RESEARCH ARTICLE

Correlations between concentrations of interleukin (IL)-17A, IL-17B and IL-17F, and endothelial cells and proangiogenic cytokines in systemic lupus erythematosus patients

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ABSTRACT. Systemic lupus erythematosus (SLE) is an autoimmune disease of multifactorial pathoaetiology. Different organs and blood vessels may be affected by chronic inflammation. A direct cause of the disease has not yet been found, so research is being carried out to this effect. The role of the recently identified helper T lymphocyte CD4+, described as Th17, and its dependent cytokines have been of particular interest. The aim of the study was to evaluate IL-17A, IL-17B, IL-17F and IL-23 in 60 SLE patients and 26 age-matched, healthy volunteers and also to investigate the correlation between levels of the investigated cytokines and VEGF, PIGF, as well as number of endothelial cells. IL-17A, IL-17B, IL-17BR and IL-17F levels were found to be higher in SLE patients than in the control group. However, only IL-17F levels showed a statistically significant correlation with the number of endothelial cells (aCEC) and disease activity. Correlations between levels of IL-17F and VEGF and PIGF as well as VEGF and IL-17A and IL-23 were statistically significant. Increased levels of the selected cytokines from the IL-17 family in SLE patients suggest a role for them not only in the inflammatory process but also in angiogenesis. This also highlights the role of IL-17F in activating vascular endothelial cells and consequently blood vessel formation, and in the relationship between the inflammatory reaction and angiogenesis in the development of SLE.

Key words: SLE, IL-17A, IL-17B, IL-17F, circulating endothelial cells

Systemic lupus erythematosus (SLE) is a chronic, systemic disease of connective tissues, the exact etiology of which is unknown. An inflammatory process within different organs and blood vessels induced by an autoimmune reaction is involved in the development of the disease. The role of the recently identified helper T lymphocytes CD4+, named Th17 after the cytokine IL-17 produced by these cells, has been of particular interest [1]. They are thought to play an important role in pathogenesis of the inflammatory process, as well as in angiogenesis. In humans, the strongest stimulating effect on the differentiation of this lymphocyte subpopulation depends on IL-1B and also IL-23 and IL-6 [2]. Since the first description of IL-17 in 1993, six subgroups from A to F within IL-17 have been identified. Despite belonging to the same family, they differ slightly in structure and function. The biological effects depend on specific transmembrane receptors, which are present on the endothelial cell surface, B and T lymphocytes, fibroblasts, as well as monocytes [3, 4]. Among Th-17 dependent cytokines – such as: IL-17, IL-21, IL-22, IL-26 and TNF-α –, a leading role belongs to interleukines which are members of IL-17 family, especially IL-17A, IL-17B and IL-17F.

IL-17A and IL-17F are most similar as regards both structure and function, taking part in antibacterial (extracellular bacteria) and antifungal defence [5]. Their activity depends on cytokine and chemokine induction influencing neutrophil activation, recruitment and migration. Apart from direct proangiogenic activity on human microvascular endothelial cells (HMVEC), IL-17 induces production and increases the proangiogenic activity of cytokines such as VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor) and HGF (hepatocyte growth factor) [6-10]. However, IL-17 also has anti-angiogenic activity [11-13]. In an experimental model of HECV human endothelial cell lines, recombinant IL-17 was found to inhibit endothelial cell migration and the formation of new blood vessels [13]. Apart from its proinflammatory activity, IL-17B is thought to have anti-angiogenic properties. The literature data also describe IL-17F anti-angiogenic activity, despite the fact that this cytokine is similar to proangiogenic IL-17A in structure and function [14, 15]. IL-17A and IL-17F expression is similar, and mRNA for both cytokines has been found on activated T lymphocytes, whereas mRNA for IL-17B has been found in the cells of spinal cord, testes, stomach, pancreas, prostate, ovaries, small intestines and chondrocytes [16, 18].

