Urinary Neutrophil Gelatinase-Associated Lipocalin to Monitor Lupus Nephritis Disease Activity

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ABSTRACT

BACKGROUND: This study was conducted to determine whether there is an association between urinary neutrophil gelatinase-associated lipocalin (uNGAL) and urinary transforming growth factor-β1 (uTGF-β1) with lupus nephritis (LN) disease activity.

METHODS: Urine samples from 18 LN patients were collected every month for six months then examined for uNGAL, uTGF-β1, and renal domain Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score.

RESULTS: The uNGAL levels were significantly different between active and inactive LN ($P < 0.05$). uTGF-β1 levels were not different between active and inactive LN ($P > 0.05$). There was a significant correlation between uNGAL levels and renal domain SLEDAI score ($r = 0.417$, $P < 0.05$). There was no correlation between uTGF-β1 levels and renal domain SLEDAI score ($r = 0.031$, $P > 0.05$).

CONCLUSION: uNGAL is better than uTGF-β1 for differentiation of active and inactive LN. uNGAL can be considered as a biomarker to monitor LN disease activity.

KEYWORDS: lupus nephritis, uNGAL, uTGF-β1, disease activity

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Introduction

Systemic lupus erythematosus (SLE) is a connective tissue disease with very diverse clinical pictures. This disease involves many organs of the body, and its course is characterized by remissions and exacerbations. Although its etiology is unknown, SLE occurrence has been linked to several predisposing factors such as genetic abnormalities, viral infections, and hormonal disorders. SLE is characterized by autoimmune lesions and formation of multiple antibodies against various organ tissues such as joints, lungs, muscles, and kidneys.[1–3]

The prevalence of renal involvement in SLE, called lupus nephritis (LN), is varied between 31% and 65% (average of 40%) at the beginning of SLE. Manifestations of renal insufficiency are often found in SLE before other clinical symptoms appear. Asians and Africans experience LN more frequently than other races. LN tends to be a chronic disease, with flares that require repeated treatments over the years. Generally, it is the most serious manifestation of SLE, with nephritis and infection as the frequent cause of death in the first ten years of disease.[1,2]

Since SLE patients with nephritis are often asymptomatic, the urinalysis should be performed on all patients suspected of having SLE. Current laboratory markers for LN such as proteinuria, urine protein creatinine ratio, creatinine clearance, anti-dsDNA, and complement levels are still unsatisfactory. Proteinuria still has some limitations, its sensitivity and specificity cannot differentiate between kidney activity and damage in LN. Persistent proteinuria does not always indicate an ongoing inflammatory process in the kidneys, which may be contributed by preexisting chronic lesion or by a new damage in the kidney during the disease course. Flare of nephritis can occur without an observable increase in the degree of proteinuria.[2,3]

LN disease course is characterized by remissions and activation of the disease, and its activity can be determined using renal domain Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score. The renal domain SLEDAI score is determined from urinalysis result. LN is defined as active when the score is >0 and defined inactive if the score is 0. A kidney biopsy is the gold standard for LN diagnosis. It also provides information of LN histological classification, relative activity, and chronicity level in the glomerulus. However, it is an invasive procedure and serial biopsy is also impractical for LN monitoring. Currently, there is a necessity for a new

biomarker that is capable of differentiating LN disease activity and severity, predicting LN flares, and monitoring treatment response and disease progression.[2,4]

Several studies have been conducted to find biomarkers that can assess LN disease activity. For example is the urinary biomarker, which samples are easy to collect and thought to be more accurate in distinguishing kidney disease from other organs.[5,6] Neutrophil gelatinase-associated lipocalin (NGAL) is a protein with a molecular weight of 25 kDa and a member of the lipocalin family. It is mainly synthesized by epithelial cells of proximal tubules, the loop of Henle, and collecting ducts of the kidney. NGAL has an important role in cell regeneration and growth after renal injury. It is found in most parts of the kidney so there is possibility that this protein can be used as a marker of kidney damage.[7–10] Transforming growth factor-β1 (TGF-β1) is an immunoregulatory (anti-inflammatory) cytokine that blocks IL-2 production (leading to inhibition of T-cell proliferation). It also regulates CD4 T-cell differentiation, decreases the production of IL-6, IL-1, and TNF-α by macrophages, and stimulates T-helper type 3 (Th3) development. Th3 exert their action primarily through TGF family cytokines, particularly beta-1. TGF-ß1 serves as a modulator and a suppressor of Th1 cytokines; therefore, Th3 cytokines play an important role in balancing Th1 and Th2 cytokines to prevent the severe cell-mediated inflammatory reaction. TGF-β1 also largely involved in renal fibrosis through its proangiogenic and profibrotic effects: induction of collagen gene expression, increased transcription of extracellular matrix receptor, and inhibition of proteins that involve in extracellular matrix breakdown.[11–15]

