Development and validation of a peripheral blood mRNA assay for the assessment of antibody-mediated kidney allograft rejection: A multicentre, prospective study

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Abstract

Background: Antibody-mediated rejection, a leading cause of renal allograft graft failure, is diagnosed by histological assessment of invasive allograft biopsies. Accurate non-invasive biomarkers are not available.

Methods: In the multicentre, prospective BIOMARGIN study, blood samples were prospectively collected at time of renal allograft biopsies between June 2011 and August 2016 and analyzed in three phases. The discovery and derivation phases of the study (N = 117 and N = 183 respectively) followed a case-control design and included whole genome transcriptomics and targeted mRNA expression analysis to construct and lock a multigene model. The primary end point was the diagnostic accuracy of the locked multigene assay for antibody-mediated rejection in a third validation cohort of serially collected blood samples (N = 387). This trial is registered with ClinicalTrials.gov, number NCT02832661.

Findings: We identified and locked an 8-gene assay (CXCL10, FCGR1A, FCGR1B, GBP1, GBP4, IL15, KLRC1, TIMP1) in blood samples from the discovery and derivation phases for discrimination between cases with (N = 49) and without (N = 134) antibody-mediated rejection. In the validation cohort, this 8-gene assay discriminated between cases with (N = 41) and without antibody-mediated rejection (N = 346) with good diagnostic accuracy (ROC AUC 79·9%; 95% CI 72·6 to 87·2, p < 0·0001). The diagnostic accuracy of the 8-gene assay was retained both at time of stable graft function and of graft dysfunction, within the first year and also later after transplantation. The 8-gene assay is correlated with microvascular inflammation and transplant glomerulopathy, but not with the histological lesions of T-cell mediated rejection.

Interpretation: We identified and validated a novel 8-gene expression assay that can be used for non-invasive diagnosis of antibody-mediated rejection.

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kidney biopsies according to the regularly updated Banff international consensus [4]. Antibody-mediated rejection can be diagnosed in clinically indicated biopsies at time of graft functional problems (rise in serum creatinine or proteinuria), but can also occur subclinically, without changes in these graft functional parameters. Subclinical antibody-mediated rejection also associates with increased risk of graft failure [5] but often remains undetected, unless protocol-specified (surveillance) kidney biopsies are performed [2,6]. Such protocol-specified biopsies are routinely performed in some centres, but not all, at varying time after transplantation.

Based on the association between antibody-mediated rejection and kidney graft failure, and the impossibility to repeatedly perform invasive protocol-specified biopsies, non-invasive diagnostic markers are needed with better sensitivity and specificity than eGFR and proteinuria [2,5,7]. Other groups have suggested non-invasive markers for antibody-mediated rejection, primarily assessed in urine samples [8–13]. Additional validation of these markers is necessary to support their potential clinical value [14–16].

Kidney allograft rejection is associated with molecular changes in renal allograft tissue, which reflect transcription changes in resident cells (e.g. interferon-gamma inducible changes in the donor endothelium) or changes in cell populations, like infiltration and activation of effector T cells and macrophages in T-cell mediated rejection or margination and activation of natural killer cells in antibody-mediated rejection [17,18]. As these graft infiltrating cells are activated primarily in lymphoid organs before travelling and infiltrating the allograft [19], we hypothesized that the molecular changes that occur in renal allograft biopsies with antibody-mediated rejection could also be reflected by changes in circulating immune cells.

Given the lack of non-invasive, sufficiently validated markers for antibody-mediated rejection, we aimed to develop and validate an mRNA-based gene set in peripheral blood that is able to non-invasively rule out or detect ongoing antibody-mediated rejection after kidney transplantation.

