Reading Human Biopsies Using mRNA Expression
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Summary: The Molecular Microscope® Diagnostic System (MMDx®)

MMDx is a central diagnostic system that uses microarrays to measure mRNA levels in transplant biopsies. The biopsy is simply placed in RNAlater® and shipped by courier at ambient temperature to the central laboratory. The mRNA levels are measured in a microarray, and algorithms based on a large Reference Set of biopsies are used to generate an automated report, usually within 29 hours. The system has been developed in kidney transplant biopsies (INTERCOMEX study (1), clinicaltrials.gov #NCT01299168) and heart endomyocardial biopsies. Lung and liver transplant biopsy MMDx adaptations are being developed.

MMDx Uses Predefined Algorithms (Classifiers) to Assign Scores That Reflect Probabilities of:

1. Rejection
   a. T cell-mediated rejection (TCMR) Score
   b. Antibody-mediated rejection (ABMR) Score
   c. Rejection Score (TCMR and/or ABMR)
2. Injury ("wounding")
   a. Acute tissue injury
   b. Irreversible damage (Atrophy-fibrosis Score)
   c. Risk of progression to failure (heavily influenced by ongoing acute parenchymal injury).
3. Consider the possibility that recent non-adherence had trigger rejection
4. Histological diagnoses and outcomes in the Reference Set biopsies most similar at the molecular level to the new biopsy

Overview: The unmet need is PRECISION

In Managing a Troubled Transplant the Clinician Must Assess Rejection and Injury in the Organ:

Rejection
T cell-mediated rejection
Antibody-mediated rejection
Under-immunosuppression/non-adherence
Guidance for therapy

Parenchymal Injury (Wounding) and Non-Rejection Diseases
Acute parenchymal injury
Irreversible injury (atrophy-fibrosis)
Risk of progression to failure
Potentially, probability of primary diseases or infections
Conventional diagnostic systems are subjective, opinion-based, and prone to inaccuracy. The error rates in conventional biopsy assessments can be estimated by the disagreement rate between pathologists in TCMR: when one diagnoses TCMR-like changes, a second will agree in 50% for kidney transplants\(^2\), 28% for heart transplants\(^3\), and 0-18% in lung transplants\(^4\). We believe that the solution lies in centralized molecular analysis measuring mRNA levels, with automated analysis to interpret the results. *This will change care.*

**Advantages and Insights from Centralized Molecular Measurements**

(see Appendix)

1. Requires less tissue than histology
2. User-friendly: simply put tissue into RNA\_later at room temperature
3. Rapid: travel (usually overnight) plus 29 hours processing (48 hours for very small biopsies)
4. Objective, quantitative, probabilistic assessment
5. Ability to correct errors in histology: recalibrate empirical histology systems.
6. Calibrate blood and urine biomarkers
7. Identify mechanisms and druggable targets
8. “Theranostic” support for drug development and use
9. Standard, worldwide assessment

**Challenges of Molecular Assessment**

1. Potential sampling error: How much is needed to represent the organ?
2. Highly focal diseases may be missed or overrepresented
3. Search for truth when conventional phenotyping is poor: no gold standard

**What is a Microarray and How Does it Measure Gene Expression?**

- Specific DNA sequences in about 500,000 tiny squares
- The sequence in each square represents part of a gene.
- Patient sample mRNA is extracted, amplified and labeled as cRNA (antisense)
- Labeled patient cRNA hybridizes to complementary cDNA sequences in specific squares
- Intensity of label in square tells how much that gene is expressed
- Total gene expression is quantified

**MMDx Project Highlights**

- Reference sets of transplant biopsies: kidneys (>1740), hearts (>800), lungs (transbronchial, >150), livers (in planning)
- International network of Key Opinion Leaders
- Easy sample submission: needle/bioptome biopsy stabilized in RNA\_later and shipped by courier
- Extensive peer-reviewed literature support (see Appendix)
- Unique 29-48 hour biopsy processing and automated reports
- New understanding of rejection and injury mechanisms
INTERCOMEX Trial Summary

The MMDx-Kidney system was developed in ongoing kidney transplant biopsy studies beginning in 2005. Insights derived from this project have changed the understanding of mechanisms of rejection and the classification of transplant diseases (see Appendix). MMDx-Kidney studies the pattern of transcript expression in kidney tissue associated with T cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR), acute parenchymal injury (AKI) and chronic parenchymal damage (atrophy-fibrosis)\(^5,6,7\), revising the conventionally defined disease classes. Classifier algorithms based on a large reference set (currently 1208 biopsies) are used to generate probability scores for each sample's diagnoses. The percent cortex in the biopsy is estimated, although MMDx can read medulla.

