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CKJ REVIEW

Kidney and urine cell transcriptomics in IgA nephropathy and lupus nephritis: a narrative review

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ABSTRACT

This narrative review sheds light on the use of transcriptomics in the analysis of kidney biopsies and urinary cell samples from patients with immunoglobulin A nephropathy or lupus nephritis. The conventional methods of examining kidney biopsy through light microscopy, immunofluorescence and electron microscopy provide valuable clinical information for diagnosis and prognosis but have some limitations that transcriptomics can address. Some recent studies have reported that kidney transcriptomics has uncovered new molecular biomarkers implicated in the inflammatory process induced by the deposition of circulating immune complexes in the investigated kidney diseases. In addition, transcriptomics applied to urinary cells mirrors the inflammatory process that occurs in the kidney. This means that we can study urinary cell transcriptomics in clinical practice to diagnose the stage of the inflammatory process. Furthermore, the transcriptomics of urinary cells can be used to make therapy decisions during patient follow-up to avoid the stress of a second kidney biopsy. The studies analyzed in this review have a significant limitation. Biomarkers have been identified in small cohorts of patients but none of them has been validated in independent external cohorts. Further prospective studies in large cohorts of patients are necessary for accurate and complete validation. Only after that can these biomarkers be widely used in clinical practice.

Keywords: IgA nephropathy, kidney, lupus nephritis, transcriptomics, urine

INTRODUCTION

Circulating immune complexes in the blood of patients are deposited in kidney tissue causing various types of glomerulonephritis which are characterized by active and chronic glomerular and tubulo-interstitial lesions. Kidney biopsy is the gold standard for diagnosing human glomerulonephritides; however, it can also be used for research purposes in the study of these diseases.

Examination of a kidney biopsy using light microscopy, immunofluorescence and electron microscopy provides accurate diagnostic and prognostic information; however, it has some critical limitations because (i) patients with similar histological lesions can have varied responses to treatment, even when similar protocols are followed in randomized clinical trials; (ii) histologic class can change overtime and repeated kidney biopsies during the course of the disease are needed to keep track of these changes; and (iii) the histologic picture is a static image that captures conditions at a specific moment but does not capture patient-specific active biologic pathways.

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Kidney transcriptomics highlights the molecular mechanisms underpinning the distinct histopathological lesions thus revealing heterogeneity of renal lesions with more clarity than other methods [1]. Furthermore, kidney transcriptomics offers the possibility of discovering new molecular biomarkers in the kidney that can be measured in a patient's urine to track active pathologic processes in real-time and monitor changes due to treatment.

KIDNEY TRANSCRIPTOMICS METHODS

Over the past two decades, various approaches have been taken in kidney transcriptomics studies focusing either on the entire transcriptome or on individual biomarkers [2].

Gene transcriptome obtained via microarray chips demonstrates many up-regulated and down-regulated genes. After extensive selection and filtering in a training set, genes are selected and validated in a testing cohort of patients using the area under the curve method. The gene data set is then used in external validation studies involving independent cohorts of patients. A biopsy transcriptome has therefore been developed and can be used in the clinical practice [3].

Next-generation RNA sequencing (RNA-seq) provides exceptional sensitivity and resolution in quantitative RNA analysis, surpassing microarrays [4]. However, bulk RNA-seq does not offer the necessary resolution to determine which cell population drives gene expression. Nevertheless, advanced computational methods can identify the specific cells playing a major role in gene expression.

Kidney transcriptome, studied via single-cell RNA seq (scRNA-seq) following laser capture microscopy which separates the glomeruli from the tubulointerstitium, is an attractive alternative method for identifying the cell types involved in the immune-inflammatory process [5]. However, single-cell transcriptomics of dissociated renal tissue disregards the heterogeneity of glomerular injury. Furthermore, scRNA-seq requires tissue dissociation, which complicates the linking of transcriptomes to specific glomeruli with defined injury states and is limited by the poor recovery of some cell types.

The possibility of studying single cells in kidney tissue enables the analysis of cell-to-cell variability. The limitation of this technique is that it requires fresh tissue samples, which can be challenging to obtain, such as additional renal core for research analysis. An alternative technique that reduces cell stress is single nucleus RNA-seq (snRNA-seq) in which nuclei are isolated from cells for sequencing [6]. However, transcriptomic findings can also be evaluated using immunohistochemistry to locate protein-related genes in kidney compartments and at the single-cell level. Furthermore, combining transcriptome and systems pharmacology can identify drug targets and guide precision medicine in a clinical setting.

