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ORIGINAL ARTICLE

Molecular assessment of antibody-mediated rejection in human pancreas allograft biopsies

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Abstract

Pancreas transplant longevity is limited by immune rejection, which is diagnosed by graft biopsy using the Banff Classification. The histological criteria for antibody-mediated rejection (AMR) are poorly reproducible and inconsistently associated with outcome. We hypothesized that a 34-gene set associated with antibody-mediated rejection in other solid organ transplants could improve diagnosis in pancreas grafts. The AMR 34-gene set, comprising endothelial, natural killer cell and inflammatory genes, was quantified using the NanoString platform in 52 formalin-fixed, paraffin-embedded pancreas transplant biopsies from 41 patients: 15 with pure AMR or mixed rejection, 22 with T cell-mediated rejection/borderline and 15 without rejection. The AMR 34-gene set was significantly increased in pure AMR and mixed rejection ($P = .001$) vs no rejection. The gene set predicted histological AMR with an area under the receiver operating characteristic curve (ROC AUC) of 0.714 ($P = .004$). The AMR 34-gene set was the only biopsy feature significantly predictive of allograft failure in univariate analysis ($P = .048$). Adding gene expression to DSA and histology increased ROC AUC for the prediction of failure from 0.736 to 0.770, but this difference did not meet statistical significance. In conclusion, assessment of transcripts has the potential to improve diagnosis and outcome prediction in pancreas graft biopsies.

KEYWORDS

antibody-mediated, Banff classification, biopsy, classification systems, expression, molecular biology, rejection, RNA and/or transcript

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1 | INTRODUCTION

Pancreas transplantation is a replacement therapy for the endocrine pancreas performed mostly in patients with type 1 diabetes, although about 10% of transplants are performed in patients with type 2 diabetes who require insulin. Whereas a simultaneous kidney-pancreas transplant will be undertaken for patients with insulin-treated diabetes and renal failure, pancreas transplant alone will be considered for those with severe hypoglycemic unawareness or severe glycemic variability and insulin-treated diabetes, but normal or near-normal renal function, and a pancreas after kidney transplant might be performed for patients with functioning kidney transplants and insulin-treated diabetes. In 2018, the United Kingdom reported 217 deceased donor pancreas transplants, representing 3.3 pancreas transplants per million population (pmp), whereas the USA reported 1,034 pancreas transplants, at 3.21 pmp (International Registry in Organ Donation and Transplantation; http://www.irodat.org/). As with other solid organ transplants, cellular and/or antibody-mediated immune responses against the transplant limit graft life span.

The gold standard for the diagnosis of rejection is histological examination of a biopsy of the grafted pancreas.[1] The Banff Classification for Allograft Pathology[2] recognizes both T cell-mediated rejection (TCMR) and antibody-mediated rejection (AMR) in the pancreas. There is an increase in rejection and graft failure in patients with a donor-specific antibody (DSA)[3-6]; and histological features of AMR (ie micro- and macro-circulation inflammation and C4d-positive staining) have been described in case reports and series.[7-12] However, interpretation of C4d immunohistochemistry[13] and recognition of interacinar capillaritis (IAC) are difficult.[2] Reports on outcomes and response to treatment in AMR are contradictory and not always clearly different between AMR and TCMR.[3,4,7,8] Therefore, although specific treatments are recommended in patients with features of AMR, a solid evidence base is lacking.[10,14]

In other transplanted solid organs (ie kidney, heart), a gene expression signature characteristic of AMR is emerging as a diagnostic tool.[15-17] Analysis of gene expression in pancreas allograft biopsies has been limited to our knowledge to a single study using qRT-PCR on 26 formalin-fixed paraffin-embedded (FFPE) biopsies.[18] The development of probe-based technology for transcript analysis designed for analysis of FFPE material has enabled an expansion of molecular analysis on this type of sample.[15,19-23]

We hypothesized that molecular assessment of transcripts associated with AMR in other organs would improve recognition of AMR and outcome prediction in pancreas grafts.

2 | MATERIALS AND METHODS

2.1 | Case selection, histopathology, and DSA

Of 129 pancreas transplant samples taken at Imperial College Healthcare Trust between 2007 and 2017, we excluded transplant pancreatectomies, and selected biopsy samples (n = 46) on the basis of (a) adequacy of sample; (b) availability of tissue in the block (visual inspection); (c) absence of advanced fibrosis, necrosis, and acute pancreatitis; and (d) a clear diagnostic label. Pancreas transplant biopsy FFPE blocks were obtained from the Imperial College Healthcare NHS Trust Tissue Bank (MREC 17/WA/0161) (approved project number R16009). Further cases of definite AMR or mixed rejection were contributed from Maryland (n = 4) and University of Nantes (n = 5), with approval for research.

Each pathologist independently classified the cases they contributed according to Banff 2015 (CR, CD and KR) into 3 diagnostic groups: cases without rejection (NR), cases with TCMR or borderline for TCMR (T), and cases with pure definite AMR (A) or "mixed" rejection (M). Mixed rejection was defined as fulfilling the criteria for TCMR plus the presence of DSA, plus at least one of IAC, vasculitis (v), and C4d-positive staining. We also further defined grades of AMR based on the 3 diagnostic components (DSA, C4d, and histological features of AMR). Cases were defined as requiring exclusion of AMR (A1), suspicious for AMR (A2), or definite AMR (A3), depending on the presence of 1, 2, or 3 of the 3 diagnostic components, respectively. A0 designates absence of all 3 diagnostic components.