TCMR has emerged as cognate T cell-antigen presenting cell engagement in the interstitium\(^8\), whereas ABMR is a natural killer cell-mediated process triggered by donor-specific antibody binding to the microcirculation\(^9\). Both share IFNG effects, which are more intense in TCMR \((10)\). ABMR has the complexity of different stages: early-stage, fully-developed, and late-stage\(^11\). The molecular changes associated with renal injury are often more extensive than suggested by histology\(^7\) and indicate that progression to graft failure is caused by continuing nephron injury, rather than autonomous fibrogenesis\(^6\). The INTERCOMEX studies are now driving toward a new classification of the disease states in biopsies using a method called archetypal analysis\(^1\).

Our clinicians indicate that when there is agreement between histology and the MMDx, it gives them greater confidence in managing the patient. When the two systems disagree, they have more faith in the molecular read than in local histology assessment\(^6\). We are iteratively refining the molecular diagnosis of TCMR and ABMR, and developing a new disease classification system. Another advantage of the MMDx system is that it can read medulla, unlike histology\(^12\) and small samples, reducing the risk of an “inadequate” or “sub-optimal” biopsy sample and the patient needing another biopsy.

The MMDx system generates an automatic report which is then signed out by a professional and transmitted to you. Biopsy processing time is usually 29 hours. Only molecular measurements and time of the biopsy post-transplant are used. The report uses the microarray measurements of the level of expression of selected genes or gene set measurements to assess the probability of TCMR, probability of ABMR, and the extent of acute and chronic injury. The possibility that recent non-adherence or under-immunosuppression has triggered rejection is raised by certain phenotypes (e.g. late-onset TCMR) and by a new classifier under development (not shown on the report).

The current report format is shown below for two different biopsies, one with TCMR and one with ABMR. This page shows the key findings (Global Disturbance (inflammation), acute kidney injury and atrophy-fibrosis scores and different rejection classifier scores) as well as the overall molecular interpretation. The relationship of the new biopsy to its nearest molecular neighbors in the Reference Set.
Current MMDx-Kidney Report (page 1) - ABMR

**General Information**

<table>
<thead>
<tr>
<th>KCL Report ID</th>
<th>Sample ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date Received (Y-M-D)</td>
<td>Time of Biopsy Post-Tx</td>
</tr>
<tr>
<td>Date Reported (Y-M-D)</td>
<td>Transplant Type</td>
</tr>
<tr>
<td>Date of Transplant (Y-M-D)</td>
<td>Biopsy Indication</td>
</tr>
<tr>
<td>Date of Biopsy (Y-M-D)</td>
<td>Primary Disease</td>
</tr>
</tbody>
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<tr>
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<td>Biopsy Indication</td>
</tr>
<tr>
<td>Date of Biopsy (Y-M-D)</td>
<td>Primary Disease</td>
</tr>
</tbody>
</table>

**Pure molecular interpretation**

Severe early-stage ABMR with g and ptc molecular features. No TCMR. Moderate atrophy-fibrosis with mild AKI.

<table>
<thead>
<tr>
<th>Classifier/gene sets</th>
<th>Biopsy score</th>
<th>Range of values</th>
<th>Upper limit of normal</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury Scores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global Disturbance Score</td>
<td>1.59</td>
<td>-3.8 - 5.8</td>
<td>0.03</td>
<td>Moderate</td>
</tr>
<tr>
<td>Acute Kidney Injury (AKI) Score</td>
<td>0.42</td>
<td>-0.6 - 1.6</td>
<td>0.39</td>
<td>Mild</td>
</tr>
<tr>
<td>Atrophy-Fibrosis Score</td>
<td>0.58</td>
<td>0.0 - 1.0</td>
<td>0.76</td>
<td>Moderate</td>
</tr>
<tr>
<td>Rejection Scores</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rejection Score</td>
<td>0.88</td>
<td>0.0 - 1.0</td>
<td>0.30</td>
<td>Severe</td>
</tr>
<tr>
<td>TCMR Score</td>
<td>0.01</td>
<td>0.0 - 1.0</td>
<td>0.10</td>
<td>Normal</td>
</tr>
<tr>
<td>ABMR Score</td>
<td>0.95</td>
<td>0.0 - 1.0</td>
<td>0.20</td>
<td>Severe</td>
</tr>
<tr>
<td>Rejection phenotype* (six scores, R1-R6, adding up to 1.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1 Non-rejection</td>
<td>0.00</td>
<td>All ABMR (Sum of R4,R5, and R6)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>R2 TCMR</td>
<td>0.00</td>
<td>R4 Early-Stage ABMR (EABMR)</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>R3 Mixed Rejection</td>
<td>0.00</td>
<td>R5 Fully-Developed ABMR (FABMR)</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R6 Late-Stage AMBR (LABMR)</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