Many studies have been done concerning urinary NGAL (uNGAL) roles on glomerulonephritis disorders, but there are a few studies investigating its role in LN. The results from uNGAL study in pediatric patients showed that uNGAL increased about three months prior to LN activation (flares).[16] TGF-β1 study with experimental animals showed that TGF-β1 could be used to predict the occurrence of LN activation (flare) and to identify advanced and irreversible kidney disease phase.[17] TGF-β1 mRNA study on urine sediment of LN patients showed a significant increase of TGF-β1 mRNA in the active disease condition, and it was decreased in parallel with reduced disease activity. However, there are also other studies reporting no correlation between urinary TGF-β1 (uTGF-β1) and LN activity status.[18]

Therefore, this study was conducted to determine uNGAL and uTGF-β1 levels in active and inactive LN patients and to find the correlation between uNGAL and uTGF-β1 levels with renal domain SLEDAI score of LN patients.

Methods

Subject and study design. This was a prospective and observational cohort study. Subjects were the healthy person and SLE patients. SLE was diagnosed according to American College of Rheumatology (ACR) criteria by a rheumatologist in Rheumatology and Immunology Division of Internal Medicine Department, Dr. Saiful Anwar General Hospital, Malang, Indonesia. All patients had at least four of 11 ACR criteria for SLE diagnosis, accompanied by additional criteria for the diagnosis of LN. LN patients are SLE patients with renal biopsy results showing histological features of LN or SLE patients with proteinuria >500 mg/day (urine dipstick >3+) or cellular casts (erythrocyte, granular, tubular, or mixed).

Samples were collected between December 2011 and 2013. This study was approved by the Ethics Committee of Medical Faculty of Brawijaya University and Dr. Saiful Anwar Hospital. Informed consent was obtained from all participants, and the research was conducted in accordance with principles of the Declaration of Helsinki. Inclusion criteria were female aged 15–50 years, who did not suffer from congenital kidney disorder, diabetes, uncontrolled hypertension, and infection, since women are more affected by lupus compared to men with a ratio of 5:1. Exclusion criteria were patients with renal trauma and other diseases that can affect kidney function.

LN disease activity. LN disease activity was determined based on renal domain SLEDAI score. Each score was obtained from urinalysis results. Renal domain SLEDAI score consists of:

- **Urinary cast**: finding of granular or erythrocyte cast – score 4; no granular or erythrocyte cast – score 0;
- **Hematuria**: erythrocyte >5/hpf – score 4; erythrocyte <5/hpf without infection, kidney stones, or others – score 0;
- **Proteinuria**: proteinuria >0.5 g/24 hours or >3+ – score 4; proteinuria <0.5 g/24 hours or ≤3+ – score 0;
- **Leukosituria/pyuria**: leukocyte >5/hpf – score 4; leukocyte <5/hpf without urinary tract infection – score 0.

The total score for renal domain SLEDAI score is 0–16 (0, 4, 8, 12, 16).[19]

LN was defined as active when the renal domain SLEDAI score was >0, while LN was defined as inactive if the renal domain SLEDAI score was 0. LN activity was assessed if renal domain SLEDAI score increased to >0 compared to the previous score. Renal domain SLEDAI score was determined monthly for six months.

