Proteomics and Metabolomics for AKI Diagnosis

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Summary: Acute kidney injury (AKI) is a severe and frequent condition in hospitalized patients. Currently, no efficient therapy of AKI is available. Therefore, efforts focus on early prevention, and potentially early initiation of renal replacement therapy to improve the outcome in AKI. The detection of AKI in hospitalized patients implies the need for early, accurate, robust, and easily accessible biomarkers of AKI evolution and outcome prediction because only a narrow window exists to implement the earlier-described measures. Even more challenging is the multifactorial origin of AKI and that the changes of molecular expression induced by AKI are difficult to distinguish from that of the diseases associated or causing AKI as shock or sepsis. During the past decade, a considerable number of protein biomarkers for AKI have been described and we expect from recent advances in the field of omics technologies that this number will increase further in the future and be extended to other sorts of biomolecules, such as RNAs, lipids, and metabolites. However, most of these biomarkers are poorly defined by their AKI-associated molecular context. In this review, we describe the state-of-the-art tissue and biofluid proteomic and metabolomic technologies and new bioinformatics approaches for proteomic and metabolomic pathway and molecular interaction analysis. In the second part of the review, we focus on AKI-associated proteomic and metabolomic biomarkers and briefly outline their pathophysiological context in AKI.

Keywords: Proteomics, metabolomics, pathway analysis, AKI diagnosis

Acute kidney injury (AKI) is the most frequent acute renal condition and is associated with increased morbidity and mortality.[1,2] Currently, AKI is defined and classified by a rapid decrease in glomerular function and/or urine output based on increases of serum creatinine or decreases of urine production.[3] This definition is applied uniformly in clinical medicine and experimental AKI. In addition, patient history and physical examination, urine chemistry and cytologic analysis, ultrasound, and very rarely kidney biopsy currently are used as diagnostic tools in AKI. However, these diagnostic methods have limitations. They do not permit early detection of AKI or prediction of the course of AKI.

To improve patient outcome, it would be critical to have clinical tools that permit early detection of patients at risk of and those with evolving AKI. It also would be critical to have markers available to determine AKI progression, assess response to therapy, subsequent requirement of renal replacement therapy, as well as the degree of renal regeneration or residual chronic kidney disease after an AKI episode. Finally, it would be ideal to have markers that also are mediators of the different pathophysiological pathways leading to AKI.

Numerous biomarkers have been reported to enable the early detection of AKI. However, most of these biomarkers are linked closely to a single pathologic process, such as tubular injury. This may explain why these markers frequently have performed poorly in AKI populations with other pathophysiological mechanisms or of heterogeneous origin. In this review, various biomarkers and the currently experimental approaches of proteomics and metabolomics in biofluids and kidney tissue are highlighted. Proteomic and metabolomic approaches may provide multimarker

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panels that answer the earlier questions that are critical in AKI.

TECHNOLOGIC ADVANCES IN PROTEOMICS

Introduction to Proteomics

Proteins and peptides constitute the main functional and structural units of the cell. Thus, the proteome and peptidome are associated with the health status of an organism. Qualitative and quantitative differences of the proteome and peptidome composition reflect pathologic conditions. Because most human diseases are characterized by a complex landscape at the molecular level it is imperative to acquire a global picture of the proteome to depict pathways and proteins with pivotal roles in pathogenesis. The introduction of high sensitivity and resolution mass spectrometry analytic approaches has enabled the identification and quantitation of proteins and peptides in tissues and biological fluids, and offered novel insights on disease-associated processes at the molecular level. The information obtained from these experiments could be exploited in the clinical setting by the introduction of biomarkers (diagnostic, predictive, prognostic) and targeted therapeutic approaches. A recent example of high-sensitivity proteomics analysis in the context of AKI is the study by Malagrino et al,[4] which resulted in the identification of 55 putative biomarkers in porcine urine. However, only one biomarker, dipeptidyl peptidase IV, was validated in the urine of human subjects with diabetic nephropathy, which is no model for AKI.[4] One of the main findings of proteomics studies to date has been that a single biomarker cannot fully account for the complexity of human diseases and thus it is preferable to use biomarker panels. Moreover, the high number of proteins contained in biological samples (several thousands) and the extended range of their concentration (spanning 12 orders of magnitude in the case of plasma) hinders the complete characterization of proteomes. In addition, post-translational modifications of specific amino acids greatly increase the complexity of proteins and peptides present in human samples and present a formidable analytic challenge.

Proteomic Technologies

Q9 Initially, two-dimensional gel electrophoresis was the principal method for protein separation before mass spectrometry (MS), but now largely is replaced by liquid chromatography (LC) and capillary electrophoresis (CE) for numerous reasons including higher-resolving capacity and ease of use.

Small proteins and peptides can be analyzed directly by LC or CE coupled to MS. Larger proteins have to be cleaved to peptides, mostly by trypsin, which cleaves polypeptide chains after lysine and arginine residues.

Many different MS methods exist but they share common principles. Peptides are ionized and then subjected to an electric or magnetic field. The subsequent ion characterization is based on its mass over charge ratio. Matrix-assisted laser desorption/ionization (MALDI), surface-enhanced laser desorption-ionization, and electron spray ionization (ESI) are the main ionization techniques that have been used in clinical proteomics. In MALDI, samples are spotted onto a plate, mixed with matrix, dried, and analyzed under high vacuum. In surface-enhanced laser desorption-ionization, the principle of MALDI is combined with selective surface binding to functionalized matrices. Different chip surfaces for hydrophobic, ionic, or affinity binding of proteins are commercially available. Before analysis, the sample is spotted on the functionalized chip matrix, and all nonadsorbed molecules are washed off. In ESI, the separation effluent is ionized online in a high-voltage field that results in desolvatization. In comparison, MALDI results in single-charged ions and readily interpretable spectra, whereas ESI generates multiply charged ions, resulting in more complex spectra but richer in information. The advantages of ESI, as compared with MALDI, are superior ionization efficiency and consequently better linear response. Furthermore, ESI is more suited for online coupling with LC or CE, whereas in MALDI fractions must be collected to be spotted on the plate. Many different approaches exist for protein mass detection, mostly in respect to amplification of ionic signals. Time of flight, Orbitrap, and Triple Quadrupoles Q10 are the most commonly used analyzers in biomarker research.

In principle, only relative quantification is possible with MS profiling techniques based on an approximate proportionality between signal intensity and the protein/peptide abundance in the sample. For comparison of different samples, normalization procedures therefore are required to compensate for biological and analytic variances (ie, by different hydration states of samples or signal suppression effects).[5] Advanced methods such as isobaric tags for relative and absolute quantification and multiple reaction monitoring (MRM) are based on exogenous synthetic peptide standards and thus are restricted to cases in which exact sequence information of the analytes to be quantified is available. Even if this is the case, these may well be suited to correct for analytic variances, but are inappropriate in correcting for differences in sample dilution. Creatinine might be a possibility to normalize the different dilutions of a set of samples, but certainly is less suited to correct for analytic variances. As shown by Jantos-Siwy et al,[6] the use of

29 high-abundant, low-variability, collagen-derived peptide fragments as internal ion signal normalization standards allowed in urinary low-molecular-weight proteome analysis (mass range, 0.8-20 kDa) both correction of analytic variances during proteomic profiling and correction for different dilution levels of individual urine samples in one single data analysis integrative step. Thus, a set of internal protein calibrants of known signal intensities might be the best option to correct for intersample variability in MS profiling experiments.

Tissue Versus Biofluid Proteomics

In respect to specimen collection, tissue is close to the origin of the disease, but its collection is invasive. Blood and urine can be collected noninvasively. Blood plasma or serum has a high dynamic range, affording depletion of most abundant proteins, and is characterized by low stability because of high proteolytic activity. Urine has a higher stability and lower complexity than blood and can be obtained easily. The use of a standardized collection protocol including deep freezing directly after urine collection avoids proteolysis by urinary protease inhibition.

Preanalytic processing steps such as centrifugation of insoluble material, depletion of most abundant proteins, ultrafiltration to remove high- or low-mass proteins, and precipitation introduce bias and add variability in the preparation of biofluids, but allow measurement of low-abundance proteins.

Methods in Tissue Proteomics

In comparison with body fluid proteomics, efficient disruption and homogenization of tissue material is a first step of the tissue proteomics pipeline. Until now, several homogenization strategies have been developed,[7,8] with the combination of mechanical and chemical methods being applied most commonly for processing tissue specimens. However, depending on the type of the tissue sample analyzed (eg, soft tissue, hard tissue), specimen size, and down-stream analytic methodology, tissue disruption and protein extraction protocols need to be adjusted and optimized (including reproducibility and efficiency of protein extraction). Despite recent advances in the proteomics field, several major limitations of tissue proteomics analysis still remain, mostly being associated with the high complexity and broad dynamic range of protein concentrations, as well as high cellular heterogeneity.[9] Because of the high complexity of tissue, and to identify low-abundance disease-related proteins, additional fractionation approaches are used (either at the level of cellular organization, protein, or peptide). Thus, studying the tissue proteome provides an opportunity to analyze specific subcellular fractions, allowing for a deeper understanding of disease-associated processes. To address the cellular tissue heterogeneity, laser-capture microdissection can be used.[10] However, the small size of the tissue sample may preclude the use of laser-capture microdissection. Moreover, considering the invasive way of collection of the tissue samples, availability of fresh-frozen specimens might be an issue. To overcome this limitation, recent improvements in the sample preparation protocols allows the analysis of formalin-fixed, paraffin-embedded tissue.[2,11,12] In addition, a tissue sample contains protein up-regulated or down-regulated at one specific time point in the course of AKI. Because it will not be possible to collect several tissue samples at different time points in human beings, this will limit the value of tissue proteomics in the dynamic process of AKI. A schematic overview comparing the advantages and limitations of tissue versus biofluid proteomics is presented in Figure 1. As indicated in Figure 1, performing both types of proteomic analyses enables complementary data cross-correlation for complete proteome expression profiling.

