

## Original article

### Membrane endothelial protein C receptor expression in renal tissue of pediatric lupus nephritis patients

**Background:** Lupus nephritis (LN) is more common and more severe in pediatric systemic lupus erythematosus (pSLE). Endothelial protein C receptor (EPCR) is an inducer of anti-apoptotic pathways in endothelial cells. Recent studies have taken elevated anti-injury biomarkers as EPCR into consideration regarding their roles to antagonize LN. **Objectives:** to evaluate the membrane expression of endothelial protein C receptor (mEPCR) in the renal microvasculature in pediatric patients with LN. **Methods** This study was conducted on 25 patients with pSLE following up at the Allergy and Immunology Clinic, Children's Hospital, Ain Shams University. The 25 patients have LN proved by a previous renal biopsy. Medical history, clinical examination and routine laboratory investigations for assessment of disease activity were done for all patients. Paraffin blocks of patients' renal biopsies were subjected to immunohistochemistry staining for the frequency of mEPCR. **Results:** mEPCR was mainly expressed in the endothelium of the peritubular capillaries. Our results showed that an equal number of patients had nil and mild marker expression (8 patients each, 32%) while 9 patients (36%) showed moderate/strong marker expression. We found that 9 out of 10 (90%) of patients with class II had nil/mild marker expression, 5 patients out of 9 (55.5%) with class III had mild/moderate marker expression, while 5 patients out of 6 (83.3%) with class IV and V had moderate/strong marker expression. We only found a significant statistical difference between the different degrees of mEPCR expression regarding 24 hours urinary proteins. No statistical significance was found between the different degrees of mEPCR expression and different immuno-suppressive therapy dose/kg or renal outcome using the renal British Isles Lupus Assessment Group (BILAG) score; in spite that most of the patients who got improved had nil/mild marker expression. **Conclusion:** mEPCR-bearing a statistically significant difference in relation to different LN classes- showed more expression in the more aggressive classes; a finding which might suggest a contribution of the endothelium of the renal parenchyma to the pathophysiology of more progressive LN. Hence the tissue marker might emerge as a potential new therapeutic target in the search for more selective treatment for SLE.

**Keywords:** p SLE, mEPCR, renal biopsy, immunohistochemistry, BILAG, lupus nephritis.

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## INTRODUCTION

The general consensus is that 60% of systemic lupus erythematosus (SLE) patients will develop nephritis at some time in the course of their illness<sup>1,2</sup>, with a reported 5–22% of these patients progressing to end-stage renal disease requiring dialysis or transplantation<sup>3</sup>.

Lupus nephritis (LN) is usually initially asymptomatic, although a few children develop gross hematuria or edema associated with nephrotic syndrome<sup>4</sup>. Nephritis is thought to be caused by local deposition of autoantibodies and immune

complexes, but there is an increasing agreement that infiltrating leukocytes also contribute to kidney damage. Histological studies have demonstrated a correlation between the extent of this infiltration, impaired renal functions and an unfavorable prognosis<sup>5</sup>.

EPCR is a prominent inducer of anti-apoptotic pathways in endothelial cells, and thus maintains vascular tone and normal blood flow in the microvasculature<sup>6</sup>. Protein C (PC) binds to EPCR which is expressed by endothelial cells and thus it has as a potent anticoagulant mechanism and potent anti-inflammatory properties<sup>7</sup>.

Recent studies have taken elevated anti-injury biomarkers (mEPCR) into consideration regarding their roles to antagonize LN. In vasculopathy as a comorbidity to LN, the persistent expression of mEPCR at peritubular capillaries may represent a response to the local cues of a deficit of active protein C<sup>8</sup>.

