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Urine Neutrophil Gelatinase-Associated Lipocalin (uNGAL) in Lupus Nephritis: A Prospective Longitudinal Study

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Abstract

Objective: Urine neutrophil gelatinase-associated lipocalin (uNGAL) is increased in active lupus nephritis (LN). In this longitudinal study, we further evaluated the role of uNGAL as a potential marker for monitoring LN response to treatment and/or early relapse.

Methods: uNGAL levels were measured at baseline and at 2- and 4-months in 100 patients with biopsy-proven LN. They were divided into 2 groups - active LN [non-remission (NR) and relapses] and inactive LN [complete remission (CR) or partial remission (PR)]. Renal function test, urinary parameters, lupus serology and renal SLE disease activity index-2K (renal SLEDAI-2K) were analyzed to determine their associations with uNGAL.

Results: At baseline, there were 47 patients in the active group (42 NR and 5 relapsed) and 53 in the inactive group (51 CR and 2 PR). With treatment, the number with active LN declined to 29 (27 NR and 2 Relapsed) at 2 months and 22 (16 NR and 6 Relapsed) at 4 months respectively. Conversely, the number in the inactive group increased to 71 (61 CR and 10 PR) at 2 months and 78 (59 CR and 19 PR) at 4 months respectively. At each visit, uNGAL levels (ng/mg creatinine) were significantly higher in the active group especially relapses and were significantly associated with proteinuria and renal SLEDAI-2K. Receiver operating characteristic (ROC) curves showed that uNGAL was a potential biomarker for LN. Nonetheless, multiple logistic regression analysis showed that only serum albumin and proteinuria and not uNGAL were independent predictors of LN activity.

Conclusions: uNGAL was increased in active LN especially flares. Although not an independent predictor for LN activity, uNGAL could serve as an adjunctive marker when the clinical diagnosis of LN especially early relapse remains uncertain. Larger and longer studies are indicated.

Keywords: Lupus nephritis; Renal disease activity; Urine neutrophil gelatinase–associated lipocalin

Introduction

Lupus nephritis (LN) is a major cause of morbidity and mortality in systemic lupus erythematosus (SLE) [1,2]. Renal biopsy is the gold standard for diagnosis of the histological severity of LN. However, multiple renal biopsies are not feasible because of its invasive nature [3].

Current markers for LN are not reliable for early detection of LN activity or flares. Hence, non invasive biomarkers are urgently needed as timely early initiation of appropriate treatment can avert permanent renal damage [4]. Neutrophil gelatinase – associated lipocalin (NGAL) is one of the more promising biomarkers. NGAL is a 25 kDa protein that was initially isolated from the supernatant of activated neutrophils [5]. However, many other cell types including kidney tubules may produce NGAL in response to a variety of injuries [6-9]. NGAL has been extensively studied in acute kidney injury [10,11]. Furthermore, urine NGAL (uNGAL) levels have been reported to be elevated in patients with LN compared to those with non-renal SLE disease and correlated significantly with disease activity [12-15].

We have previously reported that uNGAL levels do reflect LN activity [16]. In this paper, we present the results of our prospective follow up study which evaluated uNGAL as a potential marker for LN response to treatment and / or early relapse in this same LN patient cohort.

Methods

The study methodology has been detailed in the two previous reports of the baseline data on our 100 SLE patients with biopsy-proven LN [16,17]. These same 100 SLE/LN patients were followed up in a longitudinal fashion at 2 and 4 months. All patients fulfilled the ACR classification criteria for SLE [18] and eligibility included all those with biopsy-proven LN regardless of activity status at recruitment. They were divided into two groups based on the presence or absence of LN activity as detailed below. The active group included those with active renal disease as well those with non-remission (NR) or had a relapse/ flare. The inactive group included those in complete or partial remission (CR/ PR).

The calculated sample size was 100 patients [17]. The study protocol was approved by the Medical Research and Ethics Committee of the Universiti Kebangsaan Malaysia Medical Centre (UKMMC). Informed consent was obtained from all recruited subjects.

SLE disease activity index

SLE Disease Activity Index (SLEDAI-2K) [19] was used to assess the lupus disease activity. The components of this index included global (score range 0-150), extrarenal (score range 0-63) and renal (score range 0-16). The renal score corresponds to the presence of any one of the following on urinalysis: proteinuria, haematuria, leucocyturia or urinary red cell casts in the absence of stones or concurrent urinary tract infection or other causes of proteinuria [20].

Definition of LN activity

A. Active LN was defined by the presence of one or more of the following criteria:

Proteinuria with or without any of the following features [21]

Presence of haematuria and/or red cell casts.

Increase in serum creatinine or decline in eGFR.

Proteinuria was measured as spot morning urine protein creatinine index (uPCI) and was positive if the value was >100 mg/mmol creatinine (NR ≤ 20).

Renal SLEDAI score ≥ 4 (out of a total of 16) [19].

B. Relapse/ flare of LN was defined in two ways:

At recruitment, relapse was defined as recurrence of renal disease activity after a period of remission ≥ 3 months [21].

During this study with only 4 months of observation (due to time constraints), relapse was defined as an increase in proteinuria and/or haematuria and/or serum creatinine level after 4 weeks of CR /PR or decrease in serum albumin level after 4 weeks of CR /PR [21].

C. Remission was defined as absence or reduction of renal disease activity and no change in immunosuppressive therapy for at least 3 months [21].

D. Inactive LN was defined by the presence of one or more of the following criteria:

Proteinuria (uPCI) ≤ 50 mg/ mmol with/ without any of the following features [21]:

a) Serum albumin ≥ 35 g/L.

b) Inactive urine sediments (<5 red cells/HPF and no red cell casts and no leucocyturia (<5 white cells/ HPF).

c) Stable serum creatinine (unless due to other etiology eg. renin-angiotension system (RAS) blockade).

