Supplemental Documentation

A practical guide to the clinical implementation of biomarkers for subclinical rejection following kidney transplantation

Maarten Naesens MD PhD[1,2], John Friedewald MD[3], Valeria Mas MD PhD[4], Bruce Kaplan MD[5], Michael M. Abecassis MD MBA[6]

[1]Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium
[2]Department of Nephrology and Renal Transplantation, University Hospitals Leuven, Leuven, Belgium
[3]Departments of Medicine and Surgery, Comprehensive Transplant Center, Northwestern University Feinberg School of Medicine, Chicago, USA
[4]Transplant Research Institute, University of Tennessee Health Science Center, Memphis, USA
[5]Department of Transplantation, Baylor Scott and White Health System, Temple, Texas, USA
[6]Departments of Surgery and Microbiology-Immunology, Comprehensive Transplant Center, Northwestern University Feinberg School of Medicine, Chicago, USA

Abbreviations

CLIA   Clinical Laboratory Improvement Amendments

CMS   Centers for Medicare and Medicaid

COU   Context of Use

LCD   Local Coverage Determination

NPV   Negative Predictive Value

PPV   Positive Predictive Value

ROC   Receiver Operator Characteristic

subAR   Subclinical Acute Rejection

Detailed evaluation of the commercially available and promising biomarkers for subAR

Urine Gene Expression Profile (3-gene signature)

Suthanthiran et al reported a 3-gene signature in urine samples capable of detecting acute kidney transplant rejection.[1] The authors included both indication biopsies and biopsies at time of stable graft function, with matched urine samples. Cohorts with real-life prevalence of (mostly clinically evident) rejection were used for both discovery (N=206; 145 indication biopsies, 61 protocol biopsies) and validation (N=71; 36 indication biopsies and 35 protocol biopsies) cohorts. Standardized PCR assays developed in the discovery cohort were used for the validation cohort. The marker showed an area under the curve [AUC] of 0.85 (95% confidence interval [CI], 0.78 to 0.91; P<0.001) by receiver-operating-characteristic curve analysis for (primarily T-cell mediated) rejection. The number of patients with antibody-mediated rejection (AMR) was small and prevented in-depth evaluation of the value of this biomarker for diagnosing this phenotype.

The comparison with current standard of care (graft functional assessment) for rejection was not reported. The low number of rejection biopsies at time of stable graft function in this study (N=5/61 in the discovery cohort and 7/35 in the validation cohort) did not allow assessing the diagnostic performance for subAR at time of stable graft function. Specifically at time of graft dysfunction, the discovery cohort suggested clinically useful diagnostic accuracy.

Next to this diagnostic performance of the 3-gene signature, the authors also tested aspects of the performance of this test as risk biomarker for future rejection, by including a large number of urine sample analyses prior to rejection diagnosis. This analysis suggested that the 3-gene signature associated with rejection prediction, although this was not independently validated. No effort to link the 3-gene expression profile to other outcomes as prognostic biomarker (future chronic injury, graft function or graft failure) was made.

To our knowledge, there is not current effort underway to commercialize the assay and therefore no COU has been proposed.

Urine CXCL9 Protein

Hricik et al[2] reported that urine CXCL9 protein, in the absence of coincident infection, was able to detect clinically evident acute rejection (≥ Banff grade I). These authors found a bootstrapped diagnostic accuracy for acute rejection in indication biopsies (i.e. at time of graft dysfunction; N=150), with a high NPV of 92%, and still a useful PPV of 68% (ROC AC 0.86). This model was not compared to any standard-of-care clinical model. Cohorts with prevalent incidence of rejection were used for both discovery and validation cohorts. Standardized ELISA assays developed in the discovery cohort were used for the validation cohort. Several other studies have also suggested urinary CXCL9 as noninvasive biomarker for acute rejection at time of graft dysfunction, with mostly similar diagnostic accuracy, always for T-cell mediated rejection, providing sufficient external validation of the concept to measure CXCL9 protein in urine, but the thresholds were not fixed and thus not validated.[3] One study illustrated that urinary CXCL9 is not entirely specific for T-cell mediated rejection, and also associates with antibody-mediated rejection at time of graft dysfunction (N=281 indication biopsies).[4] One other study did evaluate this potential,[5] using a similar standardized ELISA assay. In a limited number of 51 urine samples matched with protocol biopsies, this study suggested that urinary CXCL9 also retains diagnostic accuracy at time of stable graft function, with a ROC AUC of 0.78. However, the small sample size and lack of independent validation limited the validity of these findings.

