2. Materials and Methods

Fifty children with LN disease and 25 healthy subjects were enrolled in this study; 25 patients were active and the other 25 patients were inactive LN. Median (range) anti-ds titers were 230 (3-1000 E/ml), median C3 and C4 were 0.55 g/l (0.05-1.03) and 0.14 g/l (0.04-0.30). Active LN was defined by at least two of the following items (a) new onset proteinuria > 0.5 g/24h, (b) an active urinary sediment representing glomerular injury (c) a renal biopsy providing evidence of active lupus nephritis.

2.1 Materials

Monoclonal antibodies (mAb) and flow cytometry.
The following mAb and reagents were used in this study: fluorescein isothiocyanate (FITC)–conjugated or peridinin chlorophyll A protein–conjugated anti-CD3 mAb, FITC-conjugated (PE)–conjugated anti-CD8 mAb, FITC - conjugated anti-CD4 mAb, PE-conjugated and anti-CD122 mAb, EDTA-blood and fresh urine samples were collected from patients. Urine samples from patients with signs of urinary tract infection were excluded.

2.2.1 Sample preparation and flow cytometry

The fresh urine sample was collected from each patient and control. For 3 ml urine was centrifuged at 2000 rpm for 20 min. The supernatant was removed and the pellet which contains lymphocyte was shaken. The lymphocytes from urine sample was Fixed with ice cold absolute alcohol 1ml for each tube and was preserved in +4 ^{o}C forever until analysis.

2.2.2 Isolation of peripheral blood

Peripheral venous blood samples were collected into tubes containing EDTA, and peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation for 20 min at 1500 rpm without break using Ficoll-Paque Plus solution. The bands of cell were pipette carefully into another centrifuge tube filled up with phosphate buffer saline (PBS) or hanks solution and mixed. After centrifugation for 10min at 1800 rpm, lymphocyte sediment were fixed with ice cold absolute alcohol 1ml and preserved at +4C^{o} until analysis.

This technique was applicable where the fluorochrome was directly linked to the primary antibody e.g.: phycoerythrin (PE) or fluorescein isothicyanate (FITC)-conjugate. 200 µl of lymphocytes were added in test tubes. The lymphocytes were washed with 1ml PBS, and then were centrifuged at 200 rpm for 5 minutes, the supernatant was discarded. The lymphocytes pellet was washed with PBS and mixed well. 3µl of required marker was added and was mixed well. The tubes were incubated at room temperature for 20 minutes. The cells were washed with 1ml PBS; then the tubes were centrifuged at 2000 rpm for 5 minutes. The supernatant was removed then 200ml of 4% paraformaldehyde were added for analysis by flow cytometer. Flow cytometric analysis was performed with FACClibur flow cytometer using Cell Quest software (Becton Dickinson, San Jose, CA). A total o10,000 to 20,000events were collected for each analysis. Lymphocyte gates were set on live cells using forward (FSC) and side scatter (SSC).

Statistical analysis: Data were explored, processed and analyzed using the statistical package for the social science, windows version 16, USA (SPSS PC+ version 16 software). Variable with normal distribution were expressed as mean± SD. In these variables, the T test was applied for group differences.

3. Results

We studied a total of 75 subjects. Their demographic and clinical data are summarized in Table (1).

Table 1: Demographic and clinical characteristics of patients and control subjects

	Control	*Lupus Nephritis patients*	
		Inactive	*Active*
Case no.	25	25	25
Sex (male\Female)	9\16	4\21	5\20
Age /years Range	9.91±3.75 5.5-17	13.41±3.66 7-17	14.7±3.48 4-19
Anti-dsDNA	7.5±5.5	50.77±23.53	247.38±168.23
creatinine mg/dl	0.44±0.12	0.59±0.4	0.96±1.1

T-cell count discriminates between LN patients and control

Table (2) detects the T-cells in the urine in all pediatric LN patients. The mean count of urinary $CD4^{+}$ %T-cells was significantly increased in pediatric LN patients in comparison to healthy subjects (34.16±7.18 vs. 14.84±4.89 respectively; P <0.0001). Insignificant differences were obtained in the mean count of urinary CD4+ T-cells in active LN patients compared to inactive LN patients ; P >0.05). The mean count of urinary $CD8^{+}$ T-cells was significantly decreased in LN patients in comparison to healthy subject (18.62±3.84 vs. 31.40±6.34, respectively; p <0.0001). Insignificant differences for urinary $CD8^{+}$ T cells (%) levels obtained from active LN as compared to inactive LN(P >0.05).