Renal SLEDAI score 0 or <4/16.

The disease course of LN

The disease course of LN over time was categorized at each visit as in Table 1 [22,23].

Complete remission (CR)

Partial remission (PR)

Non-remission (NR) or Unchanged

Relapse /flare

Laboratory assessment

Laboratory assessment included the following: full blood count, renal function test, estimated glomerular filtration rate (eGFR) (MDRD), urine analysis, urine microscopy, urine protein creatinine index (uPCI), serum complement levels (C3, C4) and anti-dsDNA antibody titres (anti-dsDNA Ab).

Criteria / Outcome	Proteinuria (uPCI)	Haematuria	Serum creatinine	Serum albumin
Complete remission (CR)	≤ 50 mg/mmol creatinine	<10 RBC×10^6/L + '0' RBC casts	Baseline or ≤ 25% increase	≥ 35 g/L
Partial remission (PR)	50% reduction in baseline or >50 ≤ 300 mg/mmol creatinine	>10<50 RBC×10^6/L + no RBC casts	Baseline or ≤ 25% increase	≥ 35 g/L
Non remission (NR)	No change or >300 mg/mmol creatinine	≥ 50<150 RBC×10^6/L ± RBC casts	≥ 25% increase	<35 g/L
Relapse /flare	Increase after 4 weeks of CR /PR	Increase after 4 weeks of CR /PR	Increase after 4 weeks of CR /PR	Decrease after 4 weeks of CR /PR
Adapted with modification from Yamaji et al. [22] and Ruiz et al. [23].				

Table 1: Criteria for the outcome of lupus nephritis.

Biomarker measurement

Urine samples were centrifuged to remove sediments and frozen in aliquots at - 8°C prior to batch processing. Urine NGAL ELISA assay: uNGAL was determined using the commercially available ELISA kit from R&D Systems (Minneapolis, Minn, USA). In brief, standard or sample was added to each well and left to incubate for 2 h at 2-8°C. The plates were washed and incubated for another 2h at 2-8°C after adding conjugate to each well. The plates were then washed and substrate solution was added to each well. After incubation for 30 min at room temperature, the stop solution was added to each well and the absorbance read at 450 nm with the correction wave length set at 540 nm. uNGAL levels were expressed in ng/mg creatinine (normalized to urinary creatinine levels).

Statistical analysis

Data was analyzed using SPSS software version 18.0. Probability (p) values of <0.05 were considered significant. Categorical variables are presented as counts (percent). Continuous variables are presented as mean (± standard deviation (SD)) if normally distributed or median (interquartile range (IQR)) if non-normally distributed. Pearson's chi-square test (χ^2) was used to compare categorical variables and a two sided independent-sample t test was used to compare normally distributed variables. The Mann–Whitney U and Kruskal –Wallis tests were used for non- normally distributed variables. Association between uNGAL levels with standard laboratory parameters were explored using Spearman's correlation coefficients. Receiver operating characteristic (ROC) curves were constructed to determine the performance characteristics of uNGAL levels for early detection of LN activity. The best cut-off value for uNGAL was calculated based on maximization of the Youden index (sensitivity + specificity – 1) [24].

Multivariate analysis was performed by means of binary logistic regression to explore for independent predictors of LN activity. uNGAL and all standard markers of LN activity of the preceding visit with a p<0.05 were included in the regression model.

Results

Baseline characteristics

A total of 100 SLE patients with biopsy proven LN were recruited and completed the 4-months study. There were 92 females and 8 males with a mean age of 36.90 ± 10.62 years. The majority had class IV± V LN (52%) followed by class III ± V (34%). The demographic, clinical and laboratory data between active and inactive LN are as detailed in Table 2.

Parameters	All subjects n=100	Active LN n=47	Inactive LN n=53	p value
Age, mean ± SD years	36.90 ± 10.62	36.40 ± 9.97	37.33 ± 11.24	0.74
- Female: no. (%)	92 (92)	43 (91.5)	49 (92.5)	0.57
- Male: no. (%)	8 (8)	4 (8.5)	4 (7.5)	
Race: no. (%)				
- Malay	41(41)	24 (51.1)	17 (32.1)	0.14
- Chinese	55 (55)	21 (44.7)	34 (64.2)	
- Indian	4 (4)	2 (4.3)	2 (3.8)	
LN duration in years	7 (1–24)	7 (1–24)	7 (1–17)	0.56
Mixed connective tissue disease (MCTD)	7 (7%)	3 (6.4)	4 (7.5)	0.82
Musculoskeletal system (MSK)	41 (41%)	20 (42.6)	21 (39.6)	0.46
- Duration of MSK in years	6 (1–27)	6.5 (1–27)	6 (1–27)	0.60
Autoimmune Haemolytic Anaemia (AIHA)	26 (26%)	14 (29.8)	12 (22.6)	0.27
- Duration of AIHA in years	4.88 ± 3.21	5.58 ± 3.44	4.28 ± 2.99	0.34
Idiopathic thrombocytopenic purpura (ITP)	9 (9%)	5 (10.6)	4 (7.5)	0.24
- Duration of ITP in years	7.5 ± 4.62	9.5 ± 5.8	5.5 ± 2.38	0.20
Thrombotic thrombocytopenic purpura (TTP)	1 (1%)	0	1 (1%)	0.53
Systolic blood pressure (mmHg)	128 ± 17.68	128 ± 13.16	120 ± 13.91	**0.001**
Diastolic blood pressure (mmHg)	75.2 ± 4.08	77.80 ± 10.31	73.68 ± 10.44	**0.04**
Classes of lupus nephritis (%)				
- WHO class I	1 (1)	1 (2.1)	0 (0)	0.71
- WHO class II ± V	6 (6)	3 (6.4)	3 (5.7)	
- WHO class III ± V	34 (34)	15 (31.9)	19 (35.8)	
- WHO class IV ± V	52 (52)	26 (55.3)	26 (49.1)	
- WHO class V	5 (5)	1 (2.1)	4 (7.5)	
- WHO class VI	2 (2)	1 (2.1)	1 (1.9)	

