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The Tie2 Receptor Antagonist Angiopoietin-2 in Systemic Lupus Erythematosus: Its Correlation with Various Disease Activity Parameters

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Background: Systemic lupus erythematosus is one of the autoimmune diseases characterized by multisystem involvement associated with autoantibody and immune complex vasculitis along with endothelial cell damage. *Objective*: to study the possible role of Angiopoietin- 2 (Ang-2) as a recently highlighted inflammatory and angiogenic mediator in the pathogenesis of SLE and its correlation with the state of another inflammatory marker, P-Selectin, as well as with various markers of the disease activity. *Patients and methods:* The present study included 3 main groups: active SLE patients (group I), inactive SLE patients (group II) and healthy normal control subjects (group III). Groups I and II were subjected to disease activity assessment using the SLEDAI scoring system and measurement of plasma Ang-2 and P-Selectin by ELISA in addition to various laboratory investigations to assess disease activity as: Complete blood count, ESR, serum creatinine, C3, C4 and 24-h urinary proteins. *Results:* The mean level of Plasma Ang-2 and P-selectin showed a high significant increase in active group compared to inactive SLE patients and control subjects ($p < 0.001$).There was a significant positive correlation between Ang-2, P-Selectin, and each of SLEDAI score and 24-h urinary proteins in all SLE patients as well as in the active group, and Ang-2 was a significant independent marker for proteinuria. A significant negative correlation was found between Ang-2, P-Selectin and each of C3, C4. Ang-2 and P-Selectin showed a high sensitivity and specificity in the patients with SLE. *Conclusion*: Our study suggests that Ang-2 may be a more useful marker than P-Selectin, C3 and C4 in the assessment of disease activity.

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INTRODUCTION

The clinical expression of SLE is the consequence of its complex immunopathology, involving the production of autoantibodies and immune complex vasculitis with endothelial cell damage (Belmont et al., 1996).

Angiogenesis plays a critical role in several pathological conditions, including atherosclerosis, proliferative retinopathies, and tumor growth. It has been reported that inflammation precedes and accompanies pathological angiogenesis, as evidenced by increased vascular permeability as well as monocyte/macrophage and neutrophil recruitment at angiogenic sites (Peter, 2000). During inflammatory processes, newly formed vessels supply inflamed tissue with oxygen and nutrients and facilitate the transport of inflammatory cells (Belmont et al., 1996; Peter, 2000).

In this process, the activational state of the endothelial layer is a major determinate for the initiation, localisation, extent and propagation of inflammatory damage (Fiedler and Augustin, 2006). Endothelial activation is characterised by phenotypic changes from a quiescent, unresponsive to a responsive state. This process is associated with increased expression of luminal adhesion molecules eg, vascular cell adhesion molecule 1 (VCAM-1), leukocyte recruitment and disassembly of cell–cell junctions, finally resulting in loss of barrier function and tissue odema (Aird, 2005).

Angiopoietins are a novel class of angiogenic growth factors that act selectively on endothelial cells (ECs) (Orfanos et al., 2007). The Angiopoietin family includes four ligands (Ang-1, Ang-2, Ang-3 and Ang-4), three of which are expressed in humans, namely Ang-1, -2, and -4. All of these act on two corresponding tyrosine kinase receptors (Tie 1 and Tie 2). Ang-1 and Ang-2 are antagonistic ligands (Jones et al., 2001).

Ang-1 has been characterized as a Tie2 agonist, having the capacity to stabilize and promote the maturation of unstable vessels in the presence of VEGF-A 165 isoform (VEGF-A)165 (Thurston et al., 1999). Conversely, Ang-2 was described initially as a natural endogenous Tie2 antagonist, thereby destabilizing existing vessels prior to VEGF-A-165 induced angiogenic sprouting (Maisonpierre et al., 1997). However, under certain circumstances, Ang-2 may induce Tie2 phosphorylation, intracellular cell signaling events, and biological activities such as endothelial cell (EC) migration and platelet-activating factor (PAF) synthesis, neutrophil activation, vascular permeability, and in vitro tubule capillary-like formation (Maliba et al., 1957; Harfouche et al., 2003;

Lemieux et al., 2005; Roviezzo et al., 2005; Bodganovic et al., 2006; Harfouche and Hussain, 2006; Brkovic et al., 2007).