Under conditions of unresolved morbidity, mEPCR may represent a physiologic attempt to limit further endothelial damage, and the observed increase in plaque and progression of LN represent an overwhelming of this reparative process by disease-provoking stimuli<sup>8</sup>. Given the prediction that shed mEPCR impairs the integrity of the endothelium and places the net balance of this protective protein in biological 'arrears', a decrease in mEPCR expression was the predicted result in patients with progressive renal injury<sup>9</sup>. However, it was found that mEPCR is highly expressed in the cortical peritubular capillaries of kidneys from patients with active LN. This profound up-regulation of mEPCR was observed even in areas with absent tubulointerstitial damage; it was therefore hypothesized that mEPCR may be an important anti-injury molecule in the cascade(s) leading to renal damage in SLE<sup>9</sup>.

## OBJECTIVES

Evaluation of the membrane expression of endothelial protein C receptor (mEPCR) in the renal microvasculature in pediatric patients with LN and correlating this to SLE disease activity and prognosis.

## METHODS

The study was an exploratory cross-sectional case control study conducted on 25 patients with LN following up at the Allergy and Immunology clinic in Children's Hospital, Ain Shams University. All patients fulfilled at least four of the revised classification criteria of The American College of Rheumatology (ACR) for diagnosis of SLE<sup>10</sup> and fulfilled the revised classification criteria of LN of the World Health Organization (WHO)<sup>11</sup>, for which they have undergone a previous renal biopsy prior to the study as a routine practical work up. A consent was obtained from each patient or their legal guardians before enrollment in the study. This study was approved by the local ethical committee of Ain Shams University. Paraffin blocks of renal biopsies of the studied patients were available at the archives of The Pathology department of Ain Shams Specialized Hospital.

### *Inclusion criteria:*

- Patients with SLE disease onset before 18 years.

### *Exclusion criteria:*

- Patients with any hepatic disease.
- Patients suffering from synovitis.
- Patients with diabetes mellitus.

All included patients were subjected to the following:

1- Detailed medical history with special emphasis on:

- Demographic data: name, age, sex, and consanguinity.
- Family history of similar condition.
- Disease onset and duration.
- History of initial renal manifestations as oliguria, hypertension, hematuria and edema.
- History of complications as avascular necrosis of head of femur, cardiac dysfunction and renal performance of peritoneal and hemodialysis.
- Clinical assessment of global disease activity using Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)<sup>12</sup> and detailed assessment of renal involvement using British Isles Lupus Assessment Group (BILAG)-2004 renal score<sup>13</sup> were done and Systemic Lupus Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index (SDI) was used to assess SLE related damage<sup>14,15</sup>.
- Activity categories have been defined on the basis of SLEDAI score:
  - No activity: 0
  - Mild activity: 1-5
  - Moderate Activity: 6-10
  - High activity: 11-19
  - Very high activity: >20

2- Thorough clinical examination laying stress on:

- Assessment of anthropometric measurements including weight, height, and body mass index with calculation of standard deviation score (SDS)<sup>16</sup>.
- 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.

3- Laboratory investigations:

- Complete blood count (CBC) using coulter counter (Coulter MAXMUG- HL -CCI) and Leishman-stained peripheral blood film examination for differential white blood cell counting.
- 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) by indirect immunofluorescence technique on HEP-2 cells.
- Anti-double stranded deoxyribonucleic acid (Anti-ds DNA) by indirect immunofluorescent microscopy (IMMCO Diagnostics, USA).
- Complement-3 (C3) was estimated initially by nephelometry and in follow up by turbidimetry (Turbiquant C3, Behring Werke Diagnostics, Marburg, Germany).
- Serum creatinine and serum urea levels were carried out on Synchron CX7 autoanalyzer (Beckman Instruments, Bera, California, USA).
- Complete urine analysis. A freshly collected random urine specimen was collected in sterile plastic container and was submitted for chemical analysis using dipsticks and microscopic examination with special emphasis on the presence of albuminuria, hematuria, pyuria, urinary casts. Urine culture and sensitivity was done in cases with pyuria to exclude urinary tract infection.
- Creatinine clearance measurement. Twenty-four hours urine was used to measure urinary creatinine, which was done using Synchron CX7 autoanalyzer (Beckman Instruments, Bera, California, USA). Creatinine clearance was then calculated using the results of urinary and serum creatinine levels.
- Twenty-four hours urinary protein measurement using Synchron CX7 autoanalyzer (Beckman Instruments, Bera, California, USA).

