Urinary Biomarkers in Acute Kidney Transplant Dysfunction

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Introduction

Kidney transplant remains the treatment of choice for end-stage renal disease (ESRD) patients; it extends their survival and improves their quality of life. Recent data showed that there are 89,707 patients registered on the kidney transplant waiting list at the United Network for Organ Sharing (UNOS) for kidney and 2,274 for kidney-pancreas transplants; kidney transplants performed during the year of 2009 were: 16,830 kidneys – 10,442 from deceased donors, and 6,388 from living donors \cite{1}.

Early diagnosis of renal allograft dysfunction is crucial for the management and long-term survival of the transplanted kidney. Acute kidney injury (AKI) of the allograft can result from different etiologies. Early after transplantation, acute tubular necrosis (ATN) manifesting as delay graft function (DGF) or slow graft function; acute rejection (AR) or drug toxicity (e.g. calcineurin inhibitor) are the leading causes of AKI. AR, ATN, and calcineurin inhibitor toxicity continue to be major causes of renal allograft dysfunction along with other causes like infections (e.g. BK and CMV viruses, pyelonephritis), obstruction, and recurrence of the original disease. Clinicians have been searching for non-invasive tools that...
would allow the diagnosis of acute renal allograft dysfunction early and accurately without performing a kidney biopsy. The following comprehensive review will describe the latest findings on urinary biomarkers in acute allograft dysfunction.

Urine Biomarkers for Acute Allograft Rejection

**Urine Proteomics**

Proteomics has become a growing field of scientific investigation, identifying new targets for therapeutic intervention and biomarkers for diagnosis and prognosis using current technology [2]. Proteomics analysis relies on the extraction and separation of the protein from the human samples, fractionation of this protein into peptides by using 2D gel electrophoresis (or using the whole protein), then performing one of the techniques: matrix-assisted laser desorption ionization (MALDI) or ionizing the whole protein by surface-enhanced laser desorption/ionization (SELDI) or capillary electrophoresis coupled to mass spectrometry (CE-MS) [3]. SELDI-TOF-MS combines matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with surface retentive chromatography [4]. MALDI-TOF-MS and the related technique, SELDI-MS, provide means for analyzing a complex mixture of proteins in solution [5, 6] (table 1).

Using MALDI-TOF-MS for analyzing urinary proteins, a recent study showed that urine samples from patients with AR had a protein peak at 11.7 kDa, correlating strongly with AR with sensitivity of 83.3% and specificity of 80%. This peak was identified as being a β₂-microglobulin [7]. In a study conducted by Schaub et al. [5], urine samples were collected on the day of the biopsy and analyzed by mass spectrometry. Three prominent peak clusters of polypeptides were found in 94% of patients with AR, 18% with stable function and in 0% of normal controls. In a different study, Schaub et al. [8] showed that patients with acute tubulointerstitial rejection had higher amounts of aspartic proteases and intact β₂-microglobulin in urine than stable kidney transplant and healthy individuals. O’Riordan et al. [9] found that 4.7- and 4.4-kDa peptides are useful in diagnosing AR. These polypeptides were identified as human β-defensin-1 (4.7 kDa) and α₁-antichymotrypsin (4.4 kDa) by mass spectrometry and ProteinChip immunoassay. Using SELDI-TOF-MS on the urine samples revealed a reduction in β-defensin-1 and increase in α₁-antichymotrypsin in patients with AR (p < 0.05) compared with stable kidney transplants.

Although proteomics-based techniques raise the potential to reveal molecular mechanisms of allograft rejection and injury, which possibly could translate into novel biomarkers, so far, no biomarkers identified by proteomics-based approach has found a clinical application. All currently published data were restricted to analysis of urine using this technology, which can only assess a limited part of the proteome. The continuous developments in this field including more sensitive mass spectrometers with higher mass accuracy, differential protein expression technology, and analysis of allograft tissue parts selected by laser-capture microdissection may allow deeper understanding of changes of the proteome associated with allograft rejection and injury [4].

