Novel transcriptomic signatures associated with premature kidney allograft failure

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Summary

Background The power to predict kidney allograft outcomes based on non-invasive assays is limited. Assessment of operational tolerance (OT) patients allows us to identify transcriptomic signatures of true non-responders for construction of predictive models.

Methods In this observational retrospective study, RNA sequencing of peripheral blood was used in a derivation cohort to identify a protective set of transcripts by comparing 15 OT patients (40% females), from the TOMOGRAM Study (NCT05124444), 14 chronic active antibody-mediated rejection (CABMR) and 23 stable graft function patients ≥15 years (STA). The selected differentially expressed transcripts between OT and CABMR were used in a validation cohort (n = 396) to predict 3-year kidney allograft loss at 3 time-points using RT-qPCR.

Findings Archetypal analysis and classifier performance of RNA sequencing data showed that OT is clearly distinguishable from CABMR, but similar to STA. Based on significant transcripts from the validation cohort in univariable analysis, 2 multivariable Cox models were created. A 3-transcript (*ADGRG3*, *ATG2A*, and *GNLY*) model from POD 7 predicted graft loss with C-statistics (C) 0.727 (95% CI, 0.638–0.820). Another 3-transcript (*IGHM*, *CD5*, *GNLY*) model from M3 predicted graft loss with C 0.786 (95% CI, 0.785–0.865). Combining 3-transcripts models with eGFR at POD 7 and M3 improved C-statistics to 0.860 (95% CI, 0.778–0.944) and 0.868 (95% CI, 0.790–0.944), respectively.

Interpretation Identification of transcripts distinguishing OT from CABMR allowed us to construct models predicting premature graft loss. Identified transcripts reflect mechanisms of injury/repair and alloimmune response when assessed at day 7 or with a loss of protective phenotype when assessed at month 3.

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Keywords: Kidney graft failure; Peripheral blood transcripts; Chronic antibody-mediated rejection; Operational tolerance; RNA sequencing

Research in context

Evidence before this study

Previous studies have demonstrated several peripheral blood transcriptomic signatures and their utility in the diagnostics of rejection after kidney transplantation. Prediction of kidney graft outcome based on peripheral blood transcripts has not been tested so far.

Added value of this study

In this study, we developed 2 models based on three peripheral blood transcripts and renal function at month 3 capable to predict premature graft loss at 3 years. Transcript selection for models was based on differential expression between unique patient cohorts with operational tolerance representing immune non responders and patients with antibody mediated rejection as high responders.

Implications of all the available evidence

Such biomarkers may help to identify low/high risk patients and to guide immunosuppression according to patients' individual needs.

Introduction

Despite improvement in short term kidney graft outcomes, the long-term graft survival had been improving only gradually,[1] largely due to chronic alloimmune graft injury.[2,3] A significant part of the posttransplant management deals with balancing the risks of alloimmune response on one hand and adverse effects of immunosuppression on the other. The tools currently available in transplantation medicine are insufficient at identifying patients at risk of premature kidney allograft loss and do not allow for safe and reliable tailoring of immunosuppression depending on individual patients' needs. Therefore, novel tools for identification of kidney transplant recipients (KTR) at risk of premature kidney allograft loss are needed.

Immune response to kidney allograft spans across a spectrum. At one end, there are patients prone to alloreactivity and development of serious rejection, most commonly chronic active antibody mediated rejection (CABMR), which leads to premature graft loss (i.e., high immune response KTR).[2] On the other end of the spectrum, there are KTR, whose graft functions remain stable and exhibit no signs of chronic alloimmune injury (i.e., low immune response KTR). A special case of low immune response KTR exists, the operationally tolerant patients, who do not develop detectable graft injury without any immunosuppression for more than 12 months.[4–6] Operationally tolerant patients could therefore help to identify novel biomarkers associated with premature graft failure. Even though the exact mechanisms of operational tolerance are yet unknown, we hypothesize that differentially expressed peripheral transcripts in the operationally tolerant and high immune response KTR may identify transcripts associated with immune hypo-responsiveness. Loss of these transcripts would lead to alloimmune injury and consequent premature graft loss. Furthermore, this novel approach resolves the problem of validation typical for operational tolerance due to it being an ultra-rare clinical phenomenon, as loss of "protective" transcripts leading to graft failure can be easily validated in large patient cohorts.

In this study we aimed to identify a set of "protective" peripheral blood transcriptomic signatures using whole transcriptome RNA sequencing of a novel European cohort of operationally tolerant patients and several comparator cohorts. The transcripts were validated in a large-scale cohort, and a predictive model based on the transcripts was constructed (Supplementary Fig. S1).

Methods

Derivation cohort

Patients

Operational tolerance (OT) patients reflecting true low immune response KTR (in fact non-responders) (n = 15) were defined as stable kidney allograft function (creatinine ≤ 150 μmol/l and proteinuria < 1 g/day) for at least 1 year after complete immunosuppression withdrawal. Those patients were identified in a previous study[7] and now the available blood samples were provided by 8 Transplant European Centers which participated in the TOMOGRAM study (NCT05124444, Fig. 1). Median time of immunosuppression-free period in the OT group was 8.9 years [min 1.1; max 22.9]. Kidney transplant recipients with stable graft function on immunosuppression (STA, n = 23) ≥ 15 years after transplantation, had stable renal function (creatinine in last 5 years < 150 μmol/l and without proteinuria, Supplementary Fig. S2) and this group reflected low-responders. Chronic active antibody mediated rejection was diagnosed based on recent Banff classification[8] but only patients ≥ 1 year after transplantation with serum creatinine plasma levels above 200 μmol/L, proteinuria > 1 g/day and transplant glomerulopathy score (cg) > 1

