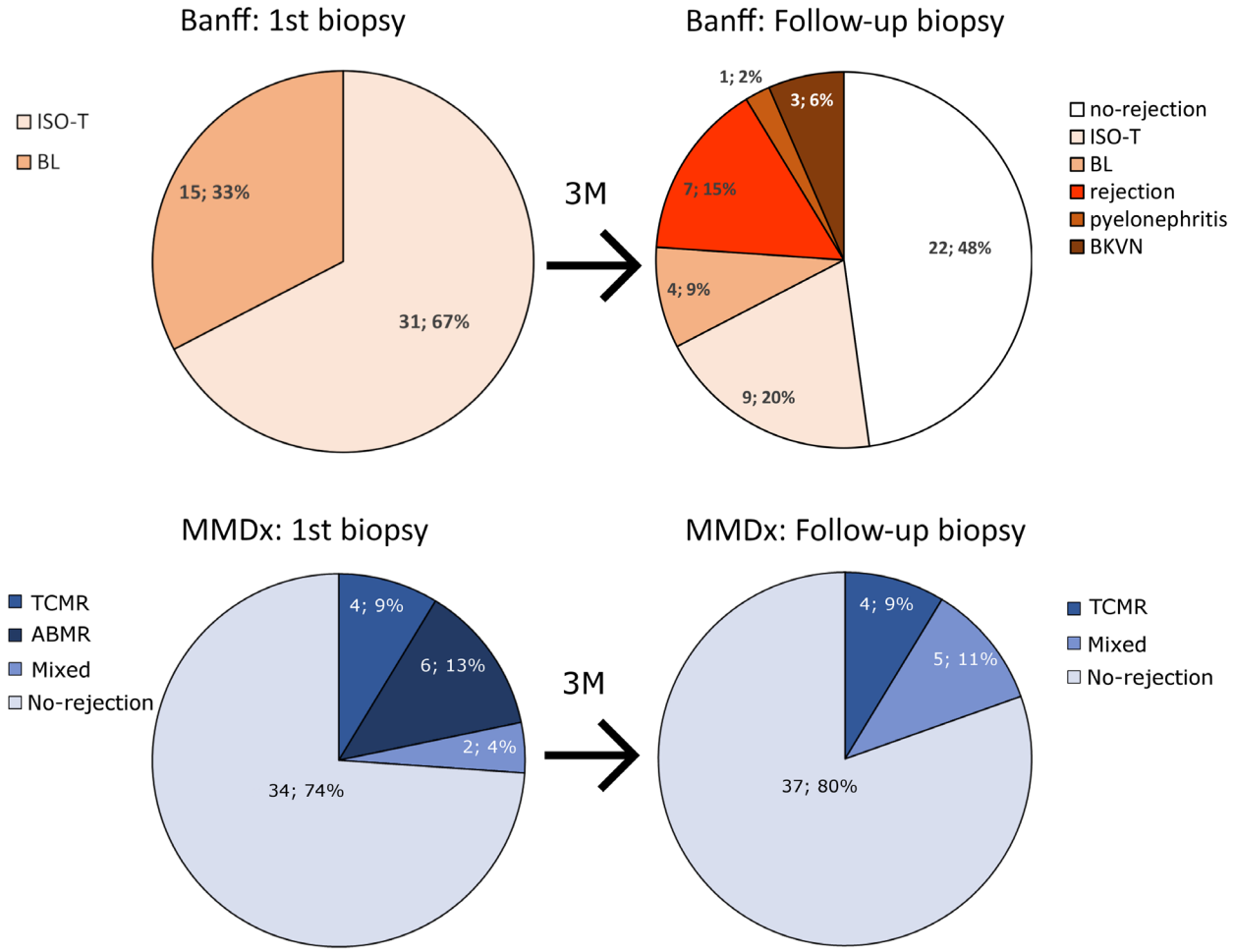

Figure 2 Histological (Banff) and MMDx results of early indication biopsies and their respective follow-up 3-month protocol biopsies.

