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## THE USE OF GENOMICS AND PATHWAY ANALYSIS IN OUR UNDERSTANDING AND PREDICTION OF CLINICAL RENAL TRANSPLANT INJURY

Madhav C. Menon<sup>1,\*</sup>, Karen L. Keung<sup>2,\*</sup>, Barbara Murphy<sup>1</sup>, and Philip J. O’Connell<sup>2</sup>

Madhav C. Menon: madhav.menon@mssm.edu; Karen L. Keung: kkeu6183@uni.sydney.edu.au; Barbara Murphy: barbara.murphy@mssm.edu; Philip J. O’Connell: philip.oconnell@sydney.edu.au

<sup>1</sup>Division of Nephrology, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, New York, USA

<sup>2</sup>Centre for Transplant and Renal Research, Westmead Millenium Institute, University of Sydney at Westmead Hospital, New South Wales 2145, Australia

### Abstract

The development and application of high-throughput molecular profiling have transformed the study of human diseases. The problem of handling large, complex datasets has been facilitated by advances in complex computational analysis. In this review the recent literature regarding the application of transcriptional genomic information to renal transplantation, with specific reference to acute rejection, acute kidney injury in allografts, chronic allograft injury and tolerance is discussed, as is the current published data regarding other “omics” strategies- proteomics, metabolomics and the micro-RNA transcriptome. These data have shed new light on our understanding of the pathogenesis of specific disease conditions following renal transplantation but their utility as a biomarker of disease has been hampered by study design and sample size. This review aims to highlight the opportunities and obstacles that exist with genomics and other related technologies in order to better understand and predict renal allograft outcome.

### Introduction

Interrogation of the genome to identify the consequences of static and dynamic genomic variation on allograft outcomes is being investigated actively in kidney transplantation (see reviews).<sup>1–4</sup> This has been made possible by the combination of ease of access of sample procurement (allograft, blood or body fluid), the availability of high-throughput technologies, and complex computational analyses. Microarray technology has been used widely to study the pathogenesis of disease processes, although its translation to clinical

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Address for correspondence: Philip O’Connell, Centre for Transplant and Renal Research, Westmead Millenium Institute, Westmead Hospital, Westmead NSW 2145, Australia.

\*Equal contribution

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Assistant Professor Madhav C. Menon<sup>1</sup>, co-author of the paper.

Dr Karen L. Keung<sup>2</sup>, co-author of the paper.

Professor Barbara Murphy<sup>1</sup>, editing of the paper.

Professor Philip O’Connell<sup>2</sup>, contributed to the writing, and editing of the paper.

practice has been limited.<sup>5</sup> Nonetheless, the simultaneous detection of quantitative gene expression and the genome-wide expression profiles (GEPs) make it possible to obtain cross-sectional information regarding the physiological and cellular regulatory environment and distinguish this from pathological events such as acute rejection and graft fibrosis. Owing to the limitations of histological diagnosis in renal allograft biopsies,<sup>6</sup> application of these analyses have been shown to improve upon,<sup>7,8</sup> further classify<sup>9</sup> and precede the histological appearance<sup>10,11</sup> of disease. Transcriptional information obtained serially could help piece together sequential events that culminate in disease phenomena such as acute rejection, chronic allograft injury, tolerance and graft loss. Events identified in this fashion can be targeted earlier with the ultimate goal of altering the course of disease. At the very least, gene expression changes, once quantified and validated, could be used as biomarkers of simultaneous or impending disease states. Heritable genomic changes in either donor or recipient (e.g polymorphisms) are relatively easier to map from tissue or blood, and can be reproduced reliably in clinical settings. When such changes correlate with allograft outcomes, they have the potential to translate into clinical assays although most assays fall well short of a validated biomarker suitable for clinical use (Figure 1).<sup>12</sup> Aside from transcripts of protein coding genes, microRNAs (miRNAs) have been identified as a regulatory layer demonstrating transcriptional and translational control on cellular protein expression and phenotype,<sup>13</sup> and recently have been investigated in transplantation.<sup>14–16</sup> Similarly, epigenetic changes (DNA- or histone-methylation patterns) have the potential to predict alterations in gene expression and correlate with specific pathogenic processes.<sup>17</sup> In this paper, the current literature regarding the application of transcriptional genomic information to renal transplantation is reviewed with specific reference to what these studies bring to our understanding of acute renal allograft rejection and chronic allograft injury.

## Transcriptome assessments

Older genomic techniques confined the study of disease phenomena to one or a few candidate genes at a given time. These genes were selected based on biological plausibility<sup>18,19</sup> or animal experimentation and the studies were hypothesis-driven.<sup>3</sup> With increased availability of high throughput technologies such as microarray and RNA-sequencing, it is possible to simultaneously identify the differential expression patterns of thousands of genes that associate with a particular outcome or disease state. Further, computational analysis of this genome-wide expression information, and its mapping to experimentally identified gene-expression information within large databases (<http://www.broadinstitute.org/> or <http://www.ncbi.nlm.nih.gov/geo/>) can help reveal the cumulative signaling pathways operational in a pathologic state. This capability has given rise to a paradigm shift from traditional hypothesis-driven experiments toward large-scale hypothesis generation experiments with potential for testing through clinical trials.<sup>4</sup>

