Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

PORTLAND PRESS

Research Article

Early isolated V-lesion may not truly represent rejection of the kidney allograft

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Intimal arteritis is known to be a negative prognostic factor for kidney allograft survival. Isolated v-lesion (IV) is defined as intimal arteritis with minimal tubulointerstitial inflammation (TI). Although the Banff classification assesses IV as T cell-mediated rejection (TCMR), clinical, and prognostic significance of early IV (early IV, eIV) with negative C4d and donor-specific antibodies (DSA) remains unclear. To help resolve if such eIV truly represents acute rejection, a molecular study was performed. The transcriptome of eIV (n=6), T cell-mediated vascular rejection with rich TI (T cell-mediated vascular rejection, TCMRV, n=4) and non-rejection histologic findings (n=8) was compared using microarrays. A total of 310 genes were identified to be deregulated in TCMRV compared with eIV. Gene enrichment analysis categorized deregulated genes to be associated primarily with T-cells associated biological processes involved in an innate and adaptive immune and inflammatory response. Comparison of deregulated gene lists between the study groups and controls showed only a 1.7% gene overlap. Unsupervised hierarchical cluster analysis revealed clear distinction of eIV from TCMRV and showed similarity with a control group. Up-regulation of immune response genes in TCMRV was validated using RT-qPCR in a different set of eIV (n=12) and TCMRV (n=8) samples. The transcriptome of early IV (< 1 month) with negative C4d and DSA is associated with a weak immune signature compared with TCMRV and shows similarity with normal findings. Such eIV may feature non-rejection origin and reflect an injury distinct from an alloimmune response. The present study supports use of molecular methods when interpreting kidney allograft biopsy findings.

Introduction

Intimal arteritis, also called v-lesion, is known to be a negative prognostic factor for kidney allograft survival [1-3]. According to the Banff classification, intimal arteritis is characterized by infiltration of mononuclear cells beneath the arterial endothelium. It is considered pathognomonic of acute rejection, either T cell- (TCMR) or antibody-mediated rejection (ABMR) [4,5].

Isolated v-lesion (IV), defined as intimal arteritis that occurs with minimal concurrent tubulointerstitial inflammation (TI), poses a challenge of unclear clinical and prognostic significance. According to the Banff classification, arterial involvement is sufficient to classify IV as type II or III acute TCMR regardless of interstitial inflammation (i) and tubulitis (t). IV could also be considered as ABMR in the presence of donor-specific antibodies (DSA) and evidence of antibody interaction with the endothelium (C4d positive staining, moderate microvascular inflammation (MI) or gene expression markers of endothelial injury in the biopsy tissue) [6].

Received: 27 August 2018
Revised: 28 September 2018
Accepted: 02 October 2018

Accepted Manuscript Online: 04 October 2018
Version of Record published: 29 October 2018

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

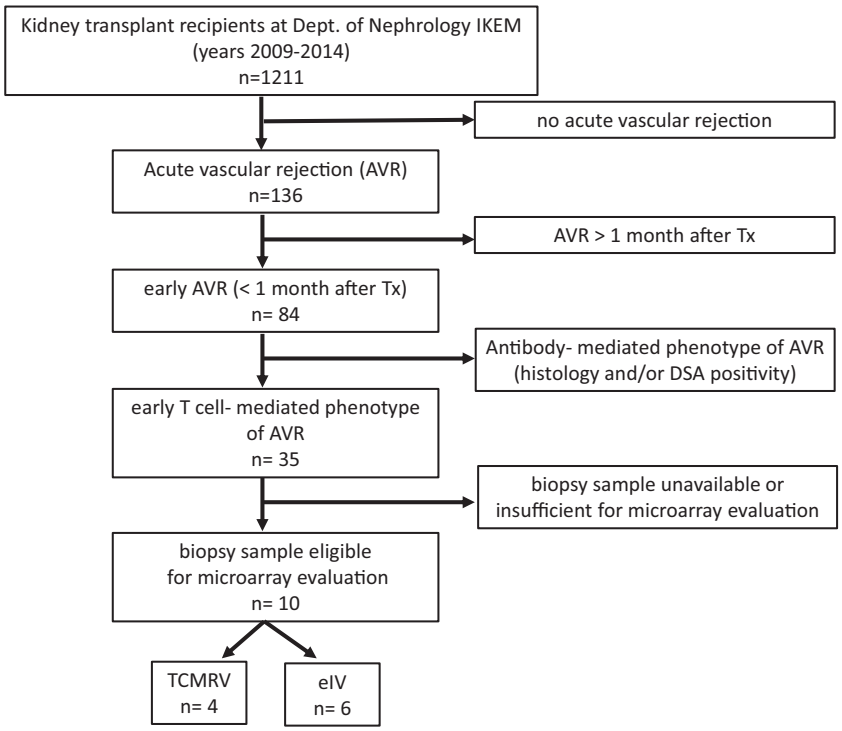

Figure 1. Study flowchart of the training set (cross-sectional screening)

All patients after kidney transplantation at our department in the years 2009–2014 were considered for inclusion in the study. Abbreviations: AVR, acute vascular rejection; DSA, donor specific antibodies; eIV, early isolated v-lesion; IKEM, Institute for Clinical and Experimental Medicine; TCMRV, T cell-mediated vascularrejection; Tx, transplantation.

However, the role of the immune response in the pathophysiology of IV remains unclear. Recent studies have reported diverse findings on IV response to antirejection treatment, clinical and prognostic significance [7-12]. While some authors believe in hidden ischemic/reperfusion injury, others are convinced of its rejection origin.

In response to the limitations of conventional histologic assessment, molecular technologies are being explored in the quest for greater precision [13]. In the transplantation field, a few genome-wide transcriptional studies have aimed to elucidate the origin of IV [14-16]. However, inclusion of heterogeneous samples regardless of DSA and time after transplantation, brought no clear findings, particularly on the non-antibody-associated phenotype of IV.

Therefore, this retrospective study was conducted to thoroughly evaluate the transcriptome of early v-lesion within the first month after kidney transplantation with no signs of ABMR (negative DSA, absence of C4d, and MI) and minimal (eIV) or rich (T cell-mediated vascular rejection, TCMRV) tubulointerstitial inflammation.

Materials and methods

Study design and patients

An observational, nested case–control study within a large single-center retrospective cohort study was performed to compare the expression profiles of renal allograft biopsies with histologic findings of eIV and TCMRV. eIV was defined as intimal arteritis ($v > 0$) with minimal tubulitis ($t \leq 1$) and interstitial inflammation ($i \leq 1$), no MI [glomerulitis (g) + peritubular capillaritis (ptc) = 0], with C4d and DSA negativity. TCMRV was defined as the presence of intimal arteritis with significant TI ($t > 1$, $i > 1$) and negative C4d, MI and DSA and served as a positive control. Only indication kidney allograft biopsies performed within the first month of post-transplant were included. As a negative control group, normal histologic findings from 3-month protocol biopsies without a history of previous rejection were included.

For a training set, 18 patients undergoing kidney transplantation at the Prague-based Institute for Clinical and Experimental Medicine (IKEM) from 2009 to 2014, with eIV (n=6), TCMRV (n=4), or normal histology (n=8) and sufficient biopsy samples for microarray evaluation were selected. A flowchart of the training set is provided in Figure 1, patients' baseline characteristics are shown in Table 1.

Clinical Science (2018) **132** 2269–2284
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Table 1 Characteristics of patients in the training set

