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# Genomic Biomarkers for Chronic Kidney Disease

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## Abstract

Chronic kidney disease (CKD) remains a major challenge in nephrology and for public health care, affecting 14–15% of the adult U.S. population and consuming significant health care resources. In the next 20 years, the number of patients with end stage renal disease is projected to increase by 50%. Ideal biomarkers that allow early identification of CKD patients at high risk of progression are urgently needed for early and targeted treatment to improve patient care. Recent success of integrating molecular approaches for personalized management of neoplastic diseases, including diagnosis, staging, prognosis, treatment selection and monitoring, has strongly encouraged kidney researchers to pursue molecular definitions of patients with kidney disease. Challenges for molecular marker identification in CKD are a high degree of cellular heterogeneity of the kidney and the paucity of human tissue availability for molecular studies. Despite these limitations potential molecular biomarker candidates have been uncovered at multiple levels along the genome – phenome continuum. Here we will review the identification and validation of potential genomic biomarker candidates of CKD and CKD progression in clinical studies. The challenges in predicting CKD progression, as well as the promises and opportunities resulting from a molecular definition of CKD will be discussed.

### Keywords

genomic biomarkers; CKD progression; GFR slope; RNA marker panel

## Definition of biomarker & genomic markers

A biological marker (biomarker) is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention(1). These characteristics exist at every level along the genome – phenome continuum, including DNA and/or RNA (genomic biomarkers), protein (proteomic biomarker) or metabolite (metabolic biomarker). The



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proteomic and metabolomic markers in renal injury are reviewed by Drs. Slocum, Heung & Pennathur in this journal issue, and therefore will not be the included in this review.

According to U.S. Department of Health and Human Services, Food and Drug Administrations (http://www.fda.gov/cber/guidelines.htm), a genomic biomarker could be a measurement of the expression, the function or the regulation of a gene. A genomic biomarker can consist of one (single marker) or more (marker panel) DNA and/or RNA characteristics.

## Definition of CKD and CKD progression

According to KDOQI guide lines(2), CKD is diagnosed if either of the following criteria is present for ≥ 3 months or more:

1. Evidence of structural or functional damage of the kidney with or without decreased glomerular filtration rate (GFR), as manifested by any of the following: pathological abnormalities; indicators of kidney damage, including abnormalities in the composition of the blood or urine, or in imaging tests.
2. GFR < 60mL/min/1.73m$^2$ that is present for ≥ 3 months with or without evidence of kidney damage.

A challenge with these CKD definitions is that a significant proportion of patients with CKD in the GFR range of 30–60mL/min/1.73m$^2$ (CKD stage III), do not show further loss of renal function over time. Therefore it becomes essential to evaluate the risk of patients to develop end stage renal disease and for interventional purpose. Progression of kidney disease is defined as either of the following by KDOQI guidelines(2):

1. Decline in the level of kidney function, estimated by measuring GFR, creatinine clearance or serum creatinine, in a patient who has been followed longitudinally with reliable (and comparable) assays of kidney function.
2. Onset of kidney failure, defined by initiation of kidney replacement therapy, either for symptoms or complications of decreased kidney function.

The initiation of kidney replacement therapy seems easy to define, whereas a clear and uniform definition of renal function decline is currently unavailable. A current definition of renal function decline requires a progressive loss of kidney function over a period of time at a rate greater than the “natural” kidney functional loss caused by aging alone. As reviewed by Levey et al.(3), GFR slope seems to be the most appropriate indicator to assess the rate of function decline/CKD progression.

Due to difficulties of modeling GFR over time, many groups use surrogates of the GFR slope or particular indices of renal damage as end points to study CKD progression(4). The surrogates of GFR slope usually include doubling of baseline serum creatinine level and/or the need for renal replacement therapy, a specified relative increase from baseline serum creatinine level, a yearly or monthly decline in GFR, GFR reduction to 50% of baseline, dialysis, kidney transplantation, or graft loss. The specific indices of renal damage include worsening of proteinuria, or development of albuminuria in patients with diabetes. The lack of a unique definition of CKD progression leads to difficulties in comparing the performance of various biomarkers across different studies. In addition, further investigations are needed to address the following questions: 1) does a change in eGFR truly represent a change in kidney function? 2) is there a causal association between GFR change over time and the clinical endpoint?

The ideal molecular markers for CKD or CKD progression, as defined above, should reflect the level of structural and functional damage of the kidney, and / or correlate with the progressive decline of kidney function.

