Original article

Value of Urinary Transferrin, Urinary Ceruloplasmin and Urinary Neutrophil Gelatinase Associated Lipocalin as biomarkers in Pediatric Lupus Nephritis

Background: *Lupus nephritis is an important cause of pediatric lupus morbidity and mortality. We sought to investigate the value of urinary transferrin (uTF), urinary ceruloplasmin (uCP) and urinary Neutrophil Gelatinase Associated Lipocalin (uNGAL) as non-invasive biomarkers of renal involvement in pediatric SLE.* ***Methods:*** *We conducted a comparative cross-sectional study over a period of 6 months at the Pediatric Allergy, Immunology and Rheumatology Unit, Ain Shams University. The study included a total number of 60 subjects; 40 patients with SLE and 20 age and sex-matched healthy subjects. Urinary levels of the three biomarkers (uTF, uCP and uNGAL) were measured by ELISA, in relation to other lupus clinical, urinary and serum parameters.* ***Results:*** *The studied patients had mean age 14.35 ± 2.3 years and 85 % (n = 34) were females. Among patients with LN (n=20), 8 (40%) had class II, 8 (40%) class III and 4 (20%) class IV LN. We observed significantly higher uTF levels, uCP expression and uNGAL levels among lupus patients compared to controls (p<0.001). In addition, the 3 studied biomarkers were significantly higher among LN group compared to SLE without LN (p<0.001). Levels of uTF, uCP and uNGAL showed significant positive correlations with 24-hour urinary proteins, serum creatinine and significant negative correlations with serum albumin, and estimated Glomerular Filtration Rate.* ***Conclusion:*** *Urinary TF, uCP and uNGAL might serve as non-invasive biomarkers of LN in pediatric and adolescent SLE. Their significant elevation in active cases may reflect a potential predictive value of renal flares and may serve as a valuable affordable tool in the follow-up of LN.*

Keywords: *Urinary Transferrin; ceruloplasmin, biomarkers, NGAL, lupus nephritis*

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INTRODUCTION

Lupus nephritis (LN) strongly affects the outcome in children with SLE. Data on the clinical course, long-term outcome and predictors of disease progression in children with LN are scant.[1] The presentation and clinical development of LN in pediatric patients vary considerably from benign, slow progressing cases to rapidly progressing disease.[2] Assessment of severity and activity of renal involvement in SLE requires a kidney biopsy, an invasive procedure with limited prognostic value. Non-invasive biomarkers are needed to help in treatment decisions and to monitor disease activity.[3]

The most readily available sources of biomarkers are urine and blood. Urine is an excellent source of biomarkers produced in the kidney and thus may give better mechanistic insight into specific renal abnormalities. Urine is less complex than serum, and thus is easier to screen for potential biomarkers.[4,5]

Human transferrin (TF) is a beta globulin that has very high affinity for iron and participates in the homeostasis of the organism since it binds to two ferric iron molecules and is responsible for transporting this metal to supply most of the body needs.[6] Urinary TF was reported to be significantly higher in pediatric SLE (PSLE) patients compared to those with juvenile idiopathic arthritis (JIA) controls.[7] Urinary TF levels were observed to be significantly higher among patients with LN compared to those without LN. The concentrations of urinary TF was significantly higher in patients with active LN than those without kidney activity. [8]

Ceruloplasmin (CP) is a 122 kDa protein that contains the majority of circulating copper. It has the function of iron oxidase and is associated with transferrin because CP oxidizes ferrous (which is toxic) to ferric that can later bind to transferrin. It has been seen that molecules associated with iron metabolism (such as CP and ferritin) have elevated levels in inflammatory diseases because their production maybe induced by the proinflammatory cytokines e.g. IL-6 and IL-1. There are few studies that have evaluated the role of urinary CP as a biomarker for LN.[5] The finding that uCP is expressed at high levels by parietal epithelial cells of Bowman's capsule suggests that uCP might be another potential biomarker for LN activity.[9]

NGAL is a 25-kDa protein belonging to the lipocalin family, produced in renal epithelia and leukocytes in response to tubular injury and systemic inflammation. High uNGAL can be used to predict AKI, discriminate intrinsic AKI from pre-renal AKI, predict renal non-recovery, in-hospital mortality, long-term CKD progression, ESRD, and mortality.[10] Prominent role of uNGAL was suggested as a potential biomarker of lupus nephritis that could serially forecast renal disease activity in SLE patients.[11] Therefore, we wanted to assess the diagnostic value of urinary TF, uCP and uNGAL as biomarkers of LN in pediatric SLE. The ultimate objective is to identify non-invasive markers of activity and/or severity of renal deterioration that would aid in the management of these cases.