Many other classifiers (page 2 of the reports – not shown for simplicity) are also run to establish details of ABMR. For example, these estimate the probability of glomerular double contours (cg) as an estimate of late-stage ABMR. For the first biopsy shown, these estimated the probability of microcirculation inflammation (ptc- and g-changes) and cg as high, indicating that this biopsy has fully-developed ABMR. The net result of all of these measurements is integrated by a novel method called “Archetypal Analysis”[^3].

[^3]: Additional references are not provided in the text.
Current MMDx-Kidney Report (page 1) - TCMR

General Information

<table>
<thead>
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<th>KCL Report ID</th>
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<td>Primary Disease</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>8.2 years</th>
<th>DD (Deceased)</th>
<th>investigate proteinuria</th>
<th>Hypertension, biopsy proven</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Pure molecular interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate TCMR. No ABMR. Moderate inflammation and atrophy-fibrosis with mild AKI.</td>
</tr>
<tr>
<td>The low ah classifier score (0.33 - page 2) raises the possibility of under-immunosuppression or non-adherence.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Injury Scores</th>
<th>Biopsy score</th>
<th>Range of values¹</th>
<th>Upper limit of normal²</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global Disturbance Score</td>
<td>1.44</td>
<td>-3.8 - 5.8</td>
<td>0.03</td>
<td>Moderate</td>
</tr>
<tr>
<td>Acute Kidney Injury (AKI) Score</td>
<td>0.53</td>
<td>-0.6 - 1.6</td>
<td>0.47</td>
<td>Mild</td>
</tr>
<tr>
<td>Atrophy-Fibrosis Score</td>
<td>0.63</td>
<td>0.0 - 1.0</td>
<td>0.39</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Rejection Scores</th>
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</tr>
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<tbody>
<tr>
<td>Rejection Score</td>
<td>0.64</td>
<td>0.0 - 1.0</td>
<td>0.30</td>
<td>Moderate</td>
</tr>
<tr>
<td>TCMR Score</td>
<td>0.36</td>
<td>0.0 - 1.0</td>
<td>0.10</td>
<td>Moderate</td>
</tr>
<tr>
<td>ABMR Score</td>
<td>0.09</td>
<td>0.0 - 1.0</td>
<td>0.20</td>
<td>Normal</td>
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</tbody>
</table>

Survival in patients with similar biopsies in the Reference Set

<table>
<thead>
<tr>
<th></th>
<th>1-year: 94%</th>
<th>3-years: 65%</th>
<th>96%</th>
</tr>
</thead>
</table>

1 - The 2.5th to 97.5th percentiles in the entire Reference Set.
2 - 90th percentile in relevant Reference Set biopsies.
3 - Scores from archetypal analysis
4 - %cortex is a quality control measure
The Molecular Microscope Diagnostic System for Heart Transplant Biopsies (MMDx-Heart)

INTERHEART Trial Summary
The current standard for histologic diagnosis of heart transplant endomyocardial biopsies (EMBs) uses the ISHLT classification, which has limited reproducibility\(^3\). To improve biopsy assessment, the Alberta Transplant Applied Genomics Centre (ATAGC) adapted the MMDx developed in kidney transplant biopsies to heart transplant endomyocardial biopsies (EMBs) in the INTERHEART study. The molecular phenotype of ABMR in heart transplants is remarkably similar to that in kidney transplants\(^{14}\). This permits us to use kidney rejection-associated transcripts to classify heart transplant biopsies. The first generation MMDx system, evaluated in EMBs from Paris, Edmonton, and Bologna, has been published\(^{15}\).