Ang-2 has been regarded as the dynamic regulator within the Ang/Tie system, since it constitutes a Weibel–Palade body-stored molecule (WPB), which is rapidly released and induced upon endothelial cells (EC) stimulation. Stimulation of EC with inflammatory mediators can promote a rapid and transient translocation of P-selectin, is one of family of selectins that include E-Selectin (Endothelial) and L- Selectin (leucocyte). Selectins are part of the broader family of cell adhesion molecules contained in Weibel-Palade bodies (WPB) to the cell surface (Bonfanti et al., 1989; Lorant et al., 1991; Rollin et al., 2004).

P-selectin functions as an adhesion molecule for a variety of leukocytes, including neutrophils, monocytes, T cells, eosinophils, basophils, platelets and some malignant cells, thereby bringing them to sites of inflammation and into contact with a range of cytokines and chemokines expressed by endothelial cells (Chen ang Geng, 2006).

Because angiogenesis and inflammation are two tightly linked processes, the search for factors that modify the inflammatory response among angiogenic growth factors seemed natural (Roviezzo et al., 2005). Kumpers et al. (2009) performed a clinical study to correlate serum concentrations of Ang-1 and Ang-2 with clinical, laboratory and histological findings in patients with SLE.

The aim of the study is to assess the level of Ang-2 and P-selectin as markers of endothelial dysfunction in SLE patients and to elucidate their correlation with various disease activity parameters.

MATERIALS AND METHODS

During the period between August 2009 and June 2010, 50 consecutive lupus patients were recruited from those attending Rheumatology and Rehabilitation & Internal Medicine Departments, Kasr El Aini Hospitals, Cairo University. The study was conducted in compliance with ethical principles rooted in the Declaration of Helsinki, and with the rules of Good Clinical Practice (GCP). All subjects consented to the study. The study comprised two groups:

Group I: consists of 50 patients with systemic Lupus (45 females and 5 males). All of them fulfilled the revised American Rheumatism Association criteria for systemic lupus erythematosus (Petri, 2005), with a mean age of 29.37 ± 8 years and mean disease duration of 5.5 ± 3.59 years. *Exclusion criteria*: patients with diabetes, neoplasia, cigarette smoking and other autoimmune disease e.g., rheumatoid arthritis were excluded from this study. According to the SLEDAI score, our SLE patients were subdivided into an "active" (SLEDAI ≥ 7) and an "inactive" SLE group (SLEDAI < 7). The group of active

SLE included 31 patients (3 males, 28 females) their age ranged from 18-45 years with a mean 27.45 $\pm$ 6.64 years. Although the group of inactive SLE included 19 patients (2 males, 17 females) their age ranged from 20-49 years with a mean 32.84 $\pm$ 9.12 years.

Group II: consists of 30 healthy volunteers (28 females and 2 males), their age ranged from 21-48 years with a mean age of 29.6 $\pm$ 8.7 years.

SLE patients were subjected to:

1. Full history taking, general examination, cardiopulmonary, abdominal, neurological and locomotor systems examination.
2. Routine laboratory investigations (CBC, liver and kidney functions, and urine analysis), immunological assays (ANA & Anti-DNA) and serum C3 and C4 levels, also 24-h urine samples were collected to estimate total urinary protein excretion levels.
3. The global disease activity was assessed by SLEDAI (Gladman et al., 2002), previously validated, reproducible and sensitive to change where it has 24 attributes grouped into 9 organ systems. The total final score falls between 0 and 105, ranging from 0 (no activity) to 105 (maximum activity). A SLEDAI score of $\geq$7 was taken as an indicator of high level of disease activity. All patients were taking steroids (dose ranged from 5–60 mg/day), 38 patients on hydroxychloroquine (dose ranged from 200–400 mg/day), 28 patients on azathioprine (dose ranged from 100–150 mg/day), 6 patients on methotrexate (dose ranged from 15-25 mg), 10 patients were receiving monthly cyclophosphamide pulse therapy (dose ranged from 600–1000 mg), and 5 patients on mycophenolate mofitil (dose ranged from 1–2 g).
4. Measurement of plasma Ang-2 and P-Selectin in all groups. Venous blood samples were drawn in fasting state in the morning. Plasma was collected using EDTA as an anticoagulant, centrifuged for 15 min at 1000x*g* within 30 min of collection, stored at $\leq$20°C. Ang-2 and P-Selectin were estimated by using enzyme immunoassay kit produced by R&D systems, Minneapolis, MN, USA (Pigott and Power, 1993; Lukasz et al., 2008).

