

## Original article

# NLR pyrin domain 3 (NLRP3) protein expression in juvenile systemic lupus erythematosus and lupus nephritis

**Background:** In systemic lupus erythematosus (SLE), the increased rate of apoptosis and inefficient clearance of apoptotic cells leads to activation of NLRP3 inflammasome. NLRP3 overstimulation is proved to be involved in the pathogenesis of lupus nephritis (LN). We sought to measure the expression of NLRP3 among pediatric patients with SLE and LN, in correlation to markers of activity. **Methods:** A pilot, cross-sectional controlled study was conducted from Jan 2022 to Jan 2024. Fifty-six patients with confirmed SLE and active LN with or without other system activity were compared to age and sex matched 56 healthy controls as regards expression of NLRP3 protein using enzyme linked immunosorbent assay (ELISA) technique (Human NLRP3 ELISA Kit). **Results:** Peripheral expression of NLRP3 was significantly higher among patients with juvenile SLE with active LN than controls ( $p$  value  $<0.001$ ). Other systemic activities were present within 27/56 patients, 11 patients had cardiac manifestations and 16 patients had neurological manifestations. SLE disease activity index (SLEDAI) was 32.4 (SD 7.6) and was significantly correlated to peripheral NLRP3. LN Class, level of C3 consumption, proteinuria and kidney functions were not correlated to levels of NLRP3 protein. **Conclusion:** Expression of NLRP3 inflammasome in the peripheral blood was significantly elevated in juvenile SLE with LN compared to controls, with significant correlation to SLEDAI, but not to LN Class or markers of activity. Comparison between pediatric patients with SLE with and without LN and comparing renal NLRP3 inflammasome to its peripheral expression in patients with LN are recommended.

**Key words:** NLRP3; inflammasome; systemic lupus erythematosus; lupus nephritis; SLEDAI

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## INTRODUCTION

The cytosolic molecular factories, namely inflammasomes, consist of a sensor protein, adaptor protein, and an apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), as well as the proinflammatory caspase, caspase-1. The inflammasome cascade can be triggered by infection or cellular stress ending with the activation of caspase-1. Active caspase-1 then processes the proinflammatory cytokines, pro-IL-1 $\beta$  and pro-IL-18 which are secreted upon caspase-1 cleavage. Inflammasome activation eventually enhances inflammatory cell death known as pyroptosis. The latter has the function of blunting intracellular pathogen replication.<sup>1</sup>

The first discovered sensor proteins that form inflammasomes are the nucleotide-binding domain (NOD) and leucine-rich repeat (LRR) containing receptor; NLR.<sup>2</sup> The NLR pyrin domain containing

(NLRP) 3 is the most thoroughly studied NLR. It can be found in myeloid cells at low levels and usually induced by Toll-like receptor (TLR) agonists and by some inflammatory cytokines, such as the tumor necrosis factor (TNF)- $\alpha$ , in an NF- $\kappa$ B-dependent manner. The NLRP3 activation signals include pathogen associated molecular patterns and toxins of bacteria, viruses, and protozoan pathogens. Also, host-derived damage-associated molecular patterns (DAMPs) such as ATP, uric acid crystals and amyloid- $\beta$  fibrils can activate NLRP3.<sup>3</sup> The interaction between NLRP3, ASC, and pro-caspase-1 forms the protein complex named inflammasome.<sup>4</sup>

In SLE, the increased rate of apoptosis together with the inefficient clearance of apoptotic cells result in accumulation of apoptotic cell debris which may progress to secondary necrosis and accumulation of DAMPs and of NLRP3. Antibody recognition of DNA and RNA antigens form

immune complexes that can act as DAMPs and activate NLRP3 through TLR activation.<sup>5</sup> In lupus nephritis (LN), renal dendritic cells and macrophages and some non-immune renal parenchymal cells such as endothelial cells, parietal epithelial cells, tubular epithelial cells and podocytes can express the NLRP3 inflammasome components after exposure to various PAMPs and DAMPs.<sup>6</sup> Moreover, ischemic reperfusion kidney injury may cause cell damage, release of endogenous DAMPs resulting in activation of the NLRP3 inflammasome. Rhabdomyolysis produce catalytic iron and myoglobin heme from damaged muscles, inducing oxidative stress in renal tubular epithelial cells enhancing NLRP3 inflammasome activation and acute kidney injury.<sup>7</sup>