Isolated glomeruli and tubulointerstitium pooled separately for molecular analysis fail to account for heterogeneity across individual biopsy samples. This limitation has been overcome by the recent digital spatial transcriptomic technique which captures tissue morphology and spatial transcriptomics on a single tissue section [7].

Digital spatial transcriptomics is a cutting-edge technology that enables the high-throughput measurement of gene expression at a single-cell resolution within a tissue. It combines tissue imaging and single-cell sequencing to create a comprehensive map of gene expression within a tissue, providing valuable insights into cellular heterogeneity and cellular function. This technique allows cells to be kept in their natural environment without inducing stress which occurs when cells are isolated from glomeruli and tubulointerstitium. Furthermore, this technique provides information on the anatomical localization and intercellular interactions involved in the pathologic process. This technique reveals the heterogeneity of glomeruli between and within biopsies by capturing individual glomeruli. Digital spatial transcriptomics overcomes this challenge of heterogeneity by providing *in situ* transcriptomic and protein expression within the context of the histologic pattern. Interestingly, the ability to detect different gene expression patterns in glomeruli at different stages of the inflammatory process allows the detection of the genes that are sequentially involved in the progression of renal damage. Finally, the possibility of applying this technique in archived kidney biopsies from randomized clinical studies or prospective longitudinal studies offers the opportunity to gather significant amounts of valuable information [8].

TRANSLATIONAL ASPECTS

Several transcriptome methods can be applied to identify and quantify gene expression patterns in glomerulonephritis. A translational approach that is suitable for clinical studies is RNA extraction from whole formalin-fixed and paraffin-embedded (FFPE) or fresh frozen (FF) kidney tissue (Fig. 1). Samples are first evaluated for RNA integrity and quality before being used for transcriptomic studies. Gene expression data analysis is then performed using various tools, including commercially available microarray plates or more sophisticated methods such as RNA-seq. These techniques generate a list of up-regulated and down-regulated genes that must be validated using real-time polymerase chain reaction (PCR). The next step is to assign biological functions to the differentially regulated genes using Gene Ontology, pathway analysis and functional networks analysis. Gene network analysis constructs gene pathways represented by nodes (genes) and edges (the biological relationship between genes), and computational methods are then used to highlight the genes prevalently involved in tissue damage. Proteins related to these genes can be investigated by immunohistochemistry or immunofluorescent technique and confocal microscopy of kidney tissue. Finally, proteins detected in the kidney can be investigated and measured in urine samples. Another step could be to correlate gene expression results with clinical or phenotype data to gain insight into the biological mechanisms driving the observed changes.

The transcriptomic process produces interesting translational aspects such as the identification of urinary protein biomarkers that can be measured in serial samples as a simple and non-invasive methodical approach to evaluating potential changes in the kidney. Gene expression data could be used to identify potential therapeutic targets such as genes involved in disease progression or drug resistance, and can be used to evaluate the effect of a personalized therapy during the clinical course of a glomerulonephritis. Furthermore, conducting transcriptomics in both kidney and urine cells may enable a comparison of immune cells in the kidney and urine, facilitating the use of urine as a reflection of the kidney.

KIDNEY TRANSCRIPTOMICS IN IGA NEPHROPATHY

Immunoglobulin A (IgA) nephropathy (IgAN) is characterized by the circulating galactose (Gal)-deficient IgA1 in the blood,