	TCMRV (*n*=4)	eIV (*n*=6)	normal (*n*=8)	*P*
Donor characteristics				
Age, years	44.5 (40.3; 57)	58.5 (51.3; 68.3)	54 (31; 58)	0.186
Gender (female)	2 (50)	5 (83.3)[2]	1 (14.3)[2]	0.045
Type of donor (deceased)	3 (75)	6 (100)	8 (100)	0.157
ECD	1 (15)	5 (83.3)	4 (57)	0.175
Hypertension	1 (15)	5 (83.3)	3 (43)	0.125
Recipient characteristics				
Age, years	55.2 (44; 66)	59.5 (53.3; 66.3)	62.5 (56.5; 66.2)	0.792
Gender (female)	1 (25)	1 (16.7)	2 (25)	0.923
Dialysis vintage, months	39.7 (24.7; 45)	30 (11; 43)	21.6 (15.6; 30)	0.678
PRA max, %	3 (0.5; 32.5)	22 (6.5; 34.75)	1 (0; 3.5)	0.062
HLA mismatch	4.5 (4; 5)	3 (2.75; 4.25)	3 (2; 4.75)	0.22
HLA at biopsy (neg./pos./not known)	2/0/2	6/0/0[2]	0/0/8[2]	0.001
DSA at biopsy (neg./pos./not known)	2/0/2	6/0/0[2]	0/0/8[2]	0.001
Transplantation characteristics				
Retransplantation	0	0	0	N/A
Cold ischemia, hours	15.25 (6.5; 19.8)	15 (14; 16)	18.1 (7.4; 19.5)	0.696
Delayed graft function	1 (15)	2 (33)	3 (43)	0.908
Biopsy characteristics				
Postoperative day	6.5 (6; 8)[3]	7.5 (7; 10)[2]	97 (93.5; 101)[2,3]	0.001
Clinical characteristics				
Induction therapy				0.005
None	3 (75)[1]	0[1]	8 (100)	
Basiliximab	1 (25)	5 (83.3)[2]	0[2]	
Thymoglobulin	0	1 (16.7)	0	
Maintenance immunosuppression				0.245
TAC/MMF/steroids	3 (75)	6	5 (62.5)	
CsA/MMF/steroids	1 (25)	0	3 (37.5)	
TAC level at the biopsy (μg/l)	14.6 (10.3; 17.2)	10.95 (8; 16.6)	8.7 (8.3; 10.4)	0.646
CsA level at the biopsy (μg/l)	231	NA	191 (176; 278)	1
Anti-rejection treatment				
Steroids	2 (50)	5 (83)	N/A	0.3745
Thymoglobulin	1 (25)	1 (17)	N/A	
Steroids + Thymoglobulin	1 (25)	0	N/A	
Serum creatinine (μmol/l)				
At biopsy	378 (307; 448)[3]	441 (226; 514)[2]	115 (97; 154)[2,3]	0.002
12 months	119 (100; 121)	161 (151; 172)	115 (93; 148)	0.171
24 months	114 (100; 121)	186 (158; 187)[2]	106 (99; 144)[2]	0.017
Proteinuria (g/24 h)				
At biopsy	0.21 (0.105; 1.37)[1]	1.52 (1.25; 2.27)[1,2]	0.36 (0.27; 0.55)[2]	0.038
12 months	0 (0; 0.11)	0.26 (0.23; 0.26)	0.26 (0.18; 0.40)	0.144
24 months	0.12 (0.06; 0.54)	0.29 (0.09; 0.3)	0.19 (0.10; 0.33)	0.913
Banff scores in diagnostic biopsy (grade)				
mm	0 (0; 0)	0 (0; 0)	0 (0; 0)	0.535
g	0 (0; 0)	0 (0; 0)	0 (0; 0)	1
cg	0 (0; 0)	0 (0; 0)	0 (0; 0)	1
i	2 (2; 2)[1,3]	0 (0; 0)[1]	0 (0; 0)[3]	0.0001
t	2.5 (2; 3)[1,3]	0 (0; 0)[1]	0 (0; 0)[2]	0.001
v	1(1; 1.75)[3]	1 (1; 1.25)[2]	0 (0; 0)[2,3]	0.0001
ptc	0 (0; 0)	0 (0; 0)	0 (0; 0)	1
ti	2 (2; 2)[3]	0 (0; 1)[2]	0 (0; 0)[2,3]	0.001
ci	0 (0; 0.75)	1 (0.75; 1)[2]	0 (0; 0)[2]	0.027
ct	0.5 (0; 1)	0 (0; 1)	1 (0; 1)	0.871
ah	1 (1; 1)	1.5 (1; 1.25)[2]	0 (0; 0.75)[2]	0.004
cv	1 (0.25; 1)	2 (1; 3)[2]	0 (0; 0)[2]	0.002
C4d positivity, n	0	0	0	N/A

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Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

Table 1 Characteristics of patients in the training set (Continued)

	TCMRV (*n*=4)	eIV (*n*=6)	normal (*n*=8)	*P*
Pathologic diagnosis				
pure TCMR	3^{3}	4^{2}	$0^{2,3}$	0.0012
TCMR + ATN	1	2	0	
Normal	0^{3}	0^{2}	$8^{2,3}$	
Number of glomeruli	8.5 (7.25; 9)	12 (7; 15)	8 (7; 12.25)	0.522

Data are presented as medians (interquartile (IQ) range) or n (%). Differences were calculated by the Kruskal–Wallis test or χ^2 Fisher exact test and significant results of *post hoc* comparisons were adjusted by the Bonferroni correction for multiple tests ([1]TCMRV compared with eIV, [2]eIV compared with control, [3]TCMRV compared with control). Abbreviations: ah, arteriolar hyaline thickening; ATN, acute tubular necrosis; cg, transplant glomerulopathy; ci, interstitial fibrosis; ct, tubular atrophy; cv, vascular intimal fibrosis; CsA, cyclosporine A; ECD, expanded criteria donor; g, glomerulitis; HLA, human leukocyte antigen; i, interstitial inflammation; mm, mesangial matrix expansion; MMF, mycophenolate mofetil; ptc, peritubular capillaritis; PRA, panel reactive antibody; t, tubulitis; TAC, tacrolimus; ti, total interstitial inflammation; v, intimal arteritis.

For a validation set, 12 eIV and 8 TCMRV biopsy samples from patients transplanted between 2010 and 2016 were retrospectively identified and validated by RT-qPCR (Table 2). The Institutional Review Board (IRB) of IKEM approved the study protocol (G09-12-20), and all patients provided informed consent to participate in the study.

Induction immunosuppression was based on the immunological risk of recipients according to panel reactive antibodies, DSA, dialysis vintage, and transplantation history. High-risk patients received depletive induction treatment with Thymoglobulin, medium-risk patients received basiliximab, and low-risk patients received no induction treatment. Maintenance immunosuppression was based on standard triple immunosuppression regimen with tacrolimus in the majority of patients. T cell-mediated rejection was treated by steroid pulses. Thymoglobulin was administered in the case of type 2B TCMR or in steroid-resistant rejections.

Biopsy of kidney allografts

All the kidney allograft biopsies were obtained under ultrasound guidance using a 16-gauge Tru-Cut needle (Somatex®; Medical Technologies GmbH, Germany). A small portion (2 mm) of the cortical zone of the biopsy specimen was immediately placed in the RNA later Stabilization Reagent (Qiagen) and stored at −20 or −80°C until RNA extraction. The remaining part of each particular biopsy was sent for histological examination and found representative.

Microarray analysis

RNA preparation and hybridization

Total RNA was isolated from 16-gauge renal biopsies using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Samples with RNA integrity number < 6 (measured using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.) were excluded from analysis. A total of 200 ng of purified RNA served as a template for the amplification and biotinylation of cRNA using the Illumina® TotalPrep™ RNA Amplification Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) according to manufacturer's instructions. Yields of labeled cRNA were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). Labeled cRNA (750 ng) was hybridized at 58°C for 16 h to Illumina HumanHT-12 v4.0 Expression BeadChips (Illumina, Inc., San Diego, CA, U.S.A.), and then washed and Cy3-stained according to manufacturer's instructions.

Scanning and microarray data analysis

After hybridization, the chip was scanned using the iScan System (Illumina), and raw data were extracted with the BeadStudio Data Analysis Software (Illumina). The lumi package in R software was used to process the raw data. The quantile method was used for normalization [17]. The given workflow represents one of the standard preprocessing pipelines for Illumina microarrays with verified applicability under similar conditions [18].

Only probes with detectable signal intensity in at least five samples were included in the analysis. After normalization, probes with log of average expression intensity less than 3.5 were removed from further analysis. Differentially expressed genes were chosen as those with a fold change > 2 and adjusted $P<0.05$ (Benjamini–Hochberg method). The affected genes were functionally annotated, and the deregulated pathways were identified using the David database (http://david.abcc.ncifcrf.gov). For comparing the lists of deregulated genes, an interactive online

Clinical Science (2018) **132** 2269–2284
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Table 2 Characteristics of patients in the validation set

	TCMRV (*n*=8)	eIV (*n*=12)	*P*
Donor characteristics			
Age, years	46 (41; 62)	57 (50; 66)	0.082
Gender (female)	4 (50)	7 (51.3)	0.535
Type of donor (deceased)	5 (62.5)	10 (83.2)	0.296
ECD	3 (37.5)	6 (50)	0.465
Recipient characteristics			
Age, years	52 (43; 62)	58 (54; 60)	0.3841
Gender (female)	1 (12.5)	2 (16.7)	0.656
Dialysis vintage, months	41 (17.5; 51)	20.4 (7.1; 25)	0.698
PRA max, %	3 (0; 10)	2 (0.5; 32.3)	0.973
HLA mismatch	5 (4; 5)	3 (3; 4.75)	0.39
HLA at biopsy (neg./pos./not known)	1/1/6	4/1/7	0.571
DSA at biopsy (neg./pos./not known)	2/0/6	5/0/7	0.392
CKD diagnosis, *n* (%)			0.339
Diabetes	1 (12.5)	5 (41.7)	
Glomerulonephritis	2 (25)	2 (16.7)	
Polycystosis	1 (12.5)	2 (16.7)	
TIN	2 (25)	0	
Hypertension	1 (12.5)	2 (16.7)	
Ischemic nephropathy	1 (12.5)	0	
Other	0	1 (8.3)	
Transplantation characteristics			
Retransplantation	1 (12.5)	1 (8.3)	0.653
Cold ischemia, hours	14 (1.25; 14)	15 (11.7; 18.2)	1
Delayed graft function	4 (50)	6 (50)	0.675
Biopsy characteristics			
Postoperative day	6.5 (6; 18)	7 (6; 13)	1
Clinical characteristics			
Induction therapy			0.966
None	1 (12.5)	2 (16.7)	
Basiliximab	5 (62.5)	7 (58.3)	
Thymoglobulin	2 (25)	3 (25)	
Maintenance immunosuppression			1
TAC/MMF/steroids	8 (100)	11 (100)	
TAC level at the biopsy (μg/l)	11.6 (8.3; 15.1)	10.3 (7.55;14.7)	0.629
Rejection treatment			0.4177
Steroids	4(50)	8 (67)	
Thymoglobulin	3 (37.5)	4 (33)	
Steroids + Thymoglobulin	1 (12.5)	0	
Serum creatinine (μmol/l)			
At biopsy	378 (166; 470)	464 (231; 609)	0.305
12 months	154 (112; 167)	154 (135; 188)	0.571
24 months	142 (118; 172)	144 (113; 184)	1.0
Banff scores in diagnostic biopsy (grade)			
mm	0 (0; 0)	0 (0; 0)	1
g	0 (0; 0)	0 (0; 0)	1
cg	0 (0; 0)	0 (0; 0)	1
i	2 (2; 2)	0 (0; 0.75)	0.000016
t	2 (1.25; 3)	0 (0; 1)	0.0002
v	1 (1; 1.75)	1 (1; 2)	0.734
ptc	0 (0; 0)	0 (0; 0)	1
ti	2 (2; 2)	0 (0; 1)	0.000016
ci	0 (0; 0.75)	0.5 (0; 1)	0.384
ct	0.5 (0; 1)	1 (0.25; 1)	0.384
ah	1 (1; 1)	1 (1; 2)	0.181
cv	1 (1; 1)	2 (1; 2)	0.02