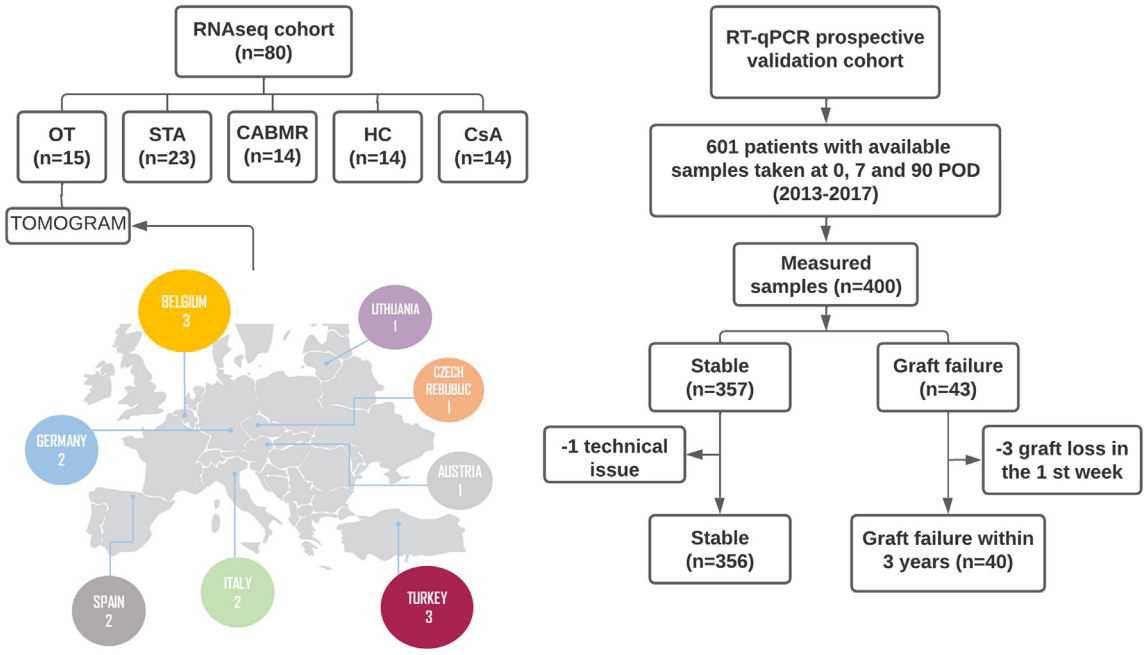

***Fig. 1:* Flow chart of the study**. OT, operationally tolerant; HC, healthy controls; STA, stable graft function; CABMR, chronic active antibody rejection; CsA, controls on CNI without graft; GF, graft failure. Blood samples from the OT patients were collected between April 2016 and March 2018 and the samples from the remaining groups were taken between June 2016 and September 2018.

were enrolled (CABMR, n = 14) to detect the worse progressive disease phenotype among high-responders. Chronic T-cell mediated rejection is considered as a relatively rare diagnosis and thus CABMR as a much frequent event was used to represent a true risk phenotype. The blood samples were taken at the same day as the biopsy was performed (Supplementary Fig. S2). No significant differences in glycemic control among OT, STA and CABMR groups were noticed and there were no intercurrent infections at the time of sampling.

Two additional control groups were used. Healthy controls (HC, n = 14) were age and sex matched to the OT group and represented otherwise healthy people without immunosuppression. Primary glomerulopathies (CKD stage 1 or 2) treated with calcineurin inhibitor immunosuppression (mostly cyclosporine A, CsA) represented another comparator with immunosuppression but without alloimmune response (n = 14) (Table 1). Transplant demographics for the OT, CABMR and STA groups are shown in Table 2 and Supplementary Fig. S3. The study protocol was approved by the Institutional Review Board of the Institute for Clinical and Experimental Medicine and Thomayer Hospital under G-18-13. All patients signed informed consent (G-18-13).

RNA isolation, library preparation and sequencing

Peripheral vein blood was drawn into PAXgene Blood RNA tubes (Qiagen, Hilden, Germany) and stored at –20 °C until analysis. Total RNA was isolated from peripheral blood (PAXgene blood RNA kit, Qiagen), concentration was measured by the Qubit fluorometer and RNA integrity number was checked using Agilent Bioanalyzer 2100. Isolated RNA (800 ng) was rRNA and globin depleted using NEBNext® Globin & rRNA Depletion Kit (New England, BioLabs, Inc). Transcriptome libraries for differential gene expression 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). Briefly, depleted RNA was randomly sheared by heat digestion in the presence of a divalent metal cation (Mg^{2+}). Sheared RNA was reversibly transcribed making 1st strand of cDNA using random hexamers as primers and reverse transcriptase. The second strand was created using dUTPs and after purification with Sample Purification Beads, NEBNext adapters were ligated. After removal of 2nd strand by uracil-DNA-dependent glycosylase, final amplification of adaptor ligated DNA using NEBNext® Multiplex Oligos for Illumina® was done. Library quality was assessed on a Bioanalyzer 2100 using Agilent DNA 1000 assay. Libraries from all 80 samples were pooled to a final concentration of 50 nmol and the quality of pooling was assessed by sequencing using MiSeq. Based on MiSeq results, final pool was enriched in 5 samples with significantly lower number of reads. High throughput sequencing of final pool was performed using NovaSeq

Group	n	Age at Tx	Age at sampling	Sex male, n (%)	Creatinine at sampling (µmol/l)	eGFR at sampling (ml/s)
Operationally tolerant (OT)	15	36 [12, 50]	56 [31, 83]	9 (60%)	99 [58, 131]	1.2 [0.8, 1.6]
Healthy controls (HC)	14	n/a	55 [32, 78]	8 (57%)	80 [47, 147]	1.6 [0.7, 2.0]
Stable graft function (STA)	23	46 [26, 76]	70 [42, 84]	14 (61%)	112 [58, 143]	0.9 [0.7, 1.5]
Chronic antibody-mediated rejection (CABMR)	14	40 [19, 65]	46 [21, 71]	12 (86%)	257 [211, 367]	0.4 [0.2, 1.1]
Controls on CNI without graft (CsA)	14	n/a	62 [26, 85]	11 (79%)	105 [62, 229]	1.0 [0.5, 1.7]

Tx, transplantation; n/a, not applicable for particular group.

Table 1: **Characteristics of compared groups.**

6000 S4 system (Illumina) with the following instrument settings: paired-end, 150 b, 4500–5000 million reads per lane. In total, 5,298,645 166 paired-end 150 b reads with high confidence were generated (mean 66.23 million reads per sample, mean read length 150 nt, mean GC content 50.68%).