Urine samples. Every urine sample was collected in a sterile container using the aseptic method, centrifuged immediately, and stored at −80 °C until analyzed. Each sample was examined for complete urinalysis, uNGAL, uTGF-β1, and renal domain SLEDAI score.

uNGAL and uTGF-β1 measurement. We used RayBio® human Lipocalin-2/NGAL enzyme-linked immunosorbent assay (ELISA) kit (ELH-Lipocalin2-001) to measure uNGAL level. It is an *in vitro* ELISA for the quantitative measurement of human Lipocalin-2 in urine. This

assay employs an antibody specific for human Lipocalin-2 coated on a 96-well plate. RnD Systems Quantikine® ELISA human TGF-β kit (DB100B) was used to measure uTGF-β1 level. This assay employs the quantitative sandwich enzyme immunoassay (ELISA) technique. A monoclonal antibody specific for TGF-β1 has been precoated onto a microplate.

Statistical analysis. Statistical analysis was performed using SPSS version 20.0 for Windows software. The correlation between uNGAL and uTGF-β1 levels and LN disease activity was analyzed with Pearson test. Differences of uNGAL and uTGF-β1 levels on LN disease activity (divided into active and inactive diseases) and control subjects were analyzed with one-way ANOVA. A *P*-value of <0.05 was considered as statistically significant.

Results

Sample characteristics. The samples in this study were LN patients and healthy subjects. Eighteen healthy subjects (female gender; mean age 28.78 years) served as a control group. All LN patients were also female with age ranging from 17 to 40 years (mean age 26.5 years) (Table 1). We performed a two-year follow-up on 30 patients with LN, but only 18 patients could be followed up regularly for six months. We could not complete the follow-up on the other 12 patients until six months because four patients died during the follow-up and eight patients went to another hospital.

During the study period, we managed to follow up 18 LN patients regularly each month. Every patient was followed for six months. Finally, we had 108 samples from 18 patients. From 108 samples, we divided LN disease activity into active and inactive LN based on the total score of renal domain SLEDAI score. Seventy-seven (71.3%) samples were active LN and 31 (28.7%) were inactive LN.

The difference in uNGAL levels between active and inactive NL patients. The monthly differences in uNGAL levels between active and inactive LN patients are shown in Table 2. Combined data of uNGAL levels between active and inactive LN patients from month 1 to month 6 gave a *P*-value of 0.001 ($P < 0.05$), showing that uNGAL levels between active and inactive LN were significantly different. The uNGAL levels for active LN were higher (mean = 11,258.04 pg/mL) than inactive LN (mean = 3,826.5 pg/mL) (Fig. 1). The uNGAL levels in control group (mean = 346.00 pg/mL) were significantly lower than LN patients ($P < 0.05$).

The difference in uTGF-β1 levels between active and inactive NL patients. The monthly differences in uTGF-β1 levels between active and inactive LN patients are shown in Table 2. Combined data of uTGF-β1 levels between active and inactive LN patients from month 1 to month 6 gave a *P*-value of 0.518 ($P > 0.05$), showing that uTGF-β1 levels between active LN (mean = 93.32 pg/mL) and inactive LN (mean = 112.11 pg/mL) were not significantly different (Fig. 2). The uTGF-β1 levels in control group (mean = 23.73

Table 1. Characteristics of subjects in this study.

	HEALTHY SUBJECTS	LN SUBJECTS
Amount of subjects	18	18
Age (in years)		
Mean ± SD	28.78 ± 3.10*	26.50 ± 6.27
Range	25.00–37.00	17.00–40.00
NGAL in urine (pg/mL)		
Healthy subjects: mean ± SD	346.00 ± 172.39**	
range	38.20–703.60	
Active LN: mean ± SD		11,258.04 ± 8751.68
range		657.00–39,440.00
Inactive LN: mean ± SD		3,826.45 ± 3506.28
range		392.00–15,784.00
TGF-β1 I in urine (pg/mL)		
Healthy subjects: mean ± SD	23.73 ± 9.75***	
range	6.13–41.88	
Active LN: mean ± SD		93.32 ± 137.09
range		11.60–1,018.80
Inactive LN: mean ± SD		112.11 ± 112.04
range		14.60–488.80
Drug treatment		
Prednisolone (n,%)		18 (100)
Immunosuppressive drugs (n,%)		12 (66.66)
Renal biopsies		
None available (n,%)		6 (33.3)
WHO class II (n,%)		3 (16.7)
WHO class III (n,%)		4 (22.2)
WHO class IV (n,%)		5 (27.8)

Notes: NGAL and TGF-β1 I in urine are presented as mean ± standard deviation. *$P > 0.05$ compared with the LN group. **$P < 0.05$ compared with the active LN and inactive LN groups. ***$P > 0.05$ compared with the active LN and inactive LN groups.