IL-23 stimulates expansion and survival of Th17 lymphocytes as well as IL-17 production [19]. Some authors

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suggest a critical role for the cytokine in the development of certain models of immune response [20, 21]. The literature data suggest that both IL-17 and IL-23 play an important role in the pathogenesis of autoimmune diseases such as systemic sclerosis, rheumatoid arthritis, psoriasis, Crohn's disease and SLE [22, 23]. These cytokines influence the immune response and stimulate the inflammatory reaction in the course of SLE. Their influence on angiogenesis in the disease is not fully understood, and the obtained results are ambiguous. The aim of this paper was to assess levels of the selected cytokines: IL-17A, IL-17B, IL-17F and IL-23 in the serum of SLE patients and to establish correlations between levels of the cytokines investigated and other factors involved in angiogenesis including VEGF, PIGF and a number of endothelial cells (ECs).

PATIENTS AND METHODS

Patients

The study was performed on 60 SLE patients, 55 females and five males; mean age 39 years. Twenty, sex and age–matched, healthy volunteers (17 women and three men) served as a control group. The diagnosis of SLE was based on the revised criteria of the American College of Rheumatology [24]. The mean duration of the disease was 66 months (range 5 months – 22 years). Nineteen patients had not been treated with immunosuppressive agents. Thirty nine patients were treated with prednisone at a dose of 5-30 mg/day during the study; five of them were additionally treated with azathioprine at a daily dose 50-150 mg. The characteristics of the patients are presented in *table 1* .

To measure the activity of the disease, the SLE Disease Activity Index 2000 (SLEDAI-2K) was used [25].This scale is a modification of a previously used SLEDAI, and is the most appropriate for clinical trials. It is possible to evaluate 24 parameters, both clinical and laboratory. SLEDAI-2K allows consideration of chronic, clinical symptoms such as active skin lesions, which can also be present on a patient's scalp, as well as mucous membranes and persistent proteinuria). In our study, each patient was evaluated twice, every four weeks. Although in the literature a score of 3 or 4 points to define active SLE has been proposed [26], we assumed, according to the proposition made by Abrahamowicz *et al.* [27], that a score of 0-5 points indicates inactive disease and a score of 6 points or more indicates active disease. A similar threshold has been established in many clinical trials. According to this definition, active disease was found in 28 patients, whereas 32 had inactive disease.

Each patient underwent a thorough physical examination performed by one of the authors. At the time of examination and collection of blood samples, the patients, as well as the control group, showed no clinical signs of infection or neoplastic disease, and had received no other medications for at least four weeks prior to blood donation. All blood samples were collected by venipuncture for routine laboratory investigations. The study was approved by the Bioethics Committee of the Medical University of Lodz, Poland. The patients were informed about the aim of the study, and all of them gave their informed consent for participation.

Table 1
Clinical and laboratory characteristics of SLE patients.

Symptoms	**Number of patients**	**100.0%**
Total	60	8.2%/ 91.8%
Age (years) mean (range)	39.2 (21 – 66)	46.7%/ 53,3%
Sex (male/female)	5/56	9.8%
Active / Inactive disease	28/32	27.7%
Immunodifusion (dsDNA antibodies)	6	80.3%
RBC ($<4.0 \times 10^6/mm^3$)	23	78.7%
HGB (<14.0 g/dL)	49	41.0%
HCT (<40.0%)	48	29.5%
Leukopenia WBC ($<4.0 \times 10^3/mm^3$)	25	16.4%
Lymphocytes ($<1.0 \times 10^3 mm^3$)	18	39.4%
Thrombocytopenia platelet ($<130 \times 10^3/mm^3$)	10	32.8%
Raised ESR (≥25mm/h)	24	27.9%
C_3 <0.9	20	6.6%
C_4 <0.1	17	39.4%
C. reactive protein (≥6.0 mg/L)	4	3.3%
Gammaglobuliny <12%	24	6.6%
AST >40 U/L	5	88.5%
ALT >40 U/L	4	6.6%
Antinuclear antibodies ANA	54	85.2%
APTT (≥40.0 s)	4	45.9%
Arthritis	52	9.8%
Skin symptoms	28	70.5%
Neurologic symptoms	6	4.9%
Hematological symptoms	43	65.6%
Renal disorder	3	
Immunosupresive treatment with steroids and/or cytotoxic agents during the study	40	

