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Transcriptomic Signatures of Antibody-mediated Rejection in Early Biopsies With Negative Histology in HLA-incompatible Kidney Transplantation

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Background. Presensitized patients with circulating donor-specific antibodies (DSAs) before transplantation are at risk for antibody-mediated rejection (AMR). Peritransplant desensitization mitigates but does not eliminate the alloimmune response. We examined the possibility that subthreshold AMR activity undetected by histology could be operating in some early biopsies. **Methods.** Transcriptome of kidney allograft biopsies performed within the first month in presensitized patients (DSA^+) who had received desensitization and did not develop active/probable AMR by histology (R^-) was compared with biopsies showing active/probable AMR (R^+/DSA^+). As negative controls, biopsies without rejection by histology in patients without DSA at transplantation were used (R^-/DSA^-). RNA sequencing from biopsies selected from the biobank was used in cohort 1 (n = 32) and microarray, including the molecular microscope (Molecular Microscope Diagnostic System [MMDx]) algorithm, in recent cohort 2 (n = 30). **Results.** The transcriptome of R^-/DSA^+ was similar to R^+/DSA^+ as these groups differed in 14 transcripts only. Contrarily, large differences were found between both DSA^+ groups and negative controls. Fast gene set enrichment analyses showed upregulation of the immune system in both DSA^+ groups (gene ontology terms: adaptive immune response, humoral immune response, antigen receptor-mediated signaling, and B-cell receptor signaling or complement activation) when compared with negative controls. MMDx assessment in cohort 2 classified 50% of R^-/DSA^+ samples as AMR and found no differences in AMR molecular scores between R^+ and R^- DSA^+ groups. In imlifidase desensitization, MMDx series showed a gradual increase in AMR scores over time. **Conclusions.** Presensitized kidney transplant recipients exhibited frequent molecular calls of AMR in biopsy-based transcript diagnostics despite desensitization therapy and negative histology.

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Presensitized kidney transplant recipients with donor-specific anti-HLA antibodies (DSAs) are at high risk of antibody-mediated rejection (AMR) that significantly affects graft outcome.[1,2] Therefore, peritransplant desensitization in HLA-incompatible transplantation has been implemented in induction protocols to decrease levels of circulating DSA

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and to overcome the HLA antibody barrier.[3] Interestingly, despite desensitization, protocol biopsies show the presence of subclinical AMR in 31% of cases after HLA-incompatible transplantation.[4] However, one-third of DSA$^+$ patients do not exhibit rejection in conventional histology,[5] which suggests that more precise diagnostics might be informative.

Biopsy-based transcript diagnostics of kidney allografts have been suggested as early as in 2013 by the Banff classification[6] to overcome the inaccuracy of histological evaluation in AMR and to predict graft prognosis. Early biopsies were not evaluated in previous studies in DSA$^+$ patients, which suggested no differences in kidney graft tissue gene expression between patients with and without AMR in histology.[5,7,8] Therefore, data from biopsy-based transcript diagnostics performed early, just after applied depletive induction and desensitization in DSA$^+$ patients, are lacking. New potent desensitization protocols were recently implemented[9,10] and emerging therapies targeting plasma cells and natural killer cells in AMR have been tested.[11,12] Rapid detection of molecular AMR despite negative histology may thus help clinicians to adapt therapy to prevent refractory graft injury.

In this retrospective cohort study in HLA-incompatible transplantation, we assessed transcriptomic profiles of biopsies performed early after desensitization and showed that biopsies in patients with DSA exhibited immune cell activation and molecular calls of AMR regardless of the presence or absence of AMR defined by histology.

MATERIALS AND METHODS

Groups Definition

In this retrospective single-center cohort study, we assessed biopsy-based transcripts in presensitized patients who received kidney allografts from HLA-incompatible donors in 2008–2024, received peritransplant desensitization, and underwent a graft biopsy within the first month after transplantation. Biopsies with no rejection finding by histology (R$^-$/DSA$^+$) were compared with biopsies showing active/probable AMR defined by the current Banff 2022[13] (R$^+$/DSA$^+$). As negative controls, biopsies were used without rejection by histology performed within the identical time frame in patients without DSAs at transplantation (R$^-$/DSA$^-$). Biopsies without rejection by histology revealed neither signs of active T cell–mediated rejection or AMR, nor microvascular inflammation (MVI; Banff glomerulitis+peritubular capillaritis [g+ptc] = 0) and significant C4d deposition (C4d ≤ 1 by immunofluorescence). Contrarily, the R$^+$/DSA$^+$ group formed presensitized patients with DSAs and the histological presence of active AMR or probable AMR. Later was defined by the presence of significant C4d staining (C4d ≥ 2) without MVI or absent C4d staining (C4d = 0) with the presence of mild MVI = 1.[13] The Banff scores for individual lesions for both cohorts are given in **Table S1** (**SDC,** http://links.lww.com/TXD/A723). Probable AMR histological diagnosis comprised 50% of cases (8/16) of rejections in cohort 1, whereas 40% of cases (2/5) in cohort 2, respectively. The cutoff for the presence of DSA was set as mean fluorescence intensity (MFI) of >2000. To improve the robustness of the study, we analyzed 2 different cohorts (cohorts 1 and 2) identically defined but assessed by different platforms, RNA sequencing (RNAseq) and microarray. The differential expression analyses were used in both cohorts. Besides that, the molecular microscope algorithm was used to generate individual MMDx reports in cohort 2 assessed by microarray. The study design is shown in **Figure S1** (**SDC,** http://links.lww.com/TXD/A723). The description of sensitization and given induction immunosuppression for both cohorts is detailed in **Table S2** (**SDC,** http://links.lww.com/TXD/A723). Notably, 5 patients in the second cohort received imlifidase-based desensitization protocol: 3 in the R$^-$/DSA$^+$ group and 2 in the R$^+$/DSA$^+$ group.