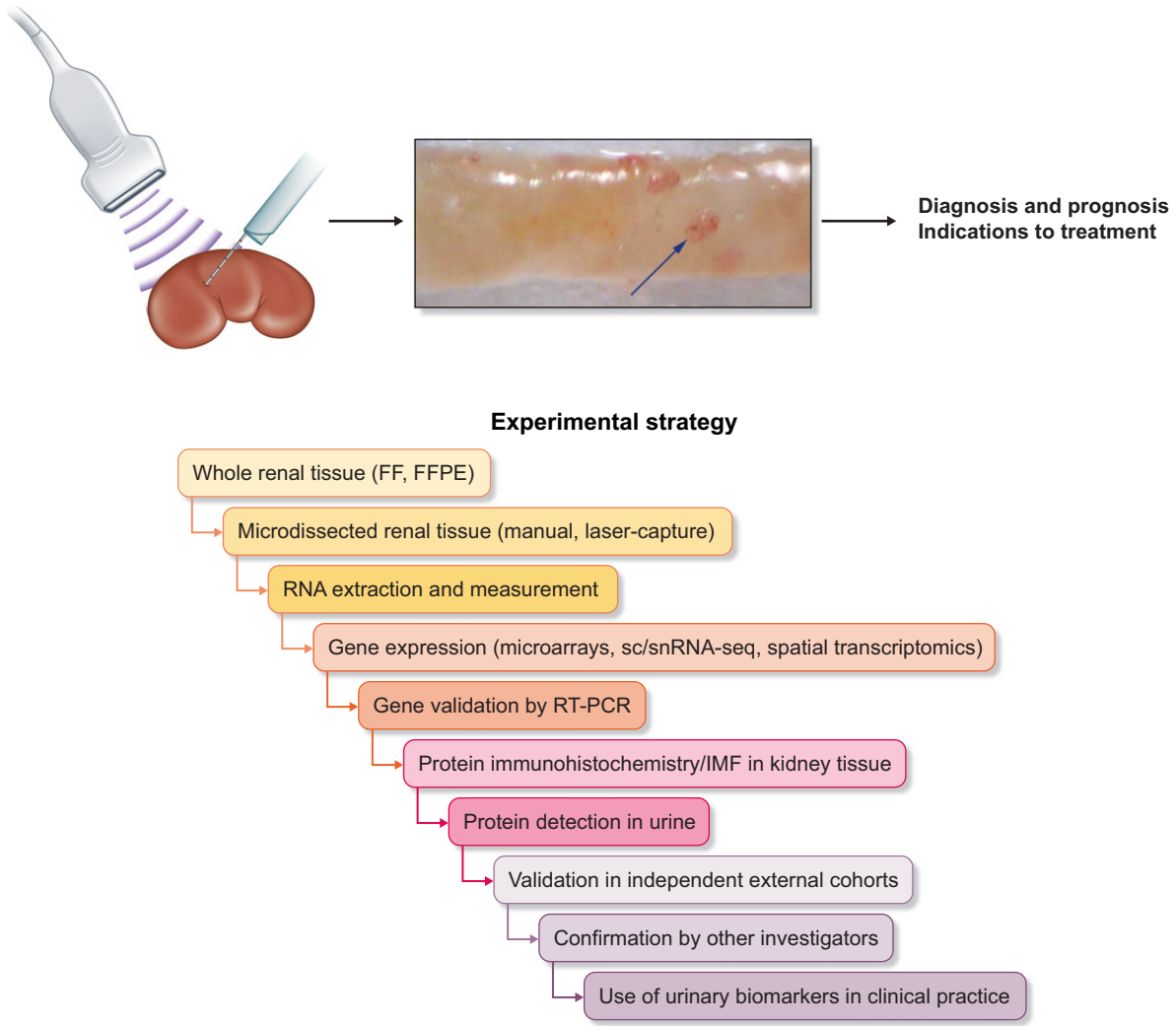

Figure 1: Medical and experimental strategies for the study of kidney biopsies. IMF: immunofluorescence.

Table 1: Transcriptomics in kidney biopsies and urine samples from IgAN patients.

Authors	Year	Renal tissue	Methods	Kidney (DEGs)	Urine	Main findings
Cox *et al.*	2020	FFPE	Microarray	*DEFA4*, *TNFAIP6*, *FAR2* in active renal lesions; *LTB*, *CXCL6*, *ITGAX* in chronic renal lesions	*TNFAIP6*, *CXCL6*	Urinary biomarker for active renal lesions; urinary biomarker for chronic renal lesions
Ju *et al.*	2015	Microdissected kidney tissue	Microarray	*NNMT*, *EGF*, *TMSB10*, *TIMP1*, *TUBA1A*, *ANXA1*	*EGF*	Urinary biomarker for chronic kidney disease