Statistical Methods

The results were analyzed using the SPSS computer software package, version 15.0 (Chicago, IL, USA). Quantitative data were expressed as mean $\pm$ SD. Differences between two groups were compared by Student's *t*-test and were compared by ANOVA test between more than two groups. Correlations between data were performed using the Pearson correlation test. Multivariate

regression analysis was used to determine the association between dependent and independent factors. Differences were considered significant at $p < 0.05$.

RESULTS

Fifty adult patients diagnosed as SLE according to the revised ARA criteria (Petri, 2005), were included in this study. They consisted of 45 females and 5 males, their demographic and clinical data were presented in Table 1.

The serum level of Ang-2 in SLE patients ranged from 2810-9100 pg/ml with a mean 4944.04 ± 1872.88 pg/ml. Although the level of p-selectin ranged from 21-60 ng/ml with a mean 36.34 ± 10.59 ng/ml. The lupus patients had a statistically significant higher mean Ang-2 and p-selectin than healthy volunteers (4944.04 ± 1872.88, 36.34 ± 10.59 vs 1734.85 ± 515.16, 27.15 ± 4.73 respectively, $p < 0.001$) (see Table 2).

Upon comparison between the SLE group ($n = 50$) and controls ($n = 30$) as regards the various demographic and laboratory variables, we found a significant increase in the mean ESR in patients group, while the mean C3 and C4 levels were significantly decrease in patients compared to controls ($p < 0.001$) (as shown in Table 2).

According to the SLEDAI score we classified our patients into active group ($SLEDAI \geq 7$, $n = 31$), and inactive group ($SLEDAI < 7$, $n = 19$). The active group consists of 27 females and 4 males their mean age was 27.45 ± 6.64 year and

Table 1: Demographic and clinical features of the study SLE group ($n = 50$).

Feature	Study SLE Group
Number	50
Sex F/M	45/5
Age	
- Range	18–49
- Mean ± SD	29.37± 8
Disease duration	
- Range	0.6–15
- Mean ± SD	5.5 ± 3.59
Fever	13/50 (26%)
Oral ulcers	24/50 (48%)
Malar rash	19/50 (38%)
Maculopapular rash	5/50 (10%)
Alopecia	4/50 (8%)
Lupus headache	10/50 (20%)
Pleural effusion	8/50 (16%)
Pericardial effusion	6/50 (12%)
Arthritis	24/50 (48%)
Lupus nephritis*	36/50 (72%)

*Lupus nephritis presented by proteinuria ≥0.5 g/24 h ± active urinary sediments.

Table 2: Comparison between SLE patients and control subjects as regards demographic and labaratory data.

	SLE (50)	Control (30)	P-value
Age (years)	29.5 ± 8.03	29.6 ± 8.71	0.96
Sex (M/F)	5/45	2/18	
Hb (g/dl)	10.65 ± 2.2	11.79 ± 1.05	0.005
WBC's (10^3/μL)	6.52 ± 2.81	6.22 ± 1.52	0.830
Platelets (10^3/μL)	267.6 ± 98.13	331.05 ± 72.91	0.01
ESR (mm/h)	58.42 ± 33.02	7.65 ± 2.39	<0.001
Creat. (mg/dl)	0.7 ± 0.17	0.63 ± 0.1	0.07
C3 (mg/dl)	82.51 ± 28.19	117.55 ± 18.58	<0.001
C4 (mg/dl)	15.05 ± 6.88	27.4 ± 6.02	<0.001
Ang-2 (pg/ml)	4944.04 ± 1872.88	1734.85 ± 515.16	<0.001
P-selectin (ng/ml)	36.34 ± 10.59	27.15 ± 4.73	<0.001

Results are expressed as mean ± SD. ***P*** = between SLE and control groups.
Hb: hemoglobin, WBCs: white blood cells, ESR: erythrocyte sedimentation rate in the first hour, Creat. Creatinine.

their main disease duration was 5.45 ± 3.53 year. Although the inactive group consists of 18 females and 1 male, their mean age was 32.84 ± 9.12 year and the mean disease duration was 5.46±3.73 year. The patients in the active group were lower age compared to inactive group ($p = 0.02$). But no significant difference was observed between them in the disease duration ($p = 0.09$).

As regards the various laboratory data we found a high significant increase in the mean level of 24-h urinary protein in the active group versus the inactive group ($p < 0.001$). But, there were no statistically significant differences between them as regards mean hemoglobin level, mean serum creatinine and mean white blood cell count.

