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REVERSE TRANSCRIPTASE MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION IN ENDOMYOCARDIAL BIOPSIES FOR THE DIAGNOSIS OF CARDIAC ALLOGRAFT REJECTION

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ABSTRACT

Background

Molecular biology has emerged as a potential companion to histology for the diagnosis of rejection after heart transplantation. Reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) is a technique of targeted gene expression analysis suitable for formalin-fixed paraffin-embedded (FFPE) biopsies. Our aim was to assess RT-MLPA for the diagnosis of allograft rejection in heart transplantation.

Methods

We performed a cross-sectional, case-control, multicenter study. After selection of a 14-transcript panel (endothelial burden, Natural-Killer cells, interferon- γ pathway, effector T-cells and antigen presentation), RT-MLPA was applied to 183 FFPE endomyocardial biopsies (EMB), randomized into a training (n=113) and a validation (n=70) series. A two-step class prediction analysis was developed (Linear prediction score - LPS1: rejection versus non-rejection; LPS2: antibody-mediated rejection -AMR- versus acute cellular rejection -ACR). A study of the agreement between pathology and RT-MLPA was performed.

Results

Overall, 48 ACR, 82 AMR, five mixed-rejection, and 48 non-rejection EMBs were analyzed. Three molecular clusters were delineated by unsupervised hierarchical analysis (molecular non-rejection, ACR and AMR). AMR was characterized by high expression of CCL4, GNLY, FCGR3, CXCL11 and ACR by high expression of CCL18 and ADAMdec. RT-MLPA and histopathology agreed in the final diagnosis in 82.2%, 67.7%, and 76.8% of the EMB in the test, validation and overall cohort, respectively. Disagreement cases were more common in the case of histological low-grade rejection and early post-transplant EMB.

Conclusion

RT-MLPA is a suitable technique for targeted gene expression analysis on FFPE EMB with a good overall agreement with the histological diagnosis of heart allograft rejection.

INTRODUCTION

Protocol endomyocardial biopsies (EMB) are a key element of the follow-up of heart transplant recipients.¹ The histological analysis of myocardial samples is the gold standard for the diagnosis of heart allograft rejections and guides therapies.^{2,3} However, this histology-based strategy is limited by several flaws. The reliability of histological analysis is nowadays questioned as inter-observer reproducibility has been reported to be as low as 28% for the diagnosis of acute cellular rejection (ACR).⁴ Besides, the histological grading may be particularly challenging for microvascular inflammation, a key criterion for the diagnosis of antibody-mediated rejection - AMR.^{5,6} Reliable, objective and disease-specific (i.e., rejection versus non-rejection and ACR versus AMR) markers of rejection are an important unmet clinical need.⁶

Gene expression analysis of the allograft tissue based on pan-genomic approaches has been increasingly recognized as a powerful tool for the understanding of the pathophysiological pathways of rejection after heart transplantation.^{7,8} However, several flaws may limit the impact of these techniques in everyday practice and their widespread use to improve the diagnosis of rejection. Targeted gene expression analysis has emerged as a potentially rapid, easy-to-use, and cost-effective alternative to pan-genomic approaches.^{9,10} Reverse Transcriptase-Multiplex Ligase-dependent Probe Amplification (RT-MLPA) is an efficient technique of targeted gene relative expression analysis suitable for formalin-fixed paraffin-embedded (FFPE) tissue.^{11,12} Our aim was to assess RT-MLPA for the diagnosis of allograft rejection in heart transplantation as a companion tool to histopathology. Using a 14-transcript RT-MLPA signature, we built a two-step class prediction analysis and compared molecular diagnosis (non-rejection, AMR, and ACR) to histopathology.

MATERIALS AND METHODS

Study design

We performed a retrospective, case-control, multicenter study. A total of 206 EMB were selected (allograft rejection: n=148, no rejection: n=58) corresponding to 206 adult heart transplant recipients monitored from five French heart transplant centers (Georges Pompidou European hospital - Paris, La Pitié-Salpêtrière - Paris, Rouen, Strasbourg and Nantes University Hospitals) between November 2005 and April 2017. All RNAs were extracted from FFPE tissues previously used for routine histopathologic diagnosis of rejection. Twenty-three biopsies (11.2 %) failed to pass RNA quality control and were excluded. The remaining 183 patients were randomized into a training series (n=113) and a validation series (n=70). A two-step class prediction analysis (Rejection versus non-rejection; AMR versus ACR) was developed from the training series and applied to the validation series. Our principal objective was to define the agreement between pathology and RT-MLPA. The study design is summarized in **Figure 1**.

