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American Journal of Physiology Cell Physiology
2022 NOV 09 ; 323(5): C1430-C1443
2022 SEP 26 (first published online)
DOI: 10.1152/ajpcell.00079.2022

Publisher: American Physiological Society

Acute kidney injury biomarkers in the single cell transcriptomic era

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Running head: AKI and transcriptomics

Abstract

Acute kidney injury (AKI) affects many hospitalized patients and is associated with increased morbidity and mortality even at milder and reversible stages. The current clinical definition relies on serum creatinine increases or a decreased urinary output. However, both parameters are of limited use because of poor sensitivity, specificity, and timeliness. Furthermore, the complex pathophysiology and diverse etiologies underlying AKI confound these issues. Precise biomarkers for specific aspects of AKI are needed. Earlier AKI biomarkers were unsuccessful in addressing these needs because they either lacked sensitivity and specificity or failed to aid in guiding clinical management. The advent of single cell transcriptomics technologies provides an unprecedented opportunity to analyze cells from urine, blood, or kidney biopsies to elucidate the detailed, cell-specific, molecular responses in AKI. These technologies uncover the cellular sources of traditional biomarkers, capture patient heterogeneity, define cell states associated with different AKI subtypes, and might eventually help to predict therapeutic response. We discuss how single cell technologies might transform diagnostic approaches to AKI by moving from single biomarkers to cell-specific molecular signatures.

Introduction

The kidneys are vital for toxin removal, electrolyte and volume homeostasis, hormone production, blood pressure regulation, and acid-base balance[1,2]. To fulfill these tasks, the kidneys require approximately 20% of the resting cardiac output and consume disproportionate amounts of oxygen and energy[3,4]. Two-hundred liters of blood are filtered every day to produce 1-2 liters of urine while circulating fluid volume, serum osmolarity, and electrolyte levels are maintained within tight ranges[5,6]. Many different, specialized cell types are required to fulfill these tasks. Circa one million functional nephrons reside in each kidney, outfitted with the glomerular filter, a tubular system for reabsorption, secretion, and adjustment, coupled to a unique vascular maintenance and exchange network is required to convert the erstwhile plasma into urine[7,8] (Fig. 1). Kidney cells are exposed to wide ranges of cellular microenvironments in which tissue oxygen partial pressures (pO2) range from 90 mmHg in the cortex to around 10 mmHg in parts of the renal medulla[9-11]. Similarly, large differences in tissue osmolality ranging from a serum-isoosmolal cortex (approximately 290 mosmol/kg H_2O) to a strongly hypertonic

inner medulla (approximately 1200 mosmol/kg H_2O in humans) must be established to regulate homeostasis[12,13].

Acute kidney injury (AKI) is a sudden loss of kidney function, is clinically defined by increases in serum creatinine levels and commonly a reduced urine output occurring over the course of seven days or less[14,15]. Creatinine is a by-product of muscle creatine metabolism, constantly produced by the body, and excreted unchanged into the urine by glomerular filtration and minimal tubular secretion[16]. Both, creatinine and urinary output are parameters of kidney function and not injury markers per se[17]. AKI occurs in up to 20% of hospitalized patients and AKI-related mortality largely exceeds that of other medical conditions, such as diabetes or breast cancer[17,18]. In addition, the economic implications of AKI are dramatic. For instance, the UK National Health Service annually spends more on AKI-related costs than on breast, lung, and skin cancers combined[19]. Even though targeted AKI therapies are lacking, early AKI detection combined with hemodynamic patient management (e. g. adequate hydration) and adaptation of the patient's medication could prevent thousands of AKI cases, thereby limiting the clinical consequences of AKI and reducing subsequent costs[19]. Identifying patients at high risk for AKI before the relatively insensitive clinical markers of increased plasma creatinine or decreased urine production become apparent could enable effective interventions. Currently available biomarkers have not yet demonstrated a general utility in this regard[20].

Apart from the early detection of AKI, biomarkers can also serve in several other aspects of AKI. This includes the assessment of a general preprocedural risk of developing AKI, the severity of AKI and the renal recovery after AKI[21]. AKI itself is limited to a duration of up to seven days by definition. However, impairment of renal function may be prolonged. Chronic kidney disease (CKD) is defined by persistent renal disease over 90 days, renal disease in the time period between AKI and CKD is called acute kidney disease (AKD)[15]. Most of the patients recover from AKI, there is however a group of patients that will develop CKD (AKI-to-CKD transition)[22]. In fact, recent research supported the notion that AKI and CKD are indeed tightly linked to each other with AKI predisposing for CKD and vice versa[23]. It is therefore necessary to prevent and monitor the development of CKD from AKI, highlighting another important application of biomarkers in AKI[24].

Recently, single cell transcriptomics has not only changed our understanding of renal cell types and cellular identities but also of renal molecular processes in health and disease[13,25-30]. Single cell transcriptomics allows the unbiased identification of cell types and cellular states in physiological or disease settings and the investigation of transcriptomic processes in single

cells or cell populations. Multiple new AKI-associated kidney cell states were identified using single cell transcriptomics, which would not have been captured by other approaches, for instance bulk RNA sequencing[13,26-30].

Many attempts have been made to replace plasma creatinine and urinary output as the clinical AKI determinants. The failure of these attempts could be due to the complexity of the underlying etiologies of AKI combined with an incomplete understanding of the molecular processes and a potential large inter-patient heterogeneity within the current stages of AKI. In fact, how many clinical and molecular subtypes of AKI exist and how large inter-patient heterogeneities confound the diagnosis, remain unclear[31]. However, these sources of variation could potentially be captured by single cell approaches, which might help to find suitable underlying targetable categories and subtypes of AKI.