Laboratory tests

On the day of blood sampling to determine CEC (circulating endothelial cells) numbers and serum levels of L-17A, IL-17B, IL-17F, IL-23, VEGF and PIGF, the following laboratory parameters were also analyzed: blood count, ESR (erythrocyte sedimentation rate), C3 (complement 3 level), C4 (complement 4 level), CRP (C reactive protein level), APTT (activated partial thromboplastin time), γ-globulin (gamma globulin level), mucoproteins, level of antinuclear antibodies (ANA), and presence of their particular types (dsDNA, RNP, Ro, La or Sm).

Endothelial cell determination

At the time of clinical assessment, venous blood samples from the patients were collected in pyrogen-free, ethylenediaminetetraacetic acid-coated (EDTA) tubes. Evalutation of circulating endothelial cells was performed using four-color flow cytometry according to the procedure of Mancuso *et al.* [28]; a panel of monoclonal antibodies was used: fluorescein isothiocyanate (FITC)-conjugated CD31 (BD Pharmingen, USA) and CD146 (Serotec, UK) phycoerythrin (PE)-conjugated CD133 (Miltenyi Biotec, Germany), CD105 (Serotec, UK), PerCP-conjugated CD36, CD45 and CD 106 (all BD Pharmingen, USA) and APC-conjugated CD34 (BD Pharmingen, USA). An appropriate analysis gate was prepared as previously described [28]. In brief, CEPCs were defined as negative

for the hematopoietic marker CD45 and positive for the endothelial cell markers CD34, CD31, and the endothelial progenitor marker CD133. Resting CEC were defined as CD45-, CD133-, CD31+, CD34+, CD146+ and negative for activation markers (CD105, CD106). CD105 or CD106 positive mature endothelial cells were classified as activated CEC (*figure 1*). To increase sensitivity and reproducibility of measurements the "lyse-no-wash" pro-