DEGs: differentially expressed genes; CXCL6: C-X-C Motif Chemokine Ligand 6; NNMT: Nicotinamide N-methyltransferase; TMSB10: Thymosin Beta 10; TUBA1A: Tubulin Alpha 1a; ANXA1: Annexin A1.

which binds to autoantibodies such as anti-Gal-deficient IgG or IgA, thus forming immune complexes that are deposited in the mesangial area of the glomeruli producing the characteristic immunofluorescent pattern of IgA associated with IgG or C3 [9]. Immune complexescause various active and/or chronic renal lesions. Therefore, the Oxford classification [10–12], based on scores of five renal lesions (mesangial proliferation, glomerular sclerosis, endothelial proliferation, extracapillary proliferation and tubule-interstitial fibrosis), is used to diagnose and determine the prognosis of the disease.

Transcriptomic studies of fresh whole renal tissue and microdissected glomeruli and tubulointerstitium, performed on kidney biopsies from IgAN patients, have been described in a recent narrative review [13]. The translational aspect of kidney transcriptomics has been limited to a few recent publications (Table 1), where the research has advanced from transcriptomics of kidney tissue to the detection of protein markers in urine samples or a transcriptomic pattern in urinary cells.

Cox *et al.* [14] designed a kidney transcriptomic study in which RNA was extracted from FFPE renal biopsies of IgAN

patients, non-IgAN patients (minimal change disease, focal segmental glomerular sclerosis and membranous nephropathy), lupus nephritis (LN) patients and living kidney donors. Genome-wide expression profiles were obtained, and biomarker identification was carried out comparing gene expression signatures in kidney biopsies of IgAN patients with active renal lesions from patients with chronic renal lesions. Bioinformatic analysis was used to identify transcripts characterizing active renal lesions [defensin alpha 4 (DEFA4), TNF-α-induced protein 6 (TNFAIP6/TSG-6) and fatty Acyl-CoA reductase 2 (FAR2)] and transcripts characterizing chronic renal lesions [lymphotoxin beta (LTB), granulocyte chemotactic protein 2 (GCP-2/CXCL6) and integrin subunit alpha X (ITGAX)]. These gene transcripts were validated by real-time PCR (RT-PCR) and then by immunohistochemistry at renal level. Finally, two of these genes, *TNFAIP6* and *CXCL6*, were detected in urine samples and confirmed in an independent cohort of IgAN patients compared with non-IgAN patients and normal subjects. A TNFAIP6/CXCL6 ratio was developed as a potential urinary biomarker for disease activity and chronicity. The next step of the study, which is ongoing, is to test these biomarkers in a longitudinal prospective randomized clinical study [15].

Ju *et al.* [16] used a renal biopsy transcriptome-driven approach to study non-invasive biomarkers in the urine of a patient cohort with chronic kidney disease. Some of them were biopsy-proven IgAN patients in the discovery cohort and in the first and second validation cohort. Kidney tissue was microdissected to separate glomeruli from the tubulo-interstitial component. Then, RNA was extracted from the tubulointerstitium and the molecular phenotypes were studied using gene chips. Among many genes, 52 candidate genes were significantly correlated with deterioration of renal function. A final panel of six transcripts was selected for having the best predictive performance. These six transcripts were further prioritized based on three parameters: (i) correlation with estimated glomerular filtration rage (eGFR), (ii) kidney-specific transcript and protein expression, and (iii) biological significance in the progression of renal damage. Principally, epidermal growth factor (*EGF*) mRNA expression was correlated with a decline of renal function expressed as a decrease in eGFR levels. Therefore, the EGF protein levels were measured in the urine of the patients and normalized to urinary creatinine. Interestingly, urinary concentration of EGF correlated with intra-renal EGF mRNA expression. This correlation was confirmed by measuring EGF levels in urine samples of three different cohort of patients, one of which consisted of only IgAN patients. In conclusion, this study confirmed EGF as a potential biomarker for monitoring the progression of renal damage, consistent with findings from previous studies using non-transcriptomic techniques [17, 18].

