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Role of Vascular Endothelial Growth Factor (VEGF) and B-Cell Lymphoma / Leukemia-2 (Bcl-2) in the Pathogenesis of Lupus Nephritis and Their Correlation to Clinicopathological Indices of Disease Activity.

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ABSTRACT

Serum concentrations of vascular endothelial growth factor(VEGF) and B cell lymphoma leukemia-2(Bcl-2) may be relevant in the pathogenesis and activity of lupus nephritis. Plasma concentrations of VEGF were found to be increased in patients with active lupus nephritis as a result of the presence of tissue hypoxia, inflammation and endothelial damage with disease activity. It was also over expressed in renal tissue from these patients. Serum levels of Bcl-2 were found to be elevated in patients with active lupus nephritis resulting in alteration in the process of apoptosis of T and B lymphocytes which may be related to the beginning of an autoimmune event that extends survival of B lymphocytes with breakdown of self-tolerance and increased production of pathogenic auto antibodies. A new biopsy index was modified from the standard National Institute of Health(NIH) activity index(AI) and chronicity index(CI) including: Glomerular activity index(GAI), Tubulointerstitial activity index(TIAI), Chronicity lesion index(CLI). Immunofluorescence index(IFI) with Addition of glomerular and tubular macrophages which were evaluated qualitatively through the macrophage marker (CD68) . The objective of this study was to assess reliability of the serum levels of VEGF and Bcl-2 in predicting disease activity and outcome in patients with lupus nephritis in addition to their clinicopathological correlation with different morphological variables from the new modified biopsy index. 40 patients with active lupus nephritis were included in this study. All patients were evaluated twice, once at time of presentation and again after 6 months from implementation of medical treatment. This evaluation included: serum samples to measure levels of VEGF and Bcl-2 by ELISA) and renal biopsy which was evaluated by: light microscopy, electron microscopy and immunohistochemistry using the new modified biopsy index in addition to staining of VEGF in collecting podocytes, distal convoluted tubules, collecting ducts by immunohistochemistry Glomerular and tubular macrophages were also stained by the macrophage marker (CD68) by immunohistochemistry.Serum VEGF levels showed a significant drop from a mean of (13.01ng/ml +/-6.45). At presentation with disease activity to a mean of (9.73 ng/ml+/-4.65)after 6 months ($p<0.05$) , whereas regarding serum Bcl-2 levels, it showed a drop from a mean of (31.6ng/m+/-40.82) at presentation to a mean of (20.82 ng/ml+/-23.59) after 6 it but months didn't reach statistical significance. Among the different morphological variables of disease activity in the new modified biopsy index, glomerular proliferation correlated significantly with urinary RBCs in a significant positive correlation both at presentation and after 6 months($P<0.05$), also glomerular macrophages correlated significantly with proteinurea in a positive correlation both at presentation and after 6 months ($P< 0.05$). Statistical analysis data showed that serum VEGF correlated significantly with tubular cell necrosis from the modified biopsy indeX in a positive correlation throughout the duration of this study($P<0.05$). Also serum VEGF levels correlated in a positive correlation with the following morphological variables:cellular crescents, interstitial inflammation and NIH (AI) both at presentation and after 6 months. Within the same study, serum VEGF levels correlated negatively with serum C3 and C4 levels both at presentation and after 6 months but all of these correlations didn't reach statistical significance. VEGF in podocytes correlated with quantitative 24hs urinary proteins both at presentation and after 6 months in a significant positive correlation($P<0.05$). Serum Bcl-2 levels correlate with karyorrhexis/fibrinoid necrosis in a positive but insignificant correlation at presentation which remained positive but turned out to be significant after 6 months($P<0.05$) .Among the distinct morphological variables denoting disease activity in new modified biopsy index, both cellular crescents and interstitial inflammation correlated with serum Bcl-2 levels both at presentation and after 6 months in a positive but insignificant correlation. Both serum VEGF and Bcl-2 can be used reliably in patients with lupus nephritis for evaluation of disease activity, prognosis and the response to medical treatment at follow up especially in presence of a baseline serum level of VEGF or Bel-2 or both and a diagnostic renal biopsy, and if the need for a second look renal biopsy arises, it is advised to apply the new modified biopsy index in view of the diagnostic and prognostic value of many of its components in an attempt to implement an appropriate line of treatment.

