PROTEOMIC BIOMARKERS FOR THE EARLY DETECTION OF ACUTE KIDNEY INJURY

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Abstract: Acute kidney injury (AKI) comprises several syndromes that are associated with a sudden decrease in renal function. AKI is a common condition especially among critically ill patients. It is typically multifactorial and of great prognostic significance. The incidence of AKI has increased while the associated mortality rate has remained unchanged over the last years. Recent definitions of AKI, namely the Risk, Injury, Failure, Loss of renal function and End-stage kidney disease (RIFLE) classification or the Acute Kidney Injury Network (AKIN) criteria, incorporate serum creatinine and urine output as the principal markers to define and detect AKI. However, elevated serum creatinine or oliguria were demonstrated to detect AKI at late stages of renal injury when preventive strategies may be less effective. Therefore, there has recently been a great scientific interest in obtaining valuable markers for early AKI detection. In the last 5 years numerous new markers such as neutrophil-gelatinase associated lipocalin, interleukin-18, cystatin C and kidney injury molecule 1 in the urine and/or serum have been studied and proposed as early detection markers of AKI. Persistently, these markers performed well in initial pilot trials. However, these promising results could often not be confirmed in later, larger multicentre trials and limitation of these biomarkers in the early diagnosis of renal injury were discovered. Furthermore, as AKI is mul-

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tifactorial and heterogeneous in origin, it seems likely that not one single marker but a panel of biomarkers will be required to detect all subtypes of AKI early during their evolution. This has initiated proteomic studies to develop panels of biomarkers which may facilitate early detection of AKI. The present review will focus on the most important clinical studies evaluating the ability of single AKI biomarkers and on those in clinical proteomics that attempted to establish panels of biomarkers in urine for early and accurate AKI diagnosis and prognosis.

Key words: acute kidney injury, diagnosis, prognosis, biomarkers, proteomics.

Clinical background

Acute kidney injury (AKI), previously termed acute renal failure, is a frequent clinical condition in critically ill patients especially, in intensive care units (ICU). It is characterized by a rapid decline or loss of renal function. Its incidence varies from 1–7% of all hospitalized patients to 30–50% of patients in ICU [1, 2]. Clinical manifestations include a rapid decrease (oliguria) or cessation (anuria) of urine output and of a glomerular filtration rate (GFR) below 10 mL/min within hours to days. AKI is further indicated by accumulation of nitrogenous-waste substances in the blood resulting in elevated serum levels of creatinine and blood urea nitrogen (BUN). It is important to differentiate AKI from chronic kidney disease (CKD), as AKI has the potential to be reversible. AKI and CKD can be differentiated by the dynamics by which nitrogenous-waste substances increase in the serum and urinary output decreases.

Irrespective of the progress being made in the understanding of the pathophysiology of AKI and its underlying processes and the advances in critical care medicine, mortality rate associated with AKI remains high especially in ICU patients at more than 50% [3]. In addition, a significant proportion of surviving patients (20%) develops CKD and end-stage renal disease, requiring chronic renal replacement therapy [4, 5]. Long-term outcome is worse for patients after recovery from AKI [6, 7], further impacting health care cost and quality of life [8].

Advances in our understanding, prevention and treatment of AKI have been hampered especially by two factors. Firstly, until recently there was a lack of uniform criteria for the definition and classification of AKI. Secondly, there is still an incomplete understanding of the pathogenesis of AKI [9]. The risk of developing AKI is determined by a patient’s susceptibility and exposure or causative factors. Patient susceptibility in developing countries varies from that of the developed countries. In developing countries, AKI is more common in young and paediatric patients, while in developed countries elderly patients are predominant [10, 11]. However, it is difficult to differentiate demographic vari-
ables which directly contribute to the risk of developing AKI from those that are more attributed to the underlying disease [9]. Conditions known to cause AKI in susceptible populations include sepsis, ischaemia, heart failure, liver disease, major surgery (especially vascular and cardiac), rhabdomyolysis, urinary tract obstruction and various nephrotoxic drugs and radiocontrast agents [12]. In critically ill patients the most common cause of AKI is sepsis, accounting for 50% of all cases [13–15].

**Diagnostic problem**

In order to standardize and detect AKI, two different sets of definition criteria have been recently established. The Acute Dialysis Quality Initiative developed the RIFLE criteria for the diagnosis of acute renal failure in critically ill patients [16] and the Acute Kidney Injury Network developed the AKIN criteria for the diagnosis of AKI [17]. Both criteria (Figure 1) for diagnosis are mainly based on measurements of urine output and serum creatinine. In clinical practice, however, AKI is predominantly detected by changes in serum creatinine [17].