## Genetic candidate markers of CKD

The heritability of GFR and albuminuria(5) and the monogenic kidney diseases, such as congenital nephrotic syndrome of the Finnish type and autosomal dominant polycystic kidney disease (ADPKD), encouraged the search for common variants associated with renal function and kidney disease phenotypes in the general population. Linkage analysis, candidate gene analysis, admixture linkage disequilibrium (6) and in particular genome-wide-association studies (GWAS), contributed greatly to the discover of over 26 candidate loci associated with CKD, which have been reviewed by O'Seaghdha and Fox in their recent paper on genetics of CKD(5). Despite these encouraging findings, determining if these leads can or cannot be developed into genetic biomarkers requires much further investigation. The odds ratios derived from the frequent variants identified by CKDGen and others are too modest (<1.2) to be useful for individual counseling at the current time. However, this did not stop commercial vendors from providing genotyping services that report these associations (i.e. variances in the uromodulin locus and CKD) to consumers. CKD loci identified through GWAS need to be linked to a specific gene variant in a causal manner, and further studies are required to establish the mechanistic link between genetic polymorphism and functional effects. The current studies of the MYH9/APOL1 association with CKD in patients of Sub-Saharan descent revealed many challenges in this field. Initial MALD studies identified multiple kidney disease-associated SNPs in an intron 23 of MYH9 (7, 8), followed by dense mapping localizing a susceptibility hotspot to a genomic area between intron 13 and 15 of MYH9 (9). However, further reports demonstrated that all attributable risk for CKD of the locus was associated with a SNPs in the last exon of the adjacent APOL1 gene, showing a stronger association with non-diabetic CKD and FSGS than MYH9 in African-Americans (10). Current studies are evaluating the functional link of APOL1 to CKD. MYH9 is known to cause monogenetic FSGS and remains an intriguing close neighbor to the SNP tagging APOL1. For a detailed discussion on strategies linking genetic polymorphism to mechanistic insight see review by Keller et al.(11).

In the recent GWAS study carried out by CKDGen consortium, which encompassed over 67,000 individuals from, mostly general population-based international cohort studies, the identified 16 loci together explain only 1.4% of population variability in eGFR (12). This strongly implies that many other genetic loci influencing kidney function exist and need to be identified using novel analytical approaches focusing i.e. on the role of rarer variants for CKD risks (5). Fine mapping of the genomic regions and adequate follow-up with functional studies to establish causal effect are required for these implicated genetic loci.

Toward this direction, Wheeler et al. (13) provided an example of how to combine the application of transcriptional profiling, expression quantitative trait mapping, and selective gene association to further empower candidates. A plausible strategy has been recommended by Keller et al. on how to integrate transcriptional network analysis to bridge the gap between GWAS identified leads and functionality (14), thereby establishing causality.

Altogether, the challenge of applying a genetic marker candidate in routine clinical practice is substantial, but emerging novel technology, increasing interest in this field, international collaborations, ongoing further functional studies and the feasibility to integrate multi-level information will undoubtedly help to overcome the current bottlenecks.

## Tissue RNA candidate markers of CKD

Biomarkers can be discovered using tissue and /or biofluid. Using the tissue manifesting the disease as a resource in the discovery phase allows organ-, tissue- or cell type-specific regulation to be captured. Here we will first review the studies using kidney tissue samples to discover biomarker candidates, and then move on to the studies using biofluid to explore non-invasive biomarker candidates.

As the kidney is a complex three-dimensional structure comprised of over 20 renal cell types, this high diversity and segment-specific gene expression regulation in renal disease can be captured by separating the different segments. The studies we review here were mostly performed using high throughput technology on either micro-dissected (15–19) or laser captured (20–22) biopsy tissues (23) from CKD patients. One study isolated glomeruli using sieve protocol (23).

The current –omics technologies not only reveal marker/marker sets in an unbiased manner, but also describe the signaling pathways/networks that are altered during disease initiation and progression. This approach has been successfully employed in translational medicine for the following reasons: 1) Common complex chronic kidney diseases are rarely caused by a single gene. Therefore, a marker panel, by providing a set of genes, is more likely to capture the complexity of the underlying pathomechanisms than studies focusing a single molecule. 2) A marker panel can provide more comprehensive assessment of treatment effects impacting multiple pathways, as a single biomarker may not reflect clinically important effects of treatment effects (24). A treatment may change the level of a single surrogate biomarker, but this change might not be associated with the hard endpoint progression of CKD. For example, treatment with nonsteroidal anti-inflammatory drugs in patients with nephrotic syndrome has been shown to decrease the CKD surrogate marker, proteinuria, however, this decrease is not a consequence of improved renal function, but rather of a decrease in glomerular perfusion associated with a significant risk of renal failure (25, 26), Such misleading effects of a single biomarker could be significantly mitigated when applying a marker panel capturing multiple aspects of the disease pathophysiology. 3) A diagnostic marker panel reflecting pathophysiology of disease can potentially provide highly promising targets for drugs or small molecules for intervention purposes.

Table 1 summarizes studies that contributed to our understanding of CKD by providing comprehensive intra-renal molecular signatures of CKD. As the most direct outcome, almost all studies revealed significantly differentially expressed genes (DEGs) or differentially expressed micro RNAs (DEmiRNAs) between patients and controls. These genes/miRNAs form a useful pool of candidates that may yield potential biomarkers in further investigations. However, the reproducibility and reliability of markers in some of the studies were limited by small sample size and lack of a validation cohort. Technical confirmation of gene expression, such as quantitative real time PCR (qRT-PCR), or immunohistochemistry staining has been applied in most(15–21), but not all of the studies. Several groups (16–19) have validated their findings using independent patient cohorts, which increased the confidence of identified marker candidates. The following studies are mentioned here for their specific strengths.