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Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

Table 2 Characteristics of patients in the validation set (Continued)

	TCMRV (*n*=8)	eIV (*n*=12)	*P*
C4d positivity, *n*	0	0	1
Pathologic diagnosis			0.2421
Pure TCMR	8	9	
TCMR + ATN	0	3	
Number of glomeruli	9 (7–19)	12 (7.5–14)	0.851

Data are presented as medians (interquartile (IQ) range) or n (%). Differences were calculated by the Mann–Whitney test or χ^2 Fisher exact test. Abbreviations: ah, arteriolar hyaline thickening; ATN, acute tubular necrosis; cg, transplant glomerulopathy; ci, interstitial fibrosis; CKD, chronic kidney disease; ct, tubular atrophy; cv, vascular intimal fibrosis; ECD, expanded criteria donor; g, glomerulitis; HLA, human leukocyte antigen; i, interstitial inflammation; mm, mesangial matrix expansion; MMF, mycophenolate mofetil; ptc, peritubular capillaritis; PRA, panel reactive antibody; t, tubulitis; TAC, tacrolimus; TCMR, T cell- mediated rejection; ti, total interstitial inflammation; TIN, tubulointerstitial nephritis; v, intimal arteritis.

tool for Venn diagrams http://bioinformatics.psb.ugent.be/webtools/Venn/ was used. Complete raw and normalized data were deposited in the NCBI Gene Expression Omnibus (GEO) database [19] and are accessible through the GEO Series accession number GSE114712 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114712).

Validation of microarray data

In order to validate the microarray results, internal validation and validation by RT-qPCR analysis was performed.

Internal validation

To identify the most relevant genes that distinguish between the TCMRV and eIV groups, the well-known support vector machine (SVM) classification algorithm [20], specifically e1071 R library implementation was applied [21]. The negative effect of overfitting caused by the small number of samples and the large number of evaluated genes was minimized by the recursive feature elimination (RFE) algorithm [22]. We first trained the classifiers on microarray data. Performance of SVM–RFE was evaluated by leave-one-out cross validation (LOOCV) [23]. Since the group sizes were not perfectly balanced in their size, the usual measure of classification accuracy (ACC) was complemented by another measure robust to skewed groups, the area under the ROC curve (AUC) [24]. The LOOCV demonstrated that TCMRV and eIV groups can be perfectly differentiated by a large scale of gene sets of various sizes, i.e., the groups can be separated by a small number of genes as well as by much larger gene sets. The gene set size ranges from 5 to 500 genes and showed absolute 100% ACC and maximum AUC (Supplementary Figure S1). However, the single small set of genes that separate the study groups was not easy to determine due to the large number of differentially expressed microarray genes. SVM–RFE perfectly split the groups with a large scale of potentially different gene sets of various sizes. For this reason, the final selection of genes for external validation was not induced directly from SVM-RFE as described below.

Validation by RT-qPCR analysis

Using a custom-made Taqman low-density array (Applied Biosystems, Foster City, CA, U.S.A.) on a validation set of patients (n=20), RT-qPCR was employed to analyze 38 genes identified by microarray to be deregulated between TCMRV and eIV and found to be of biological interest in the rejection process (Supplementary Table S1). Quantitative RT-qPCR based on TLDA technology was carried out as described elsewhere [25]. Real-time RT-qPCR data were quantified using the SDS 2.4 software package (Applied Biosystems) and relative gene expression values were determined using the comparative $2^{-\Delta\Delta Ct}$ method of the relative quantification (RQ) Manager Software v 1.2.1 (Applied Biosystems) with normalization to an endogenous control (HPRT1). The endogenous control was chosen from three candidate housekeeping genes (GAPDH-Hs99999905_m1, PGK1-Hs99999906_m1, HPRT1- Hs01003267_m1) using NormFinder (www.mdL.dk) as the gene with the most stable expression (HPRT1 with a stability value of 0.003). As a calibrator, one of the samples with a good expression profile on all of the target genes was used. All investigated mRNAs were measured in triplicate for each sample.

Risk of overfitting

In our study, we deal with the well-known $n<<P$ problem (the large number of variables and the small number of samples) that represents a specific case of ill-posed problem and may result in overfitting [26,27]. This risk is minimized by careful handling with the train, test and validation datasets. First, we employ LOOCV to split between train

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

and test sets. Both gene selection and classifier construction are performed solely on train sets, while the corresponding test sets serve for their evaluation. In particular, the SVM–RFE procedure for gene selection was re-performed with each iteration of the LOOCV procedure, so that the features are selected from each train set and applied independently to each test set. In general, this train-test split allows us to detect overfitting and avoid complex biomarkers that heavily overfit the data used for model construction. It enables to propose simple biomarkers and to smoothly distinguish between them in terms of their performance. Second, we work with the independent RT-qPCR data set that serves to validate the selected biomarkers, remove the selection bias and get an unbiased estimate of their classification accuracy (expressed in terms of AUC to compensate for unbalanced classes) [27,28].

Statistical methods

Normality of the data was tested using the Kolmogorov–Smirnov test. Nonparametric values are presented as median and interquartile range. Two groups were compared by the two-tailed Mann–Whitney U-test and three groups by the Kruskal–Wallis test with *post hoc* adjustment by the Bonferroni correction for multiple tests. For comparison of categorical data, the χ^2 Fisher exact test was used. Two-sided P-values considered statistically significant for a $P<0.05$.

In order to visualize different transcriptomes of studied samples, principal component analysis (PCA) and unsupervised hierarchical clustering (HC) were performed using R software. Both visualization tools were constructed using all genes evaluated by microarray to provide independent evidence of potential phenotype separation and to exclude circular misleading conclusion by choosing only genes deregulated between the study groups. In PCA analysis, a 3D scatter plot was produced based on the whole microarray expression profile of each sample. Calculation was done by the singular value decomposition of the centered expression matrix; no further scaling of the normalized expressions was performed.

In unsupervised HC, the Euclidean distance was selected as a similarity measure between the normalized expression profiles. The agglomerative complete-linkage HC was performed. The function hclust from R stats package was used for HC construction.

Results

Training set (microarray analysis)

Distinct microarray profiles of TCMRV and eIV

Using microarray-based gene expression profiling in the training set of patients, we identified 310 differentially regulated genes in early TCMRV compared with eIV (Supplementary Table S2). The volcano plot demonstrates deregulated genes between both groups after correction for multiple testing and shows most genes being up-regulated in TCMRV (n=288, 92.9%) compared with eIV (Figure 2A).

To get an insight into relevant biological processes, we used the DAVID database that allows gene annotation to gene ontology (GO) terms. We found that the genes regulated differentially between TCMRV and eIV were primarily associated with the innate and adaptive immune and inflammatory responses. In detail, TCMRV samples showed up-regulation of T-cell activation, costimulation, differentiation, receptor signaling pathway, positive regulation of T-cell proliferation, antigen processing, and presentation as well as B-cell activation and receptor signaling pathway, interferon-γ- and tumor necrosis factor-mediated signaling pathway and its positive regulation etc. Details of top 25 out of 65 enriched GO terms for biological processes are shown in Table 3. Activated biological processes indicate higher activation of immunity and inflammation in early TCMRV biopsies compared with eIV. The Circos plot was created based on 15 most significantly up-regulated genes between TCMRV and eIV to visualize gene association with significantly enriched pathways and GO terms that play a role in the immune response (Figure 3).

Analysis of the molecular relationships between differentially expressed genes (TCMRV compared with eIV) revealed involvement of GO terms for molecular functions highly associated with the rejection process (GO:0032395 MHC class II receptor activity, adjusted P=2.63E-07; GO:0004872 receptor activity, P=1.19E-04, GO:0005164 tumor necrosis factor receptor binding, P=2.87E-04; GO:0042605 peptide antigen binding, P=3.08E-04; GO:0045028 G-protein coupled purinergic nucleotide receptor activity, P=2.83E-03; GO:0004896 cytokine receptor activity, P=9.79E-03; GO:0019976 interleukin-2 binding, P=3.24E-02; GO:0004911 interleukin-2 receptor activity, 3.24E-02).

