Original article

CXCR 3 expression on CD4+T cells and in renal tissue of pediatric systemic lupus erythematosus patients

Background*: Pediatric systemic lupus erythematosus (pSLE) accounts for about 20% of all cases of Systemic Lupus Erythematosus (SLE), with nephritis occurring in approximately 50% of the patients.* ***Objective:*** *to evaluate the expression of CXCR3 in the kidneys and on CD4+ T cells in pSLE.* ***Methods:*** *This study was conducted on 45 patients with pSLE following up at the Allergy and Immunology Clinic, Children's Hospital, Ain Shams University and 45 age and sex matched healthy children as a control group. Medical history, clinical examination and routine laboratory investigations for assessment of disease activity were done for all patients, the frequency of CXCR3, CD4+ T cells was determined in all patients and controls. Twenty-five Paraffin blocks of patients with lupus nephritis (LN) (available at the time of the study) underwent immunohistochemistry staining for the frequencies of Chemokine C receptor (CXCR3).* ***Results:*** *The absolute level and percentage of serum CD4+CXCR3+ were significantly lower among our patients as compared to healthy controls. A significant direct correlation was found between serum CD4+CXCR3+ and both the lymphocytic count and quantitative Systemic Lupus erythematosus disease activity index (SLEDAI), as well as a significant inverse correlation between it and 24 hours urinary proteins. Variable degrees of CXCR3 expression seemed to have no impact on laboratory tests, British Isles Lupus Assessment Group (BILAG) score and cumulative doses of Immunosuppressives.* ***Conclusion:*** *Serum CD4+CXCR3+ and not renal CXCR3 may be a potential marker of LN activity.*

Keywords: : CXCR 3, Renal biopsy, pSLE , lupus nephritis.

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INTRODUCTION

Pediatric systemic lupus erythematosus (pSLE) accounts for 20% of all cases of SLE[1]**.** Renal diseases are important factors contributing to disease morbidity and mortality[2]. There is an increasing agreement that infiltrating leukocytes contribute to kidney damage. Histological studies have demonstrated a correlation between the extent of this infiltration, impaired renal functions and an unfavorable prognosis[3]. During this process, an interaction between cytokines, vasoactive substances, chemokines and their respective target cells take place. This contributes to the outcome, *i.e.,* healing or progression of the renal disorder[4].

All types of renal cells (*i.e.* endothelial, mesangial, tubular epithelial, interstitial cells, and podocytes) are able to produce chemokines in a cell- and stimulus-specific manner[4]. It was found that the proportion of circulating CD4+, CXCR3+ T cells was decreased in adult patients with active SLE as compared with controls or patients with inactive SLE. And interestingly, in the follow up analysis of patients with active SLE, the observed decrease in CD4+, CXCR3+ T cells were restored to normal levels during remission. These results suggest that the decrease in CXCR3 expression on CD4^{+} T cells of SLE patients correlates well with disease activity and could therefore represent a marker of SLE flare[5].

This study aims at evaluating the expression of CXCR3 in the kidneys and on CD4+T cells in the peripheral blood of pediatric patients with SLE.

METHODS

The study was an observational cross-sectional case- control study conducted on 45 patients with pSLE following up at the Allergy and Immunology Clinic, Children's Hospital, Ain Shams University. Only 43 patients had LN for which they have undergone a previous renal biopsy prior to the study. Another 45 age- and sex-matched healthy children were enrolled as control for serum CD4+CXCR3+ marker. A verbal consent was obtained from each patient or control or their legal guardians before enrollment in the study. This study

was approved by the local ethical committee of Ain Shams University.

Study Population:

I) Patients (group 1):

Forty-five patients were included in the study. Their age ranged from 5 to 18 years with a mean ± standard deviation (SD) of 13.6 ± 2.78 years. They were 6 (13.3%) male patients and 39 (86.6%) female patients. Group I was divided into two subgroups as follows:

Group 1 A: 25 patients who suffered from LN and whose Paraffin blocks of renal biopsies were available at the archives of Pathology department of Ain Shams Specialized Hospital. The disease activity was assessed at time of biopsy (initially) by SLEDAI (qualitative) and was as follows: 9/25 very severe activity, 12/25 severe activity and 4/25 moderate activity. Activity at the time of the study was as follows: 9/25 no activity and 16/25 mild activity

Group 1 B: 20 patients with no available renal biopsy (18 with missing paraffin blocks and 2 with no LN). The disease activity was assessed at time of biopsy (initially) by SLEDAI (qualitative) and was as follows: 2/20 very severe activity, 8/20 severe activity, 10/20 moderate activity. Activity at the time of the study was as follows: 3/20 no activity and 17/20 mild activity.