Current putative AKI biomarkers

According to the current Kidney Disease Improving Global Outcomes (KDIGO) criteria, AKI is defined by two functional kidney markers, serum creatinine (increase by 50% or ≥ 0.3mg/dl within 48 hours) and urinary output (oliguria for ≥ 6 hours), and a limited duration of seven days[14]. Hence, both parameters do not actually represent kidney injury (such as for instance troponin in myocardial infarction), but instead, renal functional impairment[17,21]. This situation naturally entails problems regarding sensitivity, specificity, and timeliness. Urine volume can be reduced for numerous reasons not related to renal function and is for instance subject to administration of diuretics. Thus, urine volume exhibits a low specificity for AKI. Serum creatinine on the other hand can underestimate the kidney function in clinical scenarios of fluid overload or reduced muscle mass and shows a delayed increase after the onset of AKI[17,19,21,32]. The relationship between creatinine and estimated glomerular filtration rate (eGFR) is hyperbolic and as a result, an almost 50% decrease in eGFR must occur (blind spot) before creatinine increases are appreciated by clinicians. Also, serum creatinine has critical limitations when assessing recovery of kidney function after AKI due to changes in volume distribution, body mass and potential renal hyperfiltration[15]. However, other biomarkers, more indicative of real kidney damage or injury than creatinine and urinary output were not included in the 2012 KDIGO guidelines[14]. It should be noted that many studies still use serum creatinine and urinary output as clinical endpoints which in itself may hinder the development and establishment of new biomarkers and interventions.

Even mild and reversible forms of AKI are associated with worsened patient outcome and increased mortality and morbidity[33-35]. Substantial efforts were (and are) being made to further improve the management of AKI including the discovery and investigation of many novel AKI biomarkers. The term “AKI biomarker” can represent different aspects of AKI. These aspects include the assessment of the risk for AKI, the onset or diagnosis and severity of AKI, the recovery from AKI and the risk of developing chronic kidney disease from AKI (risk of AKI-to-CKD transition, Table 1). However, most AKI biomarkers detect the onset and severity of AKI whereas far fewer provide an assessment of for instance preprocedural risks for developing AKI. Apart from these categories, it has to be also noted that AKI biomarkers were studied in defined clinical contexts for the mentioned different aspects of AKI (e.g. hospitalized patients, patients on intensive care units, cardiac surgery patients, pediatric patients etc.).

AKI biomarkers can be measured in the patient’s urine or blood. Cellular sources of AKI biomarkers are very diverse (Fig. 2A). AKI biomarkers include molecules specifically transcribed in response to AKI in distinct segments of the kidney tubules (e.g. KIM-1 in proximal tubules, UMOD in the thick ascending limbs, NGAL in the loop of Henle and the collecting ducts). AKI biomarkers also comprise freely filtered plasma proteins with reduced tubular reabsorption in AKI (e.g. α1- and β2-microglobulin, RBP) or tubular enzymes shed into the urine in response to cellular injury due to AKI (e.g. alanine aminopeptidase, alkaline phosphatase). Moreover, many AKI biomarkers have several (renal and extrarenal) cellular sources, which can hamper their specificity in certain clinical contexts. AKI biomarker discovery is further complicated by the multitude of etiologies leading to AKI (in the current definition) ranging from conditions of volume depletion to intrarenal processes and to post-renal obstruction which can entail profoundly different responses in the kidney[36] (Fig. 2B, C). AKI can entail kidney damage mainly affecting the glomerulus as in glomerulonephritis[37], the kidney tubules as in ischemic injury[36,38,39] or the interstitium as in certain forms of toxin-induced kidney injury[40,41]. This situation naturally complicates AKI biomarker discovery and underlines the importance of knowing the cellular sources and the pathophysiological involvement of the individual AKI biomarker candidate in the setting of AKI.

The most widely studied novel AKI biomarkers are neutrophil gelatinase-associated lipocalin (NGAL) and the combination of insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases (TIMP2)[17,21,22,42,43]. These biomarkers are not “new” per se but highly investigated and help to showcase current problems in AKI biomarker discovery including cellular sources, inter-patient heterogeneity and their functional role in AKI.

It is of note that for these biomarkers, point-of-care devices are readily available for clinical bedside testing[44-46].

NGAL is a protein with different isoforms which is expressed in and can be released by immune and kidney cells as well as multiple other non-hematopoietic cell types[47,48]. NGAL prevents iron uptake of bacteria by binding iron siderophore complexes and may thereby confer protection against infections[49,50]. In various settings of AKI including cardiac surgery, toxic damage, sepsis, or ischemia, NGAL is strongly upregulated in plasma and urine in human and animal models[38,39,51-54]. NGAL is freely filtered in the glomerulus and reabsorbed by the proximal tubules (PTs)[55]. In the injured kidney, NGAL is most upregulated in the loop of Henle and the distal tubules and secreted from the apical and basolateral sides[54,56-58]. Hence, besides increased renal production of NGAL in response to AKI, elevated levels of NGAL in the plasma can be due to a decreased glomerular filtration while elevated levels in the urine can additionally originate from reduced PT reabsorption of NGAL[24,56].

NGAL allows the diagnosis and the determination of the severity of AKI[59-61]. It was shown that NGAL can differentiate between settings of real kidney damage, where it is upregulated, from reversible states of volume depletion[62-64]. Moreover, NGAL can rise days before creatinine, making the diagnosis of AKI much timelier[65]. In first studies of NGAL for the prediction of AKI in children after cardiac surgery, urinary NGAL could predict AKI 2 hours after surgery with an area under the receiver-operating characteristic curve (AUC) of 0.998. The diagnosis of AKI in this cohort based on serum creatinine was only possible after 1-3 days[51]. These results, however, could not always be met in cohorts of adult patients where AUCs ranged between 0.5 and 0.99[48,66]. Several factors potentially contributed to this heterogeneous performance of NGAL in adult patients, including elevated levels of NGAL observed in patients with a pre-existing impairment of the kidney function (chronic kidney disease)[67,68] as well as a release of NGAL from non-kidney cells (immune and non-immune cells) especially in the context of systemic inflammation[50,69].

TIMP2 and IGFBP7 (so-called cell cycle arrest markers) were identified in a biomarker screening for the prediction of AKI[70]. It has been shown that renal cells undergo cell cycle arrest in G1 upon AKI which helps cells to prevent and repair potential DNA damages, keeping energy balance and inhibiting further cell divisions[71,72]. In the kidney, TIMP2 and IGFBP7 are expressed and released from kidney tubule cells[73] in response to cellular stress such as insufficient nutrient or blood supply, inflammation or toxins[74]. Cell cycle arrest by TIMP2 and IGFBP7 is achieved by inducing the expression of inhibitors of cyclin-dependent protein kinase complexes[74]. Additional

to kidney tubule cells, it is discussed that elevated urinary TIMP2 and IGFBP7 levels might be also due to an increased glomerular filtration and reduced PT reabsorption of TIMP2 and IGFBP7[75]. Interestingly and in contrast to NGAL, TIMP2 and IGFBP7 mRNA levels were not upregulated shortly (4 hours) after AKI onset in mouse AKI models[75]. On the other hand, urinary levels of TIMP2 and IGFBP7 were markedly increased 4 hours after AKI, which supports the notion of a tubular release of preformed TIMP2 and IGFBP in response to stress[75].