In the CTOT-01 study Hricik et al[2] reported on the urinary levels of CXCL9 protein at time of stable graft function, that preceded graft dysfunction as outcome parameter, thus suggesting urinary CXCL9 also as prognostic marker. The direct correlation with subAR in paired biopsies was not made. Rabant et al independently suggested an association between ELISA-measured urinary CXCL9 and future clinical and subclinical acute rejection, both T-cell and antibody-mediated rejection.[4] These suggestions of prognostic value were not independently validated.

Taken together, urinary CXCL9 on its own seems to be a reasonable candidate biomarker for noninvasive diagnosis of acute T-cell mediated rejection at time of graft dysfunction and with some suggestion of potential at time of stable graft function, although the latter is not validated. To our knowledge, there is not current effort underway to commercialize urinary CXCL9 protein as diagnostic biomarker to diagnose or exclude subAR.

Roedder et al reported on the performance of a PCR-based 17-Gene Expression Panel in the peripheral blood for acute rejection.[6] The study used a retrospective analysis of archived samples, and included both protocol and indication biopsies together. After evaluation in a training-set (N=143), external validation was applied to 2 independent cohorts (N=124 and N=100), with remarkably good diagnostic accuracy. The diagnostic accuracy for rejection subtypes was not assessed separately, but there was suggestion that the marker is not specific for any type of rejection, with similar levels in T-cell mediated as in antibody-mediated rejection. The excellent diagnostic performance in the validation set could be related to the reiteration of the biostatistical modelling in the validation sets,[7] although it is difficult to assess the exact contribution of this factor in the final diagnostic accuracy obtained at the validation phase.

The exact number of protocol vs. indication biopsies was not mentioned, and no sensitivity analyses on the diagnostic performance at time of stable graft function vs. at time of graft dysfunction was performed. Whether the kSORT assay works better than routine graft function tests for assessment of rejection (comparison to standard of care) was not assessed. These aspects obviate drawing conclusions on the actual potential of this multigene marker for acute rejection in specific clinical scenarios, and make it difficult to determine a clear COU as noninvasive diagnostic marker for acute rejection.

An interesting aspect of the kSORT assay is the suggestion that the kSORT test could serve also as risk marker for future rejection and as predictive marker for response to treatment after rejection. No attempt was made to associate the kSORT test result as prognostic biomarker for future graft function, chronic injury or graft survival.[6]

The kSORT test is commercialized through Immucor Dx, and the product website[8] notes that kSORT was "developed for the detection and surveillance of renal transplant rejection with goal of driving preemptive clinical interventions and improved outcomes" and "to detect renal transplant patients at high risk for acute rejection." Future studies on the performance of kSORT as diagnostic biomarker for acute rejection are necessary to evaluate its true potential in clinical routine and are underway.

Plasma Allosure ddcfDNA test (CareDx)

The detection of donor-derived cell free DNA (ddcfDNA) in the recipient's plasma has been proposed as a potential biomarker for allograft rejection, first by Snyder et al.[9] Also other studies have suggested the potential of ddcfDNA as biomarker for rejection.[10] The diagnostic company CareDx has recently developed and analytically validated the 'Allosure' test, with first clinical suggestions of association with cardiac allograft rejection (N=53 samples).[11]

Bloom et al applied this analytically validated test for ddcfDNA in 102 samples with matched kidney allograft biopsies from 14 centers, primarily at time of graft dysfunction. The diagnostic accuracy for subAR, at time of stable graft function, could not be assessed as only 1 of the limited protocol biopsies included showed subAR.[12] The ddcfDNA test discriminated rejection from no rejection, with a ROC AUC of 0.74. The test performed better for AMR versus no AMR, with a ROC AUC of 0.87. Only cases with severe TCMR had sometimes increased ddcfDNA values. Cases with grade IA TCMR did not have increased values, although low numbers warrant caution in this conclusion.