C4d staining was carried out according to the local institutes' usual protocol, in all but one case using immunohistochemistry on FFPE tissue. Further details are available in Table S1.

The anti-HLA DSA status was determined from corresponding serum samples taken within a 6-month period before or after the time of biopsy. Status was determined locally, using a Luminex single-antigen bead assay (Thermo Fisher Scientific). In cases with multiple available sera, the one closest to the biopsy date was included. An immunodominant mean fluorescence intensity (MFI) of > 500 was considered positive.

2.2 | RNA extraction from FFPE blocks

Six sections of 10 micron or 3 sections of 20 micron thickness were obtained from FFPE blocks. Equipment sterilization was performed between blocks with Ambion™ RNase Away™ Decontamination Reagent. Deparaffinization and RNA extraction were performed with the RNeasy FFPE Kit (Qiagen). RNA quantity was assessed with the NanoDrop 2000c spectrophotometer (NanoDrop Technologies).

2.3 | Gene set and quantification using NanoString nCounter

The AMR gene set was chosen using a pathomechanism-based selection,[15] derived from published genome-wide expression analysis in patients with DSA and morphological features of AMR in kidney and/or heart allografts.[24-26] NanoString nCounter (NanoString Technologies) was used to analyze a custom 38-gene set, which comprised 34 genes as the AMR signature (including 17 "endothelial genes," 9 "NK cell genes," and 8 "inflammation genes") and 4 housekeeping genes (Table 1).

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TABLE 1 Annotated list of 38 genes analyzed using NanoString nCounter Elements™ TagSet

Gene symbol	Gene name	Annotation
DARC	Duffy blood group, atypical chemokine receptor	endothelial
PECAM1	platelet/endothelial cell adhesion molecule 1	endothelial
VWF	von Willebrand factor	endothelial
CAV1	caveolin 1, caveolae protein, 22 kDa	endothelial
THBD	thrombomodulin	endothelial
CDH5	cadherin 5, type 2 (vascular endothelium)	endothelial
SELE	selectin E	endothelial
RHOJ	ras homolog family member J	endothelial
PALMD	palmdelphin	endothelial
CDH13	cadherin 13	endothelial
SOX7	SRY (sex determining region Y)-box 7	endothelial
PLAT	plasminogen activator, tissue	endothelial
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	endothelial
TEK	TEK tyrosine kinase, endothelial	endothelial
PLA1A	phospholipase A1 member A	endothelial
CD34	CD34 molecule	endothelial
PSMB10	proteasome (prosome, macropain) subunit, beta type, 10	endothelial
KLRF1	killer cell lectin-like receptor subfamily F, member 1	NK cell
GNLY	granulysin	NK cell
MYBL1	v-myb avian myeloblastosis viral oncogene homolog-like 1	NK cell
FGFBP2	fibroblast growth factor binding protein 2	NK cell
KLF4	Kruppel-like factor 4 (gut)	NK cell
TBX21	T-box 21	NK cell
GATA3	GATA binding protein 3	NK cell
CX3CR1	chemokine (C-X3-C motif) receptor 1	NK cell
SH2D1B	SH2 domain containing 1B	NK cell
IFNG	interferon, gamma	Inflammation
TNF	tumor necrosis factor	inflammation
MALL	mal, T-cell differentiation protein-like	inflammation
TRIB1	tribbles homolog 1 (Drosophila)	inflammation
CXCL11	chemokine (C-X-C motif) ligand 11	inflammation
ROBO4	roundabout, axon guidance receptor, homolog 4 (Drosophila)	inflammation
RPS6	ribosomal protein S6	inflammation
RPS6KB1	ribosomal protein S6 kinase, 70 kDa, polypeptide 1	inflammation
ACTB	actin, beta	Housekeeping
LDHA	lactate dehydrogenase A	Housekeeping
HPRT1	hypoxanthine phosphoribosyltransferase 1	Housekeeping
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Housekeeping

Gene set expression was quantified using the nCounter XT Formulation, with the NanoString nCounter FLEX Dx Analysis System as per manufacturer recommendations (with input RNA of about 100 ng).[27,28] Raw gene expression counts were quality controlled and normalized using nSolver Analysis Software version 2.0 (NanoString Technologies) and manufacturer-included positive and negative controls. Data were first normalized to the positive controls followed by normalization to the four housekeeping genes.

Normalized transcript counts were converted to z-scores (number of standard deviations away from the population mean for each gene). An aggregate AMR 34-gene set score was determined for each biopsy by adding the 34 z-scores.

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2.4 | Statistical analysis

Correlation between histological, serological, and gene expression parameters was performed using Spearman's rank correlation coefficient (rho) and heat map analysis with hierarchical clustering by Euclidean distance. Expression of the AMR 34-gene set score was compared between groups using Mann-Whitney *U* test. *P*-values < .05 were considered significant. To estimate the diagnostic accuracy of the gene set, receiver operating characteristic (ROC) curve analysis was performed.

Cox proportional hazards regression was used to estimate the effect of DSA (D), histology (H), and gene expression (G) variables on death-censored graft failure, defined as return to insulin. Variables with *P*-values < .1 in univariate analysis were further assessed with multivariate Cox analysis. These variables were then used to derive logistic regression models to simulate their relative utility in the clinical prediction of death-censored allograft failure. These models included all possible univariate and multivariate combinations of the D, H, and G categories of variables. Each model was derived using 10-fold cross-validation with 100 repeats to minimize overfitting. Model discrimination was compared using areas under the curve (AUC) in ROC curves, net reclassification index (NRI) and integrated discrimination improvement (IDI). The ability of the models to reclassify patients into low- or high-risk categories for allograft loss was compared using continuous net reclassification index (NRI).