The active group showed statistically significant high levels of Ang-2 and P-Selectin ($P < 0.001$), but low level of C4 compared to inactive group ($P = 0.039$). However, no significant difference was observed between them in the mean level of C3 ($P = 1.00$) (see Table 3).

Table 3: Comparison between active and inactive groups as regards the mean levels of C3, C4, Ang-2, and P-Selectin.

	C3 (mg/dl)	C4 (mg/dl)	Ang-2 (pg/ml)	P-Selectin (ng/ml)
Active group (n = 31)	81.60 ± 27.97	13.74 ± 6.85	5801.74 ± 1807.7	41.10 ± 8.32
p-value	1.00	0.039	<0.001	<0.001
Inactive group (n = 19)	83.99 ± 29.26	17.19 ± 6.56	3544.63 ± 876.50	28.58 ± 9.34

Results are expressed as mean ± SD. ***p*** = between active and inactive SLE. *C3, C4; complement C3 and C4; Ang-2: Angiopoietin –2.*

Regarding the correlations of plasma levels of Ang-2 and P-Selectin with some clinical and laboratory data in SLE patients. There were significant negative correlations between Ang-2 and each of C3 ($r = -0.37$, $p = 0.01$), and C4 ($r = -0.56$, $p < 0.001$). There were significant positive correlations between Ang-2 and each of the following; P-Selectin ($r = 0.90$, $p < 0.001$), SLEDAI ($r = 0.86$, $p < 0.001$), 24 h urinary proteins ($r = 0.61$, $p < 0.001$), and ESR ($r = 0.95$, $p = 0.001$). Significant positive correlations were seen also between P-Selectin and 24 h urinary proteins ($r = 0.51$, $p < 0.001$), ESR ($r = 0.53$, $p < 0.001$), Ang-2 ($r = 0.9$, $p < 0.001$) and SLEDAI ($r = 0.74$, $p < 0.001$). Significant negative correlations were seen between P-Selectin and C3 ($r = -0.31$, $p = 0.03$), C4 ($r = -.48$, $p < 0.001$). Non significant correlations were seen between each of Ang-2 and P-Selectin with age, disease duration, Hb, WBCs, platelet, and creatinine (Table 4).

We used a multivariate regression analysis to prove that Ang-2 is a significant independent marker for proteinuria ($p = 0.002$, Coefficient $\beta = 0.58$), yet C3 showed less significant value ($p = 0.013$, Coefficient $\beta = -0.3$). P-selectin and C4 are non-significant markers for proteinuria ($p = 0.47$, coefficient $\beta = -0.13$, $p = 0.29$, Coefficient $\beta = -0.12$, respectively).

To roughly estimate the potential use of Ang-2 and P- Selectin as markers for disease activity compared to the traditional markers as C3 and C4, we calculated ROC curves to identify different cut-off values for Ang-2, P-Selectin, C3 and C4 that discriminate between patients with active SLE and patients with inactive SLE. The area under the curve (AUC) of Ang-2 was 0.87 (95% CI, $p < 0.001$).A calculated Ang-2 cut-off value of 3766 pg/ml resulted in 93% sensitivity and 63% specificity in discriminating active from inactive SLE. The AUC of P-Selectin was 0.83 (95% CI, $P < 0.001$, its cut off value was 29 ng/ml resulted

Table 4: Correlations of plasma levels of Angiopoietin-2 and P-Selectin with some clinical and laboratory data in SLE patients.

	Ang-2 (pg/ml)		P-Selectin (ng/ml)	
	R	P	R	P
Age (years)	−0.17	0.23	−0.27	0.06
Dis. Dur. (years)	−0.04	0.77	0.04	0.78
24-h urine pt. (g /L/24 h)	0.61	<0.001	0.51	<0.001
Hb (g/dl)	−0.26	0.06	−0.17	0.23
WBC's ($10^3/\mu L$)	−0.28	0.05	−0.2	0.15
Platelets ($10^3/\mu L$)	−0.1	0.47	−0.05	0.72
ESR (mm/h)	0.59	<0.001	0.53	<0.001
Creat. (mg/dl)	−0.04	0.76	0.03	0.81
C3 (mg/dl)	−0.37	0.01	−0.31	0.03
C4 (mg/dl)	−0.56	<0.001	−0.48	<0.001
Ang-2 (pg/ml)			0.9	<0.001
P-Selectin (ng/ml)	0.9	<0.001		
SLEDAI	0.86	<0.001	0.74	<0.001

Table 5: Receiver operating characteristic curves (ROC) of the plasma C3, C4, Ang-2 and P-Selectin.

