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Original article

Renal expression of vascular endothelial growth factor in lupus nephritis in the pediatric age group

Background: *Vascular endothelial growth factor (VEGF) plays a crucial role in preservation of renal functions and may also serve as a useful biomarker in monitoring the progression of lupus nephritis (LN).* ***Objective:*** *We thought to correlate VEGF expression in the kidney with renal histopathology in lupus nephritis to unveil its possible relation to disease activity and severity.* ***Methods:*** *We consecutively enrolled 15 patients with lupus nephritis and ten renal biopsy specimens from patients with cystic renal diseases as controls. The study measurements included SLEDAI, SLICC/ ACR damage index and BILAG renal score. Paraffin sections from renal biopsies were subjected to routine haematoxylin and eosin staining and Immunohistochemical staining for VEGF.* ***Results:*** *Among SLE patients, 7 (46.7%) showed mild expression of VEGF, 5 (33.3%) showed moderate while 3 (20%) had strong expression of the marker. On the contrary, the control samples (100%) revealed strong marker expression. All subjects with class IV and V lupus nephritis had mild renal expression of VEGF. Renal expression of VEGF had a significant positive correlation with serum creatinine and complement C3 levels. The 24 hours' excretion of urinary proteins had a significant negative correlation with the renal expression of the marker. On the other hand, the activity indices and therapeutic modalities did not correlate with VEGF expression.* ***Conclusion:*** *This pilot study among pediatric cases of SLE revealed mild to moderate VEGF expression in most cases of proliferative LN. Further longitudinal studies are needed to investigate the consequences of this finding on the prognosis of the disease.*

Keywords: VEGF, SLE, renal biopsy, lupus nephritis.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by acute and chronic inflammation of various tissues of the body. The antibodies and accompanying cells of inflammation can affect tissues anywhere in the body. Lupus has the potential to affect a variety of areas. SLE most often harms the skin, heart, lungs, kidneys, joints and /or nervous system. The course of the disease is unpredictable with periods of illness (called flares) alternating with remission[1].

Kidney disease is one of the commonest and the most serious manifestation of SLE. Despite improvement in the medical care of SLE in the past two decades the prognosis of lupus nephritis remains unsatisfactory. Up to 25% of patients still develop end stage renal failure 10 years after the onset of renal disease[2].

Renal biopsy is the gold standard for providing information on the histological classes of lupus nephritis and the relative degree of activity and chronicity in the glomeruli[2]. There are different tissue markers that have been associated with histological classes or renal functions deterioration in lupus nephritis. Intra-renal vascular endothelial growth factor mRNA expression predicts the deterioration of renal functions[3]. Vascular endothelial growth factor (VEGF) has been shown to stabilize kidney functions in animal model of thrombotic microangiopathy[4]. The protective actions were principally mediated through preserved glomerular and peritubular capillary structures. This may help to preserve glomerular filtration rate by maintaining glomerular capillary filtration surface area as well as preventing tubulointerstitial fibrosis[5]. Hence patients with proliferative LN who had decreased intra-renal VEGF expression are at risk for a rapid decline of renal functions at the time of renal flare. The combination of renal pathology such as class III/ IV LN and reduced VEGF expression could predict poor renal survival[6]. VEGF plays a crucial role in the preservation of renal functions and may also

serve as a useful biomarker in monitoring the progression of lupus nephritis[7].

With this as a background, we were simulated to investigate VEGF expression in the kidney in relation to the renal histopathological findings in a trial to correlate its expression with activity and severity indices of the disease.

METHODS

Study Population:

This cross sectional study comprised 15 patients suffering from lupus nephritis and ten renal biopsy specimens from patients with polycystic kidney disease or solitary renal cyst were included as control specimens. Patients with lupus nephritis were enrolled consecutively from the Pediatric Allergy and Immunology Unit, Children's Hospital, Ain Shams University, Cairo, Egypt.

The patients were fulfilling at least four of the revised classification criteria for SLE of the American College of Rheumatology "ACR"[8]. LN was diagnosed and biopsy proven according to the revised classification of WHO criteria of LN[9]. Hypertension was diagnosed according to the percentiles of blood pressure[10].

Patients with diabetes mellitus, nephrectomy, cyclosporine therapy or cystic kidney diseases were excluded from the study.