II) The Control group (group 2):

Forty-five age and sex matched healthy children without clinical manifestation of any autoimmune disorders, were recruited from the outpatient clinics and pediatric surgery department, Children's Hospital, Ain Shams University. Their age ranged from 6-17 years with a mean ± SD 12.4 ± 3.88 of years. They were 37(82.2%) females and 8 (17.8%) males.

All included patients were subjected to the following:

- Detailed medical history with special emphasis on: Disease onset and duration, history of initial renal manifestations as oliguria, hypertension, hematuria and edema, drug history of immunosuppressive therapy and its duration with the calculation of the cumulative doses of these drugs. Clinical assessment of global disease activity using SLEDAI [6, 7] and detailed assessment of renal involvement using BILAG-2004 renal score[8] were done and Systemic Lupus Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index (SDI) was used to assess SLE related damage [9].

- Thorough clinical examination laying stress on: assessment of anthropometric measurements including weight, height, and body mass index with calculation of standard deviation score (SDS)[10] and complete examination including cardiac, chest, abdominal, and neurological examination to assess any organ involvement and detect the evidence of any complication related to the disease or treatment.

- Laboratory investigations:
  - Complete blood count (CBC)
  - Erythrocyte sedimentation rate (ESR) by Westergren Method.
  - C-reactive protein (CRP) using Latex agglutination test (SPINREACT, S.A. Ctra. SPAIN)
  - Serum anti-nuclear antibody (ANA)
  - Anti-double stranded deoxyribonucleic acid (Anti-ds DNA)
  - Complement-3 (C3) estimated initially by nephelometry and in follow up by turbidimetry (Turbiquant C3, Behring Werke Diagnostics, Marburg, Germany).
  - Serum creatinine and serum urea levels estimated initially by nephelometry and in follow up by turbidimetry (Turbiquant C3, Behring Werke Diagnostics, Marburg, Germany).
  - Complete urine analysis.
  - Corrected creatinine clearance: The creatinine clearance = (V) x (U)/(P) X 1.73/child's surface area (V= volume of urine ml/24 hours, U= urinary creatinine mg/dl, P= plasma creatinine mg/dl) (Burhs and Ashwood, 1999)
  - Twenty-four hours urinary proteins using Synchron CX7 autoanalyzer (Beckman Instruments, Bera, California, USA).
  - The frequency of CXCR3, CD4+ T cells using Flow cytometry. (both groups I, II)

- Immunohistochemistry studies on renal biopsy: twenty-five Paraffin blocks of patients with lupus nephritis (group IA) underwent immuno-histochemistry staining for the frequencies of Chemokine C receptor (CXCR3) cells and expression. Sections of gut tissue for Crohn's disease were regarded as positive controls for CXCR3. They were stained in each run to judge the effectiveness of the technique. Negative control slides were processed as the previous immunostaining procedure, but the primary antibody was omitted from the steps, and phosphate buffered saline (PBS) was used instead. The extent of positive staining of CXCR3 was examined in glomerular cells (glom. CXCR3) and interstitial (int. CXCR3) and extent of CXCR3 staining was graded using a scale of 0-3, where

0=no staining (-ve), 1=mild staining (+ve), 2=moderate staining (+ve), 3=strong staining (+ve) [11-14].

RESULTS

Our studied patients were 39 (86.7%) females and 6 (13.3%) males with female to male ratio 6.5:1. Only 5 patients (11.1%) had a relative family history of rheumatological diseases. Initial renal affection evaluation done for the patients showed that 40 (88.8%) patients had proteinuria, 10 (22.2%) patients were hypertensive 15, 9 (20%) patients had oliguria and 16 (35.5%) patients had hematuria. In the follow up8 (17.7%) patients had proteinuria, 5 (11.1%) patients were hypertensive and 1 (2.2%) patient still had hematuria .