As discussed above, biomarker panels add significant power, if they are related to disease pathophysiology and not solely based on correlation with outcome. The work from Henger et al.(27), Schmid et al.(16), and Lindenmeyer et al. (18) provides good examples of using prior knowledge to drive the identification of potential biomarkers. The authors applied targeted analysis on signaling pathways or networks with established association to CKD, such as cell-cell contact, matrix turn-over, cytokine and receptors associated genes (27), nuclear factor-κB (NF-κB) transcriptional programs. Most of their findings are consistent

with previous understanding of the disease process, which might be argued as a limitation, as no novel insights are revealed. However, work from Henger et al.(27) is the first to report the expression of a set of intrarenal transcripts with correlation to the progression of renal disease. Schmid et al.(16) Identified a specific NF-κB promoter module that is activated in the inflammatory stress response of progressive diabetic nephropathy (DN). This finding could lead to elucidation of novel candidates for a potential targeting strategy that share the same promoter module. Lindenmeyer and colleagues found that in contrast to what has been reported in animal models, a decrease of VEGFA expression in human DN may contribute to the progression of the disease(18). As VEGF inhibition is used to treat diabetic retinopathy this has significant potential clinical implication. In addition to dichotomizing the samples into control and patient groups, which many of the studies did, Lindenmeyer and coworkers also evaluated the relationship between the expression value of genes with clinical parameters (proteinuria and GFR) on a continuous scale. A significant and inverse correlation between VEGFA and proteinuria level, as well as EGF and proteinuria level ($r=-0.34$, $P<0.05$), was found, suggesting that decreased expression is associated with increased proteinuria.

Obtaining human renal tissue for gene expression analysis remains a significant challenge. Currently, most researchers use either frozen biopsy sections (20, 22) or RNAlater preserved biopsies at low temperature (15–19) for sample preparation to prevent loss and degradation of RNA. However, biopsies taken for routine diagnostic and staging purposes exist mainly as formalin-fixed, paraffin-embedded (FFPE) specimens. A large amount of clinical data and histopathological alterations could be correlated with comprehensive gene expression profiles, if FFPE tissue could be used for high throughput expression profiling. Extraction of RNA from FFPE tissue has been a difficult process due to chemical crosslinking of RNA and subsequent fragmentation of RNA during isolation in this material (28, 29) and thus has been used primarily for focused qRT-PCR to date (30, 31). A recent study from Hodgin and coworkers (21) demonstrates that gene expression profiling can be achieved with an exceedingly small quantity of sample available from archived FFPE renal biopsies. They identified transcripts that are significantly and differentially regulated between biopsy-proven idiopathic classic focal segmental glomerulosclerosis (FSGS) + collapsing FSGS and minimal change disease (MCD) + normal controls from laser captured glomeruli of archived material. The authors confirmed that genes that are important to the structure and function of the podocyte slit diaphragm, such as nephrin, podocin, synaptopodin, FAT tumor suppressor homolog 1, MAGI-2, and tight junction protein 1, which showed significantly reduced expression in the FSGS group versus MCD + Normal. The expression data further supports the hypothesis that FSGS might be the consequence of podocyte loss and dysregulated podocyte phenotype. The transcripts with altered expression between idiopathic ‘classic’ FSGS and collapsing FSGS will allow definition of mechanisms specific for the two different subtypes of FSGS. In addition, this study provides a resource for biomarker discovery and validation. Further validation in an expanded cohort will be necessary to test the diagnostic potential of the specific candidates as well as exploring novel candidates using this strategy.

A prognostic RNA marker set for CKD has yet to be reported mainly due to the lack of CKD patient cohorts with both gene expression data and longitudinal clinical follow up data available. Recent work from our group (19) tackled this issue by taking advantage of a prospective unbiased discovery study in a mouse model for CKD, and then validating the primary findings in human CKD patients. This strategy not only allowed our group to bypass the limitation caused by restricted human tissue availability, but also provided an experimental system to predict disease progression. Transcript levels of candidate genes were evaluated in mouse models in one kidney removed by uni-nephrectomy in 2 week-old mice. The remaining kidney was used to score the degrees of glomerulosclerosis, tubular

atrophy and interstitial inflammation at 4 weeks of age. Using this approach we were able to provide markers that are associated with renal function and subsequent disease progression in mice. These murine markers were then evaluated in humans by correlating with cross-sectional eGFR in human cohorts. Among the 10 RNA markers (AXL, BGN, COL6A1, CREB3, DKK3, ITGB5, NCF2, S100A6, SLC13A3, and MPV17L), protein signatures of 8 genes were further evaluated by immunohistochemistry staining. Four of the genes (S100A6, SLC13A3, BGN, and NCF2) were able to distinguish mice with progressive kidney phenotype from those without, and separated patients with stage III/IV from CKD stage I/II when stained in archival human biopsy samples. Again, like many other studies, further clinical development and longitudinal studies in expanded cohorts are required to move these promising candidates further toward clinically applicable biomarkers.

Most studies used micro-dissected biopsy tissue, thereby addressed the compartment-restricted injury. Woroniecka et al.(15)compared expression profile of DN patients in glomerular and tubular compartment in parallel, identified common and compartment-specific regulation in DN. Only a small number (n=330, about 18%) of overlapping transcripts were differentially regulated in both glomeruli and tubuli, most transcripts showed a compartment-specific regulation. This study not only supports the strategy for applying micro-dissection on kidney biopsies for gene expression analysis on CKD patients, but also provides important information on the mode and site of injury for targeted intervention.

Emerging interest in using miRNA as potential biomarkers in kidney tissue (32–34) has revealed several promising candidates. Work from Dai et al. (34) compared the miRNA expression in cortex of control versus patients with lupus nephritis (LN), and identified differentially expressed miRNAs. Additional studies of independent large cohorts are needed to validate these candidate markers. Another group performed qRT-PCR analysis on several selected miRNAs on total renal biopsy tissue. They identified differentially expressed miRNAs and miRNAs that are correlated with kidney function or proteinuria in IgAN (32) and hypertensive nephropathy (HTN) (33). However, tissue heterogeneity is not addressed due to the use of renal tissue homogenates in this study.