Analyses were performed in GraphPad Prism 8, MedCalc version 18.5 (MedCalc Software) and R version 3.6.0 (R Foundation for Statistical Computing).

3 | RESULTS

3.1 | RNA extraction and analysis

RNA was successfully extracted from 53 of 55 FFPE blocks (1 case each from Imperial and Nantes had insufficient RNA). Mean RNA yield was 113.96 ng/μL (minimum = 23.8 ng/μL; median = 82.4 ng/μL, maximum = 326.4 ng/μL), with purity measures of 1.55-2.05 (OD 260/280 nm). NanoString gene expression analysis was successful for all samples, but one sample was excluded due to a normalization flag encountered during nSolver data processing, leading to a total of 52 samples for analysis.

3.2 | Demographic, histological, and serological data

The 52 biopsies were from 41 pancreas transplant recipients (34 from Imperial, 4 from Maryland, 3 from Nantes). Pancreas grafts were positioned via a retroperitoneal approach with enteric exocrine drainage and systemic venous drainage into the inferior vena cava. Of the 34 cases from Imperial, 27 were SPK and 7 were PAK. Of the 4 cases from Maryland, 2 were PTA, 1 SPK and 1 PAK. Of the 3 cases from Nantes, 2 were a PTA and 1 an SPK. Immunosuppressive regimens varied. SPK recipients from imperial received induction with either alemtuzumab or IL2-receptor antagonists. PAK recipients received induction with methylprednisolone alone or with alemtuzumab depending on vintage. All patients received 1 week of corticosteroids only and maintenance immunosuppression consisted of tacrolimus with mycophenolate mofetil. Of the 4 patients from Maryland, 1 received induction with anti-thymocyte globulin followed by cyclosporin A, azathioprine, and prednisolone, 2 received induction with anti-thymocyte globulin followed by cyclosporin A, mycophenolate mofetil, and prednisolone, and 1 received induction with alemtuzumab followed by cyclosporin A, mycophenolate mofetil, and prednisolone. The 3 patients from Nantes all received induction with anti-thymocyte globulin followed by tacrolimus, mycophenolate mofetil, and prednisolone.

Of the 52 biopsies, 15 were classified as A (n = 3) or M (n = 12), 22 as T, and 15 as NR. Demographic data are provided in Table 2. We observed in cases with A + M compared to all others a younger donor age (*P* = .0097) and more pancreas transplant alone (PTA) or pancreas after kidney (PAK) (*P* = .0003).

Histological data are presented in Table 3. The most common indication for biopsy was increased amylase and/or lipase plasma level, followed by abnormal blood glucose/HBA1c or both, and occurrence of DSA. Median time to biopsy post-transplant was 226 (54-848) days, and this was shortest in cases with A + M, although the difference was not statistically significant.

Among NR cases (n = 15), 8 were normal or had mild acinar injury (Banff category 1) and 7 had graft sclerosis (category 6). Five of the 15 NR had some diagnostic criteria of AMR: 4 had a DSA; 4 had focal C4d positivity; and 1 had mild IAC.

Among T cases (n = 22), 2/22 were borderline (category 2) and 20/22 were TCMR (category 3). Among TCMR cases, 13/20 were grade 1, 3/20 were grade 2 and 4/20 were grade 3. Fifteen of the 22 T had some diagnostic criteria of AMR without fulfilling criteria for mixed rejection: 5/22 had a DSA; 3/22 had focal C4d; 7/22 had mild IAC and 3/2 had arteritis.

Among A + M cases (n = 15), 12/15 were mixed, with TCMR grade 1 in 3/12, grade 2 in 6/12, and grade 3 in 3/12 cases. Only 4/15 cases fulfilled all criteria for AMR (A3), whereas 11/15 were "suspicious" for AMR (A2), because of a positive DSA with either positive histology for AMR or a positive C4d.

Regarding the 3 diagnostic criteria of AMR, 15/52 biopsies were A0, 18/52 were A1, 15/52 were A2, and 4/52 were A3. With "histological AMR" (hAMR) defined as the presence of IAC and/or v, 22/52 showed hAMR.

When comparing biopsies from the A + M group to all other biopsies, statistically significant biopsy features were more septal eosinophils (*P* = .04), more septal (*P* = .02) and ductal (*P* = .01) neutrophils, more acinar inflammation (*P* = .005) and injury (*P* = .04), and more acinar capillaritis (*P* = .004).

Antibody data are presented in Table 4. Whereas 100% of cases in A + M had a positive DSA by definition, 23% of T cases and 27% of NR cases were also DSA+. The DSA was class I alone in 25% of cases,