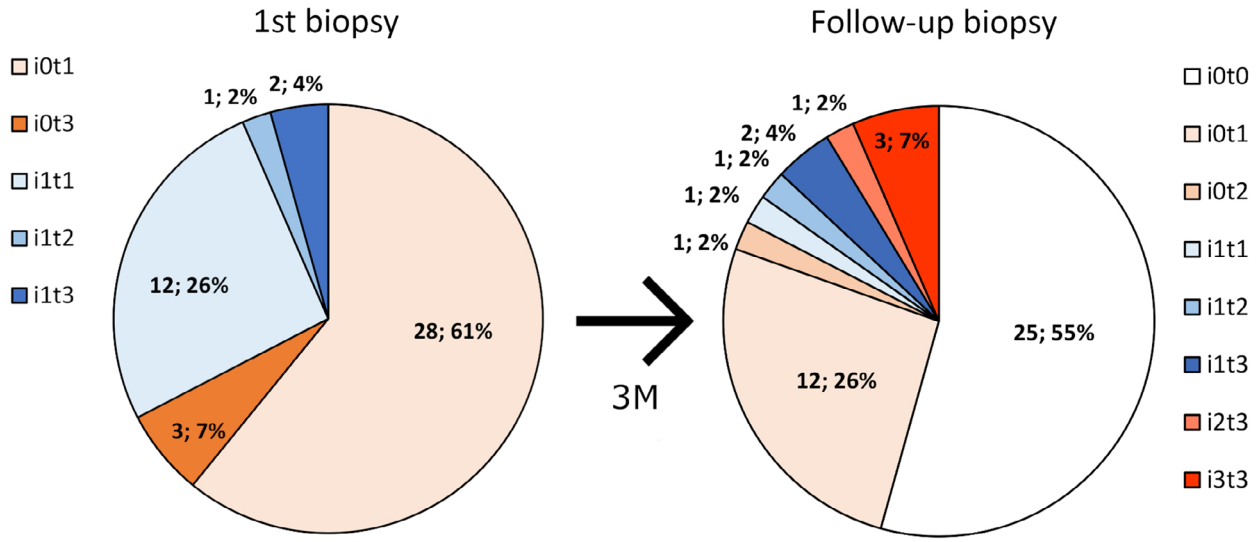

Figure 3 Development of early allograft minimal injury from early initial biopsy to 3 M follow-up biopsy.

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In two patients with mixed severe TCMR and possible early ABMR by MMDx high scores for molecular glomerulitis, peritubular capillaritis, DSA, severe interstitial inflammation and tubulitis were noted.

Molecular phenotypes of follow-up biopsies

MMDx classified 37/46 (80%) 3 M follow-up biopsies as NR and 9/46 (20%) as rejection (ABMR, TCMR, or Mixed). In the nine biopsies called rejection by MMDx, four had positive TCMR scores and five showed positive TCMR and ABMR scores (Fig. 2, Table S1).

Positive MMDx TCMR scores were recorded at 3 M in three out of six patients who had MMDx TCMR in their early initial biopsy (P29, P30, and P36, Fig. 3). Moderate TCMR persisted to 3 M follow-up biopsy in two patients (P30, P29) without improvement of graft function (Fig. 3). In one patient (P36), mild TCMR accompanied by extensive inflammation and moderate AKI was persistent until 3 M despite improved kidney graft function (creatinine 421–164 μmol/l). In a single patient (P23), the TCMR score decreased significantly but remained slightly above the cut-off (0.1).

A majority of mild early ABMR scores (P9, P4, P6, P14, P31, P39) resolved completely by 3 M, reflecting creatinine decrease. A single case (P22, graft from ECD) of early ABMR with extensive inflammation, AKI, and atrophy–fibrosis scores progressed to 3 M, showing combined severe TCMR with possible ABMR and with long-standing poor renal function (creatinine >300 μmol/l) and graft failure as early as 11 months post-transplant (Fig. 4).