Table 2: Flow cytometery markers for urinary T-cells of studied subjects included in the study (n=75)

	Control(n=25)	Inactive LN (n=25)	Active LN (n=25)
$CD4^{+}$	14.84±4.89	33.20±4.16***	35.12±9.27***
$CD8^{+}$	31.40±6.34	17.28±4.37***	19.25±3.02***
$CD8^{+}CD122^{+}$	9.06±3.41	26.46±5.15***	28.18±6.64***

*** (P value <0.001)

The mean count of urinary $CD122^{+}$Tcells among $CD8^{+}$Tcells levels obtained from LN patients was significantly increased as compared to healthy control group (27.42±5.93 vs 9.06±3.41 respectively; P <0.001). Insignificant difference was found in the $CD8^{+}$ $CD122^{+}$Tcells levels in urine obtained from inactive LN group as compared to active LN group (P >0.05)**.(Fig.1)**

Table (3) represents the flow cytometry markers of T-cells % from blood samples. $CD4^{+}$ T cells (%) levels in blood samples obtained from LN patients were significantly elevated as compared to healthy control group (26.89% ± 4.42% and 12.83%±3.17%, respectively; p <0.001). Whereas,

there were insignificant differences for $CD4^+$ T cells (%) levels in blood obtained from active LN group and inactive

Table 3: Flow cytometery markers for T-cells% from blood)

	Control (n=25)	Inactive LN (n=25)	Active LN (n=25)
$CD4^+$	12.75±3.02	26.95±6.24***	27.05±4.29***
$CD8^+$	29.04±7.11	15.16±1.68***	14.24±3.36***
$CD8^+CD122^+$	30.15±5.52	12.31±3.23***	9.5±3.57***

*** (P value <0.001)

The $CD8^+$ T cells (%) levels in blood samples obtained from active LN group were significantly decreased as compared to healthy control (15.25%±1.78% and 30.17%±7.63% respectively; P ≤0.01). There were no significant differences for $CD8^+$ T cells (%) levels in blood obtained from active LN group and inactive LN group (P >0.05). The mean count of $CD122^+$ cells among $CD8^+$ lymphocytes levels in blood samples obtained from LN patients was significantly decreased as compared to healthy control group (10.91%±3.65 versus 30.55%±5.5 respectively; P ≤0.001). On the other hand, insignificant difference was found in the $CD8^+$ $CD122^+$ T-cells levels in blood samples obtained from inactive LN group as compared to active LN group (P >0.05).(Fig.2)

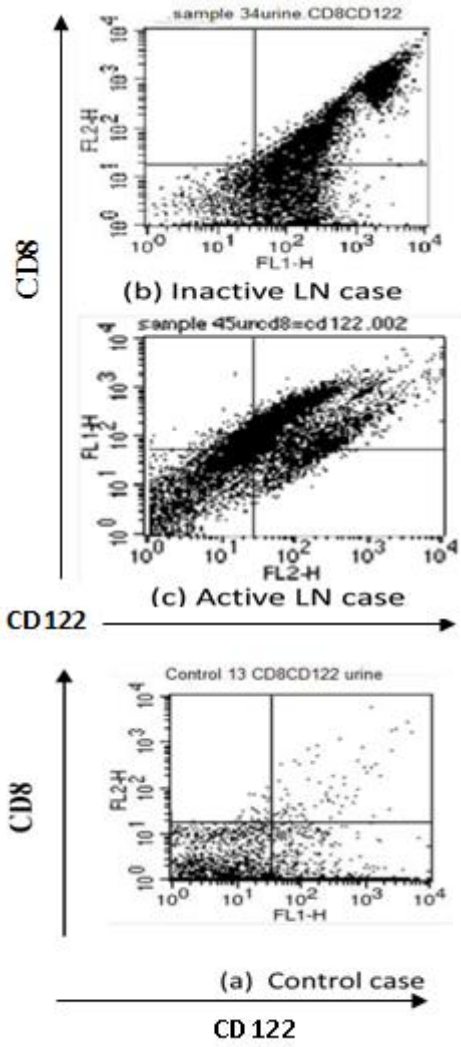

Figure 1: Comparison of flow cytometric analysis of double stain CD8 CD122T-cell populations between control and patient. Urine was stained with FITC-conjugated anti-CD8, PE-conjugated anti-CD122. Cells were gated on lymphocytes via their forward- and side-scatter properties.