Keywords: VEGF, B-Cell Lymphoma, Bcl2.

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INTRODUCTION

Serum concentrations vascular of endothelial growth factor(VEGF) may be relevant in the pathogenesis of SLE and lupus nephritis in response to the associated tissue inflammation and hypoxia, concentrations of which seem to be a marker of disease activity [10].

Results of Navarro et al showed that plasma levels of VEGF are increased in patients with lupus nephritis and is over expressed in renal tissue from these patients [9]. It is believed that alteration in the process of apoptosis of B&T lymphocytes may be related to the beginning of an autoimmune event, due to the non-elimination of auto-reactive B lymphocytes allowing auto antibodies to reach pathogenic thresholds with escape of self tolerance under the influence of serum Bcl-2(B-cel l1ymphoma / 1eukemia-2)which might be related to disease activity in patients with SLE and lupus nephritis [8]. A new biopsy index was modified from the standard NIH, activity index (AI) & chronicity index (CI) which showed a better Correlation with clinical & outcome parameters due to the addition of the tubular & immunofluorescence indices. This index included 4 elements: glomerular activity index(GAI), tubulointerstitial activity index(TIAI), chronic lesion index(CLI) & finally immunofluorescence index(IFI). Among individual morphological variables are the glomerular & tubular macrophages, the role of which in the pathology of lupus nephritis has been underappreciated,as they correlated with several clinical & outcome parameters both by semi quantitative estimate and on cell counts using the macrophage marker(Cd68) [6].

PATIENTS AND METHODS

Table 1: Components of modified biopsy index(Gary et al.,2000)

Glomerular Activity Index(GAI) • Glomerular proliferation • polymorphonuclear leukocytes • Karyorrhexis/fibrinoid necrosis • Cellular crescents • Hyaline deposits • Glomerular monocytes Maximum: 24	Scale 0-3+ 0-3+ 0-3+x2 0-3+x2 0-3+ 0-3+
Tubulointerstitial Activity Index(TIAI) • Tubular cell pyknosis • Tubular nuclear activation • Tubular cell necrosis • Tubular cell flattening • Macrophage in tubular lumen • Epithelial cells in tubular lumen • Interstitial inflammation Maximum: 21	0-3+ 0-3+ 0-3+ 0-3+ 0-3+ 0-3+ 0-3+
Chronic Lesion Index(CLI) • Glomerulosclerosis • Glomerular scars • Fibrous crescents • Tubular atrophy • Interstitial fibrosis Maximum: 15	 0-3+ 0-3+ 0-3+ 0-3+ 0-3+
Immunofluorescence Index(IFI) • Glomerular capillary immunofluorescence • Glomerular mesangial immunofluorescence • Tubulointcrstitial immunofluorescence • Vascular immunofluorescence Maximum: 96	 0-4+x6 0-4+x6 0-4+x6 0-4+x6
Biopsy index= GAI/8+CLI/5+TIAI/7+IFI/32=Max 12	

This sstudy was conducted on 40 patients (6 males and 34 females) with active SLE and lupus nephritis taken from Ain Shams University hospitals. Their ages ranged from 15-43 years. Serum samples were collected from all patients both at the time of presentation with active disease and another time after 6 months. For all 40 patients the following was done both at presentation and after 6 months: complete history

taking, clinical examination with assessment of disease activity using systemic lupus erythematosus disease activity index(SLEDAI), kidney function and liver function tests, complete blood picture, quantitative 24h. urinary proteins complete urine analysis, ANA, anti-ds-DNA,C3,C4 as well as serum VEGF & Bcl-2 which were measured by enzyme linked immunosorbent assay(ELISA).Renal biopsy was done for all patients both at presentation and after 6 months with application of the new modified biopsy index that included (GA,TIAL,CLI,IFI)in comparison to the standard NIH activity and chronicity indices. All renal biopsies were subjected to study by light microscopy, electron microscopy and immunohistochemistry(IHC). Glomerular and tubular macrophages in renal biopsy were stained by IHC using(CD68). VEGF was stained in renal biopsy in all patients both at presentation and after 6 months using IHC. Scoring of different morphological variables was done on a scale of(0-3+), whereas immune deposits (IgG,IgM,IgA,C3,C1q,fibrin)were graded on a scale of(0-4+)**(Table 1).**