Detailed history taking with special emphasis on age at onset of SLE, duration of disease and organ involvement was considered. The treatment modalities in terms of steroids dose, type and dosage of the immunosuppressive drugs received were recorded. The cumulative steroid dose was calculated. All forms of corticosteroids were converted to equivalent dose of prednisone[11].

Global disease activity was assessed by the SLE disease activity index (SLEDAI)[12], and the British Isles Lupus Assessment Group (BILAG)-2004 renal score was used to assess the degree of renal involvement[13-15]. The Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index was used to assess renal damage[16].

An informed consent was obtained from the parents or care givers of subjects before enrollment. The study protocol was approved by the ethics' committee of the department of pediatrics, Ain shams University, Cairo.

Study Measurements

The SLE patients were subjected to complete blood counting (CBC) using Coulter counter (COULTER MAXMUG-HL-CCI), erythrocyte sedimentation rate (ESR) measurement by Westergren method, complement 3 (C_3) estimation by nephelometry (minineph ™, AD 200, 6326, United kingdom), and anti DNA measurement by indirect immunofluorescent microscopy (IMMCO Diagnostics, USA). Twenty four hours urinary protein quantification was performed by the turbidimetric method (Stanbio Laboratory Inc., San Antonio, TX, USA). The serum and urinary creatinine concentrations were measured by a modified rate Jaffe method [17]. Serum creatinine and creatinine clearance assessment was carried out on Synchron CX7 autoanalyzer (Beckman Instruments, Brea, California USA).

Blood collection and processing

Five ml of venous blood were withdrawn aseptically into a sterile disposable syringe from every patient. Two ml of sample were collected in EDTA treated tubes for CBC and 1.5 ml of sample was collected in citrate tube for measuring ESR. The remaining part of sample was collected in plain tubes to be clotted and centrifuged. The yielding serum was used for detection of the required immunological markers and kidney functions.

Renal biopsies

Paraffin section already frozen and kept in pathology laboratory hospital was taken for 13 patients and all control specimens while the remaining 2 were taken fresh specimens for histological analysis. Paraffin sections were cut at 4 microns thick and subjected to routine haematoxylin and eosin staining and immunohistochemical staining for VEGF.

Immunohistochemical staining

Paraffin sections were fixed on poly-L-lysine coated slides, dried overnight in a 60°C oven. Then they were deparaffinized and dehydrated. The slides were treated in microwave oven in ready to use antigen retrieval citrate buffer for 10 minutes, then sections were left to cool at room temperature for 20 minutes. Slides were stuck to cover plates using PBS pH 7.6 and placed in sequenza center for immunostaining. Endogenous peroxidase activity was blocked by adding 2-3 drops of hydrogen peroxide blocking serum for 5 minutes at room temperature. Then sections were rinsed well with PBS for 5 minutes. Two drops of protein blocking serum were added for 10 minutes. No rinse after this step. The primary antibody was applied by adding 3 drops to each section [VEGF: A monoclonal mouse antibody (labvision, catalog No: #MS-353-R7) supplied as 1m1 of ready to use antibody 1] and incubated for 2 hours at room temperature, followed by rinsing in PBS pH 7.6. The secondary antibody was applied by adding 2

drops of biotinylated secondary antibody to each section a supersensitive immunodetection system (Bigenex, catalog No. AD 000-SL) for 30 minutes at room temperature. Slides were then rinsed in PBS pH 7.6. Two drops of peroxidase labeled streptavidin were added for 20 minutes at room temperature, then rinsing with PBS pH 7.6. Slides were incubated for 10 minutes with substrate chromogen (DAB) mixture. Slides were then rinsed with distilled water. Slides were immersed in Harris Haematoxylin for 3 seconds, rinsed in tap water, dehydrated in absolute alcohol. Lastly, slides were cleared in xylene and mounted by Canada Balsam then covered by glass cover.

Sections of breast carcinoma were regarded as positive controls for CD31. 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 PBS was used instead.

The hematoxylin & eosin stained slides were examined and scored for activity and chronicity indices. For VEGF stained slides, VEGF was expressed on podocytes and capillary endothelial cells of glomerulus and tubular epithelial cells [18]. The intensity of immunostaining was scored as 0 (no staining), 1+ (mild staining), 2+ (moderate staining), +3 (strong staining)[19].

