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Biomarkers for kidney involvement in pediatric lupus

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Abstract

Lupus nephritis (LN), the renal involvement in systemic lupus erythematosus, is currently diagnosed by histopathology obtained by percutaneous renal biopsy and is associated with increased morbidity and mortality in both adults and children. LN is more prevalent and severe in children, requiring aggressive and prolonged immunosuppression. The consequences of the diagnosis and its treatment have devastating long-term effects on the growth, well-being and quality of life of affected children. The paucity of reliable clinical indicators of the presence and severity of renal involvement have contributed to a halt in the reduction of progression to end-stage renal disease in recent years. Here, we discuss the recent development of biomarkers in the management of LN and their role as therapeutic targets.

Keywords

children; lupus nephritis; noninvasive biomarkers; pathogenesis; TWEAK

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by antibodies directed against self-antigens, resulting in multiorgan damage. Twenty percent of SLE cases are diagnosed in childhood, and 50–80% of adolescents with SLE will develop kidney involvement, lupus nephritis (LN), at some point in their disease course [1,2], compared with about 40–50% of adults with SLE. Racial differences in adult patients are associated with rate of progression and response to therapy and are prominent in pediatric disease as well [3–5]. In children, LN can be the presenting clinical feature and tends to behave more

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aggressively. Renal involvement increases morbidity due to the effects of high-dose immunosuppression, renal dysfunction and hypertension on the brain, cardiovascular system and the bones during growth and development [6]. To date, the only means to diagnose and classify LN is by percutaneous renal biopsy [7]. The kidney biopsy also allows for some risk stratification, as it reveals high activity lesions (crescentic glomerulonephritis [GN] or fibrinoid necrosis), and provides a snapshot of the degree of scarring and irreversible damage [8]. In children, the procedure is performed under sedation with ultrasound guidance. In general, two cores of tissue are obtained with a 16 or 18 gauge needle and the presence of glomeruli in the tissue is confirmed by microscopic examination. The procedure requires the involvement of several medical teams and prolonged observation post procedure, and is a source of emotional distress for the family and the child. Percutaneous renal biopsy is also associated with a risk of bleeding, infection and allergic reactions to anesthetics and sedatives. The risk of bleeding is particularly concerning in SLE patients who may have autoantibodies interfering with platelet adhesion, the effects of which cannot be predicted by routine pre-operative coagulation studies [9,10]. Once the diagnosis is made and the LN class is established, the disease is monitored through routine laboratory parameters, such as proteinuria, serum urea nitrogen and creatinine levels, and complement and anti-dsDNA antibody levels. Changes in the serum creatinine level do not reflect real-time changes in the glomerular filtration rate in the acute setting [11] particularly in children, in whom normal serum creatinine values depend on the gender, age and height of the child [12]. Nonetheless, normal complement and anti-dsDNA antibody levels make active LN improbable [13]. As a result, adjustments in clinical management are delayed. Furthermore, the optimal timing of the initial biopsy in relation to the disease course, as well as intervals for repeat biopsies to monitor disease activity when no clinical flare occurs have not been established.

Biomarkers are factors that can be objectively measured and used as diagnostic indicators of normal biological or pathogenic processes, or as prognostic indicators of the natural disease course or response to a therapeutic intervention [14,15]. Moreover, biologic mediators identified as biomarkers can also be central players in the pathogenesis of a disease and as a consequence, may become therapeutic targets. Currently, noninvasive biomarkers that distinguish between various LN classes or dynamically capture changes in LN class are not available in routine clinical care.

Recent SLE research has yielded several promising biomarkers to help in the diagnosis and monitoring of LN. However, none of the studies were conducted solely in children with SLE. Renal biomarker studies in children are hindered by the difficulty of establishing a normal age-specific profile of a given substance, as well as by developmental confounders, related to relative immaturity of the renal excretory capacity [16]. Furthermore, there may be an additional level of heterogeneity in pediatric versus adult SLE and LN, in particular [17]. Hence, pediatric studies of noninvasive biomarkers are of utmost importance. In this review, we will cover the advances in the field of biomarker research in LN over the last decade as published based on adult data and their potential applicability to pediatric LN. The reader should be aware that, unless specifically highlighted as a pediatric study, all biomarker studies discussed in this report were carried out in adult subjects.