Activity index median (IQR)	8 (0–19)	9 (0–16)	8 (0–19)	0.93
Chronicity index median (IQR)	3 (0–15)	3.58 (0–9)	3 (1–15)	0.55
CKD stage				
- Stage 1 (eGFR > 90)	61 (61)	25 (53.2)	36 (67.9)	0.06
- Stage 2 (eGFR 60 - 89)	22 (22)	10 (21.3)	12 (22.6)	
- Stage 3 (eGFR 30 - 59)	14 (14)	9 (19.1)	5 (9.4)	
- Stage 4 (eGFR 15 - 29)	3 (3)	3 (6.4)	0 (0%)	
Medications, no (%)				
- Corticosteroids	95 (95)	43 (91.5)	52 (98.1)	0.12
- Cumulative dose for previous six months (g)	1.80 (0.75–4.50)	1.80 (0.90–4.50)	1.76 (0.75–1.95)	**0.001**
- Cumulative dose from previous relapse (g)	5.040 (0.90–24.43)	4.415 (0.90–24.43)	6.685 (1.59–13.32)	**0.009**
- Time from last relapse (months)	22 (1–120)	11 (1–120)	28 (3.5–72)	**0.001**
- Cyclophosphamide	8 (8)	8 (17)	0 (0)	**0.002**
- Cyclosporine A/Tacrolimus	30 (30)	19 (40.4)	11 (20.8)	**0.03**
- Mychophenolic acid	22 (22)	12 (25.5)	10 (18.9)	0.42
- Azathioprine	36 (36)	12 (25.5)	24 (45.3)	**0.04**
- Hydroxychloroquine	42 (42)	20 (42.6)	22 (41.5)	0.91
- Renin angiotensin system blockers (ACEI / ARB/ Spironolactone)	68 (68)	29 (61.7)	39 (73.6)	0.11
- Other antihypertensive agents				
- Calcium channels blockers	28 (28)	12 (25.5)	16 (30.1)	NS
- Beta blockers	13 (13)	6 (12.7)	7 (13.2)	NS
- Alfa blockers	1 (1)	1 (1)	0 (0)	NS
- Lipid lowering agents	39 (39)	20 (42.5)	19 (35.8)	NS
- Aspirin	13 (13)	4 (8.5)	9 (17)	0.20
SD: Standard Deviation; IQR: Interquartile Range; LN: Lupus Nephritis; WHO: World Health Organization; CKD: Chronic Kidney Disease; ACEI: Angiotension Converting Enzyme Inhibitors; ARBs: Angiotensin Receptor Blockers; NS: Not Significant				

Table 2: Demographic and baseline characteristics in the Active and Inactive LN patient groups.

Course of LN in the overall study population

At recruitment, there were 47 patients in the active group (42 NR and five relapsed) and 53 in the inactive group (51 CR and 2 PR). At 2 months, 29 of the former had persistent active LN (27 NR and two relapsed) whereas the patients in the inactive group had increased from 53 to 71 (61 CR and 10 PR). At 4 months, the number of patients with active LN had declined further to 22 (16 NR and six relapsed) and the number with inactive LN had risen to 78 (59 CR and 19 PR).

In summary, with treatment over the 4 months' study period, the number of patients with active LN had decreased from 47 to 29 to 22 respectively. Conversely, the number of patients with inactive LN had increased from 53 to 71 to 78 respectively.

At each visit, there were significant differences between those with active LN compared to those with inactive renal disease in terms of serum albumin ($p<0.01$), proteinuria (uPCI, $p<0.001$), SLEDAI-2K (global) ($p<0.001$) and SLEDAI-2K (renal) ($p<0.001$). Details are shown in Table 3. There were no differences in the other markers such as ESR, anti-dsDNA Ab and serum complements (C3 and C4). However at end study, serum creatinine had risen significantly and eGFR declined in the active group only. Thus uNGAL levels were significantly higher in the active group compared to the inactive group at all time points (Table 3).

Course of LN in the group active at baseline

On follow up of the LN group active at baseline (n=47), 18 patients achieved CR/PR, two relapsed and 27 remained in NR at 2 months and 28 achieved CR/PR, three relapsed and 16 had NR at 4 months (Table 4). Two patients with NR were subjected to repeat renal biopsy. Their histological findings had deteriorated from mixed class II + V and class IV to mixed class III + V.

The uNGAL levels decreased progressively from baseline to end study in response to treatment in both those patients who achieved remission and those who remained with active LN (NR/ Relapse). However, uNGAL levels were significantly lower in those who attained remission than in those with active LN (p=0.03 and p=0.004 respectively) (Table 4).