Statistical processing

RNAseq data processing. Raw data from RNA sequencing were automatically processed by Basespace cloud interface (Illumina) in default settings. The quality of raw reads was evaluated using FastQC (v0.11.5) and MultiQC (v1.6). Clipping adaptor sequences was carried out using cutadapt (v1.18). The trimmed reads were aligned to the human cDNA reference (GeCh38) using bowtie2 (v2.1.0). The counts of reads mapped to the reference were extracted and used for differential gene expression analysis using SAMtools (v1.2). DESeq2 R library version 1.24[9] was applied to normalize read counts and to identify differences in gene expression between sample groups. First, we pre-filtered all the genes whose read count sum over all the available samples did not reach 10 as recommended in the standard workflow. 45,292 genes met this condition. Then, we normalized the read counts. DESeq2 uses the median of ratios methods that normalizes for RNA composition and sequencing depth. Next, a generalized linear DESeq2 model with default settings was applied to analyze differential expression. A multiple testing correction was performed using the Benjamini and Hochberg method. Finally, independent filtering was used to increase the detection power through omitting

	STA (n = 23)	CABMR (n = 14)	OT (n = 15)	p value
Recipient age, years	46 [26, 76]	40 [19, 65]	36 [12, 56]	0.053
Recipient sex, male, n (%)	14 (60.8%)	12 (86%)	9 (60%)	0.228
Retransplantation, n (%)	0 (0%)	5 (35.7%)	4 (26.7%)	0.021
Type of donor, deceased, n (%)	22 (95.7%)	12 (85.7%)	9 (60%)	0.011
Donor age, years	36 [12; 56]	50 [29; 73]	26 [14; 63]	0.002
Donor sex, male, n (%)	18 (78.3%)	8 (66.7%)	9 (64.2%)	0.604
Dialysis vintage, months	18 [3; 80]	16 [0; 91]	19 [3; 35]	0.796
HLA mismatch	3 [1; 4]	3 [1; 5]	0 [0; 4]	0.001
Peak PRA	8 [0; 97]	21 [0; 96]	0 [0; 70]	0.127
Cold ischemia	18 [0.9; 29]	14 [0; 23]	15 [0.8; 22]	0.013
Donor specific antibodies, n (%)	0[a]	11 (76%)	0[b]	
Original disease, n (%)				0.121
Glomerulonephritis	10 (43.4%)	4 (21.4%)	5 (33.3%)	
Polycystic kidney disease	6 (26.1%)	1 (7.1%)	1 (6.7%)	
Hypertension	0	1 (7.1%)	1 (6.7%)	
Diabetes	1 (4.3%)	1 (7.1%)	1 (6.7%)	
Pyelonephritis	6 (26.1%)	1 (7.1%)	2 (13.3%)	
Miscellaneous	0	4 (21.4%)	2 (13.3%)	
Unknown	0	2 (14.3%)	3 (20%)	
Maintenance IS at sampling				<0.001
No	0	0	15 (100%)	
CNI	12 (52.2%)	12 (85.7%)	0	
Non CNI	11 (47.8%)	2 (14.3%)	0	

PRA, panel reactive antibodies, CNI, calcineurin inhibitors. [a]In 19/23 STA patients donor specific antibodies were not examined. [b]In 9/15 OT patients donor specific antibodies were not examined.

Table 2: **Main transplant demographics of OT, STA and CABMR groups.**

genes with very low counts which may cause an increase in the number of genes with significant adjusted p-value. Gene annotation analysis was done using Enrichr.[10] Complete raw and normalized data were deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE222889.

Classifiers and archetypal analysis of RNAseq data. To evaluate the potential ability of peripheral transcripts to discriminate OT from other compared groups, we used 3 different classification algorithms, namely the generalized linear model fitted with a penalized maximum likelihood (GLMNET[11]), the nearest shrunken centroid with voom transformation (voomNSC[12]) and support vector machine with recursive feature elimination (SVM-RFE[13]). All these algorithms are well-known to be suitable for gene expression data classification, where one of the main goals is to avoid overfitting and identify a small set of transcripts that can be used as a biomarker, serve as a disease signature, etc. In all of the experiments, we use repeated 10-fold cross-validation to select a sparse model that ignores irrelevant and redundant transcripts and minimizes the classification error. To deal with unbalanced classes, the optimal numbers of transcripts (features) needed for best discrimination was checked by AUC (pROC package[14]). The transcript selection was based either on random choice (a simple uninformed benchmark) or native selection (the best combination of transcripts regardless they were differentially expressed or not, the selection methods fit the particular classification algorithms), in particular, penalized maximum likelihood for GLMNET, soft-thresholding for voomNSC and recursive feature elimination in the case of SVMs, or they were chosen as differentially expressed (DE). The experiments were carried out in the R environment (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (URL https://www.R-project.org/).

For the archetypal analysis we followed the workflow published by Reeve et al.,[15] where each sample is assigned 5 scores representing the belief of falling into each of the 5 clinical groups (OT, CABMR, STA, HC and CsA). The scores for each group were obtained using the examined classifiers (GLMNET, voomNSC and SVM-RFE). These classifiers were trained to differentiate the particular group from the rest (i.e., OT, CABMR, STA, HC and CsA, vs. rest) and their outputs combined via median to arrive at a single score. The analysis therefore involved 3 types of classifiers, each trained on 5 tasks. For the training, we used 10-fold cross-validation, where 80 samples were divided into training set (9 folds) and test set (1-fold). In contrast to Reeve et al., the features were not pre-selected as the top 20 differentially expressed genes (by p value); in our case, the feature set was selected by the classifiers themselves according to their native selection method. The decision stems from the comparative experiments conducted previously, where native feature selection often outperformed DE selection, especially in the 100 feature-size cases (for OT shown in Fig. 2A). Using DE selection however would not change significantly the conclusions of the analysis (not shown). The process of assigning 5 scores to all samples was repeated 10 times (10-fold cross validation was repeated 10 times). The final molecular score reflecting classification to a particular clinical group for each sample was calculated as the mean of all 10 repeats. These molecular scores were used as an input for principal component analysis (PCA). Eventually, archetypal analysis turned these scores into archetype scores to examine the distribution of molecular phenotypes. Archetypal analysis is an unsupervised method, similar to cluster analysis, that finds a small set of archetypes. In our case, archetypes represent the extreme points in the space of molecular scores and all the observed molecular score sample vectors can be well represented as convex combinations of the archetypes.[16] Archetype scores therefore describe each sample as a composite of underlying archetypes.[15] R package archetypes was used.[16] The whole workflow can be seen in Supplementary Fig. S4.