Future Trends in Proteomics

The MS instrumentation and methods have improved greatly during the past decade and several recent developments have indicated that proteomics analysis will greatly benefit from innovative advances.

The sensitivity of shotgun proteomics methods for untargeted proteomic profiling has increased significantly with limit-of-detection values in the low femtomolar range in the case of complex or even atomolar range in the case of simple protein mixtures. The technological advances are supplemented by computational advances in the processing and subsequent bioinformatics interpretation of the generated large MS data sets. An unresolved issue in shotgun

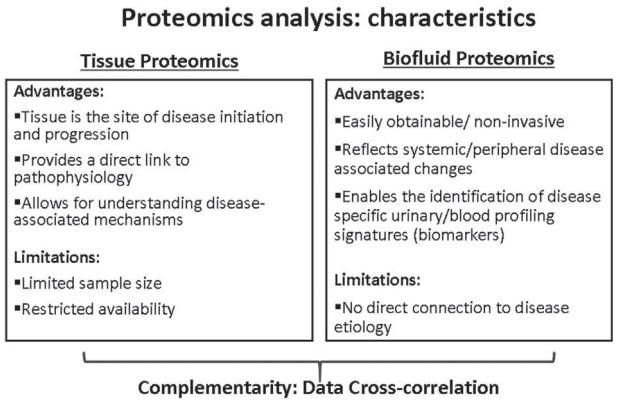

Figure 1. Advantages and limitations of tissue and biofluid proteomics.

proteomics, however, is the inability to quantify the detected protein targets directly.

New targeted approaches such as MRM, which also is called selected reaction monitoring, have emerged in recent years and allow absolute quantification of several proteins of interest in a complex mixture. MRM allows the detection and quantitation of specific peptides, and requires a triple quadrupole MS. It selects specific fragments for a known peptide of interest and precisely measures the abundance of each fragment in a subsequent step. For quantitative analyses, a known quantity of the same but isotope-labeled peptide is added to the sample before the MS run. The labeled peptide has the same amino acid sequence as the unlabeled native peptide, resulting in the same fragmentation ions but in a different mass owing to the isotope label. This enables absolute quantification of the peptide of interest, by comparing the signal intensities of corresponding labeled and unlabeled ions. Moreover, the use of stable isotope-labeled peptides ensures the specificity of the signal measured from the corresponding native peptides.[13] An alternative to using labeled peptides for MRM was investigated by Gilquin et al,[14] who introduced a technique named protein standard absolute quantification for measuring the levels of four putative AKI biomarkers in urine. The approach is based on the use of labeled protein standards and has the advantage that it takes into account all steps before MS analysis (in contrast to a typical MRM experiment that is based on the addition of a known amount of standard isotope-labeled peptides to the digested peptide mixture before MS analysis).

Q12 It is appealing to consider that specific PTMs (phosphorylation, glycosylation, acetylation, and so forth) that are associated with pathologic conditions could be used as highly specific biomarkers.[15] Moreover, novel PTMs are identified and measured with high accuracy, but their relevance to specific diseases remains to be elucidated. An interesting example is the quantification of proteins that undergo adenosine diphosphate–ribosylation upon oxidative stress in HeLa cells.[16] This highly challenging measurement was made possible by the use of a hybrid MS instrument that combines the selectivity of a triple quadrupole with the high resolution of an Orbitrap. The widespread use of this approach, named parallel reaction monitoring, will greatly facilitate the application of quantitative proteomics in research and the clinical setting. In comparison with MRM, parallel reaction monitoring can have higher sensitivity and throughput, and assays can be developed more easily.[17]

Another recent advance in proteomics analysis was the introduction of the data-independent acquisition approach named sequential window acquisition of all theoretical fragment ion spectra.[18,19] This innovative approach achieves quantitation of several hundred proteins without the use of labeled standards. It is reasonable to expect that with further improvements in instrumentation and software that we will be able in the near future to both identify and quantitate the majority of proteins contained in a biological sample.

TECHNOLOGIC ADVANCES IN METABOLOMICS

Introduction to Metabolomics

Metabolomics is defined as the analyses of molecules smaller than 1,000 Da, which are transformed as a result of, and in support of, an organism's metabolism. The metabolome is therefore a complete set of metabolites that can be produced and consumed by organisms.[20,21] Typically, the metabolome is measured in fluids or tissues.[20] In the past, these experiments focused on a single metabolite that was attributed to a specific disease or enzymatic reaction.[22] Today, technologies and computational tools allow a more extensive and wide-ranging investigation of many metabolites within a single measurement, providing a broader insight into mechanisms of diseases. In this section we present the state-of-the-art techniques in metabolomics and how they can be applied in the discovery of AKI biomarkers. A schematic overview of a metabolomics workflow is presented in Figure 2. F2

There are two main approaches in metabolomic experiments: nontargeted and targeted analyses. Targeted analysis is focused on a specific set of compounds, which often are similar in structure and chemical properties, and/or derived from the same biological pathway.[21,23] All aspects of the study, from sample preparation to data analysis, are tailored to the measurement of these specific metabolites. In contrast, nontargeted approaches take a global approach by seeking to measure as many metabolites as possible and using statistical tools to identify those that differ between healthy and ill individuals.[21,23] Nontargeted approaches are more challenging because these methods are more complex compared with targeted analysis, and novel hypotheses for diagnosis and etiology of diseases may be derived from their results.

Metabolomic Technologies

Nuclear magnetic resonance (NMR) spectroscopy and MS are the two main analytical platforms used in modern metabolomic studies. Both platforms have advantages and weakness for nontargeted and targeted approaches.

NMR is a highly robust instrument. This method is quantitative, nondestructive, and provides details of the molecular structure of metabolites detected.[23,24] High-

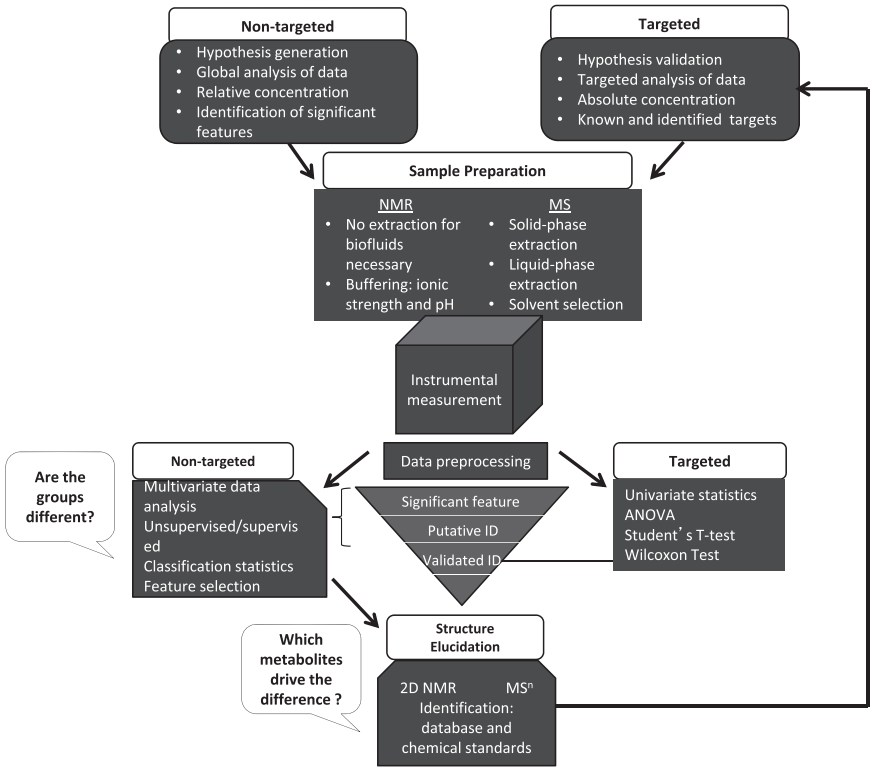

Figure 2. Schematic representation of a metabolomics workflow. Abbreviations: ANOVA, analysis of variance; 2D, two-dimensional.

resolution NMR can acquire thousands of distinct peaks and has the potential to detect and quantify hundreds of metabolites.[25,26] Because of the rather simple sample preparation and relatively short time for sample measurement, this top-down approach is well suited for high-throughput, nontargeted, metabolite fingerprinting investigations[20,24,26] and provides valuable information on the structure of compounds.[23] One drawback of NMR, however, is its relatively low sensitivity when compared with MS. Despite this limitation, NMR has been a valuable tool in a wide range of studies of human fluids,[27–29] especially of urine. Urine contains large amounts of salts that can cause intersample variability in metabolite measurements.[24,30,31] This variability has convoluting effects for statistical analyses. Therefore, both ionic strength and pH need to be well controlled by adding adequate buffer solutions.[24,31] Blood and tissue samples also can be analyzed using NMR with more extensive sample preparation.[24,32]

MS also is used widely in metabolomics. Mass spectrometers consist of an ionization source, mass analyzer, and detector. The choice of the MS system greatly may influence the quality of metabolomics data generated depending on the focus of the experiment. ESI is best suited for semipolar and polar compounds, whereas atmospheric pressure chemical ionization is better for neutral or less polar compounds.[33] Mass analyzers commonly used in metabolomics are quadrupole time-of-flight, Orbitrap, and Fourier transform. Because of their high resolving power, these instruments are ideal for distinguishing the chemical complexity of the metabolome.[34] More recently, Fourier transform ion cyclotron MS have been used more widely in metabolomics. These are the most advanced mass analyzers in terms of mass accuracy and resolving power,[34,35] helping to determine the exact molecular formulas of metabolites, and developing metabolite networks that have the potential to represent biochemical reaction networks that would be seen in nature.[34,36]

Finally, separation techniques are also an important aspect to consider. The three main techniques used in online coupling to MS are gas chromatography, liquid chromatography, and capillary electrophoresis. Separation techniques reduce matrix effects, ionization suppression, and help to separate isomers. In addition, these technologies add an extra orthogonal dimension, which is important to improve metabolite identification.[33,37]

Sample preparation is more critical in MS-based than in NMR experiments because the extraction of metabolites needs removal of unwanted proteins and salts that adversely will affect the quality of measurements as well as the instrumentation itself.[38] Solid- and liquid-phase extractions are used most commonly in

metabolomics, and applied to blood, urine, and tissues samples. Critical to all metabolite extraction methods is the choice of solvent, which are categorized according to toxicity, solubilizing power, selectivity, dissolution rate, chemical reactivity, and pH.[38] Most important is the solvent selectively, which is based on the polar index of the solvent and influences the extraction efficiency of polar or nonpolar metabolites from the original matrix.[38]

Analysis of Metabolomics Data

The large amount of acquired data in metabolomics experiments requires sophisticated data handling strategies and advanced statistical tools. In addition, before any statistical analysis, data preprocessing must take place to ensure the best possible results from the applied statistics.