Variable(s)	Cut-off value	Sensitivity (%)	Specificity (%)	Area under the curve	p-value
C3(mg/dl)	<97.60	74%	57%	0.599	0.242
C4 (mg/dl)	<19	71%	47%	0.675	0.04
Ang-2 (pg/ml)	>3766	93%	63%	0.878	<0.001
P-Selectin (ng/ml)	>29	96%	57%	0.837	<0.001

in 96% sensitivity and 57% specificity. In this regard, Ang-2 and P-Selectin outperformed the discriminatory ability of C3 (AUC 0.59,p = 0.24) and C4 (AUC 0.67, p = 0.04). (Table 5, Figures 1 and 2).

DISCUSSION

Systemic lupus erythematosus is a chronic inflammatory autoimmune disease of unknown etiology, with variable manifestations, course and prognosis (Gladman et al., 1999). The activational state of the endothelial layer is a major determinate for the initiation, localisation, extent and propagation of inflammatory damage (Fiedler and Augustin, 2006). Angiopoietin (Ang-2) has

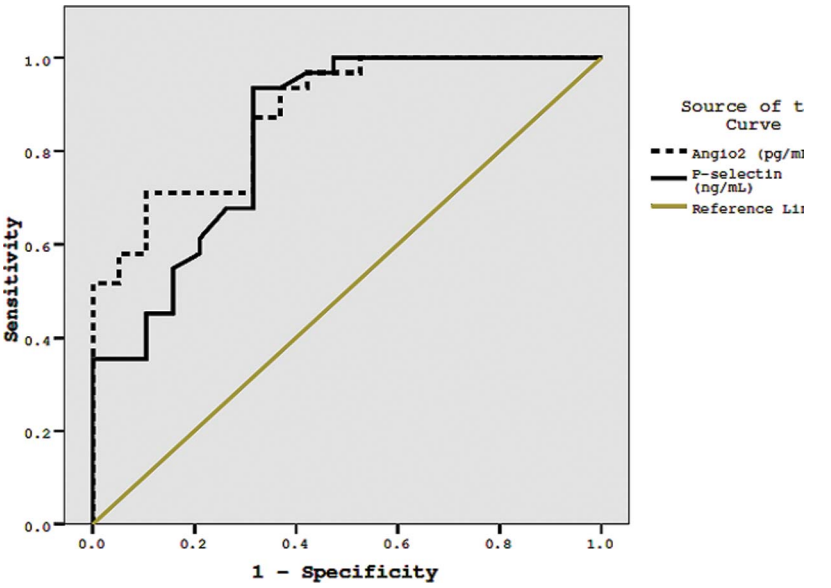

Figure 1: Receiver operating characteristic curves of Ang-2 and P- Selectin.

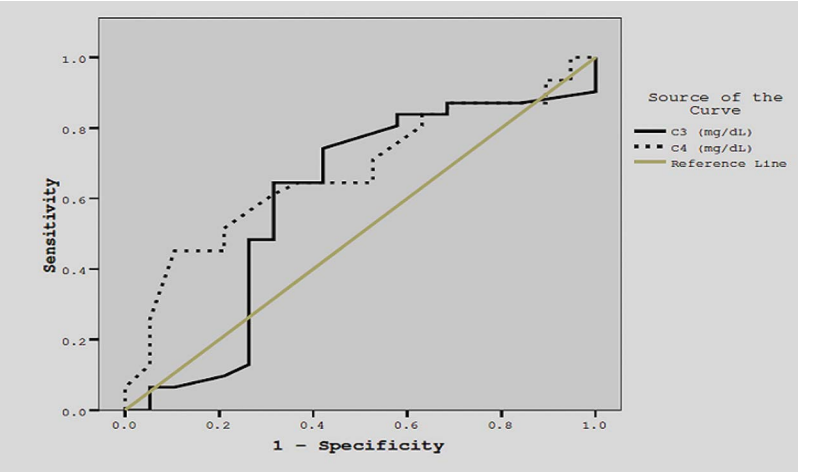

Figure 2: Receiver operating characteristic curves of C3 and C4.

emerged as a key mediator of endothelial cell activation (Fiedler et al., 2006). Binding of Ang-2 disrupts protective Ang-1/Tie2 signalling and facilitates endothelial inflammation, also it could modulate proinflammatory activities, namely P-selectin translocation, in endothelial cells (EC) (Parikh et al., 2006).