Validation cohort

Patients

Patients in the validation cohort had undergone kidney transplantation at the Institute for Clinical and Experimental Medicine in Prague between February 2013 and May 2017. PAXgene® Blood RNA tubes (Qiagen BD, Valencia, CA) have been prospectively collected from consent kidney transplant recipients (Ethical Board Approval A 13-02-01 for biobanking) at day 0 (prior the transplant surgery), and at days 7 and 90, respectively. All patients signed informed consent (A13-02-01). The sample size for the validation cohort was calculated for power = 0.8 at p = 0.05. Cohen effect size was estimated for graft failure based on *IGHM* expression at 90 post-operative day (POD).[17] The minimal sample size was set as 40 events (kidney graft failures) and 360 controls based on 90% kidney graft survival at 3 years. From 601 kidney transplant recipients who had undergone kidney transplantation in 2013–2017 and provided consent for biobanking, samples from 43 patients with graft failure at 3 years (cohort enriched for end-points) and 357 controls were analyzed (Fig. 1 Flow chart). Samples were obtained from our institutions' biobank consecutively based on date of transplant with enriched enrollment of patients with graft loss.

RT-qPCR

For the validation patient set, a custom-made Taqman low-density array (Applied Biosystems) was used for RT-qPCR to analyze 13 selected genes as described elsewhere.[18] Details are provided in Supplementary Methods.

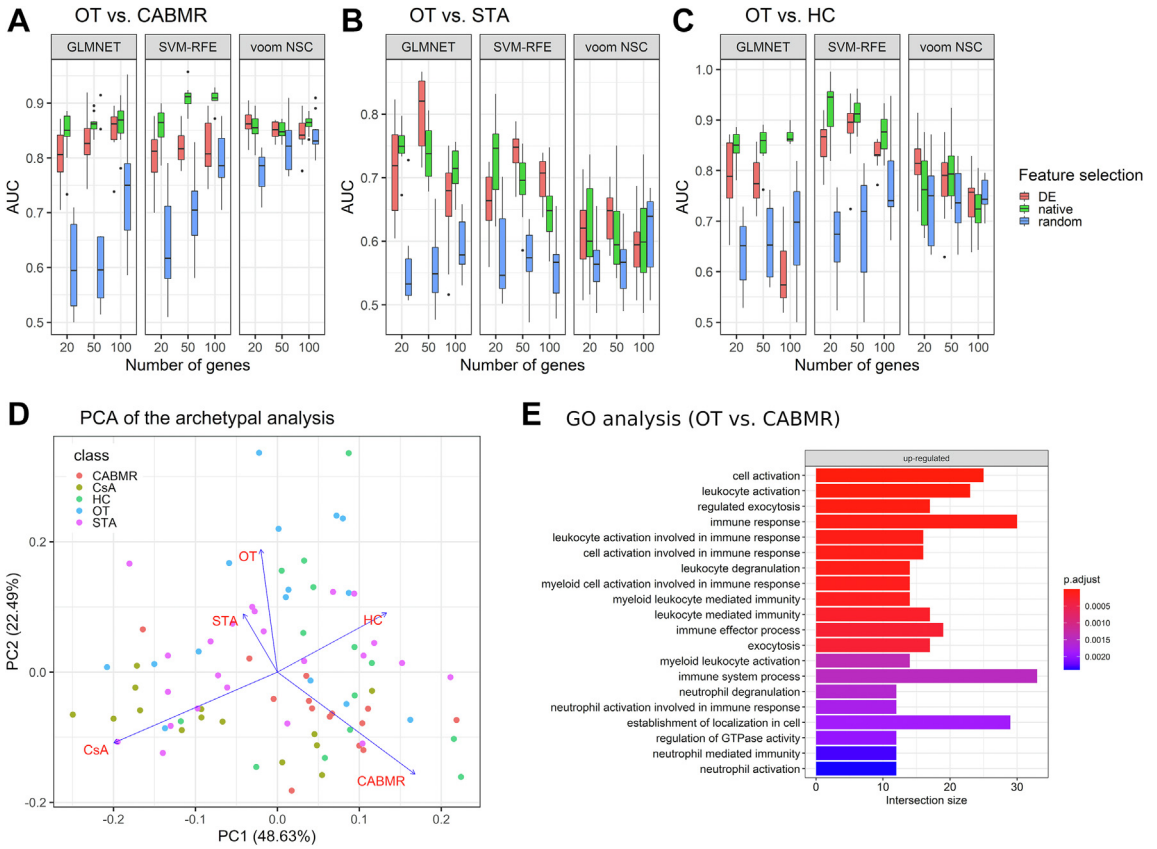

***Fig. 2:* Analysis of peripheral blood transcriptome of the 80 patients by RNA sequencing**. The ability of particular classifiers (GLMNET, voomNSC, SVM-RFE) to discriminate OT from CABMR (A), STA (B) and HC (C) based on either random, native or differential expression feature selection in 10-fold cross-validation. (D) Principal component analysis (PCA) of archetypes. For each sample, classifier scores discriminating one of 5 particular groups (OT, CABMR, STA, HC and CsA) from the rest of patients were calculated as a median of 3 classifiers (SVM-RFE, GLMNET, voomNSC) and these data were used as input for PCA. Each patient (dot) is colored by its clinical group. The superimposed arrows show the direction and relative magnitudes of the correlations between the 5 input variables and the PC scores. (E) Gene Ontology Biologic Process (GO) analysis of 107 increased transcripts in OT vs. CABMR. Intersection size displays the overlap between 107 increased transcripts and a particular GO term. GLMNET, the generalized linear model fitted with a penalized maximum likelihood; voomNSC, the nearest shrunken centroid with voom transformation; SVM-RFE, support vector machine with recursive feature elimination; AUC, area under curve; CV, cross-validation; DE, differential expression.