Among the studied patients 2 (4.4%) did not have LN, 17 (37.8%) had class II LN, 15 (33.3%) patients had class III LN, 8 (17.7%) patients had class IV LN and 3 (6.97%) patients had class V LN. [23 (51.1%) Proliferative LN (Class III and IV) and 17 (37.8%) non-proliferative LN (Class II)]. Comparison of the clinical and laboratory findings initially (at the time of renal biopsy) and at the time of the study is shown in table (1).

Table 1. Comparison between the initial and last visit as regards clinical and laboratory data of our SLE patients

Data	Initial Visit n=45		Follow up visit n=45		P
	Median	Range	Median	Range	
Weight SDS	0.01	-2.5-2.75	0.48	-1.9 -2.8	0.043
Height SDS	0.03	-2.6-2.9	-0.86	-2.3-2.83	0.00
BMI SDS	0.32	-2.6-2.4	1.19	-1.82- 2.8	0.00
Systolic blood pressure (mmHg)	110	90-150	110	90-140	0.87
Diastolic blood pressure (mmHg)	70	55-90	70	50-100	0.69
WBC(x10^9/L)	6.3	1.6-26.4	6.7	1.8-16.8	0.59
Hb (gm/dl)	8.3	7-13.6	11.9	7.5-14.8	0.00
Platelet (x10^9/L)	206.5	6-581	284	144-516	0.006
Lymphocytic count (x10^9/L)	1.8	0.6-10.2	2.5	0.18-6	0.579
Serum Creatinine (mg/dl)	0.6	0.3-6.4	0.5	0.2-1.2	0.001
BUN (mg/dl)	19	6-84	16	3-50	0.004
24 hours urinary Protein (gm/day)	0.5	60-692	0.2	0.01-3	0.00
Creatinine Clearance (mg/ml/1.73m^2)	98	692	102	34-239	0.89
C3 (mg/dl)	56.5	16-191	116	27-362	0.00
ESR (mm/hr)	92.5	15-150	30	5-130	0.00
SLEDAI	14	6-50	2	0-21	0.00

BMI :body mass index **,BUN:** Blood urea nitrogen, **C3:** Complement-3, **CBC:** Complete blood picture , **ESR:** Erythrocyte sedimentation rate , **N:**normal**, n:**number, **P <0.05**= significant, **SD:** standard deviation, **SDS:** Standard deviation score**, SLE:** Systemic lupus erythematosus, **SLEDAI:** Systemic lupus erythematosus disease activity index, **TLC:** Total Leucocytic count, **%:** percentage.

The comparison between serum levels of CD4+CXCR3+ in cases and controls is shown in table (2).

Table 2. Comparison between serum CD+ CXCR3+ in patients and controls

Data	Patients n=45		Controls n=45		
	Median	Range	Median	Range	P
Serum CD4+ CXCR3+ (x10^9/L)	0.36	0.09 - 1.49	0.66	0.05- 2.87	0.001*
Serum CD4+ CXCR3+ (%)	15.6	5.12-27.2	23.5	5.47- 48.1	0.000*
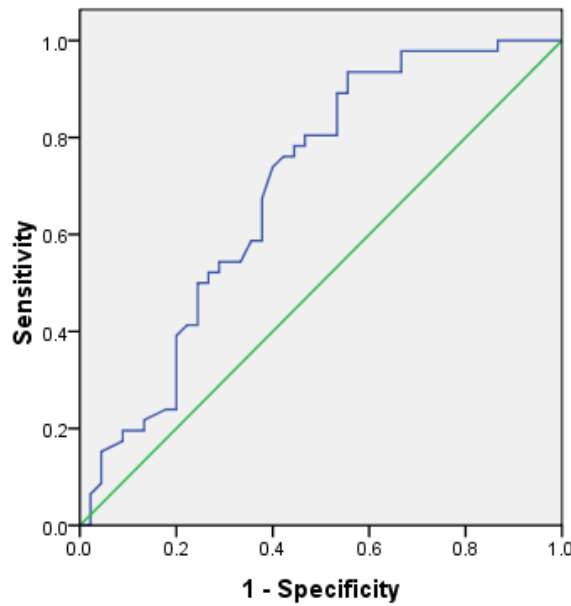
Diagonal segments are produced by ties.