Patients

Our study complies with the Declaration of Helsinki and the institutional review board approved the protocol. Written informed consent for data collection and gene expression analysis of a stored EMB was obtained at the time of enrolment in the study. Baseline donor and recipient characteristics were collected from the prospective national registry CRISTAL database (French National Agency for Organ Procurement). Immunological and immunosuppressive regimen data were retrospectively collected from the patients' charts. Codes were used to ensure strict donor and recipient anonymity. We attest our strict compliance with the International Society for Heart and Lung Transplantation (ISHLT) ethics statement.

Histopathology

A review of all the EMB was performed by two pathologists (PB and JPDVH) at the same time to obtain a consensual pathologic diagnosis for each biopsy, independently of the initial pathologic diagnosis, clinical, and molecular data. EMB specimens were formalin-fixed, paraffin-embedded, and routinely stained with hematoxylin and eosin. Immunohistochemistry was performed in tissue sections with rabbit monoclonal anti-C4d (Cliniscience, Nanterre, France) and mouse monoclonal anti-CD68 (DakoCytomation, Glostrup, Denmark) antibodies using the immunoperoxidase method, as previously described.¹³ The preparations were entirely processed on automate Ventana-Benchmark-XT (avidin-biotin-peroxidase complex method). EMB were evaluated for rejection according to the last ISHLT classification for acute cellular rejection (ACR), and to the last recommendations for the diagnosis of AMR.^{2,3,6} Mixed rejection was defined by the presence of ACR $\geq 2R$ and pAMR $\geq pAMR2$. Histological low-grade rejections were defined as ACR 1R and/or pAMR1(I+) rejections.

Detection of antibodies against donor-specific HLA molecules

HLA typing of transplant recipients was performed by molecular biology using the Innolipa HLA typing kit (Innogenetics, Belgium). Heart transplant donors, HLA-A, -B, -DR, and -DQ tissue-typing was performed using the microlymphocytotoxicity technique with tissue-typing trays (One-Lambda Inc, Canoga Park, CA). Briefly, identification of anti-HLA donor-specific antibodies (DSA) was performed by Luminex SA (LuminexLABScreen Single Antigen, One-Lambda Inc) as previously described.¹⁴ The value of 500 MFI in our laboratory was based on the criteria of validation of negative control sera.

RT-MLPA probes design

Fourteen transcripts were included in the RT-MLPA assay. Genes were selected from the Affymetrix and NanoString literature for their performance to diagnose rejection and to

distinguish between AMR and ACR.^{7,8,15,16} They included interferon-induced genes (CXCL11, CXCL9), endothelial activation related genes (PLA1A, ROBO4, ECSCR, TM4SF18, ICAM-1), NK related genes (FCGR3, CCL4), cytotoxic T lymphocytes (GNLY, CD3, CCL18), and antigen presentation (CD74 and ADAMDEC1). A housekeeper gene was added to evaluate the global gene expression level (LDHA). The probe's design has been described by Bobée et al.¹¹ Briefly, all 5' probes presented a common 5' end tail corresponding to the fluorescent labelled PCR probe illu2-FAM. In addition to the specific region, 3' probes presented a common sequence at their 3' end, a phosphorylated end for the ligation reaction at their 5' extremity and spacer sequences between the specific and common regions to allow the separation of the amplicons during the fragment size analysis. The common sequences were used to amplify all ligation products within a single PCR reaction. Probes used in the mix are detailed in **Supplementary Table 1**.

RT-MLPA procedure

The protocol and data processing of the RT-MLPA procedure were used as previously described, using MLPA reagents from MRC-Holland (Amsterdam, The Netherlands).¹¹