In addition to marker panels reviewed above, specific RNA markers derived from mechanistic insight into CKD may also be viable candidate markers. Among candidate pathways mediating CKD progression, the transforming growth factor-β (TGF-β) pathway is prominent because it controls principle pathobiological processes associated with CKD, including fibrogenesis, apoptosis, epithelial-to-mesenchymal transition (EMT), and inflammation (35, 36). TGF-beta1 overexpression in the serum has been reported to be more frequent in African American ESRD patients (n=56) compared to white ESRD (n=42) patients, suggesting it might be involved in the increased prevalence of renal failure (37). In the kidney, Yang et al. demonstrated that the levels of glomerular TGF-beta1 mRNA in renal biopsies correlated with the degree of glomerulosclerosis in 57 patients with CKD (MGN, SLE, DN, MCD and IgAN), irrespective of the disease causing ESRD(38). A correlation between glomerular TGF-beta1/beta-actin mRNA ratio with clinical parameters (urinary protein excretion and creatinine clearance) was not observed in this study. Another group reported that TGF-beta1 mRNA in tubulointerstitial-, but not in the glomerular compartment, correlated with the rate of GFR change in a cohort of 37 CKD patients, suggesting a prognostic value of TGF-beta1 mRNA (39). However, longer follow up time and independent cohort are needed for validation.

EMT has been suggested to play a role in progression of CKD on multiple levels, including podocyte damage and loss in DN (40) and FSGS (41). The RNA level of *FSP1* as a marker of EMT was found to be significantly increased in laser captured glomerular compartment of

DN or FSGS patients, in comparison with MCD patients. Immunohistochemistry staining confirmed the protein level of FSP1 in podocytes of DN or FSGS patients to be associated with more severe clinical and pathological findings of kidney injury.

The remaining challenges for tissue based mRNA marker or marker panel include: 1) limitation of the material and sample size; 2) lack of larger independent patient cohorts with longitudinal follow up data for validation; 3) invasive nature of tissue procurement. Efforts are underway to establish large patient cohorts with longitudinal follow up clinical data (described below), and will allow to test if tissue based molecular markers can also be informative in non-invasive biofluids like blood and urine.

## RNA candidate markers in biofluids

Not all tissue can be readily and repeatedly sampled without risk, whereas most biofluids can be easily obtained with minimal risk in clinical practice. Below we review the RNA marker candidates assayed in peripheral blood leukocytes and urine sediment.

**RNA candidate markers in leukocytes—**Leukocytes have been reported to play a role in initiation, development and progression of many renal diseases (42), such as IgAN, LN, and anti-neutrophil cytoplasmic autoantibody (ANCA) associated glomerulonephritis. Many studies therefore evaluated the potential of using RNA profiling of peripheral blood leukocytes or lymphocytes as a resource to develop candidate markers for CKD (42–46) (Table 2). In one of the earliest kidney disease related genome wide expression studies in leukocytes, Preston and coworkers[37] identified a set of 15 genes in circulating leukocytes, whose expression is significantly correlated with disease activity in IgAN patients, but not in patients with ANCA glomerulonephritis or LN. The authors also applied multiple regression analysis and defined a mathematical model for approximation of the serum creatinine concentration from leukocyte transcript signatures (43, 44). Subsequently, with a significantly larger patient population, Alcorta and co-workers (45) also identified and validated genes that are able to distinguish different patient populations, including healthy donor, ANCA, LN and rheumatoid arthritis (RA) patients. They then took one further step, comparing the expression pattern with in vitro-activated leukocyte subtypes from healthy donors, and discovered that genes with altered expression in ANCA are mainly expressed by neutrophils, whereas genes with altered expression in LN are expressed mainly in activated monocytes and T cells.

Recently, Cox et al. (44) reported that the leukocyte genes whose expression could distinguish IgAN patients and controls are mainly involved in WNT-β-catenin and PI3K/Akt pathways. Separating the peripheral blood mononuclear cells (PBMCs) into subpopulations (T lymphocytes, B lymphocytes and monocytes) helped to reveal that monocytes contributed to the hyperactivation of the WNT pathway.

All three studies were able to link leukocyte gene expression with renal outcomes. The challenge with this approach will be to define whether leukocytes profiles are linked to the renal phenotypes or are associated with underlying inflammatory processes affecting both leukocyte profiles and renal outcomes by independent mechanism. To answer these questions, important extra steps will be to establish a solid and specific correlation of these makers with kidney function and to investigate the expression of these genes or their downstream signaling pathways in the kidney.