2. Methods

2.1. Study design, patient population and sample collection

This part of the BIOMarkers of Renal Graft InJuries (BIOMARGIN) study (www.biomargin.eu) is a multicentre, prospective, multiphase study, performed in four European transplant centres (Hôpital Necker Paris, France; University Hospitals Leuven, Belgium; Medizinische Hochschule Hannover, Germany; and Centre Hospitalier Universitaire Limoges, France). Samples were prospectively and consecutively collected at time of renal allograft biopsies, between June 2011 and August 2016. In the four clinical centres, protocol renal allograft biopsies were performed at 3, 12, and sometimes at 24 months after transplantation, according to local centre practice, in addition to clinically indicated biopsies (biopsies at time of graft dysfunction). All adult patients who had received a single kidney allograft at these institutions and who provided written informed consent, were eligible. Recipients of combined transplantsations were excluded. All transplantations were performed with negative complement-dependent cytotoxicity cross-matches. Institutional review boards and national regulatory agencies (when required) approved the study protocol at each clinical centre.

The study was divided in three phases. In the discovery phase, blood and biopsy samples were used for genome-wide expression analysis. We selected samples based on availability and histological criteria of concomitant renal allograft biopsies (excluding cases with diagnosis of glomerulonephritis or polyomavirus nephropathy, and cases with unclear diagnosis). Based on local biopsy readings, a first selection was made, which was then further refined by judgment of the clinical courses and final confirmation by central pathology, independent from the original centre (see the Supplementary Appendix). The same study design was used for the second derivation phase, for targeted
validation of the results obtained in the discovery set, and derivation of
the multigene marker. In the validation cohort, all samples with con-
comitant adequate renal allograft biopsy histology, prospectively col-
clected according to the BIOMARGIN protocol between June 24, 2014
and July 2, 2015 were serially included. In this cohort, no selection
was made on histology, demographics, time or any other factor than
sample availability. In this validation cohort, the analyses were entirely
based on central pathology scores.

2.2. Primary and secondary end points

The primary end point was the diagnostic accuracy of a multigene
marker for antibody-mediated rejection in the validation cohort in rela-
tion to the current gold standard of tissue pathology, based on the area
under the receiver operating characteristic curve (ROC AUC). Secondary
endpoints were the diagnostic accuracy in specific clinical situations (at
time of graft dysfunction leading clinicians to perform an indication bi-
opsy versus at time of stable graft function, early (>1 year) versus later
(>1 year) after transplantation), and net benefit for clinical decision-
making.

2.3. Sample collection and biopsy scoring

Peripheral blood samples were collected at time of the renal allograft
biopsies, directly in PAXgene Blood RNA tubes® (PreAnalytiX GmbH, a
Qiagen/ BD Company, Switzerland). Two needle cores were taken at
each kidney allograft biopsy. One was used for histology, at least half
of the other one was immediately stored in Allprotect Tissue Reagent®
(Qiagen Benelux BV, Venlo, The Netherlands) for RNA expression anal-
ysis (in the discovery set). All biopsies were rescored semiquantitatively
(Qiagen Benelux BV, Venlo, The Netherlands) for RNA expression anal-
ysis using RT-PCR using OpenArray® technology on the Quantstudio
 Arrays (Affymetrix Inc., High Wycombe HP10 0HH, UK). In the deriv-
ation and validation cohorts, RNA expression analysis of mRNA extracted
from blood samples was evaluated by real-time polymerase chain reac-
tion (RT-PCR) using OpenArray® technology on the Quantstudio™ 12 K
Flex Real-Time PCR System (Life Technologies Europe BV, Ghent,
Belgium) with ACTB, GAPDH and SDHA as endogenous controls. Details
on blood and biopsy sample collection and gene expression analysis are
provided in the Supplementary Appendix.

2.4. Statistical analysis

In the discovery phase, robust-multiarray average-normalized
mRNA expression data of the 117 peripheral blood samples and 95 bi-
opies were analyzed in a statistical pipeline developed under the R
framework in an extension of the biosigner R package as developed for
this study [20], with addition of Elastic-Net and Shrunken Centroids
multivariate methods to the Sparse Partial Least Squares (SPLS), Ran-
don Forrest and Support Vector Machines-Recursive Feature Elimina-
tion (SVM-RFE) multivariate methods already available in the biosigner
package. More information on the constructed statistical pipe-
line and determination of a multivariate score for antibody-mediated
rejection (ABMR score) and T-cell mediated rejection (TCMR score) is
given in the Supplementary Appendix. A multivariate score > 0.25
was considered as specific for antibody-mediated and/or T-cell medi-
ated rejection. Ingenuity Pathway Analysis (IPA, Build: 478438 M Con-
tent version: 44691306) was used for canonical pathway enrichment
analysis.

