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Biomarkers in acute kidney injury – pathophysiological basis and clinical performance

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Abstract

Various biomarkers of acute kidney injury (AKI) have been discovered and characterized in the recent past. These molecules can be detected in urine or blood and signify structural damage to the kidney. Clinically, they are proposed as adjunct diagnostics to serum creatinine and urinary output to improve the early detection, differential diagnosis and prognostic assessment of AKI. The most obvious requirements for a biomarker include its reflection of the underlying pathophysiology of the disease. Hence, a biomarker of AKI should derive from the injured kidney and reflect a molecular process intimately connected with tissue injury. Here, we provide an overview of the basic pathophysiology, the cellular sources and the clinical performance of the most important currently proposed biomarkers of AKI: neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), liver-type fatty acid-binding protein (L-FABP), interleukin-18 (IL-18), insulin-like growth factor-binding protein 7 (IGFBP7), tissue inhibitor of metalloproteinase 2 (TIMP-2) and calprotectin (S100A8/9). We also acknowledge each biomarker's advantages and disadvantages as well as important knowledge gaps and perspectives for future studies.

Keywords

acute kidney injury; biomarkers; calprotectin; kidney injury molecule 1 (KIM-1); neutrophil gelatinase-associated lipocalin (NGAL); tissue inhibitor of metalloproteinase-2 (TIMP-2) and IGF-binding protein 7 (IGFBP7)

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Conflict of interest

Columbia University has licensed uNGAL for use in the diagnosis of AKI (applies to J. B. and K.M.S.-O.). K. Budde received research funds and/or honoraria from AiCuris, Pfizer, Novartis, Astellas, Roche, Hexal, Bristol-Myers Squibb, Veloxis Pharma, Effimune Pharma and Siemens. Other authors have reported that they have no relationships relevant to the contents of this study to disclose.

Acute kidney injury (AKI) is a common and potentially life-threatening condition (Bellomo *et al.* 2012, Kellum *et al.* 2012). It is associated with elevated short-term morbidity and mortality as well as with unfavourable long-term outcomes caused by the development of chronic kidney disease (CKD) or the occurrence of cardiovascular events (Bihorac *et al.* 2009, Chawla *et al.* 2014a,b, Wu *et al.* 2014). At the time of AKI diagnosis, a number of diagnostic and therapeutic measures are needed. These measures include the determination of the underlying cause of AKI and the initiation of specific and supportive therapeutic measures, such as antibiotic therapy for sepsis, immunosuppression for autoimmune disease, an adjustment of nephrotoxic drugs or directed fluid management (Finfer *et al.* 2004, National Heart, Lung, and Blood Institute Acute Respiratory Distress Syndrome (ARDS) Clinical Trials Network *et al.*, 2006, Murugan *et al.* 2010).

As early institution of these measures is critical for their effectiveness, efforts have been made to identify subtle insults to the kidney that do not cause measurable functional decline, that is subclinical forms of AKI, and to identify indicators of a particular risk of AKI (Kellum *et al.* 2012, Ronco *et al.* 2012, Chawla *et al.* 2015). This is also reflected in The Kidney Disease: Improving Goal Outcomes (KDIGO) clinical practice guidelines for AKI, which not only include patients with AKI but also patients who are at risk for the development of AKI (Khawaja 2012). Despite these efforts and the consensus that specific measures must be undertaken to identify patients with subclinical AKI in order to protect them from poor long-term outcomes, there is still little implementation of this knowledge in daily clinical practice. Currently, the standard diagnostic tools for the detection of AKI are monitoring of urinary output and serum creatinine concentration (sCr), both of which are markers of kidney function but not kidney injury (Waikar *et al.* 2009). Accordingly, AKI is defined by an increase of serum creatinine by $\geq 0.3 \text{ mg dL}^{-1}$ in 48 h or an increase by ≥ 1.5 -fold from a known or assumed baseline or by a decrease of urinary output to less than $0.5 \text{ mL kg}^{-1} \text{ h}^{-1}$ for 6 h (Khawaja 2012). In clinical reality, however, serial measurements of serum creatinine are often unavailable complicating the differentiation between AKI and CKD. In addition, creatinine and urinary output when measured at presentation do not always predict adverse outcomes, such as hospital mortality or a requirement for renal replacement therapy (RRT) (Mehta *et al.* 2002, Bell *et al.* 2009). Furthermore, this definition does not account for the aetiology of AKI. Most importantly, it does not differentiate between quickly reversible, volume-sensitive reductions in glomerular filtration rate (=pre-renal AKI) and primary structural injury to the kidney (=intrinsic AKI). Furthermore, the peak height of sCr, which defines AKI severity according to current criteria, may not be the best approach to understanding AKI because according to Coca *et al.*, the duration of azotaemia correlates with mortality better than the peak height of sCr (Coca *et al.* 2010). The patients with the most elevated creatinine could be those with the best muscle mass, that is the best health status at baseline. Long periods of azotaemia may correlate with time-consuming repair of the renal tubule, while short periods of azotaemia may correlate with rapidly reversible hemodynamic variation. In addition, the lack of specificity is the concern over sensitivity because a healthy renal reserve would blunt the rise in creatinine. In fact, removal or damage of a portion of a kidney may not elevate sCr, despite loss of renal mass. The shortcomings of serum creatinine as a biomarker for AKI are widely acknowledged (Devarajan 2007, Jo *et al.* 2007, Bennett *et al.* 2008).

One major advance to detect AKI at an earlier stage would be the implementation of new reliable biomarkers that identify AKI earlier than conventional tests or that detect subclinical AKI (Hoste *et al.* 2006, Cruz *et al.* 2013, Chawla *et al.* 2014a,b, Wu *et al.* 2014). Within the past years, several new potential biomarker molecules that are measurable in urine or plasma samples of patients with AKI have been discovered including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), interleukin-18 (IL-18), liver-type fatty acid-binding protein (L-FABP), tissue inhibitor of metalloproteinase-2 (TIMP-2), insulin-like growth factor-binding protein 7 (IGFBP7) and calprotectin (Heller *et al.* 2011, Kashani *et al.* 2013, Singer *et al.* 2013, Shao *et al.* 2014, Lin *et al.* 2015, Medić *et al.* 2015, Xu *et al.* 2015, Zhou *et al.* 2016). The purpose of this article is to review biological and physiological data on new biomarkers and to summarize clinical studies that investigate these new biomarkers.

NGAL

NGAL is a 25-kDa protein of the lipocalin family. Human NGAL exists as a monomer and a 45-kDa homodimer as well as it exists as a 135-kDa heterodimer where it is conjugated to gelatinase and is specific to neutrophils. The monomer is the gene product itself, which is very rapidly secreted from stimulated or damaged epithelial cells. In some cells, where the monomer is stored, the protein can homodimerize such as in neutrophils (Cai *et al.* 2010). The known functions of NGAL are related to its ability to bind iron–siderophore complexes. It exerts a bacteriostatic function of the innate immune system by sequestering iron–siderophore complexes and thereby preventing iron uptake by bacteria (Goetz *et al.* 2002, Flo *et al.* 2004). This function can also be co-opted to transport iron to the cytoplasm via catecholate–iron complexes where it activates or represses iron-responsive genes (Yang *et al.* 2002, Bao *et al.* 2010).