INTERHEART Study:
1. Expand the Reference Set for heart transplants (EMBs) with molecular, histologic, DSA, and clinical data.
2. Develop the MMDx report for EMBs, incorporating rejection and injury principles discovered in kidney.
3. Validate and refine the system by reporting in real time (<48 hours from receiving the biopsy) 300 new heart biopsies and obtain feedback from key opinion leader clinicians. This involves unselected, prospectively collected, standard-of-care EMBs from North American, European, and Australian Centers.
4. Develop and optimize a transparent and user-friendly reporting format to communicate this information to clinicians.
5. The MMDx-Heart report will express rejection as archetype scores for probability of ABMR, TCMR and non-rejection, with the scores adding up to 1.0 (see figure below).
The current standard for biopsy-based diagnoses of rejection of lung transplants is the ISHLT classification of transbronchial biopsies (TBBs), based on international consensus. This system has many weaknesses and errors, and many TBBs cannot be assessed by histology. To improve diagnostics in lung transplant biopsies, the MMDx developed in kidney transplant biopsies has been adapted to lung transplant TBBs. INTERLUNG will also explore the potential of mucosal biopsies (MB) from the third bronchial bifurcation (3B-MBs) to provide the same estimates as TBB, reducing the risks of biopsy complications.

**INTERLUNG Study:**
1. Develop a reference set of >300 TBBs with molecular, histologic, DSA, and clinical data.
2. Adapt the MMDx system to report TBBs, incorporating rejection and injury principles discovered in kidney and heart.
3. Determine from experience how many TBB bites are needed to compensate for sampling error. Current biopsies performed under research protocols usually limit MMDx to one but two or three may be needed to reduce sampling error. Because histology currently requires up to 10, MMDx-Lung can offer improved safety.
4. Report 300 TBBs in real time (<48 hours from receiving the biopsy) and obtain feedback from key opinion leader clinicians from North American, European, and Australian Centers. An example of the current biopsy reporting format is shown below, using 106 TBB as the reference set. The picture shows the emerging reference set of TBBs, and the location of the new biopsy. The use of kidney rejection-associated molecules classifies biopsies into three clusters: relatively normal; TCMR-like changes; and a third class with injury, including endothelial abnormalities of unknown significance. Clinical input from key opinion leaders will eventually establish the clinical significance of these changes (manuscript in preparation, 2017).
5. Explore the potential for a safer biopsy format by testing whether mucosal biopsies from the third bronchial bifurcation (3B-MB) can provide similar assessments to the TBB. This would offer more effective assessments at much lower risk.
The Molecular Microscope Diagnostic System for Liver Transplant Biopsies (MMDx-Liver)

INTERLIVER Summary
The Molecular Microscope will be adapted to read liver transplant biopsies for injury and rejection, using the same strategies that have been used for heart biopsies, in a new study (INTERLIVER NCT03193151). A network of liver transplant clinicians is developing the protocol, and plans are in place to have an MMDx-Liver system in place within about one year.
Insights derived from Molecular Microscope project (recent reviews (16-19))

1. Mouse models of TCMR: CATs (20); GRITs (21); injury and dedifferentiation (22-25).
2. Inflammatory/injury disturbance in kidney transplant biopsies (26), in heart transplant endomyocardial biopsies (27), and in lung transbronchial biopsies (28) is stereotyped: molecules “travel in herds”.
3. High frequency of C4d negative ABMR (29;30).
4. ABMR is the main cause of renal transplant loss and is often C4d negative (30;31).
5. High frequency of non-adherence in ABMR and graft loss (32).
6. Molecular classifier for all rejection (TCMR or ABMR) (33;34).
8. Disappearance of TCMR despite persistence of ABMR (31).
9. TCMR does not program late graft loss (31).
11. TCMR and ABMR share mechanisms (10;33).
12. Donor age is a risk factor in early ABMR (35).
14. Molecular Landscape of ABMR: potential role of NK cells and CD16a (9).
15. Molecular Landscape of ABMR in heart transplants (14).
16. Landscape of acute parenchymal injury (AKI) (7).
17. Mechanisms/landscape of atrophy-fibrosis: progression is due to injury, not autonomous fibrosis (5;38).
18. Risk score for graft failure involves AKI molecules (19).
19. Immunoglobulin transcripts (IGTs) (40) and mast cell transcripts (MCATs) (41) are features of atrophy-fibrosis.
20. TCMR-like process in polyoma virus nephropathy (PVN) (2;32).
21. Lack of TCMR in many biopsies with isolated v lesions (33;42).
22. Regression equations improve histologic prediction of TCMR (43).
23. Regression equations improve conventional diagnosis of ABMR, even without DSA (44).
24. Sub-phenotypes of ABMR: early stage, fully-developed, and late-stage sub-phenotypes (11).
25. FOXP3 expression: associated with inflammation, not outcomes (45).
27. Lack of hyalinosis may indicate non-adherence (46).
28. MMDx-Kidney can read medulla (12).
Reference List


Want More Information?

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molecular-microscope.com

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