At 3 M follow-up, three patients developed MMDx-defined mixed rejection (P5, P20, and P38) and one patient (P27) developed MMDx-defined mild TCMR with moderate AKI and extensive fibrosis (Fig. 4).

The TCMR and ABMR MMDx scores increased in two patients (P20, P38). Reduction or minimization of immunosuppression was found in one patient's chart (P20). In this patient, mild inflammation and moderate AKI despite absent significant rejection molecular scores progressed to severe TCMR along with moderate early ABMR. Kidney graft function deteriorated in this patient (creatinine 181–215 μmol/l). The other patient (P38) with negative MMDx TCMR and ABMR scores in the first biopsy progressed to severe MMDx ABMR with glomerulitis, transplant glomerulopathy (cg), and ptc-related molecular features in combination with moderate TCMR with worsening of graft function by 3 M (creatinine 134–185 μmol/l). Interestingly, this patient showed mild positive cg, ptc, and DSA molecular scores in the initial biopsy. All details describing individual courses can be found in Table S1.

The AKI score decreased ($P = 0.0011$) and atrophy–fibrosis score increased ($P = 0.0015$) between all initial and 3 M follow-up biopsies (Fig. 5), but this may simply be the natural history of AKI and atrophy–fibrosis changes reflecting the injury sustained during the transplantation process. A trending decrease in inflammation score was also observed ($P = 0.113$). In initial biopsies, AKI and inflammation scores were high (> MMDx threshold), while atrophy–fibrosis score was low (<MMDx threshold). However, rejection scores (overall rejection, TCMR, and ABMR score) in 3 M follow-up biopsies were similar to those in the initial biopsies.

When we calculated the number of biopsies with positive MMDx scores (>threshold), the majority of initial biopsies had AKI molecular score (66.7%) and inflammation molecular score (72.6%), while only 7.8% had atrophy–fibrosis molecular scores. In comparison with the first biopsy, 3 M follow-up biopsies had less inflammation molecular score (36.9%, $P < 0.001$) and acute kidney injury molecular score 23.9% ($P < 0.001$) while the number of biopsies positive for atrophy–fibrosis molecular scores increased only nonsignificantly (19.6%, $P = 0.231$; Fig. 6).

Identification of progression phenotype by MMDx

We compared the molecular scores in initial biopsies between patients with positive and negative MMDx rejection at 3 M follow-up to assess whether the initial MMDx results predicted a positive rejection score (TCMR or ABMR) at 3 M. ROC analysis was performed for all molecular scores in the first biopsy, Table 2. The best MMDx predictor of rejection molecular score at follow-up biopsy was tubulitis >1 in the first biopsy (AUC = 0.839, $P = 0.002$, sensitivity 88.9%, and specificity 70.3% at cut-off 0.075).

To select the most significant predictor of MMDx rejection at 3 M, the four most significant molecular scores from univariate binary logistic regression (inflammation score, ABMR score, ptc probability >1, and t probability >1) were entered into a multivariate model. The backward Wald binary logistic model retained the t > 1 classifier score as only significant predictor (OR = 15.9; $P = 0.016$), while adjusting for higher inflammation score (Table 3). The final model had 77.8% sensitivity and 89.2% specificity for the prediction of MMDx rejection (ABMR or TCMR). Leave-one-out 46-fold cross-validation confirmed the predictive power of the model with identical specificity and sensitivity.