Statistical Methods

Statistical analysis was performed using Statistical Package for Social Sciences, Version 17.0 (SPSS, Inc., Chicago, III, USA) for Windows. Continuous variables were expressed as mean values ± standard deviation (SD) or median (range) as appropriate. Rates and proportions were calculated for categorical data. For categorical variables, differences were analyzed with χ^2 (chi square) test and Fisher's exact test when appropriate while analysis of these data before and after treatment was done using McNemar test.

Kolmogorov-Smirnov test of normality was done to assess normality of continues variables before starting the analysis. Differences among continuous variables with normal distribution before and after treatment were analyzed by paired t test or Wilcoxon signed rank test for non-parametric data. p value of <0.05 was considered statistically significant.

RESULTS

The SLE patients were 12 females and 3 males. Their age at disease onset ranged from 5 to 13 years with a mean ± SD of 9.8 ± 1.9 years. LN was diagnosed and biopsy proven according to the revised classification of WHO criteria of LN[9]. Seven patients (46.7 %) suffered from class III LN, five suffered from class II LN (33.3%), two (13.3%) suffered from class IV LN and one patient suffered from LN class V (6.7 %).

Seven patients (46.7%) showed mild expression of VEGF in their renal biopsies, 5 (33.3%) showed moderate while three (20%) had strong expression of the marker (fig.1). On the contrary all control specimens (100%) had strong marker expression.

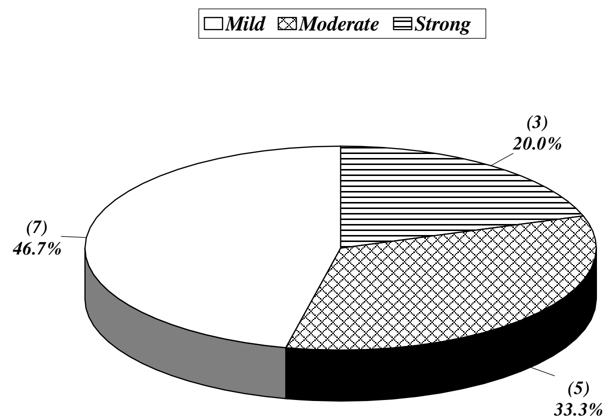

Figure 1. Frequency of VEGF expression among the studied patients.

Among our patients with class II LN, only 1 (20%) patient showed mild expression of VEGF, 3 (60%) showed moderate expression while the last 1 (20%) showed strong marker expression. The corresponding frequencies in class III LN were 3 (43%), 2 (28.5) and 2 (28.5%) respectively. As regards class IV and V LN, all (100%) of our subjects had mild renal expression of VEGF (fig.2).

Patients with mild VEGF expression had comparable age at diagnosis to those with moderate–strong expression, (p=1.000). Similarly, female predominance was observed in both groups with comparable frequency (p= 0.536).

At diagnosis, patients with mild VEGF expression had comparable serum levels of anti-dsDNA and C3 to patients with moderate or strong VEGF expression. However, the median value of anti DNA in the mild group was found to be nearly triple that of the moderate or strong groups (604 vs 213 Iu/ml). At enrolment, subjects with moderate/strong VEGF expression had significantly higher C3 levels as compared to those with mild VEGF expression. However, anti DNA levels were comparable in both groups.

Renal expression of VEGF failed to show a significant impact on renal function tests (serum

creatinine and creatinine clearance). The 24 hours urinary protein concentration tended to be higher in patients with mild VEGF expression as compared to patients with moderate or severe expression (1.5 vs 0.5 gm/day); the difference, however, did not reach statistical significance. Similarly, renal expression of VEGF did not correlate significantly with renal function test results in patients with mild versus moderate or strong levels of expression.

VEGF expression did not significantly influence the SLEDAI and renal BILAG scores of the studied patients at diagnosis. However, the median value of SLEDAI was found to be higher in patients with mild expression of VEGF.

No significant relation was detected between the type or cumulative dose of corticosteroid and/or immunosuppressive (cyclophosphamide and azathioprine) therapy in patients with mild VEGF expression as compared to those with moderate or strong levels of expression.

VEGF expression did not significantly affect activity and chronicity indices of the studied patients where both groups had comparable activity and chronicity indices. However, mild VEGF expression showed a higher activity score suggesting that a high activity index may associate decreased VEGF mRNA expression (table 1).

Renal expression of VEGF showed a significant positive correlation with serum creatinine and the level of C3 while the 24 hours urinary proteins' concentration was negatively correlated to the degree of VEGF renal expression.