Spatial-specific transcriptomic profiling was applied on FFPE kidney biopsy tissues, uncovering differences in gene expression between IgAN patients' glomeruli and those of donor controls [19]. The study pointed out the proteins of these two genes in IgAN patients with and without mesangial proliferation, but other lesions classified by the Oxford classification were not taken into consideration. Genes characterizing mesangial proliferation were related to early changes in podocytes, expression of cell adhesion molecules, modifications in transcription factor pathways, vessel development and production of extracellular matrix. This study has several limitations. The reported differentially expressed genes have a false discovery rate cut-off of 0.1, indicating that as many as 10% of the reported genes may be false positives. Additionally, the genes have not been validated in an independent dataset of patients and controls, which further limits the report by design. Despite these limitations, this methodology represents a significant step forward in the high-throughput measurement of gene expression at a single-cell level and provides a more comprehensive map of gene expression within tissues leading to a better histological classification and subsequent biomarker identification.

KIDNEY AND URINE CELL TRANSCRIPTOMICS IN LN

LN is a manifestation of the systemic lupus erythematosus (SLE) occurring in more than 50% of patients, 10%–30% of whom progress to end-stage kidney failure within 15 years despite aggressive therapy.

Interferon-alpha (IFN-α) is a molecular subphenotype which is central to the pathogenesis of the SLE [20]. IFN-α is a cytokine that works at the interface of the innate and adaptive immune systems, with the potential to break self-tolerance by activating antigen-presenting cells following the uptake of self-material. In fact, IFN-α levels are high in many SLE patients and stabilize over time [21]. Thus, IFN-α is the primary cause of the development of the disease [22]. The identification of type I IFNs as a product of IFN-stimulated genes in blood cells and the recent evidence that the resident cells in the kidneys of LN patients produce IFN provides a clear indication of the inflammatory role of IFN in the kidney damage in LN [23].

LN is caused by the deposition of circulating immune complexes (ICs) in the kidneys. The histologic classification, recently revised by the International Society of Nephrology and Renal Pathology Society [24], provides principally five main classes of glomerular lesions that are indicative of the progressive deposition of ICs in glomeruli. ICs are deposited first in the mesangium of some glomeruli (class I) inducing mesangial cell hypercellularity, matrix expansion (class II) and then an influx of leukocytes linked with the deposition of ICs in the subendothelial wall of capillaries (class III). These lesions are present in segmental areas of renal parenchyma and affect $<$50% of the glomeruli (focal LN). When the immune process involves $>$50% of the glomeruli, diffuse segmental or global lesions characterize class IV of LN. In some patients, ICs are deposited only in the subepithelial space of glomeruli which leads to a pattern of membranous LN (class V). This pattern may occur in combination with class III or IV glomerular lesions.

It has been shown that active inflammation persists in 35%–50% of LN patients following the completion of induction therapy [25, 26]. Therefore, the correlation between clinical renal remission post-induction and histologic response is poor. Hence, using the immunological biomarkers obtained in studies reviewed earlier can provide superior monitoring during the follow-up of LN patients. Furthermore, the discrepancy between a protocol or for-cause kidney biopsy and the outcome of the disease, as reported by some researchers [27], indicates that the non-invasive use of urinary immunologic biomarkers may be a good choice for monitoring patients. The reviewed biomarkers may help healthcare professionals avoid repeated kidney biopsies during disease management, and can be used to monitor the effect of therapy during the course of the disease. Finally, the specific immune system targets reviewed may be considered for newer therapies.

The scRNA-seq technique, which enables a detailed characterization of the cell populations in kidney tissue, has been applied by LN researchers [28]. This technique enables researchers to differentiate between transcripts of infiltrating cells and

Table 2: Transcriptomics in kidney biopsies and urine cell samples of LN patients.

Authors	Year	Renal tissue	Methods	Kidney	Urine	Main findings
Arazi *et al.*	2019	FF	scRNA-seq	Molecular profiling of inflammatory cell subsets	Gene expression of $CD16^+$ macrophages	Gene expression of urinary immune cells correlated with kidney inflammatory process
Fava *et al.*	2020	FF	scRNA-seq/proteomics	Molecular profiling of inflammatory cell subsets	Urinary chemokines are produced by infiltrating $CD8^+$ T cells	Urine chemokine gradient correlated with the number of kidney-infiltrating $CD8^+$ cells
Fava *et al.*	2022	FF	scRNA-seq/proteomics	IL-16-producing cells were present in kidneys	Urine IL-16 correlated with the cell infiltrate	Urine IL-16 may be a potential biomarker and target therapy
Parikh *et al.*	2022	FFPE	Microarray	Gene expression related to inflammatory cells infiltrating kidneys reduced in patients responders to therapy	Urine C5a and fibronectin decreased after therapy in responders	Several candidate genes reduced their expression after complete response to therapy