Patient group **Active LN** **(NR/Relapse)** **Inactive LN** **(CR/PR)**	**Parameter**	**Baseline** **n=47** **n=53**	**2 months** **n=29** **n=71**	**4 months** **n=22** **n=78**
Active LN Inactive LN	Serum albumin (35–50 g/L)	37.78 ± 5.54 41.88 ± 3.59	39 ± 5 41.78 ± 3.20	37.5 ± 4.97 41.02 ± 5.82
	Inter group p value	**<0.001**	**<0.001**	**0.01**
Active LN Inactive LN	Serum creatinine (44 – 80 µmol/L)	69 (IQR 33–252) 63 (IQR 41–158)	72 (IQR 30–244) 65 (IQR 37–168)	89.5 (IQR, 43–244) 63 (IQR 34–192)
	Inter group p value	0.29	0.36	**0.004**
Active LN Inactive LN	eGFR (> 60 ml /min /1.73 m^2)	93.61 ± 46.01 99.75 ± 31.54	91 ± 49.16 98 ± 32.69	75.04 ± 39.95 98.35 ± 35
	Inter group p value	0.43	0.53	**0.009**
Active LN Inactive LN	ESR	38.5 (IQR 21–91) 33 (IQR 0–46)	41 (IQR 22–92) 49 (IQR 10–103)	32 (IQR 8–105) 36 (IQR 1–78)
	Inter group p value	0.37	0.86	0.36
Active LN Inactive LN	Anti-dsDNA Ab titers (< 30 IU/dL)	35.18 (IQR 1.73–195.97) 24.24 (IQR 0.81–279.21)	30.23 (IQR 0.74–267.61) 14.37 (IQR 1.05–280)	41.53 (IQR 2.07–291.62) 18.90 (IQR 0.95–262.21)
	Inter group p value	0.84	0.89	0.73
Active LN Inactive LN	Serum C3 (79 – 152 mg /dL)	100.5 ± 36.39 109.62 ± 39.94	102.25 ± 40.53 106.37 ± 41.54	94.26 ± 26.67 104.16 ± 33.04
	Inter group p value	0.24	0.32	0.21
Active LN Inactive LN	Serum C4 (16 – 38 mg /dL)	21.46 ± 12.82 22.94 ± 11	21.14 ± 10.95 23.95 ± 13.69	22.81 ± 10.19 23.14 ± 9.48
	Inter group p value	0.54	0.32	0.89
Active LN Inactive LN	Proteinuria (uPCI) (< 20 mg/mmol creatinine)	110 (IQR 10–510) 20 (IQR 10–50)	130 (IQR 10–480) 20 (IQR 10–50)	110 (IQR 10–510) 20 (IQR 10–30)
	Inter group p value	**<0.001**	**<0.001**	**<0.001**
Active LN Inactive LN	Urinary leucocytes/HPF×10^6 /L	0 (0–20) 0 (0–5)	0 (0—20) 0 (0–15)	0 (0–20) 0 (0–10)
	Inter group p value	**<0.001**	0.30	**0.007**
Active LN Inactive LN	Urinary RBC/HPF×10^6 /L	0 (0–10) 0 (0—5)	0 (0–20) 0 (0–15)	0 (0–50) 0 (0–30)
	Inter group p value	**<0.001**	0.40	**0.03**
Active LN Inactive LN	uNGAL (ng/ mg creatinine)	195.80 (IQR 21.07–1,413) 83.66 (IQR 0–746.5)	187.94 (IQR 58.57– 993) 100.80 (IQR 8.8 0–711)	175 (IQR 40–840) 66.93 (IQR 10–605)
	Inter group p value	**0.01**	**<0.001**	**<0.001**
Active LN Inactive LN	SLEDAI-2K (global: 0 – 105)	8 (IQR 0–18) 2 (IQR 0–10)	6 (IQR 0–18) 2 (IQR 0–12)	8 (IQR 0–20) 2 (IQR 0–17)

	Inter group p value	**<0.001**	**<0.001**	**<0.001**
Active LN Inactive LN	SLEDAI-2K (renal: 0 – 16)	4 (IQR 0–16) 0 (IQR 0–3)	4 (IQR 0–12) 0 (IQR 0–8)	4 (IQR 0–16) 0 (IQR 0–12)
	Inter group p value	**<0.001**	**<0.001**	**<0.001**
Active LN Inactive LN	SLEDAI-2K (extrarenal: 0 – 89)	4 (IQR 0–12) 2 (IQR 0–10)	2 (IQR 0–10) 2 (IQR 0–9)	4 (IQR 0–12) 2 (IQR 0–8)
	Inter group p value	0.66	0.18	0.10

LN: Lupus Nephritis; eGFR: Estimated Glomerular Filtration Rate; ESR: Erythrocyte Sedimentation Rate; anti dsDNA: anti– double-stranded DNA antibody; C3: Complement 3; C4: Complement 4; uPCI: Urine Protein Creatinine Index; uNGAL: Urine Neutrophil Gelatinase-Associated Lipocalin; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index-2K

Table 3: Characteristics in the active and inactive LN groups at each time point.