Statistical methodology

Data were collected,verified,revised then edited on PC. The data were then analyzed statistically using SPSS statistical package version 12. the following tests were done:

- Description of quantitative variables as mean,standard deviation and range.
- Description of qualitative variables as number and percentage.
- Chi-square test was used to compare qualitative variables.
- Paired T-test was used to compare quantitative variables in the same group in parametric data(SD<25%of the mean).
- Will Coxon sign test was used instead of the paired T-test in comparing non parametric data(SD>25%of the mean)in the same group.
- Correlation Coefficient (r-test) was used to rank different variables against each other directly or indirectly by:

    - Paerson correlation coefficient.
    - Spaerman correlation coefficient for non-parametric data.

        * P.value>0.05(insignificant)
        * P.value>0.05(significant)
        * P.value>0.01(highly significant)

RESULTS

Serum VEGF levels showed a significant drop from a mean of(13.01ng/ml+-6.45)at presentation with disease activity to a mean of(9.73 ng/mlt+-4.65)after 6 months (P<0.05) ,whereas regarding serum Bcl-2 levels,it showed a drop from a mean of(31.6 ng/ml+-40.82)at presentation to a mean of (20.82ng/ml+-23.59)after 6 months but it didn't reach statistical significance. Immunolocalization of VEGF in collecting ducts in renal biopsy showed a significant change(P<0.05)among patients between the time of presentation with disease activity the time of follow up, as 7 patients expressed mild (+1)immune staining at a percent of(43.8%) after 6 months, 10 patients expressed moderate(+2)immune staining(62.5%)at presentation which dropped to 4 patients(25%)after 6 months,6 patients expressed intense(+3)immune staining at presentation at a percent of(37.5%)and after 6 months the number slightly dropped to 5 patients at a percent of (31.3%)(figure1).

Regarding VEGF in podocytes, it also showed a significant change(P< 0.05) between the time of presentation and after 6 months as 7 patients expressed mild(+1) immune staining at presentation at a percent of(63.6%) and their number increased slightly to 8 patients after 6 months(72.7%), Three patients expressed moderate immune staining(+2)at presentation at a percent of(27.3%), where as after 6 months,their number dropped slightly to 2 patients(18.2%) (Figure 2).

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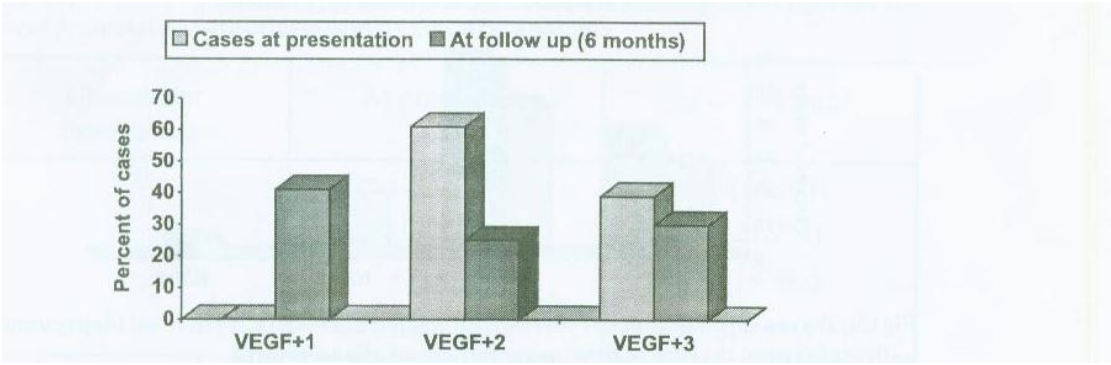