Classification of lupus nephritis

The American College of Rheumatology (ACR) criteria for the diagnosis of LN require the presence of persistent proteinuria defined as a spot urine protein/creatinine ratio of >0.5 or greater than 3+ on a urine test strip, and/or cellular casts including red blood cells, hemoglobin, granular, tubular or mixed [18]. The prevalence of LN in childhood SLE is estimated at ~60% [2]. Renal involvement can precede serological and extra-renal manifestations of SLE or can present with an incomplete set of ACR criteria for the diagnosis of SLE [19]; in both situations, targeted treatment may be delayed. Recognizing this caveat, the Systemic Lupus International Collaborating Clinics (SLICC) criteria were modified to include a renal biopsy manifesting findings consistent with LN to suffice for diagnosis of SLE, in the presence of either a positive antinuclear antibody test or anti-double-stranded DNA antibodies [20]. When compared with the ACR classification, the modified SLICC criteria have a better sensitivity [21].

LN is characterized by glomerular immune complex deposition, resulting in local inflammation and tissue damage (GN). The diagnosis is made based on visualization of immunoglobulin (IgG, IgM, IgA), complement, and C1q (so-called 'full house' staining pattern) detected by fluorescence microscopy [22]. LN classification is based on criteria developed by the WHO and further revised by the International Society of Nephrology/Renal Pathology Society (ISN/RPS) [7,23] (Table 1). The goal of the revised WHO classification was to improve the predictive role of the kidney biopsy in terms of renal outcome. However, the revisions resulted in only modest improvements [24]. Moreover, kidney biopsy tissue beyond the distinction in glomerular findings has limited use in directing therapeutic options in both adult and pediatric LN [25].

In addition to allowing classification and treatment guidance, kidney tissue provides information on the state of the tubulointerstitium and associated vascular changes, as well as indicators of nephrotoxicity from medications or hypovolemia [26]. The treatment regimen is largely based on glomerular findings as well as the racial background of the patient [27,28]. Moreover, the frequent use of cytotoxic agents for proliferative forms of LN is associated with significant risk of serious adverse events and may not be adequate for each patient. High-dose induction therapy is tapered to maintenance doses, while disease activity is monitored by the aforementioned biomarkers in the blood and urine. This practice aims to keep the disease 'in check,' but provides no assistance in determining an end point at which therapy can be safely discontinued.

While renal involvement is one of the most common presenting manifestations of pediatric SLE, ~30% of children may develop LN within the first 5 years of diagnosis, and a minority will have new-onset renal involvement later [29]. If a child presents with nephrotic-range proteinuria for example, and the biopsy reveals class V LN, any flare thereafter should be concerning for a class switch and require a subsequent biopsy [30]. While repeat biopsies are not necessarily indicated once the patient is known to have a proliferative form of LN, the patient may undergo subsequent biopsies if there is a concerning rise in serum creatinine, which may be the result of nephrotoxic drugs or ischemic injury to the kidneys [30]. Hence,

patients with LN diagnosed in childhood may undergo up to three renal biopsies during their lifetimes.

Pathogenesis of LN

In order to identify high-yield biomarkers of significant diagnostic and predictive value or those that allow monitoring of therapy and disease resolution, it is imperative to understand some of the major components implicated in the pathogenesis of LN.

In recent years, a substantial body of literature has emerged in the understanding of the development of autoimmunity in general, as well as LN. While a great deal was learned from murine models in the past, recent years have focused on accelerating the advancement of therapy development, for which animal models were unsuitable. In parallel to this effort, the application of cutting edge technology, such as next generation RNA sequencing [31] and single-cell RNA sequencing [32] to identify pathogenic mutations in several RNA species, or the development of the HpaII tiny fragment enrichment by ligation-mediated PCR assay [33] to examine DNA methylation sites have catapulted the area of genetic biomarker discovery. Nonetheless, the complex interplay of multiple factors that result in LN imply that not one single biomarker category can satisfy the aforementioned needs. In addition, the biomarkers need to be grouped such that they allow scientists and physicians alike to tailor a biomarker pattern to their current need (diagnosis, drug development, outcome prediction, and so on).