native kidney cells. Additionally, comparing transcripts of immune cells from blood, urine and kidneys may reveal markers that can be monitored in biological samples collected in longitudinal clinical studies without repeated kidney biopsies. This methodical approach has been used primarily by the Accelerating Medicines Partnership (AMP) in SLE Network [29] (Table 2).

Arazi *et al.* collected kidney biopsies, blood and midstream urine samples from a group of 24 LN patients with newly diagnosed proliferative glomerulonephritis, and no prior exposure to immunosuppressive drugs; 10 control samples were obtained from kidney biopsies of living kidney donors [23]. scRNA-seq, based on the Cel-Seq2 technique, was then performed to characterize immune cells (B and T cells, natural killer cells, macrophages, dendritic cells and other leukocytes) within the renal parenchyma. The cell-sorting process evidenced that 90% of the leukocytes were $CD45^+$ cells, while the rest were $CD45^-CD10^+$ cells. Using stepwise cell clustering of kidney cells, based on gene expression, 21 subsets of active leukocytes were identified in proliferative LN. Some of these active leukocytes were implicated in the pro-inflammatory responses while others were implicated in inflammation-resolving processes. In the first step, 10 clusters including myeloid cells, T/natural killer (NK) cells, B cells and kidney epithelial cells were identified via low resolution of gene expression. Next, the clustering of single cells evidenced disease-specific subsets of these cells and their activity. Cytotoxic T lymphocytes and NK cells exhibited an IFN signature, and these cells may be the primary source of IFN-γ and cytolytic molecules responsible for inflammation. The IFN response signature in infiltrating leukocytes was consistent with the signature found in peripheral blood cells. Two additional populations of $CD8^+$ T cells were identified using gene expression. The B cell population consisted of a broad range of activated cells, and there was a correlation between the presence of $CD16^+$ macrophages in the kidney tissue and their presence in urine. Therefore, studying gene expression in urine immune cells reflects the inflammatory processes taking place in the kidney. This non-invasive approach can be utilized for monitoring patients during therapy.

Data from the Arazi *et al.* kidney transcriptomic study were integrated into a urine proteomics study by Fava *et al.* [30]. They quantified 1000 analytes, including cytokines, growth factors and other soluble markers, in urine samples obtained from 30 LN patients at the time of kidney biopsy. Using principal component (PC) analysis of the urine proteome, patients with proliferative LN were separated from those with pure membranous LN. Furthermore, using the Gene Ontology Biological process, PC analysis for biological significance revealed the presence of 10 enriched pathways among which chemotaxis pathways were predominant. Thus, a pattern of chemokines secreted in response to IFN-γ, interleukin (IL)-1β and TNF was able to attract inflammatory cells such as monocytes, NK cells and $CD8^+$ T cells in the kidney parenchyma. Subsequently, combining data from scRNA-seq of the kidneys of 24 LN patients and their urine samples, the authors investigated whether urinary cytokines were reflective of the intrarenal cytokine production by inflammatory cells. The results demonstrated that urine chemokines can be identified based on the proteome, and derived from intrarenal chemokine production, particularly by myeloid, NK and $CD8^+$ T cells. These findings were validated in six patients, four of whom had proliferative lesions while two showed pure membranous LN. PC analysis of urine proteomics demonstrated a strong correlation with an abundance of kidney-infiltrating $CD8^+$ T cells in proliferative LN. In conclusion, LN patients with elevated expression of urinary chemokines have proliferative lesions and these cytokines are produced in the kidney by infiltrating inflammatory cells. Therefore, urine proteomics may reflect the composition of the renal cellular infiltrates. This non-invasive approach based on urine proteomics can be used in recurrent analyses during the clinical course of the disease.