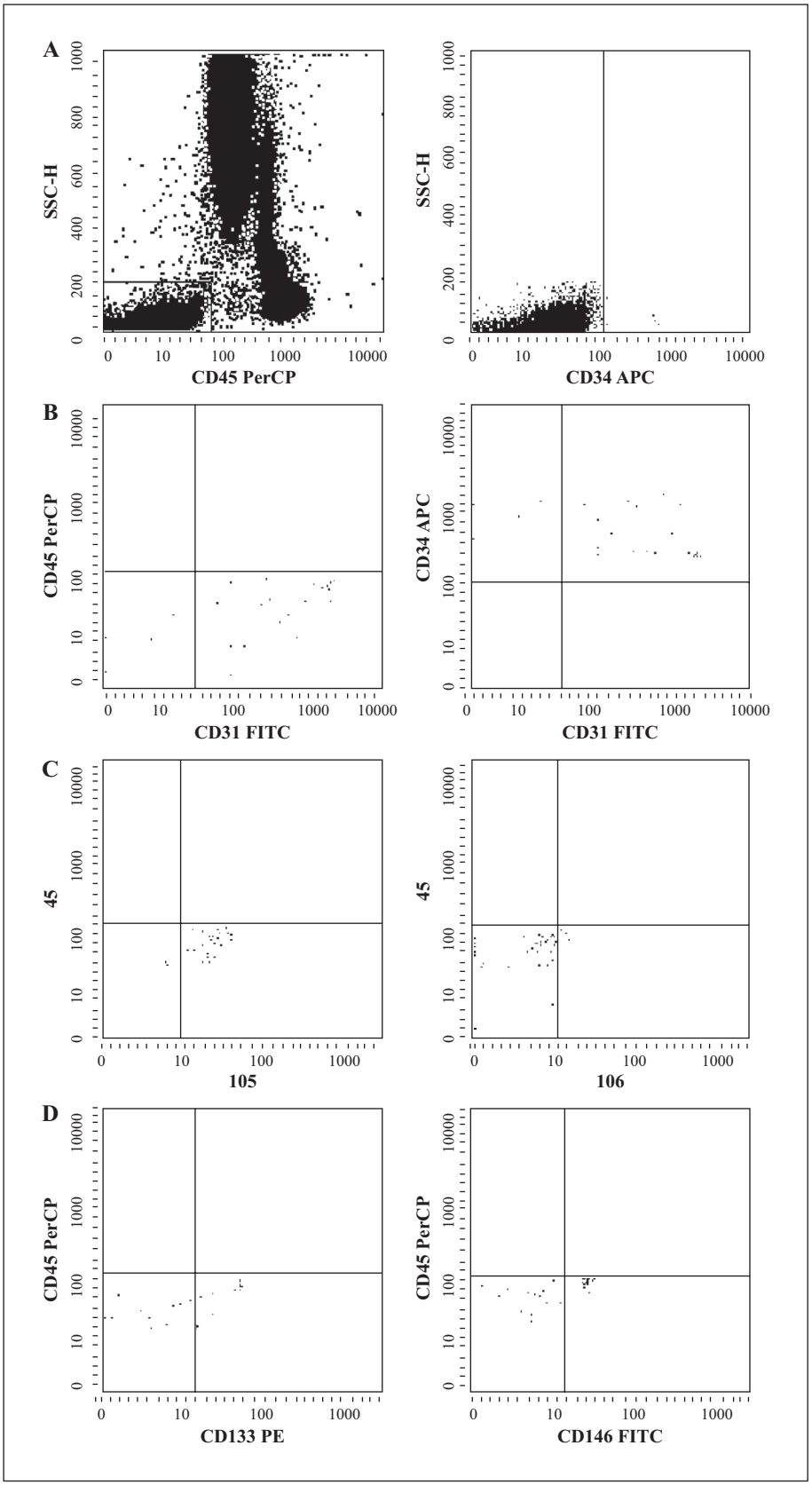

Figure 1

Detection of endothelial cells A- Four-color flow cytometry evaluation of CEC and endothelial progenitors The analysis gate used to exclude debris (including platelets and erythrocytes) and the reference beads used to obtain absolute cell count B- panels show the expression of antigens used to evaluate resting CD31 C- panels show the expression of antigens used to evaluate activated CD105 or CD106 D- panels show the expression of antigens used to evaluate progenitor endothelial cells CD133 and resting endothelial cells CD146.

cedure was performed. From each blood sample at least 100,000 cells were acquired to assure detection of low CEC levels. CEC were counted using the FAC SCalibur flow cytometer (Becton Dickinson, San Diego, CA, USA). Data obtained were analyzed using the CellQuest software program (Becton Dickinson, San Diego, CA,USA). The gating procedure has been described in detail elsewhere [29-31]. The total cell number was calculated by reference to fluorescent beads (TruCOUNT tubes, Becton Dickinson, San Diego, CA, USA), according to the so-called "single platform" method. In short, 50 μl of whole blood was added directly to the TruCOUNT tubes, containing a known number of fluorescent beads. Afterward, the FACS lysing solution (Becton Dickinson, San Jose, CA, USA) was added in order to lyse and, in this way, to eliminate interfering red blood cells. Next, appropriate monoclonal antibodies were added to the tubes for detecting particular CEC antigens. During the whole procedure no washing was performed to avoid cell loss (the method is called "lyse no wash"). The total CEC number (cell/μL) was determined by comparing the number of cellular events to bead events. This means that the number of CEC measured was divided by the number of fluorescence beads counted and multiplied by the TruCOUNT bead concentration.