4- Immunohistochemistry studies in renal biopsy: Twenty-five Paraffin blocks of patients with LN were subjected to:

- Hematoxylin & eosin to confirm the diagnosis.
- Immunohistochemistry staining for the frequency of membrane endothelial protein C receptor (mEPCR).

The extent of positive staining of mEPCR was examined in glomerular cells (glom. mEPCR) and interstitial (int. mEPCR) and extent of mEPCR 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)<sup>6</sup>.

#### Statistical methods:

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Data was presented and

suitable analysis was done according to the type of data obtained for each parameter.

## RESULTS

The study included 25 patients whose age ranged from 5 to 18 years with a mean  $\pm$  SD of  $13.44 \pm 2.78$ . They were 3 (12%) male patients and 22 (88%) female patients with female to male ratio 7.3:1. Among our patients: 10 (40%) had class II LN, 9 (36%) patients had class III LN, 3 (12%) patients had class IV LN and 3 (12%) patients had class V LN. Accordingly, they were then classified to 12 (48%) having Proliferative LN (Class III and IV) and 11 (44%) non-proliferative LN (Class II)<sup>17</sup>.

Hypertension was diagnosed according to the percentiles of blood pressure<sup>(18)</sup> and it showed that at presentation 6 (24%) patients had hypertension and on follow up only 3 (12%) had hypertension (on treatment).

The age of presentation ranged from 4 and 15 years with a mean  $\pm$  SD of  $11.02 \pm 2.71$ . Duration of illness ranged from 1 to 5 years with a mean  $\pm$  SD of  $2.54 \pm 1.63$ . Only 3 patients (12%) had a relative family history of rheumatological diseases.

Initial renal affection evaluation done for the patients showed that 21 (84%) patients had proteinuria, 6 (24%) patients were hypertensive and 16 (64%) patients had hematuria. In the follow up 4 (16%) patients only were hypertensive, 3 (12%) patient still had hematuria and 4 (16%) patients had proteinuria.

Initial anti-DNA was positive in 21 (84%) patients and negative in 4 (16%) patients. In the last follow up 10 (40%) patients had positive anti-DNA and 15 (60%) were negative.

SLEDAI done at initial presentation showed that 4 (16%) patients showed moderate activity, 12 (48%) patients showed severe activity while 9 (36%) patients showed very severe activity. Whereas, the follow up SLEDAI showed 9 (36%) patients had no activity, while 16 (64%) patients had mild disease activity, none (0%) had moderate, severe or very severe activity.

The results of initial BILAG assessment for renal affection, showed that 7 (28%) got score A, 8(32%) patients got score B, 6 (24%) patients got score C and 4 (16%) patients got score D. The follow up BILAG assessment showed that 2 (8%) got score A, 5 (20%) patients got score B, 7 (28%) patients got score C and 6 (24%) patients got score D, 5 (20%) patients got score E. The outcome of patients as regards renal affection was divided into stationary, improved and worsened. 15 (60%)

patients got improved, 10 (40%) were stationary and none (0%) worsened.

All 25 (100%) patients were on steroids therapy at a dose of (0.5-2 mg/kg/day) with a cumulative dose that ranged between 6.4 gm and 63.33 gm with a median of 20 gm. 15 (60%) patients were on cyclophosphamide at a dose of (500-750 mg/m<sup>2</sup>/month) with a cumulative dose ranging between 2.5 gm and 14.15 gm and a median of 4.1 grams. Eight (32%) patients were on azathioprine at a dose of (1.5-3mg/kg/day) with a cumulative dose that ranged between 4.5 gm and 178 gm and a median of 44.7 gm. Mycophenolate Mofetil was given to 5 (20%) patients at a dose of (600 mg/m<sup>2</sup>/day) maximum 2 gm with a cumulative dose that ranged between 150 gm and 1810 gm with a median of 213.9 gm. Six (24%) patients took baby Aspirin at the antithrombotic dose that ranged from (3-5mg/kg/day).