Important characteristics of commonly employed strategies to examine genomic information in the research settings in transplantation are summarized in Table-1. Microarrays work on the principle of DNA complementarity (see review).<sup>4</sup> Reverse transcribed, amplified cDNA is labeled with fluorescent probes and hybridized into separate arrays. The fluorescence is quantified by image analysis software and the probe-IDs (corresponding to a sequence within a gene) are identified by their location on the predesigned platform. Data

normalization and elimination of transcripts below a critical abundance threshold is followed by comparative analysis of fold changes of transcripts between control and disease conditions. Microarray results are expressed as relative fold changes in reference to a standard set of samples. Procurement and storage, RNA extraction methods and reverse transcription protocols, heterogeneity of gene chip assay used, fidelity of pre-array amplification techniques, and inaccuracy in identifying differences in low abundance transcripts are all sources of variation in the interpretation of microarray results. RNA-seq based on high throughput sequencing technologies offers key advantages over microarrays – single base resolution, absolute transcript quantification, need for lower amounts of total or fractionated RNA and a large dynamic range for detection of expression levels (8000-fold changes).<sup>20</sup>

The NanoString<sup>®</sup> nCounter system is a new technology for molecular profiling that uses a novel colour-coded barcode technology, with each barcode representing a single target molecule.<sup>21</sup> It enables digital counting of individual molecules without the need for amplification. Up to 800 transcripts can be profiled in a single reaction, and has been applied to samples of blood, raw cells and urine. Its role in transcriptome profiling in other biomedical fields such as oncology has been prominent in recent years, but only recently been used in the setting of renal transplantation.<sup>22,23</sup>

## Genomics of acute rejection

The pathogenesis of acute rejection (AR), cellular (ACR), antibody-mediated (ABMR) and mixed rejection, in renal allografts is well understood (see elegant reviews).<sup>24–26</sup> Important features to emerge are the redundancy within the immune effector mechanisms mediating ACR, the need to discern co-existent ABMR due to its prognostic and therapeutic implications,<sup>27</sup> and the paradigm of ongoing sub-clinical rejection and its impact.<sup>28–32</sup> These compound the complexity of developing unified genomic biomarkers for AR and histological diagnosis remains the gold standard.<sup>4</sup> Variability of the transcriptional milieu of ACR in the allograft and/or peripheral blood is also influenced by immunosuppressive agents, AR severity (borderline vs grade I or II), the timing of the rejection episode, the type/quality of organ, associated pathology and the representativeness of the sample used for transcriptome analysis. Most transcriptional studies have depended on clinical or for-cause biopsies for developing GEPs as biomarkers and may neglect transcriptional phenomenon in subclinical rejections. Transcriptional studies also frequently rely on “clean” phenotypes to develop and validate GEPs, ie specific pathology vs healthy controls – scenarios that are seldom encountered in pure form in routine clinical practice, making the information obtained difficult to both validate and/or to apply to patient care.<sup>33</sup> Notwithstanding these limitations, substantial progress has been made in understanding the peripheral blood and allograft transcriptomes of AR in renal transplantation.<sup>5,7,9,34–38</sup> The reader is referred to comprehensive reviews that have recently tabulated the studies employing genomics in renal allograft rejection.<sup>4,39</sup>

Prior to the widespread application of genomics, substantial progress had been made by using quantitative PCR (qPCR) to interrogate tissue from rejecting renal allografts for immune activation genes. Interestingly IL-2 gene upregulation was not associated with

rejection but investigators have demonstrated significant upregulation of other cytokines especially IP-10, CXCR3 and CD3 $\epsilon$ .<sup>18,19</sup> Other markers said to be predictive of ACR include FOXP3, IFN- $\gamma$ , T-cell receptor variable regions as well as cytotoxic T-lymphocyte effector molecules, such as perforin, granzyme B, Fas, and Fas ligand, and CD40 ligand.<sup>18,19,40</sup> Recognizing the increased allograft infiltration and subsequent urinary appearance of lymphocytes during ACR episodes, pre-selected immune transcripts (T-cell/immune related) in urinary cell pellets measured by qPCR have been reported to non-invasively differentiate ACR from non-ACR.<sup>41-50</sup> One study used 18S-ribosomal-RNA normalized 3-gene signature (CD3 $\epsilon$ , IP-10 & 18S-rRNA) to identify ACR from non-ACR with high accuracy.<sup>47</sup> The gene set was less effective at differentiating ACR from ABMR and borderline ACR (AUC – 0.78). More recently, the same investigators described the ability to distinguish AR from other causes of renal allograft dysfunction using a urinary gene signature, and subsequently applied a further transcript profile for ACR to distinguish ACR from ABMR within the AR group.<sup>49</sup> A multicenter study with qPCRs performed independently at 6 centers for 7 genes on the same urine samples showed significant correlations ( $R > 0.938$ ) in mRNA levels of all 7 genes suggesting the feasibility of urinary mRNA levels as biomarkers.<sup>50</sup> However, urine handling and storage for accurate transcript assessment has limited such studies to a small number of specialized research groups.