NGAL is expressed at very low constant levels in different cell types. NGAL is highly upregulated on mRNA and protein level after ischaemic or toxic kidney injury in human neonates, children and adults and in every animal model where it has been studied (Mishra *et al.* 2003, 2004, Supavekin *et al.* 2003). Hence, the biology of NGAL is evolutionarily conserved in AKI probably because it confers essential protection against infection (Paragas *et al.* 2014). The elevation of NGAL is detectable as early as 3 h after the injury and it peaks at approx. 6–12 h after injury depending on the severity of injury. The elevation can persist up to 5 days after the initial injury when the injury is severe (Mishra *et al.* 2005, Parikh *et al.* 2011).

In vivo data have suggested that the thick ascending loop of Henle and the intercalated cells of the collecting duct are the primary sites of NGAL production in the kidney (Schmidt-Ott *et al.* 2007, Paragas *et al.* 2014) (Fig. 1). Kidney injury leads to an apical and basolateral secretion from kidney epithelia (Mori *et al.* 2005, Schmidt-Ott *et al.* 2007, Schmidt-Ott 2011). NGAL is filtered by the glomerulus and is reabsorbed by the proximal tubule in a megalin-dependent manner (Hvidberg *et al.* 2005) (Fig. 2). A decrease in tubular reabsorption after AKI may lead to a further increase in urinary NGAL concentration. The evidence that NGAL after AKI originates from the injured kidney and not from other tissues was produced by two animal models using a luciferase-based *in vivo* monitoring technique

and by cross-transplantation of NGAL^{-/-} kidneys into NGAL^{+/+} mice and vice versa (Paragas *et al.* 2011). In a mouse model of ischaemia/reperfusion injury, the administration of a single dose of iron–siderophore-loaded NGAL (holo-NGAL) protected the kidney from ischaemic damage. In this study, it was also shown that holo-NGAL upregulates the renoprotective enzyme heme oxygenase-1 (Mori *et al.* 2005).

NGAL is the most widely studied biomarker of AKI (Singer *et al.* 2013, Ho *et al.* 2015, Zhou *et al.* 2016). It has shown its performance in various settings such as the prediction of AKI in paediatric (Dent *et al.* 2007) and adult cardiac surgery patients (Parikh *et al.* 2011, Prowle *et al.* 2015), in critically ill patients (de Geus *et al.* 2011, Cruz *et al.* 2013), in patients in the emergency room (Nickolas *et al.* 2008, 2012) as well as in the kidney transplant setting (Pajek *et al.* 2014, Pianta *et al.* 2015a, Ramirez-Sandoval *et al.* 2015). A recent meta-analysis of Ho *et al.* included 16 studies with a total of 2906 patients investigating urinary NGAL as a biomarker for the prediction of AKI after cardiac surgery in adult patients. The composite area under the curve (AUC) of urinary NGAL was 0.72. Notably, the included studies differed in terms of criteria for AKI (inclusion of urine criteria) and sample collection time (0–24 h after surgery) (Ho *et al.* 2015).

NGAL is a sensitive gene responding to tubular cell stress and damage via the Toll-like receptor 4 and nuclear factor kappa-light-chain-enhancer of activated B cells pathway triggered by sepsis (Flo *et al.* 2004). Septic damage to the kidney is the most prevalent cause of admission for AKI from the emergency room (Nickolas *et al.* 2008). Sepsis is a good example that demonstrates the utility of this biomarker – NGAL can rise days before sepsis causes sCr elevation and the need for dialysis (Parravicini *et al.* 2010).

NGAL elevates in the setting of stimuli that damage the kidney but not in the setting of rapidly reversible, fluid-sensitive volume depletion. This was shown in emergency room (ER) and in-hospital settings (Nickolas *et al.* 2008, 2012, Singer *et al.* 2011). In these studies, clinical criteria have been consulted to distinguish pre-renal from intrinsic renal failure. The AUCs for determining intrinsic AKI ranged from 0.81 to 0.95. However, NGAL was also closely associated with the duration of AKI in these studies, which is consistent with recent work from Parikh's group demonstrating a short bout of azotaemia (of any change in creatinine) is a demonstration of limited damage (Coca *et al.* 2010).

NGAL predicted death or dialysis at the time of admission to the ER. This association persisted after stratification according to sCr levels. Patients with NGAL >104 ng mL⁻¹ and sCr >1.4 mg dL⁻¹ demonstrated a 15% incidence of death or dialysis during hospitalization. Elevation of either NGAL or sCr without the other demonstrated a 5% incidence of death or dialysis. The data were reproduced by examining KIM-1 (see below) but not other biomarkers (Nickolas *et al.* 2012). Moreover, elevated urinary NGAL levels at AKI diagnosis predicted long-term adverse outcomes of end-stage renal disease or death (insert new citation: Singer E, Schrezenmeier EV, Elger A, *et al.* Urinary NGAL-positive acute kidney injury and poor long-term outcomes in hospitalized patients. *Kidney International Reports*. In press.

Koyner *et al.* (2015a) compared the ability of urine output after a standardized furosemide challenge to several AKI biomarkers (FST, urine NGAL, urine IL-18, urine KIM-1, Uromodulin urine Creatinine, urine ACR, FeNa plasma NGAL), measured just before furosemide administration, to predict several clinical outcomes in 77 patients with early AKI. While the FST was superior to all biomarkers in predicting outcomes, among the biomarkers urinary NGAL displayed the best predictive ability (AUC 0.75).

In sum, NGAL is a 'ready to go gene' that is rapidly expressed when kidney cells sense stress and/or damage. It is much more sensitive than sCr leading to the following nomenclature: NGAL- sCr- (normal), NGAL+ sCr- (damage to <50% of renal mass or early detection of severe disease), NGAL+ sCr+ (damage to >50% of renal mass) and finally NGAL- sCr+ (no renal stress or damage, but functional impairment consistent with pre-renal azotaemia). These data suggest that the AKI diagnosis based on sCr alone is inadequate and can be fractionated into different patient categories using NGAL. An important caveat is that urinary tract infection (UTI) may in some cases raise levels of urine NGAL, and in this setting, it is recommended to measure plasma NGAL (Devarajan 2007, Schmidt-Ott 2011).