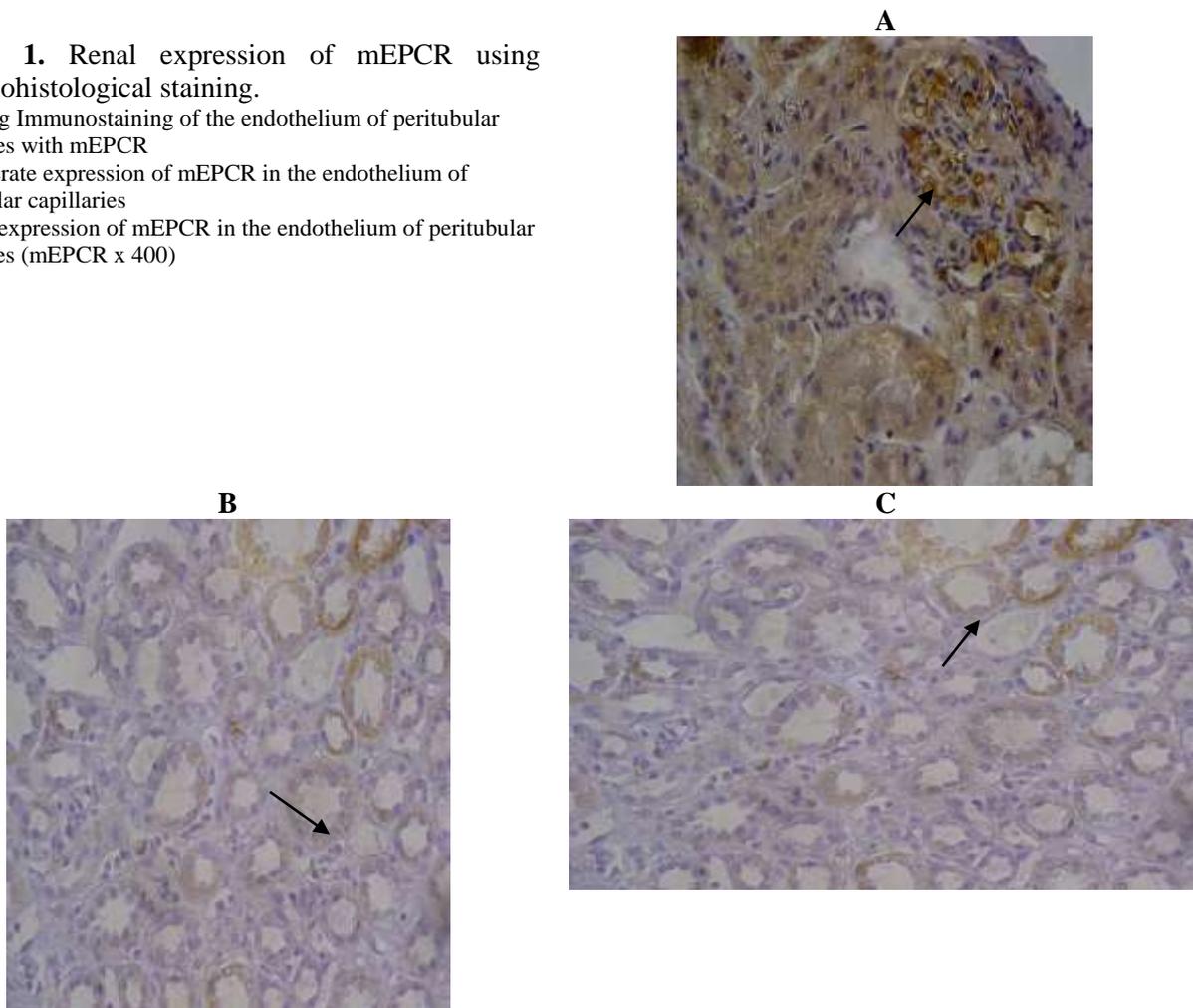
As regards the histological findings in the renal tissue of the studied patients, all patients had no thrombotic angiopathy. The median of Activity index was 4.5 with a range of (2- 15), while the