Figure 1. Receiver Operating Characteristic (ROC) curve for serum CD4+CXCR3+

Sensitivity	Specificity	Positive predictive value	Negative predictive value
60.6%	63.1%	48.8%	73.5%

The calculated area under the curve is 0.69. The natural log-transformed cut point (cut off value/threshold) that maximize the combined sensitivity and specificity for serum CD4+CXCR3+ is 0.44 x10^9/L.

The receiver-operator characteristic (ROC) curve shown in figure 2 depicted the true positive fractions (sensitivity) and false positive fractions (1-specificity) for serum CD4+CXCR3+ at various cut points in healthy subjects and patients. The calculated area under the curve was found to be 0.69 which means that the variable (serum CD4+CXCR3+) can be used to differentiate between patients and controls.

Serum CD4+CXCR3+ failed to show any significant difference between proliferative and non-proliferative LN patients although both groups had a significantly lower level than the control group.

Using the BILAG score, 27 patients were improved while 18 had a stationary course of the disease, also measuring blood pressure, 40 patients were non-hypertensive and 5 were hypertensive. No significant difference in serum CD4+CXCR3+ was found between patients with improved and stationary course as well as between hypertensive and non-hypertensive patients. Correlations of s. CD4+CXCR3+ with clinical, laboratory and drug dosing are shown in table (3).

Table 3. The relation between serum CD4+CXCR3+ and different parameters (laboratory and drug doses)

Variable	s.CD4+CXCR3+ Mean ± SD (0.42 ± 0.27)	
	r	p
Lymphocytic count	0.72	<0.001
C3	0.28	0.06
24 hr urinary protein	-0.501	<0.001
ESR	-0.07	0.07
SLEDAI	-0.43	0.006
Steroid Mean ± SD (0.81±0.99)gm/kg	-0.133	0.38
Cyclophosphamide Mean ± SD (0.11±0.05)gm/kg	-0.117	0.44
Azathioprine Mean ± SD (1.67±0.78)gm/kg	-0.112	0.46
Mycophenolate Mofetil Mean ± SD (9.97±7.86)gm/kg	-0.152	0.32

C3: complement 3, **ESR:** Erythrocyte sedimentation rate**, hr:** Hours, n: number, **P <0.05:** significant ,**SD:** Standard deviation, **SLEDAI:** Systemic lupus erythematosus disease activity index

Figure (2) demonstrates the expression of tissue CXCR3 in the renal biopsies of group 1A patients. Activity index ranged from 2-15(median 4.5), chronicity index 0-9 (median 2).
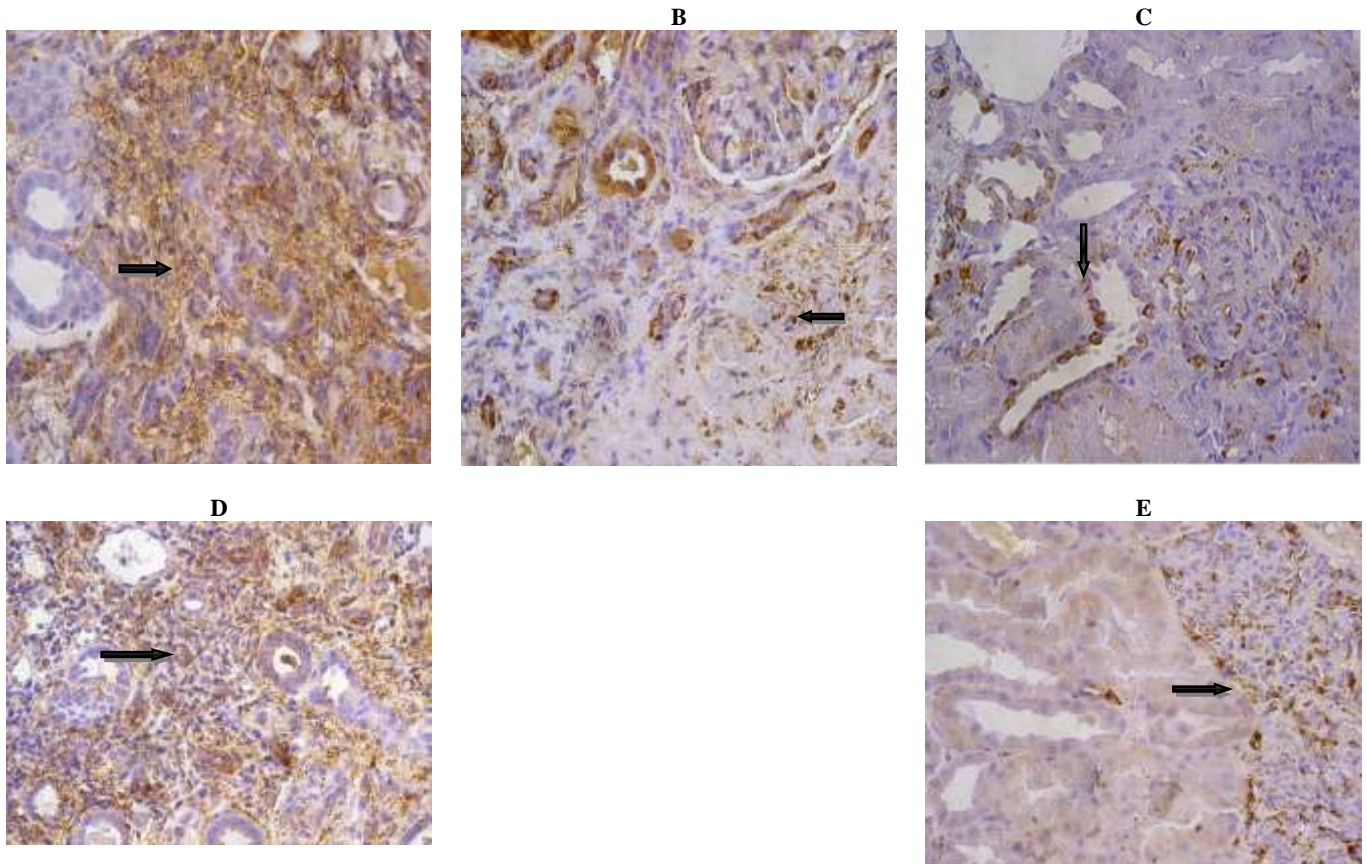