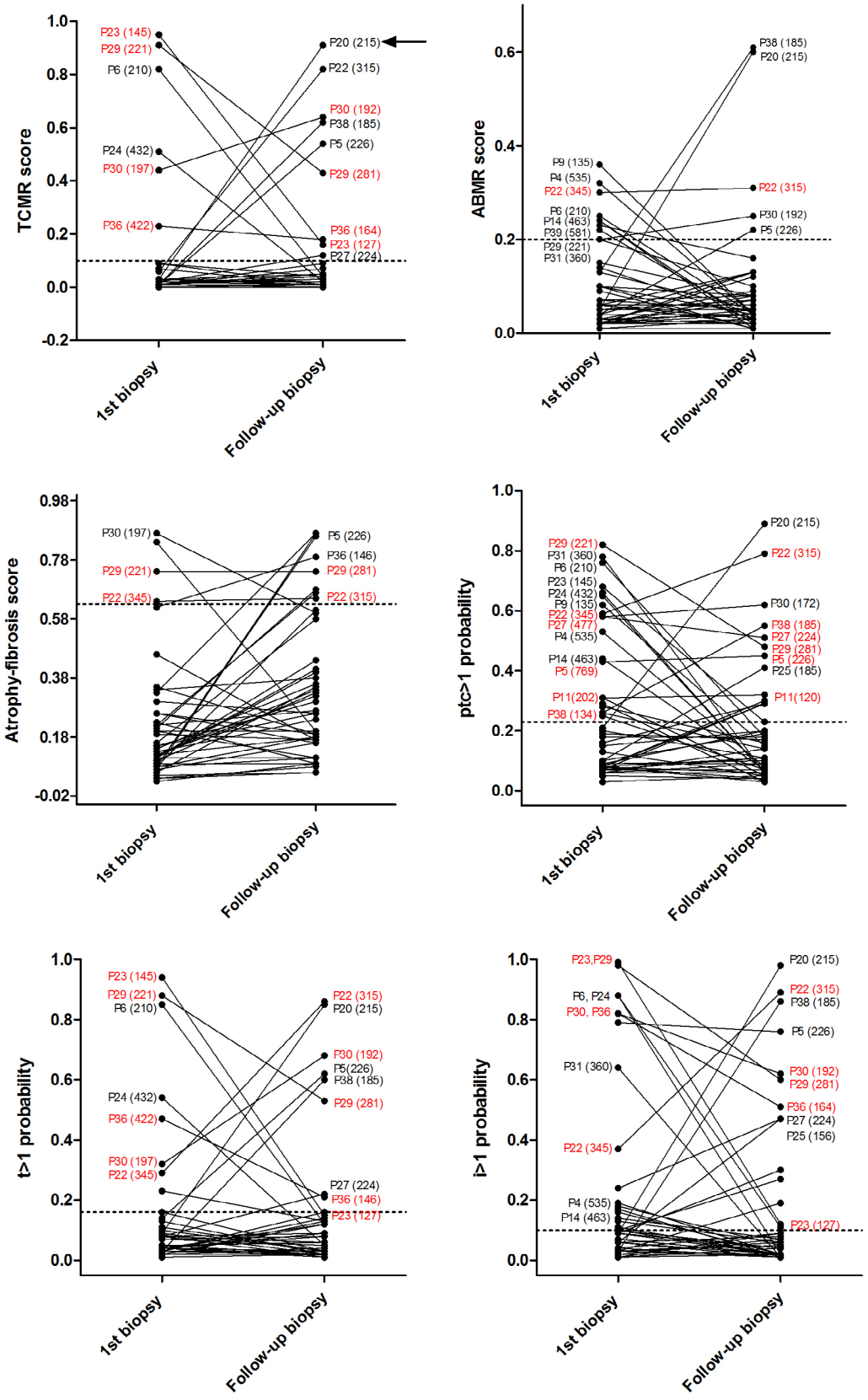

Figure 4 Development of TCMR, ABMR, atrophy–fibrosis, ptc > 1, i > 1, and t > 1 probability MMDx score from the 1st indication biopsy to the following 3 months biopsy. Patients with scores higher than cut-off suffering from any time of rejection are identified by patients' numbers and serum creatinine (μmol/l) at the time of biopsy is stated in parentheses. Noncompliant patient is indicated by an arrow. The cases with scores above the designated tresholds on both biopsies are in red.