The key steps in metabolomic data preprocessing are spectral alignment, normalization, transformation, and scaling. Normalization of data is required for accurate metabolite quantification. One common method is normalizing data based on endogenous metabolites, such as urinary creatinine. However, creatinine itself may be somewhat variable, and subject to variation owing to diseases such as AKI.[20,39] Therefore, alternative methods for normalization, such as probabilistic quotient normalization, should be considered to reduce bias.[40] This approach is based on the calculation of the most probable dilution factor from the distribution of quotients between all spectra and a reference spectrum.[39] Evaluation of a list of normalization methods can be found in a study performed in autosomal polycystic kidney disease.[41] Many of the normalization, transformation, and scaling strategies can be applied to both NMR and MS-based data, but are especially important in MS because the range in signal intensities can vary by several orders of magnitude.[42]

Metabolomics data are evaluated by univariate and multivariate analyses. For targeted metabolite analyses, univariate statistics are adequate, which analyzes each metabolite independently of other factors. These tools can include the Student *t* test, Wilcoxon test, analysis of variance, or Kruskal-Wallis one-way analysis of variance.[20,43] In nontargeted metabolomics analysis, data are analyzed in a multivariate way. Multiple metabolite features are statistically analyzed simultaneously with an attempt to evaluate patterns in metabolite data that can discriminate groups.[20,43] In multivariate statistics, both unsupervised and supervised approaches can be applied depending on added knowledge of the sample classification to control for other conditions. Unsupervised methods such as principle component analysis can be applied to summarize complex data.[20,43,44] It reduces the high dimensionality of the data by linear transformation to principle components without consideration for a y-variable (ie, classifier variable). In this analysis, score vectors of each sample can be obtained and plotted to show which components of the data best explain the differences between groups.[20,43,44] This is an important step to understand the data structure and detect outliers and review the influence of the metadata of samples such as sex. On the other hand, supervised multivariate analyses identify metabolic patterns that correlate with a particular phenotypic y-variable,[20] and can be based on the results of unsupervised methods. One frequently used method is partial least-squares analysis, used as a discriminatory or a regression analysis depending on the y-variable of interest. In this context, multivariate statistics is a powerful selection tool that narrows down data sets of thousands of variables to a manageable number that is most responsible for explaining the metabolic variation in the y-variable.[20,44,45] Once data patterns have been identified as significant, the identification of individual metabolites can be achieved by two-dimensional experiments (ie, two-dimensional NMR or MS/MS) and cross-referenced with databases such as the human metabolome database.[46] Identified metabolites then can be added to pathway enrichment analyses and merged with other omics technologies such as proteomics to gain a global picture of the metabolic processes in AKI.

Future Trends in Metabolomics

Although many novel and cutting-edge techniques have been developed, the challenge in current metabolomics is its transition to clinical use. Integrating metabolomic data with other omics data for the purpose of drug discovery and development is one direction in which this field may proceed.[47,48] Another direction undoubtedly will be personalized medicine,[49] in which nontargeted data can be used to characterize the metabolic fingerprint of individual patients. Flux analysis also is an emerging field within metabolomics,[50,51] and likely will be important in future medicine to characterize metabolic changes in real time.

MULTIVARIATE AND MULTI-OMICS DATA INTEGRATION

Multi-Omics Data Comparison

Recent advancements in genomics (next-generation sequencing) and proteomics MS technologies have shown that, although the clinical presentation may be the same, many complex and/or chronic diseases present high levels of heterogeneity at the molecular level.[52] The high heterogeneity at the molecular level is

one of the main reasons why experimental findings rarely may end in clinical biomarkers because it severely affects the statistical assessment in the preclinical and early clinical phases.[53] Therefore, one biomarker may not be applicable to all patients with a disease with consistent clinical or pathologic features. We require a better understanding of molecular mechanisms of diseases and their progression. This ultimately will lead to better diagnostic biomarkers. Q14 Appropriate analysis of -omics data and validating the results with different technologies (multi-omics comparison) provides new insights in the disease process and identifies biomarkers that are clinically useful and also disease mediators. The comparative assessment of data from different sources such as tissue, urine, and plasma also seems advantageous. In this approach, markers are identified on a molecular level in the affected tissue as well as in fluids derived from this tissue. In the case of AKI this would be biomarkers present in the kidneys as well as in the urine.

Pathway Analysis and Construction of Molecular Maps

The various omics technologies frequently give rise to long lists of modulated molecules that can be difficult to interpret or deduct what processes are involved. This is particularly a problem when integrating data associated with several categories of biomolecules such as metabolites and proteins. Although such lists are not suitable in identifying potential biomarkers and molecules of interest, the combination of shared attributes and interlinking of associated prior knowledge very often can reveal processes and molecular pathways affected or involved in the system under investigation.

A common approach is the clustering of functionality tags such as Gene Ontology or pathway names associated with individual genes or proteins based on independent and unrelated studies, whereby the occurrence of such tags in the list of molecules of interest are evaluated statistically. This can be accomplished easily Q15 using tools such as the Cytoscape[54] plugin ClueGO,[55] R-based scrips such as Gogadget,[56] and web-based solutions such as GOrilla[57] or David,[58] and many more. The results may show the common biological processes involved and to some extent potentially predict physiological end points that can be validated or used as a clinical measure. Assembling delineated molecular pathway maps, however, requires a higher level of complexity because very often individual processes such as signaling events or biochemical reactions either are not attributed to the same pathways but observed in a different context of unrelated molecular events, not contextualized at all to date, or no information has been gathered to date in terms of the functionality of individual molecules. Therefore, the construction of molecular pathway maps currently involves painstaking extraction of molecular features from the literature and other resources followed by assembly of the various components into a network of interacting and interlinking events. One particular software, PathVisio,[59] allows the manual construction of such pathway diagrams and at the same time also enables the searching and mapping of individual or groups of molecules using the Wikipathways resource.[60] This database is a public effort to assemble molecular pathways into a uniform structure, relying on literature-based evidence according to the disease or system under investigation. Alternatively, data mapping onto existing pathway collections also can be accomplished using the Kyoto Encyclopedia of Genes and Genomes database.[61] Other resources containing large-scale data of molecular pathways such as Reactome[62] or BioCyc and MetaCyc[63] also actively are engaged in developing such capabilities. In addition, independent platforms such as IMPaLA[64] that are reusing data from other resources already are available.

It already has become evident that two-dimensional representations of molecular events in complex systems and multifactorial diseases frequently are insufficient or overly complicated to follow or depict.[65] This may result in an oversimplification of pathway diagrams that can be ambiguous or misleading. One potential solution to such a problem is to avoid spatial representation and rather to establish pathway models as mathematical and computational representations.[66] Another important aspect in any data integration and pathway mapping is the risk of gaps in the discovery matrix, whereby linking molecules either were not detected or not modulated significantly in disease. GeneMania[67] allows for such an approach whereby a Q16 network is constructed using first all of the molecules in the discovery matrix, followed by addition of other connecting nodes, and, finally, pruning and removal of entries in the network diagram that are not linking to at least two other molecules found in the input list.

Ultimately, is was shown that de novo reconstruction of molecular pathways based on prior knowledge not only helps to understand the complexity of processes involved in health and disease, but is an important tool in providing the appropriate context of the observations and in identifying intervention points that potentially can be exploited pharmacologically.

Biomarkers Involved in AKI-Associated Disease Processes

We provide a short overview of biomarkers described in the literature as directly associated with AKI. To present AKI in its pathophysiological context, these AKI-specific markers were complemented with

proteins and metabolites that occupy key positions in AKI-underlying disease processes. The markers listed in this section were used in molecular interaction and pathway analysis.