In the derivation phase, we identified the multivariate combination
of transcripts that lead to the best model performance, based on the ex-
tended list of transcripts obtained in the discovery phase. This
identification of the multigene signature was done by ranking a combi-
nation of genes according to the C-statistic of logistic regression models
trained on this combination and estimated under a 3-folds cross valida-
tion. Instead of identifying the best combination as the final multigene
signature, we integrated the combinations obtained by the top K models
(see Supplementary Appendix). The best multigene signature was then
used to build a multivariable logistic regression model in a nested-cross
validation approach on the derivation cohort. The ensuing logistic re-
gression model (intercept and estimations) was then locked and repre-
seated the final multigene assay.

The diagnostic accuracy of the locked multigene signature and logis-
tic regression model calculated in the derivation phase was then eval-
uated on the validation cohort. We used receiver operating characteristic
(ROC) curves to evaluate the C-statistic (area under the curve, AUC)
of the multigene assay. The optimal marker threshold from the derivation
phase, at the highest combination of sensitivity and specificity (which
are independent of disease prevalence), was then evaluated in this val-
idation cohort. In addition, arbitrarily defined low and high thresholds
with respectively high negative and positive predictive values (which
are dependent on actual disease prevalence) were evaluated in the val-
ification cohort.

Finally, post-hoc sensitivity analyses were performed to evaluate the
accuracy of the marker in specific clinical situations. The net benefit of
the 8-gene marker for clinical decision-making was evaluated using de-
cision curve analysis [21]. To allow for comparison and assess added
value of the 8-gene assay compared with clinical parameters alone,
we built a clinical model using the 8 clinical parameters that were differ-
ently prevalent in the ABMR vs. no AMBR group. This model was built
and cross-validated in the discovery and derivation phases combined
and then assessed in the independent validation phase. The diagnostic
performances of the clinical model, the 8-gene assay and an integrated
model (adding the 8-gene assay to the clinical model) were assessed
and compared using ROC CONTRAST and random forest out-of-bag
error rates (using 500 trees). For variance analysis of continuous clinical
variables in different groups, non-parametric Wilcoxon-Mann-Whitney
U, non-parametric ANOVA and parametric one-way ANOVA were used.
Dichotomous variables were compared using the chi-square test. R [22],
SAS (version 9.4; SAS institute, Cary, NC) and GraphPad Prism (version
7; GraphPad Software, San Diego, CA) were used for data presentation.
Normalized signal intensities and. CEL files of the transcriptomic data
were deposited at the NIH Gene Expression Omnibus http://www.
cbi.nlm.nih.gov/geo under the series accession number GSE129166)
and the microarray data were handled in accordance with the MIAME
(Minimum Information About a Microarray Experiment) guidelines.
The BIOMARGIN study is registered with ClinicalTrials.gov, number
NCT02832661. The checklist according the STARD guidelines for diag-
nostic accuracy studies was completed and is included in the Supple-
mentary Appendix (Table S9).

2.5. Study approval

All patients provided informed written consent.

3. Results

3.1. Baseline characteristics

We included 687 peripheral blood samples from 630 patients ob-
tained at the time of a renal allograft biopsy, 120 with antibody-
mediated rejection and 567 without (Fig. 1). In the discovery phase,
117 blood samples and 95 biopsy samples were used for genome-
wide expression analysis from 117 patients, in a case-control study de-
sign. In the derivation phase, we included 183 samples from 183 indi-
vidual patients, again in a case-control setting. In the independent
validation cohort, 387 consecutively collected and unselected samples
from 365 patients were included in the analyses; 56 samples were
Fig. 1. Study design. Peripheral blood samples were obtained at the time of a renal allograft biopsy in four European transplant centres. In the discovery and derivation cohort, samples were selected based on availability and histological criteria of concomitant renal allograft biopsies (excluding cases with diagnosis of glomerulonephritis or polyomavirus nephropathy, and cases with unclear diagnosis), while graft function was not taken into account. In the validation cohort, all samples with concomitant adequate renal allograft biopsy histology, prospectively collected between June 24, 2014 and July 2, 2015, were serially included without selection on histology, demographics or time. The gene expression profile was not complete in seven of these samples, leading to a total of 387 cases in the validation phase. ABMR = antibody-mediated rejection.
In both genelists, there was significant enrichment in natural killer cell signaling, crosstalk between dendritic and natural killer cells, communication between innate and adaptive immune cells and antigen presentation pathways. Predicted upstream factor analysis identified interferons and interferon regulatory factors as the most likely upstream regulators (Table S3 in the Supplementary Appendix).