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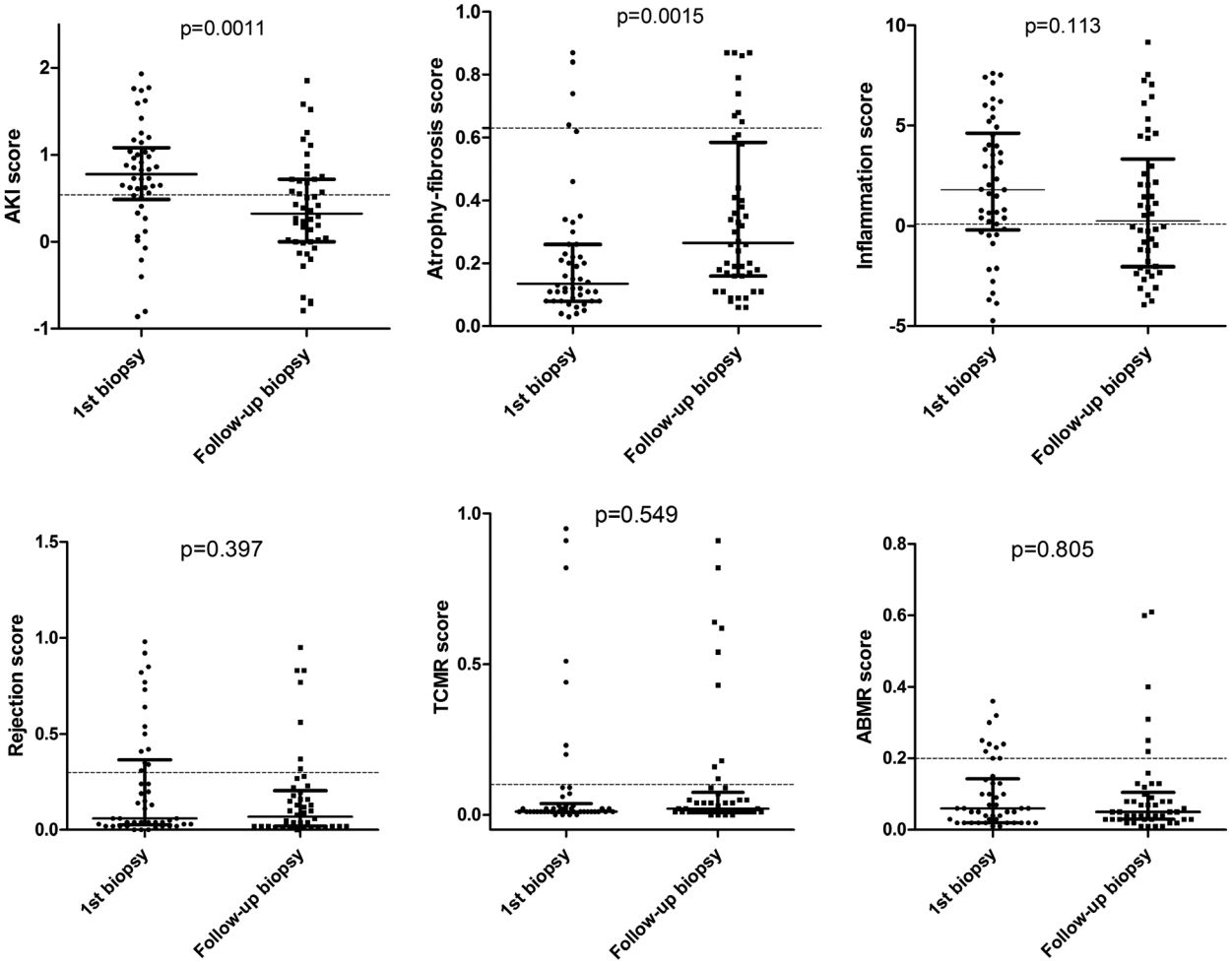

Figure 5 Developments of MMDx molecular scores at 3 months follow-up (n = 46). Groups were compared by Wilcoxon paired signed rank test. Data are presented as median and interquartile range. Dash line: MMDx threshold for particular scores.