Proteins and Peptides

Cystatin C is a 13-kDa cysteine protease inhibitor synthesized and released continuously into blood by nucleated cells. It is filtered freely by the glomerulus but, unlike creatinine, is catabolized completely by the proximal renal tubule in physiological situations. Serum cystatin C is a biomarker of glomerular filtration function whereas urinary cystatin C is a marker of proximal tubular function. In some, but not all, studies, an increase in serum cystatin C concentration could detect AKI earlier than creatinine concentration.[68–70]

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, is a 25-kDa protein[71] strongly up-regulated in urine in response to ischemic and toxic AKI.[72,73] Both a monomeric and a heterodimeric form are produced in the renal tubular epithelial cells whereas a homodimeric form is produced by neutrophils. NGAL has the ability to inhibit bacterial growth and sequestering siderophores (molecules chelating iron required, among others, for bacterial growth).[74] NGAL filtered through the glomerulus is taken up in the proximal tubule and degraded in lysosomes.[75] Serum and urinary levels have been reported to correlate with AKI, but also with sepsis and other conditions.[76]

Interleukin-18 (IL-18) is a 18-kDa proinflammatory cytokine secreted by various antigen-presenting cells. It induces interferon γ production in type 1 T-helper cells and is a mediator of ischemic injury, in particular in the kidney.[77] Urine IL-18 is increased in acute tubular necrosis and delayed kidney graft function and is predictive of mortality in cardiac surgery. Because different studies showed conflicting results on diagnostic performances of IL-18, a meta-analysis recently was performed and found that IL-18 had only a moderate diagnostic value (AUC, 0.77), performing best in predicting early AKI and AKI in pediatric patients.[78]

Kidney injury molecule-1 is a transmembrane glycoprotein that is not detectable in healthy kidneys but is expressed highly by epithelial cells of the proximal tubules after ischemic or toxic injury,[79,80] with the ectodomain being shed into the tubular lumen.[81] It functions as a phosphatidyl-serine receptor and confers a phagocytic phenotype on epithelial cells, most likely to clear cellular debris during apoptosis.[82] Kidney injury molecule-1 levels both in urine and plasma have been shown to increase in both AKI and chronic kidney disease (CKD) and to be prognostic for progression toward end-stage renal disease in diabetes.[83]

Liver-type fatty acid–binding protein 1 (FABP1) is a transport protein for free fatty acids. In the kidney, it is expressed in renal proximal tubule cells and shed into urine in response to hypoxia caused by decreased peritubular capillary blood flow. Transferring cytotoxic lipids produced from free fatty acid peroxidation into the urinary space presumably reduces their noxious cellular effects.[84] Urinary FABP1 levels are increased in both CKD and AKI. A large prospective cohort study has investigated the ability of FABP1 to predict AKI after cardiac surgery.[85] Its urine levels were not associated independently with AKI after adjusting for other kidney injury biomarkers, and even the combination with other AKI biomarkers only yielded an AUC of 0.78 to predict AKI.

N-acetyl-beta-D-glucosaminidase (NAG) is a 140-kDa hydrolytic lysosomal enzyme that breaks chemical bonds of glycosides and amino sugars in carbohydrate-rich structural components.[86] NAG is found in high concentrations in the lysosomes of proximal renal tubular cells but also under the form of a membrane-anchored molecule shed into urine during tubular damage. Although it is present in many tissues, NAG does not pass the glomerular barrier because of its high mass and urine NAG only originates from the kidney. Moreover, urinary NAG levels correlate with the severity of tubular damage.[87] In a recent large prospective observational study performed in critically ill adult intensive care unit (ICU) patients, urine NAG levels alone allowed weak to moderate prediction of AKI, severe AKI, and ICU mortality (AUCs, 0.65, 0.71, and 0.79, respectively).[88]

α-1-microglobulin (A1M) is a 27-kDa plasma protein produced by the liver. It has an immunoregulatory role and is considered to be an anti-oxidant that can scavenge pro-oxidant heme groups. The gene for A1M also codes for bikunin, a glycoprotein with a number of functions. A1M freely passes through the glomerular barrier and approximately 99% of it is reabsorbed by the megalin receptor in the proximal tubule, where it is catabolized. A1M is an indicator for proximal tubular function.[89] In a small study with nonoliguric AKI, urinary A1M (among others) best identified patients who later required renal replacement therapy,[90] but diagnostic performances for AKI in two later studies on patients undergoing cardiac surgery were modest (AUC, 0.61 and 0.62).[91,92]

Retinol binding protein (RBP) is a low-molecular-weight protein (21 kDa) synthesized mainly in the liver and transports retinol. RBP retinol circulates in the plasma bound to transthyretin,[93] a complex that prevents its glomerular filtration. Four percent to 5% of serum RBP circulates freely and passes the glomerular barrier. It subsequently is reabsorbed and degraded in

the proximal tubule. Urinary RBP is therefore a marker of proximal tubular dysfunction as in studies on AKI after heart surgery,[94] and is used as a diagnostic tool in proximal tubulopathies.[95] It may be superior to B2M because RBP is more stable at low urine pH.

Clusterin is a 75- to 80-kDa glycosylated protein that can be found in its secreted form in biological fluids. It has been suggested to play an anti-apoptotic and cell-protective role in AKI.[96] It is up-regulated in renal tissues of both human beings and experimental models by various forms of cellular stress, such as unilateral ureteral obstruction[97] and ischemia-reperfusion injury.[98] It is not filtered by the glomerula, hence urinary clusterin originates from the urinary tract. In a study on urine markers of toxic tubular dysfunction in rats, urinary clusterin had higher diagnostic power than serum creatinine (AUC, 0.88 versus 0.79) and its increase occurred before histopathologic lesions could be seen.[96]

Cysteine rich protein 61 (Cyr61) is a secreted (~40 kDa), matrix-associated, heparin-binding protein.[99] Cyr61 has been reported to control or to be involved in the cell cycle, stimulation of chemostasis, growth factor–induced effects, angiogenesis, integration of biological mechanisms of cutaneous wound healing, induction of senescence, and apoptosis in fibroblasts. CYR61 gene transcription was reported to increase rapidly (>10-fold) after experimental AKI.[72] In a recent study of 50 patients undergoing cardiac surgery with cardiopulmonary bypass, CYR61 failed to identify the patients who developed AKI.[100]

Hepatocyte growth factor (HGF) is a member of the family of neurotrophic factors, composed of a 69-kDa and a 34-kDa chain. It is a pleiotropic cytokine that is synthesized in mesenchymal cells, including leukocytes and megakaryocytes. HGF circulates as an inactive single-chain protein and is converted to the mature active form by a serine-protease homologous to Factor XII, which derives from an inactive precursor activated by thrombin.[101] In one study, urine HGF markedly increased in patients with AKI compared with normal renal function and CKD.[102] In a study on recovery after AKI, the decrease of urinary HGF in the first 2 weeks after initiation of renal replacement therapy predicted which patients ultimately would recover.[103]

Meprin A, a neutral metalloendoprotease, is composed of an α- and a β-subunit and located on apical membranes. After ischemia-reperfusion and cisplatin-induced AKI in animal models, meprin A is redistributed toward the basolateral plasma membrane, cleaved, and excreted in the urine. Meprin A is injurious to the kidney during AKI because meprin A–knockout mice and meprin inhibition provide protective roles and improve renal function. This suggests that the altered localization of meprin A may be deleterious in AKI. Meprin A may be important in AKI and could be a target for therapeutic intervention.[104] To our knowledge, urinary Meprin A has not been studied as a biomarker of AKI in human beings.

Netrin-1 is a 50- to 75-kDa laminin-like protein involved in guiding axonal growth and is a chemotropic and cell survival factor.[105] Netrin-1 is widely expressed in various tissues, including in normal renal tubular epithelial cells. However, it was found to be highly expressed and excreted in the urine after AKI in rodents. Subsequently, urinary Netrin-1 excretion was detected to increase dramatically in patients with AKI, whereas no changes were detected in healthy volunteers' urine samples.[106] In a study on AKI after sepsis and septic shock, urinary netrin-1 levels increased significantly as early as 1 hour after ICU admission and peaked at 3 to 6 hours at a seven-fold value compared with controls and baseline, with an AUC of 0.858 at 3 hours.[107]

Insulin-like growth factor-binding protein-7 (IGFBP-7) has a molecular mass of 29 kDa and is a marker of cellular stress in the early phase of tubular cell injury caused by a wide variety of insults (inflammation, ischemia, oxidative stress, drugs, and toxins).[108] Furthermore, it can initiate G1 cell-cycle arrest, which prevents cells from dividing when potentially injured.[109] Importantly, it has been described as an "alarm" protein exerting paracrine effects on adjacent cells.[110] IGFBP7 measurement proved to be a predictor of AKI in cardiac surgery patients.[111] Urine IGFBP-7 often has been studied simultaneously with tissue inhibitor of metalloproteinase-2 (TIMP-2) levels because a commercial test device is available for [TIMP-2]*[IGFBP-7]. In a recent meta-analysis the diagnostic performance of urine [TIMP-2]*[IGFBP-7] levels across 9 studies for early prediction of AKI had an AUC of 0.846.[112]

Glutathione S-transferases (α and π GSTs) are constitutive cytoplasmatic enzymes. GSTs are scavengers of free radicals and could help tubular epithelial cells to resist stress. Urine GSTs were reported to be increased after gentamycin-induced nephrotoxicity in animal models, thereby serving as tubular injury markers. Further immunohistochemistry examinations showed the localization of α and π GST in the proximal and distal renal tubules, respectively.[113] After tubular damage, GSTs are released into the urine.[114] In a recent study in AKI after cardiovascular surgery, urinary π GSTs had an AUC of 0.784 to predict advanced AKI.[115]

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is a member of the C-C chemokine family, and a potent proinflammatory chemotactic factor for monocytes. Human MCP-1 is composed of 76 amino acids and is 13 kDa in size.[116] MCP-1 levels increase in proximal tubular epithelial cells and in urine after

AKI induced by nephrotoxicity in rats.[117] In a study of patients undergoing cisplatin-based chemotherapy for lung cancer, urine levels of MCP-1 (normalized for urinary creatinine) were higher in patients who subsequently developed AKI than in those who did not (AUC, 0.85).[118]

Tumor necrosis factor (TNF)-α is a cytokine that widely is implicated in inflammatory processes. Mainly produced by activated macrophages, it exists in a soluble and membrane-bound form. TNF-α has been studied extensively for its role in sepsis, systemic inflammatory response syndrome, and other severe inflammatory and autoimmune diseases. TNF-α probably is cleared in part by the kidney, as suggested by increased blood levels in CKD patients who do not undergo dialysis. In a rat model of glycerol-induced AKI, TNF-α levels increased rapidly while kidney function decreased, and neutralizing anti–TNF-α antiserum injected before glycerol injection partly rescued kidney function.[119] In a study of Chinese patients with hemorrhagic fever with renal syndrome the course of urine TNF-α levels and other cytokines closely followed the stages of the disease and normalized at the convalescence stage.[120] Although it is a major mediator in the pathogenesis of numerous conditions causing AKI, it is not considered a specific marker of AKI itself. TNF-α receptors I and II, however, were associated strongly with the development of AKI in a study of septic patients.[121]