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TABLE 2 Demographic data

	All (n = 52)	A + M (n = 15)	T (n = 22)	NR (n = 15)	*P*-value[a]
Recipient age at transplant, median in years (IQR)	42.82 (35.2-49.2)	40.7 (31.0-49.0)	42.2 (37.9-46.0)	44.5 (36.7-49.9)	.83
Recipient sex, absolute number M;F	27; 25	6; 9	13; 9	8; 7	.36
Donor age median in years (IQR) (data missing)	38.0 (21.0-47.0) (9)	19.0 (17.0-40.0) (0)	45 (29.7-47.0) (5)	43 (31.3-49.5) (4)	**.0097**
Donor sex absolute number M;F (data missing)	25; 23 (4)	8; 7	12 ;9 (1)	5; 7 (3)	>.99
Type of pancreas transplant absolute number SPK; PTA; PAK	37; 6; 9	5; 5; 5	18; 0; 4	14; 1; 0	**.0003**[b]
Type of pancreas transplant DCD;DBD absolute number (data missing)	10; 31 (11)	4; 7 (4)	3; 15 (4)	3; 9 (3)	.41
HLA-A MM absolute number 0 ;1; 2 (data missing)	7; 19; 23 (3)	3; 4; 8	2; 9; 9 (2)	2 ;6 ;6 (1)	.62
HLA-B MM absolute number 0 ;1; 2 (data missing)	1; 16; 32 (3)	1; 5; 9	0; 7; 13 (2)	0; 4; 10 (1)	.21
HLA-DR MM absolute number 0 ;1; 2 (data missing)	1; 21; 27 (3)	0; 7; 8	1; 8; 11 (2)	0; 6; 8 (1)	.94
Total number MM, median (IQR) (data missing)	4.0 (4.0-5.5) (3)	5.0 (3.0-6.0)	4.0 (4.0-5.0) (2)	4.5 (4.0-5.0) (1)	.59
Induction therapy absolute number No; Yes [ATG; alemtuzumab; daclizumab] (missing data)	4; 44 [7; 35; 2] (4)	1; 13; [6; 7; 0] (1)	3; 18; [1; 15; 2] (1)	0; 13 [0; 13; 0] (2)	>.99
Pancreas graft survival absolute number Yes; No (%)	30; 22 (57.7%)	6; 9 (40.0%)	13; 9 (59.1%)	11; 4 (73.3%)	.13
Time to graft failure in days, median (IQR)	634 (397-1211)	429 (338-1004)	525 (288.5-927)	2556 (1783-3333)	.41
Patient survival absolute number Yes; No (%)	45; 7 (86.5%)	11; 4 (73.3%)	20; 2 (90.9%)	14; 1 (93.3%)	.17
Kidney graft survival absolute number Yes; No (Total number with kidney) (%)	36; 11 (47) (75.6%)	9; 1 (10) (90%)	16; 6 (22) (72.7%)	11; 4 (14) (78.6%)	.41

Note: Mann-Whitney *U* test was used for continuous data and Fisher's exact test was used for categorical data.

Abbreviations: A, antibody-mediated rejection, pure; ATG, anti-thymocyte globulin; DBD, deceased brain death; DCD, deceased cardiac death; F, female; IQR, interquartile range; M, male; M, mixed rejection; MM, mismatch; NR, no rejection; PAK, pancreas after kidney; PTA, pancreas transplant alone; SPK, simultaneous pancreas kidney; T, borderline and T-cell mediated rejection.

[a]Analysis for A + M vs T + NR.

[b]Analysis for SPK vs PTA + PAK.

Significant p values (< 0.05) are high-lighted in bold.

class II alone in 21%, and class I + II in 54%. The DSA was a de novo post-transplant DSA in 92%.

3.3 | Correlation between histological, serological, and gene expression parameters

Significant correlation was observed between acinar, septal and ductal inflammation, IAC and venulitis (rho = 0.322-0.628, *P* ≤ .020) (Figure 1). Chronic allograft sclerosis correlated with acinar, septal, and ductal inflammation (rho = 0.375-0.398, *P* ≤ .007). The AMR 34-gene set score correlated with acinar inflammation (rho = 0.502, *P* < .0001), septal inflammation (rho = 0.345, *P* = .012), IAC (rho = 0.347, *P* = .012), and chronic allograft sclerosis (rho = 0.382, *P* = .005).

3.4 | Diagnostic performance of the AMR 34-gene set

The AMR 34-gene set score was significantly differentially expressed between A + M and NR (*P* = .001), and between T and NR (*P* = .024) (Figure 2A). It was also significantly differentially expressed between subgroups A0 and A3 (*P* = .009) and between A1 and A3 (*P* = .014) (Figure 2B). It was significantly increased in biopsies with hAMR (IAC and/or v) compared to those without (*P* = .008), but not between biopsies from patients with or without DSA (*P* = .059), or between cases that were C4d-positive vs negative (*P* = .957). The score was significantly increased comparing diffuse C4d positivity to negative or focal staining (C4d0 vs C4d1, *P* = .058; C4d1 vs C4d2, *P* = .002; C4d0 vs C4d2, *P* = .003).

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TABLE 3 Biopsy data

	All (n = 52)	A + M (n = 15)	T (n = 22)	NR (n = 15)	Test; *P*-value[a]
Reason for Biopsy					>.99
Surveillance	2	0	0	2	
Indication	47	13	21	13	
Increased amylase	26	9	13	6	
Abnormal BMs	8	2	3	3	
Incr amylase + abnormal BMs/HbA1c	5	1	2	2	
Change in IS	3	1	0	2	
Post-treatment	1	0	1	0	
DSA (on its own/in addition to above)	0/5	0/4	1/0	0/1	
Abdominal pain in addition to above	1	1	0	0	
Unknown	3	2	1	0	
Time (days) post-transplant of biopsy					.08
Days; median (IQR)	221 (54-785)	76 (31-361)	216 (56-514)	588 (151- 1468)	
Number of acinar areas					.83
Median (IQR)	8 (7-13)	8 (6.5-12.0)	7 (6-13)	8 (7.5-13.5)	
Septal mononuclear inflammation					
No/Yes	16/36	2/13	1/21	13/2	.11
Inactive	6	3	0	2	
Active	30	10	21	0	
Septal eosinophils					
No/Yes	36/16	7/8	14/8	15/0	**.04**
Few	11	6	5	0	
Many	*5*	2	3	0	
Septal plasma cells					
No/Yes	*35/17*	6/9	14/8	15/0	.19
Few	14	8	6	0	
Many	3	1	2	0	
Septal neutrophils					
No/Yes	42/10	9/6	18/4	15/0	**.02**
Few	9	5	4	0	
Many	*1*	1	0	0	
Ductitis (mononuclear)					.33
No ducts sampled	*2*	2	0	0	
Yes	20	7	12	1	
No	30	6	10	14	
Ductitis neutrophils					**.01**
No ducts sampled	2	2	0	0	
Yes	5	4	1	0	
No	45	9	21	15	
Acinar inflammation (mononuclear)					