This work aimed at studying the possible role of Ang-2 as an inflammatory and angiogenic mediator in the pathogenesis of SLE and its correlation with the state of another inflammatory marker, P-selectin as well as various markers of disease activity.

In the present study we found that, the mean Ang -2 level was significantly higher in SLE patients than control. Also the mean Ang-2 level was significantly higher in active SLE group than inactive group. This agrees with Kumpers et al. 2009 and El-Banawy et al. (2011).

Ang-2 levels in patients with inactive SLE were still elevated compared to healthy controls. This finding implies that endothelial activation is not restricted to disease activity and represents an ongoing process during remission. Disruption of Ang-1/Tie2 signaling by excessive Ang-2 is a potential key mechanism that contributes to endothelial activation in patients with SLE (Fiedler et al., 2006). This agrees with the study of El-Banawy et al. (2011), which reported that Ang-2 can reflect the extent of endothelial activation and may be used as a biomarker of both disease activity and renal involvement in SLE (El-Banawy et al., 2011).

Accordingly, Ang-2 deficient mice did not exhibit any inflammatory response upon toxic or bacterial-induced peritonitis. Precise dissection of the underlying mechanism was reported by Fiedler and his colleagues in 2006 that Ang-2 does not primarily affect endothelial cell inflammation itself, but facilitates endothelial activation and subsequent leukocyte transmigration in the

presence of TNFα (Fiedler et al., 2006), and various other inflammatory stimuli as P–Selectin (Lemieux et al., 2005). Also, we found, a significant positive correlation between Ang-2 and SLEDAI score in all SLE patients (group I and II) ($r = 0.86$, $p < 0.001$). A similar finding was found by Kumpers et al. (2009) and El-Banawy et al. (2011), who found a strong positive correlation between Ang-2 level and SLEDAI score.

They suggested that endothelial activation in SLE represents an Ang-2 dependent process; the amount of Ang-2 within the circulation should reflect the extent of activated endothelial surface.

A significant negative correlation was found between Ang-2 and C3, C4 ($r = -0.37$, $p = 0.01$, $r = -0.56$, $p < 0.001$, respectively) in all SLE patients which also correlates with the results of Kumpers et al. (2009). This might be explained by consumption of the complement during the process of formation and fixation of immune complexes.

A significant positive correlation was found between Ang-2 and 24-h urine protein in SLE patients mainly in the active group ($p < 0.001$) and by a multivariate regression analysis we proved that Ang-2 was a significant independent marker for proteinuria ($p = 0.002$, Coefficient $\beta = 0.58$) , these findings coincide with the results of Kumpers and colleagues in 2009[20], who found that Ang-2 level was up regulated in glomeruli of patients with lupus nephritis, also agree with the results of El- Banawy et al. (2011), who found that Ang-2 level was significantly higher in patients with lupus nephritis than in patients without nephritis and Ang-2 was significantly positively correlated with 24 hours urinary protein (El-Banawy et al., 2011).

These findings are in line with increased glomerular expression of Ang-2 in preclinical models of glomerulonephritis (Lu et al., 2006). So, the current study proved that there was a close relation between circulating Ang-2 level and vascular barrier function, presented by the proteinuria as a marker for glomerular endothelial permeability. Davies and his colleagues in 2007, demonstrated that inducible glomerular Ang-2 overexpression in mice causes proteinuria and glomerular endothelial cell apoptosis indicating that Ang-2 has the capacity to modify glomerular permselectivity.

The authors provide evidence that the slit diaphragm protein nephrin, an essential component of the glomerular permselectivity barrier, is downregulated in angiopoietin-2– overexpressing mice (Davis et al., 2007). As such, Ang-2 is a candidate growth factor that might play a role in destabilizing glomerular endothelium, causing a breakdown of glomerular permselectivity in proteinuric renal diseases.

In the present study, the strong correlation between plasma level of Ang-2 and P Selectin particularly in the group of active SLE patients could reveal that Ang-2 does not primarily affect endothelial cell inflammation by itself but facilitates endothelial activation and subsequent leucocyte transmigration in the presence of various inflammatory stimuli as P-Selectin.

In our study, Ang-2 and P-Selectin showed a high sensitivity and specificity in the patients with SLE. Our study also suggests that Ang-2 may be a more useful marker than P-Selectin and the traditional markers, C3 and C4 in the assessment of disease activity. Thus, we support other groups of researchers; Kumpers et al. (2009) and El-Banawy et al. (2011), who have suggested that Ang-2 should be introduced into routine work-up in the follow-up of SLE.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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