Patient gp **Active LN** **(NR/Relapse)** **Inactive LN** **(CR/PR)**	**Parameters**	**Baseline** **n=47**	**2 months** **n=29** **n=18**	**4 months** **n=19** **n=28**
Active LN Inactive LN	Systolic blood pressure (mmHg)	128 ± 13.16	131.69 ± 12.62 121.86 ± 11.73	130.23 ± 12.51 123.33 ± 11.87
	Inter group p value		**0.01**	**0.01**
Active LN Inactive LN	Diastolic blood pressure (mmHg)	77.80 ± 10.31	80.73 ± 10.76 72.73 ± 8.25	81.15 ± 7.86 73.14 ± 9.01
	Inter group p value		**0.03**	0.77
Active LN Inactive LN	Serum albumin (35–50 g/L)	37.78 ± 5.54	37.60 ± 5.02 40.93 ± 2.54	36.92 ± 2.53 40.66 ± 3.74
	Inter group p value		**0.01**	**0.01**
Active LN Inactive LN	Serum creatinine (44–80 µmol/L)	69 (IQR 33–252)	81.97 (IQR 40–244) 67 (IQR 44–139)	86 (IQR, 48–224) 62 (IQR 41–143)
	Inter group p value		0.43	**0.01**
Active LN Inactive LN	eGFR (60 ml /min /1.73 m^2)	93.61 ± 46.01	88.56 ± 40.88 97.93 ± 31.81	71.15 ± 29.20 99 ± 38.15
	Inter group p value		0.28	**0.01**
Active LN Inactive LN	ESR	38.5 (IQR 21–91)	45 (IQR 22–92) 55 (IQR 10–103)	32 (IQR 8–105) 36.50 (IQR 1–70)
	Inter group p value		0.48	0.78
Active LN Inactive LN	Anti-dsDNA Ab titers (<30 IU)	35.18 (IQR 1.73–195.97)	38.59 (IQR 0.74–267.61) 13.82 (IQR 1.54–135.29)	13.75 (IQR 2.11–175.22) 41.53 (IQR 2.07–252.85)
	Inter group p value		0.94	0.82
Active LN Inactive LN	Serum C3 (79 – 152 mg /dL)	100.5 ± 36.39	96.44 ± 32.54 113 ± 43.05	106.06 ± 39.29 98.25 ± 21.99
	Inter group p value		0.43	0.50
Active LN	Serum C4	21.46 ± 12.82	20.08 ± 10.12	22.15 ± 19.90

Inactive LN	(16 – 38 mg /dL)		28.52 ± 16	22.93 ± 11.77
	Inter group p value		0.20	0.31
Active LN Inactive LN	Proteinuria (uPCI) (< 20 mg/mmol creatinine)	110 (IQR 10–510)	120 (IQR 10–480) 30 (IQR 10–50)	110 (IQR 10–510) 40 (IQR 10–50)
	Inter group p value		**< 0.001**	**< 0.001**
Active LN Inactive LN	Urinary leucocytes/ HPF×10^6 /L	0 (IQR 0–20)	0 (IQR 0–20) 0 (IQR 0–10)	0 (IQR 0–20) 0 (IQR 0–5)
	Inter group p value		0.31	0.009
Active LN Inactive LN	Urinary RBC/HPF×10^6 /L	0 (IQR 0–10)	0 (IQR 0–20) 0 (IQR 0–5)	0 (IQR 0–50) 0 (IQR 0–20)
	Inter group p value		0.29	0.23
Active LN Inactive LN	uNGAL (ng/ mg creatinine)	195.80 (IQR 21.07–1413)	187.33 (IQR 58.57–993) 117.9 (IQR 18.33–324)	141.6 (IQR 40–526) 74.94 (IQR 21.50–605)
	Inter group p value		**0.03**	**0.004**
Active LN Inactive LN	SLEDAI-2K (global: 0 – 105)	8 (IQR 0–18)	6 (IQR 0–18) 0 (IQR 0–12)	8 (IQR 4–16) 2 (IQR 0–12)
	Inter group p value		**< 0.001**	**< 0.001**
Active LN Inactive LN	SLEDAI-2K (renal: 0 – 16)	4 (IQR 0–16)	4 (IQR 0–12) 0 (IQR 0–8)	4 (IQR 4–16) 0 (IQR 0–12)
	Inter group p value		**<0.001**	**<0.001**
Active LN Inactive LN	SLEDAI-2K (extrarenal: 0 – 89)	4 (IQR 0–12)	2 (IQR 0–10) 0 (IQR 0–8)	4 (IQR 0–8) 2 (IQR 0–4)
	Inter group p value		0.10	0.65

Gp: group; Calcineurin inhibitors: Cyclosporine A/ Tacrolimus; eGFR: Estimated Glomerular Filtration Rate; ESR: Erythrocyte Sedimentation Rate; anti dsDNA: anti–double-stranded DNA antibody; C3: Complement 3; C4: Complement 4; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index-2K; uPCI: Urine Protein Creatinine Index; uNGAL: Urine Neutrophil Gelatinase Associated Lipocalin

Table 4: Follow up characteristics within the patient subgroup with LN active at baseline.

Lupus nephritis relapses

A total of 13 patients in the overall study population relapsed on follow up- five patients at baseline, two at 2 months and six at 4 months and were appropriately treated. Their median uNGAL levels were highest at the time of relapse compared to their pre-relapse levels and decreased in response to treatment (Figure 1).

Renal biopsy was repeated in 1/5 who relapsed at baseline, 1/2 at 2 months and none of the six relapsers at 4 months. Their histological findings had deteriorated from classes IV to class V and mixed class IV + V respectively.

Association between uNGAL with parameters of LN activity on follow up

Details of associations between uNGAL and various laboratory parameters and SLEDAI-2K measures are presented in Table 5. At each visit, uNGAL correlated directly with proteinuria (uPCI), SLEDAI-2K (global) and SLEDAI-2K (renal). uNGAL correlated with serum creatinine at 4 months only and inversely with eGFR at both 2 and 4 months. There were no associations at all time points between uNGAL with serum albumin and serological markers.

ROC curve analysis of uNGAL to identify LN activity

ROC curves were constructed to assess the potential diagnostic value of uNGAL compared with standard blood and urine markers at each visit to identify patients with active LN (Table 6).

At each visit, the area under the curve (AUC) for uNGAL was higher than those for serum albumin, serum creatinine, eGFR, anti-dsDNA Ab titres, C3, C4 for detection of LN activity (Table 6). Whereas it was lower than those for proteinuria (uPCI) and SLEDAI-2K renal score. Thus uNGAL outperformed most of the usual markers used for monitoring LN activity but was not as good as proteinuria (uPCI) and SLEDAI-2K renal score. This is illustrated by the ROC curves for uNGAL and pertinent markers at end study (Figure 2) which shows that the AUC for uNGAL was 0.79 (95% CI: 0.68 - 0.90; p<0.001). The maximum Youden index was 0.43 with a cut off value of 73.43 ng/mg creatinine, giving a sensitivity of 0.81 and specificity of 0.62 for the detection of LN activity.