Table 2. The difference in uNGAL and uTGF-β1 levels between active and inactive LN patients from month 1 to month 6.

PERIOD (MONTH)	LN PATIENT		THE DIFFERENCE BETWEEN THE CONCENTRATION OF uNGAL IN ACTIVE AND INACTIVE LN	THE DIFFERENCE BETWEEN THE CONCENTRATION OF uTGF-β1 IN ACTIVE AND INACTIVE LN
	ACTIVE LN (n)	INACTIVE LN (n)	*P* VALUE	*P* VALUE
Month 1	10	8	0.004	0.693
Month 2	11	7	0.031	0.806
Month 3	13	5	0.001	0.181
Month 4	14	4	0.001	0.376
Month 5	14	4	0.082	0.345
Month 6	15	3	0.862	0.914
Month 1–6	77	31	0.000	0.502

pg/mL) were lower than LN patients, but not significantly different ($P > 0.05$).

Correlation of uNGAL levels with renal domain SLEDAI score of LN patients. The monthly correlation between uNGAL levels and renal domain SLEDAI score of LN patients were statistically analyzed using Pearson correlation test (Table 3).

The Pearson correlation test on combined data (from month 1 to month 6) of uNGAL levels and renal domain SLEDAI score showed a correlation value (r) of 0.417 with $P < 0.05$. This result showed a significant correlation between uNGAL levels and renal domain SLEDAI score. In other words, increased uNGAL levels are correlated with increased renal domain SLEDAI score and vice versa.

Correlation of uTGF-β1 levels with renal domain SLEDAI score of LN patients. The monthly correlation between uTGF-β1 levels and renal domain SLEDAI score of LN patients was statistically analyzed using Pearson correlation test (Table 3).

The Pearson correlation test on combined data (from month 1 to month 6) of uTGF-β1 levels and renal domain SLEDAI score showed a correlation value (r) of 0.031 with $P = 0.747$ ($P > 0.05$). This result showed no significant correlation between uTGF-β1 levels and renal domain SLEDAI score. In other words, increased uTGF-β1 levels were not correlated with increased renal domain SLEDAI score and vice versa.

For more information about uNGAL levels, uTGF-β1 levels and renal domain SLEDAI score from Month 1 to Month 6, please refers to Supplementary Table 1, 2, and 3.

Discussion

The difference in uNGAL levels between active and inactive LN patients. In this study, uNGAL levels were significantly different between active and inactive LN