Comparison to standard of care creatinine testing was performed, and creatinine values did not discriminate acute rejection from no rejection, which illustrates that the ddcfDNA test performed better than standard of care, although low number of protocol biopsy cases (per definition with good graft function) limit the value of this analysis. More recently, Huang et al reported similar findings in patients undergoing for-cause biopsies (N=63), with the Allosure test being best associated solely with antibody-mediated rejection (ROC AUC 0.82) and not with T-cell mediated rejection.[13]

Bromberg et al reported on the range of ddcfDNA levels in a population of stable patients. The authors reported that 96% of levels in a prevalent population were below the positive threshold of 1%,[14] suggesting that a substantial number of patients with subAR tests could have a negative test, as the point prevalence of subAR is significantly higher than 4%.

While collectively, samples used for these studies were often paired with biopsies, most of the biopsies were performed for-cause with very few protocol biopsies in patients with stable renal function, which makes it difficult to assess the diagnostic potential of this test for subAR. Formal evaluation of the diagnostic performance of this ddcfDNA test at time of stable graft function seems warranted.

The Allosure test has not yet been evaluated for potential use as risk biomarker for future rejection, or as prognostic marker for future chronic injury, graft functional evolution or graft failure.

The Allosure test is commercially available. According to available product information on Allosure, the test's COU is to assess the probability of 'active' allograft rejection in kidney allograft recipients with clinical suspicion of rejection (i.e. at time of graft dysfunction) and to inform clinical decision-making about the necessity of renal biopsy in such patients at least 2 weeks posttransplant in conjunction with standard clinical assessment.[15] A large registry study is currently enrolling patients.

<u>Plasma Prospera ddcfDNA test (Natera)</u>

As illustrated above, there is theoretic potential and biological plausibility in the assessment of donor-derived cell-free DNA for assessment of transplant organ injury. Prospera is a ddcfDNA test commercialized by Natera, and uses a different technology platform than Allosure (see above). The analytical validity of this test was recently published.[16]

For the clinical evaluation, Sigdel et al reported on a retrospective cohort of 277 archived plasma samples from a single center, of which 217 were matched with paired biopsies (114 protocol biopsies, 103 indication biopsies; 38 with rejection and 72 with borderline changes).[17] The exact inclusion strategy of the samples was not specifically described, and it is difficult to assess whether this dataset represents real-life disease prevalence although rejection prevalence seems to be in line with previous studies (11.4% and 24.2% acute rejection in protocol and indication biopsies, respectively).

The assay differentiated acute rejection from absence of acute rejection (including borderline changes) especially at time of stable graft function (ROC AUC 0.89 in protocol biopsies), and irrespective of rejection subtype (T-cell mediated rejection, antibody-mediated rejection and mixed rejection). The test accuracy for acute rejection (ROC AUC 0.87) seemed better than standard-of-care eGFR evaluation for diagnosis of rejection (ROC AUC 0.74), with very high (95%) NPV and still relevant PPV (52%), but the added value of the test to eGFR was not explicitly assessed. The diagnostic performance at time of graft dysfunction (in indication biopsies) was not explicitly mentioned.

As these data are promising, independent validation on an external prospective cohort will be needed to validate the performance of the test and its threshold. Although no formal validation comparisons were made between Allosure and Prospera, the COU proposed in the LCD draft submission (currently pending final approval by CMS) as a 'me-too' test (compared to Allosure) stating that when used with all other clinical and laboratory data, the test detects "subclinical active rejection" and therefore may be useful in patients with significant contraindications to biopsies.[18] The LCD is pending and a registry study is planned.