In the clinical setting of AKI, the arithmetic product of urinary TIMP2 and IGFBP7 (TIMP2xIGFBP7, marketed as Nephrocheck®) is applied and was approved by the US Food and Drug Administration in 2014[70,74,76]. The initial findings for urinary TIMP2xIGFBP7 showed an AUC of 0.80 in a multi-center study in critically ill adults, some of whom had AKI stage I, for the onset of moderate to severe AKI (stage 2 or 3) within the subsequent 12 hours with a sensitivity of 92% and a specificity of 46%. In this cohort, urinary TIMP2xIGFBP7 was superior to other biomarkers including plasma and urinary NGAL[70]. The specificity could be increased at the cost of sensitivity by applying different cut off values for urinary TIMP2xIGFBP7 (>0.3 and >2 $(ng/ml)^2/1000$, the unit is often omitted). Several studies investigated the use of urinary TIMP2xIGFBP7 to guide therapeutic decisions. These decisions included nephrologist consultation, optimization of the patient's volume status or avoidance of nephrotoxic drugs if urinary TIMP2xIGFBP7 was above 0.3 (intervention group)[77-79]. These studies showed variable results including significant reductions of the occurrence of AKI in the intervention group[77,78] or no such differences[79]. These differing results might be due to different patient cohorts such as critically ill patients on intensive care units or patients admitted to the emergency room.

In summary, there is a profound need for biomarkers of different aspects of AKI. A plethora of studies helped to identify various new promising biomarkers. These studies showed that biomarkers can be sensitive and specific in defined patient cohorts (e.g., intensive care unit patients) and underlined the importance of the knowledge of the cellular sources and molecular mechanisms behind each biomarker.

				Applicability of biomarker in AKI				
Biomarker	**Biological function**	**Cellular source**	**Measured in**	**AKI risk prediction**	**AKI onset**	**AKI severity**	**Recovery from AKI**	**AKI-to-CKD transition**
Alanine aminopeptidase, alkaline phosphatase, γ-glutamyl transpeptidase[61,80]	cellular enzymes	mostly PTs but transcription is also present in other kidney tubule cell types	urine		x	x		

Albumin/protein[81,82]	plasma proteins	plasma proteins passing the glomerular filtration membrane in AKI	urine		x		x	x
C-C motif chemokine ligand 14[83]	pro-inflammatory cytokine	kidney epithelium	urine				x	
C-X-C motif chemokine 10 (CXCL10/IP-10)[81,84]	pro-inflammatory cytokine	kidney epithelium, interstitial cells, endothelial cells, leukocytes	urine		x	x		
Calprotectin[85-87]	antimicrobial protein	kidney-infiltrating inflammatory cells (neutrophils, monocytes), renal collecting duct cells	urine		x			
Chitinase 3-like protein 1[88-92]	member of glycosyl hydrolase 18 family; chemoattractant, can stimulate cell growth, proliferation and cell shape	kidney macrophages, multiple extra-renal sources and glomerular filtration	urine and plasma		x		x	x
Cystatin C[59,60,93]	cysteine protease inhibitor, produced by most of the nucleated cells	most nucleated cells, freely filtered functional marker	plasma		x	x		x
Dickkopf-3[94,95]	secreted immunomodulatory glycoprotein	kidney epithelium	urine	x				
Epidermal growth factor[96-98]	involved in tissue proliferation, differentiation and repair	thick ascending limb, distal convoluted tubule	urine				x	
Hepatocyte growth factor[81,99,100]	cellular morphogenesis, growth and motility marker	kidney mesangial cells, endothelial cells, fibroblasts, macrophages	plasma			x	x	
Hepcidin[101]	antimicrobial protein involved in iron homeostasis	liver (freely filtered), loop of Henle, collecting ducts	urine		x	x		
Interleukin-18[59,60,102]	pro-inflammatory cytokine	immune cells, PTs, intercalated cells	urine	x	x			x
Kidney injury molecule–1[24,103-105]	PT transmembrane glycoprotein involved in phagocytosis of apoptotic cells	PTs	urine	x	x	x	x	x
Liver-type fatty acid-binding protein[60]	chaperone, fatty acid transport, freely filtered and secreted from injured cells	PTs, hepatocytes	urine and plasma		x			
Matrix metalloproteinase-2 and 9[106,107]	proteolytic enzymes	kidney epithelium, interstitial cells	urine				x	
Monocyte chemoattractant peptide-1/C-C motif chemokine ligand 2[92,106,108,109]	pro-inflammatory cytokine	PTs, medullary interstitium	urine and plasma	x	x	x	x	x
N-acetyl-β-D-glucosaminidase[61,80]	cellular lysosomal enzyme	PTs	urine		x			
Netrin-1[110,111]	laminin-related protein, inhibitor of leukocyte migration	kidney epithelium, endothelial cells	urine		x			
Neutrophil gelatinase-associated lipocalin[48,54,56,58,82]	iron-binding protein involved in protection against infection, growth and differentiation factor, several isoforms	loop of Henle, collecting duct, non-hematopoietic (colon, lung) and hematopoietic cells (neutrophils)	urine and plasma		x	x		x
Osteopontin[112]	extracellular protein and cytokine for immune cell recruitment	kidney epithelium, interstitial cells, endothelial cells, leukocytes	plasma		x	x		
Procollagen type III N-terminal propeptide[113]	by-product in the synthesis of collagen type III	interstitial cells	urine and plasma				x	
Proenkephalin A[114]	endogenous opioid	freely filtered plasma protein	plasma		x	x	x	
Retinol binding protein[61]	liver-synthesized glycoprotein	plasma protein with reduced tubular reabsorption in AKI	plasma			x		
Soluble urokinase plasminogen activator receptor (suPAR)[115,116]	Cleaved from membrane-bound uPAR, involved e.g. in chemotaxis, cell migration and adhesion, at least three isoforms were reported	Endothelial cells, podocyte, immune cells, fibroblasts	plasma	x				
Tissue metalloproteinase-2 (TIMP2); insulin-like growth factor binding protein-7 (IGFBP7)[70,73,75,117]	TIMP2: inhibitor of matrix metalloproteinases, IGFBP7: regulates availability of insulin-like growth factors and cell-cell adhesion	Kidney epithelium, extrarenal sources with reduced tubular reabsorption in AKI	urine	x	x	x		x