(Continues)

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TABLE 3 (Continued)

	All (n = 52)	A + M (n = 15)	T (n = 22)	NR (n = 15)	Test; *P*-value[a]
No/Yes	19/32	1/14	4/18	14/1	**.005**
None	19	1	4	14	
Focal	15	5	9	1	
Multifocal	13	7	6	0	
Severe/extensive	5	2	3	0	
Acinar injury					
No/Yes	15/36	1/13	1/21	13/2	**.04**
None	15	1	1	13	
Single/spotty	25	8	15	2	
Multicellular confluent	11	5	6	0	
Acinar capillaritis					**.004**
No	34	5	15	14	
Yes	18	10	7	1	
Venulitis					.13
No	27	5	8	14	
Yes	25	10	14	1	
Intimal arteritis					
No arteries sampled	8	4	0	4	
No/Yes	38/6	9/3	18/3	11/0	.32
None	38	9	18	11	
Mild	1	1	0	0	
Moderate to severe intimal arteritis	0	0	0	0	
Mural necrosis	5	2	3	0	
Chronic transplant arteriopathy					
No arteries sampled	8	4	0	4	
No/Yes	33/11	9/2	16/6	8/3	.70
None	33	9	16	8	
Mild < 25%	7	1	4	2	
Moderate 26%-50%	4	1	2	1	
Severe > 50%	0	0	0	0	
Banff Diagnostic Categories					
Category 1 (Normal)	**8**	0	0	8	
Category 2 (Borderline)	**2**	0	2	0	
Category 3 (TCMR)	**31**	12/15	20/22	0/15	
Grade 1	15	3	13	0	
Grade 2	9	6	3	0	
Grade 3	7	3	4	0	
Category 4 (AMR)	**15**	15	0	0	
Definite AMR (all 3 criteria, A3)	4	4	0	0	
Suspicious for AMR (2 of 3 criteria, A2)	15	11	2	2	

(Continues)

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TABLE 3 (Continued)

	All (n = 52)	A + M (n = 15)	T (n = 22)	NR (n = 15)	Test; *P*-value[a]
Requires exclusion of AMR (1 of 3 criteria, A1)	16	0	13	3	
Category 5: Chronic allograft arteriopathy	**10**	2	6	3	
No arteries sampled	8	4	0	4	
Category 6: Chronic allograft sclerosis					
None	16	*4*	*5*	*8*	
Stage 1	26	7	12	7	
Stage 2	6	4	2	0	
Stage 3	3	0	3	0	
Category 7 + 8 (other diagnosis)	**1**	0	1 (arteriolar hyaline)	0	
C4d status					.09
Negative	36	8	19	9	
Focal	9	2	3	4	
Diffuse	5	5	0	0	
Stain not done	2	0	0	2	

Note: Mann-Whitney *U* test was used for continuous data and Fisher's exact test was used for categorical data.

Abbreviations: A, antibody-mediated rejection, pure; AMR, antibody-mediated rejection; BM, Boehringer Mannheim (test strip for blood glucose level); DSA, donor-specific antibody; IQR, interquartile range; IS, immunosuppression; M, mixed rejection; NR, no rejection;T, borderline and T cell-mediated rejection; TCMR, T cell-mediated rejection.

[a]Analysis for A + M vs T + NR.

Significant p values (< 0.05) are high-lighted in bold.

TABLE 4 anti-HLA serology data

DSA DATA	All (n = 52)	A + M (n = 15)	T (n = 22)	NR (n = 15)
DSA positive Post-transplant, absolute Number (%)	24 (46%)	15 (100%)	5 (23%)	4 (27%)
Class I only, absolute number	6	4	1	1
Class II only, absolute number	5	4	1	0
Class I + II, absolute number	13	7	3	3
Pre-transplant sensitized (anti-HLA no DSA), absolute number	11	1	5	5
Pre-transplant DSA, absolute number	2	2	0	0
de novo *DSA, absolute number*	22	13	5	4

Abbreviations: A, antibody-mediated rejection, pure; DSA, donor-specific antibody; IQR, interquartile range; M, mixed rejection; NR, no rejection; T, borderline and T cell-mediated rejection.

ROC curve analysis revealed an AUC of 0.71 (95% CI: 0.57-0.86, $P = .004$) for identifying hAMR using the AMR 34-gene set. Youden's J statistic was used to define a threshold for the gene set expression diagnostic of hAMR as > 3.73 (sensitivity = 63.6%, specificity = 80%) (Figure 3A).