To date, many of the genomic studies have attempted to identify GEPs that can separate ACR from non-ACR. This has been possible within the context of the patients studied but the results have not been applicable to broader populations, either because the GEPs lacked the required specificity and sensitivity for general use, or alternatively they have not been studied beyond the initial reference populations. Furthermore, sample sizes were small and their general applicability, much less their diagnostic utility, were not confirmed. For instance, Akalin et al initially used high-density oligonucleotide array (Affymetrix GeneChip® Hu6800) to open-endedly analyze the allograft transcriptome of patients with ACR. They identified a panel of 4 transcripts that were present in all patients but only 7 patients were evaluated.<sup>5</sup> However, the authors restricted their analysis only to those genes with a four fold or greater increased expression over baseline biopsies, and may have missed many transcripts that were changed consistently in ACR, albeit less than this threshold. Sarwal and colleagues used DNA microarrays in a systematic study of gene-expression patterns in biopsy samples from normal and dysfunctional renal allografts.<sup>9</sup> By unsupervised hierarchical clustering, they identified four clusters of gene expression patterns, which generally corresponded to the histologically identified categories. An important finding of this study was that the gene expression pattern of the 26 ACR biopsies were classifiable into a further three clusters which were not differentiated by light microscopy, which as the authors suggest may reflect unidentified distinct mechanisms in the molecular pathogenesis of rejection and may be predictive of outcome. Again this early study lacked power and has not been corroborated by other investigators. Using gene array profiles obtained from renal transplant biopsies, Flechner et al also confirmed that it was possible to separate stable function with no rejection from graft dysfunction due to ACR and graft dysfunction not related to ACR.<sup>34</sup> While AR and non-AR GEPs in PBMCS clustered separately from those of stable transplant recipients, there was very little overlap with GEPs identified within the allograft, which were associated with immune/inflammation mechanisms.

More recent studies have improved upon the design and technical problems that had prevented the development of this technology as a biomarker for AR. These problems include the lack of large cohorts to make firm conclusions, poor study design with a lack of prospective and systematic patient follow-up, heterogeneity across platforms and most significantly the lack of corroboration of findings in separate validation cohorts. Li et al used a novel approach of high throughput transcriptional profiling of total RNA from peripheral blood validated across 3 microarray platforms, followed by further selection by biological relevance and RTPCR validation, to identify a specific 5 gene-set that correlated well with AR/no AR status on simultaneously procured allograft biopsies.<sup>38</sup> The 5-gene, qPCR-based, logistic regression model was built using samples from a single center, and validated in an independent multicenter cohort that included AR episodes from transplantation to 3 years of follow-up, of varying severity. Of note, the 5-gene set was ultimately selected based on a biological candidate gene approach ie genes with enrichment in cell-specific immune responses in AR, and not in an unbiased manner. Hence, the most differentially expressed genes by magnitude may not have been selected in the final panel. This gene set also did not distinguish ACR from ABMR, and a subsequent biopsy would be required in order to make treatment decisions. Based upon this and other publications,<sup>51,52</sup> the same group of researchers has subsequently developed a 17-gene qPCR based assay (kidney solid organ response test (kSORT) assay) from peripheral blood of both adult and pediatric recipients that diagnosed AR with an AUC of 0.94 in the development cohort (n=143), and was validated with an AUC of 0.95 in a separate validation cohort (n=124).<sup>53</sup> Prospective studies using this panel are ongoing. Reeve et al studied 403 allograft biopsies (divided into development and validation cohorts) and compared transcriptional profiles of histologically confirmed ACR (with/without ABMR lesions) to biopsies with other diagnosis in a supervised manner.<sup>7</sup> They generated a T-cell mediated rejection score (TCMR-score) using the top 20 differentially regulated transcripts in each iteration. A median TCMR-score >0.1 identified pathologist-recognized ACR lesions with an accuracy of 89%. The strength of the TCMR-score was that it provided additive information to histology and allowed for accurate reclassification of ACR/non-ACR lesions, especially where there existed variation between pathologists such as when defining borderline changes of rejection (B-ACR). The major limitations of the TCMR-score were the absence of an external validation cohort, which limited generalizability of these findings, and the lack of a cohort with subclinical ACR (ie protocol biopsies).

Clinical and histological studies have identified that graft outcome for B-ACR is not as benign as originally thought with worse histological and functional 1 to 2 year outcomes than biopsies with non ACR.<sup>29-31,54</sup> Larger transcriptional datasets of blood and allograft samples on exclusive or predominant B-ACR lesions, could help clarify whether this histological readout represents a dichotomy, a continuum or a distinct entity.

The development of a diagnostic GEP for AR has been further complicated by the difficulty to differentiate ACR, ABMR and mixed AR. Acute ABMR is diagnosed with histologic evidence of acute tissue injury (microvascular or endothelial injury and/or ATN), with evidence of current/recent antibody interaction with vascular endothelium (linear C4d staining in the peritubular capillaries), and serologic evidence of donor-specific antibodies (DSAs) (HLA or other antigens). In the recent Banff classification, ABMR has been

subdivided to include biopsies that contain only one or two of these features.<sup>55</sup> To date, the most detailed genomic evaluation of ABMR has been the INTERCOM cohort study, which contained 300 biopsies. Upon review, 41% of these biopsies had features of ABMR, that were not initially diagnosed by histology.<sup>56</sup> Using an ABMR-transcriptome score developed by the same group these biopsies were diagnosed with an accuracy of 85%.<sup>36</sup> Derived from 403 indication biopsies, allografts with high ABMR-scores had a significant over-expression of endothelium-associated transcripts and correlated strongly with the presence of DSA in the serum. The ABMR score discriminated ABMR cases from both ACR and other causes of microvascular injury. In response to these data, the Banff working group has added “increased expression of gene transcripts in the biopsy tissue indicative of endothelial injury, if thoroughly validated” as an ABMR diagnostic criteria under evidence of antibody-endothelium interaction.<sup>55</sup> In both studies high ABMR scores had prognostic import and correlated independently with allograft loss.