Statistics

Cox proportional hazard regression to predict 3-year graft failure based on the expression of selected transcripts, in both uni- and multivariable analyses were used. Only transcripts from univariable analysis with $p < 0.1$ were entered into multivariable models. Multicollinearity of transcripts was checked using variance inflation factor[19] and final multivariable models included only transcripts with variance inflation factor < 1.3. Transcripts with mutually high correlation coefficients were excluded from final models (ADGRG3_7POD correlated with ARHGAP9_7POD and MX2_7POD, IGHM3_90POD correlated with TCL1A_90POD, CD5_90POD correlated with SRCAP_90POD and IGKV4_1_90POD). From mutually correlated transcripts those with lower p-value were retained in final models. The proportional hazards assumptions of all multivariable models were tested using Schoenfeld residuals using Survival package.[20] Receiver operating characteristics (ROC) and corresponding area under curves for particular Cox regression models were calculated using survivalROC package[21] and the precision recall curves (appropriate for unbalanced classes) using PRROC package.[22] Confidence intervals for the AUCs were calculated as 2.5th and 97.5th percentiles of 1000 × bootstrap resampling. Kaplan Meier plots for graft survival in patients with low/high expression of six transcripts included in final models were constructed using package survival[20] and optimal cut-off points were calculated using package cutpointr.[23]

Role of funders
The funders had no role in designing this study, data acquisition, analysis and interpretation, and decision to publish or prepare this manuscript.

Results

Classifier's ability to separate OT from other groups

We performed RNA sequencing of peripheral blood samples from OT patients aiming to discriminate them from other groups (CABMR, STA and HC). Three different methods (GLMNET, voomNSC and SVM-RFE) with 10-fold cross validation were used to calculate classifiers performance. OT is clearly separable from CABMR group based on both native and differential feature selection with AUC > 0.8 and this performance can be achieved with selection of only 20 transcripts (Fig. 2A). Furthermore, even 100 random transcripts have reasonable AUC >0.75. OT and CABMR thus differ in high number of transcripts that can be selected randomly. Although the discrimination of OT from STA group using differential or native feature selection is better than random feature selection, discrimination ability is much lower (AUC from 0.6 to 0.75) (Fig. 2B). Discrimination of OT from HC is also achievable using a small set of transcripts that are differentially expressed with AUC around 0.8 (Fig. 2C).

Archetypal analysis

Peripheral blood molecular classifiers for estimating each of 5 clinical phenotypes (OT, CABMR, STA, HC, CsA) were generated for each sample as a median of 3 classifiers (GLMNET, SVM-RFE, voomNSC) in 10-fold cross validation (Supplementary Table S1). These data were used as the input for unsupervised PCA (Fig. 2D) with the aim to identify potential clusters of molecular archetypes. PC1 and PC2 explained 71% of variance in the data. Although, we cannot see clear clustering of groups, which corresponds to poor separability of a particular comparison (OT vs. STA), the direction of correlations suggested similarity of STA and OT and their anti-correlation to CABMR. We generated 6 different archetypes (their number based on the elbow method application to the residual sum of squares curve, Supplementary Fig. S5). Although the archetypes proved to be related to formerly defined clinical classes, there was no further clarification of the relationship among them.

Top deregulated transcripts associated with non-responder phenotype

169 differentially expressed transcripts (corresponding to 136 genes) between OT and CABMR groups were found (adjusted p value < 0.05, FC > 1.3, Supplementary Fig. S6). 107 transcripts (80 genes) were increased and 62 (57 genes) decreased in OT compared to CABMR (Supplementary Table S2). Gene annotation analysis of increased transcripts revealed among the most significant GO terms leukocyte (GO:0045321, p = 5.74E-06), myeloid (GO:0002275, p = 1.28E-04) and neutrophil activation (GO:0002283, p = 1.78E-03) and regulation of GTPase activity (GO:0043087, p = 0.002). In operational tolerance, neutrophils may inhibit T cell activation[24] and inflammation.[25] Regulation of GTPase activity has been associated with operational tolerance in another 2 studies.[6,26] Down-regulated transcripts in OT showed no significant GO term enrichment (Fig. 2E). No deregulated transcripts were found between OT and STA groups which corresponds well to the poor performance of classifiers. Similarly, no deregulated transcripts were found between OT and HC groups.

Transcriptomic and clinical models associated with premature graft failure

Premature graft loss was defined as 3-years death-censored graft failure. Ten transcripts were selected as differentially expressed between OT and CABMR (adjusted p value < 0.05, FC > 1.3; *ADGRG3*, *GNLY*, *ATG2A*, *PF4*, *ARHGAP9*, *CD5*, *SRCAP*, *SLA2*, *MX2*, and *SLIRP*). All selected transcripts but *SLIRP* had higher expression in the peripheral blood in OT patients and were not substantially influenced by immunosuppression in the model adjusted for immunosuppression (see Supplementary methods) (Supplementary Fig. S7). In addition, three transcripts (*TCL1A*, *IGKV4-1*, and *IGHM*) previously described to be associated with operational tolerance[4,5,27] were evaluated. However, the expression of *IGKV4-1* and *IGHM*, was significantly influenced by immunosuppression (Supplementary Fig. S7).

All 13 transcripts were measured by RT-qPCR in a prospective validation cohort of kidney transplant recipients (n = 396) during the first 3 months after transplantation (0, 7 and 90 POD) aiming to describe a model associated with premature graft loss. Patients' demographics of the validation cohort are given in Table 3.