There were 6 cases of mixed rejection with a score below 3.73, all DSA + by definition. All 6 were A2 (ie had either hAMR or C4d but not both). Two had mild IAC; 1 had a v3 lesion with 2 low class I DSA (B55 with MFI = 400, B62 with MFI = 325) and negative C4d; and 2 had focal C4d (C4d1). Twelve NR or T cases had a score above 3.73, and in 3 cases, the score was > 20. All 3 had marked acinar inflammation.

3.5 | Outcome analysis

There were 19 death-censored graft failures among the 41 patients, at a mean of 727 days post-biopsy. If a patient had 2 or more

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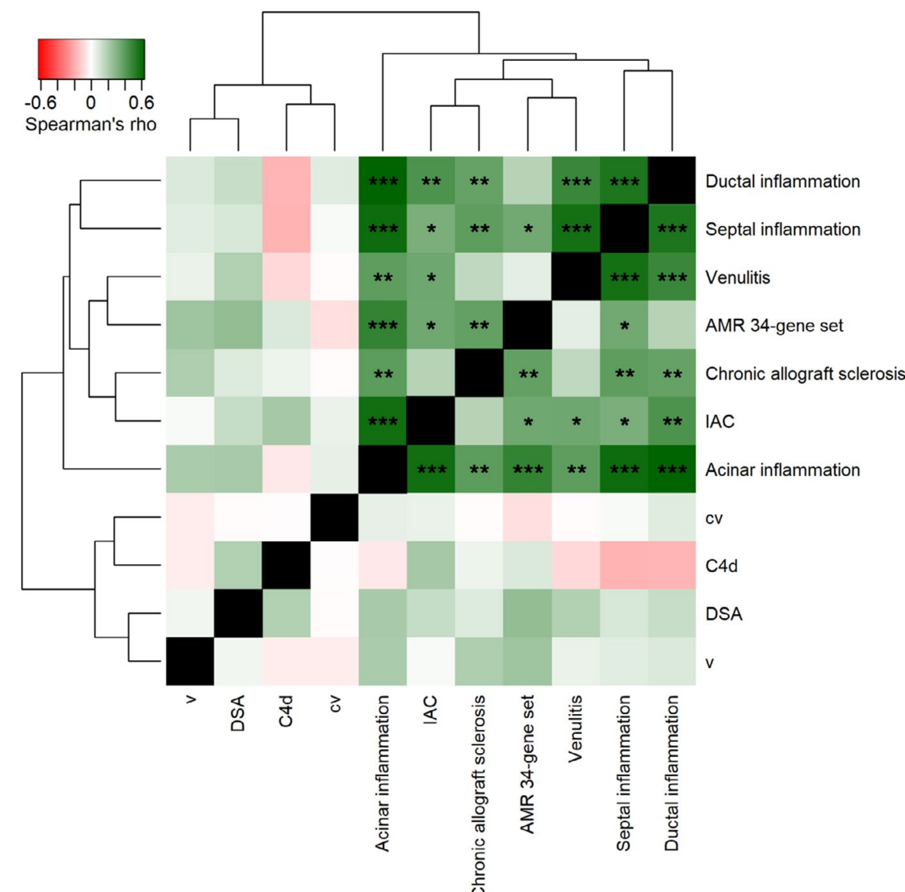

FIGURE 1 Correlation between histological, serological, and gene expression parameters. The heatmap represents correlations between parameters, color-coded by Spearman's rho statistic with green representing a positive correlation and red a negative correlation. Significant correlations are designated with asterisks (***$P < .001$, **$P < .01$, *$P < .05$). Parameters are ordered using hierarchical clustering by Euclidean distance

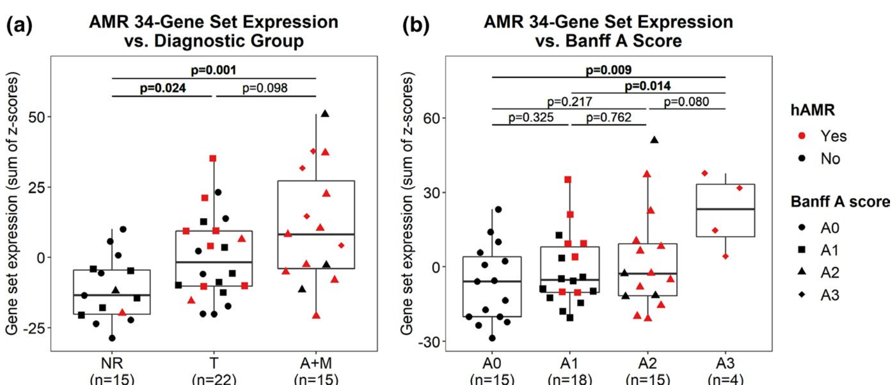

FIGURE 2 Expression of the AMR 34-gene set score comparing main diagnostic group (A) and comparing Banff A0-3 subgroups (B). Data are presented with median and interquartile range. Whiskers represent data points within 1.5× interquartile range from upper and lower box limits. Cases are color-coded in red if showing histological features of AMR (hAMR) and in black if not. Cases with all 3 features of AMR (A3) are represented in diamonds, those with 2/3 features (A2) as triangles, those with 1/3 features as squares, and those with none (A0) as circles

biopsies, data from the first biopsy with rejection were used for outcome analysis. On this basis, the 41 patients comprised 13 A + M, 9 NR, and 19 T.