Pathogenic factors can be divided into extra-renal and intra-renal components. The main extra-renal contributors are genetic and epigenetic variants that result in an abnormal response to immunogenic stimuli. Genetic variants result from changes to the sequence of nuclear DNA during meiosis and are static factors in a given patient. The most striking genetic biomarker of susceptibility for SLE is female sex, with women of reproductive age having a ninefold increased risk of developing SLE compared with men [34]. The increased risk may be related to a higher X chromosome burden, rather than hormonal factors, as studies have shown an excess representation of men with Klinefelter syndrome (XXY karyotype) among male patients with SLE compared with the general population [35]. LN-specific studies have previously shown the role of racial differences in disease severity and response to therapy [3], yet another indication, that genetic factors may play a role. While multiple genetic susceptibility loci have been implicated in lupus, the cumulative effect-size of these loci account for a small fraction of disease heritability [36]. Here is where epigenetic factors come into play [37], as they supply the link between nature and nurture, explaining for example the role of sensitivity to UV light exposure, incomplete concordance in identical twins and other disease-related phenomena [38–40]. Epigenetic changes involve DNA methylation, histone modification and RNA interference by short noncoding RNAs (miRNAs) to name the best studied [41–43]. Epigenetic changes can occur at any point in an individual's lifetime, may be reversible, and can be a consequence of exposure to certain environmental factors, such as foods or medications. The reversibility of epigenetic changes not only makes them excellent real-time biomarkers, but they can also serve as novel therapeutic targets.

The intra-renal etiology of LN involves renal deposition of immune complexes as well as antibody binding to intra-renal nuclear autoantigens, and local complement and Fc receptor activation [44–47]. The classical pathway of complement activation is thought to play an important role in SLE pathogenesis. Activation of this pathway begins when C1q binds to the Fc portion of immunoglobulin G (IgG; particularly IgG1 and IgG3) or IgM molecules that are bound to an antigen (immune complex) [48]. In turn, this leads to the start of a vicious cycle that involves the release of cytokines by resident renal cells and infiltration of immune cells with further release of inflammatory cytokines etc. While certain types of circulating dsDNA antibodies have been known to be nephritogenic for decades [49,50], it was only in recent years, utilizing more sophisticated molecular technologies, that we learned about several different pathways at play in LN pathogenesis.

The various pathways involved in the pathogenesis of LN are highly complex and elegantly described elsewhere [51,52]. A very simplified way to reiterate the most prominent pathogenic steps occurring through parallel pathways in LN is as follows: immune complexes initially deposit in the mesangium, followed by deposition in the subendothelial and subepithelial spaces of the glomerulus or in peritubular capillaries [7]. Immune complexes directly activate resident renal cells to produce inflammatory mediators [53]. These inflammatory mediators, in turn, attract other immune cells, including B cells and macrophages. Several of the intra-renal inflammatory mediators are shed into the urine and will be individually discussed below.

Current disease monitoring biomarkers (& their deficits)

After briefly reviewing mechanisms involved in the pathogenesis of LN above, one may acknowledge that biomarkers in the serum may not represent the optimal pool of disease activity indicators. Yet, clinical practice today relies for the most part on exactly those.

Serum biomarkers

Anti-dsDNA antibody titers & complement (C3 & C4) levels—Serial measurements of anti-dsDNA antibodies have been the conventional practice to monitor LN activity for almost 50 years [54]. The detection of anti-dsDNA antibodies has been incorporated into the ACR criteria for SLE. However, there are a couple of caveats one needs to be aware of when assessing its validity as a biomarker in LN; since the discovery of this antibody, detection methods have changed and become more refined, revealing that the term ‘anti-dsDNA antibodies’ encompasses a highly diverse anti-dsDNA antibody family, anti-dsDNA antibodies can occur transiently due to infections, and the mere presence of these antibodies, does not imply that they are nephritogenic [55]. In addition, depending on the detection method used, the sensitivity and specificity of the test may change [56]. In recent years, enzyme immunoassays have emerged as better methods of detection of anti-dsDNA antibodies, yet the issue of whether or not they detect the nephritogenic antibody subset remains unresolved [57].

The association of changes in levels of serum complement with SLE has been recognized in the 1950s [58] and later on, Schur and others recognized the association of low serum complement C3 and C4 with active LN [59]. The pathogenetic implications of complement

in LN where then further elucidated [60]. Conversely, normal complement levels were found to exclude renal involvement in SLE patients. Unfortunately, the assessment of the predictive value of complement levels for LN flares has yielded irreconcilable results, due to numerous factors affecting complement levels. Nonetheless, in an individual patient with LN, serial monitoring of complements levels, together with other biochemical and clinical parameters, has been the most widely used method for detection of an LN flare [61,62].

While the aforementioned markers are routinely used to monitor disease activity, one should be aware of the caveat that neither of them perfectly correlates with renal disease. Reyes-Thomas *et al.* reviewed provided some insight into the sensitivity and specificity of anti-dsDNA antibodies, complement and anti-C1q antibodies [63]. The authors also pointed out the difficulty of determining predictive values of serological biomarkers as they may vary depending on what type of correlation they were utilized for (flare, monitoring of disease at baseline, outcome prediction etc.) and the method applied for detection of the individual markers [63].