Transforming growth factor beta[106]	multifunctional cytokine	kidney epithelium, interstitial cells, endothelial cells, leukocytes	urine				x	
Tumor necrosis factor alpha[60,118,119]	pro-inflammatory cytokine	kidney epithelium, leukocytes	plasma		x			
Tumor necrosis factor receptor type 1 and 2[102,105,118-120]	cell surface receptors for tumor necrosis factors	glomeruli, endothelial cells, infiltrating leukocytes (TNFR1), distal convoluted tubules (TNFR2)	plasma				x	x
Uromodulin[92,121,122]	most abundant protein in urine of healthy individuals, involved in protection against urinary tract infections and formation of kidney stones	thick ascending limb	urine	x			x	x
Vascular endothelial growth factor[81,123]	signaling protein stimulating the formation of blood vessels	kidney epithelium, endothelial cells	urine		x			
α glutathione S-transferase[99]	cellular PT enzyme	PTs	urine		x			
α1-microglobulin[59,124,125]	freely filtered plasma protein, tubular reabsorption under physiological conditions	plasma protein with reduced tubular reabsorption in AKI	urine	x	x			
β2-microglobulin[59,126]	freely filtered plasma protein, tubular reabsorption under physiological conditions	extrarenal sources with reduced tubular reabsorption in AKI	urine		x		x	
π glutathione S-transferase[99]	cellular distal tubule enzyme	distal tubules	urine		x			

Table 1. List of selected AKI biomarkers and their clinical applicability.

Single cell technologies and molecular mechanisms of AKI

Single cell technologies enable the measurement of RNA transcripts in thousands of single cells. Several single cell RNA sequencing (scRNA-seq) platforms and techniques as well as an ever-growing number of bioinformatics tools and approaches are currently available[127,128]. Each messenger RNA transcript sequenced in a scRNA-seq experiment should be traceable back to a cell of origin of the investigated tissue. This aim is often achieved by linking cell-specific oligos (barcodes) to the messenger RNAs of the cells (Fig. 3)[129]. Transcripts can be assigned back to a single cell (of yet unknown cell type) after sequencing. As a result, an expression matrix with transcript counts for each cell barcode is produced. In a next step, cell types are assigned to each cell. For this, all cells are clustered using genes, which show high variation of expression between all cells (highly variable genes)[128]. Having the clusters of cells, it is then possible to calculate marker genes for each cluster. The identified marker genes usually show a significantly higher expression in the respective cluster, compared to other clusters. Until this step, all bioinformatics analyses are unbiased and do not assume prior knowledge. By comparing the calculated marker genes to sets of known cell type marker genes of the tissue (e.g., aquaporin 2 for kidney collecting duct principal cells, platelet cell adhesion molecule 1 for vascular endothelial cells etc.), most clusters can be assigned to known cell types. High-dimensional single cell data is usually visualized using t-distributed stochastic neighbor

embedding (t-SNE) or uniform manifold approximation and projection (UMAP) plots. These non-linear transformations place cells in two- or three-dimensional plots trying to capture gene expression similarities by spatial distance. Since the clustering process is unbiased, single cell technologies led to the discovery of new cell types in the healthy kidney and shed new light on and increased our knowledge about the richness, plasticity, and diversity of renal cell types and cell states[13,25-30,130-132]. There are several platforms which enable the analysis of RNA expression at single cell resolution[133]. The platform has to be chosen according the experimental design and research question which should be answered. In general, there are approaches which allow deep full-length RNA sequencing of a limited number of single cells and approaches which allow measuring thousands of cells by sequencing of for instance only the 3' end of a gene[133]. Single cell measurements are of course not only restricted to single cell RNA expression but can be extended with spatial resolution[134] and are also feasible for DNA[135], chromatin accessibility (ATAC-seq - Assay for Transposase-Accessible Chromatin with sequencing)[136] and proteins[137], although the developmental stages of the respective technologies are certainly different. A powerful approach is to combine single cell with single cell ATAC-seq. ATAC-seq provides information on the regions of accessible chromatin. Hence, transcription factor binding can be inferred through known binding motifs. This provides more information on the transcriptional regulation than single cell RNA sequencing alone[138,139].

One major challenge of each scRNA-seq experiment is tissue digestion. The goal is to provide a suspension of single cells with good RNA quality and gene expression profiles, which reflect the original gene expression in the tissue. For the kidney, different cold and warm digestion protocols are available[13,25,130]. Some studies using a cold digestion protocol report a reduction of some gene expression artefacts induced by warm digestion[140]. Independent of the digestion protocol, obtaining a suspension of intact single cells from each specimen is not always possible. This is in particular relevant for frozen or archived tissues. In such cases, single-nuclei RNA sequencing (snRNA-seq) can be used by applying the same techniques and methods apart from the digestion protocol[141]. In fact, even with specimens that can be used for scRNA-seq, the usage of snRNA-seq might be advantageous[142]. These advantages include a rather quick, uniform, and cold dissociation protocol for all tissues. Moreover, protocols suggested for the generation of single nuclei suspensions allow a more homogeneous dissociation of the tissue with less bias towards more easily dissociable tissue regions. For the kidney, this approach would lead to a better representation of glomerular cell types and cells from the medulla of the kidney, which are harder to dissociate than cells from the kidney cortex[13,142].

For the kidney, scRNA-seq can be performed not only on renal tissue, but also on urine samples. Urine scRNA-seq uses, as the name indicates, single cells, which are shed into the urine[143,144]. Since the availability of human specimens is limited, using scRNA-seq in urine samples provides an elegant approach to investigate transcriptional changes at cell type resolution in patients over time. However, the harsh conditions in the urine can entail high levels of noise and transcriptional deviation from the original cell state which makes clustering not as sharp as in kidney tissue and can hamper clear cell type assignments[30]. Moreover, urine usually contains a large proportion of dead cells and naturally comes with a survival bias. In many clinical settings, urine will be however the only available source of kidney cells. To overcome these difficulties, it is mandatory to establish and maintain high quality atlases in clinical settings where, both, tissue and urine data are available. This helps to provide better cell type assignments in the urine and better knowledge on how well urinary single cell transcriptomics truly reflect intrarenal processes.