**RNA candidate markers in urinary sediment—**Analysis of urinary sediment by argon laser flow cytometry allowed the identification and quantification of various cell types, including red blood cells, white blood cells, squamous epithelial cells, transitional epithelial and renal tubular cells, bacteria, hyaline and inclusional casts, yeast-like cells, crystals and

spermatozoa (47). Subsequently, urinary excretion of podocytes in health and renal disease has been evaluated many groups for its utility to assess glomerular diseases (48–52). The feasibility of isolating and quantifying RNA of specific genes in urine cells was established by Li et al., while pursuing a non-invasive approach to diagnosing acute renal rejection of allografts(53). Since then the urine sediment has become a non-invasive resource to develop biomarker candidates for chronic kidney diseases including LN, IgAN, DN and FSGS (Table 3). RNAs of the following categories of molecules have been tested in urinary sediment of CKD patients: 1) cytokines, chemokines and growth factors (54–56); 2) podocyte specific genes (57–60); 3) transcriptional factors (61, 62) and 4) extracellular matrix genes(63). One common problem in this field is the lack of validation in an independent cohort with adequate sample size, which impaired generation of reliable and reproducible results. In the above cited qRT-PCR studies it is critical to use multiple robust reference (or housekeeping) genes as the denominator for quantification to obtain expression data reflecting alterations in the target genes and not in the 'housekeeping' genes. Sato et al. (64) used a kidney-specific reference transcript that is robustly expressed even in the end-stage kidney. This allowed correction for variations in RNA quality and degradation between samples, for efficiency of the qRT-PCR step, and for the proportion of RNA recovered from kidney versus other urinary RNA sources. Using a diphtheria toxin receptor (hDTR) transgenic rat model, Sato and coworkers (64) demonstrated that a single episode of podocyte injury can trigger glomerular destabilization, resulting in persistent podocyte loss and further progress of kidney damage. The rodent model was supported by parallel human studies showing that biopsy-proven glomerular injury is associated with increased urinary podocin (NPHS2): Aquaporin 2 (AQP2) and nephrin (NPHS1): AQP2 molar ratios. This study suggests that analysis of podocyte mRNAs in urine sediment, using appropriate reference parameters, may be a useful clinical tool for the diagnosis and monitoring of glomerular disease progression.

In general, while the value of using RNAs in urine sediment to mark kidney injury has been demonstrated, the correlation of these RNA changes with intra-renal events and possible pathomechanisms needs to be carefully investigated.

## Outlook: from invasive tissue RNA markers to non-invasive protein markers

As mentioned in the previous section, each biomarker strategy currently employed in nephrology has its intrinsic advantages and limitations. The value of urinary protein as a prime resource for biomarker discovery is well-supported in the literature. As 70% of proteins excreted in the urine are of renal origin, urine should be the ideal body fluid for discovery of non-invasive biomarkers for kidney disease. However, approximately 30% of urinary proteins are plasma-derived (65), and thus may have their origin in organs other than kidney. Integrating the strengths of tissue based invasive discovery and urine based non-invasive validation strategies would enhance focus on the most promising candidates for kidney disease-specific biomarkers. In addition, information on regulation of compartment- and/or cell type–specific transcripts in CKD is important for identifying the injuries to specific kidney compartments and distinct cell types, thereby providing increased sensitivity and specificity.

## Current challenges and future opportunities

Currently, none of the above mentioned biomarker candidates are entirely validated and ready for routine clinical use. The biomarker candidates for predicting CKD progression is limited due to both the difficulties in defining progression of chronic kidney disease and the lack of longitudinal clinical study cohorts. Thoughtfully-designed, large, multicenter and prospective studies are needed to validate these potential candidates. Several concerns should be considered for CKD biomarker discovery:

1. Discovery studies need to define a priori of the clinical parameters to be predicted and then select the optimal cohorts with both biosamples and clinical outcome data (i.e. markers discovered in patients with acute renal injury (AKI) may not apply to patients with CKD; differentially expressed genes identified in cross-sectional studies may not be able to predict disease progression or outcome).
2. Biomarker statistics is rapidly developing and expert guidance is essential from designing studies with adequate power to defining thresholds and cut-offs for prediction, and effect of covariates. Including an independent validation or test cohorts into the study design will help to address the problem of overfitting large data sets on small cohort and thereby improve robustness of markers panels in subsequent studies.
3. Prospective study cohorts are needed to develop biomarkers to predict longitudinal renal outcome. High quality and sufficient details of clinical information are often the rate limiting steps in confirming the clinical utility of a biomarker.

These challenges in defining clinically useful biomarkers for CKD and CKD progression are currently addressed by several prospective cohort studies and a concerted effort by the NIDDK Chronic Kidney Disease Biomarker Discovery and Validation Consortium. In addition, improved sample collection, handling and storage, advances in proteomics technology, analytical methods and high resolution phenotype set the stage for solid discovery. Increasing availability of clinical study cohorts (66–72) (Table 4) for molecular analysis provides a resource to discover novel biomarkers and validate previously defined candidates. Many large longitudinal cohorts, such as the Chronic Renal Insufficiency Cohort (CRIC) and the German Chronic Kidney Disease Cohort (GCKD) enrolling large groups of patients (n = 3939 and n = 3549, respectively), are ideal for discovering and evaluating markers that are able to predict CKD progression, as they provide abundant clinical phenotypes over time which can be associated with their molecular phenotype at time of enrollment. Cohorts defined for molecular analysis of renal disease are being established, including, among others, the Clinical Phenotyping and Resource Biobank Core (C-PROBE), and the Nephrotic Syndrome Study Network (NEPTUNE). These networks focus on generating a resources encompassing high-resolution clinical phenotypes with a rich biospecimen repository (not only including blood and urine compartments, but also renal tissue and digital histology archives). These cohorts will allow researchers to link non-invasive biomarker candidates in urine or plasma with intra-renal pathology and pathophysiology. They are designed to serve as resources for the renal research community in defining the molecular underpinning of human renal disease (for ancillary study mechanism see https://rarediseasesnetwork.epi.usf.edu/NEPTUNE/professional/join/index.htm). The NIDDK biorepository serves as a central reference point for biosamples and data sets of established cohorts (https://www.niddkrepository.org/niddk/jsp/public/kidney2.jsp).