Figure 1: percent of change of vascular endothelial growth factor (VEGF) in collecting ducts in renal biopsy among patients between the time of presentation and after 6 months

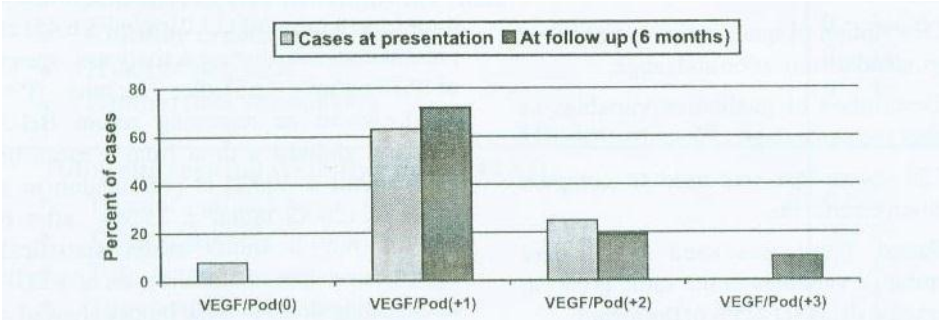

Figure 2: percent of changes of VEGF Iin podocytes in renal biopsy among patiets between the time of first presentation and after 6 months

Karyorrhexis/fibrinoid necrosis in renal biopsy as a marker of disease activity showed a significant change over the 6 month duration of this study,as 9 patients showed no kayorrhexs/fibrinoid necrosis (K/F)at presentation(27.3%) and their number increased to 12 after 6 month(36.4%),15 patients expressed mild(1+) (K/F)at presentation at a percent of(45.5%) and their number increased to 20 patients after 6 months(60.6%).Seven patients expressed moderate(2+) (K/F)at presentation(21.1%), where as after 6 months their number dropped markedly to 1 patient(3%)(Figure3)

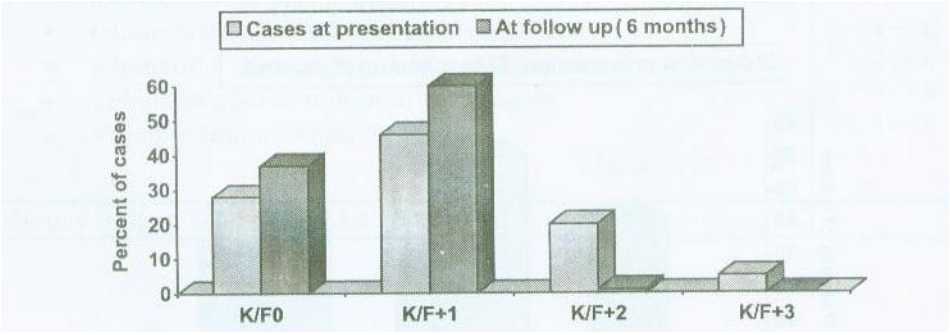

Figure 3: percent of changes of karyorrhexis / fibrinoid necrosis (K/F) in renal biopsy among patients between the time of first presentation and after 6 months

In the new modified biopsy index, glomerular proliferation correlated significantly with urinary RBCs in a significant positive correlation both at presentation and after 6 months($P < 0.05$). Glomerular macrophages correlated significantly with proteinurea in positive a correlation both at presentation and after 6 months($P<0.05$) (Figures 4& 5)

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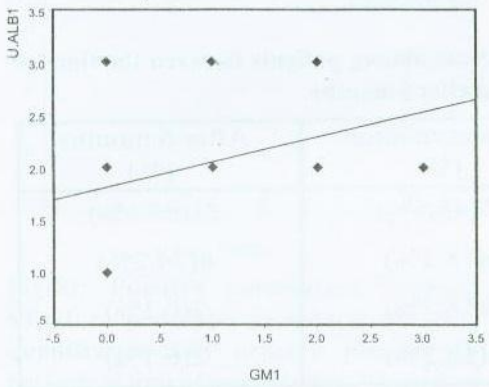

Fig (4): Positive correlation between glomerular macrophages (GM1) and urinary albumin ($U.ALB_1$) in patients at first time of presentation.