3.3. Gene signature identification and model development in the derivation cohort

From the genelists obtained in the discovery phase, we selected 44 transcripts for RT-PCR analysis in the derivation cohort, based on combinations of ABMR and TCMR scores in blood and allograft biopsies, robustness of the results with different probesets of the same gene and by their involvement in relevant canonical pathways (Table S4 in the Supplementary Appendix). First, 26 genes with ABMR score > 0.25 and TCMR score < 0.20 in blood were selected (of these selected transcripts, nine also had a high ABMR score > 0.25 in kidney biopsies). We additionally selected 17 genes with an ABMR score > 0.25 in biopsies and ABMR score > 0.20 in peripheral blood. Finally, given the biological homology of CXCL11 with CXCL10 and an ABMR score of 0.49 in biopsy samples (but only 0.08 in blood), we added CXCL11 to the gene panel in the derivation phase. The univariate associations of the expression of these 44 genes with rejection phenotypes are shown in Fig. S2 in the Supplementary Appendix. From these 44 genes, a gene signature specific for antibody-mediated rejection was identified on the samples of the derivation cohort (N = 183), and included the following eight genes: CXCL10, FCGR1A, FCGR1B, GBP1, GBP4, IL15, KLRC1, TIMP1. More information on the role of these eight genes in ABMR is provided in Table S5. Subsequently, this 8-gene signature was used to build a logistic regression model with nested loop internal cross-validation for discrimination between cases with and without antibody-mediated rejection in the derivation cohort. Applied to the samples of the derivation cohort, this gene signature and logistic regression model yielded a ROC AUC of 78.1% (95% confidence interval [CI], 70.7 to 85.6; p < 0.0001) (Fig. 2).

3.4. Diagnostic accuracy of the 8-gene assay in the validation cohort

The 8-gene signature and logistic regression model built and locked on the derivation cohort were evaluated on the 387 samples collected in the validation cohort, which contained 41 cases with antibody-mediated rejection (10.6%), representing the natural prevalence of this phenotype in the cohort of biopsies performed at the participating centres. The 8-gene assay reached a ROC AUC of 79.9% (95% CI, 72.6 to 87.2; p < 0.001) (Fig. 3 in the Supplementary Appendix). When we evaluated the diagnostic accuracy for discrimination between pure antibody-mediated rejection (N = 38) and pure borderline changes and T-cell mediated rejection (N = 13 and 3 respectively), the 8-gene assay reached a ROC AUC of 82.2% (95% CI 70.7 to 93.8, p = 0.001), and 79.3% (95% CI, 71.6 to 86.9; p < 0.001) for discrimination between pure antibody-mediated rejection (N = 38) and absence of rejection (N = 330). This 8-gene assay was not diagnostic for the group of T-cell mediated rejections (containing mostly borderline changes) (Fig. S4 in the Supplementary Appendix).

We next validated the cut-off value of the 8-gene assay that was determined in the derivation phase (Fig. S5 in the Supplementary Appendix). The optimal cut-off from the derivation phase for the 8-gene biomarker (−1.08) had a sensitivity of 73.2%, specificity of 75.7%, Youden index of 0.49, positive predictive value of 26.3% and negative predictive value of 96.0% in the validation cohort (Table 3 and Fig. S6 in the Supplementary Appendix).