## Genomics of acute kidney injury in transplant

Information regarding the molecular phenotype of AKI is difficult to procure as most episodes are self-limited and performance of native kidney biopsies for AKI episodes are not clinically indicated. Kidney transplants experience AKI and are ideally positioned to study the GEP of AKI.<sup>57</sup> cDNA microarrays were used to study GEP of 14 living-donor kidneys, 15 deceased donor kidneys with immediate function and 14 deceased donor kidneys with AKI (defined by dialysis in first week). GEPs of LD-kidneys were observed to cluster separately from deceased donor kidneys (132 genes). Further, 48 transcripts related to cell cycle regulation, cell growth/metabolism, and signal transduction were significantly differentially regulated in AKI-allografts compared to those with immediate function.<sup>58</sup> In a separate study, the same authors compared GEPs of AKI allografts with carefully selected pristine 6-week protocol biopsies, and identified 394 transcripts that were differentially expressed in AKI.<sup>37</sup> Using the geometric mean of the fold increase in the top 30 differentially regulated genes (compared with control nephrectomies), they devised an IRRAT (injury-repair-response-associated-transcript) score that correlated inversely with renal function at the time, and directly with eGFR improvement thereafter. IRRAT score was also significantly associated with DGF, deceased donor status, and interstitial inflammation but not histology. IRRAT scores have since been observed to be elevated in both ACR and ABMR suggesting the non-specific nature of this injury response signature.<sup>7,36</sup> Of note, AKI related GEPs identified by different groups in transplantation have shown significant overlaps with both mouse orthologs identified in ischemia-reperfusion models,<sup>57,59</sup> and with each other suggesting molecular homogeneity in this injury response.<sup>37,58,60</sup>

## Genomics of chronic allograft injury (CAI)

Despite the significant improvement in acute rejection rates over the last two decades, this has not translated into improved long-term graft survival. Chronic allograft injury remains a major cause of long-term graft loss, and represents an irreversible pattern of injury resulting from a number of immunological (acute and chronic rejection, HLA mismatch, donor specific antibodies), and non-immunological factors (donor age, ischemia/reperfusion injury, infection, CNI toxicity).<sup>61,62</sup> There are differing opinions as to whether allograft loss is

multifactorial, as some recent observational studies suggest that most cases of allograft loss can be attributed to a single disease entity<sup>63,64</sup> whilst other published findings suggests otherwise.<sup>65</sup> Interstitial fibrosis and tubular atrophy (IF/TA) are the histopathologic hallmarks of CAI. It has subsequently been recognised that concurrent interstitial inflammation and IFTA changes on biopsy identifies recipients at greatest risk of graft loss.<sup>66,67</sup> Despite greater understanding of the factors contributing to CAI/IFTA, the precise pathophysiological mechanisms are not fully understood. Consequently, the development of targeted therapeutic strategies has been limited. As with subclinical rejection, the presence of IFTA - a histologic diagnosis, predates the measured decline in renal function. The need for earlier detection to allow for timely therapeutic intervention has provided the impetus for numerous genome, transcriptome, and proteomic studies in the search for potential non-invasive, predictive biomarkers in CAI.

The application of microarray technology to evaluate the differential gene expression in CAI first emerged a decade ago.<sup>11</sup> Since then, a number of studies utilizing microarrays have been published to characterize the genome and transcriptome profiles of allografts with IFTA although most have involved small cohorts of patients and non-prospective in design, amongst other limitations. The concept that early transcript changes may predate the subsequent histological phenotype of CAI has been an appealing hypothesis. Scherer et al<sup>68</sup> concluded that transcriptome changes precede the observed histologic changes of IFTA, after profiling the gene expression of 3-month post transplant biopsies of 20 patients, and compared those that developed IFTA at 6 months versus those who did not. Using oligonucleotide microarray, differential transcript expression between the progressors and non-progressors was identified, with genes pertinent to T and B cell activation, the immune response profibrotic processes or in epithelial-to-mesenchymal transition featuring prominently in the progressor group. In contrast, a larger study of a retrospectively selected group of 107 allograft recipients who had their biopsy microarray data summarized into pathogenesis-based transcripts sets, found that the molecular phenotype in early (6-week post transplant) transcript expression mainly reflected the injury-repair responses to implantation stresses, without strong correlation to 6 month IFTA or renal function at 24 months.<sup>69</sup> Naesens and colleagues also profiled the gene expression of 6 month biopsies from 'progressors' and 'non-progressors' in a pediatric cohort.<sup>70</sup> These groups were defined based on 24-month CADI scores and 6 month protocol biopsies were interrogated. As a point of difference from other studies, only recipients with minimal or no chronic damage at implantation and no interval acute rejection episodes were included. This well-designed albeit highly selective study with small numbers, made a concerted effort to remove likely confounders of the molecular signature and study end point. In addition, the profiling of stable 6 month grafts seemed reasonable time point of study given that these should be devoid of the early inflammatory transcripts. 92 probe sets were significantly overexpressed in the 'progressor' group, not only in immunity related but non-immune biological functions, and shed light on the mechanistic pathways. Risk stratification for CAI based on an earlier molecular phenotype seems plausible. The disparities in these studies highlight inter-centre difference in time points of biopsy acquisition, even for 'protocol' samples, and study follow up, reinforcing the need for multicenter studies to produce more robust conclusions.