While the combination of high anti-dsDNA antibody titers and hypocomplementemia (due to immune complex-mediated activation of the classical pathway) are strongly associated with an impending LN flare, there are patients who have persistent 'serological activity' in the absence of clinically active LN [64–67].

Creatinine—An abnormal serum creatinine level at presentation is considered a negative prognostic factor for progression to end-stage renal disease (ESRD); mostly, because an acutely elevated serum creatinine level is a surrogate marker of acute, proliferative GN with or without crescent formation, particularly in the presence of hypertension, as seen in class IV LN [68]. However, an acutely elevated serum creatinine is neither diagnostic nor is it indicative of a flare, given that changes in this biomarker take several days to become appreciated and various factors impact its correlation with actual glomerular filtration rate [69]. A chronically elevated serum creatinine level is a crude indicator of advanced renal scarring, irreversible damage and reduced renal reserve [70].

Urine biomarkers

Urine sediment (leukocytes, red blood cells)—In a pediatric lupus cohort with and without renal disease at presentation, isolated sterile pyuria and hypoalbuminemia were predictive of renal disease in longitudinal analyses [71]. Isolated sterile pyuria has been noted in up to 13% adults with SLE in a cross-sectional study [72]. However, sterile pyuria can be associated with multiple etiologies besides SLE, including the use of nonsteroidal anti-inflammatory drugs.

The significance of isolated hematuria in SLE is unclear. Adult studies investigating the correlation between isolated hematuria and histopathological findings reveal conflicting data [73,74]. The resolution of hematuria and other urinary findings may take several months and should not be the sole factor to determine resolution of an LN flare.

Urinary findings, such as hematuria or pyuria, may be masked by the presence of menstrual bleeding or nonrenal causes of inflammation, respectively. Microscopic examination of the

urinary sediment in the clinical setting to distinguish those entities from LN-related changes is not always feasible.

Proteinuria—The diagnosis of proteinuria can only be accurately made in children once orthostatic ('fixed') proteinuria is ruled out. Orthostatic proteinuria is a common benign finding in children and adolescents, but can also be found in young adults [75]. This condition was described in the 1920s and renal biopsies on individuals with orthostatic proteinuria showed normal histopathology [76,77].

To rule out postural proteinuria, a urine sample has to be collected in the morning, immediately after the patient gets out of bed, minimizing the time spent in the upright position or ambulating. It is also important to advise the patient to empty the bladder on the night prior to that morning, to ensure that all urine that is collected was produced while in a recumbent position [77]. While orthostatic proteinuria in itself is a benign entity, it can significantly contribute to pre-existing proteinuria due to renal pathology. Hence, even if a patient is known to have LN, it is best advised to base disease activity on early morning urine samples only.

The presence of persistent proteinuria may be an indicator of active renal disease, but its absence does not ensure the contrary. In a recent study, Wakasugi *et al.* biopsied a cohort of 195 adult SLE patients, of whom 86 did not have clinical renal involvement. LN, other than class I was found in 58% of the SLE patients without clinical LN [78] and 15% of this subgroup had proliferative LN and this lack of quantitative correlation between the presence of proteinuria and disease severity has been demonstrated before [79].

Promising experimental biomarkers for lupus nephritis

Ideally, biomarkers that allow diagnosis, detection of a flare, outcome prediction and risk stratification should be developed to allow the clinician to initiate treatment early, particularly in those patients who are identified as at high risk for progression and would benefit from aggressive immunosuppressive treatment. However, as of the present, biomarker discovery, particularly in children, has not come even near any of these goals.

A few biomarkers that were discovered in the recent past have made their way from the experimental setting into clinical trials, and even to becoming point-of-care tests. In this section, the LN biomarkers that are currently considered to be at the forefront of research and that have been implicated in LN pathogenesis will be discussed.

Blood

C1q antibodies (& C1q deficiency)—Homozygous deficiency in C1q, an early complement component, is an exceedingly rare disorder, which was found to be associated with childhood SLE and LN in particular [80]. *In vitro*, C1q seems to have a regulatory effect on the clearance of immune complexes [81]. In adults, acquired C1q deficiency is seen due to the circulation of an anti-C1q antibody. C1q levels can drop during LN flares [82], and persistently low levels are associated with continued activity in diffuse proliferative GN [83]. This drop is thought to be caused by a circulating antibody directed

against C1q and high tissue concentrations of this antibody in LN biopsies may be suggestive of a pathogenic role in the development of LN [84]. A recent international study assessed the validity of anti-C1q antibodies as biomarkers of renal involvement in SLE while comparing to a cohort with other rheumatologic diseases. When combined with elevated anti-dsDNA antibody titers and low C3 and C4 complement components, the presence of anti-C1q antibodies increased the odds of renal involvement by 15-fold [85] in the adult SLICC SLE cohort.