To assess the potential additive value of AMR 34-gene set testing for predicting allograft failure, relative to other currently available diagnostic tools, Cox proportional hazards regression was first performed using relevant DSA, histology, and gene expression variables. Univariate Cox analysis demonstrated only AMR 34-gene set expression to be statistically significantly associated with death-censored pancreatic allograft failure (HR = 1.026, $P = .048$) (Table 5). Other variables with P-values < .1 included class II DSA (HR = 2.420, $P = .064$), acinar inflammation (HR = 1.581, $P = .072$), and IAC (HR = 2.295, $P = .080$). Assessment of these four variables in multivariate Cox analysis demonstrated none of them to be statistically significant. To simulate the clinical use of these variables, logistic regression models were then generated using different combinations of these four variables, referred to as DSA (class II DSA) (D), histology (acinar inflammation and IAC) (H), and gene expression

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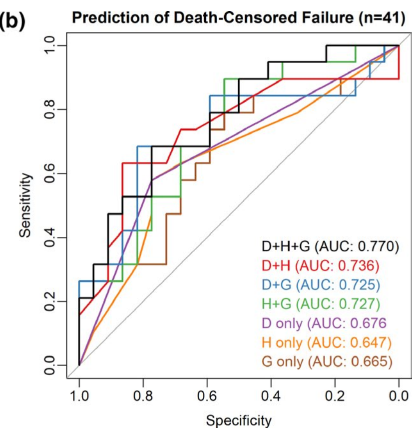

FIGURE 3 ROC curve analysis for the AMR 34-gene set score predicting hAMR status (A) and for predicting death-censored graft failure (B). (A) ROC curve analysis revealed an area under curve (AUC) of 0.71 (95% CI 0.57-0.86, $P = .004$) for identifying hAMR using the AMR gene set. Youden's J statistic was used to define a threshold for the gene set expression diagnostic of hAMR as > 3.73 (sensitivity = 63.6%, specificity = 80%). (B) ROC curve analysis revealed an area under curve (AUC) of 0.66 for G, 0.65 for H, and 0.68 for D for predicting death-censored graft failure. The full D + H + G model incorporating all of these variables demonstrated a larger ROC AUC of 0.77. D, DSA status (type II); H, histology variables (acinar inflammation and interacinar capillaritis); G, AMR 34-gene set expression

TABLE 5 Prognostic significance of serologic, histologic, and molecular parameters for predicting death-censored pancreatic allograft failure (n = 41[a])

	Univariate Analysis		Multivariate Analysis	
Variable	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Donor-specific antibodies				
Class I present	1.838 (0.728-4.640)	.198		
Class II present	2.420 (0.949-6.170)	**.064**	1.746 (0.635-4.796)	.280
Histological parameters				
Diagnostic group				
AMR (A)	0.69 (0.090-5.297)	.721		
TCMR/borderline (T)	1.029 (0.404-2.619)	.952		
Mixed AMR/TCMR (M)	1.968 (0.733-5.284)	.179		
No rejection (NR)	0.511 (0.147-1.770)	.289		
Acinar inflammation (0-3)	1.581 (0.959-2.607)	**.072**	1.198 (0.638-2.252)	.574
Septal inflammation (0-3)	1.689 (0.872-3.272)	.120		
Arteritis (v) (0-3)	0.814 (0.412-1.610)	.555		
Interacinar capillaritis (IAC) present	2.295 (0.905-5.818)	**.080**	1.219 (0.353-4.203)	.754
Ductitis present	1.631 (0.606-4.390)	.333		
Venulitis present	0.710 (0.280-1.803)	.471		
Arterial intimal fibrous thickening (cv) (0-3)	1.524 (0.735-3.158)	.257		
Chronic allograft sclerosis (0-3)	1.431 (0.843-2.429)	.185		
C4d (0-2)	1.223 (0.642-2.332)	.540		
AMR 34-gene set expression (sum of z-scores)	1.026 (1.000-1.052)	**.048**	1.018 (0.986-1.050)	.273

Abbreviations: AMR, antibody-mediated rejection; HR, hazard ratio.
[a]19/41 patients developed death-censored allograft failure.
Significant p values (< 0.05) are high-lighted in bold.

(AMR 34-gene set) (G) variables. The full D + H + G model incorporating all variables demonstrated a larger ROC AUC (0.770) than each of the reduced models, including the D + H model (AUC = 0.736) (Figure 3B), although these differences between AUCs did not meet statistical significance ($P \geq .084$) (Table 6). However, net reclassification index analysis demonstrated improved risk classification with the full D + H + G model vs the H + G (NRI = 0.703, $P = .015$), H only (NRI = 0.641, $P = .030$), and G only (NRI = 0.823, $P = .004$) reduced

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models. Integrated discrimination improvement also demonstrated superior discrimination with the D + H + G model compared with H only (IDI = 0.012, P = .028) and G only (IDI = 0.011, P = .033). These results suggest that gene expression may provide significant additive benefit for the prediction of allograft failure.

4 | DISCUSSION

Diagnostic criteria of AMR in pancreas allograft biopsies are derived from a consensus of experienced practitioners. The histological parameters used to reach a diagnosis of AMR and their cutoff values are arbitrarily set and poorly reproducible. Accordingly, outcome analysis has not always identified a robust association between histology and response to treatment or graft loss. There is an unmet clinical need for more precise diagnostic tools that are pathomechanism based, objective, and quantitative. In this study, we carry out the largest study so far of gene expression analysis in pancreas transplant biopsies, to demonstrate for the first time that a 34-gene set score is significantly increased in cases with histological features and diagnostic criteria of AMR. The score was the only feature at time of biopsy significantly associated with outcome, and the addition of the AMR 34-gene set score to other data present at the time of biopsy (DSA and histological features) improved outcome prediction.