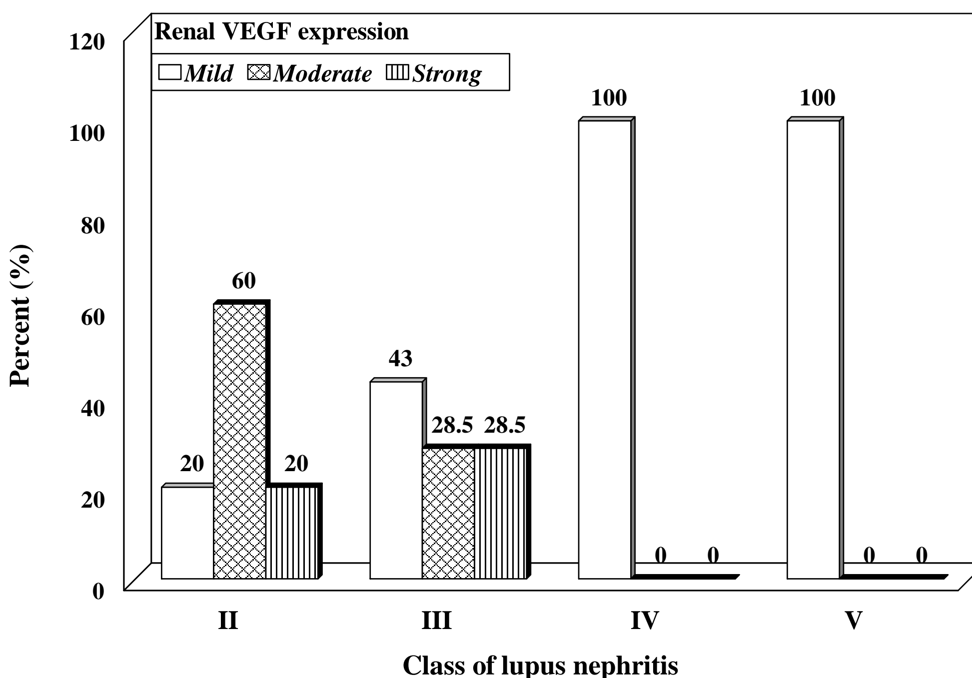

Figure 2. Variations of VEGF expression with the histological classes of LN.

Table 1. The effect of VEGF expression on activity and chronicity indices.

Activity index	Mild VEGF expression		Mod/strong VEGF expression		Test value	P value
	No	%	No	%		
Normal	1	20	4	80	Fisher = 2.143	0.182
Activity	6	60	4	40		

Mod: Moderate, P> 0.05: non-significant, No: number, %: percentage. No statistics could be done as regards Chronicity index

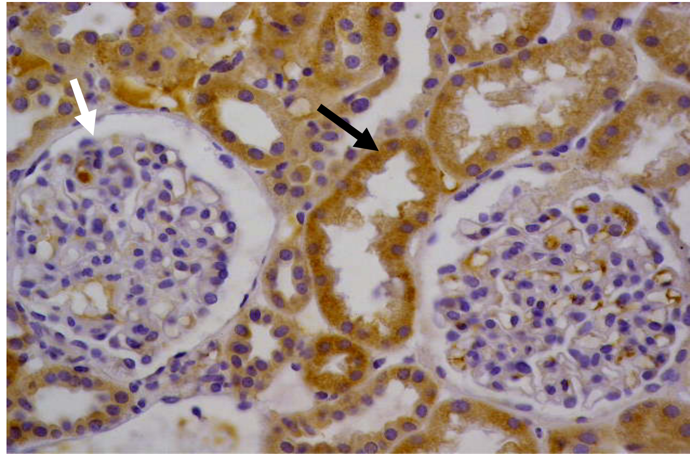

Figure (3): Marked expression of VEGF in the glomeruli (white arrow) and tubular epithelial cells (black arrow) of the kidney tissue in the control group (VEGF, X400).