3.5. Sensitivity analysis

The 8-gene assay retained its accuracy for antibody-mediated rejection in patients with stable graft function and at time of graft

| Variable | Mean (median) ± standard deviation (min – max) or no. (%) |
|----------|----------------------------------------------------------|
| Transplant characteristics (N = 365 patients) | |
| Recipient age at transplantation (years) | 50·2 (52·2) ± 15·3 (2·7–78·5) |
| Recipient age at time of biopsy (years) | 52·4 (54·1) ± 14·4 (19·0–79·6) |
| Recipient sex (male/female) | 224/141 (61·4–38·6%) |
| Repeat transplantation | 64 (17·5%) |
| Recipient ethnicity (European/Asian/African/Other) | 318/3·6/35* (87·8%–90·8%–1·7%–9·7%) |
| Donor age (years) | 50·6 (52·0) ± 15·5* (5·0–91·0) |
| Donor sex (male/female) | 177/180* (50·4–49·6) |
| Deceased/Living donor | 287/83* (77·0%–23·0%) |
| Heart-beating/Non-heart-beating donor | 250/28* (89·9–10·1) |
| Cold ischemia time (hours) | 12·1* (12·6) ± 7·8 (0·27–35·8) |

| Biopsy characteristics (N = 387 biopsies) | |
| Indication/protocol biopsy | 134/253 (34·6%–65·4%) |
| Time after transplantation (days) | 908 (359) ± 1733 (6–12,564) |
| Biopsy time after transplantation | 207 (53·5%) |
| < 1 year | 180 (46·5%) |
| > 1 year | 127 (32·7%) |
| MDRD eGFR, (mL/min/1·73 m²) | 43·0 (41·8) ± 17·5 (5·8–96·2) |
| Proteinuria (g/g creatinine) | 0·4–0·1 ± 1·0 (0·0–8·0) |

| Immunosuppression at time of biopsy | |
| Cyclosporine | 40 (10·3%) |
| Tacrolimus | 331 (85·5%) |
| Mycophenolate | 320 (82·7%) |
| Azathioprine | 9 (2·3%) |
| mTOR inhibitor | 49 (12·7%) |
| Corticosteroids | 352 (91·0%) |
| Histological diagnosis | |
| No rejection | 330 (85·3%) |
| Borderline changes | 15 (3·9%) |
| Grade 1 or 2 | 4 (1·0%) |
| Antibody-mediated rejection | 41 (10·6%) |
| Mixed rejection | 3 (8·0%) |
| Interstitial fibrosis/tubular atrophy | |
| Grade 0 | 182 (47·0%) |
| Grade 1 | 93 (24·0%) |
| Grade 2 | 64 (16·5%) |
| Grade 3 | 48 (12·4%) |
| Polymavirus-associated nephropathy | 14 (3·6%) |
| De novo/recurrent glomerulonephritis | 26 (6·7%) |

* Mixed rejection cases are defined by co-occurrence of antibody-mediated rejection and T-cell mediated rejection. * Missing data on donor age, donor gender, donor type, cold ischemia time, proteinuria, recipient ethnicity and induction therapy.

excluded, 49 because of inadequate biopsy histology and seven because of incompleteness of the gene expression profile. Patients’ demographics and clinical characteristics of the three independent peripheral blood sample sets are provided in Table 1 and Table S1 in the Supplementary Appendix. There were significant differences between patients with versus without antibody-mediated rejection in some baseline characteristics, reflecting the background risk for development of antibody-mediated rejection as shown in Table 2. Details on the clinical characteristics of the biopsy samples used for micro-array gene expression (N = 95) were provided separately [18]. Histological characteristics of the biopsies in the validation set are provided in Table S2.

3.2. Discovery of transcripts specific for antibody-mediated rejection

In peripheral blood and biopsy samples, respectively 970 and 783 probesets (730 and 576 individual genes) had an ABMR score > 0.25. Pathway enrichment analysis of the biopsy signature was previously published [18]. Based on ABMR scores in peripheral blood and biopsies, 2 genelists were determined (Fig. S1 in the Supplementary Appendix).
dysfunction, within the first year and also later after transplantation (Table 3, Fig. 3, Table S6 in the Supplementary Appendix). In all scenarios, the 8-gene assay allowed to rule out ongoing antibody-mediated rejection with high negative predictive values. However, the power was low in these sub-analyses, and these accuracy parameters should be interpreted cautiously. The 8-gene assay reached universal high specificities for antibody-mediated rejection, but the positive predictive value was lower due to the low prevalence of this disease in the validation cohort, especially in protocol-specific antibodies and proteinuria.