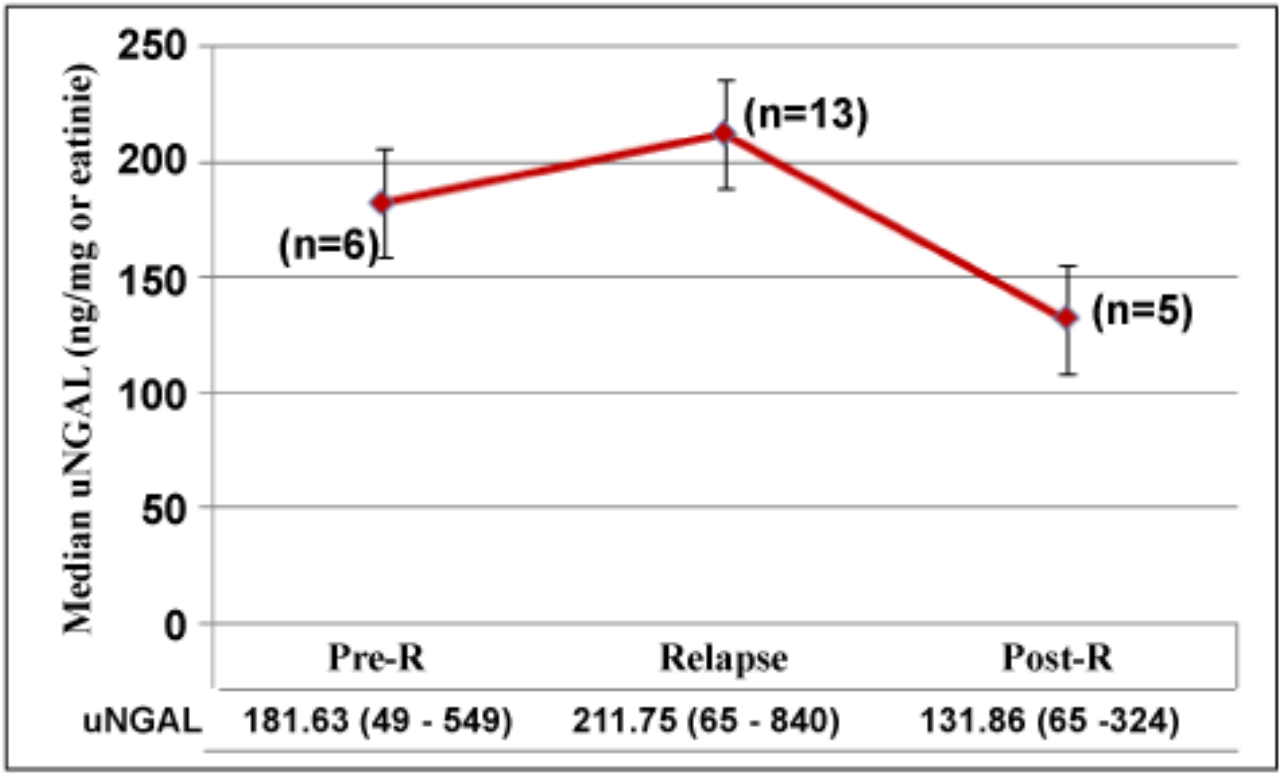

Figure 1: Median uNGAL levels in LN relapse compared to pre and post relapse levels. Pre-R : Pre-Relapse; Post-R : Post-Relapse.

Spearman's rho variable (Active: Inactive LN)	Baseline (47:53)		2 months (28:71)		4 months (22:78)	
	r_{sp}	p value	r_{sp}	p value	r_{sp}	p value
Serum albumin	-0.11	0.27	-0.17	0.08	-0.16	0.10
Serum creatinine	0.17	0.09	0.16	0.11	0.22	**0.03**
eGFR	-0.18	0.07	-0.20	**0.04**	-0.25	**0.01**
Anti-dsDNA Ab titres (IU)	0.17	0.09	-0.03	0.72	0.06	0.56
C3 (mg/dl)	-0.09	0.34	-0.08	0.38	-0.19	0.06
C4 (mg/dl)	-0.02	0.97	-0.09	0.38	-0.19	0.06
Proteinuria (uPCI)	0.34	**0.001**	0.31	**0.002**	0.37	**<0.001**
Leucocyturia	0.21	**0.03**	0.19	0.06	0.13	0.19
Haematuria	0.10	0.29	0.04	0.64	0.06	0.50
SLEDAI-2K global score	0.19	**0.05**	0.29	**0.004**	0.33	**0.001**
SLEDAI -2K renal score	0.32	**0.001**	0.31	**0.001**	0.35	**0.001**
SLEDAI-2K-extra renal score	-0.12	0.22	0.05	0.60	0.17	0.08

Table 5: Associations between uNGAL with parameters of lupus nephritis (LN) activity on follow up.