All patients received triple-drug maintenance immunosuppression based on tacrolimus (target trough levels within the first 14 days at 8–15 ng/mL), mycophenolate mofetil (2 g/d) or mycophenolic acid (1440 mg/d), and tapered prednisone (20 mg initially).

The ethics committee of the Institute for Clinical and Experimental Medicine approved the study protocol (approval No. A13-02-01), with all patients giving their informed consent to participate in the study.

Cohort 1

Biopsy specimens for RNA sequencing were selected from prospectively collected biobanks in patients with known pretransplant DSA status if samples exhibited sufficient RNA quality and biopsies were performed within the first month. Patients' demographics are given in Table 1 (cohort 1, n = 32). All but 1 patient in the R$^-$/DSA$^+$ group (n = 8) received kidney allografts from deceased donors. Patients in the R$^+$/DSA$^+$ group (n = 16) experienced more previous transplantations, had the highest calculated panel-reactive antibody ($P < 0.001$), and remained longer on dialysis before transplantation ($P = 0.004$; Table 1).

Cohort 2

Biopsies for microarray were selected from 257 ones with available MMDx reports. Seventy-seven MMDx assessments were available in biopsies performed within the first month and 30 from them were included in the study, 10 in R$^-$/DSA$^+$, 5 in R$^+$/DSA$^+$, and 15 in R$^-$/DSA$^-$ groups, respectively. MMDx assessments used for routine diagnostics were performed either in Prague (since 2022, n = 29) or in Edmonton (before 2022, n = 1). Transplant demographics did not differ among groups (Table 1).

Biopsies

Renal biopsies were obtained using a 16-G biopsy needle under percutaneous ultrasound guidance. The majority of the biopsy core was used for histological examination. A small piece of the biopsy specimen (2–4 mm) cut from the middle of the biopsy core was immediately placed in RNAlater, kept at 5 °C for 24 h, and stored at −80 °C in the institutional biobank for transcriptomic analysis. Since 2022, when MMDx assessment has become routine in the center, another core biopsy was kept in RNAlater. Histological assessment of all biopsies was classified or reclassified according to the latest Banff report.[13]

Libraries Preparation and RNA Sequencing

After sample homogenization in 0.5 mL of Trizol reagent (Thermo Fisher Scientific, Carlsbad, CA), total RNA was extracted using phenol-chloroform and purified using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA concentration was measured by Qubit fluorometer, and RNA quality was checked by measuring RNA integrity number using Agilent Bioanalyzer 2100. From 400 ng of total RNA, mRNA

TABLE 1.

Transplant demographics

Demographics	R⁻/DSA⁺	R⁺/DSA⁺	R⁻/DSA⁻	*P*
Cohort 1				
n	8	16	8	
Recipient age, y	48 (35–60)	55 (44–60)	47 (32–52)	0.147
Recipient sex, male	3 (38%)	8 (50%)	6 (75%)	0.304
Donor age, y	51 (50–58)	52 (40–58)	58 (54–62)	0.184
Transplantation 1st/2nd/3rd/4th	6/1/0/1	3/7/5/1	8/0/0/0	0.005
cPRA	64.8 (13–88)	92.7 (79–99)	0 (0–0)	<0.001
HLA mismatch	4 (4–4.8)	4 (2.2–5)	3 (3–3.8)	0.098
Dialysis vintage, mo	41 (12–106)	61 (27–84)	12 (8–20)	0.004
Cold ischemia, h	16 (2–19)	15 (14–17)	16 (13–19)	0.965
Biopsy POD	7 (7–7)	9.5 (8–12)	7.5 (7–11)	0.025
Cohort 2				
n	10	5	15	
Recipient age, y	51 (37–63)	52 (48–57)	42 (32–62)	0.567
Recipient sex, male	3 (30%)	3 (60%)	7 (47%)	0.507
Donor age, y	44 (38–66)	48 (32–64)	49 (47–63)	0.595
Transplantation 1st/2nd/3rd/4th/6th	4/4/1/1/0	1/1/2/0/1	10/2/2/1/0	0.186
cPRA	94 (0–100)	86 (49.5–94)	0 (0–98)	0.208
HLA mismatch	4 (2.8–4.3)	3 (1–4.5)	3 (2–3)	0.124
Dialysis vintage, mo	47 (22–76)	66 (36–156)	23 (10–50)	0.111
Cold ischemia, h	14.5 (12.1–19.9)	17.6 (12.3–22.9)	14.1 (10.6–16.2)	0.19
Biopsy POD	7 (5–11)	18.0 (12–25)	7 (6–10)	0.052