In the setting of AKI, scRNA-seq revealed the existence of new AKI-associated cell states in kidney scRNA-seq and snRNA-seq data from mouse and human AKI sampes[26-30,145,146]. Ischemia-reperfusion injury (IRI) is a commonly used AKI model in mice. For IRI, kidney injury is induced by clamping the kidney artery for a defined amount of time followed by reperfusion[38,39]. Kirita et al.[26] performed snRNA-seq on IRI mouse kidneys at five different time points after injury (4, 12 hours and 2, 14, 42 days) and controls. This study discovered distinct AKI-associated cell states which were assignable to major cell types of the kidney tubule but additionally showed specific gene expression profiles deviating from healthy kidney epithelium. These cell states induced by IRI were found among PT cells. The healthy PT consists of at least three discernible anatomic segments, the S1, S2 and S3 segments[147]. Using bioinformatics approaches including trajectory analysis and marker gene analysis, these novel AKI-associated cell states could be assigned to injured PT cells from different healthy PT segments. Having samples from different time points after injury also allowed the investigators to check whether cells from AKI-associated cell states are able to regenerate back to healthy kidney epithelium. Such regeneration is particularly important as non-regenerative cell states mean loss of healthy nephrons and therefore kidney function. Kirita et al. suggested that not all AKI-associated cell states will regenerate back to normal kidney epithelium. Particularly, cells from an AKI-associated cell state they labeled “failed repair PT” are thought to be unable to regenerate back to healthy PTs. These “failed repair PTs” are characterized by a distinct gene expression profile including downregulation of PT marker genes (dedifferentiation) and upregulation pro-fibrotic and pro-inflammatory genes such as vascular cell adhesion molecule-1 (VCAM-1)[26]. This study

did not systematically analyze all available AKI biomarker candidates within their data. However, the investigators presented expression of the well-investigated AKI biomarker gene kidney injury molecule-1 (KIM-1)[104], which was mainly expressed in injured PT cells of the S3 segment. This finding gives KIM-1 new annotations as it seems to be associated with an injured cell state which can still regenerate back to normal kidney epithelium (not failed repair) and a specific anatomic segment of the kidney tubule (PT S3 segment). Also, other scRNA-seq mouse IRI studies discovered distinct PT AKI-related cell states[27,28]. Additional scRNA-seq studies further helped to refine injured PT cells of the failed repair cluster[28,139]. A recent study found that PT cells expressing VCAM-1 (a defining marker gene of failed repair PT cells) are also present - although at much lower abundance than in AKI - in PTs from healthy kidneys[139].

Animal studies represent important first insights into the pathogenesis of a disease but naturally come with certain limitations, especially regarding the translation to patients. In the case of AKI, mouse IRI studies, also from studies not involving any scRNA-seq or snRNA-seq, usually report the most pronounced damage in the kidney in the PTs[36,38,39,148,149]. In fact, this process is a highly debated topic and if this picture truly represents the conditions in human AKI remains unclear[148,149]. Recent human snRNA-seq data indicate an involvement of multiple cell types of the kidney tubule in AKI associated with critical illness[29]. This study compared kidney biopsy tissue from post mortem biopsies collected within 2 hours post mortem of AKI to control kidney smaples from post mortem biopsies of different time points and normal kidney tissue from tumor nephrectomies. This study also found a VCAM-1-expressing AKI-associated cluster of dedifferentiated PT cells. These human AKI-associated PT cells did also show a high expression of epithelial mesenchymal transition signaling and overlapping marker gene expression with the earlier presented murine AKI failed repair cells (human failed repair cells). Surprisingly, very comparable AKI-associated clusters (failed repair clusters) could also be identified in other renal cell types such as the thick ascending limbs or the distal convoluted tubules[29]. All included AKI patients had moderate to severe AKI within five days prior to sampling in the setting of severe pneumonia. However, abundances of failed repair PT cells varied heavily between the patients[29].

Current scRNA-seq and snRNA-seq studies allow unprecedented insights into the molecular mechanisms of AKI. However, the current studies show a need for data from human AKI samples in controlled clinical settings which is currently limited. Mouse and human AKI kidney data indicate that kidney PT cells (and other cell types) undergo defined gene expression programs including pro-fibrotic states of failed repair. Whether individual abundances of AKI-

associated cell states correlate with renal and global patient outcome, or whether they are present in different etiologies of AKI and vary between patients of the same etiology and stage of AKI is not yet clear.

Single cell technologies can change the approach to AKI biomarker discovery

Even though NGAL and IGFBP7/TIMP2 can provide useful additional information on risk, onset and severity of AKI, some reported limitations of these AKI biomarkers originated from systemic non-renal secretion, unclear cut off values and usability in different clinical settings (e.g. critically ill patients on ICUs versus patients in the emergency room)[24,48,79]. In other words, these limitations are linked to uncertainties regarding cellular sources and involvement of these biomarkers in different forms or settings of AKI. Single cell or single nuclei transcriptomics might help to some extent in these regards. Concerning the origin of gene transcripts, single cell or nuclei approaches provide cell type-specific information on gene expression. Moreover, there is a growing number of publicly available scRNA-seq datasets and platforms with control samples and samples from disease settings -renal and non-renal tissue - which can provide direct useful information on the expression domain of a gene of interest[150-152]. Potential AKI biomarker candidates can be filtered, ranked, or quantitated on an RNA level using these resources.

Regarding the involvement and relevance of existing AKI biomarkers in different settings or forms of AKI, single cell transcriptomic data is still limited but promises to be useful. For instance, the AKI biomarkers IGFBP7/TIMP2, interleukin 18 (IL18) and KIM-1 are all biomarkers which can be used to define the onset of AKI (Table 1). Additionally, they are all reported to be expressed in PTs[75,153,154]. However, scRNA-seq and snRNA-seq studies indicate distinct and extended expression domains. IGFBP7 is expressed predominantly in PT cells and TIMP2 in distal tubule cells (but also PT) in AKI[73,75]. Consistently, in scRNA-seq data from human pneumonia-associated AKI, IGFBP7 was shown to be upregulated in PTs but also in thick ascending limbs and podocytes[29]. On the other hand, TIMP2 showed upregulation in thick ascending limbs and distal convoluted tubules. Interestingly, in PTs, IGFBP7 was mainly expressed in the previously mentioned failed repair PTs[26]. Notably, failed repair PTs (and therefore IGFBP7 expression) showed pronounced variation in abundances among individuals with moderate to severe AKI. IGFBP7 might therefore also reflect the abundance of failed repair cells in PTs.