Comparison of the study groups with normal histologic findings

By comparing eIV with normal histologic findings, 28 up- and 22 down-regulated genes were identified (Figure 2B, Supplementary Table S3). Comparison of eIV with normal indicates involvement of down-regulated genes in GO terms for molecular functions highly associated with ion binding (GO:0046872, metal ion binding, P=6.0E-3;

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

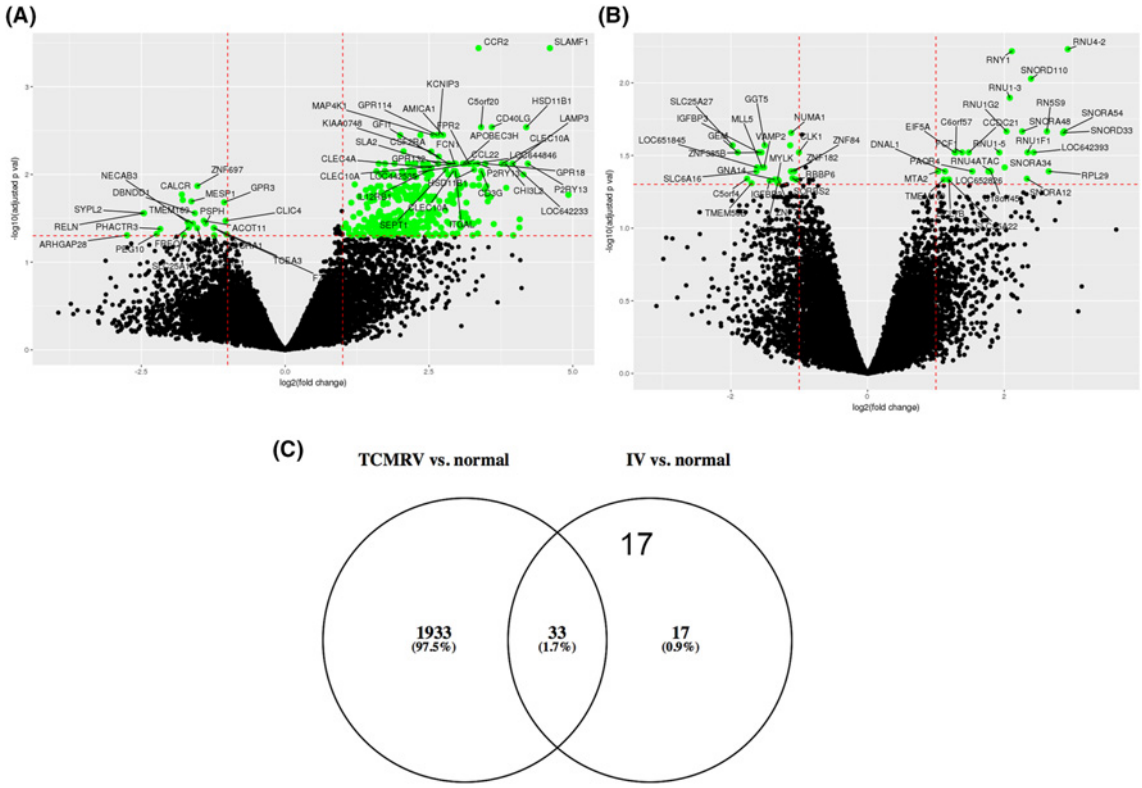

Figure 2. Genes with different mRNA expression in studied groups.

The volcano plot analysis showing differences in mRNA expression values between TCMRV and eIV (**A**) and between eIV and normal (**B**) considering an adjusted *P*-value cut-off = 0.05 and a fold-change cut-off = 2. The data for all genes are plotted as $\log_2$-fold change compared with the -log10 of the p-value. Thresholds are shown as dashed lines. (**C**) Venn diagram of shared deregulated genes between TCMRV compared with normal and eIV compared with normal. Comparison of deregulated gene lists between the study groups and controls shows a 1.7% (*n*=33) gene overlap with no association with any gene ontology (GO) term.

GO:0043169, cation binding, *P*=6.4E-3; GO:0043167, ion binding, *P*=7.2E-3; GO:0046914, transition metal ion binding, *P*=26.1E-3; GO:0008270, zinc ion binding, *P*=36.7E-3, unadjusted *P*-values shown). Genes up-regulated in eIV compared with normal histology showed association with translation (GO:0006412, unadjusted *P*=8.39E-03) and RNA binding (GO:0003723, unadjusted *P*=3.95E-02) reflecting the early post-transplant time period of eIV findings (median 8 days in eIV compared with 97 days in control group).

On the other hand, comparison of TCMRV to the control group revealed a large number of significantly deregulated genes (*n*=1966). Gene enrichment analysis found 239 GO terms for the biological process, predominantly from an immune response, to be significantly associated with deregulated genes (Supplementary Table S4) and confirmed true rejection origin of TCMRV. Comparison of deregulated gene lists between the study groups and controls using a Venn diagram showed only a 1.7% (*n*=33) gene overlap (Figure 2C, Supplementary Table S5). The genes shared between those two comparisons (TCMRV compared with normal and eIV compared with normal) were not significantly associated with any GO term. Taken together, the transcriptome of eIV showed relative similarity with the control group but was dramatically different from that of TCMRV.

Visualization of microarray data

The 3D PCA analysis applied to the whole transcriptome data demonstrated clear separation of TCMRV from eIV kidney allografts (Figure 4). Control samples were spread among eIVs demonstrating similarity of both groups' transcriptome. In addition, unsupervised HC analysis was applied to the whole microarray data and a similar pattern was obtained (Figure 5). Three main subclusters were formed. The first cluster (left) was formed by an individual eIV patient (eIV_5) undergoing successful steroid treatment of early IV but a low level of DSA class I (MFI 1100) was detected retrospectively with no impact on graft function or later development of ABMR. The second cluster

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

Table 3 Top 25 GO terms for biological process enriched in TCMRV compared with eIV

	GO term	Count	*P*
GO:0006955	Immune response	54	4.28E-31
GO:0031295	T-cell costimulation	21	7.03E-17
GO:0050853	T-cell receptor signaling pathway	25	8.95E-16
GO:0006954	Inflammatory response	36	9.93E-16
GO:0002250	Adaptive immune response	21	1.61E-11
GO:0042110	T-cell activation	14	2.44E-11
GO:0045087	Innate immune response	31	3.24E-10
GO:0007165	Signal transduction	50	3.01E-09
GO:0060333	Interferon-γ-mediated signaling pathway	14	4.92E-09
GO:0002504	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	8	3.39E-07
GO:0050776	Regulation of the immune response	17	1.17E-06
GO:0032729	Positive regulation of interferon-γ production	10	2.31E-06
GO:0007166	Cell surface receptor signaling pathway	20	2.84E-06
GO:0033209	Tumor necrosis factor-mediated signaling pathway	13	1.77E-05
GO:0042102	Positive regulation of T-cell proliferation	10	1.94E-05
GO:0030217	T-cell differentiation	8	2.01E-05
GO:0006935	Chemotaxis	13	2.10E-05
GO:0006915	Apoptotic process	27	2.53E-05
GO:0030168	Platelet activation	12	7.54E-05
GO:0050853	B-cell receptor signaling pathway	9	7.81E-05
GO:0043547	Positive regulation of GTPase activity	26	7.88E-05
GO:0001816	Cytokine production	7	8.71E-05
GO:0050900	Leukocyte migration	12	1.18E-04
GO:0042113	B-cell activation	7	1.96E-04
GO:0006968	Cellular defense response	9	2.01E-04

The genes with different expression in TCMRV and eIV after correction for multiple comparisons (n=310) were entered into the DAVID gene ontology database. Top 25 out of 65 GO terms are shown.

included exclusively TCMRV samples. In the third cluster, we were not able to distinguish between eIV and control samples mirroring the groups' transcriptome similarity. Taken together, unsupervised HC analysis distinguished TCMRV from eIV samples but failed to distinguish between eIV and controls.

Validation set (RT-qPCR analysis)

RT-qPCR analysis of the validation set confirmed up-regulation of the immune response in TCMRV compared with eIV

We verified these findings in a validation set of patients (n=20) using a different technique. The clinical characteristics of the validation cohort are similar to that of the training set (Table 2). A total of 38 genes were selected for RT-qPCR validation (Supplementary Table S1). These genes exhibited at least two-fold change and adjusted P-value <0.05 in microarray expression and were of biological interest for TCMRV and eIV comparison.

RT-qPCR analysis confirmed that early indication biopsy tissues of TCMRV had statistically significantly higher expression of *BCL11B, BTLA, CCL17, CCR7, CD2, CTLA4, CXCL13, GIMAP5, IL21R, KLRG1, LAX1, LCK, LTA, LTB, SLA2, SLAMF1, TNFRSF4* and *ZAP70* compared with eIV (Figure 6). The validated genes are significantly involved in regulation of immune system process, T-cell differentiation, activation, proliferation, B-cell activation, overall lymphocyte and leukocyte activation, immune response-regulating cell signal transduction, and apoptosis.