Continuous variables are presented as medians with interquartile range.
cPRA, calculated panel-reactive antibody; POD, postoperative day.

was isolated using poly (A) magnetic selection NEBNext Poly (A) mRNA magnetic isolation module (New England, BioLabs, Inc). Transcriptome libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep with Sample Purification Beads according to the manufacturer's protocol (New England, BioLabs, Inc). In brief, mRNA was randomly fragmented by heat digestion in the presence of a divalent metal cation ($Mg2^+$). Sheared RNA was reversibly transcribed, making first strand of cDNA using random hexamers as primers and reverse transcriptase. The second strand was created using dUTPs, purified with NEBNext Sample Purification Beads, and ligated with diluted NEBNext adapters. After the removal of the second strand by uracil-DNA-dependent glycosylase, the final amplification of adaptor-ligated DNA was done using NEBNext Multiplex Oligos for Illumina. Library was purified with NEBNext Sample Purification Beads and its quality was assessed on a Bioanalyzer 2100 using the Agilent DNA 1000 assay. Libraries from all 32 samples were pooled to a final concentration of 35 nmol, and the pooling quality was assessed by sequencing using MiSeq. High throughput sequencing of the final pool was performed using the NovaSeq6000 S4 system (Illumina) with the following instrument settings: paired end, 150 b, 300–400 million reads per lane. In total, 1 076 758 297 paired-end 150 b reads were generated. Raw data were automatically processed by the Basespace cloud interface (Illumina) in default settings. The base-calling, adapter clipping, and quality filtering were carried out using bcl2fastq version 2.20.0.422 Conversion Software (Illumina).

RNA Sequencing Data Analysis Protocol

The quality of raw reads was evaluated using FastQC (version 0.11.8) and MultiQC (version 1.7). Clipping adaptor sequences were carried out using cutadapt (version 1.18). The trimmed reads were aligned to the human transcriptome reference (GRCh38) using bowtie2 (version 2.3.4.3). The alignments were evaluated using qualimap2 (version 2.2.2). The counts of reads mapped to the reference were extracted and used for differential gene expression analysis using SAMtools (version 1.9). The differential gene expression analysis was performed using the DESEq2 package in R (version 4.2.2). For differential expression analysis, only transcripts with a sum of read in all samples of >50 and a maximum number of reads of >10 were considered. The differentially expressed transcripts had to meet the condition of having nonzero expression in >5 samples of just compared groups in comparison of R⁻/DSA⁺ (n = 8) versus R⁻/DSA⁻ (n = 8) and >9 samples of just compared groups in R⁺/DSA⁺ (n = 16) versus R⁻/DSA⁺ (n = 8) and AMR (n = 16) versus R⁻/DSA⁻ (n = 8). The transcripts with log fold change >1 or <–1 and with *P* values of <0.05 were considered as significantly differentially expressed. As the number of differentially expressed genes between R⁻/DSA⁺ and R⁺/DSA⁺ was small, the lists ranked according to t-statistic from the "fsgea" package in R were used for gene annotation. Upset plot of shared gene ontology (GO) terms among particular group comparisons was constructed using the UpsetR library.[14] Complete raw and normalized data were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and can be accessed using the GEO Series accession number GSE276593.

Microarray Sample Processing and Data Analysis

RNA was extracted from biopsy tissues using a TRIzol-chloroform method,[15] RNA concentration was measured using a Nanodrop 2000 and RNA quality was assessed by the Agilent 2100 Bioanalyzer. RNA was labeled using the Thermo