Since NLRP3 inflammasome has been frequently implicated in the pathogenesis of renal tissue damage, its inhibition might prove to be a therapeutic target in LN.<sup>8</sup> We, therefore, sought to assess the value of NLRP3 peripheral expression as a relatively non-invasive and easy to perform test in the assessment of pediatric lupus nephritis in relation to some conventional clinical and laboratory markers of activity.

## METHODS

**Study settings:** This pilot, comparative cross-sectional study was conducted on 56 pediatric patients diagnosed as systemic lupus erythematosus (SLE) according to the Systemic Lupus International Collaborating Clinics (SLICC) criteria of diagnosis.<sup>9</sup> SLE activity was assessed according to SLE disease activity index (SLEDAI)-2K.<sup>10</sup> Lupus nephritis (LN) activity was diagnosed and categorized according to the British Isles lupus assessment group (BILAG) 2004 criteria.<sup>11</sup> The patients' group was compared to 56 age and sex matched healthy subjects as a control group. Patients had active LN either with or without other systemic activities; they were recruited from the Pediatric Allergy, Immunology and Rheumatology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt during the period from Jan 2022 to Jan 2023.

### Inclusion criteria:

- Patients with biopsy-confirmed LN according to World Health Organization (WHO) and the International Society of Nephrology/Renal Pathology Society's recommendations.<sup>12</sup>
- Ages between 5 and 16 years old from both genders.
- SLE/LN activity status within one week prior to enrollment.

### Exclusion criteria:

- Patients with evidence of autoinflammatory disorders.
- Patient who had macrophage activation syndrome three months or less before enrollment.

**Ethical considerations:** The study gained approval of the Research Ethics Committee of the Department of Pediatrics, Faculty of Medicine, Ain Shams University. Ethical approval number is FMASU 000017585. Verbal consent was obtained from the guardians of all the enrolled individuals.

**Sampling method:** Consecutive enrolment.

**Study tools:** All patients were subjected to:

1. *Clinical history taking* including age, gender, onset, duration and diagnostic lag, and system/organ affection.
2. *Anthropometric measurements:* weight, height, and body mass index (BMI)
3. *Clinical examination:* including arterial blood pressure measuring and other vital signs, presence of enlarged lymph nodes, liver or spleen, presence of any cardiac, neurological, or musculoskeletal signs.
4. *Medical records* were revised for the histopathological classification of renal biopsy, and the last laboratory evaluation (within 4 weeks) including complete blood counts (CBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), C3, anti-ds-DNA, 24-hour urinary proteins, urine analysis, and serum creatinine and urea levels.
5. *Laboratory investigations:* Both patients and controls were subjected to assessment of their NLRP3 protein level in the peripheral blood using enzyme linked immunosorbent assay (ELISA) technique (Human NLRP3 ELISA Kit) according to the manufacturer's instructions.

### Statistical methods:

Data was collected, revised, coded and entered to the statistical package for social science (IBM SPSS) version 23. The quantitative data were presented as mean, standard deviations and ranges. Categorical variables were presented as number and percentages. The suitable corresponding tests for comparison and correlation were used. Receiver-operating characteristic (ROC) curve was used to examine the discriminative value of urinary or serum CC16. The area under the ROC curve (AUC) was interpreted as follows: AUC < 0.6 = fail, 0.6 to 0.69 = poor, 0.7 to 0.79 = fair, 0.8 to 0.89 = good, ≥ 0.9 = excellent. P value < 0.05 was considered significant.