Agreement between microarray and RT-qPCR data

Validation of reference genes in the validation set was defined as both qualitative (direction) and quantitative agreement between microarray and RT-qPCR measurements. The direction of RT-qPCR gene expressions agreed with the microarray technique in 100% of validated genes. Quantitative agreement between microarray and RT-qPCR was confirmed by a significant correlation of normalized data (Pearson $r = 0.663$, P=0.00006) (Supplementary Figure

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

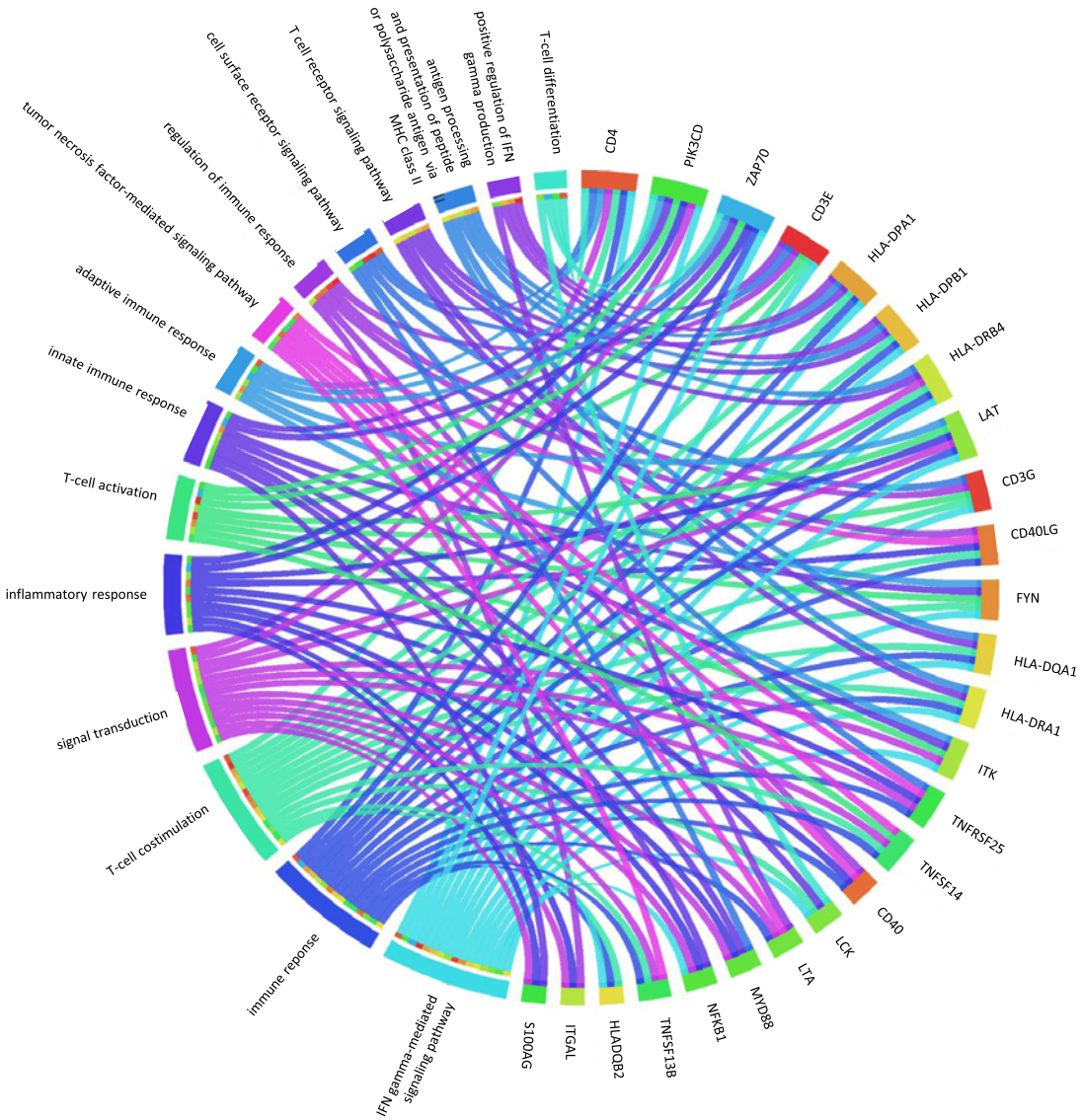

Figure 3. The Circos plot represents significantly enriched pathways and GO terms for biological process associated with the 15 most significant up-regulated genes between TCMRV compared with eIV samples, detected using the DAVID database

Outside the circle, dysregulated genes and significantly enriched pathways together with GO terms are indicated.

S2). To further validate differences in the transcriptome of the study groups, the SVM–RFE classifiers were trained on RT-qPCR data. LOOCV confirmed that the genes selected for validation from microarray data showed around 80% accuracy (ACC) and a 0.75 area under the curve (AUC) (Supplementary Figure S3) thus confirming reasonable gene selection for external RT-qPCR validation.

Discussion

In the present study, we investigated the transcriptome of eIV with paucity of TI and TCMRV with rich TI. Our main results are that the transcriptome of eIV revealed a weak immunologic signature compared with TCMRV and showed similarity with non-rejection 3-month protocol biopsy. Based on our results, eIV may feature a non-rejection phenotype and reflect peritransplant injury. As the current Banff histopathological criteria consider intimal arteritis (after exclusion of ABMR) to be at least type II of TCMR irrespective of TI, our results agree with calls for reassessment of the current approach in histology interpretation. Furthermore, difference in non-rejection phenotype of DSA- and

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

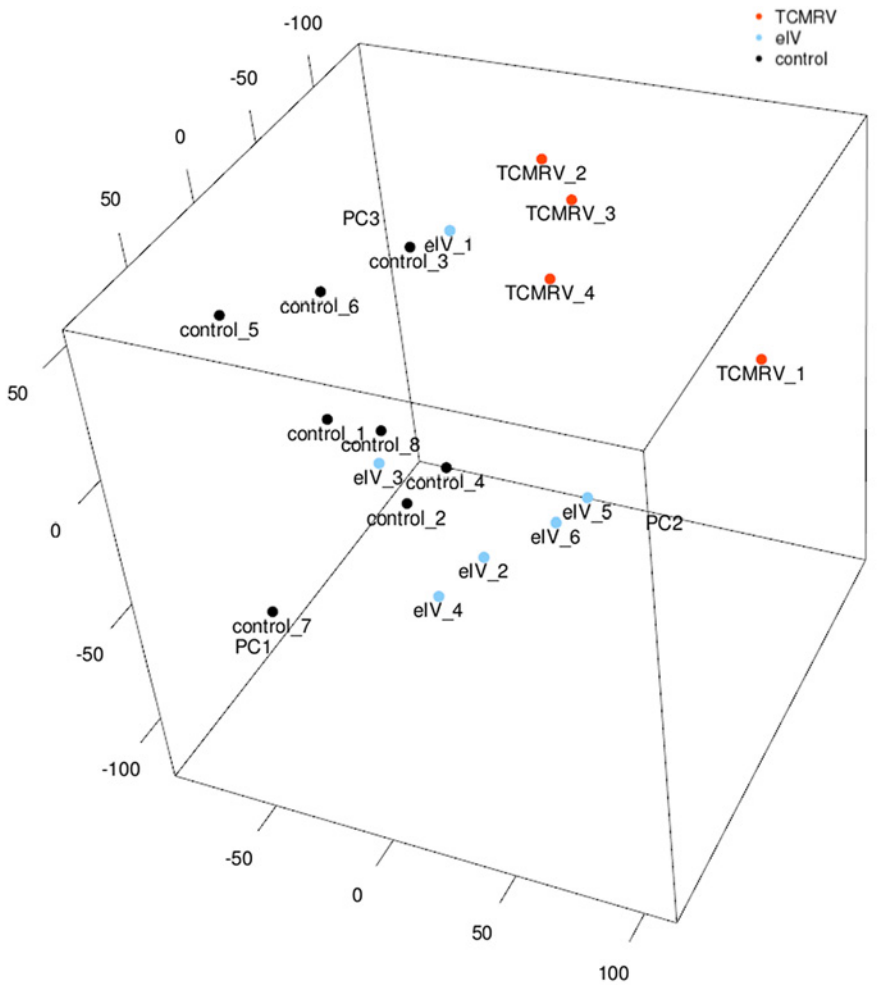

Figure 4. 3D PCA applied to the whole transcriptome of TCMRV, eIV, and control samples (training set)