**Urine Genomics**

Genomics refers to the analysis of the genomes; on the other hand, functional genomics is the field that uses global approaches to understand the functions of genes and proteins. Most human sequenced genes and genomes’ biological functions remain unknown. Human genomes are encoded in the DNA, which is copied into RNA; the RNA molecules are used to make proteins. The functions of unknown genes can be determined by repeated measurements of their RNA transcripts. Quantifying mRNAs can be done by Northern blotting or quantitative polymerase chain reaction (PCR), which measures a few genes at a time. DNA microarray allows a rapid analysis of gene expression; it is based on the principle that complemen-

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**Table 1. Proteomics analysis methods**

<table>
<thead>
<tr>
<th>Methods</th>
<th>Full name</th>
<th>Biomarker</th>
<th>Reference (first author)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SELDI-TOF-MS</td>
<td>surface-enhanced laser desorption/ionization time-of-flight mass spectrometry</td>
<td>cleaved β₂-microglobulin</td>
<td>Schaub [5, 8]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β-defensin-1, α₁-antichymotrypsin</td>
<td>O’Riordan [9]</td>
</tr>
<tr>
<td>MALDI-TOF-MS</td>
<td>matrix-assisted laser desorption/ionization time-of-flight MS</td>
<td>β₂-microglobulin</td>
<td>Oetting [7]</td>
</tr>
<tr>
<td>CE-MS</td>
<td>capillary electrophoresis mass spectrometry</td>
<td>urinary poly-peptide pattern</td>
<td>Schaub [5]</td>
</tr>
</tbody>
</table>

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Alachkar/Rabb/Jaar

Urinary Biomarkers in Acute Kidney Transplant Dysfunction

CD103 mRNA

CD103 is a member of the integrin family heterodimers that play diverse roles in T-cell activation, homing, and delivery of effectors function [11]. It expresses on T cells in the gut mucosa [12] at high levels by >95% of intestinal intraepithelial lymphocytes and 40% of lamina propria lymphocytes [13, 14]. CD103, a known receptor for the epithelial cell-specific ligand E-cadherin, is expressed by a major subset of CD8+ cytotoxic T cells elicited in response to allogenic renal epithelial cells [15]. Ding et al. [16] found that CD103 mRNA levels were higher in urinary samples from 30 patients with AR compared with the levels in 12 patients with other findings on renal allograft biopsy (table 2), and 25 patients with stable graft function (p = 0.001).

Granulysin mRNA

Kotsch et al. [17] monitored the urinary mRNA expression in 221 specimens from 26 patients by real-time PCR for 3 months. An increase in granulysin mRNA was detected during 11 of 14 episodes of AR. Follow-up studies also showed its predictive value for delayed AR episodes weeks before rising serum creatinine.

Perforin, Granzyme B and Fas-Ligand mRNA

Target mRNA was measured with a quantitative PCR assay in AR and other clinical complications in kidney transplant in 162 urine samples from 37 transplant recipients. Perforin (P), granzyme B (GB) and Fas-ligand (FAS-L) gene expression in urine were upregulated in AR episodes [18].

mRNA Encoding Interferon-Inducible Protein-10

Tatapudi et al. [19] studied mRNA encoding interferon-inducible protein-10 (IP-10) and the chemokine receptor CXCR3 in urinary cells in 63 urine specimens in patients with kidney transplantation. In this study, mRNA levels of IP-10 and CXCR3 (table 2) predicted acute cellular rejection.