RNA extraction: All RNAs were extracted from FFPE tissues previously used for routine histopathologic diagnosis of rejection. RNA isolation from paraffin blocks was performed using the 16 LEV FFPE Maxwell kit and the Maxwell 16 automation system (*Promega, Madison WI*) using the manufacturer's instructions. **Reverse transcription:** 2 mL of total RNA from the FFPE EMB were added to 3.75 mL of reverse transcription mix containing random hexamer primers. Next, 0.5 mL of Moloney murine leukemia virus reverse transcriptase was added, and the samples were incubated for 15 minutes at 37°C for cDNA synthesis, heated for 2 minutes at 98 °C, and cooled at 4 °C. **Ligation :** 3 mL of RT-MLPA probe mix was added (1.5 mL of SALSA-MLPA buffer and 1.5 mL of final dilution probe mix) before denaturation at 95°C for 2 minutes and hybridization at 60 °C for one hour. Ligation of the annealed oligonucleotides was performed

at 54 °C for 15 minutes, adding 32 mL of ligation mix, and heated for 5 minutes at 98 °C. **PCR:** 2.5 mL of the ligation mixture was added to 7.5 mL of Salsa PCR master mix containing the labeled forward primer and the unlabeled reverse primer. **Fragment analysis:** The resulting MLPA amplicons were analyzed by fragment analysis using an ABI 3130 XL capillary electrophoresis analyzer (Applied Biosystems, Foster City, CA).

Statistical methods

We report means and standard deviations to describe continuous variables, numbers and percentages for categorical variables. Comparisons between groups were performed using parametric Student t-test or ANOVA, non-parametric Kruskal-Wallis, Willcoxon and χ^2 test. Unsupervised clustering analysis and dendrograms of the molecular profiles obtained after RT-MLPA procedures were performed with the hcluster module of the amap package in the R software. Linear predictor scores were obtained using the LPS package in R software (<https://cran.r-project.org/web/packages/LPS/index.html>), implemented from Wright et al.¹⁷

A class prediction analysis was developed to assign each molecular profile obtained by RT-MLPA with a molecular diagnosis: mNR (molecular non-rejecting), mACR (molecular ACR) and mAMR (molecular AMR). A first linear predictor score (LPS1) was built to identify rejection cases (rejection vs mNR) and a second (LPS2) to discriminate mAMR from mACR cases. Each LPS was first developed in the training series that was randomly divided into two subgroups. The first subgroup ($\frac{2}{3}$ of the profiles = training set) and the second subgroup ($\frac{1}{3}$ of the profiles = validation set) were used to create the model and to assess its internal validation, respectively. The threshold was fixed to a probability of 90 %. External validation was then performed using the validation series (**Figure 1**). Statistical significance was set at $p \leq 0.05$. All tests were two-sided. Statistical analyses were performed using STATA 15.0 software (StataCorp LP, College Station, TX) and R software.

RESULTS

Patients characteristics and histopathology

The clinical and histopathological characteristics of the 183 included patients are summarized in Table 1. In brief, patients were mostly males (66%) and mean age at transplant was 46.7 ± 14 years old. Almost three-quarters of the patients received an induction therapy based on anti-thymocyte globulin (ATG) infusions. Most of the patients were on triple immunosuppressive therapy at the time of EMB (calcineurin inhibitors, mycophenolate mofetil, and corticosteroids) and an anti-HLA DSA was present in 41.5% of the cases. Pathology analysis showed 48 ACR, 82 AMR, five mixed-rejection, and 48 non-rejecting EMBs. The median time between heart transplantation and EMB was 60.7 ± 15 months, and 16% of EMB were performed in the six weeks following heart transplantation. Time between heart transplantation and EMB was significantly shorter for ACR EMB (18.2 ± 40.5 months) compared to non-rejecting (54.2 ± 68.6 months, $p=0.02$) and AMR EMB (49.3 ± 62.4 months, $p=0.03$). Quilty lesion was present in 44 EMB. The presence of DSA was more frequent in case of histological AMR compared to other diagnoses (65.8, 23.4 and 25.5 for AMR, ACR and no rejection, respectively, $p<0.001$). In patients with DSA, the MFI was higher in patients diagnosed with histological AMR compared to other diagnoses ($8,200 \pm 5,500$, $2,700 \pm 2,200$, $3,000 \pm 2,500$ for AMR, ACR and no rejection respectively, $p=0.005$). Baseline characteristics were similar between the training and the validation series.

RT-MLPA profiles on FFPE endomyocardial biopsies

Examples of RT-MLPA profiles in EMB with a pathological diagnosis of AMR, ACR and no rejection are given in **Figure 2A-C**. AMR was characterized by high expression of CCL4, PLA1A, GNLY, CXCL9, FCGR3, and CXCL11. ACR was characterized by high relative expression of CCL18 and ADAMdec (**Figure 3**).

Unsupervised hierarchical analysis of these profiles is given in **Figure 4A**. Three molecular clusters were delineated, that corresponded to the pathological diagnosis of the non-rejection, ACR, and AMR, respectively.