median of chronicity index was 2 with a range of (0-9). The rest of the histological data are demonstrated in table 1. Figure 1 (A, B, C) demonstrates the expression of mEPCR in the renal biopsies of patients. An equal number of patients had nil and mild marker expression (8 patients each, 32%) while 9 patients (36%) showed moderate/strong marker expression. In a trial to examine the renal expression of mEPCR in relation to LN classes, we found that 9 out of 10 (90%) patients with class II had nil/mild marker expression, 5 out of 9 patients (55.5%) with class III had mild/moderate marker expression, while 5 out of 6 patients (83.3%) with class IV and V had moderate/strong marker expression. A finding which bore a significant difference (P=0.01).

Table 2 represents a comparison between groups with different degrees of mEPCR expression regarding some clinical and laboratory variables, table 3, 4 compare proliferative and non-proliferative LN regarding histological variants, activity and chronicity indices

**Figure 1.** Renal expression of mEPCR using Immunohistological staining.

- A: Strong Immunostaining of the endothelium of peritubular capillaries with mEPCR
- B: Moderate expression of mEPCR in the endothelium of peritubular capillaries
- C: Mild expression of mEPCR in the endothelium of peritubular capillaries (mEPCR x 400)



**Table 1.** Descriptive histological data found in renal biopsies of patients.

Variable	Patients (n=25)			
	Nil	Mild	Moderate	Strong
Fibrinoid necrosis	22 (88%)	2 (8%)	1 (4%)	-
Glomerular Neutrophils	13 (52%)	9 (36%)	3 (12%)	-
Endocapillary proliferation	13 (52%)	8 (32%)	2 (8%)	2 (8%)
Crescent Formation	22 (88%)	2 (8%)	1 (4%)	-

**Table 2.** Comparison between groups with different degrees of mEPCR expression as regards some clinical and laboratory variables.

Variable		mEPCR		P
		Nil/Mild	Moderate/Strong	
BILAG	Improved (n=15) n(%)	10 (66.6%)	5 (33.3%)	0.73
	Stationary (n=10) n(%)	6 (60%)	4 (40%)	
BILAG score (at the time of renal biopsy)	A (n=7) n(%)	3 (37.5%)	4 (57.1%)	0.597
	B (n=8) n(%)	5 (62.5%)	3 (37.5%)	
	C (n=6) n(%)	5 (83.3%)	1 (16.7%)	
	D (n=4) n(%)	3 (75%)	1 (25%)	
Drugs	Steroid (gm/kg) Median (range)	0.45 (0.05- 0.71) (n=16)	0.46 (0.26- 3.1) (n=9)	0.45
	Cyclophosphamide (gm/kg) Median (range)	0.1 (0.05- 0.21) (n=8)	0.1 (0.07-0.16) (n=7)	0.53
Indices	Activity Median (range)	5 (2-15)	4 (2-10)	0.69
	Chronicity Median (range)	1 (0-2)	3 (0-3)	0.01
Lab. Data	Lymphocytic count ( $\times 10^3 /\mu 1$ )	1.5 (0.6-4.3)	1.6 (0.6-4.37)	0.535
	s. creatinine (mg/dl)	0.7 (0.3-6.4)	0.6 (0.3-5.3)	0.252
	24 hrs urinary protein (gm)	0.5 (0.1-3.4)	1.4 (0.28-4)	0.049

P <0.05: significant

**Table 3.** Comparison between activity and chronicity indices in both proliferative and non-proliferative LN

Variable	Patients with Proliferative LN n=12		Patients with non-proliferative LN n=10		P
	Median	Range	Median	Range	
Activity Index	6	2-15	3	2-5	0.004
Chronicity Index	2	0-9	2	0-9	0.56