### Table 2. Performance of selected urinary biomarkers in acute rejection

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>ROC</th>
<th>Reference (first author)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine proteomics</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>β2-Microglobulin</td>
<td>83.3</td>
<td>80</td>
<td>NA</td>
<td>Oetting [7]</td>
</tr>
<tr>
<td>β-Defensin-1 α1-antichymotrypsin</td>
<td>NA</td>
<td>NA</td>
<td>0.749</td>
<td>O’Riordan [9]</td>
</tr>
<tr>
<td><strong>Urine genomics</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CD103 mRNA</td>
<td>59</td>
<td>75</td>
<td>NA</td>
<td>Ding [16]</td>
</tr>
<tr>
<td>IP-10 mRNA</td>
<td>100</td>
<td>78</td>
<td>NA</td>
<td>Tatapudi [19]</td>
</tr>
<tr>
<td>CXCR3 mRNA</td>
<td>63</td>
<td>83</td>
<td>NA</td>
<td>Tatapudi [19]</td>
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<tr>
<td><strong>Cytokines and binding receptors</strong></td>
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<td></td>
<td></td>
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<tr>
<td>IL-10</td>
<td>88</td>
<td>100</td>
<td>NA</td>
<td>Jiménez [41]</td>
</tr>
<tr>
<td>IFN-λ</td>
<td>77</td>
<td>100</td>
<td>NA</td>
<td>Jiménez [41]</td>
</tr>
<tr>
<td>IP-10</td>
<td>93</td>
<td>89</td>
<td>NA</td>
<td>Hauser [49]</td>
</tr>
<tr>
<td>MIG</td>
<td>86.4</td>
<td>91.3</td>
<td>NA</td>
<td>Hu [50]</td>
</tr>
<tr>
<td><strong>Growth factors and others</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>85.7</td>
<td>78.3</td>
<td>NA</td>
<td>Peng [61]</td>
</tr>
</tbody>
</table>

NA = Not available.
FOXP3 mRNA

Regulatory T cells (Tregs) have been implicated in the homeostasis of the immune response, control of alloimmune responses, and induction and maintenance of transplant tolerance in vivo [20]. Tregs are a subset of T cells with CD4+CD25high FOXP3+ phenotype [21]. The transcription factor forkhead box P3 (FOXP3) is an X-linked forkhead/winged helix transcription factor, has been recognized as the master regulator of Tregs [22]. Zheng et al. [23] demonstrated that FOXP3 acts as a transcriptional activator and a repressor that amplifies and stabilizes gene expression in Tregs and thus maintains the homeostasis in these cells. They also produce cytokines such as interleukin (IL)-10 and transforming growth factor-β (TGF-β) [24, 25]. Recently, chemokine receptors have also been implicated in the function and the regulatory capacity of Tregs. It has been demonstrated in animals [26, 27] and humans [28, 29] that chemokines and their receptors are important molecules for the regulation of immune responses and tolerance induction. Schneider et al. [30] reported that CC chemokine receptor 7 (CCR7) is required for in vivo function of CD4+CD25+ Tregs in a murine model, and Uhea et al. [26] and others [27] have proposed that CCR7 expression is involved in the organization of thymic architecture and function and lymph node homing of naive Tregs [31]. Some data support that the inducible CD4+CD25+FoxP3+ Treg cells are present in recipients who developed tolerance spontaneously to a kidney transplant [32, 33].

Muthukumar et al. [34] found higher levels of FOXP3 mRNA in urine of renal allograft recipients undergoing AR compared with stable patients or healthy controls. Urine specimens of 36 subjects with AR, 18 subjects with chronic allograft nephropathy, and 29 subjects with normal biopsy were analyzed. Also, Aquino-Dias et al. [35] demonstrated that the level of FOXP3 mRNA in the urine was diagnostic of AR in patients with DGF.

Neutrophil Gelatinase-Associated Lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is an extracellular 25-kDa ligand-binding protein, a member of the lipocalin family, expressed at low levels in several human tissues, including the kidney. NGAL is one of the earliest induced genes and proteins in a kidney undergoing ischemic or nephrotoxic injury [36]. Because of its small molecular size and resistance to degradation, NGAL is readily excreted and detected in urine. It has also been evaluated as a biomarker of acute injury in kidney transplantation. In a recent study, urine NGAL concentration significantly increased in patients with tubulitis or other tubular pathologies [37]. Urine NGAL also found to be increased with subclinical tubulitis in the early months following kidney transplantation [38].

Cytokines and Binding Receptors

Chemokines play a role in control leukocyte migration during inflammatory processes by interaction with their respective G-protein-coupled receptors. The chemokine receptors comprise two groups, the CC receptors 1–10 (CCR1 to CCR10), which bind CC chemokines, and the CXC receptors 1–5 (CXCR1 to CXCR5), which bind CXC chemokines [39, 40]. The following are examples of cytokines studied in kidney transplantation:

Using a cytometric bead array technique, the concentrations of six human cytokines (IL-2, IL-4, IL-5, IL-10, tumor necrosis factor-α (TNF-α) and interferon (IFN)-λ) were measured in the urine. Results showed (table 2) that patients who developed kidney allograft rejection had high levels of IL-10 and IFN-λ cytokines in urine compared to patients without rejection [41].