Class Prediction Analysis

The steps of class prediction analysis are summarized in **Figure 4B**. A two-step linear predictor score (LPS) method was applied to train two consecutive Bayesian predictors. The first (LPS1) was built to identify rejection cases (Rejection vs. mNR) and the second (LPS2) to discriminate mAMR from mACR cases. Each LPS was first developed in the training series and applied in the validation series. The details of the performance of LPS1 and LPS2 in the training series is given in **Supplementary Figure 1**. LPS1 identified 28 (24.8%) and 10 (14.3%) mNR, 69 (61.1%) and 47 (67.1%) Rejection, in the training and validation series, respectively. LPS2 identified 31 (44.9%) and 15 (31.9%) mAMR, 31 (44.9%) and 21 (44.7%) mACR, in the training and validation series, respectively. Overall 29 cases (15.8%) and 18 (15.5%) cases were left unclassified by LPS1 and LPS2, respectively.

Agreement between histology and RT-MLPA

Table 2 details the results of the agreement between the histopathology and RT-MLPA in the training series (n=97), validation series (n=57), and overall cohort (n=154) after exclusion of the cases left unclassified by the class prediction analysis. RT-MLPA and histopathology agreed for the diagnosis of rejection in 91.8%, 82.9%, and 92.2% of the EMB in the training, validation, and overall cohort, respectively. In rejecting cases, an agreement for the type of rejection (AMR or ACR) was achieved in 86.7%, 66.7%, and 79.2% of the EMB in the test (n=60), validation (n=36), and overall cohort (n=96), respectively. Finally, RT-MLPA and histopathology agreed for

a final diagnosis (No rejection, AMR, or ACR) in 82.2%, 66.7%, and 76.8% of the EMB in test, validation and overall cohort, respectively.

Disagreement between histology and RT-MLPA

A summary of the disagreements between histopathology and RT-MLPA for the diagnosis of rejection (n=12) and for the type of rejection (n=20) is given in **Table 3**. Details of the clinical, biological, and pathological characteristics are provided in **Supplementary Table 2**. Twelve cases (6.5%) were discordant between histology and molecular diagnosis by LPS1 including eight cases of mNR diagnosis with histological rejection (mostly low-grade rejection, n = 5 (62.5%): pAMR1(I+): n=4; ACR 1R: n=1) and four cases of hNR with molecular rejection. Interestingly, graft function was severely impaired at the time of EMB in two out of four of the hNR cases with molecular rejection. The histological grade of rejection was significantly associated with the risk of discordance by LPS1, which was higher in case of the histological low-grade rejection ($p < 0.01$, **Supplementary Table 3**).

Twenty cases (17.2%) were discordant between the histology and molecular diagnosis by LPS2 including 13 cases (65%) of hAMR classified as mACR. The time post-transplant was significantly associated with the risk of discordance by LPS2, which was higher in patients with EMB < six weeks post-transplant ($p = 0.02$, **Supplementary Table 3**).

DISCUSSION

We described a first evaluation of the diagnostic performance of RT-MLPA, a technique of targeted gene expression analysis suitable for FFPE EMB to detect biopsy-proven rejection in heart transplantation. By studying the relative expression of 14 selected transcripts (endothelial burden, NK cells, interferon- γ pathway, effector T-cell and antigen presentation) on 183 EMB, the unsupervised analysis identified three clusters of gene expression. A two-step class prediction analysis was developed to assign each molecular profile obtained by RT-MLPA with a molecular diagnosis: molecular non-rejection (mNR), mACR and mAMR. Overall agreement between RT-MLPA (mNR, mACR and mAMR) and histopathology (hNR, hACR and hAMR) were 92.2%, 79.2% and 76.8% for the diagnosis or rejection, the type of rejection and the final diagnosis, respectively.