Among all new biomarkers, NGAL is the most widely investigated new biomarker. Different investigators analysed diverse patient populations and determined the performance of NGAL (Singer *et al.* 2013, Ho *et al.* 2015, Ramirez-Sandoval *et al.* 2015). It is clear that NGAL is intensely expressed in a dose-dependent fashion with the severity of kidney disease and is activated at the time of patient presentation, for example in the emergency department (Nickolas *et al.* 2012). It is also clear that it separates volume depletion and intrinsic damage, two clinically separate entities that have been lumped together in the AKI diagnosis. Progress with this biomarker requires clear cut-off values in different settings [Nickolas suggested 104 ng mL^{-1} and increasing levels of creatinine, i.e. RIFLE level >R best correlate (Nickolas *et al.* 2012)]. Reference values for a healthy adult population for urinary NGAL were established, revealing increasing NGAL levels with age and higher NGAL levels in women compared to men (Cullen *et al.* 2012, Pennemans *et al.* 2013).

The pathophysiology and the assignment to the kidney have been shown in animal models (Paragas *et al.* 2011). Compared to other biomarkers (e.g. cell cycle arrest biomarkers,) the relationship to AKI and mechanistic understanding of NGAL is much more advanced.

KIM-1

KIM-1 is a 38.7-kDa type I transmembrane glycoprotein with an extracellular immunoglobulin-like domain topping a long mucin-like domain (Ichimura *et al.* 1998). It is expressed at low levels in the normal kidney as well as in other organs, but its expression is dramatically up-regulated in the kidney post-ischaemia/reperfusion injury in rats (Ichimura *et al.* 1998) as well as in rodent models of drug-induced AKI (Amin *et al.* 2004, Prozialeck *et al.* 2007). The expression is mainly upregulated in proximal tubule cells both in rodents (Ichimura *et al.* 2004) and in humans (Han *et al.* 2002). The extracellular domain of KIM-1 is shed from the cell surface by a metalloproteinase-dependent process (Bailly *et al.* 2002) (Fig. 3). This shedding together with an increased intrarenal synthesis of KIM-1 mRNA and

protein is most likely the cause of the increase of KIM-1 in the urine after AKI (Han *et al.* 2002, Ichimura *et al.* 2004). It has been proposed that KIM-1 plays an important role in kidney recovery and tubular regeneration because it was shown that it acts as a phosphatidylserine receptor and thereby mediates the phagocytosis of apoptotic bodies and cell debris into cultured renal epithelial cells (Ichimura *et al.* 2008) (Fig. 3). Mice with a mutation in the KIM-1 mucin domain had greater functional impairment of renal function and a stronger inflammatory response after cisplatin-induced AKI and in ischaemia/reperfusion injury (Yang *et al.* 2009). Ismail *et al.* showed that KIM-1-deficient cultured tubular epithelial cells were virtually incapable of taking up apoptotic cells (Ismail *et al.* 2015a). They also showed that this process is mediated by binding of KIM-1 to the alpha subunit of heterotrimeric G12 protein (G α 12). Just recently, data have been published demonstrating that KIM-1 inhibits G α 12 activation and that endogenous KIM-1 protects mice against renal ischaemia/reperfusion injury (Ismail *et al.* 2015b) (Fig. 3).

In comparison with the protective effect of KIM-1 in AKI, it has an unfavourable effect in CKD. Conditional expression of KIM-1 in renal epithelial cells in mice leads to spontaneous and progressive interstitial kidney inflammation and fibrosis (Humphreys *et al.* 2013). In CKD, KIM-1 is colocalized with areas of inflammation and fibrosis (van Timmeren *et al.* 2007) and it correlates directly with the degree of interstitial fibrosis in renal allografts before reperfusion (Schröppel *et al.* 2010). Studies investigating KIM-1 as a biomarker of AKI have overall shown variable results (Hall *et al.* 2011, Arthur *et al.* 2014). Combination of KIM-1 with IL-18 achieved an AUC of 0.92 in a study of 32 urine biomarkers in AKI after cardiac surgery and the combination outperformed other biomarkers (Arthur *et al.* 2014). A detailed summary of available studies in different disease settings was just recently given (Shao *et al.* 2014, Medić *et al.* 2015). In addition, KIM-1 might be useful for the detection of nephrotoxicity in pre-clinical and early phase I and II clinical studies (Dieterle *et al.* 2010, Vaidya *et al.* 2010). A lateral flow dipstick for KIM-1 has been introduced providing a simplified way of assessing KIM-1 levels (Fuchs *et al.* 2012). Reference ranges of urinary KIM-1 in healthy individuals were studied, indicating a linear increase of KIM-1 with age and higher KIM-1 values in males (Pennemans *et al.* 2013).

L-FABP

L-FABP is a 14 kDa protein from the large superfamily of lipid-binding proteins (Tan *et al.* 2002). The family contains nine members with tissue-specific distribution being named after the tissue where they first have been discovered: liver (L), intestine (I), muscle and heart (H), adipocytes (A), epidermal tissue (E), ileum (IL), brain (B), testis (T) and myelin (M).

The common function of all members of this family is the regulation of fatty acids uptake and the intracellular transport (Chmurzyńska 2006) (Fig. 4). L-FABP, which is not only expressed in the liver but also in the intestine, stomach, lung and kidney (Smathers & Petersen 2011), binds to fatty acids and transports them to the mitochondria and peroxisomes where β -oxidation takes place providing energy for tubular cells (Sweetser *et al.* 1987). Besides its transport function, L-FABP also protects cells from oxidative stress induced by H₂O₂ (Wang *et al.* 2005). L-FABP expression is inducible by hypoxia because the human L-FABP gene contains a hypoxia-inducible factor 1 α response element

(Yamamoto *et al.* 2007, Noiri *et al.* 2009). In concordance with this finding, the level of L-FABP was shown to directly correlate with ischaemic time in kidney transplant recipients (Yamamoto *et al.* 2007).

Within the kidney, it is predominantly located in the proximal tubule and is excreted into the tubular lumen together with bound toxic peroxisomal products, which accumulate otherwise (Maatman *et al.* 1991, Yamamoto *et al.* 2007) (Fig. 4). L-FABP is not expressed in mice; therefore, human-L-FABP transgenic mice were used to investigate the role of L-FABP in AKI (Kamijo-Ikemori *et al.* 2011). These mice showed less histological changes and lower blood urea nitrogen levels after ischaemia and reperfusion (Yamamoto *et al.* 2007, Noiri *et al.* 2009). The peroxisome proliferator activated receptor- α (PPAR- α) upregulates L-FABP gene expression (Landrier *et al.* 2004). Fibrates are known agonists of PPAR- α and were therefore used in models of cisplatin-induced AKI in rodents. In these models, fibrates were able to attenuate AKI (Negishi *et al.* 2007, 2008). Nevertheless, human observational studies reveal an increased rather than a decreased risk of AKI with fibrate use (Attridge *et al.* 2012, Zhao *et al.* 2012).