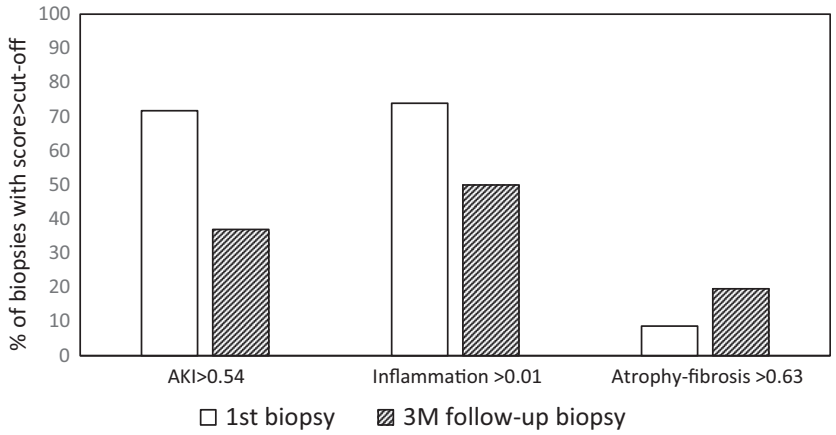

Figure 6 The number of biopsies with positive (>threshold) AKI, inflammation, and atrophy–fibrosis scores in the early initial and 3 months follow-up biopsy.

Similarly, we analyzed prediction of Banff-defined rejection in the follow-up biopsy (n = 7) based on molecular scores in initial biopsy. Only molecular rejection score predicted Banff rejection in the follow-up biopsy (AUC = 0.742; P = 0.044, Table S3). When prediction was performed for Banff rejection including

Table 3. Prediction of MMDx rejection (defined by MMDx score for either TCMR or ABMR) at 3 months based on MMDx scores from indication biopsies in univariate and multivariate binary logistic regression model.

	Univariate analysis			Multivariate analysis		
	P value	OR	95% CI	*P* value	OR	95% CI
t > 1 probability	0.009	18.91	2.1–169.8	0.016	15.9	1.7–150.1
ptc > 1 probability	0.016	14.8	1.66–131.4			
Inflammation score	0.032	6.46	1.17–35.7	0.093	4.97	0.77–32.3
ABMR score	0.050	4.73	0.99–22.4			
i > 1 probability	0.060	5.13	0.94–28.18			
TCMR score	0.060	5.13	0.94–28.18			
Rejection score	0.072	8.52	0.83–87.9			

category of borderline suspicious for rejection (Banff 2019, excluding isolated tubulitis), Banff rejection was predicted by higher molecular atrophy–fibrosis (AUC = 0.788, $P = 0.004$), TCMR (AUC = 0.742, $P = 0.017$), and ct scores (AUC = 0.726, $P = 0.025$, Table S4). The correlation between Banff and molecular scores in all 92 analyzed biopsies is given in Table S5.

Five of 12 patients with positive MMDx TCMR and/or ABMR score on the initial biopsy displayed rejection molecular scores also in the follow-up biopsies. These patients had significantly higher atrophy–fibrosis score (and tubular atrophy >1 probability) in the first biopsy, and according to ROC analysis, higher atrophy–fibrosis predicted persistence of rejection molecular scores with high probability (AUC = 0.957, with cut-off >0.24 at 100% sensitivity and 71.4% specificity; $P = 0.009$; Fig. S1).

In 4/34 patients with negative ABMR and/or TCMR rejection scores on the initial biopsy, a higher ptc > 1 probability predicted MMDx rejection at 3 M follow-up (AUC = 0.90; sensitivity 100% and specificity 80% at cut-off = 0.20; $P = 0.011$; Fig. S1).