Reliable, objective and disease-specific (i.e., cellular vs. antibody-mediated) markers of rejection are an important unmet clinical need in the field of heart transplantation. Gene expression analysis of the allograft tissue based on pan-genomic approaches has been increasingly recognized as a powerful tool for the discovery and the understanding of the pathophysiological pathways of rejection in solid organ transplant.^{18–20} These techniques provided new insights into the pathophysiology of rejection after heart transplantation.⁸ Recently, the analysis of the expression of a 34-gene set using the NanoString nCounter demonstrated the efficiency of this technology in the field of AMR in heart transplantation.¹⁵ As underlined by the Banff 2017 meeting report, the molecular assessment using new methods of targeted-gene expression of a selected gene-set from FFPE routine biopsies is the next step forward for a multicenter validation of the contribution of molecular pathology in the daily clinical practice.²¹

Within this context, we evaluated the diagnostic performance of RT-MLPA, a new cost-effective, robust and easy-to-use technique of multiplex PCR able to analyze the profiles of expression of

a selected-gene set from FFPE-biopsies.¹¹ We aimed to introduce a two-step approach allowing us to discriminate non-rejection from rejection and ACR from AMR. We believe this approach fits clinical practice. The selection of our 14-transcript set was based on the Affimetrix and NanoString literature. A major interest of our technique is its applicability to FFPE EMB. First, this allows histopathological assessment of the myocardial samples analyzed, excluding the non-specific lesions such as fibrosis, fibrin clot, previous biopsy site or pericardial tissue. Second, this technique can be included in a standard of care procedure since an additional biopsy for a fresh-frozen sample is not required, thus limiting the risk of potential procedural complications. Third, we can analyze simultaneously all myocardial samples of a paraffin block. The focal distribution of the rejection accounts for the high variability of myocardial samples and the need for at least three samples to accurately assess rejection.^{2,3} Finally, RT-MLPA is a cost-effective, strong and easy-to-use technique based on common instruments and reagents and could thus easily be implemented into routine diagnosis workflows.

Using the RT-MLPA procedure and class prediction analysis, the agreement between molecular and pathology diagnoses was high (92.2%, 79.2% and 76.8% for the diagnosis of rejection, the type of rejection and the final diagnosis, respectively). However, we observed several discordant cases. At the level of diagnosis of rejection (LPS1), discordant cases included mainly cases with low-grade of rejection (pAMR1(I+), ACR1R) classified as non-rejection by RT-MLPA. Such low-grade rejections have previously been identified as molecularly quiescent according to Affimetrix and NanoString nCounter studies.^{8,15,22} Moreover, the clinical relevance of isolated subclinical pAMR1(I+) and ACR 1R is debated and international guidelines currently do not recommend their treatment.^{1,5} Other discordant cases included cases with normal histology (hNR) that were classified as molecular rejection. Interestingly, two of these cases had severe graft dysfunction at the time of biopsy and could correspond to “biopsy-negative” rejections as described by Fishbein and coll.²³ Disagreements between histology and RT-MLPA for the diagnosis of the type of rejection (ACR versus AMR) corresponded mostly to hAMR being classified as mACR.

Early post-transplant EMB were at higher risk of disagreement at the LPS2 level. Induction therapy, ischemia-reperfusion lesions, inflammatory post-transplant burden might modify gene expression and make it more difficult to distinguish AMR from ACR. Thus, time post-transplant is an important caveat and should be taken into account for data interpretation. Further studies are needed to clarify this point. Another potential confounding factor could be the presence of Quilty lesion. The present study was not designed to assess the molecular profile of Quilty lesion. However, we did not find any association between Quilty lesion and disagreements between the molecular and the histologic diagnosis (data not shown).

The aim of this study was to evaluate a simple targeted molecular method as a companion of histopathology, and not to replace the pathologist's eyes. This is why we decided to study the agreement between histological and molecular diagnoses, with the statement that neither histology nor molecular biology is the absolute truth. Thus, agreement between the two methods strongly reinforces the pathologist's diagnosis. Regarding the disagreement between histopathology and RT-MLPA, we believe that molecular data could provide relevant complementary information for the final diagnosis, from molecular quiescence in a subset of histological low-grade rejection to molecular activity in suspected biopsy-negative rejections. Our study highlights the fact that molecular studies and histopathology are complementary.

The present study should be interpreted within the context of its limitations. First, our study was not designed to assess the performance of RT-MLPA as compared to the molecular gold standard (microarray analysis). However, the molecular expression comparison between these two techniques has already been performed.¹¹ Second, the matching percentage between RT-MLPA and histopathology was lower in the validation series compared to the training series. On the one hand, despite the randomization of EMBs, two parameters that influence the agreement between molecular and histological diagnoses were found slightly and not statistically significantly imbalanced between the two series to the detriment of the validation cohort: the

time between transplantation and EMB tended to be shorter and low grade rejections more frequent in the validation series. On the other hand, the limited sample size of the validation series, smaller than the training series, might have increased the risk of sample bias and potentially limit the overall performance of our test in this cohort. Finally, the findings of this retrospective cross-sectional case-control study must be further validated. The next step will be to analyze longitudinally the targeted-gene expression profile of rejections and to evaluate the dynamic changes before rejection and after its treatment.