Blood TruGraf Gene Expression Profile (Viracor-Eurofins)

The TruGraf v1 assay is a microarray-based test that analyzes a Gene Expression Profile in the peripheral blood, which makes use of 200 transcripts.[19] The test associates with either a normal kidney biopsy (Transplant eXcellent – TX) or the absence of a normal biopsy (not-TX) in patients with stable graft function.

All aspects of discovery and external validation of the TruGraf test were performed on blood samples paired with protocol biopsies from prevalent cohorts. For the purpose of validation, the model (classifiers) derived from preselected bioinformatics and the threshold used to test performance on the discovery cohort were locked. PPV of Trugraf for TX was 89% and NPV 45%, illustrating that the test is able to identify patients without ongoing injury (thus not needing protocol biopsies). The lower PPV of this test for subAR limits the value of a single positive test. Clinical utility was assessed through both retrospective and prospective surveys of physician decision impact.[20]

A recent study has suggested the prognostic potential of this test by demonstrating a correlation with graft outcomes at 24 months following kidney transplantation.[21] Comparison with current standard of care (graft functional assessment) is irrelevant in this setting as per study design only patients with stable graft function were included. How the test performs at time of graft dysfunction (indication biopsies) was not assessed. It was not yet studied whether the kinetics of the test offer any predictive value, and whether therapeutic decisions on e.g. increasing immunosuppression could be informed by the single value of the test or its kinetics.

The TruGraf v1 blood test is a laboratory-developed test performed as a service available exclusively through the CLIA certified laboratory at Transplant Genomics Inc. The COU proposed in the LCD draft submission (currently pending final approval by CMS) states that "The TruGraf test is intended for use in kidney transplant recipients with stable renal function as an alternative to surveillance biopsies in facilities that utilize surveillance biopsies."[22] While primarily used to rule out subAR, it is expected that both centers that perform or do not perform protocol biopsies can use the TruGraf test to inform the use of a protocol biopsy in a relatively small number of stable patients.[23] A registry study is planned.

Blood 7-Gene Expression Panel

Recently, Christakoudi et al[24] took an in silico approach to biomarker development wherein they identified a gene expression panel from the literature, and applied this Gene Expression Panel to a first case-control study comparing mRNA profiles of peripheral blood samples of T-cell mediated rejection in indication biopsies (N=27) with stable graft function (but without biopsy confirmation; N=335). Following this, using qPCR they tested the performance of the gene expression profile, which consisted of 7 transcripts, on both cross-sectional and longitudinal samples, comparing patients with 'stable samples' (no paired protocol biopsies) and patients with rejection (in indication biopsies, i.e. at time of graft dysfunction). The diagnostic accuracy for rejection specific at time of graft dysfunction was not assessed. Also the value of the test for subAR could not be assessed, inherent to the study design as no protocol biopsies were performed.

Given that graft function was used as a selection criterion, comparison of the biomarker performance to standard of care eGFR evaluation for noninvasive diagnosis of rejection could not be performed, as this study design led to an artificially perfect diagnostic accuracy of eGFR.

Next to the diagnostic value, the longitudinal study suggested that this 7-gene expression panel could also be used as risk biomarker for future rejection, and as predictive biomarker for therapeutic response.

There is no evidence of an attempt to commercialize, or statement of COU, or plans for a registry study.

<u>Pretransplant blood 23-Gene Expression Profile (RenalytixAI).</u>

Zhang et al used samples from a prospective multicenter cohort to develop a 23 gene set derived from whole blood RNA sequencing prior to transplantation to predict early acute rejection after kidney transplantation.[25] This test was not developed as diagnostic test for ongoing rejection, but is a risk biomarker for future acute rejection (including borderline changes and antibody-mediated rejection), irrespective of its impact of renal function (both indication and protocol biopsies were included). Instead of using an independent external cohort of patients for validation, the cohort was randomly split into 2 cohorts: a discovery (N=81) and a validation (N=74) cohort. The accuracy of the marker in the validation set reached a ROC AUC of 0.74.