**RIFLE criteria (Bellomo et al., Crit Care 2004, [16])**

<table>
<thead>
<tr>
<th>Stage</th>
<th>S-creatinine ↑ or GFR ↓</th>
<th>Urine output ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk</td>
<td>150 % or &gt; 25 – 50 %</td>
<td>&lt; 0.5 mL/kg/h for 6 h</td>
</tr>
<tr>
<td>Injury</td>
<td>200 % or &gt; 50 – 75 %</td>
<td>&lt; 0.5 mL/kg/h for 12 h</td>
</tr>
<tr>
<td>Failure</td>
<td>300 % or ≥ 4 mg/dL or &gt; 75 %</td>
<td>&lt; 0.3 mL/kg/h for 24 h or anuria for 12 h</td>
</tr>
<tr>
<td>Loss</td>
<td>Persistent AKI = Complete loss of renal function &gt; 4 wk</td>
<td></td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease = Complete loss of renal function &gt; 3 mo</td>
<td></td>
</tr>
</tbody>
</table>

**AKIN criteria (Mehta et al., Crit Care 2007, [17])**

<table>
<thead>
<tr>
<th>Stage</th>
<th>S-creatinine ↑</th>
<th>Urine output ↓</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>≥ 0.3 mg/dL or 150 – 200 %</td>
<td>&lt; 0.5 mL/kg/h within 6 – 12 h</td>
</tr>
<tr>
<td>2</td>
<td>201 – 300 %</td>
<td>&lt; 0.5 mL/kg/h for &gt; 12 h</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 300 % or dialysis</td>
<td>&lt; 0.3 mL/kg/h for &gt; 24 h or anuria for &gt; 12 h</td>
</tr>
</tbody>
</table>

*Figure 1 – AKI staging according to RIFLE and AKIN criteria*
Creatinine is a 113 Dalton molecule derived from creatine metabolism after creatinine’s release from the muscle. As creatinine is freely filtered by the glomerulus and excreted without significant metabolic changes or reabsorption by the kidney, this molecule has been a useful indicator of kidney function. However, serum creatinine has important limitations as a tool for assessing GFR. Firstly, creatinine levels are affected by a variety of non-renal factors such as age, gender, muscle mass, diet and nutritional status [18]. Although equations have been developed to correct for some of these factors, these are only applicable to CKD but not to AKI, as they require a stable creatinine metabolism [19]. Secondly, serum creatinine concentration and its value is influenced by its volume of distribution that can be substantially affected by volume overload, a common situation in AKI [20]. Finally and probably most importantly, serum creatinine increases only after substantial loss of GFR resulting in a lag phase in the temporal relationship between serum creatinine increase and loss of GFR. As a result, current clinical diagnosis of AKI based on creatinine limits its early detection in clinical routine as well as the early implementation of preventive measures. Therefore, the development of new AKI biomarkers has had high priority in the nephrological community during the last years with the aim of identifying markers that are superior to serum creatinine in the early detection of AKI.

In the following part we will briefly summarise the most promising single biomarkers for AKI.

**Single biomarkers for AKI**

New biomarkers for AKI can be categorized as inflammatory mediators, excreted tubular proteins and surrogate markers indicative of tubular damage (albumin, alpha 1-microglobulin, beta 2-glycoprotein, plasma retinol binding protein, N-acetyl-β-D-glucosaminidase (NAG)) and liver-type fatty acid binding protein (L-FABP) [21, 22]. The most promising of the AKI biomarker candidates, namely neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), interleukin-18 (IL-18) and serum cystatin C (CysC) will be presented in more detail:

**Neutrophil gelatinase-associated lipocalin (NGAL)**

*Function:* NGAL, also known as lipocalin-2, is a 25-kDa protein strongly up-regulated by interleukin-1 during inflammation. NGAL has the ability of sequestering siderophores, microbial iron-chelating required for bacterial growth, and prevents urinary tract infection.
Diagnostic evidence: NGAL has been presented as a biomarker for early detection of AKI and for AKI prognosis [23]. The first study that pointed out the association of NGAL with AKI development was performed by Mishra and colleagues in 2005 [24]. In this prospective study urine and plasma NGAL rose significantly in children developing AKI after cardiac surgery within 2 h postoperatively. However, the classification performance of NGAL decreased in similar studies performed in adults also having cardiac surgery [25] possibly due to confounding variables and comorbid conditions that accumulate with age. Other positive results have been obtained when NGAL has been tested as a biomarker of AKI in kidney transplantation and the subsequent development of delayed graft function [26]. Among other things, NGAL was tested in haemolytic uraemic syndrome [27], urinary tract infections [28], critically ill children and adults [29, 30] and also CKD [31]. Taken together, NGAL is the most promising novel renal biomarker in urine and also in plasma. However, as pointed out by Chawla and Kellum [32], NGAL is expressed in multiple organs affording further studies to understand how non-kidney sources of NGAL have an impact on urinary NGAL. As a consequence, prospective multicentre studies are urgently required to determine the performance of plasma and urinary NGAL in unselected ICU patient populations including patients with preexisting CKD [33, 34]. Since the reported cut-off values for NGAL differ across a wide range it seems reasonable to speculate that each clinical setting may require different cut-off values [35].

Interleukin-18 (IL-18)

Function: IL-18 is an 18-kDa proinflammatory cytokine secreted by macrophages and other antigen-presenting cells. It has the ability to induce interferon gamma production in type-1 T helper cells and is a sensitive mediator of ischaemic injury in different organs such as the heart, brain and kidney [36].

Diagnostic evidence: A first evidence for IL-18’s role in ischaemic AKI was given by animal studies [37, 38]. Later studies in humans suggested that urinary IL-18 may serve as marker of AKI development after cardiac surgery, of graft function after kidney allograft transplantation, and of mortality in acute respiratory distress syndrome [39–41]. Siew et al. [42] reported that urinary IL-18, when measured within 48 h of AKI development, could not reliably predict AKI in a broadly selected, critically ill adult patient cohort. Despite this negative result, urinary IL-18 remained predictive in this study for worse clinical outcomes such as death and acute dialysis within 28 days of ascertainment independently of other factors [42]. A matter of concern is that IL-18 increases in a variety of pathophysiological conditions, such as sepsis, inflammatory arthritis, inflammatory bowel diseases, systemic lupus erythematosus, psoriasis, hepatitis and multiple sclerosis. This property significantly limits its application, due to reduced sensitivity and specificity [43].
**Kidney injury molecule-1 (KIM-1)**

*Function:* KIM-1 is a type 1 transmembrane glycoprotein that is undetectable in normal kidneys but highly expressed by proximal tubules epithelial cells after ischaemic or toxic injury [44, 45] with the ectodomain being shedded into the tubular lumen [46]. It functions as a phosphatidyl-serine receptor and confers a phagocytic phenotype on epithelial cells, most likely to clear cellular debris during enhanced apoptosis [47].

*Diagnostic evidence:* In previous studies in adults, KIM-1 was able to discriminate patients with acute tubular necrosis from those without, and predicted AKI in adults undergoing cardiac surgery [48–50]. In another prospective study on 201 hospital patients with AKI, an increase of urinary KIM-1 was associated with increased mortality or dialysis requirement [51]. Its potential use as an early marker is based so far on limited data: a rise in its urinary levels was detectable before the increase of BUN and creatinine in plasma during cadmium-induced renal damage [52] as well as its expression in biopsy sections of kidney allograft recipients before histological signs of acute tubular necrosis became evident [53].

**Cystatin C (CysC)**

*Function:* CysC is a cysteine protease inhibitor that is synthesized and continuously released into the blood by nucleated cells. Its levels are not significantly affected by age, gender, infection, liver disease or muscle mass in contrast to serum creatinine. This molecule is freely filtered by the glomerulus but, unlike creatinine, reabsorbed and metabolized by the proximal tubule. Therefore, elevated levels of serum CysC in serum correlate inversely with GFR while increased urinary CysC indicates renal tubular damage [54] and in fact the diagnostic accuracy of serum CysC to reflect GFR has been shown to be superior compared to serum creatinine since its levels are less influenced by inflammation, infection, body mass, diet and drugs [55].