In a study conducted by Smith et al. [42], soluble ILs, cyclic GMP (cGMP), nitrate, and nitrite were measured in 192 urine samples collected from 13 patients during the first 3 months of renal transplantation. For follow-up analysis, 67 urine samples were collected randomly from 24 patients. TNF-α, IL-6, and IL-8 were found to increase at the time of rejection (p ≤ 0.01).

A study investigated the kidney gene, protein expression and the urinary excretion rate of IL-6 and epidermal growth factor (EGF) in 29 renal transplant recipients: 16 with AR and 13 with acute tubular damage or cyclosporine toxicity. Urinary IL-6/EGF ratio was markedly increased in AR, and only slightly increased in the other group [43].

Urine IL-6 levels were also measured daily in another study in 101 recipients during post-transplant hospital stay. DGF was associated with very high urine IL-6 levels. Steroid-sensitive AR was associated with significantly increasing urine IL-6 [44]. Levels of soluble IL-2-receptor (sIL-2R), IL-6 and IL-8 were measured in serum and urine of 79 patients with kidney transplantation; AR was associated with an increase in the urine levels of IL-6 and sIL-2R [45].

The concentration of monocyte chemoattractant protein-1 (MCP-1) in urine and serum of 19 renal transplant patients was investigated by Prodjosudjadi et al. [46] who found that urinary excretion of MCP-1 was increased in patients with AR. Urine samples were collected in an-
other study from 35 patients with AR and 65 with a stable graft function within the first 6 months after transplantation. MCP-1 level was tenfold higher in AR than in patients with a stable graft function [47].

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine and a potent activator of macrophages and T cells. Brown et al. [48] have used ELISA to detect MIF in 9 allograft patients who had serial urine MIF concentrations measured in the first 14 days after transplantation. Levels increased on day 1 post-transplantation and subsequently fell in parallel with the serum creatinine, also increased before episodes of AR.

Chemokine monokine induced by IFN-γ (MIG) (CXCL9) and IFN-γ-inducible protein 10 (IP-10) (CXCL10) are studied as early markers of AR in renal transplantation. In a prospective study of 69 patients, urine samples were collected for a median of 29 days after transplant. Urine MIG and IP-10 were analyzed by ELISA and were elevated in 14 patients who had biopsy-proven AR. Urinary MIG predicted AR (table 2) with a sensitivity of 93% and a specificity of 89% [49]. In a study of 73 patients with renal allograft dysfunction and 26 patients with stable graft function, urinary levels of CXCR3-binding chemokines, monokine induced by IFN-c (MIG/CXCL9), IFN-c-induced protein of 10 kDa (IP-10/CXCL10), and IFN-inducible T-cell chemoattractant (I-TAC/CXCL11) were measured by a particle-based triplex assay. IP-10, MIG and I-TAC were significantly (table 2) elevated in renal allograft recipients with AR with 86.4% sensitivity and 91.3% specificity [50]. Panzer et al. [51] demonstrated that urinary chemokine IP-10 excretion was significantly upregulated in patients with AR, whereas MIG and regulated upon activation, normal T cell expressed and secreted (RANTES) showed only a non-significant tendency.

A new lymphokine called HILDA (human interleukin for DA cells) was analyzed in serum and urine of transplant recipients. HILDA/LIF increased the urine but not in the serum of kidney graft recipients during AR, but not in stable renal function [52].