Other investigators have utilized gene arrays to interrogate protocol biopsies within the first year of transplantation.<sup>71,72</sup> They showed that a combination of immunological and tissue remodeling gene patterns preceded the onset of fibrosis but specific gene pathways associated with expected biological process such as EMT and the TGF- $\beta$  pathway were missing. The identification of macrophage-related genes in the 12 month protocol biopsies demonstrating the most fibrosis was of interest and led to a third study where the role of macrophages in renal transplant fibrosis was examined.<sup>73</sup> In this study of 46 patients, two unexpected features were identified. Firstly the overwhelming majority of infiltrating macrophages were identified as M2 phenotype by immunohistochemistry. Paradoxically the gene expression patterns from arrays performed on their 12 month protocol biopsies demonstrated an alloimmune gene expression pattern with upregulation of interferon-gamma response genes in those grafts with progressive fibrosis despite there being no history of rejection and no evidence of it on histology. Whilst the study did show that there was a strong association of macrophages with fibrosis it did not clarify their role in the process. However it does highlight the importance of an ongoing subclinical alloimmune response as a driver of fibrosis in the first 12 months after transplant, even in those grafts where there was no histological evidence of it. In addition to immune related events, renal parenchymal factors have been identified as having an impact on renal graft fibrosis. In the prospective multicentre Genomics of Chronic Allograft Rejection (GoCAR) study expression of the SHROOM3 gene, which is found in tubular cells and podocytes in the kidney and encodes an actin-binding protein, in allografts at 3 months post transplant or the presence of the SHROOM3 risk allele in the donor, was associated with increased allograft fibrosis at 1 year.<sup>74</sup>

There have been few meta-analyses of genomic studies in chronic allograft injury,<sup>75-78</sup> There are difficulties in undertaking such analyses as there are many confounders including the timing and reasons for the biopsy (protocol or for cause biopsies), the timing of clinical and histological follow up and the type of genomic platform used. One of the earlier meta-analysis<sup>76</sup> utilized a novel non-parametric approach for combining data from multiple independent microarray studies, and pooled two published datasets comprising 40 transplant biopsies samples (27 with CAI and the remainder with normal histology).<sup>79,80</sup> Both studies used Affymetrix microarray GeneChips, which eliminated cross-platform confounders. Amongst the identified differentially expressed genes for CAI, there was an over-representation of six KEGG pathways including oxidative phosphorylation, ATP synthesis, citrate cycle, reductive carboxylate cycle, methionine metabolism.<sup>76</sup> More recently a meta-analysis of separate data sets were undertaken to evaluate the role of growth factors and integrin pathways in CAI.<sup>78</sup> Three data sets were pooled and all were hybridized on Affymetrix arrays. Although the results were of interest with regard to the pathogenesis of CAI they were not robust in differentiating between mild, moderate and severe CAN/IFTA. IGF1 was the only growth factor pathway that was differentially expressed with progressive IFTA severity. Hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) signaling pathways were significantly upregulated in early CAN/IFTA (Banff 0 vs 1) and were expressed in moderate/severe CAN/IFTA (Banff 0 vs Banff 2, 3). However, they were not able to differentiate between mild CAN/IFTA (Banff 1) and moderate and severe grades (Banff 2 and 3 respectively).



Studies in CAI that have put forth a limited gene set with potential as a non-invasive diagnostic biomarker panel have been limited. Of note, one such study aimed to build a multi-gene prediction model around vimentin, chosen based on other published work.<sup>81</sup> In this cross-sectional investigation, indication or protocol biopsies of 114 patients were classified as having IFTA or normal histology, and urinary cell RNA was extracted from urine collected at the time of biopsy. Samples were divided prior to analysis at a 2:1 ratio into a discovery (76 patients, 32 with allograft fibrosis and 44 with normal histology) and validation set (38 patients, 16 with fibrosis and 22 normal histology). The levels of 22 selected mRNAs were measured using their pre-amplification enhanced kinetic quantitative PCR assay. Following adjustments only 2 mRNAs were found to be predictive of fibrosis, resulting in a 4- gene model comprised of vimentin, NKCC2, E-cadherin and 18S rRNA, which yielded a composite score that was highly associated with IF/TA. The gene set performed well in the validation cohort with an AUC of 0.89 ( $p < 0.0001$ ); specificity was 77.3% and sensitivity 87.5% for diagnosis of IFTA at the composite score cut-point (same as discovery set) of 4.5. However the timing of biopsies was heterogeneous, and results are potentially confounded by a statistically significant difference between the time to biopsy in the fibrosis and non-fibrosis groups. These results require confirmation in a more uniform study protocol. Further, whether this profile is predictive rather than diagnostic of fibrosis remains to be determined.