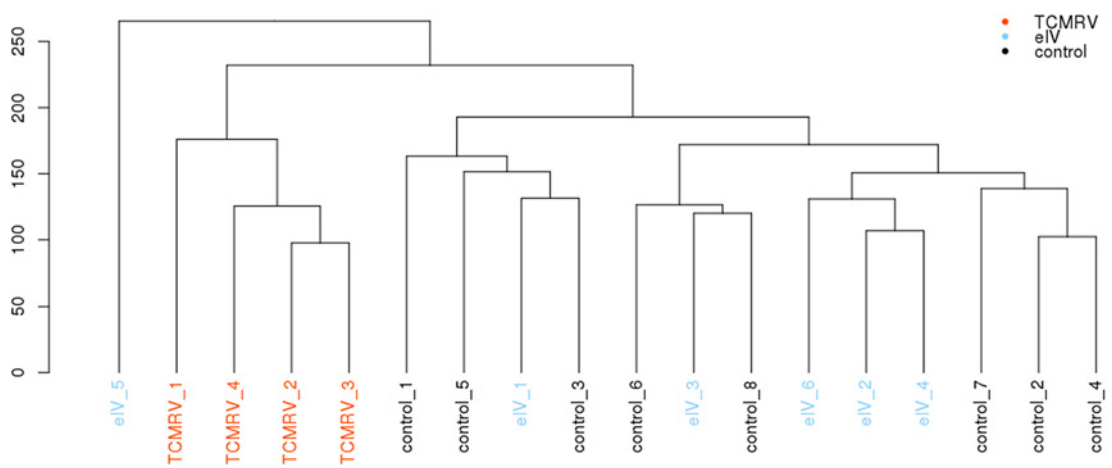

Figure 5. Unsupervised hierarchical cluster analysis applied to the whole transcriptome of TCMRV, eIV and control samples (training set)

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

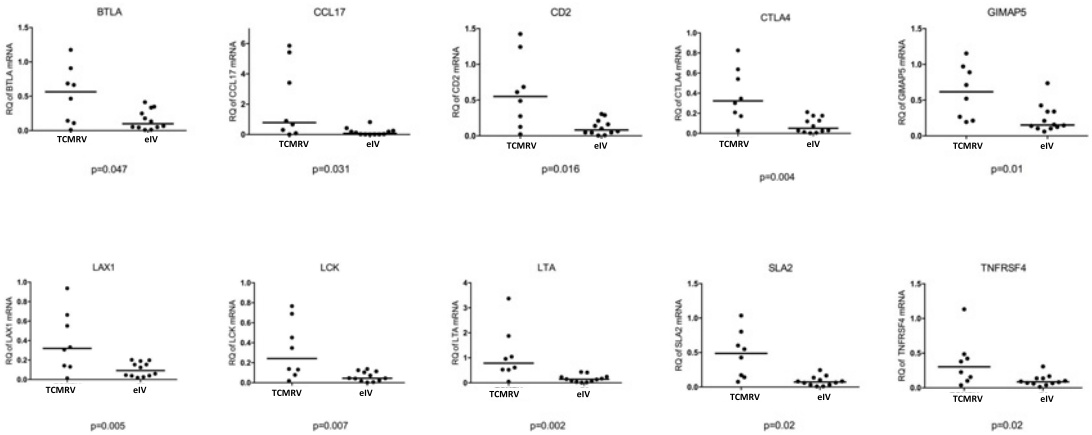

Figure 6. Validation of microarray analysis by RT-qPCR of early indication biopsy samples
Scatter plots show top 10 deregulated genes between TCMRV and eIV.

C4d-negative eIV described in our study and rejection phenotype of late and often DSA positive IV [16] supports use of a molecular microscope in the case of an IV finding as recommended by the last Banff Kidney Meeting report [6].

Our first goal was to find out whether eIV represents a true TCMR. To do so, we included early intimal arteritis with minimal TI (eIV) and rich TI (TCMRV) and excluded cases with any MI, and C4d or DSA positivity. With these inclusion/exclusion criteria, only not anti-HLA-antibody-mediated intimal arteritis was evaluated. We used microarray technology to allow for investigation at the molecular level. Because the gene expression patterns change over time after transplantation and may vary widely, even among tissues with similar morphological changes [29], we decided to include only early indication kidney allograft biopsies into the study groups.

We found that the transcriptome of eIV was significantly different from that of TCMRV. In general, the genes deregulated between TCMRV and eIV were annotated to GO terms associated with the innate and adaptive immune and inflammatory responses. This is in accordance with other studies which identified relevant biological processes for acute rejection to be up-regulated in T cell-mediated rejection and led to development of molecular test for T cell-mediated rejection (TCMR score) [30,31]. In detail, up-regulated genes in our TCMRV samples were associated with T-cell activation, differentiation, costimulation, T-cell receptor signaling pathway, signal transduction, interferon-γ- and tumor necrosis factor-mediated signaling pathway, antigen processing and presentation, apoptosis, and others as listed in Table 3. Vice versa, eIV revealed significant down-regulation of T cell-mediated processes similarly to recent study showing negative molecular TCMR score in majority of IV in contrast to positive TCMR score in 95% of v-lesions with rich TI and proposing that IV in absence of DSA should not be used to diagnose TCMR [16].

Another interesting finding of our study is that PCA and HC indicate a rather clear distinction of TCMRV from eIV and controls at the molecular level. The separation is not perfect as all microarray probes, not only the deregulated ones, were used. The standalone eIV_5 patient had retrospectively detected DSA and his separation might reflect the fact that molecular changes precede the histologic ones.

The Venn diagram comparing deregulated genes in TCMRV and eIV compared with controls demonstrated that the shared genes were not annotated to GO terms associated with rejection or the immune response. Moreover, eIV samples revealed relative similarity to normal histologic findings. So, if TCMRV represents true tubulointerstitial rejection, then eIV with a distinct transcriptome profile is probably of non-rejection origin.

Interestingly, by comparison of eIV and controls only 50 genes were found to be differentially regulated and found to be associated with down-regulation of zinc ion binding in early IV. It is well known that zinc plays an important role in ischemia/reperfusion injury and has potential antioxidant properties [32]. Recent work shows that oral zinc chloride supplementation mitigates renal ischemia/reperfusion injury in rats by modulating oxidative stress, endoplasmic reticulum stress, and autophagy [33]. Therefore, we speculate that down-regulation of zinc ion binding genes in eIV might reflect exhaustion of zinc metabolism in early post-transplant biopsies with potential ongoing ischemia/reperfusion injury compared with quiescent 3-month protocol biopsies with normal histology and no signs of acute kidney injury.

Another important finding in the search of IV origin was the identification of several potential target molecules that could have important roles in understanding of IV process. Therefore, we evaluated set of deregulated genes from

microarray study with potential biological interest in a validation set of samples using different molecular diagnostic technique. RT-qPCR analysis revealed significant up-regulation of several genes with an important role in rejection process in TCMRV compared with IV, such as regulation of immune system process, T-cell differentiation, activation, proliferation, B-cell activation, overall lymphocyte and leukocyte activation, immune response-regulating cell signal transduction and apoptosis and confirmed stronger activation of an innate and adaptive immune responses in TCMRV compared with eIV.

Our findings agree with other microarray studies which showed a discrepancy between conventional histologic and molecular assessment of TCMR [14-16,31,34]. The discrepancy was particularly marked in IV, where most cases exhibited low TCMR scores [31]. Intimal arteritis has been shown to have less importance in the diagnostics of TCMR than tubulitis and interstitial infiltrate [15]. Salazar reported that only a minority of IV exhibited the molecular signature of TCMR. Particularly early biopsies performed in the first year post-transplant in DSA-negative patients, usually did not reflect rejection, while late IV often associated with DSA was indicative of ABMR. Taken together, current diagnostic conventions for v-lesion tend to overdiagnose rejections as many biopsies with no molecular TCMR score are traditionally assessed as being TCMR [16].

Our and others' results suggest that early post-transplant IV might reflect mechanisms different from rejection, i.e., injury-repair response to implantation stresses, endothelial injury related to ischemia-reperfusion [7]. The presence of intimal arteritis in early non-rejection biopsies might be explainable by increased vascular permeability and facilitated extravasation of leukocytes by interrupting the integrity of the renal vascular endothelium triggered by renal injury [35,36].

However, our conclusions differ from those of the few clinical studies analyzing IV. Wu et al. [37] reported intimal arteritis to be an independent histologic risk factor of long-term graft loss regardless of the timing of finding and the extent of TI and considered IV to be an acute rejection of kidney allograft with a poor long-term outcome. Sis provided clinical evidence that IV behaves as true acute rejection because it is associated with functional improvement after anti-rejection treatment and represents an independent risk factor for allograft failure [38]. Such a putative discrepancy might be explainable by few facts. First, these studies analyzed the clinical course of IV at different time points after transplantation ignoring the fact that it is the time of biopsy that is critical for understanding IV. Nevertheless, most early IV specimens have no signature of molecular rejection and are DSA negative, whereas late IV ones are often DSA positive and show molecular ABMR [16]. Second, all discrepant studies were based on the clinical presentation of intimal arteritis, not on transcriptome analysis and evaluated the response to anti-rejection treatment in a very heterogeneous cohort. Third, clinical studies reporting the severity of IV analyzed biopsies were from earlier eras when aggressive TCMR was common and ABMR poorly recognized. Historically, IV was believed to actually imply rejection and had a negative effect on kidney graft survival. However, the nature and significance of IV have changed over time and v-lesions from a decade ago must be reinterpreted in the current era [39]. Nowadays, IV represents a rare finding in indication biopsies. At our center, 2.5% of kidney allografts show IV, mostly during the first 3 months post-transplant, with a mild clinical course and a favorable outcome [40].