Urinary excretion of soluble ICAM-1 was measured in 136 allograft recipients during the first 1–6 weeks post-transplant: 30 patients had AR and 106 patients had stable function. Increased urinary excretion of sICAM-1 was demonstrated a few days before AR [53]. In a different study, urinary concentrations of sICAM-1, sVCAM-1 and complement degradation product C4d were determined in 75 allografts recipients and 29 healthy controls. The transplant recipients were divided in four groups: group 1: steroid-sensitive AR; group 2: steroid-resistant AR; group 3: chronic allograft dysfunction, and group 4: stable graft function. Groups 1–3 had significantly higher urinary C4d compared with healthy controls and group 4 (p < 0.05). The urinary levels of sVCAM-1 were higher in group 2 and healthy controls than other groups (p < 0.05) [54].

Vascular Endothelial Growth Factor
Vascular endothelial growth factor (VEGF) mRNA and protein are detected in glomerular podocytes, distal tubules, and in some proximal tubules [55]. In AR, these cells express VEGF ligand and VEGF receptors are up-regulated [56]. VEGF expression is increased in pathological conditions associated with macrophage infiltration [57]. When VEGF increases, macrophage and leukocyte infiltration is facilitated by the increasing of capillary permeability [58] and leukocyte migration is augmented to the allograft, which may lead to rejection [59]. Fifteen different sequence polymorphisms have been identified within the VEGF gene. The correlation among VEGF gene polymorphisms, VEGF production and AR has been investigated. Homozygotes with –1154G/G genotype and –2578C/C genotype showed the greatest risk of rejection and had the highest production of VEGF, as compared with –1154A/A and –2578A/A, heterozygotes with –1154G/A and –2578C/A [60]. In a recent study (table 2), urinary VEGF was determined by ELISA in 215 allograft recipients and 80 healthy controls. Patients with AR (n = 67) excreted urinary VEGF at a significantly higher level [61].

Urokinase Plasminogen Activator
In a study by Roelofs et al. [62], blood, urine, and tissue samples were collected from 33 patients with AR and from 14 without rejection. Blood and urine were obtained from 10 healthy volunteers. Low levels of urokinase plasminogen activator in urine were detectable in healthy volunteers, increased in non-rejecting allograft recipients and much higher in patients with AR. Table 2 shows a summary of selected reviewed biomarkers.

Urine Biomarkers for Acute Allograft Ischemic Injury
Neutrophil Gelatinase-Associated Lipocalin
Although some data showed that NGAL was increased in tubulitis, Parikh et al. [63] showed that NGAL was increased in DGF. Urine samples were collected on day 0 from recipients of living donor kidneys (n = 23), deceased
donor kidneys with prompt graft function (n = 20) and deceased donor kidneys with DGF (n = 10). Urine NGAL was significantly different in the three groups on day 0, with maximally elevated levels noted in the DGF group (p < 0.0001). The ROC curve for prediction of DGF based on urine NGAL was 0.9.

Cytokines and Binding Receptors
A soluble form of CXC chemokine ligand 16 (CXCL16) was investigated in patients with renal transplant. Acute tubular damage in renal allografts was associated with elevated urinary CXCL16 [64]. In the same study mentioned above [50], Hu et al. [65] showed that IP-10, MIG and I-TAC were significantly elevated in ATN; sensitivity was 86.4% and specificity was 91.3%. Urine specimens from 40 renal allograft recipients were analyzed for actin, /H9253-glutamyl transpeptidase, lactate dehydrogenase, IL-6, and IL-8 during the first post-transplant week. On day 0, urinary actin, /H9253-glutamyl transpeptidase, IL-6, and IL-8 were elevated in recipients who had sustained acute renal failure after ischemia compared with those who recovered. In contrast, urinary lactate dehydrogenase and TNF-α increased in recipients with recovering renal function compared with those who had sustained acute renal failure. Table 3 shows selective biomarkers of acute ischemic injury.

Urine Biomarkers Associated with Infections
Some urine biomarkers are also correlated with infections in kidney transplant recipients. Perforin (P), granzyme B (GB) and Fas-ligand (FAS-L) gene expression in urine were upregulated in urinary tract infections (UTI) and CMV infections [18].