In conclusion, the accumulated knowledge from basic science, clinical research and translational medicine, together with the growing resources of patients' bio-specimens and clinical information from large multicenter study cohorts will facilitate the identification of biomarkers for CKD and CKD progression. Molecular biomarkers will provide novel information to stratify patients into mechanistically defined subgroups on top of our current knowledge gained from decades of clinical experience. This progress in the biomarker field will inevitably lead to a better understanding of pathomechanisms of CKD and will contribute substantially to targeted, rational intervention in CKD patient care.

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## Abbreviations

| | |
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| **CKD** | chronic kidney disease |
| **ADPKD** | autosomal dominant polycystic kidney disease |
| **FFPE** | formalin-fixed, paraffin-embedded |
| **GFR** | glomerular filtration rate |
| **GWAS** | genome-wide-association studies |
| **KDOQI** | Kidney Disease Outcomes Quality Initiative |
| **NF-κB** | nuclear factor-κB |
| **qRT-PCR** | quantitative real time PCR |
| **PBMC** | peripheral blood mononuclear cell |

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**Table 1**

Studies investigated RNA candidate markers in kidney biopsies of CKD patients.

| Authors | Study Characteristic | Tissue | Marker Panel | Discovery Cohort | Study Outcomes | Verification & Validation |
|---|---|---|---|---|---|---|
| Henger et al.[27] | targeted analysis on cell-cell contact, matrix turnover, cytokines, chemokine and their receptors | whole renal tissue | 6Ckine, MMP-9, MMP-3, MMP-7, Integrin-β4, Pleiotrophin & urokinase R | HN (n=9), CTRL (n=3) | DEGs that can effectively separate patients according to clinical outcome. | qRT-PCR for 9 genes in biopsies of independent CKD cohort (n=32) |
| Peterson et al.[20] | unbiased | glomeruli | 88 ↑ and 89 ↓ in LN vs. CTRL | SLE (n=11), CTRL (n=2), LN (n=3), CTRL (n=2) | DEGs | qRT-PCR for CTSC, ISG15 and MX1 from samples of the same cohort |
| Baelde et al.[23] | unbiased | glomeruli | 96 ↑ and 519 ↓ in DN vs. CTRL | DN (n=2), CTRL (n=2) | DEGs | RT-PCR on NPHS1, VEGF, TGF-b1 in independent group: DN (n=5), CTRL (n=8) |
| Schmid et al.[16] | unbiased + targeted analysis of NF-kb pathway analysis | tubulo-interstitium | 1,349 genes differentially regulated among CTRL, MCD and DN | DN (n=13), CTRL (n=7), MCD (n=4) | DEGs Identified a specific NF-KB promoter module activated in the inflammation stress response | qRT-PCR on CXCL10, RANTES, EDN1 and IFNB1 in independent cohort: DN (n=22), CTRL (n=17) |
| Lindenmeyer et al.[18] | targeted common hypothesis analysis | tubulo-interstitium | 38 ↑ and 11 ↓ in DN vs. CTRL | DN (n=11), CTRL (n=3), MCD (n=4) | DEGs altered pathways, correlation of VEGF-A & EGF with proteinuria | qRT-PCR for VEGFA, EGF, FN in original cohort plus additional DN (n=17), additional CTRL (n=4), additional MCD (n=3); IHC for VEGFA, EGF |
| Bennett et al.[22] | unbiased | glomeruli | 124 ↑ and 4 ↓ in FSGS vs. CTRL | FSGS (n=4), CTRL (n=3) | DEGs | IHC staining of CD24 and CXCL1 |
| Dai et al. [34] | unbiased | cortex | 36 ↑ and 30 ↓ in SLE vs. CTRL | SLE (n=5), CTRL (n=3) | DEmiRNAs | qRT-PCR for 2 of the miRNAs in the same cohort |
| Wang et al.[32] | targeted analysis | whole renal tissue | miR-200C↓ miR-141↑, miR-205↑ in IgAN vs. CTRL and GS | IgAN (n=43), CTRL (n=20) GS (n=15) | identified GFR, proteinuria and GFR decline correlated with miRNAs | |
| Wang et al.[33] | targeted analysis | whole renal tissue | miR-200a&b, miR-141, mir-429, miR-205 and Mir-192 all ↑ in HTN | HTN (n=34), CTRL (n=20) | identified GFR and proteinuria correlated with miRNAs | |
| Ju et al.[19] | unbiased discovery in mouse model + targeted validation in patients | tubulo-interstitium | NCF2, BGN, ITGB5, COL6A1, S100A6, SLC13A3, DKK3, MPV17L, AXL, CREB3 | Tgfb1 transgenic mouse model | disease progression related genes identified in mouse model are validated in two cohorts of CKD patients by array & IHC | HTN (n=19), IgAN (n=21), MCD (n=1), TMN (n=6), TN (n=3) IHC in independent CKD cohort (n=20) |
| Hodgin et al.[21] | unbiased | glomeruli | 316 genes | FSGS (n=8), COLL (n=6), MCD (n=7), CTRL (n=9) | DEGs between FSGS +COLL vs. MCD+CTRL;FSGS vs. COLL; MCD vs. CTRL. altered GO categories | qRT-PCR for 10 genes from samples of the same cohort |