Recently studies examining the genome, transcriptome and/or proteome profile on the same biological samples in an integrated manner using high-throughput technologies have emerged. Kurian and colleagues<sup>82</sup> used DNA microarrays along with tandem mass spectrometry to evaluate the gene and protein expression in the PBMCs of patients with biopsy proven IF/TA of varying severity. The aim was to identify diagnostic biomarkers of IF/TA in the peripheral blood. This proof-of-concept study identified transcript and protein signatures, which appeared to accurately stratify patients into the different IF/TA grades. However, as the authors identified, the patients were pooled from two independent sets, and were clinically heterogeneous with variation in the timing of sample collection and methods of transcript purification. In a separate study<sup>83</sup> the proteome and genome expression was performed in parallel on allograft tissue from the same patients in the aforementioned study. Multiple sets of genes were mapped to different functional pathways, with levels appearing to correlate with histologic severity of IF/TA. These studies show that genomics has the ability to shed light on the pathogenesis of CAI whilst holding potential as an assay that is diagnostic and/or predictive of future fibrosis. However to date the utility of the findings is limited by small sample size, lack of homogeneity of the study population and were mostly non- prospective in design. Therefore carefully coordinated collaborative efforts between centers will be required to allow for robust validation settings.

## Genomics of tolerance

The first study published on gene expression profiling in operationally tolerant renal graft recipients using microarray came from Brouard and colleagues.<sup>84</sup> A subset of patients in their study was stratified into three clinical phenotypes: tolerant patients without immunosuppression for at least 2 years, chronic rejection and age-matched controls. Using a customized cDNA microarray platform (Lymphochip), a 'tolerant footprint' of 49 genes was

identified from PBMCs. Thirty-three of these differentiated operationally tolerant from chronic rejection with 99% specificity and 86% sensitivity. There was under-expression of co-stimulatory genes and Th1/Th2 related cytokines in the tolerant group. In addition, 27% of these genes were regulated by TGF- $\beta$ . In a later study from the same group, 20 of these were selected and formed a gene set which the authors proposed could potentially identify those patients at 'minimal risk' of rejection, where reduction of immunosuppression could be considered.<sup>85</sup> In a cohort of 144 patients who were at least 5 years post-transplant with stable graft function, 3.5% displayed a tolerance profile in the peripheral blood based on this 20-gene set. However, these findings were not obtained in parallel with kidney biopsies to verify the absence of immune reactivity and to date no study has been published that tests their hypothesis.

The bias towards B cells in the tolerant state was also identified by multicenter studies by Newell<sup>86</sup> and Sagoo.<sup>87</sup> The patients in the tolerance cohort from each study were from the US Immune Tolerance Network (25 patients) and the European Indices of Tolerance consortium (11 patients). The two studies used samples from each other's cohort for validation of their findings. Newell and colleagues used microarray for whole blood gene profiling, and found 30 genes over-expressed by at least two fold in 19 operationally tolerant patients (more than 12 months off immunosuppression) versus patients with stable graft function with ongoing triple immunosuppression. Twenty two of these genes were B-cell specific. However, this GEP could not differentiate between tolerant patients and healthy (non-transplanted) controls. Further analysis identified a three-gene signature that was able to distinguish operationally tolerant patients from those with stable function on immunosuppression with 100% sensitivity and 83% specificity in a test set of 12 patients divided equally into the two groups. The expansion of B-cell transcripts was not only identified in the blood, but also in the urine sediment, which exhibited upregulation of CD20 mRNA in the tolerant group. Using a different microarray platform Sagoo and colleagues was able to distinguish operationally tolerant recipients from chronic rejection, stable patients on immunosuppression and normal controls.<sup>87</sup> Again a predominance of B-cell related genes and associated molecular pathways were identified in their tolerance footprint. However, it is possible that the strong representation of B cells in the aforementioned studies may reflect the absence of immunosuppression, rather than the state of tolerance per se. Newell was unable to identify any of the three genes in their predictive set in Sagoo's platform, questioning the robustness of the findings.<sup>86</sup> More recently, Baron and colleagues<sup>88</sup> compared the gene lists derived from the five tolerance-related blood transcriptomic studies, and were unable to identify a gene signature to differentiate tolerant subjects from stable controls on immunosuppression.<sup>84,86,87,89,90</sup> To enhance the study power, the microarray data from the 96 operationally tolerant patients were integrated into one dataset. The authors made efforts to overcome systematic biases derived from merging microarray data obtained from different platforms by renormalization and standardization of the data prior to integration, and only the 1846 consensus genes were retained for analysis. Their meta-analysis identified a robust differential gene signature when compared to subjects who were stable on immunosuppression. The most discriminating gene clusters were linked to B-cells, monocytes and CD4 T cells at various stages of differentiation. Following cross validation, a selection of the 20 top-ranked genes with the majority

overexpressed in tolerance and pertaining to B cells was revalidated in an independent cohort of 18 new tolerance samples of which 6 were new cases, with 91.7% accuracy (94.4% sensitivity, 90% specificity, 85% PPV and 96.4% NPV). This meta-analysis reaffirms the prominent role of B cells in the tolerant state as identified in earlier studies, but whether their strong presence is causative or consequent remains unanswered.