Discussion

Molecular assessment of kidney allografts using the innovative MMDx platform presents an opportunity to improve post-transplant diagnostics and to re-examine histological findings of minimal injury which does not meet criteria for TCMR. Pathologists have long known that low level tubulitis is common and nonspecific in many renal diseases, but clinicians have been worried that it represents mild or early stage TCMR and treat them with steroids as rejection.

In these analyses, we evaluated molecular profiles of 46 early indication biopsy with minimal kidney allograft injury and their corresponding follow-up biopsies at 3 months. We found that early minimal allograft injury represents a heterogeneous cohort according to MMDx evaluation but is mostly nonrejection. In 30/46 patients (65%), MMDx rejection scores were below the cut-offs for ABMR and/or TCMR scores and remained negative in the 3-month biopsies. Of note, both TCMR and ABMR molecular rejection scores increased substantially in four patients; one of these patients was found to be nonadherent. The rejection score decreased in 9/46 patients at 3 months (three initially positive TCMR scores and six initially positive ABMR scores, respectively). Interestingly, in 5/12 patients with initially positive MMDx rejection scores (TCMR and/or ABMR), persistence of MMDx signals of rejection at follow-up biopsy was noticed.

Early features of ABMR by MMDx resolved in 5/6 cases in 3-month follow-up biopsy. This resolution was not associated with pretransplant DSA. Of note, molecular ABMR scores in early indication biopsies were only slightly above the cut-off for positivity and therefore steroid pulses were probably sufficient to eliminate such signals. Early ABMR archetype was previously found in 12% of borderline changes [17]. Nevertheless, it was previously shown that graft failure is associated with acute kidney injury rather than ABMR rejection scores [19]. Despite similar short-term outcomes, patients with persistent MMDx rejection scores at follow-up may be at risk if their biopsies continue to have smoldering inflammation. However, whether persistence of inflammation has clinical consequences remains a question: if most minimal injury is nonspecific, then it has no long-term consequence. Moreover, the number of patients in this cohort was too small to observe a reasonable number of graft losses over long-term follow-up.

By comparison, MMDx scores increased the understanding of the consequences of peri-transplant injury. Paired comparison of MMDx scores in first and follow-

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up biopsies shows that MMDx AKI scores decreased in most patients while the tubular atrophy–fibrosis scores significantly increased. This observation demonstrates that ischemic and other peri-transplant injuries affect IFTA development as early as in 3 M post-transplantation.

Our results found that minimal allograft injury is seldom related to TCMR, and 74% of them had a nonrejection phenotype in early indication biopsies corresponding to previous findings diagnosed at a later time post-transplant [2,3,10]. A transcriptomic approach using MMDx revealed in histologically classified minimal injury various diagnoses including no-rejection, phenotypes, or severe AKI associated with longer ischemia and higher donor age but not associated with rejection.

Our results indicate that the clinical outcomes after early minimal histological injury, that is present tubulitis with or without interstitial inflammation, are heterogeneous reflecting the diverse underlying processes. In works of others, only 28% of untreated borderline changes ($N = 65$) progressed to acute rejection within 40 days postbiopsy [20], renal function at 1 and 2 years and graft survival were similar in patients with either treated ($N = 49$) or untreated borderline changes ($N = 42$) [21]. Recent study showed that clinical outcomes of borderline changes range from rejection to nonspecific inflammation [22].

In our study, no Banff histological score in the first indication biopsies predicted rejection in the 3 M follow-up biopsy. It is likely that transcriptomic tools to predict clinical outcomes may better describe underlying rejection-associated processes. A prior study measured the expression of several rejection-associated transcripts and found lower expression of *FOXP3* in untreated borderline changes with deteriorating renal function at 40 days postbiopsy [23]. Our previous study using a microarray approach found increased expression of transcripts associated with immunity, inflammation, and fibrosis in borderline changes in early indication biopsies with further progression of graft dysfunction [6]. In the current study, the MMDx-defined progressive phenotype was predicted by a higher MMDx tubulitis classifier score on the initial biopsy in a model adjusted for inflammation score. Nevertheless, this finding is limited by low numbers of patients who progressed to rejection in the follow-up biopsy. Contrary to previous studies, the progression was defined by occurrence of MMDx rejection scores at 3 M follow-up biopsies. So far, the transcriptomic approach has been used for prediction of future outcomes, defined by renal function [6], graft loss [24], or histopathological diagnosis of rejection [25,26]. In sequential biopsy series, only Banff histopathological scores were compared [12,27].

