Non-invasive biomarkers to guide management following renal transplantation: the need for a multiplatform approach

Paramit Chowdhury\textsuperscript{a,b} and Maria P. Hernandez-Fuentes\textsuperscript{b}

Purpose of review
Balancing the level of anti-rejection therapy is the key challenge facing clinicians managing recipients of a solid organ transplant. Identification of biomarkers in non-invasive samples will allow serial monitoring which can prompt changes in therapy at an earlier stage without the need for invasive tests, and before significant damage to the graft or side effects have occurred. In this review we provide an update on the present status of such biomarker discovery in renal transplantation.

Recent findings
Previous studies focusing on candidate biomarkers have identified a number of genes that have the potential to identify patients undergoing rejection. Advances in technology now allow screening of samples for markers which have led to identification of further genes as well as adding microRNAs and proteins to the list. Similarly, by studying patients in whom anti-rejection therapy has been withdrawn, a gene signature for operational tolerance has been described.

Summary
It has become clear that to obtain a test suitable for clinical purposes, combinations of markers are required to identify specific clinical phenotypes. Identification and validation of such marker sets in large cohorts is urgently required to allow progression to clinical trials with the ultimate goal of offering recipients personalized anti-rejection therapy regimes.

Keywords
biomarker, microRNA, mRNA, proteomics, transplantation

INTRODUCTION
Transplantation remains the optimal treatment for patients with end-stage kidney disease, with survival, quality of life and economic benefits \cite{1,2}. However, key challenges that need to be overcome include prolonging the lifespan of the transplanted organ beyond the present half life of around 10–20 years, and reducing the morbidity and mortality caused by the side effects of anti-rejection therapy. Overcoming these obstacles requires the discovery and validation of novel biomarkers to allow earlier recognition of any immune response against the kidney, a better understanding of rejection processes to allow more directed therapy and identification of patients where anti-rejection therapy can be minimized, thus reducing side effects. The ultimate goal will be the ability to tailor anti-rejection therapy for an individual donor and recipient combination.

GENETIC BIOMARKERS IN NON-INVASIVE SAMPLES
As molecular events precede the development of the immune response they provide an ideal opportunity to detect host responses before significant damage to the graft has occurred. Whereas such changes can be detected in tissue from biopsies, the ability to detect a signal in non-invasive samples such as peripheral blood and urine has added advantage of allowing more practical serial monitoring of
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KEY POINTS

- Biomarkers in non-invasive samples such as blood and urine offer the opportunity for serial monitoring of the immune status of renal transplant recipients.
- Mass screening of samples includes identifying new mRNA, microRNA and protein markers to add to the previous list of candidate markers that could potentially predict acute rejection or development of operational tolerance.
- Combination of multiple markers will hopefully provide levels of sensitivity and specificity that will allow translation into clinical practice.

patients. Advances in reverse transcriptase polymerase chain reaction (RT-PCR) and array technology now allow reliable and cost-effective analysis of multiple genes in a single sample. A number of candidate markers in blood and urine specimens, reviewed below, have previously been identified, but are yet to make it into routine clinical practice.

mRNA in blood

Since Vasconcellos et al. first published their finding that granzyme B (GZB), perforin and Fas ligand messenger RNA (mRNA) levels were increased in peripheral blood mononuclear cells at the time of rejection, a number of small single-centre studies have followed (reviewed in [3]). However, not all subsequent studies have confirmed this finding. Elevated mRNA levels of these markers have also been described in other inflammatory processes affecting the kidney. Heidt et al. [4] have recently reported that they found similarly elevated levels of GZB and perforin mRNA in peripheral blood during both rejection and cytomegalovirus infection. Advances in technology now allow discovery of genes using platforms offering high throughput profiling. Li et al. [5] performed gene microarray studies using three different platforms on blood samples taken from paediatric and young adult recipients at the time of biopsy. Using subsequent quantitative PCR in verification and validation steps, they selected a set of five genes which were then applied to a separate test set of 198 samples for patients from 12 different United States centres, containing 32 taken in patients with acute rejection, 94 with stable function and 72 where the biopsy showed no rejection but demonstrated other abnormalities. Using this five-gene model they were able to distinguish acute rejection from stable patients with 91% sensitivity and 94% specificity [5]. Perhaps most interestingly using this gene set 8 out of 12 patients with biopsies classified as ‘borderline’ were classified as having acute rejection. In assessing markers, the timing of a rejection episode remains an unclear entity. While conventionally this is taken as the time of development of graft dysfunction leading to a biopsy, data from protocol biopsies have revealed a high incidence of sub-clinical and hence potentially unrecognized rejection [6]. A further complicating factor is our increasing recognition that rejection represents a heterogeneous process [7]. The effect of different anti-rejection therapy on these markers also needs further evaluation. In particular, the use of induction agents such as antithymocyte globulin (ATG) and alemtuzumab (Campath-1H) not only cause depletion of lymphocytes, the cells from which many of these markers are derived for several months, but also affect the nature of the reconstituting lymphocyte pool [8,9]. Clearly further large multicentre longitudinal studies of genetic biomarkers are needed with careful evaluation of rejection subgroups and anti-rejection therapy when interpreting results. The clinical feasibility of using genetic biomarkers in peripheral blood has been demonstrated in the field of cardiac transplantation [10]. The use of a commercially available gene expression profiling test was used in patients to monitor for rejection post cardiac transplant and compared with the standard approach of routine biopsy. For patients at low risk, the strategy of monitoring using gene expression profiling was not associated with an increased risk of adverse outcomes and resulted in fewer biopsies being performed and greater patient satisfaction.