## Other 'omics: Proteomics, Transcriptomics and Metabolomics

A number of other biomic disciplines have emerged in the last decade. These disciplines, including proteomics, transcriptomics and metabolomics refer to the high throughput application to profile the proteome, transcriptome, and metabolome respectively.

**Proteomics** is the large-scale study of proteins. Previously, profiling of the proteome was limited by its complexity, but has gained significant momentum with technological advancements, particularly in relation to mass spectrometry (MS). Identification and quantitative strategies using MS have been aided by improvements in protein separation methods, both gel based (eg 2-dimensional differential in-gel electrophoresis (2D-DIGE)) and non-gel based (eg high performance liquid chromatography (HPLC)) whilst a range of isotope-based labelling methods allow for relative and absolute protein quantification. Urine has been frequently utilized in both non-targeted and targeted proteomic studies of the renal allograft, as has peripheral blood.<sup>48,91–98</sup>

Earlier studies utilising mass spectrometry-based technology in urine proteomic profiling identified protein signals that were present in subjects with acute rejection, but did not further characterise the protein associated with these signals.<sup>99,100</sup> In a proof of principle study,<sup>100</sup> SELDI-TOF-MS was used to discover urine protein peaks associated with acute cellular rejection. A follow up study revealed that these were protein derivatives of non-tryptic cleaved forms of  $\beta$ -2 microglobulin.<sup>101</sup> However, a multicenter study did not identify a statistically significant correlation of intact or cleaved  $\beta$ -2 microglobulin in urine in the presence of AR.<sup>48</sup> This prospective targeted observational study conducted through the Clinical Trials in Organ Transplantation-01 (CTOT-1) protocol, aimed to evaluate and validate a panel of candidate urinary mRNA and protein biomarkers chosen from other published work for diagnosing and predicting acute rejection. These included CCR1, CCR5, CXCR3, CCL5 (RANTES), CXCL9, CXCL10, IL-8, perforin and granzyme B which were studied in this cohort of 280 adult and pediatric first transplant recipients. The most robust findings were for CXCL9, both its urinary mRNA and protein yielded PPVs of 61–67% for AR, but more impressively were the NPVs of 83% and 92% respectively. In addition, low urinary CXCL9 protein at 6 months identified those at low risk of subsequent AR. These findings suggest that urinary CXCL9 protein could aid clinical decision-making by ruling out AR in those with renal function impairment, and identify patients at low risk of subsequent AR where tapering of immunosuppression could therefore be considered. Other studies have applied a variety of proteomic techniques to identify protein expression in an unbiased high-throughput manner in allograft rejection, before utilizing targeted ELISA for validation of biomarker panels.<sup>93,97,102</sup> However as with the majority of promising proteomic studies to date, multicenter prospective trials are required to validate these candidate markers before meaningful conclusions can be made.

**Transcriptomics** is the study of the RNA transcripts encoded by the genome. Whilst much of the work in the last decade has been in mRNA evaluation, studies in miRNA expression profiling in the setting of renal transplantation, has increased exponentially in recent years. MiRNA are small, non-coding RNAs, which regulate gene expression of target mRNAs. Similar to genome and mRNA profiling, large scale sequencing of miRNAs has been dominated by microarray applications. One of the advantages of this technology is that there are far fewer miRNA than genes and hence the computational analysis is simpler and there remains the possibility that a more robust marker could be identified as a predictor of either AR or IF/TA. Scian and Mas<sup>103,104</sup> have elegantly summarised miRNA profiling studies of the renal allograft in their reviews. To date no conclusive patterns of miRNA expression have been associated with AR<sup>14,105,106</sup> or IF/TA.<sup>16,107,108</sup>

**Metabolomics** relates to the global profiling of small molecule (<1500 Da) metabolites. Since the genome, transcriptome and proteome can undergo further changes after translation, the metabolome reflects the downstream products of these processes. There have been advances in separation techniques, such as capillary electrophoresis and ultra-high pressure liquid chromatography (UPLC) systems, and improvements in analytic approaches, being mass spectrometry- based methods and high-resolution nuclear magnetic resonance (NMR) spectroscopy. This has enabled the simultaneous study of dozens of small molecule metabolites in tissue and biological fluids. Whilst the use of non-targeted metabolomic approaches in the study of renal diseases and drug metabolism have been well documented,<sup>109–111</sup> studies demonstrating its potential role in monitoring kidney transplants are limited. It has been used to identify borderline tubulitis and acute TCMR in pediatric renal transplant recipients.<sup>112</sup> Smaller studies have also been published evaluating the differential urinary and serum metabolomic profile in adult allograft recipients with AR using mass spectrometry based methods,<sup>113,114</sup> and NMR spectroscopy was used to profile the urine metabolome in patients with IFTA versus IFTA with inflammation in the DeKAF study.<sup>115</sup>