The diagnostic performance of the 23-gene set for paired biopsies, either at time of stable function or graft dysfunction, was not assessed. Until now, the data have not been externally validated. The performance of the 23-gene marker was not compared to standard-of-care risk prediction for rejection, like the number and type of HLA antigen or eplet mismatches, but was better than recipient age and original kidney disease, which did not contribute to the acute rejection risk prediction in this study.

Next to its interesting performance as risk marker for acute rejection, this 23-gene panel also predicted graft failure at 2 and 5 years after transplantation.

The authors mention a US Provisional Patent Application related to this biomarker, suggesting potential for further commercialization of the test, likely through the involvement of <u>RenalytixAI (FractalDx)</u>. The potential COU could be inferred from the authors' statement that the test, performed at the time of transplant, may risk-stratify patients in terms of immune-reactivity following KT.

Blood 17-Gene Expression Profile for acute rejection at 3 months independent of graft function (RenalytixAI)

Using the same cohort of patients as in their previous study[25] where they identified a pretransplant 23 gene set to predict graft rejection and outcome, Zhang et al recently identified a posttransplant 17-gene set in blood that associates with T-cell mediated rejection (including borderline changes) at 3 months, independent of graft function (both protocol biopsies and indication biopsies obtained at 3 months were included).[26] Similarly to their previous study,[25] they randomly attributed patients to the discovery (N=88) and first validation cohort (N=65).

After initial genome-wide studies, targeted analysis of the 17-gene set was applied using a custom-made kit, first on a cross-validated training set (N=113 derived from the initial set used for gene set selection; ROC AUC 0.83), and then and the on an independent validation cohort (N=110; ROC AUC not reported). For the purpose of validation, the model (classifiers) derived from preselected bioinformatics in the training set were locked, together with the thresholds used to stratify the patients into 3 probability groups (high, intermediate and low risk). In the independent validation cohort, these tertile probability cutoffs were applied and the high threshold yielded a PPV of 73% and the low threshold a NPV of 0.89%, which is less than the values in the training set, but still clinically relevant.

All samples were paired with concomitant biopsies. However, as the biopsies were mixed protocol and indication biopsies, comparison of the diagnostic performance of the 17-gene set with a standard clinical model was needed. The cross-validated performance of a more standard clinical model of donor age, induction therapy and 3-month creatinine values remained weaker (ROC AUC 0.67) than the diagnostic value of the 17-gene panel. The diagnostic performance of the combination of the 17-gene panel with this clinical model was not assessed.

The lack of sufficient cases of antibody-mediated rejection obviated assessing the diagnostic performance of the 17-gene set for this phenotype. The test performance at time points other than 3 months, the evolution over time and predictive performance for therapy response, were not assessed.

Next to its diagnostic potential, also some prognostic potential of this test was suggested, as the 17-gene set results correlated with subclinical T-cell mediated rejection at later time points and lower death-censored graft survival.

The authors mention a US Provisional Patent Application related to this biomarker, suggesting potential for further commercialization of the test, likely through the involvement of RenalytixAI (FractalDx). No COU has been proposed, other than a statement by the authors that the "assay offers the potential to be used as an immune-monitoring tool to guide the use of immunosuppression".

Urine 11-Gene Expression Profile (Common Rejection Module)

Sigdel et al recently suggested the diagnostic potential of the Common Rejection Module (CRM) geneset for noninvasive diagnosis of acute rejection in urine samples.[27] The CRM is a set of 11 genes that was developed in a meta-analysis of publicly available microarray datasets of tissue biopsies of solid organ transplants (not restricted to kidney transplantation).[28] Applied to a set of 150 urine samples with paired biopsies in a case-control study design, the 11 CRM geneset discriminated patients with biopsy-confirmed acute rejection (in indication biopsies) from "stable" biopsies (normal protocol biopsies).