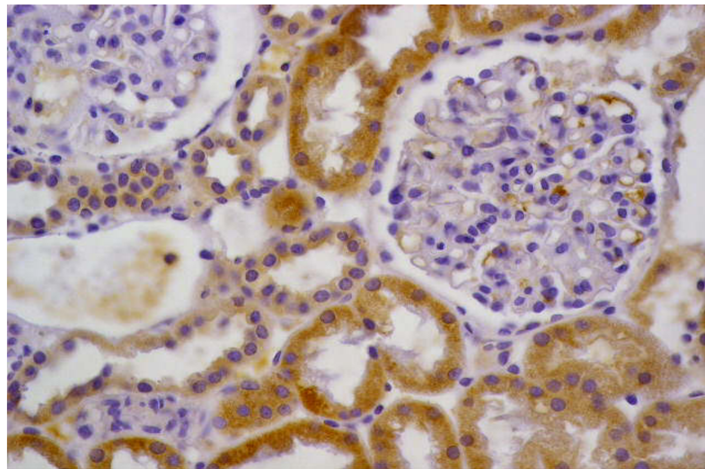

Figure (4): VEGF expression was decreased in the glomeruli and tubules of kidneys of patients with lupus nephritis class II (VEGF, X400).

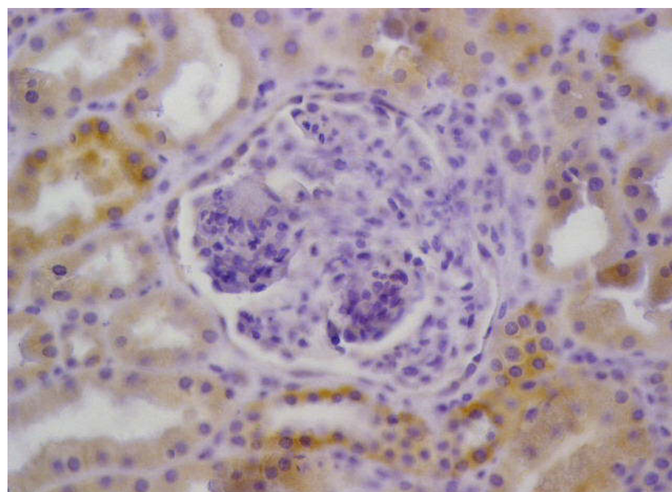

Figure (5): VEGF protein was absent in glomeruli and markedly reduced in the tubules of kidney of patients with lupus nephritis class IV (VEGF, X400).

DISCUSSION

VEGF plays a crucial role in preservation of renal functions and may also serve as a useful biomarker in monitoring the progression of LN [7]. Intra renal VEGF mRNA expression predicts the deterioration of renal functions[3].

In our series, renal expression of VEGF was mild in about 47% of subjects and moderate in 33% (both together represent 80% of the studied sample) while only 20% of the SLE subjects studied had strong levels of expression which could have been considered a favorable marker.

The integrity of the glomerular and peri-tubular capillaries is vital for renal functions. Progressive capillary loss, with obliteration of the microvasculature, frequently accompanies fibrosis, which is a characteristic feature of progressive renal disease. Progression of glomerulopathy is due to loss of glomerular integrity. Vascular endothelial growth factor (VEGF) promotes survival, proliferation, and differentiation of glomerular endothelial cells. The protective actions were principally mediated through preservation of glomerular and peritubular capillary structures[20].

All of our control specimens (100%) had strong expression of the marker as VEGF is known to have an angiogenic role in the pathogenesis of cystic kidney disease and is involved in the development of the pericystic circulation which may be necessary for cyst cells to grow[21].

A previous study [3] that investigated biopsy specimens from 35 adult patients (age 31± 1.27 years) with class III: IV LN (ISN/RPS categorization) reported that in a lupus mouse model as well as in LN patients, renal expression of VEGF was decreased and associated with a worse prognosis due to rapid decline of renal functions as compared with the control patients. The authors suggested that VEGF may serve as a molecular marker of renal damage and may be a predictive factor for short term loss of kidney functions in patients with LN. Similarly, Thacker and associates [22] revealed that the immunohistochemistry of renal biopsies from patients with LN had down regulation of VEGF and decreased glomerular and blood vessel capillary density compared with controls.

We used specimens from known patients with cystic kidney disease to control our results and all of them showed strong expression of renal VEGF. This was in agreement with Yoshida and associates[23] who reported that VEGF protein expression was increased in the inner strips of the outer medulla where cystic alterations were prominent. A relevant study identified increased angiogenesis in and around the cyst walls of 14 patients with autosomal dominant polycystic kidney disease (ADPKD). In addition VEGF expression was increased in the epithelial cells of cysts and in some capillaries surrounding the cysts and some glomeruli[21].