Conclusion

We demonstrated that RT-MLPA is a suitable method to analyze FFPE EMB in the setting of a clinical transplant practice. A signature of 14 transcripts could distinguish between ACR, AMR, and absence of rejection. Integration of molecular assays to conventional histopathology represent the next step for the development of a reliable gold standard of rejection diagnosis.

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CONFLICT OF INTEREST

None of the authors had a conflict of interest to disclose.

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FIGURE LEGENDS

Figure 1: Flowchart of the study

183 endomyocardial biopsies corresponding to 183 heart allograft recipients were selected for the study and randomized into a training (n=113) and a validation series (n=70).

hACR: histological diagnosis of acute cellular rejection, hAMR: histological diagnosis of antibody-mediated rejection, hMixed: histological diagnosis of mixed rejection and hNR: histological diagnosis of non-rejection.

Figure 2: RT-MLPA profiles

Molecular profiles of antibody-mediated rejection (A) with high expression of CCL4, PLA1A, GNLY, CXCL9, FCGR3 and CXCL11 and of acute cellular rejection (B) with high expression of ADAMdec and CCL18 as compared to non-rejecting control EMB (C).

Figure 3: Expression of transcripts according to histological diagnosis

Boxplots represent the relative level expression for each marker. Points correspond to samples. Corrected p-values are drawn to compare expression level between groups (hAMR: histological antibody-mediated rejection n=82, hACR: histological cellular rejection n=48, hNR: histological non-rejection n=48. Mixed rejection (n=5) is not shown).

Non parametric tests were performed, Kruskal-Wallis test for global group comparisons, and Mann-Whitney test for pairwise comparisons. P-values are corrected for multiple testing using Holm's correction).

CCL4, PLA1A, CXCL9, FCGR3, CXCL11, and TN4SF18 were overexpressed in hAMR biopsies compared to histological non-rejection. CD8A, CXCL9, CCL18, and ADAMdec were overexpressed in hACR biopsies compared to histological non-rejection. ECSCR, ROBO4, and

TM4SF18 were under expressed in histological rejection, either AMR or ACR, compared to histological non-rejection.

Kruskal-Wallis test: CCL4, ECSCR, CXCL9, PLA1A, CD8A, CCL18, CXCL11, ROBO4, ADAMdec, TM4SF18: $p < 10^{-15}$, GNLY: $p = 0.005$, CD74: $p < 0.0001$, FCGR3: $p = 0.0002$.

Figure 4. - Unsupervised hierarchical and class prediction analysis

A. Unsupervised hierarchical analysis of RT-MLPA analysis from the 183 EMB. Histological diagnosis is provided on the upper line (Pathology). Three clusters of molecular patterns are delimited by the relative expression of the 14 selected transcripts. The lower line (RTMLPA) provides the molecular diagnosis according to the two steps in the class prediction analysis. Unclassified: Cases unclassified by the first linear prediction scores (LPS1). Unclassified rejection: cases classified as rejection by LPS1 but unclassified by LPS2 (see below).

B. The principle of the RT-MLPA prediction model for rejection. The RT-MLPA assay consists of two consecutive LPS. The first LPS is designed to identify rejection cases among all EMB, whereas the second is designed to discriminate mAMR from mACR.

LPS means Linear prediction score, mACR: molecular acute cellular rejection, mAMR: molecular antibody-mediated rejection, mNR: molecular non-rejection.

Supplementary Figure 1: Class Prediction Analysis

“Heat maps represent training (A – B) and validation (C – D) sets, along with the expected histopathological classes (middle panel called “truth”) and the predicted probability to belong to each of the groups (top panel). Samples (columns) are arranged by increasing LPS scores. Genes (rows) are ranked by their discriminating power (t statistics showed on the right). Black vertical bars split samples according to the group prediction (A and C: rejection-non rejection, B and D: mAMR-mACR). The confidence threshold to classify a sample was set at 90 percent.

A and C: Non rejection - rejection predictor (LPS1) in the training (A) and validation (C) sets, discriminated by differential expression of ROBO4/ECSCR/TM4SF18 (down in the rejection group) and CCL4/CXCL11/CXCL9 (up in the rejection group).