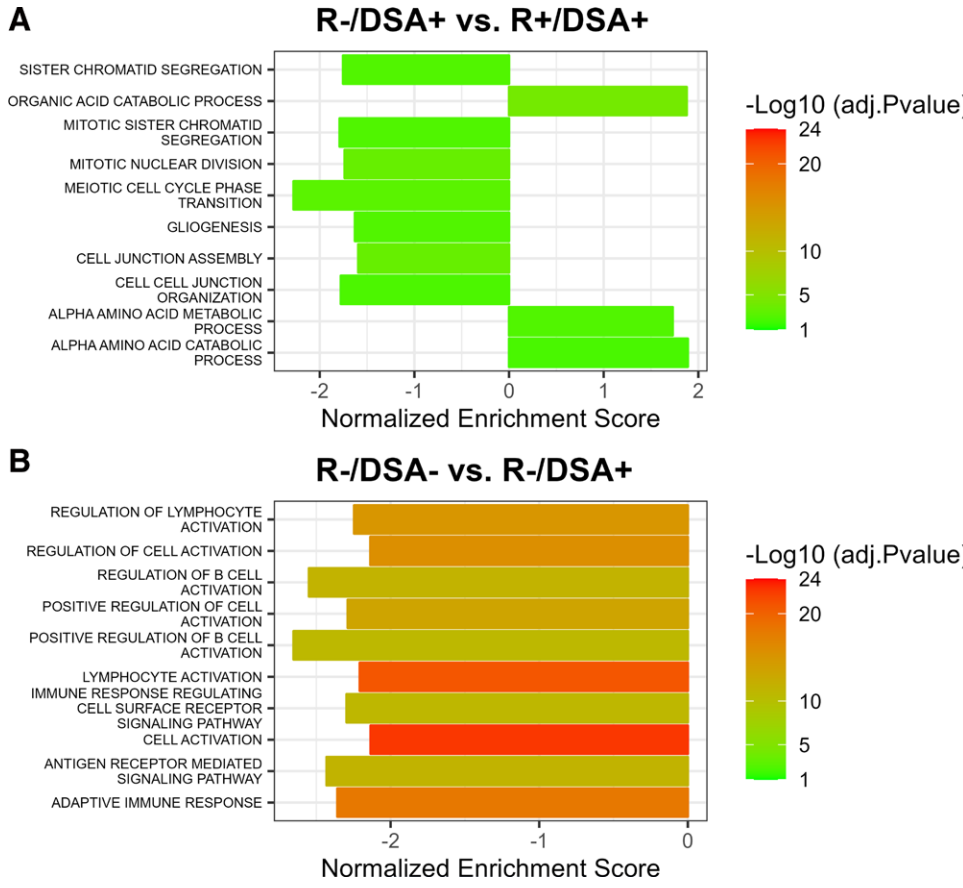

FIGURE 1. Significantly enriched GO terms in differentially expressed genes between R^-/DSA^+ vs R^-/DSA^+ (A) and R^-/DSA^- vs R^-/DSA^+ (B) in cohort 1. Fast gene set enrichment analysis was performed using the list of preranked transcripts based on the results of the t-statistic. The 10 top increased GO terms are displayed by bar plots where the color reflects the increasing adjusted *P* value from red to green. The top 10 increased GO terms ranked according to adjusted *P* value with an absolute value of NES > 2 (or >1.5 in comparison of R^-/DSA^+ vs R^+/DSA^+) are displayed. The GO terms with NES > 0 are enriched in genes upregulated in the R^+/DSA^+ group and those with NES < 0 are enriched in genes upregulated in the R^-/DSA^+ group. DSA, donor-specific antibody; GO, gene ontology; NES, normalized enrichment score; R^-, no rejection by histology; R^+, rejection by histology.

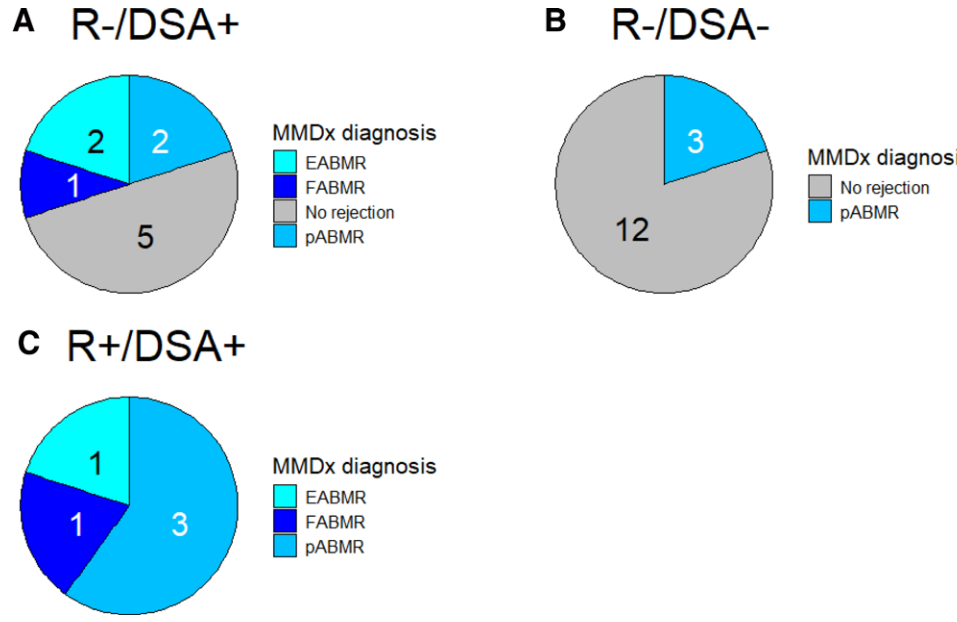

FIGURE 2. The proportions of MMDx rejection diagnoses in R^-/DSA^+ (n = 10) (A); R^-/DSA^- (n = 15) (B); and R^+/DSA^+ (n = 5) (C) in biopsies performed within the first month after transplantation in cohort 2. AMR, antibody-mediated rejection; DSA, donor-specific antibody; EAMR, early-stage AMR; FAMR, fully developed AMR; MMDx, Molecular Microscope Diagnostic System; pAMR, possible/probable AMR; pMixed, possible mixed rejection; R^-, no rejection by histology; R^+, rejection by histology.