| Authors | Study Characteristic | Tissue | Marker Panel | Discovery Cohort | Study Outcomes | Verification & Validation |
|---|---|---|---|---|---|---|
| Neusser et al.[17] | targeted analysis of HIF target genes | glomeruli | 290 DEGs of 476 HIF target genes | NSC (n=18), TN (n=4) | DEGs can distinguish NSC from CTRL. | qRT-PCR of LOXL2, FN1, CXCR4 was confirmed in an independent cohort NSC (n=13), FSGS (n=18), IgAN (n=15); MCD (n=12) LD (n=6) |
| Woroniecka et al.[15] | unbiased | glomeruli | 1,700 differentially expressed transcripts | DN (n=9), CTRL (n=13) | DEGs altered pathways | qRT-PCR and IHC for top gene C3 |
| | | tubulo-interstitium | 1,831 differentially expressed transcripts | DN (n=10), CTRL (n=12) | DEGs altered pathways | qRT-PCR and IHC for top gene C3 |

Abbreviations: control (CTRL), differentially expressed genes (DEGs), differentially expressed miRNAs (DEmiRNAs), diabetic nephropathy (DN), focal segmental glomerulosclerosis (FSGS), collapsing FSGS (COLL), Gene Ontology (GO), hydronephrotic (HN), hypertensive nephropathy (HTN), IgA nephritis (IgAN), immunohistochemistry (IHC), lupus nephritis (LN), minimal change disease (MCD), nephrosclerosis (NSC), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), thin basement membrane disease (TMN), tumor nephrectomy (TN).

**Table 2**

Studies investigated RNA candidate markers in leukocytes of CKD patients.

| Authors | Study Characteristic | Marker Panel | Discovery Cohort | Study Outcomes | Verification & Validation |
|---|---|---|---|---|---|
| Preston et al.[43] | unbiased | 15 genes significantly correlated with IgAN activity, but not ANCA | IgAN (n=9), CTRL (n=12), ANCA (N=5), FSGS (n=5), MCD (n=6) | DEGs' signature reflects kidney function. | qRT-PCR in independent cohort: IgAN (n=15) & CTRL (n=20). |
| Alcorta et al.[45] | unbiased | 16 genes correlated with clinical activity of ANCA | ANCA (n=22), SLE (40), RA (n=26), CTRL (n=28) | Identified DEGs that can distinguish donor source. | qRT-PCR in enlarged cohort: ANCA (n=69), LN (n=48) & CTRL (n=49) |
| Cox et al.[44] | unbiased | 21 genes separating IgAN from CTRL. | IgAN (n=12), CTRL (n=8), | DEGs are involved in WNT-b-catenin and PI3K/Akt pathways | qRT-PCR and western blot in IgAN (n=16) & CTRL (n=16). Subpopulation study in a cohort of IgAN (n=10) & CTRL (n=10). |

Abbreviations: antineutrophil cytoplasmic autoantibody-positive glomerulonephritis (ANCA).

**Table 3**

Studies investigated RNA candidate markers in urine sediment of CKD patients.

| Authors | Marker panel | Discovery cohort | Study duration | Study outcome(s) |
|---|---|---|---|---|
| Szeto et al.[59] | NPHS1/SYNPO ratio, NPHS2/ SYNPO ratio | DN (n=9), IgAN (n=10), MCD(n=5), MN(n=5), DN with microalbuminuria (n=10), CTRL (n=9) | average 23 months | Both ratios significantly correlated with rate of GFR decline |
| Szeto et al.[55] | TGF-β1, CTGF, HGF, VEGF, MCP1, FN, CASP3,ASMA, COL-1, COL-3, COL-4 | IgAN (n=12), GS (n=17), CTRL (n=10) | cross sectional | TGF-β1 correlates with GFR, histological damage. CTGF and collagen 1 correlates with GFR decline |
| Szeto et al.[54] | TGF-β1, CTGF, HGF, VEGF, MCP1, FN, CASP3, ASMA, COL-1, COL-3, COL-4 | CKD (n=131) | average 27 months | CTGF, MCP1, and Collagen III correlate with histological damage. HGF predicts primary end point (doubling of SCr. or enter ESRD). |
| Wang et al.[58] | NPHS1, NPHS2, WT1, ACTN4, SYNPO | DN (n=21), CTRL (n=9) | average 25 months | Differential expression between patients and CTRL. Correlation between gene expression and clinical phenotype |
| Tsugawa et al.[62] | T-bet, GATA-3, FOXP3, RIG1 | LN (n=14), IgAN (n=13), CTRL (n=12) | cross sectional | Expression differs between LN and IgAN. |
| Kwan et al.[56] | IL-17, IL-27, IL-23, ROR-γ | LN active (n=23), LN remission (n=25), SLE no history of kidney involvement, CTRL (n=8) | cross sectional | Correlation between gene expression and clinical phenotype |
| Wang et al.[61] | FOXP3 | LN active (n=25), LN inactive (n=17), CTRL (n=7) | cross sectional | FOXP3 is up-regulated in patients with active LN; level of expression is correlated with disease activity. |
| Sato et al.[64] | NPHS1/AQP2 ratio, NPHS2/AQP2 ratio | hDTR transgenic rat | 56 days in rats, cross sectional in CKD patients (n=4) | Biopsy-proven glomerular injury is associated with increased urinary NPHS2/AQP2 and NPHS1/AQP2 molar ratios |
| Zheng et al.[57] | NPHS2, CD2AP, ACTN4, SYNPO, PODXL | DN (n=51), CTRL (n=13) | cross sectional | Differential expression between patients and CTRL. Correlation between gene expression and clinical phenotype |
| Zheng et al.[63] | ACTA1, FN, FSP1, MMP9 | DN (n=44), CTRL (n=12) | cross sectional | Differential expression between patients and CTRL. Correlation between gene expression and clinical phenotype |
| Navarro-Munoz et al.[60] | B7-1, NPHS1, NPHS2, SYNPO | MCD (n=11), FSGS (n=11), MGN (n=17), MPGN (n=7), IgAN (n=23), CTRL (n=14) | cross sectional | B7-1 and NPHS1 may distinguish MCD and FSGS |