### 3.6. Correlation of the 8-gene assay with histological and clinical variables

The 8-gene assay correlated with graft functional parameters like eGFR and proteinuria, and with histological lesions diagnostic for antibody-mediated rejection like glomerulitis, peritubular capillaritis, microvascular inflammation, and transplant glomerulopathy in the validation cohort (Table S7 in the Supplementary Appendix). Distribution of the 8-gene assay score per histological lesion grade is shown in Fig. 4.

#### 3.7. Comparison with traditional biomarkers and added clinical value of the 8-gene assay

The 8-gene assay associated with diagnosis of antibody-mediated rejection, independent of traditional factors associating with antibody-mediated rejection (female gender, recipient age, time after transplantation, presence of donor-specific antibodies and proteinuria) (Table S8 in the Supplementary Appendix). We next built and internally cross-validated a clinical model on the data from the discovery and derivation phase combined, which consisted of the 8 clinical parameters that differed between ABMR vs. no ABMR cases (donor-specific...
antibodies, proteinuria, eGFR, time after transplantation, recipient age at time of transplantation, donor age, recipient sex and protocol vs. indication biopsy). In the validation set, this clinical model reached an AUC of 77·3% (95% CI 68·5 to 86·0, \( p < 0·0001 \)). Adding the result of the 8-gene assay to the clinical model increased the diagnostic accuracy for ABMR to 88·0% (95% CI, 82·7 to 93·3; \( p < 0·0001 \)) (Fig. S7 in the Supplementary Appendix). Random forest out-of-bag error rates in the validation set were comparable for the 8-gene assay and the clinical model (14·99% and 14·73%, respectively). When adding the 8-gene assay to the clinical model the error rate dropped to 11·37%. In protocol biopsies (at time of stable graft function), the clinical model was not contributive (AUC of 55·7%, 95% CI, 37·2–74·2). Both in indication and protocol biopsies the 8-gene assay had added diagnostic value on top of the clinical model (Fig. S7 in the Supplementary Appendix). Decision curve analysis confirmed the net benefit of using the 8-gene assay for diagnosis of antibody-mediated rejection across the range of probability thresholds between 5% and 35% (Fig. S8 in the Supplementary Appendix).

### Table 3

| Population (N = ABMR/total) | Diagnostic Accuracy (ROC AUC) | Accuracy (Total correct/total) | Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value |
|-----------------------------|-----------------------------|-------------------------------|------------|------------|--------------------------|--------------------------|
| All biopsies (N = 41/387)   | 79·9% (72·7–87·2)           | 35·1%                         | 95·1%      | 28·0%      | 13·5%                    | 98·0%                    |
| Low threshold (−2·50)       | \( p < 0·0001 \)            | 75·5%                         | 73·2%      | 75·7%      | 26·3%                    | 96·0%                    |
| Optimal threshold (−1·08)   |                            | 89·1%                         | 22·0%      | 97·1%      | 47·4%                    | 91·3%                    |
| High threshold (0·50)       |                            |                               |            |            |                          |                          |
| Post-hoc sensitivity analyses |                           |                               |            |            |                          |                          |
| Early biopsies <1 year (N = 10/207) | 90·9% (85·3–96·4) | 33·8%                         | 100%       | 30·5%      | 6·8%                     | 100%                     |
| Low threshold (−2·50)       | \( p < 0·0001 \)            | 79·2%                         | 90·0%      | 78·7%      | 17·6%                    | 99·4%                    |
| Optimal threshold (−1·08)   |                            | 93·2%                         | 20·0%      | 97·0%      | 25·0%                    | 96·0%                    |
| High threshold (0·50)       |                            |                               |            |            |                          |                          |
| Late biopsies > 1 year (N = 31/180) | 73·5% (63·6–83·4) | 36·7%                         | 93·5%      | 24·8%      | 20·6%                    | 94·9%                    |
| Low threshold (−2·50)       | \( p < 0·0001 \)            | 71·1%                         | 67·7%      | 71·8%      | 33·3%                    | 91·5%                    |
| Optimal threshold (−1·08)   |                            | 84·4%                         | 22·6%      | 97·3%      | 63·6%                    | 85·8%                    |
| High threshold (0·50)       |                            |                               |            |            |                          |                          |
| Biopsies at time of graft dysfunction (N = 30/134) | 83·4% (75·4–91·3) | 34·4%                         | 100%       | 31·4%      | 6·2%                     | 100%                     |
| Low threshold (−2·50)       | \( p < 0·0001 \)            | 77·5%                         | 72·7%      | 77·7%      | 12·9%                    | 94·8%                    |
| Optimal threshold (−1·08)   |                            | 79·1%                         | 26·7%      | 94·2%      | 57·1%                    | 81·7%                    |
| High threshold (0·50)       |                            |                               |            |            |                          |                          |