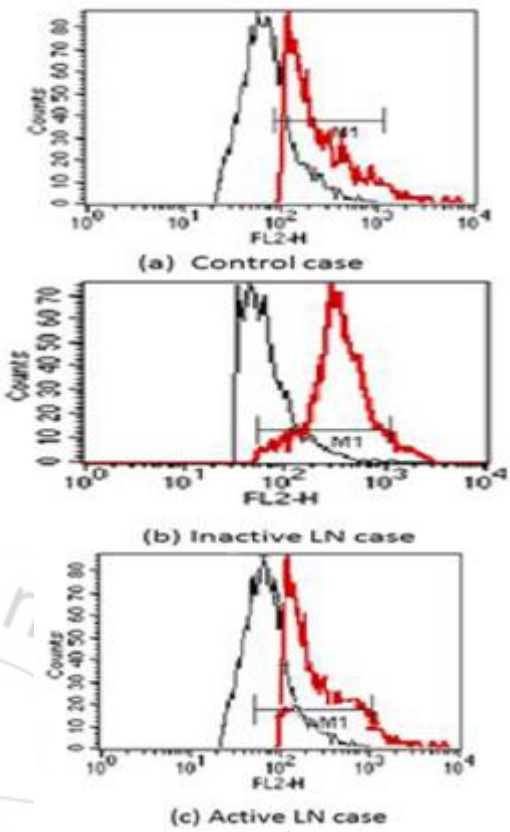

Figure 2: Flow cytometry histograms for $CD8^+CD122^+$ show positive expression for both two marker according to M1 (positive expression value) (black color CD8, red color CD122)

4. Discussion

In lupus nephritis, the severity of renal involvement depends on activated T cells and macrophages, which secrete a variety of inflammatory mediators into the kidney **[17]. Chan [18]** further supported the view that lupus nephritis involves intrarenal infiltration, activation, and alteration of the secretory phenotype of lymphocytes toward the Th1 pathway

Fritsch [19] hypothesis that effector memories T-cells migrate during active renal disease from the peripheral blood to the kidney and appear in the urinary sediment. The sediment was composed mainly of red blood cells, lymphocytes, and tubular epithelial cells.

Our data demonstrated the distribution frequency of $CD8^+$, $CD4^+$ and CD8/CD122 T-cells in the peripheral blood and urine of LN patients. In our findings the surface markers $CD4^+$ T-cells levels in urine samples obtained from LN patients were significantly elevated as compared to healthy control group. This result was confirmed by data obtained by **Enghard [20]** who demonstrated that urinary CD4 T-cells are a highly sensitive and specific marker for detecting proliferative LN in patients with SLE.

The surface markers $CD8^+$T-cells levels in urine samples obtained from LN patients were significantly decreased as compared to healthy control group. **Dolff [6]** showed that the

percentage of circulating CD8+ TEM cells in SLE patients significantly decreased versus control (33.9 ±18.3 % vs. 42.9 ±11.0 %, p=0.008). On the other hand, **Dolff [8]** study demonstrated the analysis of the urinary sediment in active renal disease showed an increased number of CD8+ T-cells and absence of these cells during remission.

In the present study, the surface markers $CD4^+$T-cells levels in urine samples obtained from LN patients were significantly elevated as compared to healthy control group (15.14±5.73 and 34.53±8.17) respectively (p<0.001). This result was confirmed by data obtained by **Enghard [20]. Enghard [20]** demonstrated that urinary CD4 T-cells are a highly sensitive and specific marker for detecting proliferative LN in patients with SLE.

In the current study, The mean count of urinary $CD122^+$Tcells among $CD8^+$Tcells levels obtained from LN patients was significantly increased as compared to healthy control group (9.06±3.41 vs 27.42±5.93 respectively; P <0.001).
The present study is the first investigation $CD8^+CD122^+$ in the peripheral blood and urine of LN patients. The data provide strong evidence that $CD8^+$ $CD122^+$T-cells migrate from the peripheral blood to the kidney and appear in the urine during LN flares. Therefore, $CD8^+$ $CD122^+$Tcells may be a useful monitoring tool in LN patients with renal involvement.

Moreover, this analysis will be especially helpful in LN patients with a recent history of LN. This relates to the fact that persistent proteinuria is a frequent observation which limits the use of this marker for the judgment of renal activity. Measuring urinary CD8+ CD122+T-cells helps to discriminate between LN and healthy subjects. To introduce this test into routine diagnostics, further studies are needed to confirm these results in a larger cohort of biopsy proven LN patients.