Figure 2. Renal expression of CXCR3 using Immuno-histological staining

A: Strong CXCR3 expression in the interstitial inflammatory cellular infiltrate, **B:** Moderate expression of CXCR3 using Immunostaining of the periglomerular inflammatory cells, **C:** Mild CXCR3 immunostaining, **D:** Diffuse interstitial infiltrate of CXCR3 positive cells, **E:** Nodular interstitial infiltrate of CXCR3 positive cells **(CXCR3 X 400).**

Dividing the patients according to the extent of CXCR3 renal expression into Nil/Mild and Moderate/Strong, we compared the 2 groups regarding clinical and laboratory parameters in table (4).

Table 4. Comparing different degrees of CXCR3 renal expression as regards clinical and laboratory parameters

		CXCR3		P
		Nil/Mild	**Moderate/Strong**	
BILAG	**Improved (n = 15)**	7 (46.6%)	8 (53.3%)	0.74
	Stationary (n = 10)	4 (40%)	6 (60%)	
Initial BILAG	A (n = 7)	4(57.1%)	3(37.5%)	0.3
	B (n = 8)	5(62.5%)	3 (37.5%)	
	C (n = 6)	1(16.7%)	5 (83.3%)	
	D (n = 4)	1(25%)	3 (75%)	
Indices	**Activity median (range)**	4(2-15)	5(2-10)	0.14
	Chronicity median (range)	2 (0-9)	1 (0-9)	1.73
Initial Lab. Data	**Lymphocytic count ($\times 10^3$/μl)**	1.3 (0.6-4.3)	1.9(0.6- 2.5)	0.305
	s. creatinine (mg/dl)	0.8(0.3-5.3)	0.6 (0.3-6.4)	0.12
	24 hrs. urinary protein (gm)	1.2 (0.25-3.4)	0.5 (0.1-4)	0.23

n= number, **p <0.05:** significant.

Among our 25 patients (with available renal biopsy paraffin blocks), 10 patients had class II, 9 patients had class III, 3 patients had class IV and 3 patients had class V. Most of our patients with mild/moderate CXCR3 expression were distributed among classes II and III LN (66.6% and 100% respectively). Two thirds of our patients with a strong marker expression were class IV (2 = 66.6%) and one was class V (33.3%).

DISCUSSION

Considering the inflammatory nature of lupus nephritis and the assumed role of cytokines in the disease progress, we aimed at studying the expression of CXCR3 in the kidneys of pediatric patients with lupus nephritis and the expression of CXCR3 on CD4+T cells in their blood.