*The optimal threshold for the ROC curve was chosen in the derivation phase at maximum sensitivity and specificity. §Low and high thresholds were arbitrarily selected in the independent validation cohort. ROC AUC = area under the receiver operating characteristic curve.

Fig. 3. Diagnostic accuracy of the 8-gene assay for antibody-mediated rejection in specific subgroups in the validation set (N = 387). Post-hoc sensitivity analysis of the 8-gene marker according to time after transplantation is shown in panel A and according to stable graft function vs. graft dysfunction in panel B.
4. Discussion

In this multicentre, prospective, multiphase study we identified and validated an 8-gene expression assay in peripheral blood samples with good diagnostic accuracy for non-invasive diagnosis of antibody-mediated rejection. The 8-gene assay retained this accuracy both at time of stable graft function and at time of graft dysfunction, within the first year and also later after transplantation. The diagnostic accuracy of the 8-gene assay for antibody-mediated rejection was superior to that of clinical indicators, and the assay offered benefit in clinical decisions to perform or not perform a biopsy for diagnosis of antibody-mediated rejection.

This study is a landmark in the field of biomarker discovery and development in renal transplantation in several aspects. First, its multiphase study design with independent discovery, derivation and validation sets allowed for robust biomarker development and validation [15,16]. After nested loop internal cross-validation in the derivation set, we performed independent external validation of the locked and fully specified model in a representative population with natural prevalence of antibody-mediated rejection, using the same technology platform. Second, stringent phenotypic selection with central reassessment was applied, minimizing the interobserver variability in the current gold standard for diagnosis of rejection and reference standard for performance of the biomarker. Third, the comparison with an internally cross-validated and externally validated clinical model, and assessment of the net benefit of using this 8-gene assay indicate the clinical usefulness of this marker on top of routinely available clinical markers.

The unbiased transcriptomic analysis on the discovery set illustrated that the molecular changes that occur in renal allograft biopsies with antibody-mediated rejection, primarily related to immune regulation, are also reflected in transcriptional differences in peripheral blood. The selected gene transcripts in our study come from biologically plausible molecular pathways, based on gene pathway enrichment analyses. Especially natural killer cells and their interaction with dendritic cells are known to be notorious players in humoral rejection [23]. The significant enrichment of these pathways in our gene transcripts corroborates the specificity of our marker for antibody-mediated rejection. Furthermore, the eight individual genes have relevant pathophysiological functions and most have references to rejection as found from proposed gene transcript lists [4,17].

In addition, we assessed the accuracy of this biomarker in different clinical scenarios. The clinical value of a biomarker in renal transplantation depends on the setting in which biopsies are performed, as we also observed in our post-hoc sensitivity analyses. The better diagnostic accuracy of our marker in the first year after transplantation is relevant, as therapeutic implications will be greatest when antibody-mediated rejection is detected early, before chronic damage has developed and the disease becomes irreversible [3,24]. The high negative predictive value and high sensitivity of our 8-gene assay in all settings indicates that the assay can be used to rule out antibody-mediated rejection, both at time of graft dysfunction and at time of stable graft function.