METHODS

This was a cross sectional study conducted over a period of 6 months (from August 2019 to March 2020) in the Pediatric Allergy, Immunology and Rheumatology Unit, Children's Hospital, Ain Shams University. The study included 40 cases of juvenile SLE (below the age of 16) patients and 20 age and sex matched healthy subjects as a control group. Sample size was calculated using PASS 11 program, setting power at 80%, alpha error 5. Based on this sample size of 60 patients is enough to achieve the study objective. Subjects with the following features were excluded from the study: Underlying congenital renal anomalies e.g. obstructive uropathy with renal impairment**,** history of recent intake of nephrotoxic drugs, associated other immunologic disorders with possible renal involvement such as mixed connective tissue disease, acute kidney injury. e.g., septic shock, dehydration, etc and patients suffering from any chronic liver disease.

Ethical consideration: A written informed consent was obtained from the guardians of all participants before inclusion in the study, the whole study design was approved by the Institutional Review Board (IRB), Faculty of Medicine, Ain Shams University. Confidentiality and personal privacy were respected in all levels of the study and guardians felt free to withdraw from the study at any time without any consequences.

Study Methods:

I. **Complete history taking and detailed systemic examination** for disease onset, duration, different organ affection by SLE and detection of symptoms and signs of renal involvement including hypertension, oliguria, pitting edema, puffiness of eyelids, in addition to medications used. Lupus disease activity was assessed according to Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).[13] Renal biopsies results were retrieved from patients' medical records and reviewed and evaluated according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis.[14]

II. **Laboratory evaluation:** Complete blood count (CBC) was examined by Sysmex XN-1000, (Sysmex Corporation, Japan), Erythrocyte sedimentation rate (ESR) by Westergren method, C-reactive protein, serum albumin assay, serum urea and serum creatinine were measured using on the automated Beckman Coulter AU480 analyzer (Beckman Coulter, USA)]. Serum complement components (C3 and C4) were measured by turbidimetric assay (DIALAB GmbH, Austria). Anti- Nuclear Antibodies

(ANA) and Anti-dsDNA were measured by enzyme-linked immunoassay (ELISA) on the automated CHROMATE awareness technology INC. 24 hours urinary protein was measured using the automated Beckman Coulter AU480 analyzer (Beckman Coulter, USA). Urine was manually examined by dipsticks and microscopic examination.

Urinary biomarkers:

Urine was collected in sterile cups, centrifuged in sterile tubes at 2000-3000 RPM for approximately 20 minutes and then stored at -20°C. The samples were then analyzed for the following biomarkers:

Urinary transferrin (uTF): The assay was measured by ELISA using a commercially available kit supplied bBioassay Technology Laboratory (1008 Junjiang Inter. Bldg. 228 Ningguo, Shanghai, China).

Urinary ceruloplasmin (Ucp): Quantitative measurement of uCP concentration was applied to all cases and controls using sandwich ELISA technique (Bioassay Technology Laboratory, 1008 Junjiang Inter. Bldg. 228 Ningguo, Shanghai, China).

Urinary Neutrophil Gelatinase Associated Lipocalin (uNGAL): by sandwich ELISA technique using a commercially available kit supplied by Elabscience company (14780 Memorial Drive, Suite 216, Houston, Texas 77079, USA).

Statistical analysis: The collected data were revised, coded and tabulated using Statistical package for Social Science (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 26. Armonk, NY: IBM Corp). Data were presented and suitable analysis was done according to the type of data obtained for each parameter. Kolmogrov Smirnov test was done to test the normality of data distribution. Mean and Standard deviation (± SD) were calculated for parametric numerical data. Median and interquartile range were calculated for non-parametric numerical data. Frequency and percentage were calculated for non-numerical data. Student T Test was used to assess the statistical significance of the difference between two study groups of normally distributed data. Mann Whitney test for abnormally distributed quantitative variables, to compare between 2 groups. Chi-Square test was used to examine the relationship between two qualitative variables. Fisher's exact test was used to examine the relationship between two qualitative variables when the expected count is less than 5 in more than 20% of cells. Spearman rank correlation coefficient (rs) – a non-parametric equivalent to Pearson correlation coefficient – was calculated to indicate strength and direction of association between two numerical variables, both are continuous but not normally distributed or at least one of them is ordinal. In all applied tests, the probability values are set at 0.05 so that a p value < 0.05 is considered significant.