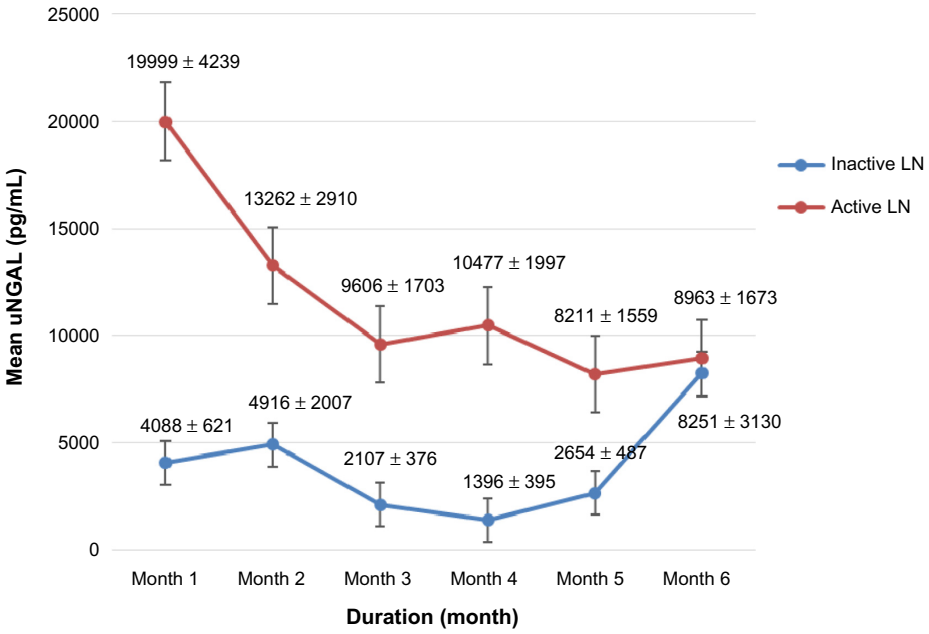

Figure 1. Comparison of uNGAL levels (mean ± SEM) between active and inactive LN patients.

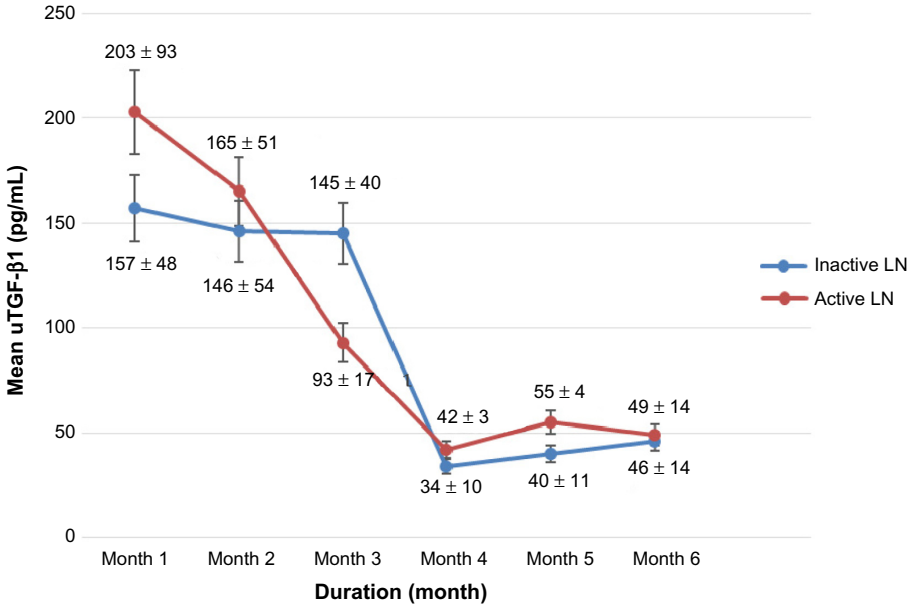

Figure 2. Comparison of uTGF-β1 levels (mean ± SEM) between active and inactive LN patients.

patients. This result is crucial to evaluate LN disease activity. If uNGAL levels are increased than the previous results, we can assume that LN is active and it will need a proper intervention. uNGAL is known as a biomarker for acute kidney injury (AKI) with high sensitivity and specificity.[20–22] Renal epithelial cells will express and excrete very large number of NGAL in urine within 30 minutes in the event of damage caused by ischemia-reperfusion injury, nephrotoxins, sepsis and chronic-progressive changes.[23] An earlier study by Pitashny et al on 70 adult patients with SLE (32 of them with the active renal disease) showed that uNGAL levels were significantly higher in LN than non-renal SLE patients or healthy controls.[24] Brunner et al also found that among patients with SLE, higher uNGAL levels could distinguish patients with or without active kidney disease.[25]