In a subsequent study, Fava *et al.* [31] used urine proteomics to explore other potential biomarkers that reflect the inflammatory processes in the kidneys of LN patients and to monitor potential treatment. Among the 237 urinary biomarkers associated with LN, IL-16 was the most significantly enriched urine protein in patients with proliferative LN compared with pure membranous LN. The levels of this cytokine strongly correlated with the scores of the National Institutes of Health LN activity index and there was a drop in its levels in the urine of patients after complete or partial remission in response to therapy. Next, the authors investigated the relative intrarenal gene expression of this cytokine by analyzing data from scRNA-seq performed on kidney biopsies. They found that IL-16 transcript was expressed by resident and inflammatory cells, with immune cells being the primary source. Additionally, immunohistochemistry revealed that the IL-16 protein levels were higher in the kidneys of proliferative LN patients when compared with those with pure membranous LN. This further suggests that urine proteomics have the potential to help physicians to monitor the effectiveness of treatment for patients with proliferative LN.

A recent scientific contribution from research combining data from kidney and urine transcriptomics has been done by Parikh *et al.*, who performed transcriptomic analysis on protocol kidney biopsy at flare and then after therapy in 58 LN patients with proliferative renal lesions [32]. RNA samples, extracted from FFPE sections of kidney biopsy tissue after laser microdissection of glomeruli and tubulointerstitium, were analyzed using a Nanostring nCounter GX human Immunology Panel (Nanostring Technologies) which contained 579 immune response genes and controls. The immune profiling of the gene transcripts at flare biopsy, obtained after comparison with normal renal tissue from 10 kidney living donors, showed overexpression of monocytes, integrin and complement components at glomerular level and increased genes related to T cells and complement components at tubular interstitial level. The differences related to transcripts in patients with complete remission compared with those with no-remission involved primarily integrins, extracellular matrix and adhesion molecules. In contrast, gene transcripts of pro-inflammatory cytokines and chemokines and neutrophil surface markers were dominant in the kidneys of non-responders. Ingenuity Pathway Analysis showed persistent overexpression of IFN and JAK-STAT signaling at the glomerular level in responder patients, and inflammatory chemokines and adhesion molecules at the tubular level in kidney of non-responders. This trend was confirmed by RT-PCR. The Delta Correlation Analysis showed alterations in the expression of monocyte and IFN transcripts at the glomerular level among individuals who responded to therapy. The changes in other transcripts were associated with variations in proteinuria. Next, the investigators confirmed the presence of some proteins encoding highly overexpressed transcripts at kidney level by immunohistochemistry. Finally, some of the detected proteins were investigated in urine samples. High concentrations of C5a, VCAM, ICAM-1 and FN1 were found in LN at flare, and their concentrations were reduced in patients who were responders to therapy. The kidney transcriptome analysis revealed elevated expression of certain genes involved in various pathways (neutrophil and T-cell infiltrate in the glomerular area and lymphoid aggregates in the tubulointerstitium) during flares, which decreased after therapy. This pattern was also evident in urine samples. Therefore, these findings suggest that urine from LN patients with proliferative lesions can be used to evaluate specific biomarkers of intrarenal inflammation and assess patient's response to therapy.

CONCLUSIONS

Applying transcriptomic techniques to the kidney biopsies of patients with IgAN or LN, researchers have identified the phenotypes of infiltrating cells implicated in the inflammatory processes and changes in resident cells. The detection of similar cells in urine and other biological biomarkers suggests that a non-invasive approach can be used in the clinical management of these diseases without protocol or for-cause kidney biopsies. The studies reviewed show molecular characteristics of kidney biopsies that match classical histological patterns. Combining molecular analysis with renal histology may improve our understanding of these diseases and improve their histological classification, leading to personalized treatments. These candidate biomarkers can be used in combination with the traditional clinical variables. However, there is a major limitation to these studies. The described biomarkers have been identified in small cohorts of patients and none has been validated in independent external cohorts. Finally, extensive patient heterogeneity gives rise to vastly different transcriptomic profiles, thus studies with large patient groups are necessary for validation before these biomarkers can be used in clinical practice, avoiding repetitive kidney biopsies and empirical treatments for patients who cannot undergo biopsy.

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CONFLICT OF INTEREST STATEMENT

F.P.S. is member of the *CKJ* editorial board. The other authors have no conflicts of interests to disclose.

DATA AVAILABILITY STATEMENT

No new data were generated or analyzed in support of this research.

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