Measurement of cytokine levels

Venous blood samples were collected into pyrogen-free tubes at the time of clinical assessment. They were allowed to clot at -4°C for 1 hour and centrifuged at 2000 g for 10 min. The serum obtained was divided into aliquots and stored at -80°C until assayed for IL-17A, IL-17B, sIL-17BR, IL-17F, IL-23, VEGF and PIGF. The patient and healthy volunteer serum samples were analysed using ELISA sandwich kits (BioVendor GmbH, D-69120 Heidelberg, Germany and R&D Systems Inc, Minneapolis, USA), in accordance with the manufacturer's instructions. All samples were measured in duplicate. The procedure has been described in detail elsewhere [32]. Both standards and samples were evaluated in duplicate and the inter-assay variations were shown to be within the range given by the manufacturer. Assay sensitivity was 0.5 pg/mL for IL-17A, 15.5 pg/mL for IL17F, 6.8 pg/mL for IL-23, 9.0 pg/mL for VEGF, and 0.6 ng/mL for PIGF. Test sensitivity for IL-17B and IL-17BR was not provided by the producer because of the different well coating.

Statistical analysis

For statistical analysis of the data the median and range of the variables measured are given (min–max). The Shapiro-Wilk's test was used to evaluate distribution. The differences in median values in more than two subgroups were evaluated with the Kruskal-Wallis test or by the analysis of variance. In the case of significant differences, the comparison for all possible pairs was performed. The comparison of variable values in two groups was performed, depending on the distribution of features, using the Mann-Whitney test, the test for two means for independent samples, the Cochran-Cox or the Tukey tests. The correlation between features was evaluated using the Spearman rank coefficient p. Comparisons and correlations were considered significant when $p<0.05$.

RESULTS

IL-17A, IL-17B, IL-17BR and IL-17F levels were significantly higher in SLE patients than in the control group ($p<0.001$; for IL-17F $p<0.03$; *table 2*). The highest levels were found in the active phase of the disease compared with patients in an inactive phase and the control group. However, only the level of IL-17F correlated with the activity of the disease (*figure 2*). Levels of IL-23 in the serum of SLE patients and the control group did not differ significantly (*table 2*). A statistically significant difference was found between IL-23 and IL-17A levels ($p<0.016$). Moreover, a positive correlation was also found between IL-17B and its receptor IL-17BR ($p<0.001$) (*figure 3*). Nevertheless, no significant correlation was found between IL-17A and IL-

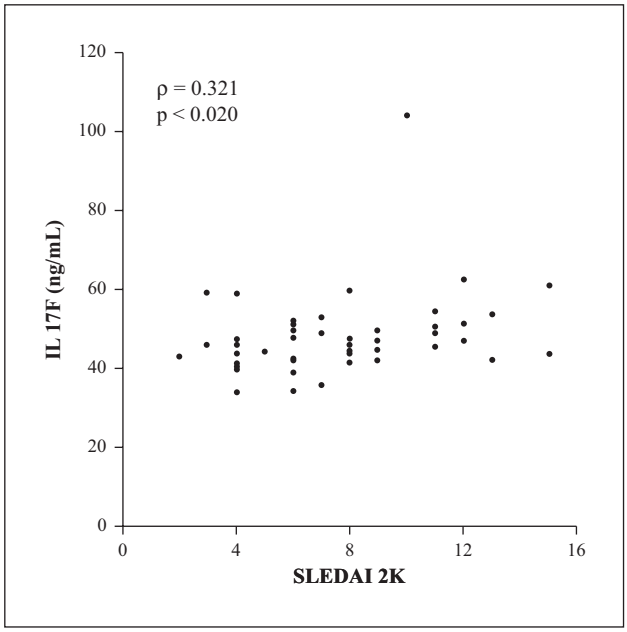

Figure 2
Correlation between IL-17F level and SLE activity.