The difference in uTGF-β1 levels between active and inactive LN patients. Our results showed no significant difference in uTGF-β1 levels between active and inactive LN patients. It showed that uTGF-β1 levels are more likely to describe the chronic condition of kidney damage in LN patients, characterized by fibrosis of kidney tissue. Increased TGF-β1 in target organ can cause dysregulation of tissue repair, progressive fibrogenesis, and end-organ damage.[14,17] The previous study by Saxena et al measured the TGF-β1 expression in lupus-prone mice, both in lymphoid tissues (spleen) and target organ (kidney). They found no correlation between uTGF-β1 levels and glomerular activity index, but there was a strong correlation between uTGF-β1 levels, chronicity index, and tubulointerstitial index, which describes local fibrosis. Their data also showed that tissue and urinary TGF-β1 levels correlated with the degree of organ damage in the kidneys, especially with chronic fibrotic lesions but not with inflammatory lesions. The role of TGF-β1 in fibrosis process also has been reported by Yamamoto and Loskutoff. They found increased expression of TGF-β mRNA and PAI-1 mRNA over time in kidney tissue of lupus-prone mice after three hours of intraperitoneal injection with TGF-β. TGF-β and PAI-1 may contribute to the kidney pathology

Table 3. Pearson correlation test results between uNGAL and uTGF-β1 levels with renal domain SLEDAI score of LN patients from month 1 to month 6.

PERIOD	SAMPLE (n)	uNGAL		uTGF-β1	
		PEARSON CORRELATION (r)	*P* VALUE	PEARSON CORRELATION (r)	*P* VALUE
Month 1	18	0.394	0.106	0.029	0.908
Month 2	18	0.510	0.031	0.156	0.537
Month 3	18	0.434	0.072	−0.175	0.488
Month 4	18	0.752	0.000	0.242	0.333
Month 5	18	0.322	0.192	0.135	0.594
Month 6	18	0.378	0.121	0.289	0.244
Month 1–6	108	0.417	0.000	0.031	0.747

by stimulating extracellular matrix accumulation and trigger sclerotic and fibrotic changes.[26]

Our findings are different from the previous study by Hammad et al. In their study, latent and active TGF-β1 levels were assessed in the plasma and urine of all patients (32 children with active SLE and 15 healthy children as control group) before starting immunosuppressive therapy. They found that patients with symptomatic nephritis had significantly elevated urinary active TGF-β1 levels in comparison to those with silent nephritis.[26] These contradictory results were correlated with those of Tamaki et al, who found that only latent TGF-β1 levels increased in chronic renal fibrosis. Its levels were elevated in parallel with the degree of renal fibrosis, while active TGF-β1 levels were low, suggesting that in acute nephritis, TGF-β1 may be activated shortly after being secreted, while in the chronic phase, most TGF-β1 may exist for a long time in tissues as the latent type and may then be activated little by little.[27–29]

TGF-β1 is secreted from many cellular sources as a latent form and activated mainly by proteases to exert its biological function.[30] It is known that the production of both active and latent TGF-β1 was impaired in lymphocytes isolated from SLE patients.[31] A later study showed that in more than half of the patients with SLE, the isolated T-cell population had no TGF-β1 mRNA expression and at least one member of the TGF-β1 pathway was also missing.[32] In addition, normal TGF-β1 activation was lower in SLE patients with severe organ damage than those with a Systemic Lupus International Collaborating Clinics (SLICC) score of 0.[30] The correlation between lower TGF-β1 levels and decreased CD4+, CD8+, and NK cell frequency suggests that the reduction of these cell subsets might be responsible for the decreased levels of TGF-β1.[33] The counterregulatory mechanisms between latent and active TGF-β1 remain largely unknown; it will need further investigation to demonstrate the functional complexity of latent vs. active TGF-β1 in renal fibrosis and inflammation.

Correlation of uNGAL levels with renal domain SLEDAI score of LN patients. Overall, this study showed that uNGAL levels were positively correlated with renal domain SLEDAI score in LN patients, suggesting that uNGAL may be useful as a marker for monitoring LN disease activity. Our study showed significant correlations between uNGAL and renal domain SLEDAI score of LN patient only on months 2 and 4. One may assume that the SLEDAI score was not sensitive enough to every renal flare in patients who already manifest baseline indicators of nephritis. For instance, patients who have a renal flare manifested by an increase in urinary protein excretion from already significant baseline levels of proteinuria will not be picked up by SLEDAI, which has already awarded the maximal score possible for proteinuria.[10] The other reason is that NGAL measurements may be influenced by a number of coexisting variables, including chronic kidney disease, chronic hypertension, systemic infections, inflammatory conditions, anemia, hypoxia, and malignancies. In this context, NGAL might be a useful biomarker for kidney disease, but comorbid conditions when evaluating NGAL values should be considered because it can affect serum and urine NGAL levels.[34,35]