While IGFBP7 showed maximum expression in failed repair PT cells, IL18 expression peaked in a different AKI-associated PT subpopulation which displayed increased hypoxia response signaling[29]. This subcluster could be associated with mouse IRI injured S3 cells and showed some degrees of inter-patient heterogeneity although not as pronounced as the failed repair cluster[26,29]. These findings are not contradictive to the fact that IGFBP7 and IL18 can indeed be used to make the diagnosis of AKI but support a notion of potentially different expression domains (early injured PT S3 versus PT failed repair cells). KIM-1 was also mainly expressed in injured S3 cells in AKI[26,29]. A systematic analysis of all available AKI biomarkers is unfortunately not present in all studies. Published kidney AKI data can, however, provide new AKI biomarker candidates for different injured subclusters.

Apart from providing new AKI biomarker candidates or evaluating known AKI biomarkers on RNA levels, single cell transcriptomics might be helpful in providing new AKI subtype categories. As Fig. 2B indicates, many clinical conditions can lead to AKI. It is however unclear if these different conditions entail different molecular responses in the kidney. It has been shown that mice show very different gene expression responses to IRI and states of dehydration which can also lead to AKI[36]. Although a variety of factors can lead to AKI, it is unclear how many molecular subtypes of AKI truly exist[17,31]. This would be important to know as different molecular mechanisms might entail different AKI biomarkers and different therapeutic measures. Our current classification of AKI and groups of patients observed in clinical studies (e.g. moderate to severe AKI in critically ill patients[77,79]) might not reflect the underlying molecular mechanisms. This unknown heterogeneity can hamper the usability and discovery of AKI biomarkers. It might be that patients with moderate to severe AKI in our current definition present very heterogeneous abundances of for instance failed repair cells or injured PT S3 cells or even completely new tubular cell states and consecutively show heterogeneous blood or urine AKI biomarker levels. These assumptions will need, however, more single cell-resolved kidney AKI data from independent studies.

Clearly single cell transcriptomics alone are not able to capture the full range of possible AKI biomarkers (Fig. 2A) as only measurable differences in RNA production can be determined. However, single cell transcriptomics represents a new tool in the chain of biomarker discovery. This new tool can be used in a direct manner for biomarker discovery from scRNA-seq or snRNA-seq but also in an indirect (and potentially more important) manner providing AKI molecular subtype classification. Reevaluating new and known AKI biomarkers in a context of

molecular signaling-driven AKI classification might further strengthen the role of other AKI biomarkers apart from serum creatinine and urinary output.

Conclusions

Single cell transcriptomics enables unprecedented insights into the molecular mechanisms of AKI. In the field of AKI biomarkers, single cell transcriptomics can be helpful in several aspects. Transcriptomics can help to provide cellular sources of AKI biomarker gene expression, to discover new AKI biomarker candidates, and to restructure or at least expand our current AKI classification. Potential future new AKI biomarker candidates from single cell transcriptomics can be linked to AKI-associated cell states and distinct molecular signaling instead of AKI stage and patient cohorts. In addition to in depth animal studies, further studies require the inclusion of more human data. Such an approach will help to overcome certain limitations of AKI animal models and will additionally provide information on inter-patient heterogeneity and transcriptomic variance in comparable clinical settings of AKI. New therapeutic interventions could thereby become reality.

Acknowledgments

This work was supported by grants to KMSO from the Deutsche Forschungsgemeinschaft (DFG; SFB 1365, GRK 2318 and FOR 2841), to C. H. from the DFG (HI 2238/2-1) and the Berlin Institute of Health (BIH) and MDC (BIH and MDC Focus Area Translational Vascular Biomedicine), and by the Urological Research Foundation (Berlin). We thank Friedrich C. Luft for his valuable input to the manuscript. All figures were created with Biorender.com. Figure 1 was adapted from “Nephron in kidney section (labeled)” and “Nephron (cortical)”, figure 2 was adapted from “Renal corpuscle” and “Kidney (cross-section)”, figure 3 was adapted from templates “Single-cell sequencing”, “CITE-seq” and “Heatmap 2 (generic)”, by Biorender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