This study expands on work by others showing a distinctive transcriptional profile of AMR in solid organ transplants.[15,26,29] The ability to detect this profile is particularly important in the pancreas allograft, where each of the 2 typical histological features of AMR pose problems: IAC in the context of severe acinar inflammation in TCMR may be over- or underestimated, whereas a v-lesion could be due to TCMR, AMR, or both. Most of the cases with hAMR in our cohort also showed features of TCMR and were classified as mixed rejection, and there was no statistically significant difference in AMR 34-gene set score between mixed rejection and TCMR categories. In some cases, the inability to identify a DSA may have led to cases of TCMR with histological features of AMR being classified as T; conversely some cases classified as mixed rejection because of the presence of a DSA and tenuous histological features (mild IAC or focal C4d) may in fact not have an AMR component. Across the whole cohort, 3 biopsies (2 M and 1 NR) had a DSA and focal C4d, without histological features of AMR, all of which had a low value for the AMR 34-gene set score, below threshold value for AMR. This finding suggests that the combination of DSA and C4d1 might best not be considered suspicious for ABMR.

Although the lack of association of the Banff diagnostic category with outcome in our cohort is in line with previous reports in the literature, other studies have shown an association between C4d staining and/or DSA and outcome, which we did not find.[5,6,8,9] In the case of DSA, the P-value was borderline and our negative observation could partly be due to small sample size. In addition, we selected a low cutoff value for DSA positivity (MFI > 500) and, because of the retrospective nature of our study, a wide time window of 6 months around the biopsy to capture matching DSA data. In the case of C4d, interpretation is difficult in the pancreas, plus we adhered to the more recent definition of C4d positivity (>1%), whereas other studies have used a threshold of 5%.[7,9] We also found no significant association between DSA and C4d, which may also be explained by the elements cited above.

We acknowledge that our study has limitations. Despite our multicenter, retrospective design recruiting cases as far back as 2006, we were only able to include a limited number of samples. Surgical technique, induction and baseline immunosuppression,

TABLE 6 Comparison of logistic regression model performance for predicting death-censored pancreatic allograft failure (n = 41[a])

	Logistic Regression Models						
Performance Measure[b]	1. D + H + G	2. D + H	3. D + G	4. H + G	5. D only	6. H only	7. G only
AUC, %	77.0	73.6	72.5	72.7	67.6	64.7	66.5
95% CI	(62.6-91.5)	(57.1-90.0)	(55.8-89.2)	(57.1-88.4)	(53.1-82.1)	(47.5-82.0)	(49.2-83.8)
P-value (vs D + H + G)		.543	.341	.425	.084	.146	.100
NRI, %		32.5	36.8	70.3	43.1	64.1	82.3
95% CI		(−27.7-92.8)	(−22.3-96.0)	(13.8-126.9)	(−16.8-103.0)	(6.1-122.1)	(26.3-138.3)
P-value (vs D + H + G)		.290	.222	**.015**	.159	**.030**	**.004**
IDI, %		4.2	2.7	6.1	8.7	12.0	11.0
95% CI		(−2.9-11.4)	(−3.0-8.4)	(−1.6-13.8)	(−0.5-17.9)	(1.3-22.7)	(0.9-21.1)
P-value (vs D + H + G)		.244	.355	.120	.065	**.028**	**.033**

Abbreviations: AUC, area under the receiver operating characteristic curve; D, DSA status (type II); G, AMR 34-gene set expression; H, histology variables (acinar inflammation and interacinar capillaritis); IDI, integrated discrimination improvement; NRI, net reclassification index.

[a]19/41 patients developed death-censored allograft failure.

[b]Models 2 through 7 were each compared with model 1. For model comparisons, NRI and IDI values greater than 0 indicate better performance with the full model 1 than the reduced models.

Significant p values (< 0.05) are high-lighted in bold.

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and post-biopsy treatment varied between cases and centers, and granular data on important serological markers (amylase, lipase, HbA1c, glucose, and C-peptide) were incomplete and non-standardized; therefore, the effect of these on outcome could not be tested. The AMR 34-gene set we used was derived from renal gene datasets[19,26] and may not represent the optimal panel for diagnosis of AMR in the pancreas. Application of whole transcriptome analysis and/or use of consensus commercial panels (eg nCounter Human Organ Transplant Panel) will help refine an ideal diagnostic panel for pancreas transplants, and these would be best investigated in the context of prospective, multi-center studies with complete and standardized data collection for demographic, histological, and serological data. We did not test promising emerging alternative molecular tests, including other rejection-related gene sets, or donor-derived, cell-free DNA, which may complement the gene set data, and allow improved differentiation of TCMR from AMR.

This study demonstrates for the first time the presence of a canonical AMR-related gene expression profile in pancreas allograft biopsies, which is predictive of poor outcome. It lays the groundwork for the necessary comprehensive multicenter validation of molecular testing by demonstrating the usefulness of gene analysis and the potential to use archives of well-annotated FFPE samples with long-term follow-up. The lack of a true diagnostic "gold standard" for diagnosing AMR requires such collaborative validation studies as the next step toward the adoption of molecular diagnostics into routine transplantation pathology.

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CONFLICT OF INTEREST

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation. M. Mengel received consultancy honoraria from Vitaeris Inc, Novartis, and CSL Behring.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, C.R., upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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