	Baseline				2 months				4 months			
			95% CI				95% CI				95% CI	
Variables	AUC	p	LB	UB	AUC	p	LB	UB	AUC	p	LB	UB
uNGAL	0.83	**0.001**	0.74	0.92	0.70	**0.002**	0.59	0.81	0.79	**<0.001**	0.68	0.90
Serum albumin	0.23	**0.004**	0.13	0.33	0.24	**<0.001**	0.14	0.35	0.23	**<0.001**	0.12	0.34
Serum creatinine	0.59	0.14	0.46	0.72	0.57	0.25	0.43	0.71	0.70	**0.003**	0.57	0.84
eGFR	0.40	0.15	0.27	0.53	0.40	0.16	0.27	0.54	0.29	**0.004**	0.16	0.43
Anti dsDNA Ab titres	0.48	0.78	0.35	0.61	0.47	0.74	0.34	0.61	0.53	0.71	0.35	0.70
Serum C3	0.39	0.09	0.26	0.51	0.46	0.56	0.33	0.58	0.39	0.18	0.25	0.53
Serum C4	0.43	0.35	0.30	0.56	0.42	0.27	0.30	0.55	0.46	0.67	0.30	0.62
Haematuria	0.62	0.08	0.48	0.76	0.54	0.51	0.41	0.67	0.62	0.08	0.48	0.76
Proteinuria (uPCI)	0.94	**<0.001**	0.90	0.99	0.92	**<0.001**	0.85	0.99	0.88	**<0.001**	0.78	0.97
SLEDAI-2K (renal score)	0.96	**<0.001**	0.90	1	0.84	**<0.001**	0.75	0.92	0.84	**<0.001**	0.75	0.93

AUC: Area Under the Curve; CI: Confidence Interval; LB: Lower Bound; UB: Upper Bound; C3: Complement 3; C4: Complement 4; uPCI: Urine Protein Creatinine Index; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index-2K

Table 6: Area under the curve (AUC) of ROC curves for uNGAL and standard biomarkers for lupus nephritis activity on follow up.

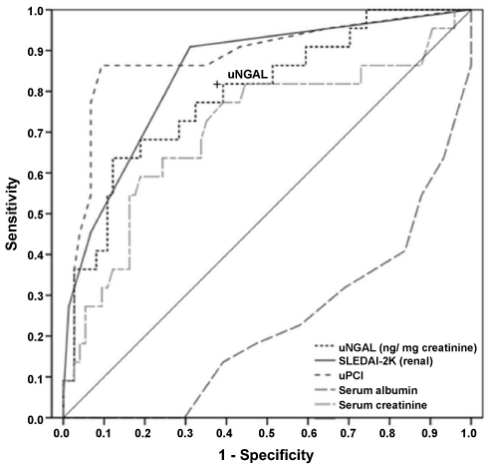

Figure 2: Receiver operating characteristic curve (ROC) of urine neutrophil gelatinase-associated lipocalin (uNGAL) compared with those of Systemic Lupus Erythematosus Disease Activity Index-2K renal score (SLEDAI-2K renal), urine protein creatinine index (uPCI), serum albumin and serum creatinine for the diagnosis of lupus nephritis (LN) activity. The area under the curve (AUC) for uNGAL was 0.79 (p<0.001). The symbol (+) represents the best cut-off value for uNGAL (73.43 ng/mg creatinine) with a sensitivity of 0.81 and a specificity of 0.62. The AUC for SLEDAI-2K (renal) was 0.84 (p<0.001) and that for proteinuria was 0.88 (p<0.001). The AUC for serum albumin was 0.23 (p<0.001) and that for serum creatinine was 0.70 (p=0.003). Thus, uNGAL outperformed serum albumin and serum creatinine but was not as good as proteinuria (uPCI) and SLEDAI-2K renal score for the detection of LN activity.

					95% CI for EXP(B)	
	B	S.E	p	OR	Lower	Upper
uNGAL	0.001	0.002	0.48	1.001	0.99	1.005
Serum albumin	-0.19	0.08	**0.02**	0.82	0.70	0.96
Serum creatinine	0.001	0.07	0.95	1.001	0.96	1.03
eGFR	-0.008	0.01	0.54	0.99	0.96	1.01
Proteinuria (uPCI)	5.17	2.14	**0.03**	3.97	3.20	4.68
SLEDAI-2K (renal score)	0.11	0.12	0.35	1.12	0.88	1.41

R^2 0.35 (Hosmer & Lemeshow's), 0 .31 (Cox & Snell), 0.48 (Nagelkerke). Model x^2=35.13, p<0.001. B: Beta; SE: Standard Error; OR: Odds Ratio = Exp(B); CI: Confidence Interval; uNGAL: Urine Neutrophil Gelatinase-Associated Lipocalin; eGFR: Estimated Glomerular Filtration Rate; uPCI: Urine Protein Creatinine Index; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index-2K.

Table 7: Predictors of Lupus Nephritis outcome at last follow up.

Independent predictors of LN activity

Binary logistic regression was performed to assess independent predictors of LN activity. uNGAL and all clinical variables with a p value ≤ 0.05 (Table 3) at 2 months were entered into the regression model to predict LN outcome at 4 months. These included serum albumin, serum creatinine, eGFR, proteinuria (uPCI) and SLEDAI-2K (renal) (Table 7).

Only a fall in the serum albumin (odds ratio (OR)=0.82, 95% CI, 0.70 - 0.96, p=0.02) and increasing proteinuria (uPCI) (OR=3.97, 95% CI, 3.20-4.68, p=0.03) emerged as independent predictors of LN activity.

Discussion

The potential role for uNGAL in monitoring and predicting renal disease activity in LN has been explored in both murine and human LN. In murine models of LN, NGAL has been found to induce apoptosis in mesangial cells and facilitate recruitment of inflammatory cells in the kidney through the upregulation of pro-inflammatory mediators [25].

In humans, many cross-sectional studies have examined the role of NGAL in SLE patients for detection of LN activity and relapses [12,13,16]. We too have previously demonstrated in a cross-sectional study that uNGAL levels in patients with active LN were higher than in those with inactive renal disease [16]. We also found no association between uNGAL with prednisolone and other immunosuppressive medications [16].