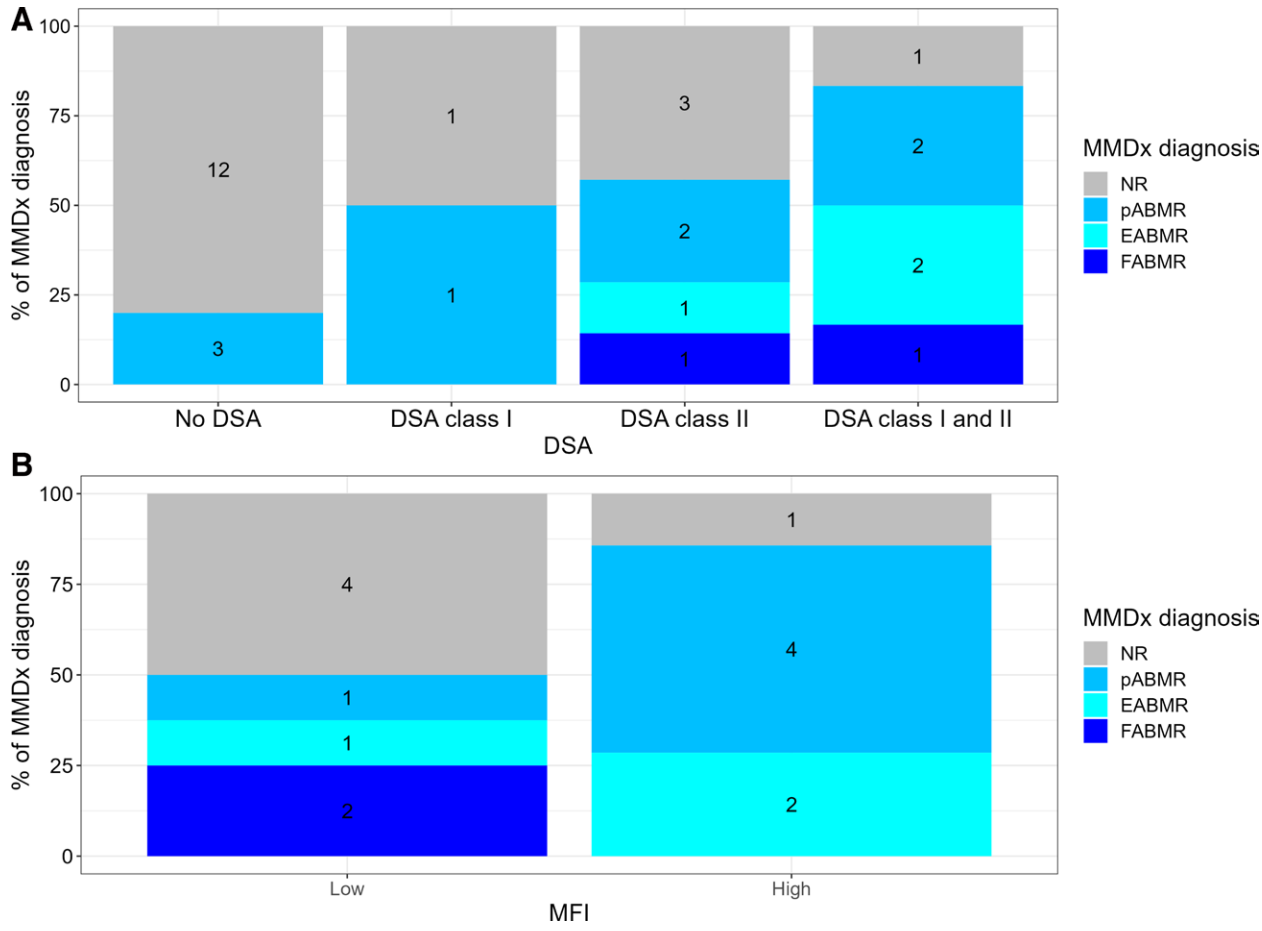

FIGURE 3. The percentage of individual MMDx diagnoses according to DSA class (A) and maximal DSA level (MFI) (B) at biopsy irrespective of histology diagnosis. High, MFI ≥5000; low, MFI <5000. On the y-axis are percentages, whereas the numbers in bars represent n. DSA, donor-specific antibody; MFI, mean fluorescence intensity; MMDx, Molecular Microscope Diagnostic System.