mRNA in urine

Analysis of gene expression in urinary cells has the potential advantage of targeting changes more specific to the response against the kidney. A number of potential candidates have been identified in urinary samples from patients undergoing acute rejection. Li et al. [11] found elevated levels of GZB and perforin mRNA in urine samples from recipients undergoing acute rejection. In addition, bacterial urinary tract infection did not confound the diagnosis of acute rejection made using urinary GZB mRNA. Muthukumar et al. [12] analysed urinary cell mRNA for serine protease inhibitor-9 (PI-9), an endogenous agonist of the GZB/perforin lytic pathway. They found higher levels of PI-9, GZB and perforin mRNA in urinary samples taken at time of biopsy in 33 cases of acute rejection compared with a similar number of samples from patients with stable function, or compared with two other groups consisting of patients with a biopsy diagnosis other than rejection or those with a biopsy diagnosis of
chronic allograft nephropathy. The same group later reported that not only were mRNA levels of FOXP3, a specification and functional factor for regulatory T lymphocytes, elevated in patients undergoing acute rejection, but that levels were also able to predict response to treatment [13]. More recently, Renesto et al. [14] demonstrated that mRNA for the Th1-related molecule T-cell immunoglobulin mucin-3 was more highly expressed in urinary samples taken at time of biopsy in 30 cases of rejection compared with samples from stable patients. Manfro et al. [15] have extending this finding and showed that both peripheral blood and urinary TIM-3 mRNA was higher in patients with acute rejection compared with than in patients with other causes of graft dysfunction.

In a subgroup of patients with delayed graft function, TIM-3 mRNA was able to distinguish acute rejection from those with acute tubular necrosis alone with 100% accuracy [15]. Aquino-Dias et al. [16] also examined gene expression in the biopsy samples, peripheral monocytes and urinary cells from 35 patients with delayed graft function, and found that diagnostic accuracy for acute rejection was highest for FOXP3 in both blood and urine, with ranging from 94 to 100%. These latter two studies suggest that peripheral sampling has the potential to replace the routine biopsies required in patients with delayed graft function to monitor for rejection.

As well as mRNA for molecules related to T-cell activation and killing, elevated levels of mRNA of molecules involved in cell trafficking having also been demonstrated during acute rejection. Tatapudi et al. [17] reported elevated levels of urinary mRNA for the chemokine receptor CXCR3 and its ligand interferon-inducible protein-10 (IP-10) in 27 cases of acute rejection compared with urine samples from 27 samples from patients with stable graft function. Ding et al. [18] demonstrated elevated mRNA levels of urinary cell CD103 in samples from 30 recipients with acute rejection compared with patients with other findings on biopsy or stable function.

The predictive power of urinary markers has been investigated by a number of groups. Kotsch et al. [19] reported that serial analyses of urinary cell granulysin mRNA predicted the development of acute rejection, with increases in granulysin mRNA preceding the rise in serum creatinine by several weeks. Seller et al. [20] have reported elevated mRNA levels of NKG2D, a co-stimulatory CD8+ lymphocyte receptor, in sequential urine samples collected prior to the diagnosis of acute rejection. More recently, preliminary data from the Clinical Trials in Organ Transplantation Programme (CTOT-04) in the USA have shown that urinary cell mRNA levels of perforin and PI-9 can predict the development of rejection 60–90 days beforehand with and area under the curve (AUC) of 0.88 sing a combination of six genes increased sensitivity (AUC 0.93) [21].