Abbreviations that have not been defined in the previous tables: focal glomerulosclerosis (GS), human diphtheria toxin receptor (hDTR), membranous glomerulonephritis (MGN), membranous nephropathy (MN), membranoproliferative glomerulonephritis (MPGN). Serum Creatinine (SCr)

**Table 4**

Multi-center study cohorts with biospecimens available for chronic kidney disease-related biomarker research.

| Study | Participants | Enrollment Status | Enrollment (or Target) | Biospecimen available | Geographic Region | References |
|---|---|---|---|---|---|---|
| **AASK** | African Americans, ages 18–70, diastolic BP ≥ 95 mm Hg, and GFR 20–65 mL/min/1.73 $m^2$ | Completed | 1094 | Buffy coat, serum, urine | US | Sika et al.[66] |
| **CRIC** | Racially diverse, ages 21–74, age-based eGFR 20–70 mL/min/1.73 $m^2$ | Completed | 3939 | Blood, urine | US | Lash et al. [67] |
| **EDIC** | Ages 13–39 years, with type 1 diabetes for 1–15 years. | Complete | 1274 | DNA, plasma, serum, urine | North America | NIDDK Central Repository (11/28/11): https://www.niddkrepository.org/niddk/home.do |
| **GoKIND** | Type 1 diabetes ≥ 10 years and severe DN. Type 1 diabetes ≥ 15 year and normoalbuminuria | Completed | 3079 | DNA, urine, plasma, serum | US | Mueller et al. [68] |
| **MDRD** | MAP ≤ 125 mmHg, GFR 13–55 ml/min/1.73$m^2$ | Completed | 840 | Buffy coat, plasma, serum, urine | US | NIDDK Central Repository (11/28/11): https://www.niddkrepository.org/niddk/home.do |
| **MMCKD** | Caucasians, ages 19–65, and non-diabetic CKD stage 1–5 | Completed | 227 | Blood, urine | Europe | Fliser et al.[69] |
| **CPROBE** | CKD stage I–IV | Ongoing | 635 (1500) | Biopsy, blood, urine | US | http://kidneycenter.med.umich.edu/cprobe |
| **CKID** | Children, ages 1–16 years, and eGFR 30–90 mL/min/1.73$m^2$ | Ongoing | 830 | Blood, urine | US | Furth et al.[72] |
| **FIND** | Ages ≥ 18 years, and diabetes or diabetic nephropathy. | Ongoing | 4846 | DNA, serum, urine, whole blood | US | NIDDK Central Repository (11/28/11): https://www.niddkrepository.org/niddk/home.do |
| **NEPTUNE** | Nephrotic syndrome, a planned kidney biopsy, and proteinuria ≥ 500 | Ongoing | 142 (450) | Biopsy, blood, urine | North America | https://rarediseasesnetwork.epi.usf.edu/NEPTUNE/studies/6801-all.htm (11/28/11) |

| Study | Participants | Enrollment Status | Enrollment (or Target) | Biospecimen available | Geographic Region | References |
|---|---|---|---|---|---|---|
| | mg/24hr within 3 months of enrollment. | | | | | |
| **CKD-JAC** | Japanese and Asians living in Japan, ages 20–75, and eGFR 10–59 ml/min/1.73m$^2$ | Completed | 3084 | Plasma, Urine | Japan | Imai et al.[70] |
| **ERCB** | Renal disease with kidney biopsy | Ongoing | >2500 | Biopsy | Europe | http://www.forschungsportal.ch/unizh/p9291.htm (11/28/11) |
| **GCKD** | eGFR 30–60 ml/min/1.73 m$^2$ or overt proteinuria and eGFR > 60 ml/min/1.73m$^2$ | Ongoing | 3549 (5000) | Blood, Urine | Germany | Eckardt et al. [71] |

Abbreviations: African American Study of Kidney Disease and Hypertension (AASK), Modification of Diet in Renal Disease (MDRD), Chronic Renal Insufficiency Cohort (CRIC), Genetics of Kidney Disease in Diabetes (GoKIND) Study, Mild to Moderate Kidney Disease Study (MMKD), Clinical Phenotyping Resource and Biobank Core (C-PROBE), Chronic Kidney Disease in Children (CKID) Study, Epidemiology of Diabetes Interventions and Complications (EDIC), Family Investigation of Nephropathy of Diabetes (FIND) Consortium, Nephrotic Syndrome Study Network (NEPTUNE), Chronic Kidney Disease Japanese Cohort (CKD-JAC), European cDNA Bank (ERCB), German Chronic Kidney Disease (GCKD) Study.

Mean arterial pressure (MAP), Urinary albumin concentration (UAC).