TIMP-2 has a molecular weight of approximately 24 kDa. TIMP-2 is expressed constitutively in renal tubules and glomeruli.[122] It is involved in G1 cell-cycle arrest during the early phases of cell injury.[109] Renal tubular cells enter a short period of G1 cell-cycle arrest after ischemic insult, explaining enhanced TIMP-2 expression in this pathologic pathway of AKI. In several studies urine IGFBP-7 has been studied in parallel with TIMP-2 as another G1 cell arrest marker.[112]

Osteopontin (OPN) has chemokine-like features and plays a critical role in the formation of bone and calcified extracellular matrix. It is expressed and up-regulated during inflammation and various biological processes.[123] OPN is present foremost in the loop of Henle and in distal nephrons in healthy kidneys, but after renal damage its expression may be increased significantly in all tubular segments and in the glomeruli.[124] The role of OPN in inhibiting kidney stone formation has been highlighted. In a study on AKI in critically ill patients, the levels of serum OPN predicted the outcome of renal replacement therapy (weaning versus maintaining renal replacement therapy).[125] In another study on AKI and mortality in very-low-birth-weight infants, urinary OPN levels were tested along with other urine biomarkers and had an AUC of 0.83 for AKI, but the study size was very small (30 subjects altogether).[126]

Fibroblast growth factor (FGF) 23 and C-terminal FGF23 have a molecular weight of approximately 31 kDa. It is produced in bone, controls renal phosphate reabsorption, and has been considered the most potent phosphaturic hormone. It influences the production of parathyroid hormone and 1,25-(OH)2-vitamin D[127] and participates in mineral homeostasis.[128] FGF23 is increased dramatically in advanced CKD. FGF23 circulates both as the full-length intact protein and as a C-terminal fragment (cFGF23) after proteolytic cleavage.[129] In a murine folic acid–induced AKI model, cFGF23 levels increased by 24 hours after induction of AKI, and remained unchanged in controls. Although cFGF23 started to increase as early as 1 hour after induction of AKI, intact FGF23 started to increase 1 hour later. In human beings, several reports have suggested that FGF23 and cFGF23 are predictive of AKI and mortality. In a prospective study, 350 critically ill patients were admitted to the ICU and urinary and plasma FGF23 levels were measured within 24 hours of admission, among other measurements.[130] Urinary and plasma FGF23 levels, but not levels of other mineral metabolites, were associated significantly with mortality and AKI. In multivariate analyses, ICU patients with the highest versus the lowest quartile of urinary FGF23 had a 3.9 greater odds (95% confidence interval, 1.6-9.5) of dying and of AKI. The mechanisms underlying the early increase of FGF23 remain to be elucidated and may be independent of phosphorus metabolism.

Angiotensinogen (AGT) is related structurally to serin protease inhibitors and has a molecular mass of 55 to 60 kDa. It is the substrate of renin in the renin-angiotensin-aldosterone system blood pressure regulating system. Although systemic AGT is produced mostly in the liver, intrarenal AGT is synthesized primarily in cells of the proximal tubule and is secreted from the apical surface into the lumen. Urinary AGT (uAGT) levels have been shown to reflect the intrarenal RAS system activation. Several studies have suggested that uAGT may be an early biomarker of AKI in the context of acute cardiorenal syndrome after acute decompensated heart failure. In a large prospective cohort study on acute decompensated heart failure patients, daily uAGT levels were analyzed consecutively and peaked on day 1 in the patients who later developed AKI. After multivariable adjustment, the top quartile of uAGT had a 50-fold higher risk of AKI compared with the last quartile, resulting in an AUC of 0.84 for predicting AKI. One-year mortality also strongly was associated with uAGT levels.[131] Similar outcomes have been reported for uAGT in patients undergoing cardiac surgery.[132]

Prostaglandin-H2 D-isomerase (PTGDS), also designated as β-trace protein, is a 23- to 29-kDa secreted protein, which receives its name from the conversion of prostaglandin H2 into the isoform D2. Besides this isomerase activity, it can bind to small lipophilic molecules such as bilirubin or retinoic acid. Moreover, it functions as a scavenger for hydrophobic molecules and inhibits platelet aggregation. Similar to other small proteins, PTGDS is taken up by tubular cells from the circulation.[133] In this respect, fractional clearance of PTGDS from the blood serves as a marker for impaired glomerular filtration and shows the same diagnostic accuracy as creatinine, cystatin C, and B2M.[134–137]

γ-Glutamyl transpeptidase (GGT) is an approximately 99-kDa heavily glycosylated cell membrane protein composed of a heavy and light polypeptide chain that transfers γ-glutamyl groups mainly from the antioxidant glutathione to amino acids, peptides, and water, and is an essential component of the γ-glutamyl cycle for detoxification of xenobiotics.[138] It is expressed in all cells and tissues, with the liver being the major source of circulating GGT.[139] In normal kidneys, GGT is not filtered via the glomerulus. Therefore, urinary GGT released by proximal tubular cells is indicative of tubular damage.[140] In a study of patients after liver transplantation, absolute urinary GGT levels taken directly after surgery enabled prediction of AKI with an AUC value of 0.74.[141]

Alanine aminopeptidase (AAP) is a 109-kDa proteolytic enzyme that hydrolyzes oligopeptides derived from protein degradation. Besides this nonspecific substrate selectivity, AAP also catalyzes the conversion of various peptides such as peptide hormones, neuropeptides, and chemokines from their precursor into their biologically active forms. In the kidney, AAP is released into urine by damaged tubular cells during acute renal tubular necrosis, renal graft rejection, or nephrotoxic action of immunosuppressive drugs.[142–144] In an experimental cisplatin-induced AKI model in the rat, AAP showed good predictive accuracy for AKI with an AUC value of 0.89.[145]

Lactate dehydrogenase (LDH) is a 140-kDa ubiquitous enzyme that catalyzes the anaerobic, nicotinamide adenine dinucleotide phosphate-dependent interconversion of pyruvate and lactate. High LDH levels in the blood are indicative of cell death and tissue damage.[146] As reported in a recent study, plasma and especially urinary levels of LDH released from the renal cortex correlated with the severity of renal damage in different experimental AKI models.[147]

Erythrocyte superoxide dismutase 1 (SOD1) is the most abundant member of the family of antioxidant enzymes, representing approximately 90% of SODs.[148,149] It is a major defense against reactive oxygen species and primarily is intracellular, where it is found in the copper–zinc bound form. SOD1 long has been known to be involved in ischemic AKI in animal models because its administration to rats combined with sucrose reduced histologic and functional lesions.[150] Similar results also were observed in kidney transplantation.[151] More recently, SOD1 activity in erythrocytes has been shown to be associated with septic AKI in critically ill patients, although the diagnostic performance was weak (AUC, 0.69).[149]

Semaphorin-3A is a 89-kDa secreted protein with short-range context-dependent chemorepulsive and chemoattractive properties. Initially described in the context of axon guidance, it later became clear that semaphorin-3A also plays a role in vascular growth, angiogenesis, and immune cell regulation.[152] In the kidney, semaphorin-3A and its receptor complex, consisting of neuropilin 1 and plexin A1 or A3,[153,154] are expressed in developing nephrons and mature podocytes and collecting tubules.[155] In pediatric AKI after cardiopulmonary bypass, it was found that semaphorin-3A levels increase within 2 to 6 hours after cardiopulmonary bypass, allowed prediction of AKI with an AUC of 0.88 at 2 hours after cardiopulmonary bypass, and correlated with AKI duration and severity.[156]

Transforming growth factor-β1 (TGF-β1) is a 44-kDa multifunctional cytokine with broad growth factor stimulation, cell proliferation, and cell differentiation properties. The most notable functions of TGF-β1 are stimulation of osteoblastic bone formation,[157] stimulation of collagen production during wound healing and fibrosis,[158] T-helper cell 17 and regulatory T-cell differentiation,[159] and induction of epithelial-to-mesenchymal cell transition.[160] In the kidney, both TGF-β1 and its receptor are expressed in high levels by proximal tubular cells.[161] The role of TGF-β1 in AKI still is not clear. On the one hand TGF-β1 stimulates epithelial de-differentiation as a first step of cellular repair after kidney injury,[162,163] whereas on the other hand TGF-β1 induces proximal tubule apoptosis,[164,165] inhibits proximal tubule proliferation, and slows re-differentiation.[166,167] In several studies on patients with sickle-cell disease, a hematologic disease frequently associated with episodes of kidney injury, urinary TGF-β1 increased in parallel to serum creatinine level and with increasing anemia.[168,169]

IL-6 is a 23-kDa cytokine with a variety of functions mainly in the context of cell differentiation and acute-phase response. Depending on the mode of signaling, trans-signaling via binding to a soluble IL-6–receptor isoform or classic membrane-bound IL-6–receptor signaling, IL-6 possesses proinflammatory or anti-inflammatory properties.[169,170] IL-6 is secreted by endothelial cells in response to proinflammatory stimuli such as TNF-α.[171] IL-6 activates target cells via membrane-bound or soluble IL-6 receptor by association with the signal transducer gp130 and induction of

the Janus kinase/signal transducers and activators of transcription signaling pathway.[172] IL-6 blood levels have been associated with higher AKI-associated mortality in human beings[173] and in mice.[174]

Chemokine C-X3-C motif ligand 1 (CX3CL1, fractalkine) is a 43-kDa chemo-attractant factor for macrophages expressed mainly by endothelial cells.[175] Up-regulation of CX3CL1 was observed in ischemia/reperfusion-induced AKI. After renal injury, CX3CL1 promoted interstitial fibrosis.[176] Inhibition of the CX3CL1-receptor CX3CR1 reduced the number of macrophages in the injured kidney and thus may have therapeutic potential in AKI.[177,178] Other studies confirmed that CX3CL1 is an important target in anti-inflammation therapy for ischemic AKI.[179] In a study on AKI after cardiopulmonary bypass, numerous candidate biomarkers for AKI including CX3CL1 were tested and this molecule reached an AUC of 0.73.[180]