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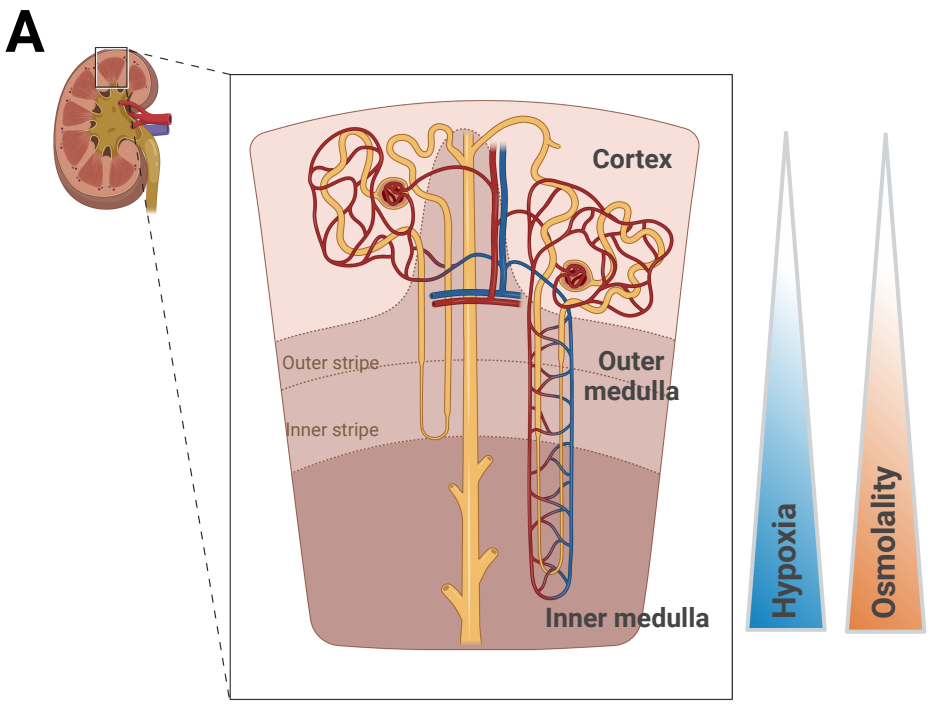
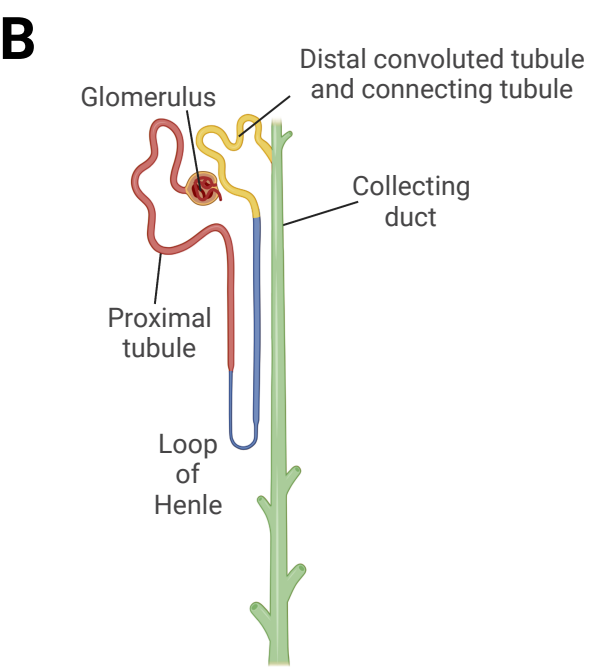
Figure legends

Figure 1. The basic anatomical structure of the kidney and physiological properties. A. *Schematic overview of the structure of the kidney. The kidney regions cortex, outer and inner medulla are shown. In addition, a cortical (left) and a juxtamedullary (right) nephron are shown. The gradient-colored triangles for hypoxia and osmolality should indicate the increasing tissue osmolality and hypoxia towards the inner medulla.* ***B.*** *A more detailed and magnified scheme of the (cortical) nephron shown in A. Major kidney tubular structures are color-coded as indicated.*

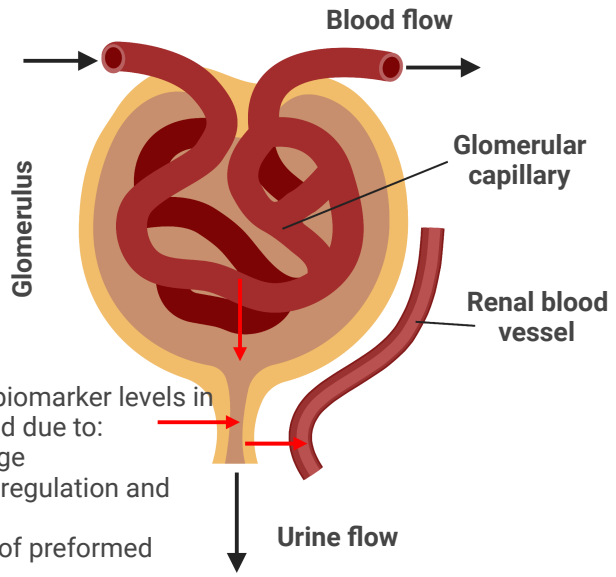
Figure 2. Sources of AKI biomarkers and pathophysiological conditions leading to AKI. A. *Scheme of a glomerulus (see Fig. 1) depicting potential sources for elevated levels of AKI biomarkers in blood and urine.* ***B.*** *Scheme showing potential clinical settings and pathophysiological conditions which can lead to the diagnosis of AKI.* ***C.*** *Table with the clinical settings and pathophysiological conditions depicted in B. and corresponding, potential therapeutic decisions.*

Figure 3. Basic steps in preparing and evaluating single cell data. *Points 1-6 indicate major steps for the preparation, implementation and analysis of single cell experiments for a droplet-based approach. The lower right panel shows the general structure of the tagged microbeads (used in point 2) which allows the assignment of individual mRNA molecules to their respective cell. For this, tagged microbeads carry a cell barcode (cellular identity, identical for all oligos on one microbead, different between different microbeads) and unique molecular identifiers (transcript identity to avoid counting PCR duplicates, different for each oligo on each microbead).*


A
Cortex
Outer stripe
Outer medulla
Inner stripe
Inner medulla
Hypoxia
Osmolality

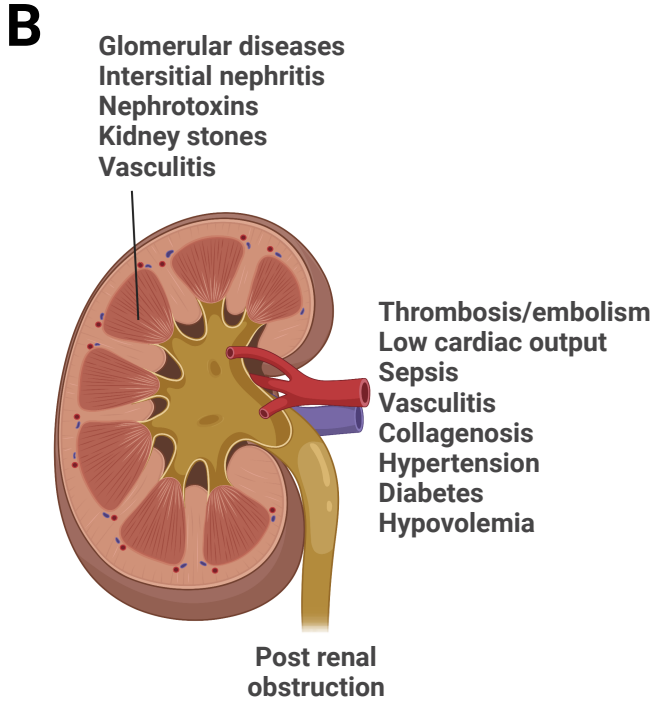

B
Glomerulus
Distal convoluted tubule and connecting tubule
Collecting duct
Proximal tubule
Loop of Henle