Fisher Scientific 3′IVT Plus Labeling Kit and analyzed using PrimeView Gene Chip microarrays (Affymetrix, Santa Clara, CA) according to standard operation protocols of Molecular Microscope Diagnostic System (MMDx) as previously described.[15] The resulting CEL files, consisting of a matrix of measurements of all probes included on the array, were used either for differential expression analysis or MMDx report generation.

Differential gene expression analysis was performed using the "limma" package,[16] and volcano plots were constructed using "EnhancedVolcano" package.[17] CEL files are available on the GEO website (GSE276194).

Classifiers related to rejection (AMR, T cell-mediated rejection, and all rejection) or acute kidney injury, inflammation, and chronic injury (atrophy/fibrosis score) were generated using a recently published reference set of 1208 biopsy specimens.[18]

RESULTS

Cohort 1

The transcriptome in the R$^-$/DSA$^+$ group was similar to the R$^+$/DSA$^+$ group, as there were only 14 differentially expressed transcripts between them. Fast gene set enrichment analysis identified 12 significantly increased and 7 decreased GO terms between R$^-$/DSA$^+$ versus R$^+$/DSA$^+$ groups. GO terms which were increased in the R$^-$/DSA$^+$ group were associated with cell junction or glial cell differentiation while with amino acid and organic acid metabolism in the R$^+$/DSA$^+$ group (**Table S3, SDC,** http://links.lww.com/TXD/A723; Figure 1A). Therefore, DSA$^+$ patients exhibited similar transcriptomic profiles by RNA sequencing regardless of the presence of active/probable AMR by histology.

Interestingly, when comparing biopsies in R$^-$/DSA$^-$ with both DSA$^+$ groups, 275 and 651 differentially expressed transcripts were found in cases with and without active/probable AMR, respectively. In both DSA$^+$ groups, 131 identical GO terms were found (**Figure S2, SDC,** http://links.lww.com/TXD/A723) in comparison with DSA$^-$ controls. These GO terms were associated with the activation of immune response (adaptive immune response, antigen receptor-mediated signaling, B-cell receptor signaling, and complement activation). A complete list of GO terms is shown in **Tables S4 and S5 (SDC,** http://links.lww.com/TXD/A723). The top 10 GO terms with increased transcripts in the cohort of DSA$^+$ patients without rejection compared with the cohort of DSA$^-$ patients ranked according to adjusted *P* value are displayed in Figure 1B.

After categorizing the DSA$^+$ patients according to MFI level[19] and DSA class, 39 differentially expressed transcripts were found in comparison of patients with MFI >6000 (n = 12) and those with MFI ≤6000 (n = 12; **Table S6, SDC,** http://links.lww.com/TXD/A723) and only 2 transcripts (*MDH2*, malate