## RESULTS

Fifty-six pediatric patients who were diagnosed as SLE with LN in activity were recruited. Their mean age was 12.41 (SD 2.53) years and they included 8/56 (14.29%) males and 48/56 (85.71%) females. Patients were compared to 56 healthy age ( $P = 0.56$ ) and sex ( $p = 0.67$ ) matched controls. Obesity was documented within 21/56 (37.5%) of the patients, and underweight was found in 1/56 (1.7%) according to their body mass index (BMI) ( $>95$ th and  $<5$ th percentile respectively). All obese subjects had corticosteroids-induced obesity. The underweight patient had the least SLEDAI index. Eight patients (14.4%) had systolic and diastolic hypertension and one patient had diastolic hypertension only.

The median disease duration was 24 months, ranging from 2 to 84 months. LN histopathological classification revealed class II LN (39.3%), class III (37.5%), class IV (16.1%) and class V LN (7.1%). Three patients needed regular renal dialysis. Associated lupus carditis was detected among 11 (19.6%) patients, confirmed by echocardiography with or without abnormal levels of cardiac enzymes (carditis in 81.8% and pericardial effusion in 18.2%). Neurological lupus was evident in another 16 patients (28.6%), 10 of them had cerebritis and the other 6 had peripheral neuritis. SLE disease activity index (SLEDAI) mean value was 32.4 (SD 7.6), ranging between 18 and 48 (Table 1).

Laboratory data showed consumed C3 (median 60.35 mg/dl, IQR 39.5 - 71) and proteinuria (median 341 mg, IQR 224.5 - 932). Hematuria was recorded among 5 patients, granular casts among 10 patients, elevated anti-ds DNA titre among 46 patients and elevated serum creatinine among three patients. Lupus anticoagulant index was high in 22/56 patients and anti-cardiolipin antibodies were positive in 16/56 patients. CBC values were unremarkable except for one patient who had leukopenia and autoimmune hemolysis (Table 2).

NLRP3 protein was significantly higher among SLE patients 1940.5 ng/L (1725 - 2205.5) than healthy control 249.75 ng/L (143.8 - 380.75) ( $Z = 9.12$ ,  $p < 0.001$ ). The predictive value of NLRP3 protein was evaluated using the receiver operating characteristic (ROC) curve which showed that a level higher than 963.9 ng/L was a dependable differentiator between SLE and controls with a sensitivity and specificity of 100% for both with area under the curve = one. The 95% confidence index = 0.97-1 (Figure 1).

NLRP3 protein expression was not correlated with the patients' age, BMI, blood pressure, disease duration or diagnosis lag (Table 3). Although NLRP3 expression was significantly correlated to the SLEDAI-2K ( $P < 0.001$ ), it was not correlated to LN Class or any laboratory parameters except for hemoglobin and serum urea levels (Table 4).

**Table 1.** Clinical characteristics of the SLE patients

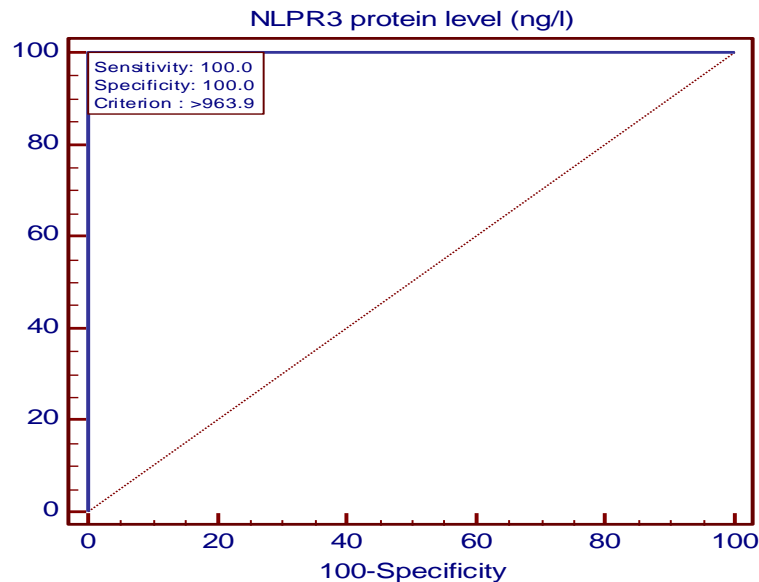
Data	N	%	
Renal Dialysis	No	53	94.64
	Yes	3	5.36
Lupus nephritis Class	2	22	39.29
	3	21	37.5
	4	9	16.07
	5	4	7.14
Cardiac activity (n = 11)	No	45	80.36
	Yes	11	19.64
Type of cardiac affection	Carditis	9	81.8
	Pericardial effusion	2	18.2
Neurological activity (n = 16)	No	40	71.43
	Yes	16	28.57
Type of neurological affection	Cerebritis	10	62.5
	Peripheral neuritis	6	37.5
SLEDAI-2K	Mean $\pm$ SD	Median (IQR)	Range
	32.14 $\pm$ 7.57	33 (27 - 38)	18 - 48
Duration of the disease (mo.)	28.61 $\pm$ 19.17	24 (12 - 36)	2 - 84
Diagnosis lag (mo.)	4.16 $\pm$ 2.97	4 (2 - 6)	1 - 12