## Future directions

The human genome project was the catalyst for what is now recognised as the ‘Omics revolution. Molecular profiling strategies have evolved at an unprecedented rate as a response to the demand for high throughput technology. This has been accompanied by significant advancements in mass spectrometry-based techniques in the field of proteomics, which has further contributed to our understanding of the biological complexities of the allograft recipient. These profiling strategies have the power, not only to shed light on the mechanistic pathways of disease, but also to identify potential diagnostic and predictive biological markers, as well as lead on to the development of therapeutic targets. However, it is also important to recognise the potential limitations of such tools. Beyond this, the major challenge of validating such biomarkers remains. There is a pressing need for multicentre collaboration to allow for large-scale, prospective studies, with better study design and comparative histological evaluation at defined time points. Only then can such discoveries transition from the bench to potentially become diagnostic and prognostic clinical tools, and ultimately achieve the common goal of improving graft longevity and patient survival.

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## ABBREVIATIONS

<b>GEP</b>	genome-wide expression profiles
<b>ABMR</b>	antibody mediated rejection
<b>ACR</b>	acute cellular rejection
<b>AR</b>	acute rejection
<b>CAI</b>	chronic allograft injury
<b>CAN</b>	chronic allograft nephropathy
<b>DSA</b>	donor specific antibodies
<b>IF/TA</b>	interstitial fibrosis/tubular atrophy
<b>miRNA</b>	microRNA
<b>MS</b>	mass spectrometry
<b>PBMC</b>	peripheral blood mononuclear cells
<b>qPCR</b>	quantitative polymerase chain reaction
<b>TCMR</b>	T-cell mediated rejection

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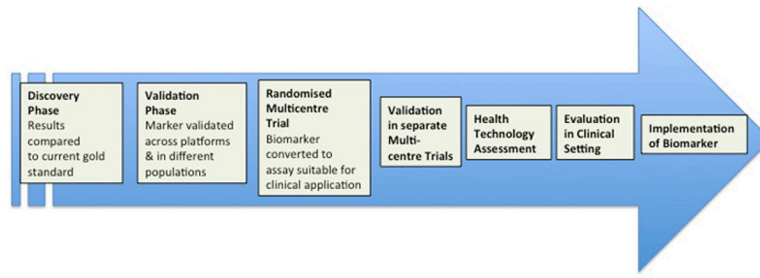
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**Figure 1.** Steps required to translate research findings predictive of a clinical outcome into a validated biomarker suitable for clinical use. *Adapted from Willis and Lord. Nature Reviews Immunology. May 2015;15(5):323–329*<sup>12</sup>.

**Table 1**

Summary of advantages and disadvantages of current transcriptome assessment technologies

	Advantages	Disadvantages
QUANTITATIVE RT-PCR	<ul style="list-style-type: none"> <li>Established 'gold standard' of quantitative expression profiling</li> <li>High sensitivity</li> <li>High dynamic range</li> <li>High reproducibility</li> </ul>	<ul style="list-style-type: none"> <li>Low throughput- limited number of genes/transcripts can be evaluated in a single experiment-often used for validation.</li> <li>Larger amounts of sample required compared to other methods</li> <li>Depends on fidelity of "housekeeping genes"</li> </ul>
MICROARRAY	<ul style="list-style-type: none"> <li>Well established method</li> <li>Widely available</li> <li>Medium to high throughput- thousands of genes/transcripts can be profiled in a single experiment</li> <li>High reproducibility (at mid to high expression range)</li> <li>Publicly available databases on thousands of microarray studies</li> <li>Relatively low cost</li> </ul>	<ul style="list-style-type: none"> <li>Reference genome required ie determines relative and not absolute expression</li> <li>Only detects sequences complementary to those on the array</li> <li>Lower dynamic range</li> <li>Lower specificity and sensitivity compared to other techniques</li> <li>Analysis can be difficult comparing datasets from different microarray platforms</li> </ul>
NEXT GENERATION SEQUENCING	<ul style="list-style-type: none"> <li>High throughput- entire transcriptome can be profiled in a single experiment</li> <li>Can identify novel transcripts</li> <li>Direct digital counting</li> <li>High dynamic range</li> <li>High sensitivity</li> <li>High reproducibility</li> </ul>	<ul style="list-style-type: none"> <li>Comparatively more expensive per sample</li> <li>Sequence-specific biases</li> <li>Complex /time-consuming computational data analysis</li> </ul>
NANOSTRING® nCOUNTER ANALYSIS SYSTEM	<ul style="list-style-type: none"> <li>Medium throughput- up to 800 genes/transcripts in a single experiment</li> <li>Direct digital counting</li> <li>No reverse transcription or RNA amplification step, therefore reducing technical biases</li> <li>High sensitivity and specificity</li> <li>High dynamic range</li> <li>High reproducibility</li> <li>Paraffin embedded tissues can be used</li> </ul>	<ul style="list-style-type: none"> <li>High upfront costs of code sets and consumables</li> <li>Prior knowledge of genes required</li> <li>Not ideal for non-targeted gene discovery studies-fewer genes/transcripts per experiment compared to NGS and/or microarray</li> </ul>

	<b>Advantages</b>	<b>Disadvantages</b>
	<ul style="list-style-type: none"><li>• Rapid turnaround time (from sample processing to data acquisition)</li></ul>	

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