Studies that investigate L-FABP as predictor of AKI in patients after cardiac surgery have shown AUCs between 0.52 and 0.85 with a composite AUC of 0.72 when summarizing six studies including 1700 patients (Ho *et al.* 2015). Patients with high L-FABP measured by the time of ICU admission had greater risk for the development of AKI within 1 week (Doi *et al.* 2011). Therefore, L-FABP can be used to identify patients with a high susceptibility for renal stress. There are no data available on the prediction of long-term mortality or ESRD for L-FABP. A recent review article summarizes available study results on L-FABP in detail (Xu *et al.* 2015).

IL-18

Interleukin-18, also known as interferon- γ inducing factor, is a 24-kDa cytokine that belongs to the interleukin-1 superfamily (Novick *et al.* 2013). IL-18 is first synthesized as an inactive precursor without a signal peptide, and it remains intracellular until its cleavage by caspase-1 and its subsequent secretion by monocytes/macrophages (Fantuzzi *et al.* 1999) (Fig. 5). Caspase-1 acts as a component of the so-called inflammasome, a cytosolic protein complex that mediates cleavage and release of interleukins in response to extrinsic stimuli. Several cell surface receptors such as toll-like receptors (TLRs), retinoic acid-induced gene-like receptors, nucleotide-binding domain-leucine-rich repeat, scavenger receptors and C-type lectins can start the cascade (Chang *et al.* 2014) (Fig. 5). Cleaved IL-18 exerts a proinflammatory effect by signal transduction through the IL-18 receptor/IL-18 receptor accessory protein heterodimer (Cheung *et al.* 2005). IL-18 is also produced by the intercalated cells of the collecting ducts in the healthy kidney (Gauer *et al.* 2007) but appears to be induced more broadly in injured tubular epithelial cells (Franke *et al.* 2012).

Because of the pathophysiological plausibility of IL-18 in the development and progression of AKI, which was reported in different rodent animal models, IL-18 was suggested to be a new biomarker in AKI. IL-18-deficient mice are protected from ischemia/reperfusion-induced AKI (Wu *et al.* 2008). Similarly, caspase-deficient mice, which are not able to

cleave IL-18, develop less severe AKI after ischaemia/reperfusion (Melnikov *et al.* 2001). Inhibition of caspase-1 attenuates glycerol-induced acute renal failure in rats (Homsy *et al.* 2006). In 1999, a naturally occurring interleukin-18 binding protein was identified and it was shown to abolish IL-18 induction of interferon-gamma (Novick *et al.* 1999). The administration of this IL-18 binding protein just before ischaemia/reperfusion injury ameliorated kidney damage in rats (Wang *et al.* 2012). Overall, only few clinical studies have tested the utility of IL-18 as a biomarker for AKI (Lin *et al.* 2015). The results showed moderate results for AKI in paediatric patients after cardiac surgery (Krawczeski *et al.* 2011, Zheng *et al.* 2013), but it failed to show reliable prediction in the general ICU population (Nisula *et al.* 2015) or in the ER (Nickolas *et al.* 2012). In a study assaying different biomarkers for the prediction of delayed graft function, urinary IL-18 measured 4 h post-transplantation was among the most promising markers contributing independently to a clinical predictive model (Pianta *et al.* 2015b). In addition to human studies, several animal models have shown utility of using IL-18 in AKI (Melnikov *et al.* 2001, Homsy *et al.* 2006, Wu *et al.* 2008).

Even if the prognostic and diagnostic value of IL-18 is limited (Zhao *et al.* 2012, Zheng *et al.* 2013, Nisula *et al.* 2015), the use of anti-IL-18 treatment may be a potential future AKI treatment option, which may make urinary IL-18 an important adjunctive biomarker. It would be plausible that patients with high urine IL-18 benefit most from anti-IL-18 treatment, whereas patients with low IL-18 levels do not.

IGFBP7 and TIMP-2 (the so-called cell cycle arrest biomarkers)

Cell cycle arrest in G1 phase may be cellular mechanism to escape from situations when potential DNA damage can occur (Rodier *et al.* 2007). In the setting of ischaemic or septic kidney injury, renal epithelial cells have been shown to undergo G1 cell cycle arrest (Witzgall *et al.* 1994, Fraker *et al.* 1995, Yang *et al.* 2009). The cyclin-dependent kinase-inhibitor p21 prevents cell cycle progression from G1 to S phase. p21-deficient mice are more susceptible to cisplatin-induced kidney injury, develop a more severe morphologic damage, and have a higher mortality, suggesting that cell cycle arrest is important in limiting the consequences of AKI (Megyesi *et al.* 1998).

In 2013, IGFBP7 and TIMP-2 were identified as AKI biomarkers by Kashani *et al.* in a screen of 340 candidate biomarkers to predict AKI based on creatinine criteria (Kashani *et al.* 2013). In this study, the product of IGFBP7 and TIMP-2 concentrations was superior to several other biomarkers, which was later validated in additional cohorts (see below). TIMP-2, a 21-kDa protein, is a member of the tissue inhibitor of metalloproteinase (TIMP) family, which are endogenous inhibitors of metalloproteinase activities. IGFBP7, a 29-kDa secreted protein, is known to bind to and inhibit signalling through IGF-1 receptors (Evdokimova *et al.* 2012).

The cellular sources of IGFBP7 and TIMP-2 in AKI are poorly understood. Increased levels of IGFBP7 mRNA were found in urinary nitrate-induced acute renal failure in mice (Taulan *et al.* 2006). Apart from this report and the finding that IGFBP7 and TIMP-2 are enriched in the urine of patients at risk of AKI, the site of synthesis of these molecules in the setting of

AKI is unknown. While Kashani *et al.* speculated that IGFBP7 and TIMP-2 are synthesized by renal tubular cells (Kashani *et al.* 2013), there is no scientific evidence to support this.

Furthermore, the physiological roles of IGFBP7 and TIMP-2 in the kidney are unknown. Kashani *et al.* pointed out that IGFBP7 and TIMP2 were known to induce G1 cell cycle arrest and even designated them as ‘cell cycle arrest biomarkers’ (Kashani *et al.* 2013). Nevertheless, the notion that IGFBP7 or TIMP-2 are associated with cell cycle arrest in the kidney is unsubstantiated by experimental evidence. Data supporting the involvement of TIMP-2 in cell cycle arrest come from an *in vitro* study in human microvascular cells, not kidney cells (Seo *et al.* 2006). IGFBP7 has been shown to induce cell cycle arrest in colon cancer cell lines (Ma *et al.* 2008) and in breast cancer cell lines (Zuo *et al.* 2012), but not in the kidney.