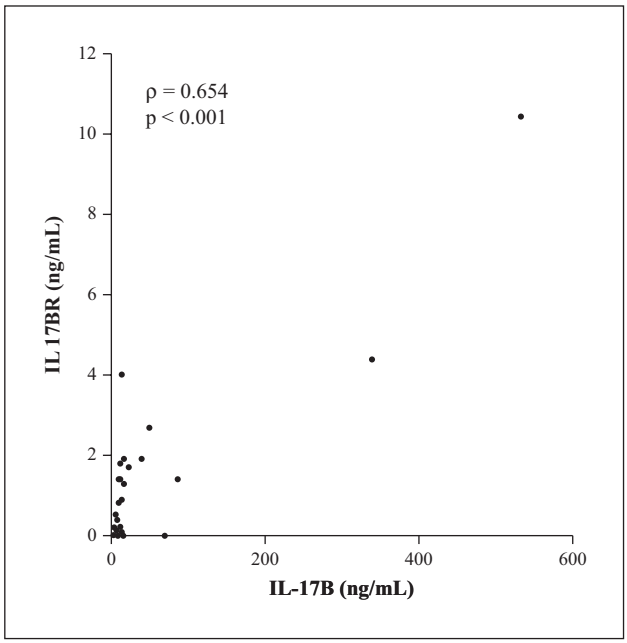

Figure 3
Correlation between levels of IL-17B and IL-17BR.

Table 2
Serum levels of IL-17A, IL-17B, IL-17BR,IL-17F, IL-23, PEIGF in patients with SLE and control group.

Factor	All SLE n = 60 (a)	Active SLE n = 28 (b)	Inactive SLE n = 32 (c)	Control group n = 20 (d)	Statisticcally significiant comparision
IL-17A					(a)-(d) p<0.001
$\bar{x} \pm s$	2.14 ± 3.82	2.89 ± 5.12	1.30 ± 0.89	0.43 ± 0.19	(b)-(c)-(d) p<0.001
Me	1.20	1.20	1.00	0.40	(b)-(d) p<0.001
Range	(0.6-23.0)	(0.6-23.0)	(0.6-5.2)	(0.2-1.1)	(c)-(d) p<0.001
IL-17B					(a)-(d) p<0,001
$\bar{x} \pm s$	29.42 ± 86.38	41.50 ± 116.79	15.84 ± 20.93	2.76 ± 0.84	(b)-(c)-(d) p<0.001
Me	8.60	8.10	9.35	2.80	(b)-(d) p<0.001
Range	(2.0-531.0)	(2.0-531.0)	(2.0-87.4)	(1.0-4.0)	(c)-(d) p<0.001
IL-17BR					(a)-(d) p<0.001
$\bar{x} \pm s$	0.78 ± 1.71	0.92 ± 2.20	0.63 ± 0.98	0.00 ± 0.00	(b)-(c)-(d) p<0.001
Me	0.10	0.00	0.10	0.00	(b)-(d) p<0.02
Range	(0.0-10.4)	(0.0-10.4)	(0.0-4.0)	(0.0-0.0)	(c)-(d) p<0.002
IL-17F					(a)-(d) p<0.03
$\bar{x} \pm s$	47.98 ± 10.09	50.23 ± 12.19	45.46 ± 6.41	43.11 ± 5.41	(b)-(c)-(d) p<0.02
Me	46.20	47.30	44.20	42.80	(b)-(d) p<0.02
Range	(34.0-104.2)	(35.6-104.2)	(34.0-59.2)	(35.6-52.4)	
PEIGF					(a)-(d) p<0.03
$\bar{x} \pm s$	14.38 ± 5.42	14.99 ± 5.98	13.68 ± 4.70	11.23 ± 3.57	
Me	13.55	15.10	12.90	12.00	
Range	(4.4-32.2)	(4.4-32.2)	(4.9-25.10)	(4.7-17.0)	
VEGF					(a)-(d) p<0.001
$\bar{x} \pm s$	431.9 ± 311.6	469.4 2 ± 325.34	388.93 ± 295.12	202.5 ± 117.6	(b)-(c)-(d) p<0.003
Me	335.70	411.60	319.40	211.80	(b)-(d) p<0.002
Range	(26.1-1438.4)	(62.5-1438.4)	(26.1-1221.3)	(13.4-399.0)	(c)-(d) p = 0.05
IL-23					
$\bar{x} \pm s$	13.80 ± 38.85	15.81 ± 44.74	11.62 ± 32.08	10.31 ± 30.95	
Me	1.40	1.40	1.40	0.00	
Range	(0.0-229.4)	(0.0-229.4)	(0.0-158.6)	(0.0-139.3)	