All subjects with class IV/V LN had mild expression of renal VEGF as compared to 20% and 43% of those with class II and III respectively suggesting the decline of this marker with LN severity. This agrees with the results of a relevant study on 35 adult patients with class III or IV LN (ISN/RPS categorization) that reported lower levels of intra renal VEGF expression as compared to 10 donor kidneys sampled at the time of allograft reperfusion. On the contrary, Frieriand associates[19] found no correlation between LN class severity and intensity of staining for VEGF expression. However, most of their patients were of the proliferative LN and other classes were not evenly expressed. Another study reported that VEGF was increased in plasma and renal tissue of patients with LN and a moderate degree of renal failure and assumed that overexpression of this angiogenic factor could be related to endothelial repair in this instance[24].

In our series, level of renal VEGF expression did not vary with the age at onset of SLE. The same conclusion was previously reported[19]. Also we did not find a specific gender predilection for VEGF expression, where both males and females had comparable levels of expression and again this was previously concluded from a relevant study[3].

Although the serum anti-ds DNA levels did not vary significantly with the level VEGF expression in our series, the median value of anti-ds DNA in subjects with mild VEGF expression was nearly triple that of the moderate to strong group at diagnosis. Also, serum C3 level at enrollment was significantly lower in patients with mild VEGF expression as compared to those with moderate/strong expression. This may relate VEGF expression with the immune alternations in SLE. The conclusions are however limited by the sample size. Binding of Anti-DNA antibodies from lupus patients to proximal tubulo-epithelial cells leads to rapid internalization and triggers an inflammatory response characterized by increased synthesis of interleukin-6 and tumor necrosis factor. This inflammatory response in epithelial cells is accompanied by decreased VEGF synthesis[25].

Our patients with mild VEGF expression were comparable to those with moderate and severe VEGF expression in terms of renal function testing at presentation. However, renal VEGF levels showed a positive linear correlation to the serum creatinine and C3 concentrations. Also, the 24 hour urinary protein levels both at diagnosis and

enrollment were found to be higher in patients with mild VEGF expression as compared to the moderate to strong group and showed a significant negative linear correlation with the renal expression of the marker (p= 0.012). This might suggest that VEGF expression is related to severity of nephrosis. Reduction in renal VEGF could contribute to endothelial cell injury causing capillary rarefaction and leading to tubular atrophy, interstitial fibrosis and ultimately proteinuria[26]

In a trial to evaluate the effect of VEGF on global disease activity and specific organ involvement, SLEDAI, BILAG and SLICC/ACR were plotted for all patients at the time of diagnosis as well as at enrollment. VEGF renal expression did not significantly influence the SLEDAI or BILAG scores of our SLE patients. However the median SLEDAI value tended to be higher in the group with mild renal expression of VEGF. Moreover, SLICC/ACR was found to be 0 in all of our SLE patients. This observation could be attributed to the small sample size or the uneven distribution of the classes of LN in our sample. VEGF expression did not significantly affect activity and chronicity indices of the studied patients where both groups had comparable activity and chronicity indices. However, mild VEGF expression showed a higher activity score suggesting that a high activity index may be associated with decreased VEGF mRNA expression.

Avihingsanon and colleagues[3] found that 53% of his patients with low VEGF expression but none of the patients with high levels experienced loss of their renal functions within 12 months. They also reported that there was an inverse relationship between intra-renal VEGF and histological activity indices as higher activity indices were associated with decreased VEGF mRNA expression. Samples with activity score ≥ 3 of total 24 expressed lower VEGF expression.

We did not find any significant relation between the type or cumulative dose of immunosuppressive therapy received (azathioprine and cyclophosphamide) and the level of renal VEGF expression. The variation of patients' characteristics and immunosuppressive treatments may be considered confounding factors.

In conclusion, VEGF showed mild/moderate expression in the majority of our pediatric SLE patients especially those with class IV and V lupus nephritis. Patients with lower grades of renal involvement tended to have higher levels of VEGF expression. This might link higher levels of VEGF expression to proliferative LN. The deleterious effect of decreased VEGF expression could not be significantly highlighted among our sample. However, the significant positive correlation between VEGF expression and the serum C3 probably supports its link to the inflammatory consequences of SLE. The small sample size is indeed a limiting factor in the analysis.

Further longitudinal studies on a wider scale are needed to verify the exact pathogenetic relation between VEGF renal expression and proliferative LN (ISN/ RPS class III or IV). This marker may prove to be a potential candidate for targeting therapy.

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