A



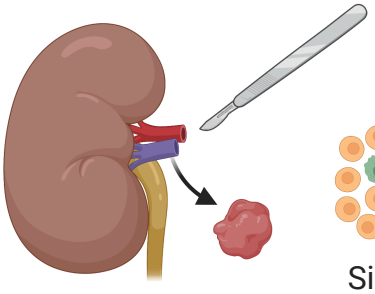
Altered AKI biomarker levels in urine or blood due to:
1. cell damage
2. tubular upregulation and secretion
3. secretion of preformed proteins
4. altered tubular reabsorption
5. altered glomerular filtration
6. secretion from invading or resident non-kideny tubule cells

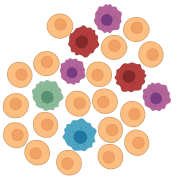
B



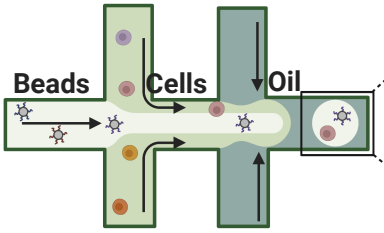
C

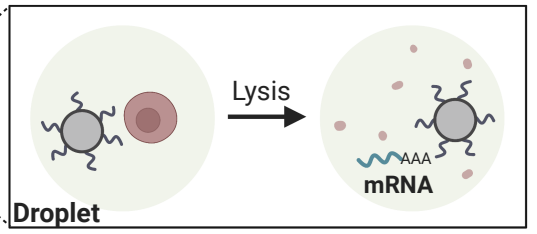
Clinical diagnosis	Potential therapeutic decisions
Thrombosis/embolism	Anticoagulation/lysis, angiographic/surgical intervention
Low cardiac output	Optimization of heart failure therapy
Sepsis	Antibiosis, volume therapy, ICU therapy (if necessary)
Hypertension	Optimization of antihypertensive therapy
Diabetes	Optimization of antidiabetic therapy
Hypovolemia (prerenal azotemia)	Volume therapy
Collagenosis/glomerular diseases /vasculitis	Immunosuppressive therapy (if necessary), optimization of cardiovascular risk factors
Interstitial nephritis	Discontinuation of potential noxious agents, glucocorticoids (if necessary)
Nephrotoxins	Discontinuation of noxious agents
Kidney stones	medical expulsive therapy, lithotripsy, ureteral splint, analysis of stone composition,
Postrenal obstruction	(Surgical) removal of obstruction, ureteral splint





Single-cell suspension

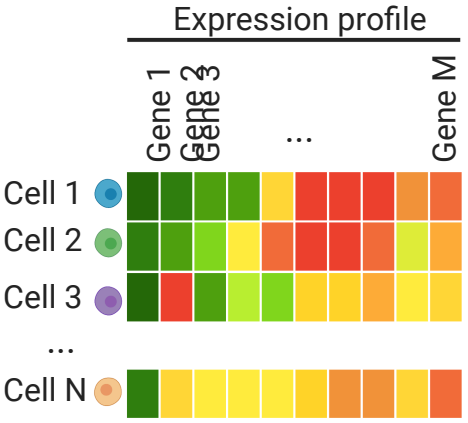




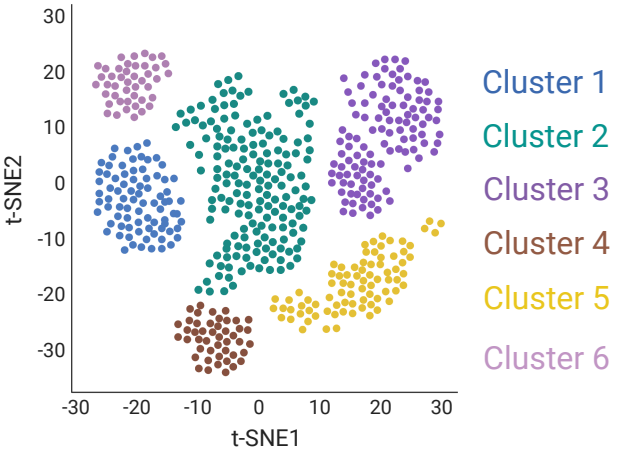
1 Tissue prepartion and generation of single cell suspension

2 Tagged microbeads and cells are encapsulated within droplets

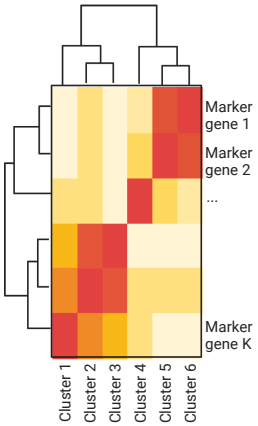
3 Single cell sequencing after library prep

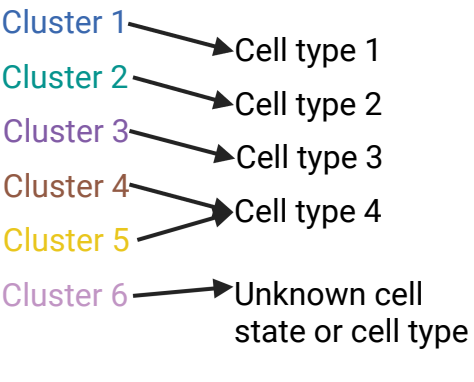


4 Single cell expression profile after alignment

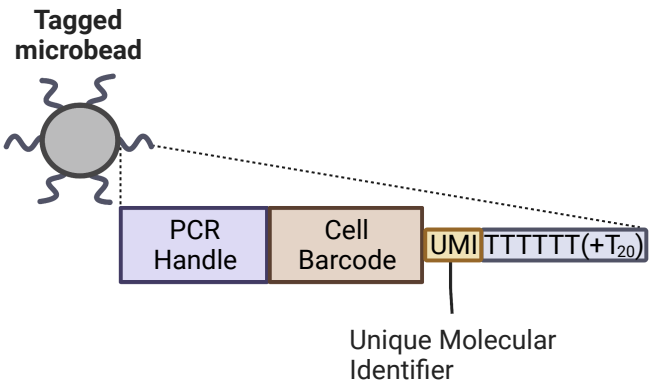


5 Unbiased clustering





6 Cell type assignment using marker genes



Structure of tagged microbead