	Graft failure within 3 years		p value
	Yes (n = 40)	No (n = 356)	
Recipient age, years	56 [25; 79]	57 [21; 78]	0.721
Donor age, years	57 [26; 79]	55 [15; 83]	0.327
HLA mismatch	4 [0; 6]	3 [0; 6]	0.002
Peak PRA	8 [0; 100]	8 [0; 100]	0.983
Cold ischemia, hours	16 [0; 27]	15 [0; 25]	0.8
Dialysis vintage, months	34 [0; 137]	28 [0; 173]	0.278
Thymoglobulin induction	27 (68%)	226 (64%)	0.729
Delayed graft function	27 (68%)	110 (28%)	<0.001
Rejection by the 1st week	12 (30%)	15 (4%)	<0.001
Rejection by the 3rd month	26 (65%)	70 (20%)	<0.001

29 out 40 patients lost their grafts based on rejections (ABMR, n = 22; TCMR, n = 7). Three patients lost graft due to diabetic nephropathy, 1 had IgAN recurrence, 2 BK nephropathy in combination with chronic rejection, 1 cardiorenal syndrome, 2 infectious complications, and 2 had bad renal function with progression from transplantation. PRA, panel reactive antibodies.

Table 3: **Transplant demographic of validation cohort (n = 396).**

Patients with graft failure at 3 years experienced poorer HLA match, higher rate of delayed graft function and acute rejection at 7 and 90 days, respectively.

A Cox proportional hazard model was built to predict 3-year graft failure at first in univariable analysis of all measured transcripts at each time-point (Supplementary Table S3). Based on the significance of transcripts in univariable analysis and after exclusion of correlated transcripts final models were generated (Fig. 3). Three transcripts' signatures from day 7 (*ADGRG3*, *ATG2A* and *GNLY*) and from month 3 (*IGHM*, *CD5*, *GNLY*) predicted graft failure with AUC = 0.727 (95% CI, 0.638–0.820) and 0.786 (95% CI, 0.785–0.865), respectively (Fig. 3,A1 and B1, Supplementary Table S4A and B). Graft survival was significantly worse in patients with high expression of *ADGRG3* and *ATG2A* or low expression of *GNLY*, *IGHM* and *CD5* as shown by Kaplan–Meier plots (Supplementary Fig. S8).

In a model predicting 3-year graft failure, based on the most significant variables from univariable analysis, clinical variables known at transplantation (recipient and donor age, HLA mismatch, peak PRA, cold ischemia, dialysis vintage, retransplantation) only HLA mismatch remained significant (HR = 1.49, p = 0.002) and AUC reached 0.664 (95% CI, 0.570–0.755) which is less than our model of 3 transcripts at months 3 (AUC = 0.786) (Supplementary Table S4C). Renal function at 3 months (estimated as eGFR in ml/s/1.73 m^2) predicted 3-year graft failure with AUC = 0.789 (95% CI, 0.703–0.875) which is comparable to our 3 transcripts model at 3 months (Supplementary Table S4E).

In univariable analysis, delayed graft function and rejection within first 3 months predicted graft failure with AUC 0.676 and 0.717, respectively. In multivariable analysis of clinical variables known at month 3 (HLA

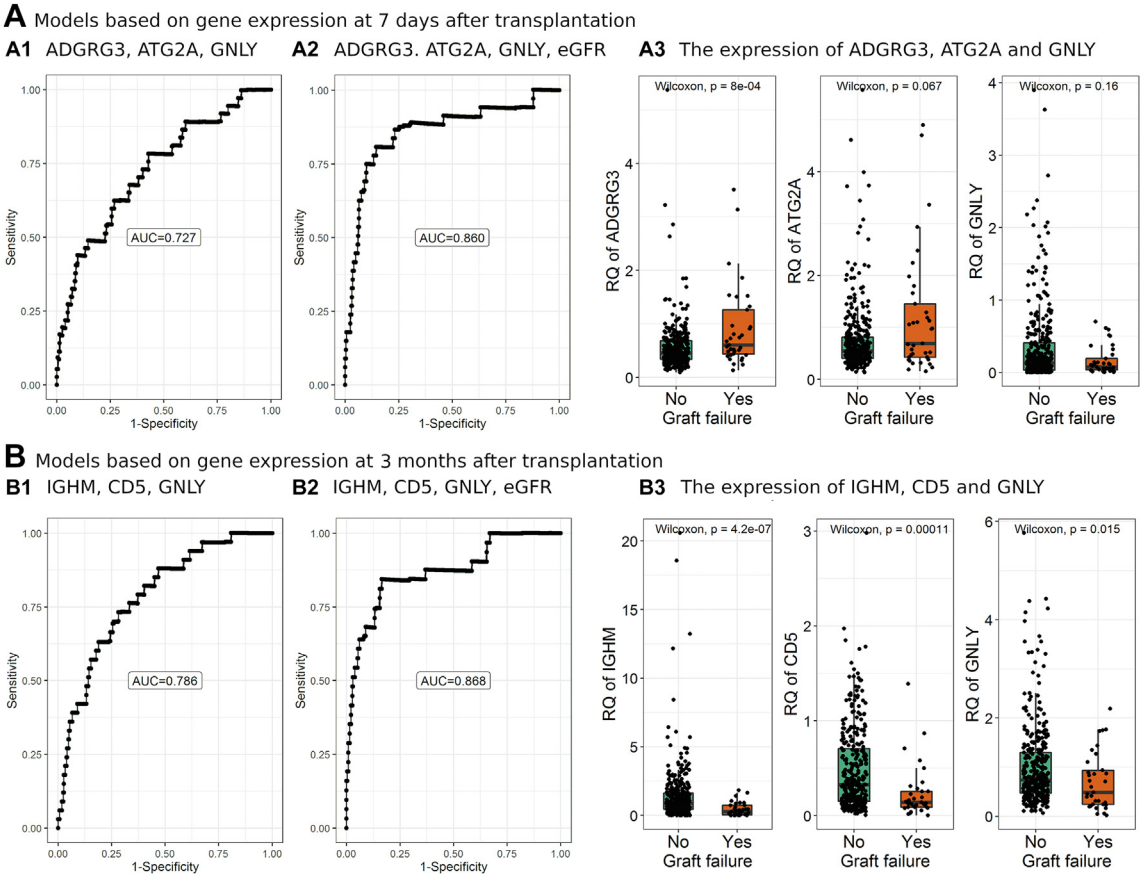

***Fig. 3*: Cox regression models predicting 3 - year graft failure based on peripheral blood 3 - transcripts signatures at 7 days (A) or 3 months (B) after transplantation**. ROC analysis according to the multivariable Cox regression model consisting of the expression of *ADGRG3*, *ATG2A*, and *GNLY* (A1) and *ADGRG3*, *ATG2A*, *GNLY* and eGFR at 7 POD (A2). The expression of *ADGRG3*, *ATG2A*, and *GNLY* at 7 days after transplantation in patients with/without graft failure in 3 years (A3). ROC analysis according to the multivariable Cox regression model consisting of the expression of *IGHM*, *CD5*, and *GNLY* (B1) and *IGHM*, *CD5*, *GNLY*, and eGFR at 3 months (B2). The expression of *IGHM*, *CD5*, and *GNLY* at 3 months after transplantation in patients with/without graft failure in 3 years (B3). ROC, Receiver operating characteristic; AUC, area under curve; eGFR, estimated glomerular filtration rate (ml/s/1.73 m^2).