Potential limitations of our study include its retrospective design and small sample size. However, the study limitations reflect IV in the real-life setting where IV is a rare finding, with heterogeneous interpretation and treatment. We realize that most of the samples in our study had mild to moderate arteritis (v1) and were found in early indication biopsies within the first month post-transplant. Therefore, our study results indicating a potential non-rejection origin of eIV are applicable exclusively to this specific cohort. We are also aware that all findings of intimal arteritis were treated by steroids or Thymoglobulin according to our historical standards of care for TCMR, which might have influenced graft prognosis. However, the renal allograft transcriptome was analyzed before initiating any anti-rejection treatment. Moreover, the question whether eIV patients should be given anti-rejection treatment, augmentation of immunosuppression or whether they should be followed by periodic surveillance biopsies, cannot be answered by the present study and calls for appropriate randomized controlled clinical trials. We are aware of fact that 2 eIV and 1 TCMRV kidney allografts exhibited intimal arteritis in addition to acute tubular necrosis which may have affected mRNA expression and graft function development. Additionally, the eIV group showed higher rates of anti-IL2 blockade (basiliximab) which may have had a potential impact on milder histologic finding and the molecular phenotype of eIV. While, admittedly, mRNA expression evaluation using laser capture microdissection would be of relevance in molecular analysis of highly focal changes, this approach is limited by the tendency of laser capture to damage the RNA, high costs and complexity, making it technically challenging [41]. Moreover, molecular assessments are able to assess an ongoing pathological process as rejection or injury even in the absence of glomeruli and arteries, are independent of cortex proportion and, although histology cannot assess the medulla, rejection does occur in the medulla as well as cortex [42]. Therefore, in our opinion, it still seems reasonable to evaluate vascular rejection from non-dissected tissue.

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

The strength of the present study is careful selection of AVR samples with the histology of the T cell-mediated phenotype and exclusion of ABMR according to the updated Banff classification [4]. Our study is further strengthened by the uniform approach of a high-volume transplant center and well-selected study groups, where only patients early after kidney transplantation within the first month post-transplant, with no rejection history, no surgical complications, similar immunological risk, and incidence of delayed graft function were enrolled into study groups. We included only indication biopsies and excluded subclinical, late, and DSA positive findings. Furthermore, microarray analysis was performed based on recent recommendations to avoid common errors in the implementation and interpretation of microarray studies [43]. Only genes that passed through correction for multiple testing were judged as significantly deregulated. To avoid circular logic, PCA and cluster analyses were based on the whole microarray data, not only on deregulated genes between the two groups, which allow to show the real separation of the distinct transcriptomes of eIV and TCMRV.

Our microarray study of DSA- and C4d-negative eIV revealed a weaker immune signature compared with TCMRV and showed similarity with normal histologic findings. Our results suggest that mild eIV occurring early after kidney transplantation in a not highly sensitized population may feature non-rejection origin. It seems more likely that inflammation seen in eIV might reflect endothelial injury from the transplantation process distinct from an alloimmune response. The evaluation of problematic biopsies with IV should combine histology and serologic HLA antibody assessment with use of molecular techniques as microarray to ensure correct diagnosis assignment. This is important particularly in case of IV which often show a discrepancy between conventional histology and microarrays. Using current conventions, many cases of IV are interpreted by pathologists as T-cell mediated rejection but exhibit low molecular TCMR score by molecular assessment, especially if DSA negative and observed early after kidney transplantation. Incorporation of molecular microscope into clinical praxis of IV on top of conventional histology and HLA antibody assessment might lower the rate of false positive rejections and help with therapeutic approach. Further prospective studies are warranted to delineate the pathogenesis and prognosis of this rare and heterogeneous entity before any reassessment of guidelines for diagnostic interpretation and treatment of IV.

Clinical perspectives

- **Background as to why the study was undertaken:** IV with minimal TI represents a challenging clinical situation after kidney transplantation, especially when occurring early and in the absence of DSA and C4d. To find out if early IV (eIV) represents a true rejection, the present study compared the transcriptome of eIV and T-cell mediated vascular rejection (TCMRV).

- **A brief summary of the results:** The transcriptome of early IV (<1 month) with negative C4d and DSA is associated with a weak immune signature compared with TCMRV and shows similarity with normal findings. Such eIV may feature non-rejection origin and reflect an injury distinct from an alloimmune response.

- **The potential significance of the results to human health and disease:** Although intimal arteritis has been known to have a deleterious impact on kidney graft survival regardless of TI, our results agree with recent studies impeaching the rejection origin and negative prognosis of eIV. The present study supports reassessment of the current approach in interpretation of kidney allograft biopsy and supports use of molecular methods.

Acknowledgements
We thank study co-ordinator Marie Kolarova for her participation in research performance and the transplant laboratory team for their contribution to biopsy samples processing.

Competing interests
The author declares that there are no competing interests associated with the manuscript.

Funding
This work was supported by the Ministry of Health of the Czech Republic [grant numbers 15-26519A and 17-29992A]; and the OP VVV MEYS funded project 'Research Center for Informatics' [grant number CZ.02.1.01/0.0/0.0/16_019/0000765].

Author contribution

M.W. participated in design and performance of the research, data analysis, writing of the paper. P.H. participated in processing of tissue samples. J.K. and V.S. contributed to statistical analysis. M.N. participated in performance of the research. E.H. participated in histologic assessment. Z.K. participated in microarray evaluation. P.V. participated in immunogenetic examination. O.V. contributed to the research design and writing of the paper

Abbreviations

ABMR, antibody-mediated rejection; ACC, classification accuracy; ah, arteriolar hyaline thickening; ATN, acute tubular necrosis; AUC, area under the ROC curve; AVR, acute vascular rejection; DSA, donor-specific antibody; ECD, expanded criteria donor; eIV, early isolated v-lesion; GEO, Gene Expression Omnibus; GO, gene ontology; HC, hierarchical clustering; HPRT, hypoxanthine-guanine phosphoribosyltransferaze; IKEM, Institute for Clinical and Experimental Medicine; IV, isolated v-lesion; LOOCV, leave-one-out cross validation; MI, microvascular inflammation; PCA, principal component analysis; PRA, panel reactive antibodies; RFE, recursive feature elimination; ROC, receiver operating characteristic; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SVM, support vector machine; TCMR, T cell-mediated rejection; TCMRV, T cell-mediated vascular rejection; TI, tubulointerstitial inflammation; TLDA, TaqMan Low Density Array.

References

1 Lefaucheur, C., Loupy, A., Vernerey, D., Duong-Van-Huyen, J.P., Suberbielle, C., Anglicheau, D. et al. (2013) Antibody-mediated vascular rejection of kidney allografts: a population-based study. *Lancet* **381**, 313–319, https://doi.org/10.1016/S0140-6736(12)61265-3
2 Wu, K., Budde, K., Schmidt, D., Neumayer, H.H. and Rudolph, B. (2015) The relationship of the severity and category of acute rejection with intimal arteritis defined in banff classification to clinical outcomes. *Transplantation* **99**, e105–e114, https://doi.org/10.1097/TP.0000000000000640
3 Mueller, A., Schnuelle, P., Waldherr, R. and van der Woude, F.J. (2000) Impact of the Banff '97 classification for histological diagnosis of rejection on clinical outcome and renal function parameters after kidney transplantation. *Transplantation* **69**, 1123–1127, https://doi.org/10.1097/00007890-200003270-00017
4 Djamali, A., Kaufman, D.B., Ellis, T.M., Zhong, W., Matas, A. and Samaniego, M. (2014) Diagnosis and management of antibody-mediated rejection: current status and novel approaches. *Am. J. Transplant.* **14**, 255–271, https://doi.org/10.1111/ajt.12589
5 Haas, M., Sis, B., Racusen, L.C., Solez, K., Glotz, D., Colvin, R.B. et al. (2014) Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. *Am. J. Transplant.* **14**, 272–283, https://doi.org/10.1111/ajt.12590
6 Haas, M., Loupy, A., Lefaucheur, C., Roufosse, C., Glotz, D., Seron, D. et al. (2018) The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell-mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. *Am. J. Transplant.* **18**, 293–307, https://doi.org/10.1111/ajt.14625
7 Mueller, T.F., Einecke, G., Reeve, J., Sis, B., Mengel, M., Jhangri, G.S. et al. (2007) Microarray analysis of rejection in human kidney transplants using pathogenesis-based transcript sets. *Am. J. Transplant.* **7**, 2712–2722, https://doi.org/10.1111/j.1600-6143.2007.02005.x
8 Teo, R.Z., Wong, G., Russ, G.R. and Lim, W.H. (2016) Cell-mediated and humoral acute vascular rejection and graft loss: a registry study. *Nephrology (Carlton)* **21**, 147–155, https://doi.org/10.1111/nep.12577
9 Sis, B., Bagnasco, S.M., Cornell, L.D., Randhawa, P., Haas, M., Lategan, B. et al. (2015) Isolated endarteritis and kidney transplant survival: a multicenter collaborative study. *J. Am. Soc. Nephrol.* **26**, 1216–1227, https://doi.org/10.1681/ASN.2014020157
10 Salazar, I.D., Merino López, M., Chang, J. and Halloran, P.F. (2015) Reassessing the significance of intimal arteritis in kidney transplant biopsy specimens. *J. Am. Soc. Nephrol.* **26**, 3190–3198, https://doi.org/10.1681/ASN.2014111064
11 Wu, K.Y., Budde, K., Schmidt, D., Neumayer, H.H. and Rudolph, B. (2014) Acute cellular rejection with isolated v-lesions is not associated with more favorable outcomes than vascular rejection with more tubulointerstitial inflammations. *Clin. Transplant.* **28**, 410–418, https://doi.org/10.1111/ctr.12333
12 Rabant, M., Boullenger, F., Gnemmi, V., Pelle, G., Glowacki, F., Hertig, A. et al. (2017) Isolated v-lesion in kidney transplant recipients: Characteristics, association with DSA, and histological follow-up. *Am. J. Transplant.* **18**, 972–981
13 Menon, M.C., Keung, K.L., Murphy, B. and O'Connell, P.J. (2016) The use of genomics and pathway analysis in our understanding and prediction of clinical renal transplant injury. *Transplantation* **100**, 1405–1414, https://doi.org/10.1097/TP.0000000000000943
14 Reeve, J., Einecke, G., Mengel, M., Sis, B., Kayser, N., Kaplan, B. et al. (2009) Diagnosing rejection in renal transplants: a comparison of molecular- and histopathology-based approaches. *Am. J. Transplant.* **9**, 1802–1810, https://doi.org/10.1111/j.1600-6143.2009.02694.x
15 Reeve, J., Chang, J., Salazar, I.D., Lopez, M.M. and Halloran, P.F. (2016) Using molecular phenotyping to guide improvements in the histologic diagnosis of t cell-mediated rejection. *Am. J. Transplant.* **16**, 1183–1192, https://doi.org/10.1111/ajt.13572
16 Salazar, I.D., Merino Lopez, M., Chang, J. and Halloran, P.F. (2015) Reassessing the Significance of intimal arteritis in kidney transplant biopsy specimens. *J. Am. Soc. Nephrol.* **26**, 3190–3198, https://doi.org/10.1681/ASN.2014111064
17 Bolstad, B.M., Irizarry, R.A., Astrand, M. and Speed, T.P. (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185–193, https://doi.org/10.1093/bioinformatics/19.2.185
18 Schmid, R., Baum, P., Ittrich, C., Fundel-Clemens, K., Huber, W., Brors, B. et al. (2010) Comparison of normalization methods for Illumina BeadChip HumanHT-12 v3. *BMC Genomics* **11**, 349, https://doi.org/10.1186/1471-2164-11-349
19 Edgar, R., Domrachev, M. and Lash, A.E. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **30**, 207–210, https://doi.org/10.1093/nar/30.1.207