RESULTS

The study included 60 subjects: 40 patients and 20 controls. The 40 patients were divided into 20 systemic lupus erythematosus (SLE) with lupus nephritis (LN) (10 active and 10 inactive LN according to renal systemic lupus erythematosus disease activity index rSLEDAI) and the other 20 were SLE without clinical or laboratory evidence of LN. The ages of the patients ranged between 9 and 17 years with mean age 14.35 ± 2.3 years. The majority of them were females; n = 34 (85%), and the rest were males; n = 6 (15%). The ages of the control group ranged between 7 and 17 years with mean age 12.5 ± 2.7 years. They were 15 females (75%) and 5 males (15%). Lupus disease duration in the patients ranged between 1 and 78 months with mean duration 27.4 ± 20.2 months. In the SLE group without LN (n = 20), SLE duration ranged between 1 and 38 months with mean ± SD 15.25 ± 11.24 months. Clinical manifestations of enrolled patients are shown in **(table 1).**

In the SLE group with LN (n = 20), LN duration ranged between 2 and 60 months with mean ± SD 26.5 ± 17.7. Eight patients (40%) had class II, 8 (40%) had class III and 4 (20%) had class IV LN according to renal histopathological finding based on the classification revised by the International

Society of Nephrology / Renal Pathology Society (ISN/RPS), while none of the included patients had class V or class VI LN.

All the 3 studied urinary biomarkers ceruloplasmin, transferrin and N-Gal were significantly higher among the enrolled lupus patients in comparison to the healthy controls ($p< 0.001$, table 2). The urinary levels of these biomarkers were significantly higher among lupus patients with nephritis versus those without ($p< 0.001$, table 3). However, the enrolled patients with lupus nephritis classes II, III and IV had comparable levels of the 3 studied biomarkers, as shown in table 4.

All the 3 studied biomarkers showed significant positive correlations with the degree of proteinuria and serum creatinine levels and negative correlations with glomerular filtration rate and serum albumin levels. Other correlations with different lupus clinical and laboratory parameters are shown in table 5. The 3 urinary biomarkers showed good discriminative value between patients with and without LN (table 6) and also between patients with active versus inactive LN (table 7).

Table 1. Frequency of organs affection and immunological findings among SLE group with and without LN at enrollment.

Organs and laboratory	SLE without LN		SLE with LN	
	N = 20	(%)	N = 20	(%)
Constitutional	5	25%	4	20%
Mucocutaneous	10	50%	7	35%
Musculoskeletal	8	40%	6	30%
Arthritis	3	15%	4	20%
Cerebritis	2	10%	3	15%
Carditis	4	20%	5	25%
Generalized edema	0	0%	2	10%
Low C3	9	45%	10	50%
Positive anti-dsDNA	12	60%	14	70%

Anti-dsDNA: Anti-double stranded DNA; C3: Complement component 3; LN: lupus nephritis; SLE: systemic lupus erythematosus.

Table 2. Comparison of the studied urinary biomarkers between patients and controls

Urinary biomarker	**Patients (Mean±SD)**	**Controls (Mean±SD)**	**Z**	**P**
Ceruloplasmin (ng/ml)	134.38±73.774	15.70±4.824	-6.281	0.000*
Transferrin (mg/dL)	3.8 ± 1.72	0.22 ± 0.05	13	0.000
N-Gal (ng/ml)	3.046±1.4925	0.290±0.0857	-6.282	0.000*

LN: Lupus nephritis; Max: Maximum; Min: Minimum; SD: Standard deviation; SLE: systemic lupus erythematosus.

Table 3. Comparison of the studied urinary biomarkers between patients with versus without lupus nephritis

Urinary biomarker	**Patients with LN (Mean±SD)**	**Patients without LN (Mean±SD)**	**Z**	**P**
Ceruloplasmin (ng/ml)	193.75±56.239	75±24.225	-5.296	0.000*
Transferrin (mg/dL)	5 ± 1.7	2.64 ± 0.8	5.5	0.000
N-Gal (ng/ml)	4.085±1.3370	2.008±0.7164	-4.330	0.000*

Table 4. Comparison of the studied urinary biomarkers among different classes of LN.

Urinary biomarker	Class II LN (Mean±SD)	Class III LN (Mean±SD)	Class IV LN (Mean±SD)	F	P
Ceruloplasmin (ng/ml)	201.88±70.3	195±56.9	175±16.8	0.285	0.756
Transferrin (mg/dL)	5.7 ± 2.4	4.35 ± 0.8	4.4 ± 0.3	1.611	0.229
N-Gal (ng/ml)	4.388±1.51	4.038±1.3	3.6±1.2	0.473	0.631

Table 5. Correlations between the studied urinary biomarkers and different clinical and laboratory parameters.