Biomarkers related to B cells & plasma cells—Infiltrating autoreactive B cells have been shown to play an important role in the perpetuation of LN [86]. The cytokines and chemokines that are shed by resident renal cells play an important role in the migration and survival of B cells [87] and the development of a plasma cell niche within the kidney [88]. Long-lived plasma cells, migrate to deeper parts of the renal cortex, exit the cell cycle and continuously produce autoantibodies [68,89]. This process becomes uncoupled from circulating mediators of immune responses (autoantibodies and B cells) [90–92].

BAFF is a cytokine that belongs to the TNF ligand family [93]. A monoclonal antibody against BAFF (belimumab) was the first new drug to be approved for the treatment of SLE in several decades, but no specific benefit for LN could be demonstrated so far. This may be related to the fact that intra-renal BAFF expression does not correlate with plasma BAFF levels, with the former being higher and contributing to the infiltration of B cells into the renal interstitium [89]. Hence, plasma levels of BAFF might not be an appropriate biomarker to identify LN patients that may benefit from treatment with Belimumab, and urinary BAFF detection should be explored instead.

APRIL is another cytokine from the TNF ligand family, which has also been associated with the development of LN. However, while serum BAFF levels did not correlate with severity of renal disease, Treamtrakanpon *et al.* provided intriguing evidence that serum levels of APRIL could predict treatment resistance in adult patients with LN – a finding which, if validated, could be critical in therapy decisions for individual LN patients [94].

Biomarkers related to macrophages—Macrophages are an important part of the repertoire of immune cells that infiltrate the kidneys in response to glomerular immune complex deposition and have been used as markers of disease activity in kidney tissue [95,96]. However, it was not until very recently that the importance of macrophages in the progression of LN was demonstrated. The identification of a unique macrophage activation phenotype in a murine LN model permitted a higher level of understanding of the pathogenetic implications, which were subsequently shown to play a role in human LN as well [97,98].

One of the cytokines responsible for the development, survival, proliferation and activation of macrophages, as well as macrophage influx in LN, is CSF-1 [99]. Renal tubular epithelial cells express CSF-1, and intra-renal CSF-1 expression increases with progressive LN [100–102]. Recently, Menke *et al.* demonstrated that elevated serum CSF-1 heralded the initial onset of LN in adult SLE patients, and a rise in serum or urine CSF-1 predicted recurrences of LN before conventional serologic measures, in patients with known LN [103].

Chalmers *et al.* took this discovery one step further and administrated a selective inhibitor of the CSF-1 receptor kinase in a mouse model of immune complex-mediated nephritis. This intervention led to the amelioration of LN and improved renal function, reduction in proteinuria and lower expression levels of inflammatory cytokines [104].

Urine

The differential concentrations of cytokines within the kidney versus peripheral blood have led to an increased effort in searching for biomarkers in the urine, as it is believed to better reflect the intra-renal milieu [105]. Inflammatory cytokines have been recognized as important mediators in LN pathogenesis [106–108], which are also conveniently being shed in the urine. Coupled together, these developments have resulted in a significant rise in urinary biomarker discovery and accelerated the discovery of new therapeutic targets in both pediatric and adult LN.

TWEAK—A pioneering development in LN research was initiated by the recognition of the downstream effects of interactions between TWEAK, a member of the TNF superfamily and its receptor Fn14 [109]. TWEAK's receptor, Fn14, is present on mesangial cells, podocytes, endothelial cells and tubular cells, and is upregulated in LN [110]. TWEAK is secreted by resident renal cells and leads to the infiltration of immune cells and secretion of other, highly relevant cytokines in LN [110–112]. Cytokines/chemokines that are upregulated in response to the TWEAK/Fn14 interaction include: CCL5/RANTES, MCP-1/CCL2, IP-10 and VCAM-1. Interestingly, blocking the interaction between TWEAK and Fn14 in murine inflammatory kidney diseases leads to amelioration of the disease [109,113]. Most recently, Xia *et al.* demonstrated that Fn14 deficiency resulted in an ameliorated clinical picture of a lupus-prone mouse strain [114]. Hence, the TWEAK-Fn14 interaction takes a sentinel position in the intra-renal inflammatory cascade in LN. These findings resulted in the development of a human anti-TWEAK antibody as an adjuvant treatment to the standard-of-care, which is being tested in a multicenter Phase II clinical trial (anti-TWEAK in LN, ClinicalTrials.gov identifier: NCT01499355).