Another strength of our study is the comparison with readily available clinical information, where we illustrated the added value of the 8-gene assay both at time of stable graft function and at time of graft dysfunction. As protocol biopsies are not guided by clinical information, the 8-gene assay could be used to avoid performing the biopsy in such cases. This indicates the usefulness of the biomarker and possibility to adapt cutoff values of the biomarker according to centre preference, as was illustrated using arbitrarily chosen low and high cutoffs. For further clinical implementation, validation of these cutoffs in the same way the optimal cutoff was validated, is still warranted.

Finally, the high specificity for antibody-mediated rejection at all instances is clinically meaningful, although the positive predictive value is lower, primarily related to the low prevalence of antibody-mediated rejection in this cohort. Yet, the decision curve analysis of our 8-gene assay confirmed that the test offers benefit in clinical decision making over the range of clinically reasonable thresholds for performing a biopsy. It can be anticipated that the positive predictive value of our test would be even higher in higher-risk cohorts, as was illustrated in our subanalysis of cases with donor-specific antibodies. In further clinical validation of this biomarker, the diagnostic performance in specific risk groups, at different time points and in different clinical situations will need to be addressed in larger populations.

Our study has several limitations. Definition of antibody-mediated rejection is a topic of active discussion, which makes our reference standard of histology imperfect [4]. This may have affected the final diagnostic accuracy of our 8-gene assay and re-evaluation of the diagnostic performance of our assay in updated versions of the Banff diagnostic classification will be needed. Moreover, given the inherent difficulties with histological diagnosis of antibody-mediated rejection as gold standard for diagnosis of antibody-mediated rejection (reproducibility, sampling error), better diagnostic accuracy of any test is not expected. In addition, the study design of the validation cohort did not allow evaluating the prognostic performance of the assay for future antibody-mediated rejection, or for prediction of outcome of patients with antibody-mediated rejection. Also, in clinical practice, the combination of our 8-gene assay with non-invasive mRNA markers for T-cell mediated rejection and chronic tubulo-interstitial injury, as were previously proposed [25–29], would be interesting to assess different graft injury processes simultaneously. Correlations of the marker with lesions like transplant glomerulopathy are rather weak, leaving biopsies in some cases indispensable for determining the degree of injury and the utility of treatment. As the vast majority of our population was treated with a calcineurin inhibitor-based immunosuppressive regimen, further studies are necessary to assess whether the 8-gene assay is also valuable in patients on other immunosuppressive regimens. The same remark applies for the fact that the majority of our population was of Caucasian ethnicity, requiring further studies to assess whether the 8-gene assay is also valuable in patients of other ethnic groups. Also, our validation cohort with real-life disease prevalence had very low incidence of TCN1, with a majority of the TCN1 samples meeting only criteria for borderline changes. Although this reflects the natural disease prevalence in our clinical centres, this could differ from other clinical centres.
with different clinical practice, and perhaps overestimate the discriminative performance of our marker for ABMR versus TCMR.

In conclusion, we present a novel 8-gene biomarker with robust accuracy for non-invasive diagnosis of antibody-mediated rejection after kidney transplantation. Further evaluation and validation is warranted in larger prospective studies.

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Data sharing statement

All of the individual de-identified participant data that underlie the results reported in this article (text, tables, figures, and appendices) can be made available on a collaborative basis following institutional review board approval, immediately following publication, without end date and for any purpose. Proposals should be directed at maarten.naens@kuleuven.be.

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Declaration of interests

This manuscript is related to a European patent application (Title: mRNA-based biomarkers for antibody-mediated transplant rejection; Application Number EP19152365.3), which has been filed on January 17, 2019.

Author contributions

MN, PM, DA, WG and ME conceived and designed the study. EL, HDl, L-HL, BS, DK, ME, WG, DG and MN collected the clinical data and samples. SY, HDl, LVL, FS and MN performed the gene expression analyses. EVL, SG, SY, MC, LT and MN did the statistical analyses and interpreted the data. EVL, SY and MN wrote the draft of the report. All authors revised the report.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.028.

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