17B, IL-17BR and IL-17F levels, or between IL-17F and IL-17B, IL-17BR levels, or between IL-23 and IL-17B, IL-17BR and IL-17F.

Assessment of associations between EC numbers and levels IL-17 family cytokines, showed that the correlation between IL-17F levels and the number of activated endothelial cells (aCEC) was statistically significant (p<0.04) (*figure 4*). There was no statistically significant correlation between IL-17A, IL-17B, IL-17BR, IL-23 levels and the number of ECs (p>0.05). Moreover, there was also no correlation between the levels of the cytokines investigated and other endothelial cell subpopulations (rCEC, pCEC). Nevertheless, statistically significant differences were found between VEGF and IL-17A (p<0.04; *figure 5*), VEGF and IL-17F (p<0.002; *figure 6*), VEGF and IL-23 (p<0.025; *figure 7*), and also PIGF and IL-17 (p<0.05; *figure 8*). No significant correlation was found between Il-17B, IL-17BR levels and VEGF, PIGF (p>0.05), or between IL-17A, IL-23 levels and PIGF(p>0.05).

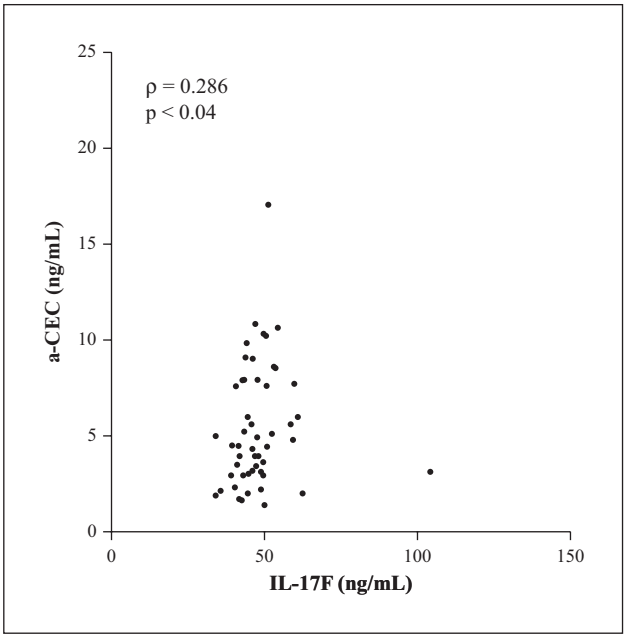

Figure 4
Correlation between number of aCEC and IL-17F level.

DISCUSSION

The high levels of IL-17 family cytokines in SLE patients found by many authors suggest their role in the pathogenesis of SLE. These cytokines are produced by helper T lymphocytes responsible for immune response, which may become dysregulated in the impaired immune process [33-35]. Moreover, other cells involved in the immune