SLEDAI-2K= SLE disease activity index 2000, SD= standard deviation, IQR= interquartile range

**Table 2.** Laboratory data of the SLE patients

Data		Mean $\pm$ SD	Median (IQR)	Range
ESR (mm/h)		56.45 $\pm$ 42.98	37 (23 – 95)	12 – 167
CRP (mg/l)		14.23 $\pm$ 16.76	6 (6 – 20)	0.7 – 100
C3 (mg/dl)		56.57 $\pm$ 19.67	60.35 (39.5 – 71)	15 – 90
Anti-ds-DNA	Negative	10 (17.86%)		
	Positive	46 (82.14%)		
Lupus anti-coagulant	Normal	34 (60.71%)		
	Elevated	22 (39.29%)		
Anti-cardiolipin antibody	Negative	40 (71.43%)		
	Positive	16 (28.57%)		
24-hour urinary proteins (mg)		745.95 $\pm$ 1069.23	341 (224.5 – 932)	60 – 6180
Urine granular casts	Negative	46 (82.14%)		
	Positive	10 (17.86%)		
Urine RBCs	Insignificant	51 (91.07%)		
	Significant	5 (8.93%)		
Serum creatinine (mg/dl)		0.66 $\pm$ 0.42	0.55 (0.5 – 0.8)	0.3 – 3.1
Urea levels (mg/dl)		21.71 $\pm$ 9.1	19.5 (15 – 24)	9 – 43
TLC ( $\times 10^9/l$ )		8.8 $\pm$ 4.66	8.45 (4.8 – 11.4)	2.2 – 25.5
ANL ( $\times 10^9/l$ )		5.61 $\pm$ 3.41	5.4 (2.8 – 7)	0.7 – 17.5
ALC ( $\times 10^9/l$ )		2.46 $\pm$ 1.64	1.95 (1.4 – 3.05)	0.6 – 7.1
Hb (gm/dl)		11.14 $\pm$ 1.25	11.35 (10.2 – 12)	7.7 – 13
MCV (fl)		80.52 $\pm$ 3.92	80.9 (78 – 83)	70.8 – 89
MCH (pg)		25.08 $\pm$ 2.73	24.55 (23 – 26)	22 – 38.5
PLT ( $\times 10^9/l$ )		280.86 $\pm$ 72.62	285 (223 – 323.5)	143 – 472

ALC= absolute lymphocytic count, ANL= absolute neutrophil count, Anti-ds-DNA= deoxyribonuclease, CRP= C-reactive protein, C3= complement protein 3, ESR= erythrocyte sedimentation rate, IQR= interquartile range, HB= hemoglobin, MCV= mean corpuscular volume, MCH= mean corpuscular hemoglobin, PLT=platelet, SD= standard deviation, , RBCs= red blood cell, TLC= total leucocytic count

**Figure 1.** ROC curve analysis of NLRP3 in SLE and control groups

**Table 3.** Correlations between the NLRP3 results and some clinical data of the SLE patients

Data	NLRP3 protein level (ng/L)	
	Spearman's rho	p-value
Age (Year)	-0.213	0.114
BMI	0.023	0.868
SBP	-0.078	0.568
DBP	0.169	0.214
Duration of the disease (Month)	0.258	0.055
Diagnosis lag (Month)	0.228	0.091