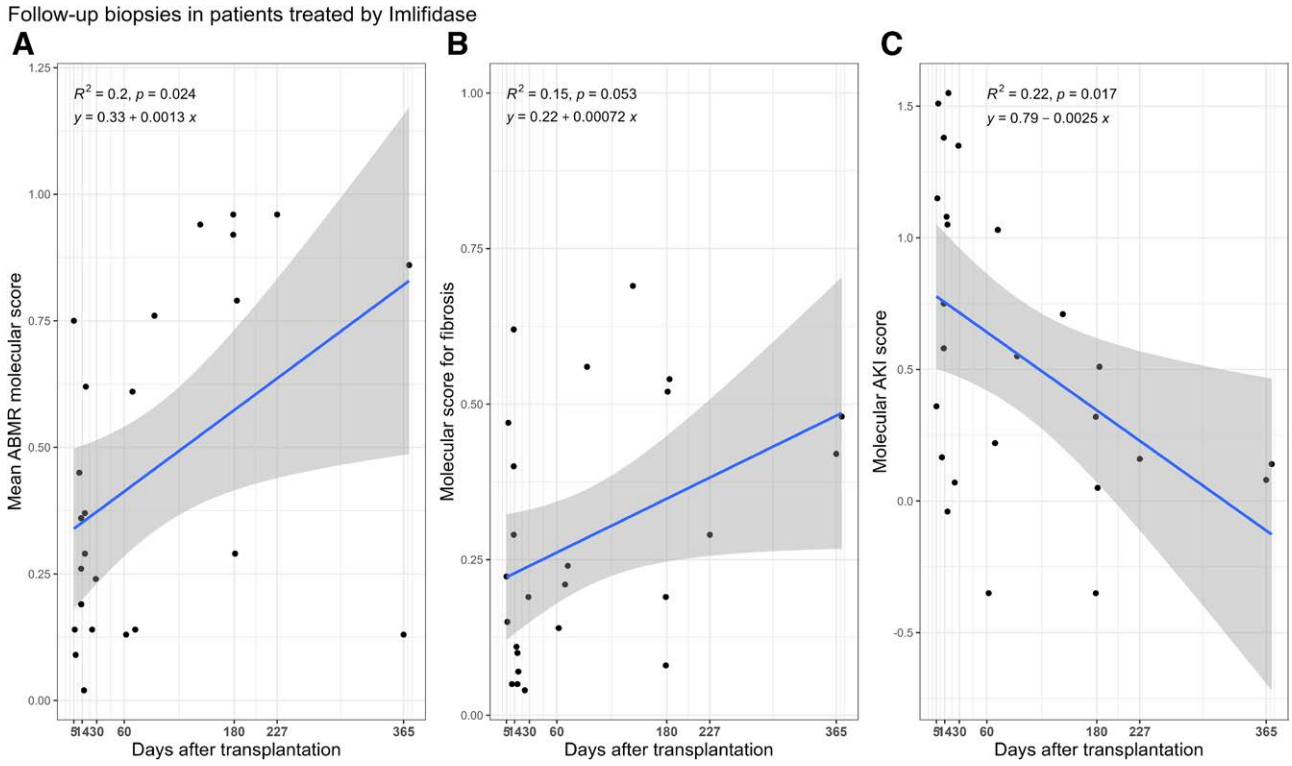

FIGURE 4. Evolution of molecular scores after imlifidase desensitization: mean AMR score (A), atrophy fibrosis score (B), and AKI score (C) by MMDx in biopsy series indicated for cause or per-protocol (8 patients, 25 biopsies). AKI, acute kidney injury; AMR, antibody-mediated rejection; MMDx, Molecular Microscope Diagnostic System.

dehydrogenase, adjusted P = 0.022 and *DPT*, dermatopontin, adjusted P = 0.032) in comparison of patients with DSA class 1 (n = 10) versus DSA class 2 (n = 9). GO-enrichment analysis of transcripts decreased in DSA$^+$ with MFI >6000 showed enrichment of only 1 GO term (collagen-containing extracellular matrix; transcripts [*MFAP5, HNRNPM, SFRP2, CXCL12, MMRN1, SERPINF1, DPT, COL10A1, WNT2*]; adjusted $P = 1.6 \times 10^{-6}$).

Cohort 2

Biopsies in cohort 2 (n = 30) were processed using the Affymetrix microarray platform, and the difference in gene expression between particular groups was analyzed using differential expression. Besides that, MMDx algorithm was used to create MMDx sign outs for individual patients.

No differentially expressed transcripts were found among all 3 groups after adjustment to multiple testing. When a nonadjusted P value was used, 27 transcripts were differentially expressed between both DSA$^+$ cohorts, that is, with and without histological signs of active/probable AMR. Seventeen differentially expressed transcripts were found between R$^-$/DSA$^+$ and R$^-$/DSA$^-$ and 28 transcripts between R$^+$/DSA$^+$ and R$^-$/DSA$^-$, respectively (nonadjusted $P < 0.001$, fold change > 1.5; **Figure S3, SDC,** http://links.lww.com/TXD/A723).

Using the MMDx algorithm, 5 of 10 biopsies in the R$^-$/DSA$^+$ group were classified as molecular no rejection (Figure 2A). In the other 5 biopsies, the rejection or possible rejection phenotype was diagnosed: 2 cases of early AMR, 2 of possible AMR, and 1 of fully developed AMR.

In 12 of 15 cases (80%) in the R$^-$/DSA$^-$ group, no molecular rejection was confirmed by MMDx. In the remaining 3 cases, the MMDx reported possible AMR (Figure 2B). On the contrary, in all biopsies from the R$^+$/DSA$^+$ group, the MMDx confirmed histological diagnosis (Figure 2C).

There were no significant differences in molecular rejection and injury-related scores from the MMDx report between both DSA$^+$ groups (**Figures S4 and S5, SDC,** http://links.lww.com/TXD/A723).

Interestingly, the severity of molecular rejection was higher in patients with DSA class II compared with those with DSA class I (Figure 3) and in those with higher values of DSA (MFI >5000), irrespective of histology findings.

Follow-up Biopsies in DSA$^+$ Patients

In 9 of 18 patients (50%) in the R$^-$/DSA$^+$ group and in 15 of 21 patients (71%) in the R$^+$/DSA$^+$ group, follow-up biopsies revealed rejection by histology in up to 12 mo, respectively. All but 1 rejection was AMR type.

Interestingly, in patients who experienced imlifidase desensitization and received rATG, rituximab, and high-dose IVIG as induction, a gradual increase of both molecular AMR and atrophy fibrosis scores and a decrease of molecular acute kidney injury score, all defined by MMDx, were detected (Figure 4).