The diagnostic value of TIMP-2 and IGFBP7 was validated in the Sapphire study (Kashani *et al.* 2013). In this study, the product of TIMP-2 and IGFBP7 ([TIMP-2]·[IGFBP7]) was superior to seven other biomarkers (urine NGAL, plasma Cystatin C, urine KIM-1, plasma NGAL, urine IL-18, urine pi-GST, urine L-FABP) and to TIMP-2 and IGFBP7 alone in predicting stage 2 or 3 AKI (AUC-receiver operating characteristic (ROC) 0.8). Follow-up studies by the same authors also conducted in the ICU population using [TIMP-2]·[IGFBP7] determined an AUC-ROC of 0.82 for the prediction of stage 2 or 3 AKI (Bihorac *et al.* 2014) and 0.79 (Hoste *et al.* 2014), respectively. This indicated that [TIMP-2]·[IGFBP7] are moderate to good biomarkers in predicting severe AKI within 12 h in the ICU setting. Conversely, an independent group of researchers failed to replicate the predictive performance of [TIMP-2]·[IGFBP7] in a similar ICU setting (Bell *et al.* 2015). [TIMP-2]·[IGFBP7] was also investigated in cardiac surgery patients (Meersch *et al.* 2014, Pilarczyk *et al.* 2015, Wetz *et al.* 2015), in a mixed collective including cardiac and non-cardiac surgery patients (Gunnerson *et al.* 2016), and in major surgery patients only (Gocze *et al.* 2015) showing AUCs between 0.7 and 0.85 for the prediction of AKI.

Koyner *et al.*, in a secondary long-term follow-up of the Sapphire study, showed that [TIMP-2]·[IGFBP7] concentrations at the time of ICU admission were predictive of a composite long-term outcome of death or RRT requirement over the next 9 months only in patients who developed AKI, but not in those who did not develop AKI (Koyner *et al.* 2015b). Currently, no information is available regarding the performance of [TIMP-2]·[IGFBP7] in patients outside the ICU/perioperative setting, nor is it known whether these biomarkers associate with intrinsic injury to the kidney as opposed to pre-renal AKI. In a healthy patient population, [TIMP-2]·[IGFBP7] did not show significant differences between male and female subjects, but a weak inverse correlation with age (Chindarkar *et al.* 2016).

In sum, data on the so-called cell cycle arrest biomarkers are emerging, but too little is known about their cellular sources and mechanism of action to link them with the pathophysiology of AKI. Given the promising performance of these markers in some clinical studies, research into their pathophysiology will be a major priority.

Calprotectin

Calprotectin is a 24 kDa heterodimer formed from the two monomers S100A8 (10 835 Da) and S100A9 (13 242 Da) (Stríz & Trebichavský 2004). It has initially been identified as an antimicrobial protein in the cytoplasm of neutrophil granulocytes (Dale *et al.* 1983). Intracellular calprotectin's main function is to interact with the cytoskeleton whereas when it is secreted by activated immune cells it acts as a danger-associated molecular pattern protein (Ehrchen *et al.* 2009) (Fig. 6). There is no singular receptor that exerts calprotectin signal transduction (Ehrchen *et al.* 2009), but it has been shown that S100A8 and S100A9 are endogenous activators of toll-like receptor 4 (Vogl *et al.* 2007) (Fig. 6).

Concerning the involvement of calprotectin in renal pathophysiology, it has been shown that renal collecting duct epithelial cells produce S100A8 and S100A9 in a model of kidney injury in response to unilateral ureteral obstruction (UUO) (Fujii *et al.* 2011). S100A8 and S100A9 attract CD11b+Ly-6C+ inflammatory monocytes to the kidneys, which then contribute to the cells' differentiation into M1-type CD11b+F4/80^{lo} cells. These cells promote renal epithelial injury and inflammation. S100A8 and S100A9 are also induced in response to ischaemia reperfusion injury in mice (Dessing *et al.* 2015). Infiltrating kidney neutrophils are the main source of S100A8/9 in the ischaemic kidney. S100A9-knockout mice, which lack active calprotectin, show an increased transition to renal fibrosis in response to ischaemia reperfusion injury, while the initial renal injury is similar to wild-type mice. This is associated with an enhanced formation of alternatively activated M2 macrophages in the damaged kidney (Dessing *et al.* 2015).

Ebbing *et al.* investigated time-dependent changes of calprotectin in patients undergoing nephron-sparing surgery for kidney tumours, which leads to iatrogenic renal ischaemia reperfusion injury due to transient clamping of the renal artery. Calprotectin concentrations started to be significantly increased at the end of the operation (approx. 2 h after ischaemia) and reached maximal levels 48 h post-surgery, with a 69-fold increase over baseline in calprotectin levels. Calprotectin was still significantly increased 5 days after surgery (Ebbing *et al.* 2016). Elevation of calprotectin has been described in several other diseases such as rheumatoid arthritis (Hammer *et al.* 2007), inflammatory bowel disease (Foell *et al.* 2008), myocardial infarction (Altwegg *et al.* 2007), cancer (Müller *et al.* 2008) and several others (Seeliger *et al.* 2003, Brun *et al.* 2005, Payen *et al.* 2008). In the clinical interpretation of urinary calprotectin findings, one should be aware that there are two clinical settings other than AKI that lead to an increase of calprotectin: as calprotectin is predominantly derived from neutrophils and monocytes, pyuria substantially increases urinary calprotectin. Moreover, urothelial carcinoma is associated with increased concentrations (Ebbing *et al.* 2014).

There are three studies investigating the diagnostic accuracy of calprotectin in its ability of distinguishing pre-renal from intrinsic AKI (Heller *et al.* 2011, Seibert *et al.* 2013, Chang *et al.* 2015). Calprotectin showed a very high accuracy in predicting intrinsic AKI with an AUC ranging from 0.92 to 0.97 in these studies. In a study on calprotectin measured in renal allograft recipients, a significant but weak ($r = -0.33$) inverse association of urinary calprotectin concentrations measured directly after surgery and eGFR 4 weeks after

transplantation was observed (Tepel *et al.* 2014). A recent multi-centre study analysed the diagnostic accuracy of calprotectin in the differentiation of pre-renal and intrinsic acute allograft injury (Seibert *et al.* 2016). Notably, urinary calprotectin concentrations of subjects with intrinsic AKI were 36 times higher than in pre-renal allograft injury yielding an AUC of 0.94. Immunohistochemical stainings in this study indicated calprotectin was produced primarily by infiltrating inflammatory cells confirming previous observations in the mouse ischaemic kidney (Fig. 6).

Summary and perspectives

A desirable biomarker should be non-invasive, detectable at early stages of the disease and prognostically relevant, but it should also be specific for a tissue type and have a close pathophysiological relation to the disease. Several potential biomarkers for AKI have been introduced, and each of these biomarkers has its advantages and disadvantages (for an overview, see Table 1). Today there is no perfect biomarker of AKI. It seems unlikely that the ‘kidney troponin’ will be found, but this is in part due to the shortcomings and the heterogenous nature of the current definition of AKI, which is itself based on surrogate markers of kidney function.

Among the individual markers, KIM-1 and L-FABP are derived from proximal tubules, NGAL is derived from the distal nephron and collecting duct, while IL-18 and calprotectin are probably largely derived from immune cells infiltrating the injured kidney (Fig. 1). The sources of IGFBP7 and TIMP-2 are currently unknown.