The geneset was derived from preexisting literature in tissue biopsies, and this study therefore provides suggestion that the 11-gene list is also potentially interesting for noninvasive monitoring of rejection. The urinary CRM (uCRM) score was derived using Variable Selection Using Random Forests (VSURF), and validated by splitting the data into a training set (80%) and testing set (20%). The threshold of the score was derived using a decision tree classification model. Given the low number of samples in the testing set, no diagnostic performance was provided in the testing set separately. As noted by the authors, expansion of the number of patients will be necessary in an independent validation study.[27]

The comparison between biopsy-proven acute rejection in indication biopsies and stable patients (normal protocol biopsies) make it difficult to determine a clear COU as noninvasive diagnostic marker for acute rejection. Given that graft function was thus used as a selection criterion, comparison of the biomarker performance to standard of care eGFR evaluation for noninvasive diagnosis of rejection could not be performed. To define a potential COU, larger validation studies would need evaluation of the uCRM score in relation to biopsy histology, either at time of stable graft function or at time of indication biopsies separately.

In comparison to the other assays reported in this manuscript, the uCRM assay is less advanced in its development as potential diagnostic marker, and there is no evidence of an attempt to commercialize, or statement of COU of the uCRM score.

<u>Urine 4-metabolite profile (Numares)</u>

The Numares AXINON renalTX-SCORE assay is a test that analyzes a urinary metabolite profile by the measurement of 4 metabolites (alanine, citrate, lactate and urea) by NMR in spot urine samples, normalized to creatinine.[29] The test compared acute/active rejection versus no rejection, defined according to the Banff 97 classification (which thus encompasses acute TCMR (Banff 2017) but not borderline changes or antibody-mediated rejection. The control group consisted of samples obtained maximum 7 days prior or at at the day of biopsies, and samples obtained in patients without concomitant biopsies. The dataset was split in a "strict set", consisting of samples with concomitant biopsies, and an "extended" set that included control samples obtained without a concomitant biopsy. The "test set" consisted of prospectively collected, prevalent and repetitive samples obtained in the same single center as the training cohort, but in a completely different time period. The authors report on a third, prospective clinical validation cohort, but no data are yet available on this validation study.

As also samples without concomitant biopsies are included (in the "extended set), it could be deducted that also samples at time of stable graft function were included in this study. However, no data are presented on the inclusion of protocol biopsies, which makes it unclear whether this test could be used for subAR or is positioned as test for clinical rejection. The (unpublished) details in the Numares documentation suggest using the score in combination with eGFR to classify the patients in 3 different risk categories for acute kidney rejection, from low to high,[30] which suggests that the test is able to both detect subAR as rejection at time of graft dysfunction.

The diagnostic accuracy remained stable in the independent test set (ROC AUC 0.72-0.74), but it is relevant to note that the training and test sets were combined for final model selection, and independent validation of the model is not yet done. Thresholds have not been proposed for this test, implicating that also the PPV and NPV remain unclear. The diagnostic accuracy was not affected by exclusion of samples that were not paired with biopsies. Comparison with current standard of care (graft functional assessment) is not reported. Although the test set contained repetitive sampling in patients and the website mentions the value in predicting rejection in the following days, no data of the kinetics of the test was available.

The AXINON renalTX-SCORE assay has received CE-marking in the European Union as in vitro diagnostic test, and is a Research-Use-Only product in the United States. The COU of the AXINON renalTX-SCORE mentioned on the product website states that this "is a non-invasive test intended to support the diagnosis of a kidney allograft rejection in conjunction with other measurements and clinical evaluations."[30] Also, it is mentioned that the test can indicate rejection 1 to 7 days before a documented rejection, could be used for evaluating therapeutic success, and monitor the response to anti-rejection therapy. An independent, prospective validation study is ongoing.

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