*Diagnostic evidence:* Serum CysC is not a biomarker of AKI since its levels are not a direct marker of renal injury, and rather serves as a GFR marker [36]. Several studies have focused on the diagnostic accuracy of CysC in predicting AKI. Unfortunately, results have been conflicting. In high-risk patients serum CysC detected AKI 1–2 days earlier than serum creatinine [56]. However, in a mixed heterogeneous, multicentre ICU population serum and urinary CysC were poor predictors of AKI and the need for renal replacement therapy [57]. In a meta-analysis performed by Zhang et al. [58] using the data of 19 studies from 11 countries and 3,336 patients, it was found that serum CysC could be used as a reliable marker with an odds ratio of 23.5 in the prediction of AKI whereas urinary CysC showed only moderate diagnostic accuracy with an odds ratio of only 2.6.
Endre et al. [59] in a recent prospective observational study of 529 ICU patients and Lameire et al. [60] in a commentary on this work reach a sobering conclusion on the diagnostic and prognostic performance of these single AKI markers. In the study by Endre et al. [59] none of these markers reached an AUC value above 0.7 for the prediction of AKI on ICU entry and of death in 7 days, while urinary NGAL, CysC and IL-18 predicted dialysis in 7 days with AUC’s of 0.79, 0.71 and 0.73, respectively. This is in contrast to some previous studies with AUC values above 0.9 [61, 24] which was attributed to their selection of homogeneous study populations [60]. In conclusion, the single AKI markers performed well in selected, predominantly homogenous patient cohorts, whereas they failed for the most part in multicentre, heterogenous cohorts which rather represent clinical routine (see Table 1 for a listing of clinical studies). Due to this, multimarker patterns were suggested by experts in this field for which proteomic technologies are predestined.

Proteomic approaches and biomarkers profiles for AKI

The main rationale for the application of proteome analysis in the context of AKI is that AKI is a multifactorial and heterogeneous process. Due to the diversity of pathological processes leading to AKI, it is highly unlikely that one single diagnostic marker may serve as a reliable predictor for all AKI forms. A broadly applicable, multimarker diagnostic model will avoid this. The advantage of such a multimarker strategy is that it allows compensation for potential biological, pre-analytical and analytical variances of single biomarkers.

Mass spectrometry combined with chromatographic separation techniques has advanced exceptionally in recent years and has become a valuable tool for profiling of human proteomes and a systematic search of protein and peptide markers indicative of various renal and non-renal diseases without the need for a hypothesis-driven propagation process [62–65].

While proteome analysis aiming at biomarkers for renal disease can be focused on urine, plasma, or serum, urine seems to be the most attractive body fluid for several reasons. Firstly, urine can be obtained in large quantities in a non-invasive manner. Secondly, the urinary proteome is relative stable since it is retained in the bladder for several hours, providing sufficient time for complete proteolytic processing by endogenous proteases. The low molecular mass proteome of the urine does not undergo any significant change if urine is stored for up to 3 days at 4°C or 6 h at room temperature [66, 67].
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For these reasons, several groups have embraced the search for urinary proteomic biomarkers for the early detection of AKI using different analytical platforms. In an early study conducted in 2005, Nguyen et al. [68] identified 4 proteins with a mass-to-charge ratio (m/z) of 6.4, 28.5, 43 and 66 kDa, being increased at baseline and at 2 and 6 h post-operation in the urine of children who developed ischaemic kidney injury 2–3 days after a cardiopulmonary bypass (CPB). These proteins in combination allowed detection of AKI in this small patient cohort with 100% sensitivity and specificity. One of these proteins (m/z 6.4) was later identified as aprotinin [69], a very basic polypeptide with serine protease inhibitory activity, negatively affecting both coagulation and fibrinolysis [70]. The other 3 peaks were identified as the acute-phase proteins alpha 1-microglobulin (28.5 kDa), alpha 1-acid glycoprotein (43 kDa) and albumin (66 kDa) [71].

Using the same analytical platform Ho and colleagues identified, besides known up-regulated tubular injury markers, two novel peptide peaks at 2.43 and 2.78 kDa that were significantly increased in patients after CPB surgery not developing AKI [72]. The authors were able to resolve one of these peptides as hepcidin-25, the active form of hepcidin, which is secreted by the liver to maintain iron homeostasis and which is up-regulated during acute phase response [73].