Fischer et al. [45] found that IL-6 and IL-8 levels did not change in CMV infections or systemic extrarenal bacterial infections; however, acute pyelonephritis resulted in a higher serum IL 6 level. In another study, the level of IL-8 in urine was elevated in patients with UTI. Patients with higher concentrations of serum creatinine during UTI had high urine levels of IL-8 [66]. Smith et al. [42] found that IL-2 was decreased in UTI 1–5 days prior to diagnosis but increased 1–5 days after diagnosis; the same study showed that cGMP increased and nitrate decreased with UTI.

Limitations
Although the above discussed urinary biomarkers data is promising, there have been some limitations to expand the application of these markers from clinical research to clinical practice. In general, these clinical studies included small numbers of participants, making the statistical analysis underpowered and not significantly robust. On the other hand, in spite of the advances in the proteomic and genomic techniques, data has not been sufficient to yield using these expensive methods in clinical practice. Also, Bioplex multiple ELISA and Luminex techniques have been extensively used in cytokines and other substances analysis resulting in the detection of few biomarkers; most of these data could not provide a strong evidence on the causality and correlations between the biomarkers and the clinical outcomes, resulting in suboptimal clinical benefits of these markers. However, despite these limitations, the discovery of biomarkers in renal transplantation is an evolving field of significant clinical implication. Larger and more comprehensive multi-center studies are urgently needed.

Clinical Implications
In spite of the limitations of these techniques, based on frequency and well-established data, we suggest that a combined measurement of granzyme B and perforin mRNA in blood or urine samples and urinary IP-10 mRNA or protein can be a relatively sensitive and specific tool to detect acute cellular rejection. Also, mRNA expression analysis via proteomics methods can offer a sensitive and specific monitoring tool of allograft function that may detect rejection.

In clinical practice, measurements of granzyme B, perforin and IP-10 can be non-invasive monitoring methods in kidney transplant recipients, during the first year post-transplantation that may help predict ARs. Alternatively, proteomic and possibly genomic analysis may play a significant role in monitoring allograft function. Additionally, data have been expanding on the use

<table>
<thead>
<tr>
<th>Table 3. Performance of selected urinary biomarkers in acute ischemic injury</th>
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<tbody>
<tr>
<td>Biomarkers</td>
</tr>
<tr>
<td>NGAL</td>
</tr>
<tr>
<td>IL-18</td>
</tr>
<tr>
<td>IP-10</td>
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<tr>
<td>MIG</td>
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</table>

NA = Not available.
of NGAL as a non-invasive surrogate biomarker that highly indicates acute tubular injury. If one of these non-invasive tests suggests AR, an allograft kidney biopsy is indicated. However, in cases of allograft dysfunction that are not explained by other causes, a biopsy is indicated in spite of having negative biomarkers, as some cellular and antibody-mediated rejection types have not been correlated with a well-studied non-invasive biomarker.

**Conclusion**

Acute renal allograft injury secondary to AR, acute ischemic injury or drug toxicity has a significant short-and long-term impact on graft function and graft survival in kidney transplant patients. Timely detection and treatment of acute allograft injury is a critical step in saving allograft function and improving allograft survival. In spite of advancing our understanding of immunology, immunogenetics and proteomics along with the new generations of techniques that allow for such investigations, this essential area of research is still evolving and there is a great need for more sensitive and specific urinary biomarkers that can be obtained promptly and easily during clinical practice.

**Disclosure Statement**

None.
The review of the literature on biomarkers of acute renal dysfunction after transplantation by Alachkar and her colleagues from Johns Hopkins in Baltimore is timely in view of the ever growing interest in the field of biomarkers in nephrology. When exploring the relevance of biomarkers, it is clearly important to determine the perceived relevance and clinical importance of the putative biomarkers in a given condition; emphasis may be on diagnosis, pathophysiology, response to therapy or prognosis. The nephrology literature is rapidly growing in this area of interest and research, with little focus on the potential advantage of a given 'new' biomarker over previously well-established markers of disease. Also biomarkers are not biofactors and, as mentioned in the review, do not necessarily imply causality. Besides the potential value of a given biomarker, attention should also be paid to the practicalities of using such a test and the associated cost. Research for biomarkers in AKI and acute graft dysfunction goes on, but few are likely to fully substitute for good clinical judgement and timely measurement and interpretation of traditional kidney function tests such as serum creatinine.