**MicroRNAs**

More recent discoveries of genetic biomarkers have occurred in the field of microRNAs (miRNAs). These are small non-coding nucleotides which regulate expression of target genes, primarily by translational repression or mRNA degradation [22]. A single miRNA is able to control hundreds of target genes. Sui et al. [23] performed microarray analysis and found 20 miRNAs that were differentially expressed in acute rejection. Anglicheau et al. [24] studied the expression of intragraft miRNAs during rejection. They found a subset of 17 miRNAs differentially expressed between rejection and normal biopsies in a training set. In a validation set they found miR142-5p, 155 and 223 were very significantly over-expressed, whereas miR-10b, 30a-3p and let-7C were under-expressed. Using receiver-operating curves (ROCs) they found a 100% sensitivity and 95% specificity for diagnosing acute rejection for miR142-5p. Lorenzen et al. [25] have studied mRNA levels in the urine of patients with rejection. They found levels of miR-10b and 210 were down-regulated and miR-10a up-regulated in the urine of patients with acute rejection compared to controls. However, only miR-210 was able to distinguish between patients with rejection compared those with urinary tract infection. These initial studies support the hypothesis that miRNA levels can add to the ability to predict and diagnose rejection.

**PROTEOMICS**

The advent of protein mass spectrometry now allows large-scale profiling of the protein content of biological fluids to allow identification of putative protein biomarkers of disease processes. Such profiling has revealed the presence of over 1000 proteins in human urine specimens [26]. Several preliminary studies have tried to identify a protein ‘signature’ in urine that is diagnostic of acute rejection. Using surface-enhanced laser desorption/ionization-coupled to mass spectrometry (SELDI-TOF), Clarke et al. [27] analysed urine samples from 17 patients with biopsy-prove acute rejection and 15 patients without rejection. Analysis of spectra revealed 45 peaks that differed in urine samples from rejecting and non-rejecting. Using two candidates they obtained a sensitivity of 83% and specificity of 100%. Using a similar
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A set of biomarkers that allows us to predict acute rejection before there is evidence of tissue damage would be of clinical use in the early-phase post-transplantation, as it would allow us to adjust immunosuppression accordingly. A critical gap in our ability to personalize immunosuppression therapy is the lack of available tests or biomarkers to indicate when a patient has become partially or totally tolerant to their graft. The availability of this information in clinical practice would enable the decrease or even withdrawal of an individual’s immunosuppressive therapy [34,35]. For this elusive condition, we have coined the term ‘the hidden phenotype’ [36].

A critical limitation to the study of tolerance to the transplanted kidney in humans is the paucity of available patients. A very limited number of patients that stop their anti-rejection therapy are able to maintain good graft function for longer than a few months. These rare patients have been central to the description of biomarkers that are indicative of tolerance. Collaboration between Nantes and Stanford obtained the first published molecular signature of tolerance. They identified a set of 33 genes using expression arrays. The expression of these genes could distinguish with high specificity operationally tolerant kidney transplant recipients [37]. Two further studies have used a set of biomarkers to define kidney transplantation tolerance: the Indices of Tolerance (IoT) consortium and the Immune Tolerance network (ITN) [38**,39**]. Using gene expression analysis on whole peripheral blood, both studies found a strong B-cell signature associated with tolerance despite differences in the genetic makeup and degree of human leukocyte antigen matching between the cohorts. This signature was accompanied by an expansion of B cells in peripheral blood, mainly of the naive and/or transitional phenotype. More recently, a group in Brazil has described a predominant regulatory gene expression profile with higher gene expression of GATA3, FOXP3, TGFB1 and TGFB receptor 1 compared with other kidney transplant recipients as well as a reduced activation of the IL-4/STAT6 pathway in monocytes [40,41].

Between them these studies have used a number of platforms to identify transplantation tolerance in kidney recipients. In general the cross-platform approach adopted towards biomarker identification has highlighted a particularly prominent role for B cells within transplantation tolerance, which have very recently re-emerged with newly defined roles within inflammation and immunity [42–44]. In this issue an extensive summary of the implications of these discoveries can be found.

**CONCLUSION**

Some studies have identified the potential of both genetic and protein biomarkers in non-invasive blood and urine samples for identifying patients at risk of rejection, or those in whom there might be the potential to reduce or withdraw anti-rejection therapy.

With all the high-throughput techniques and the new bioinformatic tools now available, it is becoming easier to combine information emerging from genetic studies, gene expression studies, proteomics and other techniques. The next necessary step to translate this wealth of information into a clinically useful test is to assess the benefits of including this information into post-transplant clinical care, with adequately designed biomarker-lead clinical trials, with the ultimate goal of being able to offer recipients individual tailoring of anti-rejection therapy in the future.
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Conflicts of interest

None declared.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 111–112).


Interesting study performed using paediatric and young adults’ samples.


This is the first example of the clinical use of a peripheral gene biomarker set for post-transplant monitoring.


This article and [39-42], provided the original link between transitional or immature B lymphocytes and transplantation tolerance, that is now the focus of several further lines of basic research.