mismatch, delayed graft function, rejection by month 3, renal function at 3 months), AUC reached 0.856 (95% CI, 0.781–0.932, Supplementary Table S4D). The multivariable model based on clinical variables known at day 7 (HLA mismatch, delayed graft function and rejection within the first week after transplantation) reached AUC 0.817 (95% CI, 0.747–0.886, Supplementary Table S4F).

When eGFR at 3 months was added to multivariable 3-transripts models, the AUC increased to 0.860 (95% CI, 0.778–0.944) for a model predicting outcome from transcripts measured at 7 days after transplantation (Fig. 3,A2, Supplementary Table S4H) and 0.868 (95% CI, 0.790–0.944) for a model predicting outcome from transcripts measured at 3 months after transplantation (Fig. 3,B2, Supplementary Table S4G). The effect of potential confounders known before transplantation (HLA mismatch, donor age and gender, donor type, recipient age and gender, retransplanation and cold ischemia time) on the expression of transcripts included in final models was negligible (Supplementary Table S5).

As our validation cohort consisted of unbalanced classes, performance of models was evaluated also by calculating area under curve of the precision recall curves (AUPRC). Similarly, as using AUROC (area under receiver operating curves) the top performing AUPRC had models combining the expression of 3 transcripts and renal function at month 3 (AUPRC = 0.553, 95% CI 0.379–0.727 for expression of *GNLY*, *CD5* and *IGHM* at month 3 and AUPRC = 0.478, 95% CI 0.314–0.661 for transcripts *ADGRG3*, *ATG2A* and *GNLY* expressed at day 7 (Table 4).

The intragraft expression of selected transcripts

To answer the question whether peripheral blood transcriptome is mirrored in the graft tissue, the intragraft expression of all 13 validated transcripts in paired samples from blood and protocol kidney graft biopsies at 3 months in 26 patients were assessed. We found all transcripts to be detectable in kidney allograft biopsies, and most of them (9/13) have significantly lower expression in the biopsy as compared to blood (Supplementary Fig. S9A). However, three transcripts, *ATG2A*, *SLIRP* and *SRCAP*, were significantly more expressed in the biopsy (Supplementary Fig. S9B), and the expression of *IGKV4-1* did not differ between biopsy and peripheral blood. When looking at peripheral transcripts which predicted premature graft loss, the expression of *GNLY* in kidney graft tissue was higher in patients who lost their grafts at 3 years in comparison with those who did not (Supplementary Fig. S9C).

Discussion

Reliable prediction of the risks of adverse alloimmune events in kidney transplant recipients may help clinicians to adapt therapeutic approaches and monitoring strategies. In this retrospective cohort study, we identified a set of "protective" peripheral transcripts typical for immunologically non-reactive individuals and validated these findings in a large cohort. Finally, we constructed several models that predict 3-year graft failure and are based on peripheral transcripts analyzed either at 7 days or at 3 months post-transplant.

In comparison with previous reports, we used a different approach to the prediction of kidney graft survival in our current study. Most importantly, we have identified a large cohort of new European patients with operational tolerance and founded a European multicenter study[7] under the umbrella of DESCARTES, the European Renal Association Working Group, to study transcriptomic and genetic abnormalities in this cohort.[28] By comparing the peripheral blood transcriptomic signatures of operationally tolerant patients with several comparator cohorts, including high responders with CABMR, we were able to identify a set of "protective" peripheral transcripts. We were then able to validate the findings in a large cohort that consisted of 396 patients, including 40 who experienced graft loss within a 3-year follow-up. Based on these results, we constructed several prediction models of kidney allograft failure.

Model	AUROC	95% CI	AUPRC	95% CI
ADGRG3, *ATG2A* and *GNLY* at day 7	0.727	0.636–0.817	0.291	0.148–0.443
ADGRG3, *ATG2A* and *GNLY* at day 7 and renal function at month 3	0.862	0.784–0.940	0.478	0.314–0.661
IGHM, *CD5* and *GNLY* at month 3	0.783	0.704–0.863	0.285	0.149–0.431
IGHM, *CD5* and *GNLY* at month 3 and renal function at month 3	0.865	0.788–0.942	0.553	0.379–0.727
Clinical variables known at transplantation[a]	0.666	0.578–0.755	0.183	0.104–0.285
Clinical variables known at month 3[b]	0.836	0.770–0.901	0.335	0.218–0.465
Clinical variables known at day 7[c]	0.815	0.745–0.886	0.343	0.210–0.502

[a]HLA mismatch, donor age, retransplantation, dialysis vintage, recipient age, cold ischemia time, peak PRA. [b]HLA mismatch, delayed graft function, rejection by month 3, renal function at month 3 (eGFR). [c]HLA mismatch, delayed graft function, rejection by week 1.

Table 4: **AUROC and AUPRC of individual models predicting 3 years graft failure.**

Predictive models of kidney graft failure have been given a lot of attention recently, even to the point of proposing such models as a surrogate endpoints in clinical studies.[29] Interestingly, a recently described model utilizing histological, immunological, and clinical variables obtained at indication biopsy in a much larger cohort[30] predicts graft failure at 3-years with C statistics of 0.835. Compared to our models iBox estimates graft survival at any time after transplantation, but includes Banff scores of biopsy results and donor specific antibodies as strong predictors of graft loss. Both our models, constructed only from three selected transcripts and eGFR, are based on a non-invasive assessment only.