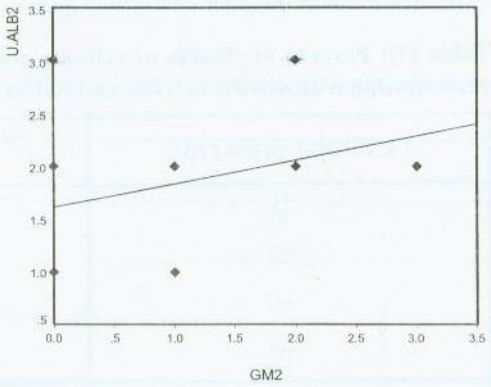

Fig (5): Positive correlation between glomerular macrophages (GM2) and urinary albumin ($U.LAB_2$) in patients after 6 months.

Also glomerular macrophages in this study correlated positively with ESR, quantitative 24 hour urinary proteins both at presentation and after 6 months. Within the same study, glomerular macrophages correlated negatively with serum C3 and C4 levels throughout the entire duration of this study, yet none of these correlations reached statistical significance

Table (2): Percent of change of glomerular macrophages among patients between the time of presentation with disease activity and after 6 months.

Glomerular macrophages	At presentation	After 6 months
0	12(36.4%)	18(54.5%)
+1	10(30.3%)	7(21.2%)
+2	6(18.2%)	5(15.2%)
+3	5(15.2%)	3(9.1%)
$X^2 = 3.47$ $P > 0.05$ (N.S.)		

Cellular crescents as distinct morphological variable from the modified biopsy index denoting disease activity. correlated in insignificant positive correlation with VEGF in podocytes. collecting ducts and podocytes both at time of presentation and after 6 months. Also cellular crescents correlated positively with ESR and negatively with C3 levels which persisted although the duration of this study, yet neither of which reached statistical significance.

Table 3: Percent of change of cellular crescents among patients between th time of presentation with disease activity and follow up after 6 months

Cellular crescents	At presentation (%)	After 6 months (%)
0	15(45.5%)	21(63.6%)
+1	6(18.2%)	8(24.2%)
+2	6(18.2%)	2(6.1%)
+3	6(18.2%)	2(6.1%)
$X^2 = 8.23$ $P > 0.05$ (N.S.)		

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Statistical analysis of data showed that serum VEGF correlated significantly with tubular cell necrosis(TCN)from the modified biopsy index in a positive correlation throughout the duration of this study($P<0.05$).

Also serum VEGF levels correlated in a positive correlation with following morphological variables: cellular crescents,interstitial inflammation and the NIH(AI) both at the time of presentation with disease activity and after 6 months.Within the same study, serum VEGF levels correlated negatively with serum C3 and C4 levels although the whole duration of this study, but all of these correlations didn't reach statistical significance. Regarding VEGF in the collecting ducts in renal biopsy, it correlated significantly($P< 0.05$)with serum C4 levels in a negative correlation both at presentation(Figure 6) and after 6 months(Figure 7)

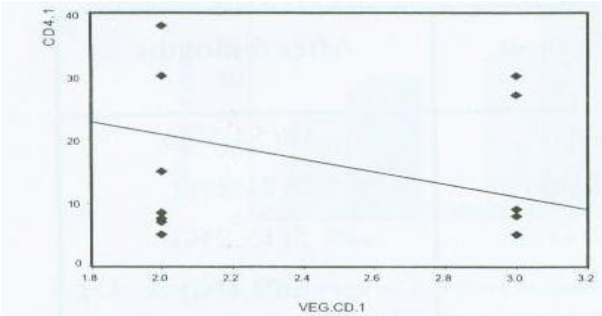


Fig (6): Negative correlation between VEGF in collecting ducts (CD) and serum C4 levels at time of presentation $(C4)_1$.