P-/E-selectin are lectin cell adhesion molecules that bind sialyl Lewis groups and related terminal glycans on the surface of other cells.[181] Because of a low binding affinity to their ligands, selectins mediate the process of rolling of lymphocytes and platelets on the vessel wall, promoting lymphocyte homing as well as lymphocyte and platelet aggregation and extravasation.[181] In response to the inflammatory cytokines IL-1β, TNF-α, and interferon-γ or other inflammatory stimuli, the 90-kDa P-selectin is translocated rapidly from secretory granules to the surface of platelets and endothelial cells.[182] The earlier-described inflammatory cytokines also induce de novo synthesis and expression of the 66-kDa E-selectin on endothelial cells.[182] During renal ischemia, up-regulation of P- but not L-selectin was observed, which was accompanied by enhanced adhesion of neutrophils to the renal endothelium.[183] Various animal studies have shown beneficial effects of P- and E-selectin inhibition on the progression and outcome of ischemia/reperfusion- and endotoxin-induced AKI.[184–187] Recently, a study of patients with severe sepsis admitted to the emergency department showed that serum E-selectin was an independent and powerful predictor of early AKI.[188]

Basigin is a 42-kDa cell-surface glycoprotein belonging to the Ig superfamily. It is expressed on many cell types and owing to a broad spectrum of ligands is involved in a variety of physiological processes. Most important in the context of AKI is its binding to E-selectin.[189] Basigin-deficient mice showed significantly lower numbers of neutrophils in the kidney and less renal damage after induction of ischemia compared with wild-type mice.[189] In biopsy specimens of patients with acute tubular necrosis, basigin expression was found on inflammatory cells in the interstitium and was absent in damaged tubules.[190] Moreover, it was found in patients after abdominal aortic aneurysm surgery that serum and urine levels of basigin on postoperative day 1 was higher in patients who developed AKI than in those who did not and had a similar profile as that of urinary L-FABP.[190]

Intercellular adhesion molecule-1 is a 58-kDa cell-surface glycoprotein of the Ig superfamily expressed mainly on endothelial and some immune cells. It functions as an adhesion molecule on endothelial cells that upon activation by the cytokines IL-1 and TNF-α or reactive oxygen species allow attachment of circulating neutrophils and leukocytes via their β_2 integrin receptors LFA-1 and Mac-1.[191] Attachment by this molecular interaction represents the essential first step for transmigration of the inflammatory cells through the endothelial layer on their way to the sites of infection.[192,193] Intercellular adhesion molecule-1 was connected to ischemic AKI by the finding that intercellular adhesion molecule-1–deficient mice showed decreased structural and functional damage to the kidney upon induction of ischemia compared with normal mice.[194]

Toll-like receptors (TLRs) are pattern-recognition receptors expressed on leukocytes, fibroblasts, and epithelial and endothelial cells. They are the responsible elements for the induction of the innate immune system against pathogens by binding conserved pathogen-associated molecular patterns.[195] Most importantly in the context of AKI are TLR-2, TLR-4, and TLR-9. TLR-2 mainly recognizes lipid structures on gram-positive bacteria,[196] whereas TLR-4 binds to lipopolysaccharide on gram-negative bacteria[197] and TLR-9 to bacterial CpG-DNA sequence motifs.[198] It was shown that TLR-2– and TLR-4–deficient mice are less susceptible to ischemic renal injury.[199,200] Mice deficient for the Tamm-Horsfall protein had more severe renal damage after induction of ischemia than their wild-type counterparts and this was associated with increased TLR-4 expression.[201] Furthermore, inhibition of TLR-9 by chloroquine or CpG-DNA antagonists, same as TLR-9 knockout, protect mice from sepsis-induced AKI.[202]

Macrophage inflammatory protein-2 is an 8-kDa cytokine that is secreted by macrophages to act via the chemokine receptor CXCR2 as a chemotactic agent for neutrophils.[203] It is involved in the early phase of an innate immune response against pathogens after TLR-mediated activation of tissue macrophages.[204] Its implication in ischemia-reperfusion injury of the kidney is shown by findings in mouse models that CXCR2 inhibition and macrophage inflammatory protein-2 antibody neutralization prevents interstitial infiltration of neutrophils and further results in decreased progression of kidney injury and increased animal survival rates compared with untreated controls.[205,206]

Caspase-1 is a 45-kDa zymogen that cleaves in its inflammasome-assembled autoactivated form

precursors of the inflammatory cytokines IL-1β and IL-18.[207,208] The latter subsequently are released by infected cells during pyroptotic cell death to initiate local inflammatory responses.[209] In transgenic mice increased caspase-1 expression in the kidney resulted in acute tubular necrosis via proinflammatory cytokine activation and granulocyte recruitment. This indicates that caspase-1 may be a marker for ischemic or cisplatin-induced AKI.[210,211]

C-terminal agrin fragment (CAF) is the 22-kDa c-terminal fragment of agrin, which is the major heparin sulfate proteoglycan in the glomerular basement membrane.[212,213] CAF is generated by cleavage of agrin by the serine protease neurotrypsin.[214] In human beings, CAF was detected both in urine and blood, suggesting its role as a marker for renal clearance with increased serum levels indicating impaired kidney function.[215] In a study of 61 patients with severe sepsis and septic shock, serum levels of CAF were associated with AKI (AUC, 0.721) independent of sepsis.[216]

Q34 Hepcidin is a 25–amino acid, cystein-rich antimicrobial peptide that elicits its function by regulation of iron metabolism. It inhibits iron export from gut enterocytes and macrophages by its binding to the iron channel ferroportin.[217] Increased hepcidin serum levels therefore prevent iron release into circulation and results in reduced iron availability to invading pathogens. Hepcidin levels increase during inflammation in response to bone morphogenetic protein-6 and IL-6 activation.[218,219] On days 1 and 5 after cardiopulmonary bypass surgery the urinary hepcidin to creatinine ratio was associated inversely with mild to moderate AKI (AUC, 0.77 and 0.84).[220]

C-X-C motif chemokine 5 (CXCL5) is a 10-kDa CXC chemokine also known as epithelial-derived neutrophil-activating peptide 78. It is expressed by highly specialized resident epithelial and mononuclear cells upon induction by the proinflammatory cytokines TFN-α or IL-1 and shows chemoattractant and angiogenic properties on neutrophils by binding to the chemokine receptor CXCR2.[221,222] In a sepsis-induced AKI mouse model, CXCL5 expression was induced in kidney tubular cells during an IL-17–mediated immune response, which consequently resulted in the recruitment of neutrophils and induction of renal injury.[223]

A urinary peptide marker panel composed of 20 naturally occurring peptides from serum albumin, α-1-antitrypsin, β-2-microglobulin, fibrinogen α chain, and the collagen chains 1 α (I) and 1 α (III) was established by support vector machine modeling and allowed prediction of AKI in ICU patients and in leukemia patients after hematopoietic stem cell transplantation in blinded validation with AUC values of 0.84 and 0.90, respectively.[224] In a subsequent case-control validation study, the peptide marker panel was applied to patients after cardiac surgery and in this patient cohort showed an AUC of 0.81 for the prediction of AKI.[225]

Metabolites

Creatinine is a 113-D molecule derived from creatine metabolism, mainly in the muscle. Creatinine is filtered freely by the glomerulus and excreted without significant metabolic changes or reabsorption by the kidney. Serum creatinine has been used clinically and experimentally as a marker of glomerular filtration rate for more than 50 years. It is limited by a late increase in AKI, its variable production under conditions such as sepsis, and tubular secretion, which increases with decreasing glomerular filtration rate.

Uric acid is a 168-D final oxidation product of purine metabolism and undergoes glomerular filtration.[226] Therefore, increased serum uric acid levels are seen in patients with reduced glomerular filtration rate. However, in recent years, it has been proposed that uric acid itself plays a causal role in the pathophysiology of CKD and possibly in AKI. Uric acid is known to cause endothelial dysfunction, increased IL-6 synthesis, and impairment of nitric oxide production, all of which may contribute to AKI and its progression. It remains unclear whether these cellular changes related to uric acid are reversible upon treatment of hyperuricemia. It also remains unclear whether uric acid levels can be a marker of AKI.[227]

Asymmetric dimethylarginine (ADMA) is a 202-D methylated analogue of the amino acid arginine. It is generated by arginine methyltransferase–mediated post-translational protein methylation and constitutive release during protein metabolism. It is eliminated from the blood by urinary excretion and dimethylarginine dimethylaminohydrolase degradation.[228] ADMA is an endogenous inhibitor of nitric oxide synthase. The negative effect on the vasoactive function of nitric oxide synthase provides the reason that increased ADMA blood levels are associated with endothelial dysfunction and progression of kidney injury.[229–231] Moreover, because of the impact of reactive oxygen species on the expression and activity of arginine methyltransferase and dimethylarginine dimethylaminohydrolase,[232] ADMA accumulates in the kidney during oxidative stress, and by nitric oxide synthase inhibition exacerbates ischemic damage to the kidney.[233]

Urea is a 60-D molecule that is produced by the liver in the urea cycle during degradation of proteins. The blood urea nitrogen (BUN) test measures the content of nitrogen from blood urea. BUN is used widely in combination with creatinine for the diagnosis of AKI. A BUN to creatinine ratio of greater than 20 may indicate prerenal AKI. Similar to creatinine, BUN is limited in its diagnostic use because it is influenced

strongly by nonrenal factors, such as catabolic state and food and fluid intake.[234]

Renal osmolytes are critical to renal medullary health in protecting cells and proteins from the harsh osmotic gradients needed to produce concentrated urine.[235] An extensive review by Burg et al[236] outlines in detail the role that osmolytes play in renal health. In recent metabolomic experiments using NMR, renal osmolytes such as betaine, taurine, and myo-inositol were found to be dysregulated significantly in AKI mouse models.[237,238] In both cases, decreased osmolyte concentrations in kidney tissue were observed and these findings can shed light on potential diagnostic, prognostic, and treatment strategies for AKI.