Studies in mice have shown that NGAL, KIM-1, IL-18 and calprotectin participate critically in the pathogenesis of AKI, while IGFBP7 and TIMP2 have not been linked with AKI in model organisms.

Each of the described biomarkers is not entirely specific for AKI. This is reflected by imperfect test characteristics of each biomarker with the best AUC-ROCs ranging between 0.75 and 0.85. The pathophysiologic basis for this lack of sensitivity and specificity is only partially understood. For instance, NGAL, IL-18 and calprotectin are known to be produced in immune cells and display associations with UTIs and sepsis, which are independent from AKI. NGAL, KIM-1 and IL-18 are elevated in patients with CKD. TIMP-2 and IGFBP7 have not been thoroughly tested in settings outside the ICU, and their pathophysiological roles are currently unclear. NGAL and calprotectin are closely associated with intrinsic AKI and display much lower levels in pre-renal AKI, which makes them potentially suitable in the differential diagnosis of patients with established AKI. All new biomarkers have in common that if they are once elevated after AKI they stay elevated for a long time. Therefore, an assignment to a phase of AKI is difficult (Koyner *et al.* 2012). Notably however, the expression of NGAL, including the length of time of expression, is dose dependent on disease severity so these parameters must be reviewed in greater detail.

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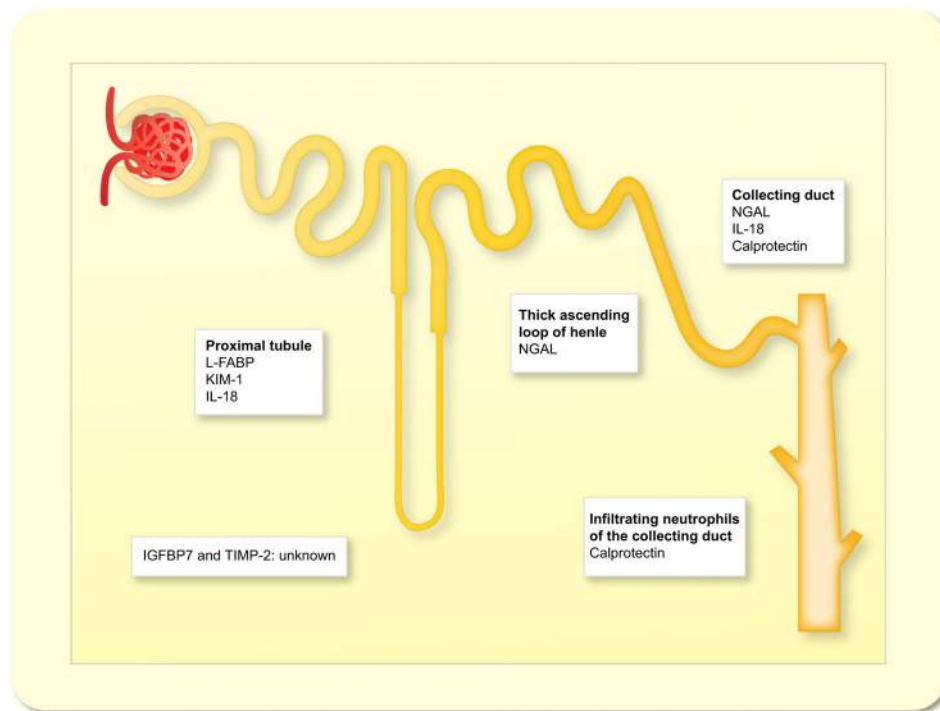


Figure 1.
Sites of origin of biomarkers of acute kidney injury.

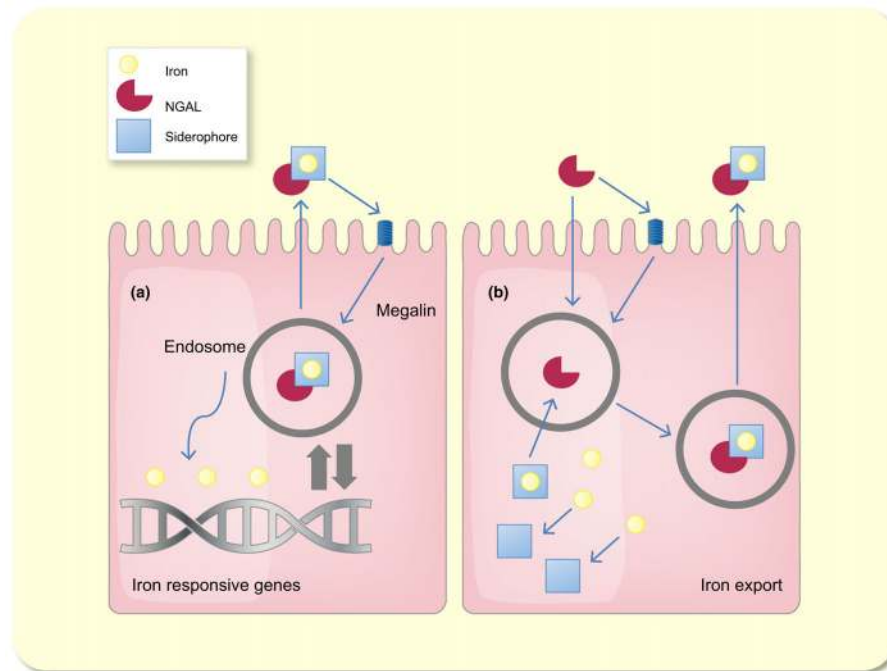


Figure 2. Schematic model of the functions of neutrophil gelatinase-associated lipocalin (NGAL) (a) Siderophore–iron associated NGAL delivers iron into the cell in a megalin-dependent manner. After the uptake, NGAL traffics into the endosome from where iron is released. This results in a regulation of iron-responsive genes. (b) Siderophore-free NGAL captures siderophore-bound iron and transports it into the extracellular space.