BMI= Body mass index, SBP= systolic blood pressure, DBP= diastolic blood pressure

**Table 4.** Variation of the NLRP3 protein levels with the SLE patients' clinical and laboratory data

Data		NLRP3 protein level (ng/L)	Test of Significance	
		Median (IQR)	Value	p-Value
Lupus nephritis class	2	2155.5 (1853 - 3129)	H= 5.641	0.130
	3	1886 (1552 - 2033)		
	4	1938 (1804 - 2126)		
	5	1732 (1536.5 - 2047.5)		
Lupus carditis	No	1902 (1715 - 2177)	Z= -1.547	0.122
	Yes	2180 (1891 - 3227)		
Neurological lupus	No	1899.5 (1612 - 2200.5)	Z= -1.152	0.249
	Yes	2083.5 (1878.5 - 2232)		
Anti-ds-DNA	Negative	1853 (1735 - 2000)	Z= -1.070	0.285
	Positive	1955.5 (1715 - 2255)		
Lupus Anti-coagulant	Negative	1971.5 (1755 - 2560)	Z= -1.175	0.240
	Positive	1874 (1651 - 2177)		
Anti-cardiolipin antibody	Negative	1940.5 (1745 - 2239.5)	Z= -0.653	0.514
	Positive	1900 (1569 - 2162)		
Urine granular casts	Negative	1896 (1715 - 2213)	Z= -1.134	0.257
	Positive	2130 (1943 - 2198)		
Urine RBCs	Normal	1947 (1735 - 2255)	Z= -1.221	0.222
	Increased	1804 (1657 - 1943)		
ESR (mm/h)			r= -0.014	0.918
CRP (mg/l)			r= -0.141	0.301
C3 (mg/dl)			r= 0.124	0.361
24-hour urinary proteins (mg)			r= 0.249	0.064
Serum creatinine (mg/dl)			r= 0.211	0.118
Urea levels (mg/dl)			r= 0.488	<0.001 (significant)
TLC (*10 <sup>9</sup> /l)			r= 0.047	0.732
ANL (*10 <sup>9</sup> /l)			r= 0.041	0.762
ALC (*10 <sup>9</sup> /l)			r= 0.097	0.475
Hb (gm/dl)			r= 0.264	0.050
MCV (fl)			r= 0.154	0.259
MCH (pg)			r= 0.161	0.237
PLT (*10 <sup>9</sup> /l)			r= -0.146	0.284
SLEDAI-2K			r= 0.508	<0.001 (significant)

ALC= absolute lymphocytic count, ANL= absolute neutrophil count, Anti-ds-DNA= deoxyribonuclease, CRP= C-reactive protein, C3= complement protein 3, ESR= erythrocyte sedimentation rate, HB= hemoglobin, MCV= mean corpuscular volume, MCH= mean corpuscular hemoglobin, PLT=platelet, RBCs= red blood cell, SLEDAI-2K= SLE disease activity index 2000. TLC= total leucocytic count Mann-Whitney test of significance (Z); Kruskal Wallis test of significance (H); Spearman's rho (r)

## DISCUSSION

In the current study, some of the LN patients had other than system affection; namely cardiac and neurological. Such multisystem affection had its impact on the disease activity score (SLEDI) mounting up to  $32.14 \pm 7.57$ . Although NLRP3 protein expression was not correlated to LN activity, LN Class, laboratory markers of LN activity, or presence of other system affection, it was positively correlated to the SLEDAI-2K ( $r=0.508$ ,  $p\text{-value}<0.001$ ). This may indicate a relation of the inflammasome expression to the overall activity of SLE rather than LN in particular. It might be attributed to the progression of the inflammatory condition during the active serositis, neuritis, cerebritis, carditis and even nephritis.<sup>13</sup>