B and D: mAMR - mACR predictor (LPS2) in the training (B) and validation (D) sets discriminated by differential expression of CCL18/CD74/CD8A/ADAMDEC1 (down in the mAMR) and PLA1A/TM4SF18/CXCL11 (up in the mAMR).”

**Pathology & mRNA
extraction**
n = 183

Training series
n = 113

hNR: n = 33
hACR: n = 32
hAMR: n = 48
hMixed: n = 2

Validation series
n = 70

hNR: n = 15
hACR: n = 16
hAMR: n = 36
hMixed: n = 3

RT-MLPA

**Class Prediction
Analysis**

Training set
(2/3)

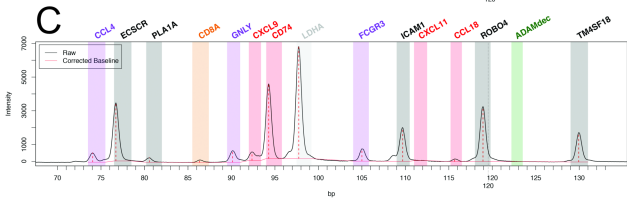
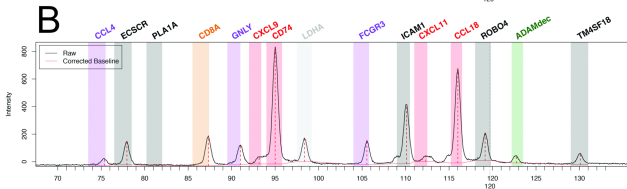
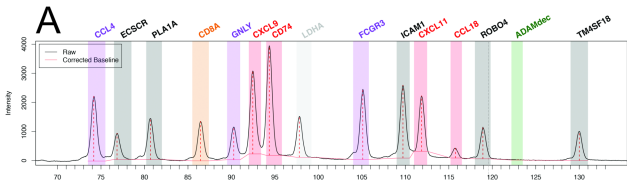
Validation set
(1/3)

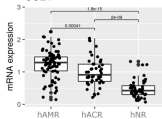
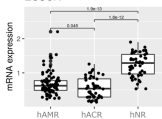
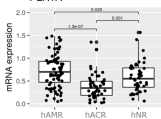
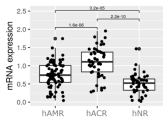
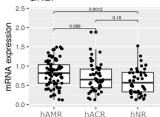
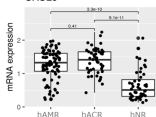
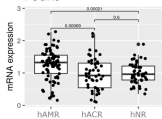
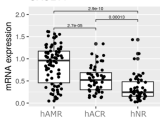
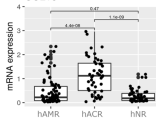
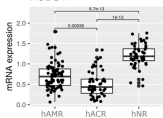
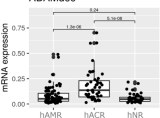
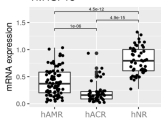
Internal
validation

External
validation

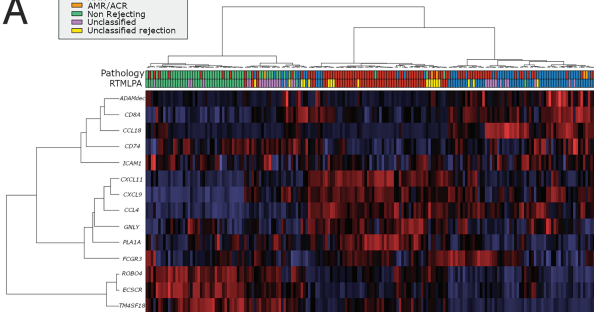
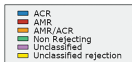
**Class Prediction
Analysis**