Our findings indicate that MMDx has increased sensitivity for identifying ptc-related changes on a molecular level before any visible changes are seen by conventional microscopy. In 4/34 patients (11.8%) with negative rejection scores on the first biopsy and positive rejection scores at follow-up, higher peritubular capillaritis scores were found in the indication biopsy. A minimal number of indication biopsies displayed Banff peritubular capillaritis score higher than 1 (in our cohort 1 out of 46), while an MMDx ptc molecular score higher than threshold was found in 20 (43%) patients.

Finally, our analysis of persistent MMDx rejection scores indicates a relationship to atrophy–fibrosis. In 5/12 patients with positive MMDx rejection scores in the first biopsy (42%), the MMDx rejection scores did not drop under the threshold at follow-up biopsy. Previously detected intimal arteritis, severe tubulitis [28], or more extensive peritubular capillaritis leukocyte infiltration [29] were associated with decreased therapy responsiveness. In our patients classified by MMDx in the first biopsy as rejection, higher atrophy–fibrosis score predicted persistence of rejection scores. Of note, a higher atrophy–fibrosis score often reflects lower kidney graft quality originating from ECDs.

In conclusion, sequential MMDx evaluation of early indication biopsies with histological minimal injury and their follow-up biopsies showed that molecular rejection phenotype persisted in some of them despite given steroids to all patients. Molecular tubulitis score at initial biopsy has the potential to discriminate patients at risk for molecular scores for rejection at follow-up biopsy. Longitudinal studies are, however, needed to show whether MMDx or other molecular classifiers along with important clinical variables outperform recent prediction tools for graft outcomes such as iBox which are based on conventional histology [30].

Authorship

PH: involved in research design, the performance of the research, data analysis, and writing the paper. KMT: involved in performance of the research and writing the paper. MM, JM, LV and JS: involved in the performance of the research. PFH: involved in research design and writing of the paper. OV: involved in research design, the performance of the research, and writing of the paper.

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Conflicts of interest

P F Halloran holds shares in Transcriptome Sciences Inc., a University of Alberta research company with an interest in molecular diagnostics; has given lectures for Thermo Fisher, and is a consultant for CSL Behring. The other authors of this manuscript have no conflict of interest to disclose.

SUPPORTING INFORMATION

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

Figure S1. Prediction of MMDx rejection at 3 M based on MMDx scores of initial biopsy A/higher atrophy–fibrosis score in initial biopsy predicted MMDx rejection at M3 in 5/12 patients in which MMDx recorded rejection already in the first biopsy B/higher ptc probability score in initial biopsy predicted MMDx rejection at M3 in 4/34 with negative TCMR and/or ABMR score in initial biopsy.

Table S1. Detailed description of individual Banff histological scores, MMDx scores, and creatinine for both early initial and follow-up biopsies in each patient.

Table S2. Prediction of Banff histological 3-month subclinical rejection (chronic active ABMR, TCMR, and chronic active TCMR) on the basis of Banff histological scores in the early initial biopsy.

Table S3. Prediction of Banff-defined rejection at 3 months based on MMDx scores in the initial biopsy with minimal allograft injury.

Table S4. Prediction of Banff-defined rejection including category of borderline suspicious for rejection (Banff 2019, excluding isolated tubulitis) at 3 months based on MMDx scores in the initial biopsy with minimal allograft injury.

Table S5. Correlation of Banff histological scores with molecular scores for all 96 analyzed biopsies (Spearman rank correlation).

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