Clinical Science (2018) **132** 2269–2284
https://doi.org/10.1042/CS20180745

20 Vapnik, V.N.V. (1998) *Statistical Learning Theory*, Wiley, New York
21 Meyer, D., Hornik, K., Weingessel, A. and Leisch, F. (2017), e1071: Misc Functions of the Department of Statistics, Probability Theory Group. R package version 1.6-8. 2017 [Available from: https://CRAN.R-project.org/package=e1071]
22 Guyon, I.W.J., Barnhill, S. and Vapnik, V.N. (2002) Gene selection for cancer classification using support vector machines. *Machine Learning* **46**, 389–422, https://doi.org/10.1023/A:1012487302797
23 Molinaro, A.M., Simon, R. and Pfeiffer, R.M. (2005) Prediction error estimation: a comparison of resampling methods. *Bioinformatics* **21**, 3301–3307, https://doi.org/10.1093/bioinformatics/bti499
24 Zweig, M.H. and Campbell, G. (1993) Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clin. Chem.* **39**, 561–577
25 Wohlfahrtova, M., Brabcova, I., Zelezny, F., Balaz, P., Janousek, L., Honsova, E. et al. (2014) Tubular atrophy and low netrin-1 gene expression are associated with delayed kidney allograft function. *Transplantation* **97**, 176–183, https://doi.org/10.1097/TP.0b013e3182a95d04
26 Simon, R., Radmacher, M.D., Dobbin, K. and McShane, L.M. (2003) Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. *J. Natl. Cancer Inst.* **95**, 14–18, https://doi.org/10.1093/jnci/95.1.14
27 Ambroise, C. and McLachlan, G.J. (2002) Selection bias in gene extraction on the basis of microarray gene-expression data. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 6562–6566, https://doi.org/10.1073/pnas.102102699
28 Krawczuk, J. and Lukaszuk, T. (2016) The feature selection bias problem in relation to high-dimensional gene data. *Artif. Intell. Med.* **66**, 63–71, https://doi.org/10.1016/j.artmed.2015.11.001
29 Hruba, P., Brabcova, I., Gueler, F., Krejcik, Z., Stranecky, V., Svobodova, E. et al. (2015) Molecular diagnostics identifies risks for graft dysfunction despite borderline histologic changes. *Kidney Int.* **88**, 785–795, https://doi.org/10.1038/ki.2015.211
30 Saint-Mezard, P., Berthier, C.C., Zhang, H., Hertig, A., Kaiser, S., Schumacher, M. et al. (2009) Analysis of independent microarray datasets of renal biopsies identifies a robust transcript signature of acute allograft rejection. *Transpl. Int.* **22**, 293–302, https://doi.org/10.1111/j.1432-2277.2008.00790.x
31 Reeve, J., Sellares, J., Mengel, M., Sis, B., Skene, A., Hidalgo, L. et al. (2013) Molecular diagnosis of T cell-mediated rejection in human kidney transplant biopsies. *Am. J. Transplant.* **13**, 645–655, https://doi.org/10.1111/ajt.12079
32 Bulbuloglu, E., Yildiz, H., Senoglu, N., Coskuner, I., Yuzbasioglu, M.F., Kilinc, M. et al. (2011) Protective effects of zinc, pentoxifylline, and N-acetylcysteine in an animal model of laparoscopy-induced ischemia/reperfusion injury of the small intestine. *J. Laparoendosc. Adv. Surg. Tech. A* **21**, 947–951, https://doi.org/10.1089/lap.2011.0194
33 Hadj Abdallah, N., Baulies, A., Bouhlel, A., Bejaoui, M., Zaouali, M.A., Ben Mimouna, S. et al. (2018) Zinc mitigates renal ischemia-reperfusion injury in rats by modulating oxidative stress, endoplasmic reticulum stress, and autophagy. *J. Cell. Physiol.*, https://doi.org/10.1002/jcp.26747
34 Halloran, P.F., Pereira, A.B., Chang, J., Matas, A., Picton, M., De Freitas, D. et al. (2013) Potential impact of microarray diagnosis of T cell-mediated rejection in kidney transplants: the INTERCOM study. *Am. J. Transplant.* **13**, 2352–2363, https://doi.org/10.1111/ajt.12387
35 Jang, H.R., Ko, G.J., Wasowska, B.A. and Rabb, H. (2009) The interaction between ischemia-reperfusion and immune responses in the kidney. *J. Mol. Med. (Berl.)* **87**, 859–864, https://doi.org/10.1007/s00109-009-0491-y
36 Ponticelli, C. (2014) Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. *Nephrol. Dial. Transplant.* **29**, 1134–1140, https://doi.org/10.1093/ndt/gft488
37 Wu, K., Budde, K., Lu, H., Schmidt, D., Liefeldt, L., Glander, P. et al. (2014) The severity of acute cellular rejection defined by Banff classification is associated with kidney allograft outcomes. *Transplantation* **97**, 1146–1154, https://doi.org/10.1097/01.TP.0000441094.32217.05
38 Sis, B., Bagnasco, S.M., Cornell, L.D., Randhawa, P., Haas, M., Lategan, B. et al. (2015) Isolated endarteritis and kidney transplant survival: a multicenter collaborative study. *J. Am. Soc. Nephrol.* **26**, 1216–1227, https://doi.org/10.1681/ASN.2014020157
39 Sollinger, H.W. (1995) Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation* **60**, 225–232, https://doi.org/10.1097/00007890-199508000-00003
40 Novotny, M., Hruba, P., Vichova, P., Maluskova, J., Honsova, E., Viklicky, O. et al. (2018) Isolated v-lesion represents a benign phenotype of vascular rejection of the kidney allograft- a retrospective study. *Transpl. Int.* **31**, 1153–1163, https://doi.org/10.1111/tri.13286
41 Halloran, P.F., Famulski, K.S. and Reeve, J. (2016) Molecular assessment of disease states in kidney transplant biopsy samples. *Nat. Rev. Nephrol.* **12**, 534–548, https://doi.org/10.1038/nrneph.2016.85
42 Madill-Thomsen, K.S., Wiggins, R.C., Eskandary, F., Bohmig, G.A. and Halloran, P.F. (2017) The effect of cortex/medulla proportions on molecular diagnoses in kidney transplant biopsies: rejection and injury can be assessed in medulla. *Am. J. Transpl.* **17**, 2117–2128, https://doi.org/10.1111/ajt.14233
43 Reeve, J., Halloran, P.F. and Kaplan, B. (2015) Common errors in the implementation and interpretation of microarray studies. *Transplantation* **99**, 470–475, https://doi.org/10.1097/TP.0000000000000691