P<0.05:significant

**Table 4.** Comparison between proliferative and non-proliferative LN as regards histological variants.

Variables	Patients with Proliferative LN n= 12				Patients with non-proliferative LN n=10				P
	Nil	Mild	Moderate	Strong	Nil	Mild	Moderate	Strong	
Fibrinoid necrosis	10 (83.3%)	1 (8.3%)	1 (8.3%)	-	9 (90%)	1(10%)	0(0%)	-	0.53
Glomerular Neutrophils	3 (25%)	6(50%)	3 (25%)	-	7(70%)	3 (30%)	0(0%)	-	0.014
Endocapillary proliferation	1 (8.3%)	7(58.3%)	2 (16.7%)	2 (16.7%)	9(90%)	1 (10%)	0(0%)	0(0%)	0.00
Crescent Formation	9 (75%)	2 (16.7%)	1 (8.3%)	-	10 (100%)	0(0%)	0(0%)	-	0.13
Renal mEPCR	3 (25%)	4 (33.3%)	3 (25%)	2 (16.7%)	5(50%)	4(40%)	0(0%)	2(20%)	0.42

P<0.05:significant

## DISCUSSION

LN has been described as the most serious complication of SLE and the strongest predictor of poor outcome<sup>19</sup>. It affects up to 80% of the patients with SLE<sup>20</sup>. There has been growing evidence suggesting that infiltration of T lymphocytes and other leucocytes at sites of inflammation plays a critical role in organ involvement in SLE<sup>21</sup>. In addition, cytokines were found to participate in the local inflammatory process that mediates tissue insult in SLE<sup>22,23</sup>.

The contribution of the vascular endothelium to the pathogenesis of renal injury has not been emphasized in LN. The state of microvasculature has never been identified in the definition of the WHO classification, National Institutes of Health chronicity and activity indices, nor in recent International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 pathological classifications of LN. Recent murine data based on microarray analysis suggests that endothelial activation is a feature observed in progressive glomerulosclerosis but not in non-progressive glomerulosclerosis<sup>6</sup>.

The Endothelial Protein C Receptor (EPCR) is expressed on leukocytes, on endothelium of large blood vessels and to a less extent on capillaries. Membrane bound EPCR plays an important role in the activation of protein C that has anticoagulant, anti-inflammatory and cytoprotective effects<sup>24</sup>. Accordingly, we examined the site and distribution of mEPCR expression in our patients' renal biopsies. The main site of expression was in the endothelium of the peritubular capillaries. Such finding was confirmed by Izmirly and colleagues (2009) who conducted a study on 49 patients with LN and controls and reported that positive staining for mEPCR was in the peritubular capillaries in areas with tubulointerstitial damage, while most of the glomeruli were negative<sup>6</sup>.

Given the assumption that shed mEPCR impairs the integrity of the endothelium and places the net balance of this protective protein in biological 'arrears', a decrease in mEPCR expression was the predicted result in patients with progressive renal injury. However, it was found that mEPCR is highly expressed in the cortical peritubular capillaries of kidneys from patients with active LN<sup>9</sup>. Such unexpected behavior was explained by 2 hypotheses: The first one stated that the increase in mEPCR expression represent a physiological defense mechanism done by the endothelium, while the other hypothesis stated that the circulating immune complex deposited on the endothelium activates the classical complement pathway which

in turn binds to protein S impairing its ability to generate active protein C and accordingly up regulating the receptor<sup>6</sup>.

Moreover, mEPCR marker expression failed to show a significant difference among patients with proliferative and those with non-proliferative LN. However, we found that the marker expression was still more in the proliferative group as compared to those patients within the non-proliferative group. This goes in accordance with Mendez and colleagues (2013) who did not find any association between mEPCR expression and ISN/RPS classification when they examined the renal biopsies of 34 adult patients with LN<sup>25</sup>. Same was found by Shabaan et al, 2018<sup>26</sup>.

On the contrary, Izmirly and colleagues (2012) examined non-lesional non-sun exposed areas of skin of 27 SLE patients with LN and 5 healthy controls. Their study showed that the median % of mEPCR positive staining in the dermal blood vessels was significantly higher in patients than controls (94% versus 59%;  $P=0.046$ ). There was a higher median level of mEPCR expression in patients with class III and IV and with biopsies with high immune complex deposition versus those of class V (96% versus 60%;  $P=0.029$ ). This median % increased in patients with active nephritis (96% versus 59%) in comparison to those with inactive LN<sup>27</sup>.