A longitudinal study was performed by Suzuki et al in 85 pediatric SLE patients. They found that uNGAL (rather than plasma NGAL) was correlated to worsening of global or renal disease activity as assessed by SLEDAI in SLE patients, and urinary NGAL may be an important biomarker for assessing renal disease activity in SLE patients.[16] Another longitudinal study was performed by Hinze et al in 111 pediatric SLE patients. They assessed SLE disease activity by using three standard indices of disease activity. Plasma and urinary NGAL levels were measured every three months. Their study found a significant increase in urinary NGAL/creatinine levels that were detected three months prior to LN worsening that was assessed on three disease activity indices.[36]

Correlation of uTGF-β1 levels with renal domain SLEDAI score of LN patients. In this study, we found no correlation between uTGF-β1 levels with renal domain SLEDAI score of LN patients. A study by Hammad et al also found that increased uTGF-β1 levels did not represent systemic disease activity because its levels were not correlated with SLEDAI. Increased uTGF-β1 levels are not a simple epiphenomenon of increased excretion of proteinuria; uTGF-β1 levels in patients with symptomatic nephritis did not correlate with proteinuria degree.[27]

Jin et al found no significant correlation between total TGF-β1 in serum with SLEDAI score. This was probably because of huge variability and diversity in the type of involvement by SLE. It is known that the use of immunosuppressive therapies, for example, glucocorticosteroids, upregulates TGF-β1 production by immune cells and expands regulatory T-cells. Thus, more aggressive immunosuppressive therapy in patients with high disease activity may increase total TGF-β1 levels to almost the same levels in patients with low or moderate disease activity. However, it remains unclear why active TGF-β1, which might be biologically more meaningful than total TGF-β1, has no association with SLICC and degree of renal damage.[30]

Effects of corticosteroid on uTGF-β1 levels were also investigated by Goumenos et al and Haramaki et al. They found that uTGF-β1 levels were significantly lower in patients who showed a significant improvement in renal function with immunosuppressive therapy when compared with patients with no signs of improvement. Levels of uTGF-β1 correlated positively with cellular crescents in kidney biopsy. According to their results, very high uTGF-β1 levels are followed by poor response to immunosuppressive therapy and probably represent development of irreversible damage in kidney tissue of patients with crescentic nephritis.[37–39]

Study limitation. This study had several limitations. First, the number of enrolled patients was relatively small. Second, samples for assessment of LN disease activity were highly varied every month because of treatment and other factors that

could not be controlled by the researcher. Third, assessment on LN disease activity was based only on clinical and laboratory findings, not renal biopsy, which was the gold standard.

Conclusion

Between active and inactive LN patients in this study, significant differences were only found in uNGAL levels, but not in uTGF-β1 levels. Significant correlation with renal domain SLEDAI score was also found only with uNGAL levels. Based on these results, we suggest that uNGAL can be considered as a biomarker to assess and monitor LN disease activity.

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Author Contributions

Conceived and designed the experiments: HS, JWW, KH, HK. Analyzed the data: HS, JWW. Wrote the first draft of the manuscript: HS, JWW. Contributed to the writing of the manuscript: HS, AR, JWW, AG, KH, HK. Agreed with the results and conclusions of the manuscript: KH, HS, AG, AR. Jointly developed the structure and arguments for the paper: HS, JWW, AG, KH, AR. Made critical revisions and approved the final version: HS, KH, HK, AR. All the authors reviewed and approved the final manuscript.

Supplementary Material

Supplementary Table 1. The uNGAL levels of LN patients from month 1 to month 6.

Supplementary Table 2. The uTGF-β1 levels of LN patients from month 1 to month 6.

Supplementary Table 3. The SLEDAI scores of LN patients from month 1 to month 6.

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