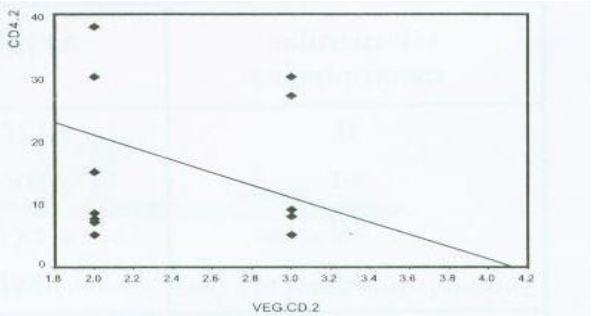
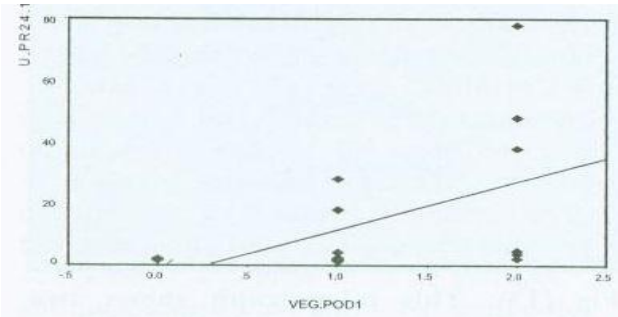


Fig (7): Negative correlation between VEGF in collecting ducts (CD) and serum C4 levels after 6 months $(C4)_2$.

Vascular endothelial growth factor in podocytes correlated with quantitative 24 hour urinary proteins both at presentation With disease activity and after 6 months in a significant positive correlation ($p<0.05$) (figures 8&9).



Fig(8): Positive correlation between VEGF in podocytes in renal biopsy and quantitative 24h urinary proteins in patients at time of quantitative 24h urinary proteins in patients presentation (U.PR24.1).

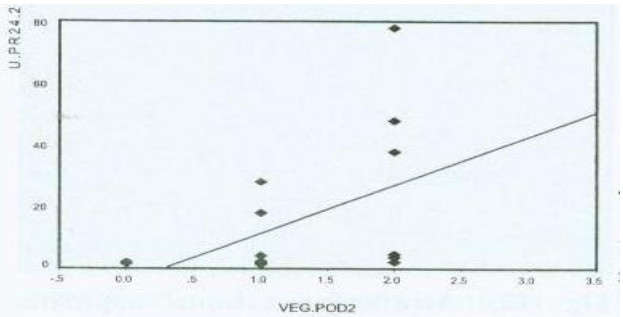


Fig (9): Positive correlation between VEGF in podocytes in renal biopsy and quantitative 24h urinary proteins in patients after 6 months (U.PR24.2)

Serum Bcl-2 levels correlated with both cellular crescents and interstitial inflammation from the new modified biopsy index both at the time of presentation With disease activity and after 6 months in a significant positive correlation , yet it reached no Statistical significance . Also serum Bc1-2 levels correlated with NIH (AI) in a positive correlation both at the time of presentation and after 6 months but it didn't reach statistical significance .

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DISCUSSION

Renal manifestations of SLE range from asymptomatic hematuria or proteinurea to overt nephritic or nephrotic syndromes, rapidly progressive glomerulonephritis and chronic renal failure. Subclinical nephropathy both during presentation and during monitoring of disease activity is frequently missed because of the unreliability of routine screening tests Depression of classic complement pathway components and high titers of anti-DNA antinucleosome or anti-Clq antibodies identify patients whom are at increased risk of renal involvement or flares of nephritis Several disease activity and damage indices are available,but they are mostly used in clinical setting and research none has achieved wide use for standard clinical practice [2].