Monocyte chemoattractant protein (MCP)-1/CCL2—MCP-1, which is expressed by mesangial cells, podocytes and monocytes, recruits monocytes, memory T cells and dendritic cells to the sites of inflammation, through a leukocyte chemotactic effect. Mouse models of LN show that MCP-1 is involved in the induction of inflammation and injury [115]. Conversely, if MCP-1 is either knocked out or inhibited, mice with LN have prolonged survival and less proteinuria [116,117].

MCP-1, as measured by either urine mRNA levels or urinary protein concentrations, has been shown to be a sensitive and reliable biomarker for LN [118–122]. Some evidence suggests that this biomarker could help to distinguish specific classes of LN, as its levels are significantly higher in patients with proliferative LN than in patients with other classes [123,124]. However, studies assessing the potential use of urinary CCL2 (or its mRNA) levels to predict flares have provided conflicting results [119,125,126].

CCL5/RANTES—RANTES is an important chemotactic molecule for T cells and plays an active role in leukocyte recruitment and the development of LN, as demonstrated in a RANTES-deficient murine model, which developed milder renal disease upon induction of an immune complex-mediated nephritis [127]. Along those lines, urinary CD4⁺ T cells were recently described as an additional tool for the identification of LN in adult SLE patients [128].

IP-10—IP-10 was found to promote mesangial cell expansion in adult patients with proliferative GN, a quality, which may give it a significant advantage as a biomarker [129] over other candidates, since proliferative GN is associated with a higher risk of progression to ESRD [27]. Due to its implication in mesangial proliferation, *IP-10* mRNA isolated from urine cells may be able to distinguish diffuse proliferative GN (WHO class IV) from other classes of LN [130].

VCAM-1—VCAM-1 is a cell surface protein, which mediates adherence of inflammatory cells to target cells. VCAM-1 expression was found to be elevated in murine models of LN, particularly in endothelial cells, cortical tubules and glomerular cells and contributes to the infiltration of the kidneys with T cells and macrophages [131].

Urinary VCAM-1 was found to strongly associate with the renal pathology activity index and GN class IV in renal biopsies from LN patients and correlate negatively with the chronicity index [132]. In clinical practice, having a biomarker that allows the clinician to distinguish between active disease causing an acute decrease in renal function, and progressive fibrosis, would be tremendously informative to clinical management.

NGAL—NGAL was first described as highly expressed in renal tissue of mice after ischemia/reperfusion injury [133]. Subsequently, high levels of NGAL were shown in a variety of kidney diseases [134,135]. In 2006, Brunner *et al.* showed that urinary NGAL is a promising potential biomarker of childhood-onset SLE nephritis [136]. High urinary NGAL levels were subsequently also validated in an adult cohort of LN patients compared with SLE patients without LN or normal controls in a cross-sectional study [137]. In 2010, Rubinstein *et al.* demonstrated that urinary NGAL predicts renal flare in patients with a history of biopsy-proven nephritis with a higher sensitivity and specificity than dsDNA antibody titers [138]. Later, NGAL was found to play a role in the pathogenesis of LN in a pristane-induced murine model. NGAL-deficient mice were more susceptible to pristane-induced autoimmunity by developing a higher number of antibody-producing cells and higher levels of inflammatory markers in the spleen than their NGAL-sufficient counterparts. Surprisingly, they displayed a milder form of LN, revealing a tissue-specific role [139].

Genetic & epigenetic variants

Intuitively, biomarkers related to genetic susceptibility may be of significant relevance in pediatric LN, considering the over-representation of LN among children with SLE. Although there are studies delineating genetic susceptibility markers related to childhood SLE [140]. LN-specific studies directed at exploring the existence and role of susceptibility

genes in childhood-onset LN have not been undertaken in a systematic way yet. Here, we discuss a select few adult studies that show great promise to be applicable in the pediatric LN population.

APOL1 polymorphisms

Recently, a gene polymorphism in the *APOL1* gene revealed a risk allele (G1/G2) that was associated with an increased risk for progression to ESRD in patients with hypertension, diabetes mellitus or focal segmental glomerulosclerosis [141,142]. The latter study included 139 children, but interestingly, patients found to carry the risk allele tended to be older. Given that LN is more prevalent among African-Americans than among European-Americans, a recent large multicenter study demonstrated that the same *APOL1* alleles strongly impact the risk of LN-ESRD in African-Americans, as well as the time to progression to ESRD [143].