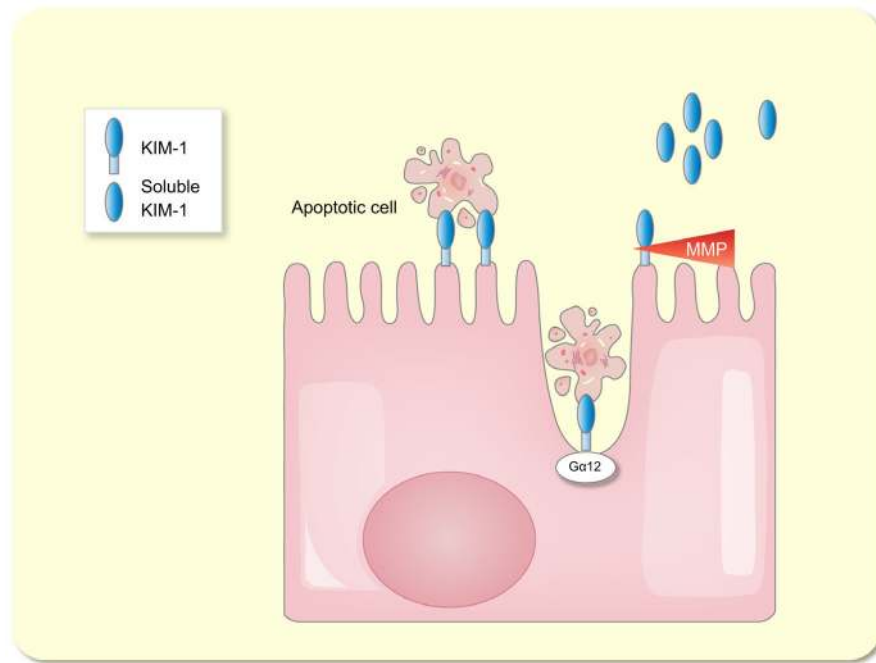


Figure 3. Schematic model of the functions kidney injury molecule-1 (KIM-1). KIM-1 acts as a phosphatidylserine receptor and binds apoptotic cell bodies. KIM-1 binds to the alpha subunit of heterotrimeric G12 protein (G α 12), thereby mediating phagocytosis of apoptotic cell bodies. The extracellular domain of KIM-1 is shed from the cell surface by a metalloproteinase (MMP)-dependent process.

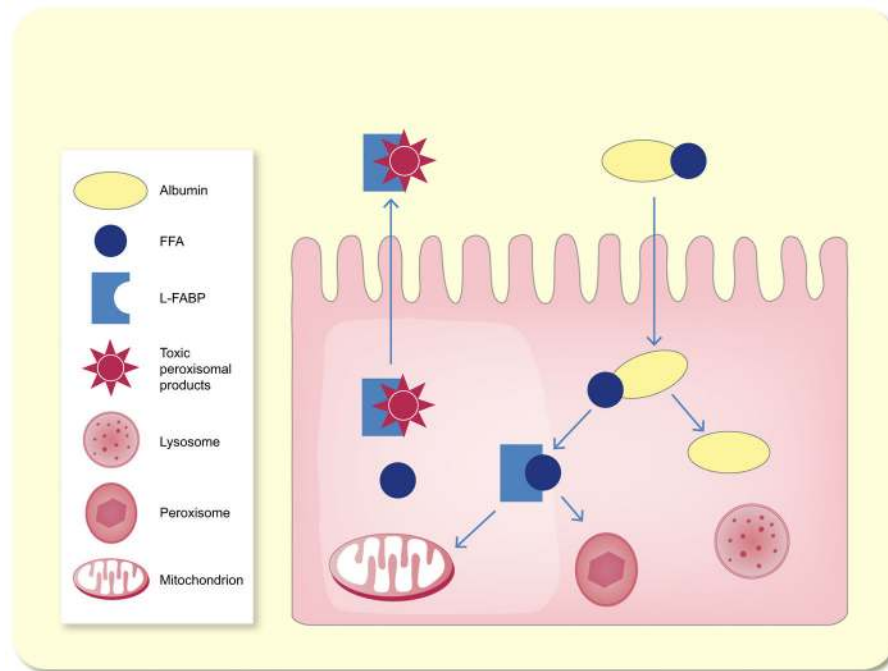


Figure 4. Schematic model of the functions of liver-type fatty acid-binding protein (L-FABP). L-FABP transports albumin bound free fatty acids (FFA) to mitochondria and peroxisomes to be metabolized. L-FABP is excreted into the tubular lumen together with bound toxic peroxisomal products, which accumulate otherwise.

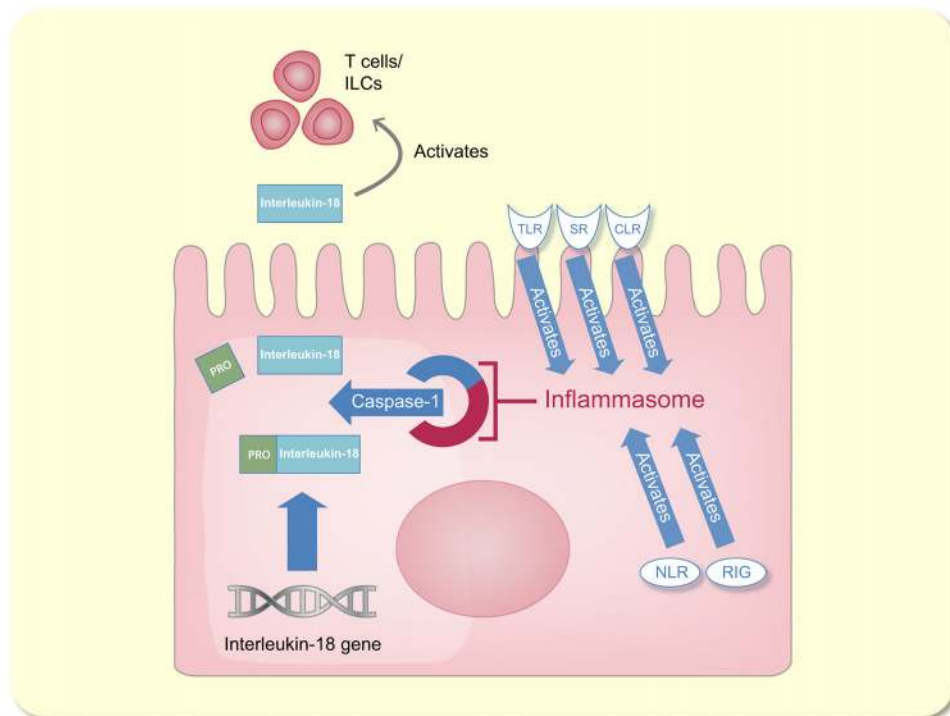


Figure 5. Schematic model of the functions of interleukin-18 (IL-18). IL-18 is synthesized as an inactive precursor without a signal peptide. It is cleaved by caspase-1, which is a part of the inflammasome. Toll-like receptors (TLRs), retinoic acid-induced gene-like receptors (RIG), nucleotide-binding domain-leucine-rich repeat (NLR), scavenger receptors (SR) and C-type lectins (CLR) can activate the inflammasome. Cleaved IL-18 exerts a proinflammatory effect by activating T cells and innate lymphoid cells.