Metzger et al. [33] used capillary electrophoresis mass spectrometry to identify urinary peptide markers predictive of AKI in urine samples obtained from ICU patients that later developed AKI defined by a serum creatinine increase ≥ 50% in ≤ 48 hours (maximum 5 days prior AKI) or remained normal in kidney function. The 20 statistically most significant peptide markers in a comparative group analysis (Figure 2) were combined to a support vector machine-based classifier, which allowed classification of a blinded test set of ICU patient samples (n = 20, 9 case and 11 controls) with 89% sensitivity and 82% specificity. In order to evaluate general applicability, this classifier was further applied to the classification of urine samples from haematopoietic stem cell transplanted patients of whom 13 developed AKI after transplantation and 16 did not. AUC in this validation set was 0.90 with sensitivity and specificity values of 94 and 82%, respectively. The 20 polypeptides were identified by amino acid sequencing as degradation products of 6 proteins. Fragments of albumin, alpha 1-antitrypsin and beta 2-microglobulin were up-regulated, fibrinogen alpha chain, collagen 1 alpha (I) and collagen 1 alpha (III) were down-regulated in AKI. The alterations of these polypeptides identified in the urine may be attributed to differences in production rates, increased assembly into filaments, increased proteolysis in the plasma or urine, abnormal renal function, or a combination of the above, and may be relevant at different points of the disease process as outlined in Figure 3.
Figure 2 – Distribution of urinary peptides included in the AKI-specific biomarker panel. AUC’s for ROC comparison of AKI and non-AKI within the ICU and HSCT patient groups are shown in the insets. Abbreviations: AKI, acute kidney injury; AUC, area under the curve; HSCT, haematopoietic stem cell transplantation; ICU, intensive care unit; ROC, receiver operating characteristics. Modified from data shown in Metzger et al., 2010 [33]

Figure 3 – Pathophysiological relevance of parent proteins from the peptides included in the AKI-specific proteomic biomarker model of Metzger et al., 2010 [33]

Abbreviations: A1AT, alpha 1-antitrypsin; ALBU, albumin; B2MG, beta 2-microglobulin; CO1A1, collagen alpha-1(I) chain; CO1A3, collagen alpha-1(III) chain; ECM, extracellular matrix; FIBA, fibrinogen alpha chain; MMP, matrix metalloproteinases.
A recent study of Maddens et al. [74] using LTQ-OrbiTRAP for mass spectrometry analysis identified urinary NGAL, thioredoxin, gelsolin, chitinase 3-like protein 1 and 3 and acidic mammalian chitinase as being the most discriminatory markers for experimental sepsis-induced AKI in mice. Differential expression was verified by immunoblot analysis in urine, plasma and renal tissue homogenates. In a small set of human septic patients the authors detected possible differences in excretion levels of the human homologue of chitinase 3-like protein 1 and acidic mammalian chitinase protein between patients with, compared to those without, AKI. However, the study was too small to draw any conclusions. The potential use of chitinase proteins as sensitive markers for diagnosis of septic-induced AKI is limited, as the authors stated, mostly by the fact that increased levels are also detectable during inflammatory responses, such as asthma or inflammatory bowel disease, liver fibrosis and also for non-AKI patients of the AKI study group without recognizable comorbidities.

Gel-based proteomics has also been tested as a platform in the search for biomarkers of AKI. Using this methodological approach, Aregger et al. [75] identified 3 proteins in a cohort of 36 patients undergoing CPB to be differentially regulated between patients who developed AKI and those who did not. The identified proteins were albumin, being upregulated, and zinc alpha 2-glycoprotein and adrenomedullin-binding protein, both down-regulated. Limiting the results of the study, only zinc alpha 2-glycoprotein was applied to a validation set of 22 patients with AKI and 46 patients without testing its diagnostic performance in immunoblot and ELISA. An AUC value of 0.68 revealed that zinc alpha 2-glycoprotein is only a weak predictor of AKI.

Conclusion

It is evident that an effective prevention or intervention strategy for patients particularly in the ICU (with the possible therapeutic options depicted in Figure 4) relies on accurate and early detection of AKI. Considering the heterogeneity and complexity of AKI, a multiple marker approach seems to be more favourable than single markers. A multimarker approach will not rely on particular, single aspects of AKI, i.e. tubular damage, fibrosis, inflammation, necrosis or apoptosis, but combines the significant findings indicative of specific etiologies, ideally enabling detection of AKI independent of the underlying cause.

Irrespective of the approach, large, prospective, multicentre clinical trials on unselected patient populations are required to validate the different proposed biomarkers or classifiers. By analogy with a recent editorial by Vlahou [76], such a large study would best be performed in a way that allows testing all the biomarkers currently proposed. Unfortunately, neither industry, nor government agencies currently see the need for such a large trial.
Once suitable biomarkers and classifiers for accurate, early detection of AKI have been identified, it is essential to design an easy-to-apply analytical test based on these markers that is suitable for routine laboratory use ideally as a point of care device in intensive care units. One promising approach is the application of MALDI-MS as a robust and fast platform for efficient analysis of urinary biomarkers, which has been demonstrated feasible in CKD [77].

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