Navarro et al. [9] performed a study on 28 with SLE with a mean age of(36.6+16.1), and 24 healthy controls.The study showed significant increase in serum vascular endothelial growth factor(VEGF) levels in patients with SLE and active nephritis in comparison to healthy controls.Within the same study, VEGF immunolocalization in patients with lupus nephritis was found to be most evident in distal convoluted tubules,collecting ducts as well as some podocytes.These results were in agreement with results of this study except for the fact that in the former study the assessment of disease activity was performed only once at presentation with disease activity,whereas in this study, the assessment of disease activity was done twice,once at presentation and another time after 6 months. This study also showed several correlations between serum VEGF and several markers of disease activity as:ESR,C3andC4 levels.

In fact serum VEGF correlated positively with ESR and negatively with serum C3 and C4 levels throughout the whole duration of this study but neither of which reached statistical significance. These results were in disagreement with the results of the study of Ewa et al. [4] on 60 patients with SLE with a mean age of 40 years and each patient was examined twice 2-4 weeks apart. The former study revealed a significant positive correlation between serum VEGF and ESR C3, C4 levels at both occasions.

Miret et al. [8]performed a study on 68 patients with SLE with a mean age of 38 years and the systemic lupus erythematosus disease activity index(SLEDAI)was used to assess disease activity and serum Bcl-2 levels were measured and correlated with different components of the (SLEDAI)at the time of presentation. The study showed increased levels of Bcl-2 in patients with active SLE which was highly significant($P< 0.001$). Furthermore,serum Bcl-2 levels showed a highly significant positive correlation with different SLEDAI values. Whereas in this study, serum Bcl-2 levels were measured twice in 40 patients with lupus nephritis at two separate occasions 6 months apart and it showed several correlation with different markers of disease activity but none of them reached statistical significance as a positive correlation with ESR and a negative correlation with serum C3 and C4 levels all of which persisted over the 6 months duration of this study.

Graninger, [7] performed a study on 24 females with SLE with a mean age of 33 years,the SLEDAI was implemented and the mRNA of bcl-2 in peripheral of blood mononuclear cells in SLE patients was measured and correlated with SLEDAI values. In 19 patients, the concentrations of bcl-2 mRNA were higher than those obtained from healthy Individuals .Within the same study,there was a positive highly significant correlation between amount of bc1-2 mRNA in lymphocytes from SLE patients and the SLEDAI values($P<0.001$).In comparison to our study,which was conducted on patients with lupus nephritis in addition to the fact that bcl-2 levels were measured in serum of patients using ELISA) over the duration of 6 months and they correlated with several markers of disease activity as ESR,C3 and C4 levels but all of which reached no statistical significance.

The role of glomerular macrophages in the pathology of lupus nephritis has been underappreciated. In the study performed by Gary et al. [5], glomerular macrophages showed a apositive significant correlation with proteinurea at first time of presentation($P < 0.05$) and a positive highly significant correlation after 6 months($P<0.001$). This was in agreement with the results of our study, except for the fact that glomenular macrophages correlated with other variables as:ESR quantitative 24h urinary proteins and urinary RBCs but with no statistical significance,which was in favor of glomerular macrophages as a useful individual morphological predictor of disease activity in patients with lupus nephritis. Among the individual morphological variables in renal biopsy, subendothelial deposits have shown the best correlation with outcome. Infacat their persistence at the second biopsy was associated with poor survival which was in accordance with the results of Tateno et al. [11], Esdaile et al. [3], Gary et al. [5] and finally this study.

Also in this study,cellular crescents emerged as a strong predictive pathological feature of disease activity both at presentation with disease activity and another time after 6months as they correlated with several variables

As:ESR and C3 levels,which was in agreement with the study performed Austin by et al. [1]

CONCLUSION

Finally, we can conclude that in presence of a diagnostic first renal biopsy and a baseline serum level of VEGF or Bcl-2 or both in patients with lupus nephritis, both new markers can be used reliably for evaluation of disease progression, prognosis and the response to treatment at follow up,and if the need for a second look renal biopsy arises, it is advised to apply the new modified biopsy index in view of the diagnostic and prognostic value of many of its components especially glomerular and tubular macrophages through their specific marker(CD68).

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