To date, only four longitudinal studies have been reported on the usefulness of uNGAL as a biomarker for active LN or predictor for impending relapse/ flares. Of these four studies, three were performed in pediatric patients and only one in adults. Suzuki et al. [15] in a longitudinal study of 85 pediatric patients with SLE showed that uNGAL levels were a novel biomarker for relapse of LN. Hinze et al. [26] in another longitudinal study of children with SLE (n=111) found that uNGAL levels predicted the impending relapse of LN. An increase in plasma NGAL levels also predicted worsening of global and renal disease activity. In a recent longitudinal study of childhood–onset SLE patients (n=64), Watson et al. [27] demonstrated uNGAL to be a good predictor of worsening renal disease activity.

In the only reported longitudinal study of 107 adult SLE patients of whom only 25 had biopsy-proven LN, Rubinstein et al. [14] also found that uNGAL was a significant predictor of renal flare and outperformed anti-ds DNA Ab.

Unlike all the above longitudinal studies, all our adult SLE patients were 'homogenous' for biopsy-proven LN albeit with varying grades of activity. These 100 LN patients were followed at shorter 2 monthly intervals over 3 visits in an attempt to diagnose and/or predict 'early' flares. uNGAL levels were again higher in patients with active LN compared to those with inactive LN.

With treatment, majority of patients with active LN at baseline (28/47, 60%) achieved remission at end study, a third remained with NR (16/47, 34%) and three patients (6%) relapsed. uNGAL levels fell significantly in all patients in response to treatment in both CR/PR as well as in the persistent NR patients.

In those patients who relapsed (13%), uNGAL levels increased concurrently with LN relapse but decreased progressively with treatment. However, in the single one nonresponder throughout the study, uNGAL increased further on follow up in tandem with nephrotic range proteinuria and rising serum creatinine levels. This patient was subjected to repeat renal biopsy which showed that although the activity index of her class IV LN had decreased from 14/24 to 3/24, she had developed an added membranous component i.e. class IV+V. More intensive remission-induction therapy was instituted. Just like SLE its parent disease, LN can also undergo multiple episodes of relapses-remissions and to perform repeated renal biopsies is such patients is not only highly traumatic, may lead to complications and is possibly unethical. Thus serial uNGAL monitoring in conjunction with the usual clinical parameters can obviate repeated 'invasive' renal biopsies as is the current clinical nephrology practice.

Similar to findings reported by Suzuki et al. [15], uNGAL levels in our study also correlated with proteinuria, SLEDAI-2K global and renal scores. These data suggest that the source of increased uNGAL in LN is most likely due to increased production by the kidneys and not due to extrarenal disease per se. The correlation between uNGAL levels with SLEDAI-2K global score can be explained by the renal components included as criteria in the said score. Like Pitashny et al. [13] and Suzuki et al. [15], we too found no correlation between uNGAL levels with SLEDAI-2K extrarenal score or anti-dsDNA Ab titres and serum complements. The role of anti-dsDNA Ab titres and C3 and C4 levels as indicators of LN disease activity or LN outcome remains controversial [28]. Similar to other studies [29,30], we found no association between anti-dsDNA Ab titres and serum complement levels with LN activity.

At all time points, the ROC curves for uNGAL showed it is to be a good noninvasive marker for detection of LN activity and performed better than many of the usual blood and urinary markers such as serum albumin, serum creatinine, eGFR and haematuria. However, it was not as good as proteinuria and SLEDAI-2K renal score for the detection of clinical LN activity. This fact may be due to that the proteinuria and SLEDAI-2K renal score were included as major criteria in the definition of LN activity. uNGAL also outperformed the usual serological markers i.e. anti-dsDNA Ab titres, serum complements for diagnosis of LN activity. This lack of correlation between anti-dsDNA Ab titres and LN activity corroborates with that reported by Rubinstein et al. [14]. Whereas these same authors [14] found that using ROC curves, uNGAL showed equivalent performance to those of C3 and C4 for predicting LN flares in patients with a past history of biopsy-proven LN.

Multiple logistic regression analysis showed that only serum albumin and proteinuria were independent predictors of LN activity or relapse but not uNGAL. This is in contradiction to findings by Rubinstein et al. [14] that uNGAL remained a significant predictor for LN activity even after adjustment for age, sex, and race, class of LN and anti-dsDNA Ab titres.

The major limitation of this study was that the association between uNGAL with the various histological classes of LN were not obtained concurrently. This was due to the delay between urine collection for NGAL and renal biopsies. Perhaps more conclusive results could have been obtained if urine samples for the NGAL were taken concurrently with renal biopsies for those patients who had LN flares or who had persistently active LN. Another limitation was the short follow up of 4 months only due to cost (predominantly) and time constraints. Notwithstanding these shortfalls, this study was purposely designed with the shorter observation intervals of 2 months over the 3 visits as this would have had a better chance of diagnosing/ picking up early renal flares as well as provide closer monitoring of treatment response. A longer follow up for at least 2 - 3 years would be ideal.

In conclusion, uNGAL was significantly increased in active LN especially in LN flares. It had good diagnostic performances with good sensitivities and moderate specificities for detection of LN activity and/ or relapse. Perhaps the sensitivities and specificities of these biomarkers could be improved by incorporating several new biomarkers currently also under study into a panel of biomarkers for assessing LN activity similar to that proposed for acute kidney injury (AKI), interleukin-8 (IL-18), kidney injury molecule-1 (KIM-1) and liver-type fatty acid-binding protein (L-FABP) [31]. Although uNGAL was not an independent predictor for LN activity, it could serve as an adjunctive marker for the diagnosis of

subclinical and early relapses. Larger prospective longitudinal studies for longer periods are indicated.

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The manuscript has been seen and approved by all authors and it is not under consideration for publication elsewhere in a similar form.

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Conflict of Interest

The authors declare no conflict of interest.

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