The interferon signature

The important role of the type I IFN cytokine family in the pathogenesis of SLE began to be recognized in the 1970s [144]. Although it is beyond the scope of this report to discuss the various up- and downstream effects of the IFN signaling pathways, it is worth mentioning a study by Miyake *et al.* that demonstrated an interesting association of IFN- γ gene microsatellite polymorphisms that allowed the distinction between WHO class IV versus class V LN in adult SLE patients [145].

miRNAs

miRNAs are small noncoding RNAs that modulate gene expression at the translational level [146]. The last few years have introduced us to the important role miRNAs play in LN [147].

The involvement of miRNAs has been shown to not only affect the expression of genes encoding inflammatory cytokines for example, but also those involved in other inflammatory mechanisms in SLE patients, for example, T-cell DNA methylation in SLE patients [148], thereby making miRNAs the 'master controllers' of inflammatory processes. Profiling miRNA expression in urine samples of an adult and pediatric cross-sectional cohort of known LN patients during active and inactive disease revealed a set of miRNAs that correlated with disease activity. In addition, this study revealed miRNAs that distinguished proliferative from nonproliferative forms of LN [149].

miRNAs were also shown to affect cytokine and immune cell function and production [150–152]. Selective inhibition of miRNAs using antisense morpholino oligonucleotides is a relatively new therapeutic approach [153], and will likely begin to be deployed, once the exact role of individual miRNAs in LN pathogenesis will be elucidated.

Conclusion

SLE is an extremely complex disease, which is difficult to diagnose, difficult to treat and difficult to keep in check. Given the complexity, ever since the first publications on SLE

appeared [154], physicians treating these patients sought support for their clinical decision-making process in biomarkers of the disease. In fact, even in making the diagnosis of SLE, the ACR criteria rely on biomarkers (Table 2) [18]. This is true for very few other diseases.

What started in the late 1940s with the astute observation by Hargraves *et al.* of what they called the lupus erythematosus cell [154], has now culminated in a body of literature that is continuously growing, relying no longer only on astute clinical observations, but on cutting edge technology.

When looking back at the biomarker discoveries over the last decade, it is intriguing to see, to what extent our understanding of the pathogenesis of several novel molecules have the potential to transform the way LN patients are treated and followed (Figure 1).

Yet, there is still a lot of work to do, before we can tuck away the renal biopsy needles. We now know that the repertoire of cytokines that make up the intra-renal inflammatory milieu is not optimally reflected in the serum of patients. Therefore, our attention has shifted to the urine, which presumably has been in contact with the deepest parts of the kidneys, flowing through the medulla, as it is being transformed to a tertiary lymphoid organ and becoming disconnected from the peripheral blood. The urine may be the Holy Grail that harbors all the messages collected along the way. We now just have to find a way to translate those messages and put them together into a text that allows us to understand what class of LN we deal with, how it should be treated, and what we can tell the patient to expect.

As we compare these urinary biomarkers, that can be measured at the precision of micromolar scales, to the current gold standard marker for LN, the kidney biopsy, we must question whether the biopsy is too rough a measure to guide clinical judgment. Perhaps it is time for us to re-think our classification system and not merely judge the LN kidney by the appearance of a few glomeruli, but go deeper into intricate molecular details of cellular function and inflammation. This is for the next decade of biomarker discoveries to teach us.

Future perspective

While past biomarker research relied on astute clinical observation and limited technological capabilities, the past decade has brought on the opposite; cutting-edge technology allows detection of the most minuscule molecule in a minute amount of a given biological specimen. This development can, to a certain extent be compared with social media, while in the past we had to find a landline phone and call a number to talk to a particular person, we now are able to know what any person we ever met and ‘befriended’ is currently doing anywhere in the world. It remains to be determined what we make of all this information. Interestingly, when looking at biomarkers that have emerged recently and quickly made their way from being a name on the row of a large table of data, to becoming a therapeutic target in clinical trials, it is remarkable to note that the rapid achievement of the progress was made based on a hybrid approach. The initial findings were further rigorously analyzed, validated and studied for potential mechanistic implications. Taking this work further by validating the biomarkers in children is the next logical (and probably most efficient step). The aforementioned approach will allow us to rapidly improve the message-to-noise ratio of the current biomarker research world and prevent the majority of emerging biomarkers from

joining the group of the exclusive few to define the signature of biomarkers in pediatric LN. The combination of biomarker panels that will reliably diagnose, allow monitoring, and predict outcomes and response to therapy may differ between adult and pediatric LN. However, in order for this statement to be confirmed or refuted, the studies have to be carried out.