In our study we measured the serum CD4+CXCR3+ in both studied groups (patients and controls) and found that the serum level was significantly lower in the patients' group in comparison to our controls, both in the absolute count and the percentage *(P=0.001, 0.000 respectively)*. Such lower level of circulating serum CD4+CXCR3+ is probably because of CXCR3 prevalent role in inflammatory control by mediating the recruitment of Th1 CD4+ T cells into the inflamed tissue[16]. This comes in accordance with several other studies[17,5,3].

Such results were not only found in SLE patients, but in other autoimmune diseases, where serum CD4+CXCR3+ was found lower in patients with rheumatoid arthritis in comparison to healthy controls and a very high number of CD4+CXCR3+ was found in the synovial tissue of patients[18]. Similarly, CXCR3 was found to accumulate at sites of inflammation; skin in discoid lupus[19], Sarcodosis[20], Sjogren Syndrome and dermatomyositis[21]. Opposite results were seen in some studies [22, 23].

A similar comparison was done between proliferative and non-proliferative LN in relation to CD4+CXCR3+. However, it failed to show a significant difference *(P=0.46);* despite that the mean level was lower in the proliferative group which might indicate a more aggressive inflammatory process. The absence of the statistical significance could be attributed to the uneven patient distribution and to the small sample size.

In a trial to find a link between serum CD4+CXCR3+ and different drug modalities used in treatment of our patients, we correlated serum level of CD4+CXCR3+ and the cumulative doses of different immunosuppressive drugs/kg (Steroids, Cyclophosphamide, Azathioprine and Mycophenolate Mofetil), but there was no significant correlation *(P= 0.38, 0.44, 0.46, 0.32 respectively)*. The same was found in one study that did not find any significant difference in the serum level of CD4+CXCR+ in between treated and untreated patients[5]. This can be explained by the hypothesis that serum CD4+CXCR3+ correlates with disease flare irrespective to treatment.

On the contrary, a study detected a significant difference in the serum level of CD4+CXCR3+ in between patients with active LN taking cyclophosphamide and inactive LN*,* but this difference could be either due to treatment or disease activity. However, they could not find any difference in the frequency of CD4+CXCD3+ in between children with nephrotic syndrome treated with prednisone, MMF or cyclosporine and healthy controls[17].

We studied serum CD4+CXCR3+ in relation to patients' blood pressure (hypertensive and non-hypertensive). No statistical difference was found between the serum level in non-hypertensive and hypertensive patients *(P= 0.47)*. Such finding could be attributed to the fact that only 5 patients were hypertensive. Available literature is conflicting; an interesting study found that CXCR3 deficient mice had a potentiality for hypertension, with a concomitant increase in expression of angiotensin II type 1 receptor[24]. On the contrary, another study found that the level of circulating CXCR3+ chemokines in newly diagnosed hypertensive patients was higher in comparison to controls[25].

In our study serum CD4+CXCR3+ had a significantly direct correlation with the absolute lymphocytic count *(P= 0.001)*. A significant negative correlation was plotted between the marker and 24 hours urinary protein and SLEDAI *(P=0.001, P=0.006)*. This can be explained by the role of chemokines in the inflammatory process and tissue damage through lymphocyte trafficking[26], in particular IP-10 chemokine via its interaction with its receptor CXCR3[27]. This agrees with several studies that stated that there was an inverse statistically significant correlation between circulating CD4+CXCR3+ and renal SLEDAI done in patients with pSLE[17,5,3, 23].

Depending on the significant difference between the serum level of CD4+CXCR3+ in our patients and the control group, we were able to calculate the cut off level for serum CD4+CXCR3+ that was 0.44×10^9/L with a sensitivity of 60.6% and a specificity of 63.1% (area under the curve 0.69). Up to our best knowledge this is by far the first cut off level to be documented in the pediatric age group and in adults. However; Avihingsanon and colleagues, (2006) were able to get a cut off level for messenger ribonucleic acid (mRNA) CXCR3 in urine using the ROC curve. The calculated area

under the curve was 0.79. The cut off level was 1.65 copies/µ of the total RNA copies and it had a sensitivity of 65% and a specificity of 83%[27].