This wide spread up-regulation in inflamed and non-inflamed tissues was explained by the role of mEPCR in both endothelial activation and repair. This was confirmed by Kurosawa and colleagues (1998) who found a significant elevation of the soluble form of EPCR in comparison to controls and it was also associated with exclusive expression of mEPCR on large blood vessels<sup>28</sup>.

To find a relation between the degree of marker expression and disease activity we compared the different laboratory markers at time of renal biopsy to the degree of mEPCR expression (lymphocytic count, serum creatinine and 24 hours urinary protein) and BILAG score done at time of renal biopsy. The results showed a statistically significant elevated 24 hours urinary protein in moderate/severe mEPCR expression compared to mild/moderate ( $P=0.049$ ). This could reflect the damage occurring in the vascular integrity because of the coagulation defect and the subsequent protein loss in urine. This goes in accordance with Izmirly and colleagues (2008) as they found that there was an increase in marker expression with active disease<sup>29</sup>. The contrary was stated by Mendez and colleagues (2013) that could not find any

association with mEPCR expression and serum creatinine, abnormal GFR and proteinuria<sup>25</sup>.

In a trial to explore the prognostic effect of mEPCR we compared the degree of mEPCR expression to the cumulative doses of different immunosuppressive therapy (Steroid, Cyclophosphamide), but we could not find any significant difference (P= 0.45, 0.6 respectively). This could be attributed to small sample size and uneven immunosuppressive intake in patients with SLE that could be related to other organ affection.

The elevated expression of the marker mEPCR represents an attempt to preserve an antithrombotic state of the microvasculature to generate free active protein C to protect the endothelial integrity. Accordingly, we compared the degree of its expression to activity and chronicity indices, which showed that there was an inverse significant correlation with chronicity index and not with activity index (P= 0.01, 0.69 respectively). This supports the hypothesis that mEPCR is an important anti-injury molecule in the cascade(s) leading to renal damage in SLE<sup>9</sup>. However, Mendez and colleagues (2013) did not find any statistical significance between it and both activity and chronicity indices<sup>25</sup>. The same was found by Izmirly and colleagues (2009) who stated a non-significant correlation for activity and chronicity indices<sup>6</sup>.

In spite that most of the patients who got improved had nil/mild marker expression, no significant difference was reported between renal expression of mEPCR and renal outcome using the renal BILAG.

Mendez and colleagues (2013) found that 75% of patients with peritubular staining > 25% did not respond to therapy, while 100% of patients with staining <25% attained good response<sup>25</sup>. The same was confirmed by Izmirly and colleagues (2009) who stated that 84.6% of patients with a score of >2 did not respond to treatment versus 28.6% in those with a score of < 2 (P=0.0018)<sup>6</sup>. In addition, Izmirly and colleagues (2012) stated that the median of mEPCR was significantly higher in patients with a renal prognostic score >3 than in those with a score <3 (P=0.036)<sup>27</sup>.

For further proof of its bad prognostic effect in renal injury other than LN, Lattenist and colleagues (2013) conducted a study on 81 patients with renal transplants and assessed acute rejection of kidney allografts. They divided those with allograft into T-cell-mediated rejection (26 patients) and antibody-mediated rejection (22 patients) and 33 patients without rejection. Renal EPCR expression was

higher in patients with rejection than in control patients. Antibody mediated rejection patients had more EPCR expression on glomeruli and in peritubular capillaries which was explained to be protective behavior from the graft<sup>24</sup>.

In conclusion, renal expression of mEPCR bore a statistically significant difference in relation to different LN classes showing more expression in the more aggressive classes; a finding which might suggest a contribution of the endothelium of the renal parenchyma to the pathophysiology of more progressive LN. Hence the tissue marker might emerge as a potential new therapeutic target in the search for more selective treatment for SLE that could replace the wide range of immunosuppressive agents that are currently used in the SLE treatment regimen.

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