The reader should take a few minutes and search the literature for a couple of biomarkers of their choice that have not made it past the LN association stage, and try to track them over time. Is it not remarkable how many of them were enthusiastically ‘befriended’ only to be left behind and not investigated further? There are many orphan biomarkers left behind out there, expecting a kind scientist to pick them up and nurture them. Who knows what potential they may harbor?

References

Papers of special note have been highlighted as:

- of interest;
- of considerable interest

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Executive summary

Lupus nephritis classification

- Currently based on kidney tissue obtained by percutaneous biopsy.
 - Six classes developed in 1974 by the WHO and further modified by the ISN/RPS with the aim to improve outcome prediction;
 - Modified classification has only modestly improved outcome prediction.

Lupus nephritis in pediatric lupus

- More common and more severe than in adults with lupus.
- Diagnostic biopsy is burdened with anxiety, pain and potential side effects from sedation that is required with procedure.
- Class switches along the course of the disease may require multiple biopsies during the lifetime of a patient.

Established biomarkers for lupus nephritis

- Active urinary sediment (sterile pyuria, hematuria and proteinuria).
- Elevated anti-dsDNA antibody levels and hypocomplementemia are suggestive of active renal disease.
- Elevated blood pressure, serum creatinine and blood urea nitrogen levels are associated with a severe form of lupus nephritis.

Experimental biomarkers for lupus nephritis

- Urinary biomarkers better reflect the degree of intra-renal inflammation than those measured in the serum.
- Urine is easily obtainable in large quantities even in children.
- Urinary TWEAK, NGAL and MCP-1 correlate with disease activity.
- TWEAK is now in clinical trials as a novel therapeutic target.
- microRNAs, DNA methylation and histone acetylation represent epigenetic alterations that not only have biomarker potential, but also may be targeted and reversed by alteration of environmental factors (chemical exposures and dietary changes).
- Gene polymorphisms that are not pathogenic in and of themselves, but may alter the disease course.

Figure 1.
Biomarkers in lupus nephritis.

Table 1

WHO classification of glomerular lesions in lupus nephritis.

WHO class	Description
I	Minimal mesangial immune deposits on IF with normal light microscopy
II	Mesangial hypercellularity or matrix expansion on light microscopy with immune deposits confined to mesangium on IF
III	Focal LN, subendothelial immune deposits and proliferative changes in <50% of glomeruli
IV	Diffuse LN, subendothelial deposits and proliferative glomerular changes involving 50% of glomeruli
V	Membranous LN, subepithelial immune deposits and membranous thickening of glomerular capillaries
VI	Advanced sclerosing LN, sclerosis of >90% of glomeruli

Classes III and IV are divided into active, chronic or active on chronic lesions to designate the likelihood of responding to escalation of immunosuppression [26,28].

IF: Immunofluorescence; LN: Lupus nephritis.

Table 2

Summary of biomarkers and applicability to pediatric lupus nephritis.

Biomarker	Biological compartment of detection	Detection method	Relevance to pediatric LN	Ref.
Albumin	Urine	ELISA	Standard clinical practice	[75–79]
Red blood cells and white blood cells	Urine	Microscopic visualization	Standard clinical practice	[71–73]
C3/C4 complement level	Serum	ELISA	Standard clinical practice	[58–62]
Anti-dsDNA antibodies	Serum	ELISA	Standard clinical practice	[54–57]
APRIL	Serum	ELISA	Medium	[94]
TWEAK	Urine	ELISA	High	[109–114]
MCP-1	Urine	qRT-PCR; ELISA	Medium	[118–122]
RANTES/CD4 ⁺ T cells	Urine	ELISA; FACS	Medium	[127,128]
VCAM-1	Urine	ELISA; qRT-PCR	Medium	[132]
NGAL	Urine	ELISA	High – point-of-care testing available	[133–139]
miRNAs	Urine; blood PBMCs	qRT-PCR; hybridization; NGS	High	[146–153]

ELISA: Enzyme-linked immunosorbent assay; FACS: Fluorescence-activated cell sorting; LN: Lupus nephritis; NGS: Next-generation sequencing; PBMC: Peripheral blood mononuclear cell; qRT-PCR: Quantitative real-time polymerase chain reaction.