Adenosine triphosphate (ATP) metabolites arise from the degradation of the cellular energy transporter ATP. Hypoxia, ischemia/reperfusion, and nephrotoxic agents can have rapid and sustained effects on intracellular ATP levels by causing ATP depletion.[239–242] Currently, it is not possible to monitor changes in intracellular ATP levels in patients at risk for AKI in a clinical setting. The ATP metabolites adenosine monophosphate, hypoxanthine, and inosine, which freely diffuse out of renal proximal tubular epithelial cells, however, might be used in the future as noninvasive markers for AKI-associated alterations in intracellular ATP metabolism. Another potential target is 2',3'-cAMP, which is produced in the kidney in response to energy depletion, and is a potent opener of apoptosis and necrosis, mediating mitochondrial permeability transition pores.[243,244]

Integration of Established AKI Biomarkers in Molecular Interaction Graphs

In a proof-of-concept approach, we evaluated the integration of the earlier-described biomarkers for AKI and its underlying disease conditions in pathway and protein interaction analysis. Our aim was to select a sufficiently large and representative set of AKI-associated markers. By doing so, we wanted to investigate which AKI-related pathways were enriched and which additional proteins were essential to bridge gaps in the interaction graph.

We selected predominately those biomarkers for our molecular interaction analysis that were involved directly and critical in the different pathophysiological pathways of AKI. We excluded markers of AKI that merely represent functional changes but are not linked to AKI pathogenesis as the glomerular filtration rate markers serum creatinine and cystatin C. The same applies to markers of glomerular filtration barrier and tubular cell dysfunction as urinary albumin, α-1-antitrypsin, and B2M. Some markers and substances that are incorporated in the molecular interaction analysis may not or only in part be considered biomarkers of AKI. As examples, IL-6 and TNF-α are key factors in all major pathways of injury (ischemia, ischemia-reperfusion, proinflammation, apoptosis, necrosis, oxidative stress, direct cell toxicity) of diseases causing AKI. These may be shock, sepsis, cardiac failure, drug toxicity, radio contrast, and others. We included both in our molecular interaction analysis because their role in these underlying conditions cannot be differentiated from their function in similar pathways that occur intrarenally during AKI.

The gap-bridging proteins in Figure 3 identified by GeneMania are involved predominantly in cell surface–receptor signaling either as ligands such as the chemokines CXCL1, CXCL3, CXCL8, CCL3, and CCL4, and the cytokine IL-1β, as cell surface (co) receptors such as peroxisome proliferator activated receptor α, antigen-presenting glycoprotein CD1d, lymphocyte antigen 96, natriuretic peptide receptor 1, and erythropoietin receptor, or as intracellular proteins for receptor-associated signal transduction, such as TNF-receptor superfamily member 1A and receptor-interacting serine/threonine kinase 1. As proof for the validity of our molecular interaction model, many of the (patho)physiological processes mediated by these cell surface–receptor complexes were described also in the context of AKI or its underlying disease conditions.[245–252] This also accounts for all other proteins included in the molecular interaction graph not involved in receptor signaling such as the flavin adenine dinucleotide-linked sulfhydryl oxidase ALR, for which renoprotective effects were described in ischemia/reperfusion,[253] the peptidylprolyl isomerase F, a mitochondrial protein found to be involved in ischemia/reperfusion-induced cell death,[254] the calcitonin gene-related peptide 2, a vasodilator that increases susceptibility to AKI,[255] and superoxide dismutase 3, an extracellular oxidoreductase that decreases oxidative stress and injury after ischemia/reperfusion-induced AKI.[256] The connection of all gap-bridging proteins to AKI was interpreted as a sign for the high integrity of the molecular interaction graph.

By combining all previously described biomarkers for AKI-associated disease processes with the gap-bridging proteins in our molecular interaction analysis (with the results presented in Fig. 4A), neutrophil and platelet degranulation as well as IL-4/-13 and -10 signaling came up as the most significant molecular processes. It seems that these are late events most likely caused by oxidative stress or other danger signals in tubular epithelial cells. Danger signals are transduced by inflammatory mediators and ILs to circulating immune cells. Immune cells pass through the vascular wall of the renal microvasculature into the inflamed site via cell contact to activated endothelium.[257,258] A morphological result of oxidative

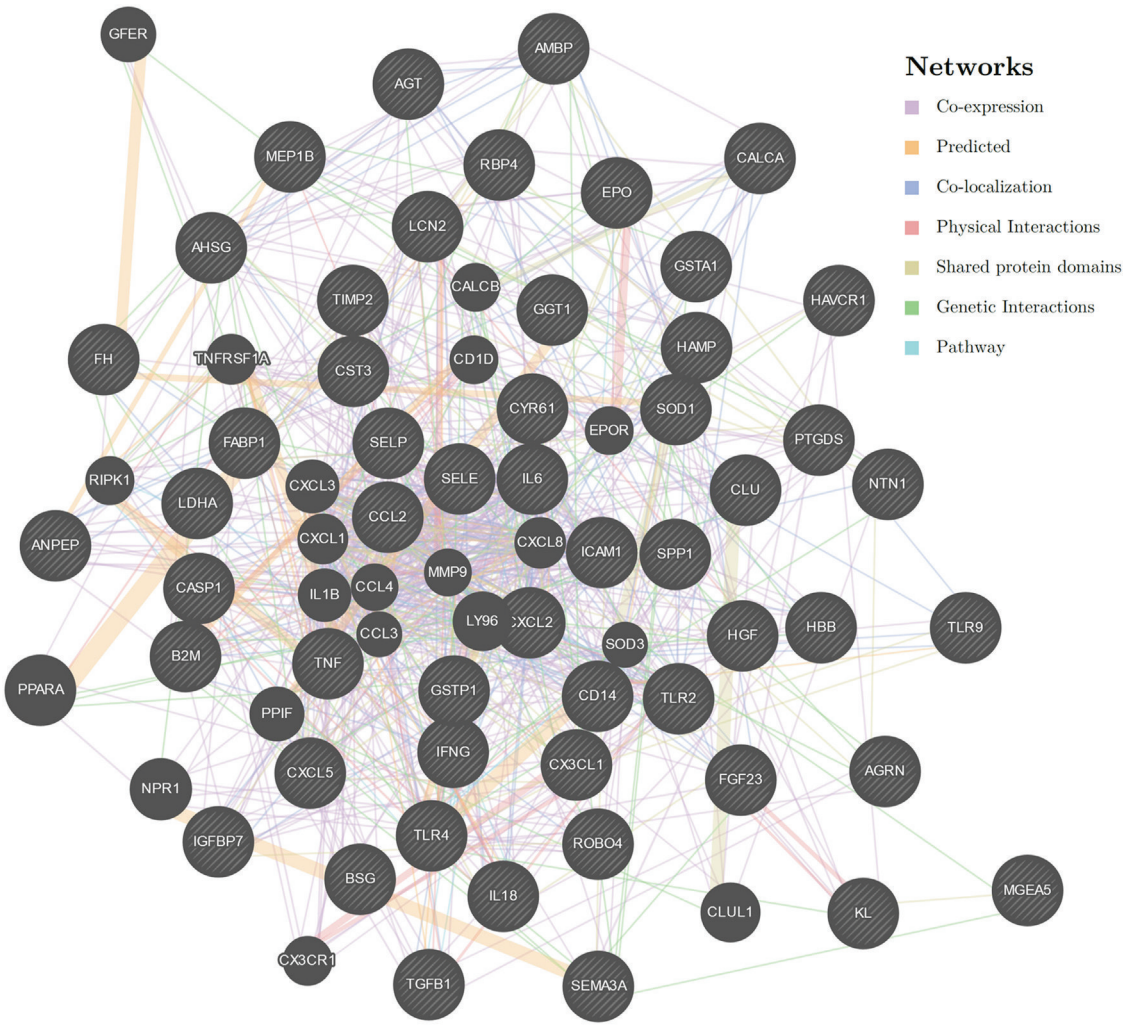

Figure 3. GeneMania network analysis of AKI-modulated molecules. Modulated proteins were analyzed by network construction allowing for gap-filling of missing molecules. Input proteins are shaded and imputed ones are colored uniformly. Connecting edges are colored according to the network legend.

stress is increased autophagy in tubular cells,[259] which is expressed on the molecular level by a disinhibition of SNARE complex formation, with the latter being essential for the fusion of cellular vesicles.[260] A linkage between oxidative stress and inflammation pathways is provided by TLRs and their response to heat shock protein 70 released by the affected tubular epithelial cells as the damage-associated molecular pattern molecule.[261] Other significant TLR-mediated processes in AKI according to the molecular interaction analysis are induction of apoptosis by caspase-8 activation[262,263] and programmed necrosis mediated by IKK and RIP1.[264] Infiltration of neutrophils and neutrophil degranulation in the kidney as well as apoptosis and programmed necrosis of tubular epithelial cells result in protease-mediated degradation of the extracellular matrix and collagen fibrils. Therefore, specific peptide fragments in urine may serve as a surrogate marker for altered activity of certain proteases in AKI (ie, MMP-9 or cathepsin D).[224,265,266]

As shown in Figure 4B, the danger signal sent out by tubular epithelial cells (ie, owing to oxidative and/or fluid shear stress or advanced glycation end product formation), is transduced on the signal transduction level by Toll-like and advanced glycation end product pattern recognition receptors into inflammatory and cell survival responses.[267] Induction of the NF-κB and the NOD-like receptor signaling pathways by TLRs result in the production of various proinflammatory cytokines and chemokines, such as IL-1β, IL-17A, and IL-18.[268,269] In consequence, effector cells such as neutrophils are recruited to the site of inflammation via chemokine and cytokine receptor interactions. Cytokine signaling pathways in the effector cells, such as the TNF and IL-17 signaling pathways, lead to further amplification of the inflammatory response. Because of the release of hematopoietic growth factors, such as