We found a set of 3 different protective transcripts at day 7 (*ADGRG3*, *ATG2A* and *GNLY*) and at month 3 (*IGHM*, *CD5*, and *GNLY*). Granulysin (*GNLY*) transcripts from both the day 7 and month 3 were selected into final model. *GNLY* transcripts originating from both T and NK cells are released upon antigen stimulation and were shown to be increased in the graft tissue as well as in the peripheral blood[31] or urine sediments[32] during acute allograft rejection. The increased *GNLY* transcripts were found also in kidney biopsies from patients with donor-specific antibodies and ABMR,[33] and prediction of graft loss based on this transcript has been also recently discussed.[34] Another selected transcript, *ADGRG3*, encodes the adhesion G protein-coupled transmembrane receptor transcribed in immune cells.[35] A role of *ADGRG3* was suggested in the B cell maturation[36] and acute kidney injury[37] and may thus be associated with premature allograft loss. *ATG2A* (autophagy related 2A protein) was significant in our model as well. Autophagy plays an important role in normal proximal tubule function and recovery from acute ischemic kidney injury.[38] Therefore, two out of three transcripts used in the model from day 7 are associated with ischemic injury, which is supported by the fact that cold ischemia time and delayed graft function are well-established factors of impaired kidney allograft survival.[39] In the discovery cohort, increased expression of *ADGRG3* and *ATG2A* was observed in patients with operational tolerance. Contrary, in the validation cohort, a higher expression of those transcripts measured at day 7 predicted risk of graft failure. While early after transplantation such transcripts may be associated with peritransplant ischemic injury, later, they may reflect changes in B cell compartment, well described in OT patients.[4,18]

For the month 3 model another two transcripts, *CD5* and *IGHM*, were selected. CD5 transcripts were decreased among the patients with allograft loss at 3 years in our study. This finding is in line with the evidence that peripheral CD5+ B lymphocytes producing IL-10 were detected among operationally tolerant patients.[40] Furthermore, Cherukuri et al. found the CD5 antigen on the surface of transitional B cells[41] that were shown to be more frequent among patients without acute rejection.[42] Accordingly, CD5 transcripts were decreased among patients with allograft loss at 3 years in our study.

IGHM (Immunoglobulin Heavy Constant Mu) peripheral transcripts were previously shown to be reduced in patients with acute rejection compared to operationally tolerant patients.[27] However, as *IGHM* expression is affected by immunosuppression,[43] it cannot be used for detection of operational tolerance. However, it may be used to detect low immune response recipients. Both *CD5* and *IGHM* seem to play a protective role and lower expression of these transcripts may signal higher alloimmune reactivity.

Interestingly, principal component analysis of molecular archetypes showed no difference between operationally tolerant and low immune response recipients with stable graft function and no proteinuria many years after transplantation. This is in contrast to previously published studies.[4,5] In our study, a new cohort of 15 patients with operational tolerance was used, but the low incidence of this phenomenon can lead to bias and overfitting. Therefore, we selected 3 previously described transcripts associated with OT (*TCL1A*, *IGHM* and *IGKV4-1*) and showed that they also predicted graft loss in a validation cohort similarly to transcripts selected in our study. Final models were created using the most significant transcripts while eliminating their collinearity.

The strength of this study is the unique design and large validation cohort. Furthermore, we have studied transcriptomic signatures from peripheral blood, which is non-invasive and can therefore be repeated and does not expose the patients to the potential adverse events associated with allograft biopsy. Finally, our validation prospective cohort was enrolled in a single center. This ensures a homogenous approach with regards to immunosuppression protocols and rejection treatment strategies.

There are several limitations of this study. First, the numbers of operationally tolerant patients were small due to extremely low incidence.[7] Second, it is well known that some peripheral transcripts are affected by immunosuppression. To avoid this bias, we used a previously described drug-adjusted approach[44] in the discovery cohort. However, in our setting of relatively small sample size and high numbers of measured transcripts this approach may have ultimately led to exaggerated adjusted coefficients. Therefore, it was decided that the selection of 10 transcripts for validation should be based mainly on transcripts not influenced by immunosuppression. As other potential confounders concerning, only HLA mismatch adjustment affected the significance of candidate genes in a discovery cohort (data not shown). Clearly, such analysis was

underpowered, however HLA mismatch is among the most important variables affecting kidney graft outcome which must be taken into account while interpreting our results. In the validation cohort which was much larger, however, transcripts used in final models remained significant even after adjustment for clinical variables including HLA mismatch. In the validation cohort, donor specific antibodies were measured only in high-risk patients and thus this variable could not be included in the model. Finally, several transcriptomic studies on tolerant or immune quiescence patients described different sets of protective genes.[4–6,45] The lack of reproducibility can be explained by different cohorts used as comparators.

In conclusion, using a unique cohort of operationally tolerant patients, we identified a set of peripheral transcripts typical for non-immune response recipients and after validation in a much larger cohort, we constructed two models based on 3 peripheral transcripts from early post-transplant period, that predict premature graft loss at 3 years with a good performance. The studied transcripts may in the future help to identify kidney transplant recipients at risk of rejection and premature graft failure and ultimately allow for better immunosuppression tailoring.

Contributors

OV, PH, DA, AM and MA developed the concept and designed the study. PH, PM and EG performed RNA sequencing and RT-qPCR measurements. JK, AW-Le and PH did the statistical analysis. AM coordinated blood sampling and data collection from patients with operational tolerance. GBP, LB, ARP, MAG, NS, MSS, LW, AM, KB, BW, MM and OV have recruited operationally tolerant patients and provided their clinical data. DM recruited majority of CKD patients from control CsA group and provided their clinical data. LV provided pathological results. OV, PH, JK, IZ drafted the manuscript. All authors contributed to data interpretation and the critical revision of the manuscript. All authors read and approved the final version of the manuscript. The underlaying data were verified by OV, PH and JK.

Data sharing statement

Data from RNA sequencing are available on Gene expression Omnibus under accession number GSE222889. Data from validation will be made available on reasonable request to the corresponding author, after approval by the local ethics committee.

Declaration of interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/disclosure-of-interest/. All authors declare no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104782.

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