Clinical and laboratory parameters.	Urinary Ceruloplasmin	Urinary Transferrin dL)/(mg	Urinary N-Gal
	r	r	r
Age (years)	0.457*	0.5*	0.324*
Lupus disease duration (months)	0.785*	0.55*	0.668*
LN duration (months)	0.775*	0.065	0.686*
ESR (mm/hr)	0.596*	0.5*	0.483*
Anti-dsDNA (IU/mL)	-0.148	0.4*	0.051
S.Albumin (g/dL)	-0.667*	- 0.65*	- 0.480*
HB (g/dL)	-0.096	- 0.12	- 0.072
TLC X10^3/uL	-0.209	- 0.22	- 0.178
PLT X10^3/uL	0.030	0.15	0.092
24-hr urinary proteins (mg/dL)	0.784*	0.8*	0.692*
S.Creatinine (mg/dL)	0.708*	0.61*	0.686*
e.GFR (mL/min/1.73 m^2)	-0.703*	- 0.63*	- 0.685*

Anti-dsDNA: Anti-double stranded DNA antibodies; e.GFR: Estimated Glomerular Filtration Rate (Revised Bedside Schwartz Formula); ESR: erythrocyte sedimentation rate; HB: Hemoglobin; hr: hour; LN: Lupus nephritis; PLT: platelet; S: Serum; TLC: Total leukocyte count.

Table 6: ROC curve analysis to discriminate between patients with and without lupus nephritis.

Urinary biomarker	Ceruloplasmin (ng/ml)	dL)/Transferrin (mg	N-Gal (ng/ml)
Cut-off level	107.50	3.2	2.350
AUC	0.989	0.958	0.899
Sensitivity	95%	90%	90%
specificity	100%	85%	65%

Table 7: ROC curve analysis to discriminate between patients with active and non-active lupus nephritis.

Urinary biomarker	**Ceruloplasmin (ng/ml)**	**Transferrin dL)/(mg**	**N-Gal (ng/ml)**
Cut-off level	187.5	4.8	4.150
AUC	1	0.9	0.99
Sensitivity	100%	70%	100%
specificity	100%	100%	90%

DISCUSSION

Involvement of the kidneys by LN is one of the most severe clinical manifestations seen in SLE. Lupus nephritis affects 50-75% of all children with systemic lupus erythematosus and represents a major contributor to morbidity and mortality in childhood onset lupus.[15] The presentation and clinical development of LN in pediatric patients vary considerably from benign, slow progressing cases to rapidly progressing disease.[3]

Preventing renal functional decline (RFD) and chronic kidney disease is central to improving health outcomes in LN. To achieve this goal, having predictive biomarkers to identify patients at risk for RFD early in their disease course would be ideal.[7] There is a need for high-quality accurate biomarkers to judge LN activity and renal damage with SLE. TF, CP and NGAL were isolated in the urine of pediatric patients with active LN, using proteomics studies.[7,16] Unlike other sample sources (serum or tissue), obtaining urine is not an invasive procedure, and it can be easily collected, allowing for sequential sampling. In the case of LN, the urine is physically close to the site of activity of the disease, being an interesting sample for monitoring of patients with LN.[17] Therefore, uTF, uCP and uNGAL were assessed in our study among a group of pediatric lupus patients in relation to disease activity.

In our study, uTF was found to be significantly higher in SLE patients with and without nephritis compared to controls. A previous work reported that urinary concentration of TF ($p < 0.0001$) was markedly higher in children with SLE than those with JIA.[18] Also, in our study urinary TF was found to be significantly higher in SLE with LN compared to SLE without LN and in active LN group compared to inactive LN group. Similar observations were previously published.[5]

Our results and previous published data suggest a significant link between urinary TF and renal lupus disease, particularly during activity. These data need to be further confirmed through prospective studies to assess the value of TF in predicting disease flares and in reflecting the response to LN treatment as well.