References

[1] A.A. Salloum “Lupus nephritis in childhood” Saudi J Kidney Dis Transplant, 14(1), pp43-56, 2003.
[2] B. E. Gilliam, Ombrello A. K, Burlingame R. W, Pepmueller P. H. and MooreT. L “Measurement of Autoantibodies in Pediatric-Onset Systemic Lupus Erythematosus and Their Relationship with Disease-Associated Manifestations” j.semarthrit, pp. 840-848, 2012.
[3] L.D. Chiewchengcho , Murphy R,Morgan T, Edwards SW, Leone V, Friswell M,. “Mucocutaneous manifestations in a UK national cohort of juvenile-onset systemic lupus erythematosus patients” Rheumatology (Oxford), 53(8), pp. 1504–12, 2014.
[4] G. Contreras, Pardo V, Leclercq B, Lenz O, Tozman E, O'Nan P, Roth D “Sequential therapies for proliferative lupus nephritis” N Engl J Med, 350(10),pp. 971-80 , 2004.
[5] J.J. Weening, D'Agati V.D, Schwartz M.M, Seshan S.V, Alpers C.E, Appel G.B, Balow J.E, Bruijn J.A, Cook T, Ferrario F, Fogo A.B, Ginzler E.M, Hebert L, Hill G, Hill P, Jennette J.C, Kong N.C, Lesavre P, Lockshin M, Looi L.M, Makino H, Moura L.A, Nagata M. “The classification of glomerulonephritis in systemic lupus erythematosus revisited” J Am Soc Nephrol , 15, pp. 241-250, 2004.
[6] S. Dolff, Berden J.H, Bijl M. “Treatment of lupus nephritis” Expert Rev Clin Immunol, 6, pp.901-911, 2010.
[7] Y .Wang, Yu F, Song D, Wang S.X, Zhao M.H “ Podocyte involvement in lupus nephritis based on the 2003 ISN/RPS system: A large cohort study from a single centre” Rheumatology (Oxford), 53, pp .1235–1244, 2014).
[8] S .Dolff, Abdulahad W.H, van Dijk M.C, Limburg P.C, Kallenberg C.G, Bijl M. “Urinary T cells in active lupus nephritis show an effector memory phenotype” Ann Rheum Dis, 69, pp.2034-2041, 2010.
[9] L .Zhihong, Haitao Zhang, Zhangsuo Liu, Changying Xing, Ping Fu, Zhaohui Ni, Jianghua Chen, Hongli Lin, Fuyou Liu, Yongcheng He, Yani He, Lining Miao, Nan Chen, Ying Li, Yong Gu, Wei Shi, Weixin Hu, Zhengzhao Liu, Hao Bao, Caihong Zeng, and Minlin Zhou “Multitarget Therapy for Induction Treatment of Lupus Nephritis: A Randomized Trial” *Ann Intern Med.* , 162(1), pp.18-26 ,2015.
[10] J.E. Balow “ Clinical presentation and monitoring of lupus nephritis” Lupus,14, pp.25-30, 2005.
[11] G.K. Bertsias, Ioannidis J.P, Aringer M . “EULAR recommendations for the management of systemic lupus erythematosus with neuropsychiatric manifestations: report of a task force of the EULAR standing committee for clinical affairs” Ann Rheum Dis , 69, pp. 2074-2082, 2010.
[12] C.C. Najafi, Korbet S.M, Lewis E.J, Schwartz M.M, Reichlin .M, Evans. J. “Significance of histologic patterns of glomerular injury upon long-term prognosis in severe lupus glomerulonephritis” Kidney Int, 59, pp. 2156-2163, 2001.
[13] R.W. Chan, Lai F.M, Li E.K, Tam L.S, Chung K.Y, Chow K.M, Li P.K, Szeto C.C. “Urinary mononuclear cell and disease activity of systemic lupus erythematosus”. Lupus, 15, pp .262-267, 2006.
[14] R. Gupta, Yadav A, Misra R, Aggarwal A “Urinary sCD25 as a biomarker of lupus nephritis disease activity” Lupus, 24, pp. 273–79,2015.
[15] W.H. Abdulahad, Kallenberg C.G, Limburg P.C, Stegeman C.A. “Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis” Arthritis Rheum, 60, pp. 2830-2838, 2009.
[16] F. Zahran, Al-haggar M, Derbala S.A “Regulatory T Cells in Pediatric Lupus Nephritis” Indian journal of applied research, 3(10), pp. 2249-555X, 2013.
[17] E.G. Neilson, Couser W.G, Immunologic renal diseases. 2nd ed, Philadelphia, Lippincott Williams & Wilkins, 2001.
[18] R. Chan, Tam L, S., Li E, Lai F, Chow K., Lai K, Li P, and Szeto C.C “Inflammatory Cytokine Gene Expression in the Urinary Sediment of Patients With Lupus Nephritis” Arthritis Rheum, 48, (5), pp .1326–1331 , 2003.
[19] R.D. Fritsch, Shen X, G.Illei G, Yarboro C.H, Prussin C, Hathcock K.S, Hodes R.J, and Lipsky P.E. “Abnormal differentiation of memory T cells in systemic lupus

erythematosus" Arthritis Rheum, 54, pp .2184-2197, 2006.

[20] P. Enghard , Rieder C, Kopetschke K, Klocke J.R, Undeutsch R, Biesen R, Dragun D, Gollasch M, Schneider U, Aupperle K, Humrich J.Y, Hiepe F, Backhaus M, Radbruch A.H, Burmester G.R, Riemekasten G "Urinary CD4 T cells identify SLE patients with proliferative lupus nephritis and can be used to monitor treatment response" Ann Rheum Dis, 73(1), pp. 277-83, 2014.