The lack of NLRP3 correlation with LN histopathological classes could be due to the uneven distribution of classes in the current study. Class II LN was present in 39.29%, Class III in 37.5%, Class IV in 16.1% and Class V LN in 7.1% of the study sample. Three patients had end-stage kidney disease (ESKD) and necessitated regular dialysis. Commonly, Class III and IV are detected in up to 75% of LN patients, carrying the worse prognosis.<sup>14</sup> Therefore, the current study results may not precisely reflect the influence of histopathological renal changes on the expression of NLRP3. An NLRP3 inflammasome significant tissue expression was reported in a previous study in biopsies of LN patients.<sup>15</sup>

Another confounding factor in our study was the influence of obesity and prolonged use of corticosteroids on NLRP3 expression. Obesity-associated systemic inflammation is characterized by increased circulating proinflammatory cytokines and activation of several kinases that regulate inflammation. Obesity-induced inflammation is mediated primarily by immune cells such as macrophages and T lymphocytes that resides in the metabolic tissues. Adipose tissue derived cells can produce inflammatory cytokines, such as tumor necrosis factor alpha, interleukin (IL) 6, and IL-10 suggesting increased risk of obesity and metabolic imbalance in patients with SLE.<sup>16</sup> However, there was no significant correlation between BMI and NLRP3 protein expression in our patients, denoting that neither obesity nor prolonged use of corticosteroids augmented the expression of NLRP3. The finding is indeed limited by the sample size.

Previous investigations of tissue versus peripheral blood expression of NLRP3 in LN revealed considerable data. A Chinese study conducted on

86 patients with LN reported elevated renal tubular expression of NLRP3 among patients with LN Class IV with a positive correlation to SLEDAI.<sup>17</sup> Another group reported significant correlation between NLRP3 and renal pathological activity index.<sup>18</sup> Yang et al. could prove that NLRP3 inflammasome is hyperactivated in macrophages of SLE patients.<sup>19</sup> LN associated podocyte dysfunction has been shown to promote inflammation and inflammasome activation and expression.<sup>20</sup> The cell membrane disruption caused by the release of inflammatory metabolites and endotoxins, or the interrupted binding of ATP to the P2X7 receptor on the cell membrane can trigger inflammasome cascade, specifically NLRP3.<sup>21,22</sup>

Hutton et al suggested that the major effect of NLRP3 inflammasome on the adaptive immunity is through switching T helper cell subsets towards the Th17 and Th1 varieties.<sup>23</sup> Since few T cells are present within the glomeruli and most infiltrating renal T cells are located in the peri-glomerular region, the effects of intraglomerular NLRP3 inflammasome activation may not endorse much effect on the overall intrarenal T cell subsets but rather on tubular and interstitial T cells. This observation may support our finding of positive correlation between NLRP3 expression and blood urea level ( $r=0.488$ ,  $p\text{-value}<0.001$ ), as the latter is one of the intracellular inducers of inflammation and promoters of cellular stress, leading to stimulation of NLRP3 expression and cleavage of the caspase cascade.<sup>24</sup>

Our study is limited by the sample size and uneven distribution of LN Classes due to the consecutive manner of subjects' inclusion. Also, the lack of comparison to an SLE group without renal involvement is another limitation. However, the strength of the study comes from its pilot nature in pediatric SLE and the documentation of LN in the subjects by prior biopsy results.

**In conclusion,** NLRP3 protein expression was significantly elevated in patients with LN compared to the healthy control group. NLRP3 protein was significantly correlated to SLE activity index values and blood urea levels denoting the value of the inflammasome in SLE activity and LN with or without other system involvement. Comparing NLRP3 patients with LN to SLE patients without LN is recommended, and studying NLRP3 protein expression in relation to various therapeutic options may provide a potential input to therapeutic protocols.

## AUTHORS' CONTRIBUTION

G.S. brought the idea to the team and shared A.H. and E.E. in designing the study protocol, following up the data collection and data analysis. G.S. shared in data interpretation with A.L., A.H. and K.A. and discussed the results. E.E. was the one in direct contact with the subjects; she collected the blood samples, sent them to A.A. for laboratory analysis, tabulated the data for statistical analysis and wrote the initial manuscript. G.S. finalized the manuscript. All authors revised and approved the final manuscript.

## CONFLICTS OF INTEREST

Authors declare they have no conflicts of interest related to this work.

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