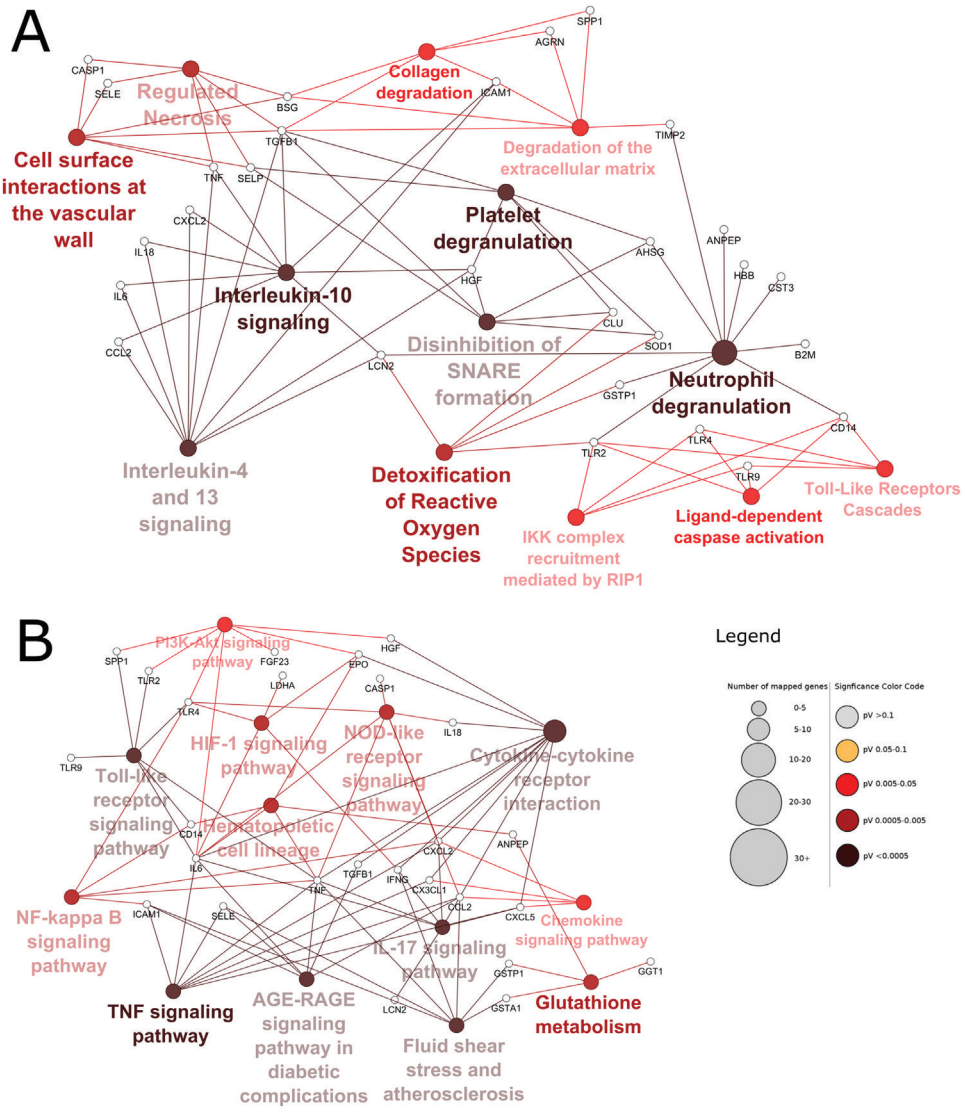

Figure 4. Term cluster analysis of AKI-modulated molecules. Proteins for which quantitative changes were reported in the literature were analyzed using the Cytoscape plug-in ClueGO and CluePedia and terms derived from the (A) reactome database of pathways or (B) Kyoto Encyclopedia of Genes and Genomes pathway maps. Molecules are shown as open circles and pathway terms are shown as shaded nodes according to the legend. Thresholding settings were $\kappa = 0.4$ for network diagram generation and $P = .05$ for all pathway term clusters shown. Disease-specific nodes that relate only to particular diseases unrelated to AKI were removed. Abbreviations: AGE-RAGE, _____; HIF-1, _____; IKK, _____; NF-κB, nuclear factor κB; NOD, _____; PI3K-Akt, _____; RIP1, _____.

G-CSF, inflammation is accompanied by cell maturation processes (ie, granulopoiesis)[270] of intermediate effector cells. In addition to these signaling pathways associated with inflammation there are other activated pathways, such as the HIF-1 and the PI3K-Akt signaling pathway, which mediate cell survival.[271,272] One survival response of the tubular epithelial cells to oxidative stress is increased synthesis of the endogenous antioxidant glutathione, a cellular process that is regulated by the PI3K-Akt signaling pathway.[273]

AKI Biomarker Patterns for Molecular Pathways, a New Conceptual Approach for Phenotype-Specific AKI Diagnosis

New biomarkers for AKI would have large clinical implication as elucidated earlier. For more than 10 years a large number of studies attempted to identify new biomarkers of AKI. Numerous biomarkers, including plasma and urinary NGAL, urinary IL-18, TIMP-2, and IGFBP-7, often have shown promising

results in pilot studies, detecting AKI and its severity and progression. Studies with these biomarkers provided important information with regard to pathophysiological mechanisms and some permitted early detection in some forms of AKI. Despite intense investigation, the clinical use and relevance of these and other markers in AKI has in general remained inconclusive. Furthermore, most clinicians worldwide do not have access to these assays because they are not commercially available or are expensive. The principal goal of most studies attempting to identify AKI biomarkers has been to develop one universal urinary or serum biomarker that would permit both valid determination of risk, diagnosis, severity and/or outcome of AKI, discriminate between etiologies of AKI, and monitor its course. However, the initial optimism to find such a universal marker now has subsided. Most large studies in heterogeneous patient cohorts could not confirm the predominantly excellent results of initial, small-scale, pilot studies in homogenous cohorts.

Independent of the diagnostic accuracy of any biomarker for AKI, in clinical practice the early detection of AKI has no impact on clinical decisions to date. Moreover, therapeutic possibilities for AKI are very limited and the benefit of an early or late start of renal replacement therapy is not clear. Therefore, the early or late detection of AKI in most cases does not have any influence on the clinical course of the patient. This partially may explain why most biomarkers have not been included in the panel of classic laboratory parameters used in the clinical setting.

There are numerous requirements to an ideal biomarker in AKI as for any other marker in laboratory medicine. In addition to good pre-analytical properties, an ideal marker would be both sensitive and specific, precise and reliable, react rapidly to any AKI, and its measurement should be standardized, simple, rapid, and inexpensive. The biomarkers presently available, whether commercially or only experimentally, do not fulfill the majority of these requirements.

On closer view, one single ideal and universal AKI marker likely never will be discovered. Perhaps the goal to discover such a molecule should be abandoned altogether. AKI is a nonuniform, very complex condition with a wide spectrum of causes and pathophysiological mechanisms. On the one hand, a single causative factor may damage the kidneys by several pathomechanisms. On the other hand, AKI, especially in critically ill patients, frequently is caused by numerous factors. AKI is characterized by many different courses, variable severity, and responses to preventive measures and therapies with ultimately different outcomes. One form of AKI may evolve into another one over time, such as postrenal AKI into intrarenal AKI with tubular damage when obstruction persists for a long time. The particular point in the course of AKI also needs to be considered, when severity and outcome of AKI are assessed.

This is why we require several biomarkers that cover different aspects of AKI. Marker panels could provide us with accurate and detailed information on specific causes, sensitive detection of an acute decrease in kidney function and injury in various renal structures, and quantifying the degree of renal injury. Ideally, these markers also should be mediators of different disease pathways in AKI. Thereby, these markers may lead to a better understanding of the pathologic mechanisms, indicate the etiology of AKI, provide targets for future therapies, and permit monitoring of therapy.

To use the specific marker or combination of markers for the respective diagnostic appropriate question, it is necessary to identify the conditions causing AKI. AKI may be categorized in several different ways. We chose an approach to categorize by phenotype. Because most conditions causing AKI as well as AKI markers are specific to the anatomic sites of renal injury, it seems intuitive to categorize according to this phenotype. Furthermore, most conditions and diseases causing AKI preferably have one specific disease mechanism. Therefore, we used these mechanisms as a second dimension to categorize the conditions causing AKI. Of note, some conditions have several mechanisms and affect more than one anatomic site, whereas AKI in some clinical settings, as in critically ill patients, has numerous causes. This presentation only includes the predominant mechanisms, anatomic sites, and conditions. It is not an attempt to cover the complete and complex picture of AKI, but rather to simplify it. It is limited because the anatomic sites of injury and the mechanisms may vary as AKI persists or progresses. This may occur (eg, in therapy-resistant shock) when initial function changes are superseded by ischemia as the principal mechanism of injury. Further potential dimensions to categorize AKI also were not taken into account to prevent unnecessary complexity and because we considered these of lesser importance for the question of biomarkers. However, AKI also could be categorized by mechanisms of injury (ischemia, ischemia-reperfusion, proinflammation, apoptosis, necrosis, oxidative stress, direct cell toxicity), severity of injury, regeneration, and the time point in the course of AKI.

Our review shows parallel disease pathways present in conditions causing AKI, as in the kidneys in AKI itself. This is shown by the parallels of markers and mediators identified in our molecular interaction analysis. Future research should attempt to differentiate between the two and elucidate which part is systemic and which is derived from renal injury directly. This may aid to further improve diagnostic panels because they are expected to be as specific to the injured organ as possible.

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