We assessed the degree of CXCR3 expression in the renal tissue and found 11 patients having nil/mild expression for CXCR3, whereas, 11 patients had moderate expression and 3 patients had strong expression in their renal tissue biopsies. The distribution of CXCR3 expression was mainly in the interstitium. This could explain the renal damage and mesangial expansion occurring by Th1 cells recruitment. This goes in accordance with many studies[3,28-30]. Many documented abundant expression of CXCR3 and its ligands CXCL10 in the renal interstitium of adult patients with LN. The main site of distribution was mainly periglomerular and perivascular, but not glomerular. On the contrary, other studies conducted on patients with LN[31] and mice[32] described that CXCR3 expression was distributed in both the tubulointerstitial tissue and the glomeruli.

On comparing the extent of CXCR3 expression in different classes of LN, it was found that most of our patients with mild/moderate marker expression were distributed among classes II and III LN(66.6% and 100% for mild and moderate expression respectively). Two thirds of our patients with strong marker expression were of class IV "2 patients" (66.6%) and one was class V LN (33.3%). However, this failed to have a statistical significance *(P=0.2).* Similarly, Lu and colleagues, (2011) could not find any difference in the degree of expression in relation to the different classes of LN. Such results made them reach a hypothesis that CXCR3 expression is important to determine disease severity rather than the histological pattern of LN[33].

On the contrary, a study was able to differentiate class IV LN from other classes by assessing the value of mRNA for CXCR3 in the urine of patients with LN as its level was significantly higher than that found in other classes[27]. The same was found by Merchant and Klein, 2011 who stated that the expression of CXCR3 in renal tissue was higher in patients with class IV in comparison to other classes[33].

Upon comparing different laboratory tests and BILAG score done at time of renal biopsy in the different degrees of CXCR3 renal expression, no statistical difference was found. However, Segerer et al,(2004) found significant correlation between CXCR3 expression and serum creatinine, BUN and proteinuria[29]. In addition, several experiments involving mice[32,34,35] found that CXCR3+/+ mice with nephritis had a significantly elevated

creatinine, BUN and albuminuria in comparison to CXCR3-/- mice.

In the current study, the cumulative doses of steroid and cyclophosphamide were compared in the different degrees of CXCR3 renal expression, but no statistical difference was found *(P= 0.45, 0.68 respectively).* We relate this to uneven immuno-suppressive intake by our patients, as LN was not the only indication for such immunosuppressive treatment. Also, no follow up biopsy was done to compare the degree of expression after treatment.

This agrees with Lu et al, (2011) who found no correlation between the gene expression of CXCR3 and the dosage of different immunosuppressive drugs (prednisolone and azathioprine)[31]. However, Avihingsanon et al, (2006) assessed the level of mRNA for CXCR3 baseline and after treatment in 10 patients who responded to treatment and 4 who did not respond. Initially before treatment there was no difference in the level of expression, but after treatment it tended to be markedly less in the responder group than in the non-responder *(P= 0.66 versus 0.05.* [27]

We compared activity and chronicity indices of renal biopsy in the different degrees of CXCR3 renal expression and to our patient's renal outcome using the renal BILAG score and neither showed statistical significance. Comparable number of patients were found to have improved and stationary disease course *(P=0.74),* despite the fact that most of the patients (60%) who had a stationary course showed moderate/strong expression and those who got improved had an equal number of patients having nil/mild expression and moderate/strong expression.

However, Segerer et al, (2004) stated that severe tubulointerstitial injury was associated with elevated number of CXCR3 expression and cellular infiltration. They also found a positive significant correlation between the globally sclerosed glomeruli and the number of CXCR3+ cells infiltration and tubulointerstitial damage[29]. Several animal studies found the same[32, 34, 35].

CONCLUSION
Serum CD4+CXCR3+ was found significantly lower in patients with pSLE as compared to healthy controls. Its level did not vary significantly among proliferative and non-proliferative LN although it was found higher in patients with an improved renal course of disease guided by BILAG score in comparison to those with stationary course. Renal expression of CXCR3 was mainly interstitial. However, no statistically significant variation could

be found among different LN classes, although its expression was less in those with improved renal course as evaluated by renal BILAG score. Hence Serum CD4+CXCR3+ may be a promising marker of LN activity and improved disease course. Less can be said about renal expression of CXCR3 though many consider the marker as a therapeutic target. Both offered no help in pointing out the class of LN.

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