DISCUSSION

Presensitized patients who receive HLA-incompatible kidney transplantation are at risk of AMR and premature graft loss despite peritransplant desensitization and depletive immunosuppression. With the advent of molecular pathology, classical histology has been accompanied by biopsy-based transcript diagnostics in several centers, including

ours, to improve diagnostics robustness. Herein, we aimed to assess 2 cohorts: patients in whom part of core biopsies were stored and retrospectively assessed by RNAseq and recent ones where biopsy-based transcript diagnostics were already clinically indicated and results available. We show that DSA+ patients exhibit in biopsies that were performed very early after transplantation such profound transcriptomic changes (defined by RNAseq) or molecular calls of AMR by biopsy-based transcript diagnostics, which were independent of the presence or absence of active/probable AMR in routine histology. We believe this observation has important clinical translation as early diagnosis of AMR may help clinicians adapt therapy[12] and monitoring[20,21] and potentially change outcomes. Moreover, a significant part of patients with negative histology in initial biopsy experienced frequent AMR or mixed rejections in follow-up biopsies. Our observation thus accompanies recent data on the effective treatment of AMR[11,12] and suggests that molecular diagnostics may be an important addition to biopsy assessment for guiding therapeutic decisions.

To study subtle changes of gene expression that may reflect incipient AMR, RNA sequencing was used because it detects a higher number of differentially expressed genes, especially those with low expression.[22] In our study, the biopsy-derived transcriptome in all DSA+ patients was similar, albeit identical to the RNA sequencing.

A microarray-based approach, analyzed either by differential expression or by the MMDx algorithm,[18] was used in cohort 2. MMDx is a robust method that interprets the gene expression results of thousands of transcripts using ensembles of machine learning algorithms to discriminate various rejection types,[23] and it benefits from clinical validation and already routine clinical application. The differential expression analysis revealed no differences between DSA+ groups in both cohorts. When using the MMDx algorithm, no differences in molecular scores for AMR were found. However, MMDx sign outs which are the result of multiple classifier and archetype analyses showed 50% molecular rejection among DSA+ patients without histological AMR features, whereas molecular rejection was present in every patient with probable/active AMR. In the large INTERCOMEX study, MMDx showed a gradual increase in AMR-related classifiers from DSA− and AMR− patients to DSA+ and AMR+ patients.[5] Despite being large, biopsies in the INTERCOMEX studies were performed frequently late, also involved subclinical findings as protocol biopsies were often used, and data on given therapy were not available.

Patients with high levels of DSAs, presence of class II DSAs, and higher number of DSAs were shown to be at higher risk of premature graft failure.[24-26] These observations are in line with the results of our study, where patients with DSA class II and higher DSA MFI experienced more frequent molecular rejections. In the RNA sequencing cohort, no marked differences in gene expression in subcategories of DSA class or DSA MFI intensity were found. This result may, however, be influenced by the small sample size of our study and by selected MFI cutoff or Luminex inter-lab variability.

In our study, we merged active AMR and probable AMR into a single group to adhere to changes suggested by the last Banff classification.[13] The current Banff 2022 definition of active AMR is based on the presence of circulating DSA, C4d staining, MVI, and biopsy-based transcripts diagnostics. To fulfill the criteria for active AMR, biopsies with at least moderate MVI (g+ptc ≥ 2) require C4d positivity and/or DSA positivity, whereas biopsies with mild MVI (g+ptc = 1) require C4d positivity irrespective of DSA status. In our study, the groups were defined on the basis of histological criteria, and biopsy-based transcripts were not taken into account because, in the former cohort, the molecular assessment was not available for clinical use. In the study of Dominy et al,[27] the expression of selected AMR-associated transcripts, evaluated by the Nanostring platform, was similar between C4d positive and negative biopsies without histological signs of rejection. In that study, the C4d positive group consisted of only 43% of DSA+ patients (18/42) and biopsies were performed later (median 46 d posttransplant). In contrast, in our study, all biopsies with C4d positivity and without MVI were performed early after transplantation. Similarly, the increased intragraft rejection-associated gene transcripts in DSA+ patients with negative histology compared with DSA− patients were reported by Hayde et al[28] later posttransplant. Clearly, probable AMR without signs of MVI represents less severe rejection. However, early after desensitization in HLA-incompatible transplantation, even probable AMR is of concern because it may represent the beginning of a continuous rejection process.

The limitation of our study is the relatively small sample size and retrospective design. Despite that, our data are the first one focused only on indication biopsies performed in the first month in patients who received desensitization; thus, the study cohort is homogenous in comparison with others and eliminates a time bias. Both cohorts differed in desensitization regimens as the patients in the second cohort were enrolled recently, when new drugs, including imlifidase, and similarly biopsy-based transcript diagnostics were available for routine use.

Frequent observation of histological rejection in consecutive biopsies clearly underlines the importance of molecular assessments in cases with negative histology in early biopsies in DSA+ transplantation. Early detection of molecular calls of rejection is necessary to adapt immunosuppression early and to treat subclinical injury before structural changes develop and graft function deteriorates. The use of noninvasive innovative biomarkers such as donor-derived cell-free DNA (dd-cfDNA) early after transplantation may be problematic; however, later on, as shown by Trifecta study,[29] there is a good correlation between dd-cfDNA and molecular diagnostics.[20,21] Such approaches seem to be critical in highly sensitized patients whose chances for repetitive transplantation are minimal.

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