Study of agreement with histopathology

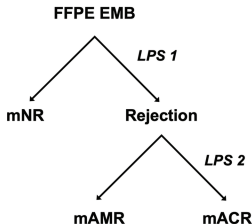


CCL4**ECSCR****PLA1A****CD8A****GNLY****CXCL9****FCGR3****CXCL11****CCL18****ROBO4****ADAMdec****TM4SF18**

A



B



Training, n = 113

hNR=33, hACR=32, hAMR=46, hMixed=2

Validation, n = 70

hNR=15, hACR=16, hAMR=36, hMixed=3

Training, n = 69

hNR=2, hACR=25, hAMR=40, hMixed=2

Validation, n = 47

hNR=2, hACR=14, hAMR=29, hMixed=2

Table 1: Characteristics of patients and endomyocardial biopsies

	Training series	Validation series	p value
	(n=113)	(n=70)	
Donor age (years)	40.9±14	41.6±13.3	0.76
Donor gender (male: n, %)	75 (67.6)	46 (65.7)	0.48
Recipient age (years)	47.5±14.3	45.6±13.4	0.37
Recipient gender (male: n, %)	85 (75.2)	51 (72.9)	0.72
Cold ischemic time (min)	180±59	187±61	0.48
Primary Heart disease			
- Dilated cardiomyopathy (n,%)	60 (53.1)	33 (47.1)	
- Ischemic cardiomyopathy (n,%)	33 (29.2)	24 (34.3)	0.68
- Other (n,%)	20 (17.7)	13 (18.6)	
Induction regimen			
- ATG (n,%)	76 (67.3)	58 (82.9)	0.04
- Basiliximab (n,%)	33 (29.2)	12 (17.1)	
- Other (n,%)	4 (3.5)	0 (0)	
IS regimen at biopsy			
- Steroids (n,%)	103 (91.1)	67 (97.1)	0.12
- Calcineurin inhibitors (n,%)	108 (95.6)	65 (94.2)	0.68
- MMF (n,%)	96 (85)	60 (87)	0.71
- mTOR inhibitors	42 (37.2)	17 (24.3)	0.07
Time post-transplant (months)	48±64	34±55	0.12
Time between transplant and EMB < 6 weeks (n,%)	16 (14.2)	14 (20)	0.30
Pathology			
No rejection (n,%)	33 (29.2)	15 (21.4)	

hAMR (n,%)	46 (40.7)	36 (51.4)	0.31
- <i>pAMR1(I+)</i> (n,%)	7 (6.2)	4 (5.7)	
- <i>pAMR1 (H+)</i> (n,%)	8 (7.1)	8 (11.4)	
- <i>pAMR2-3</i> (n,%)	31 (27.4)	24 (34.3)	
hACR (n,%)	32 (28.3)	16 (22.9)	
- <i>1R</i> (n,%)	5 (4.4)	8 (11.4)	
- <i>2R</i> (n,%)	15 (13.3)	6 (8.6)	
- <i>3R</i> (n,%)	12 (10.6)	2 (2.9)	
hMixed rejection* (n,%)	2 (1.8)	3 (4.3)	

hACR means histological diagnosis of acute cellular rejection, mAMR: histological diagnosis of antibody-mediated rejection, hMixed rejection: histological diagnosis of mixed rejection, IS regimen: immunosuppressive regimen.

Table 2: Agreement between histopathology and RT-MLPA

Agreement for the diagnosis of rejection	
- Test series (n=97)	89 (91.8%)
- Validation series (n=57)	53 (82.9%)
- Overall (n=154)	142 (92.2%)
Agreement for the type of rejection	
- Test series (n=60)	52 (86.7%)
- Validation series (n=36)	24 (66.7%)
- Overall (n=96)	76 (79.2%)
Agreement for final diagnosis	
- Test series (n=90)	74 (82.2%)
- Validation series (n=48)	32 (66.7%)
- Overall (n=138)	106 (76.8%)

Table 3: Details of disagreement between histopathology and RT-MLPA

Pathology	RT-MLPA	n (%)
<i>for the diagnosis of rejection (n=12)</i>		
No rejection	mAMR	2 (16.7%)
No rejection	mACR	0 (0%)
No rejection	Rejection	2 (16.7%)
pAMR1(I+)	mNR	4 (33.3%)
pAMR1(H+)	mNR	1 (8.3%)
pAMR2-3	mNR	1 (8.3%)
ACR 1R	mNR	1 (8.3%)
ACR 2R	mNR	1 (8.3%)
ACR 3R	mNR	0 (0%)
Mixed	mNR	0 (0%)
<i>for the type of rejection (n=20)</i>		
pAMR1(I+)	mACR	2 (10%)
pAMR1(H+)	mACR	2 (10%)
pAMR2-3	mACR	13 (65%)
ACR 1R	mAMR	2 (10%)
ACR 2R	mAMR	1 (5%)
ACR 3R	mAMR	0 (0%)

ACR means acute cellular rejection, AMR: antibody-mediated rejection, mACR: molecular diagnosis of acute cellular rejection, mAMR: molecular diagnosis of antibody-mediated rejection, mNR: molecular diagnosis of non-rejection.