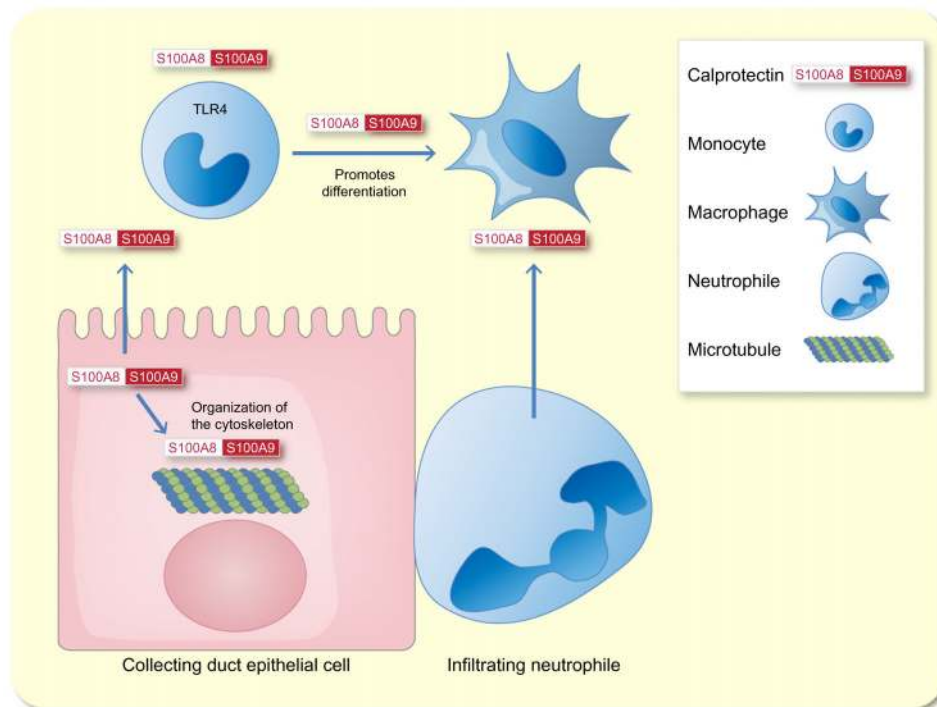


Figure 6. Schematic model of the functions of calprotectin. The two monomers S100A8 and S100A9 form calprotectin. Intracellular calprotectin's main function is to interact with the cytoskeleton. When calprotectin is secreted by activated immune cells, it acts as a danger-associated molecular pattern protein. S100A8 and S100A9 are endogenous activators of toll-like receptor 4 (TLR4). This promotes the differentiation of monocytes to macrophages.

Table 1

Overview of origin, physiological function in the kidney, animal models, disadvantages and key clinical studies in different settings of biomarkers of acute kidney injury (AKI)

	NGAL	KIM-1	L-FABP	IL-18	IGFBP7 and TIMP-2	Calprotectin
Origin	Thick ascending loop of Henle and the intercalated cells of the collecting duct (Schmidt-Ott <i>et al.</i> 2007, Paragas <i>et al.</i> 2014)	Proximal tubule cells (Han <i>et al.</i> 2002, Ichimura <i>et al.</i> 2008)	Proximal tubule cells (Yamamoto <i>et al.</i> 2007)	Collecting duct (Gauer <i>et al.</i> 2007) Tubular epithelial cells (Franke <i>et al.</i> 2012)	Unknown	Collecting duct and in filtrating immune cells (Fujiu <i>et al.</i> 2011, Seibert <i>et al.</i> 2016)
Physiological function in the kidney	Bacteriostatic function in the innate immune system, iron delivery to mammalian cells (Goetz <i>et al.</i> 2002, Flo <i>et al.</i> 2004, Bao <i>et al.</i> 2010)	Tubular regeneration by mediating phagocytosis of apoptotic bodies (Ichimura <i>et al.</i> 2008)	Regulation of fatty acids uptake and the intracellular transport (Chmurzyńska 2006)	Proinflammatory effect (Cheung <i>et al.</i> 2005)	Unknown	Polarization of M2 macrophages, promotion of repair after injury (Dessing <i>et al.</i> 2015)
Animal models	Holo-NGAL protects kidney from damage in response to ischaemia reperfusion injury (Mori <i>et al.</i> 2005)	KIM-1 knockout protects against damage in response to ischaemia reperfusion injury (Ismail <i>et al.</i> 2015b)	Human-L-FABP transgenic mice have less damage after ischaemia and reperfusion (Yamamoto <i>et al.</i> 2007)	IL-18-deficient mice are protected from ischaemia reperfusion-induced AKI (Wu <i>et al.</i> 2008)	Unknown	A lack of active calprotectin leads to more fibrosis after inn response to ischaemia reperfusion injury (Dessing <i>et al.</i> 2015)
Disadvantages	AKI-independent association with sepsis, CKD, UTI (Devarajan 2007, Schmidt-Ott 2011)	Is induced in various chronic proteinuric, inflammatory diseases (Smith <i>et al.</i> 2006)	Association of L-FABP with anaemia (Imai <i>et al.</i> 2015)	No reliable prediction of AKI	Unclear cellular sources and pathophysiology	Elevated in UTI (Heller <i>et al.</i> 2011) Elevated in urothelial carcinoma (Ebbing <i>et al.</i> 2014)
Key clinical studies						
Diagnosis of intrinsic AKI	AUC 0.87 (Singer <i>et al.</i> 2011) AUC 0.81 (Nickolas <i>et al.</i> 2012) AUC 0.95 (Nickolas <i>et al.</i> 2008)	AUC 0.71 (Nickolas <i>et al.</i> 2012)	AUC 0.70 (Nickolas <i>et al.</i> 2012)	AUC 0.64 (Nickolas <i>et al.</i> 2012)		AUC 0.97 (Heller <i>et al.</i> 2011) AUC 0.99 (Seibert <i>et al.</i> 2013) AUC 0.94 (Seibert <i>et al.</i> 2016) AUC 0.94 (Chang <i>et al.</i> 2015)
Early prediction of AKI	AUC 0.67 (Parikh <i>et al.</i> 2011) AUC 0.72 (Koyner <i>et al.</i> 2010)	AUC 0.69 (Koyner <i>et al.</i> 2010) AUC 0.71 (Parikh <i>et al.</i> 2013)	AUC 0.66 (Parikh <i>et al.</i> 2013) AUC 0.69 (Prowle <i>et al.</i> 2015)	AUC 0.75 (Parikh <i>et al.</i> 2013) AUC 0.55 (Haase <i>et al.</i> 2008)	AUC 0.85 (Bihorac <i>et al.</i> 2014) AUC 0.80 (Kashani <i>et al.</i> 2013)	
Prediction of in-hospital death	Hall <i>et al.</i> (2011), Singer <i>et al.</i> (2011), Nickolas <i>et al.</i> (2012)	Hall <i>et al.</i> (2011), Gonzalez & Vincent (2012), Nickolas <i>et al.</i> (2012)	Doi <i>et al.</i> (2011), Nickolas <i>et al.</i> (2012)	Doi <i>et al.</i> (2011), Hall <i>et al.</i> (2011)		
Prediction of long-term ESRD/mortality	Bolligiano <i>et al.</i> (2009), Ralib <i>et al.</i> (2012), Coca <i>et al.</i> (2014)				Koyner <i>et al.</i> (2015b)	