We observed that urinary TF levels were comparable between proliferative and non-proliferative LN classes. Thus, although urinary TF could successfully reflect renal activity, yet it did not show significant value in differentiating between the different histopathological classes of LN. Worth to note that, our study didn't represent all classes of LN. We had 8 patients with class II and 12 patients with proliferative LN (8 class III and 4 class IV) while the rest of classes were not represented. In view of the small sample size our conclusion in this point is limited. Although Urrego et al., 2020 [8] supported our results in their work yet Brunner et al., 2012 [18] had different observation in their study that showed elevated levels of transferrin are associated with mesangial proliferation ($p= 0.024$), capillary proliferation ($p = 0.017$) and the formation of cellular glomerular crescents ($p= 0.024$), and also reported that TF increased with wire-loops but changes didn't reach statistical significance. TF significantly increased with high Biopsy Activity Index Scores (BAI) ($p= 0.004$). Also, Brunner et al, in 2016 reported a relation between elevated level

of urinary TF and endocapillary hypercellularity (62%) according to National Institutes of Health Activity Index (NIH-AI) but that association didn't reach statistical significance. [19]

Our study showed significant positive correlations between urinary transferrin and age, lupus disease duration, erythrocyte sedimentation rate (ESR), anti-double stranded DNA antibodies (Anti-dsDNA), 24-hour urinary proteins, and serum creatinine and significant negative correlations with serum albumin and estimated Glomerular Filtration Rate. Thus, TF seems to correlate with measures of lupus disease activity in general and renal involvement, in particular. Similarly, a statistically significant positive correlation between concentrations of urinary TF and 24 hours proteinuria were previously reported.[8]

In the current study, we also observed a significant increase of urinary CP levels in the SLE patients as compared to the control group and this was clearly obvious in cases with renal involvement being significantly higher than its levels in those without renal involvement. Similar observations were reported by Urrego et al [8] who reported elevated levels of urinary CP in conjunction with LN in adult patients. An earlier relevant study concluded that SLE patients had much higher levels in patients with pediatric immune vasculitis syndromes as compared to the healthy control subjects especially patients with biopsy evidence of nephritis.[20]

Among the SLE patients in the current study, urinary CP excretion was positively correlated to age, SLE duration, ESR, 24-hour urinary proteins and serum creatinine and was negatively correlated to the serum albumin and eGFR. These findings suggest that this non-invasive marker is indicative of renal damage in juvenile SLE. Bennett et al [21] reported an association between age and ceruloplasmin in a pediatric group of LN patients. It was more of an inverted U-shaped association, increasing from age 3 - < 5 up to 10 - < 15, but then decreasing in the age group 15 - < 18. In urine, CP increased in response to infections, acting as a molecular source of copper which can inhibit bacterial growth. A relevant study observed that urinary CP levels were weakly correlated with the urinary protein/creatinine ratio and that the eGFR was totally unrelated to the levels of CP.[20] A relevant study did not prove any correlation between urinary concentrations of CP and the range of proteinuria in an adult study population.[8] The difference is obviously due to the diverse study designs and study samples.

In the current study, we observed a significant increase of urinary NGAL levels in the SLE patients as compared to the control group. This was also observed in 61 juvenile and adult SLE patients as compared to a matched control group.[22] Urinary NGAL was associated with active flares of LN in 16 adult SLE patients in comparison to 14 matched SLE cases without renal involvement.[12] Urinary NGAL was reported to be higher in 85 pediatric SLE patients as compared to a group of 30 patients with juvenile idiopathic arthritis (JIA) probably reflecting the higher frequency of renal affection in SLE.[23]

The increased urinary NGAL in our patients is probably due to excessive production by the kidneys rather by being produced elsewhere. It was postulated that the NGAL production in LN is due to increased glomerular protein loss, disturbed reabsorption in the proximal nephron segment and increased intrarenal production.[12] In other words, NGAL excretion is associated with renal affection much stronger than with global disease activity and that renal epithelial cells are the major source of NGAL detected in urine.[18]

In the current study, urinary NGAL expression showed positive correlations with age, SLE duration, LN duration, ESR, 24-hour urinary proteins and serum creatinine and negative correlation with serum albumin and eGFR. These findings support our assumption that this non-invasive marker is closely related to SLE activity and renal damage in pediatric SLE.

Our conclusions are indeed limited by the sample size. Also, the cross-sectional study design hindered proper interpretation of the effect of therapeutic modalities or the exact

influence of disease flares. The consecutive manner of case recruitment did not allow for the proportionate distribution of the sample in terms of classes of LN, not the adequate representation of all LN classes. Despite these limitations, our data involved many parameters of disease activity and severity which allowed correlation analysis with the markers under study.

CONCLUSIONS

In conclusion, uTF, uCP and uNGAL could serve as non-invasive biomarkers of LN in the pediatric age group. Their significant elevation in active cases may point to a possible predictive value of renal flares and may serve as a valuable affordable tool in the follow-up of children with LN. Our findings need to be validated through wider scale prospective studies involving different classes of LN with serial assessment of the levels of these biomarkers in relation to renal disease progression and histopathological findings.

CONFLICTS OF INTEREST

Authors declare they have no conflicts of interest.

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