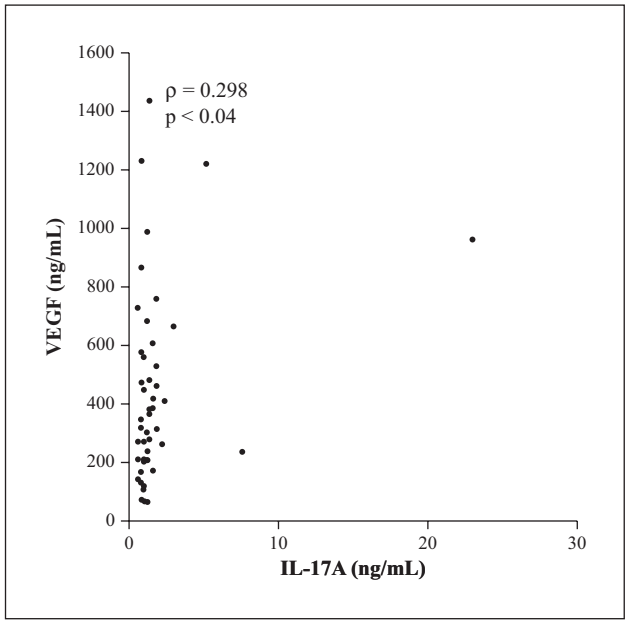

Figure 5
Correlation between VEGF and IL-17A level.

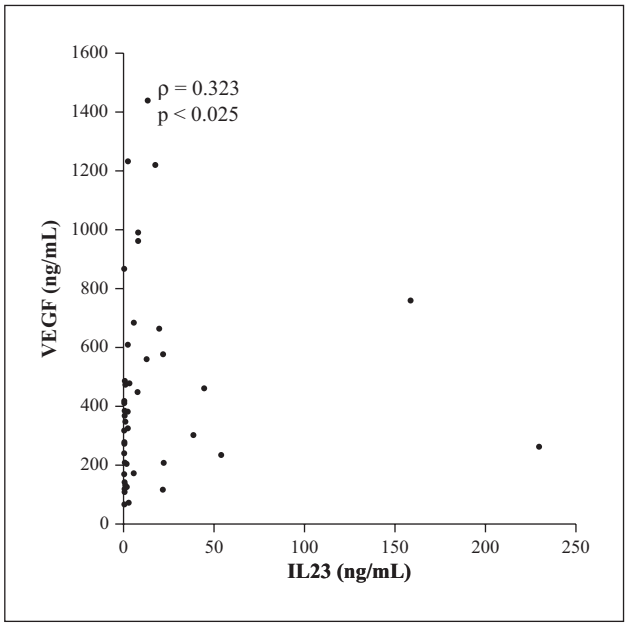

Figure 7
Correlation between levels of VEGF and IL-23.

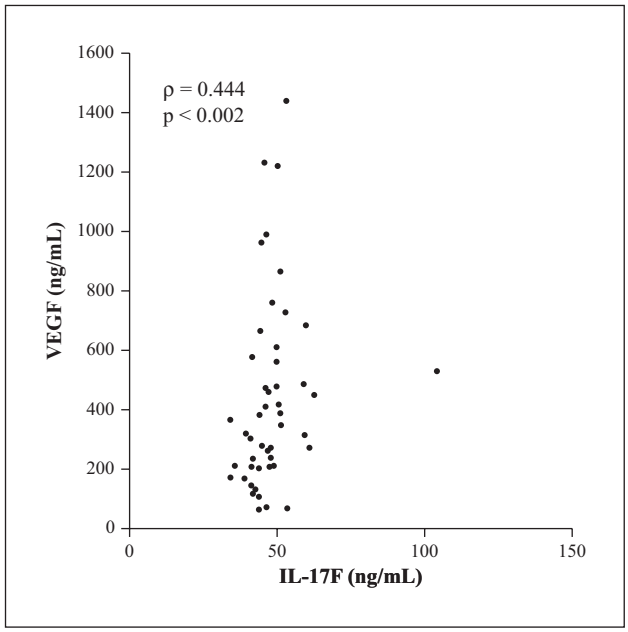

Figure 6
Correlation between levels of VEGF and IL-17F.

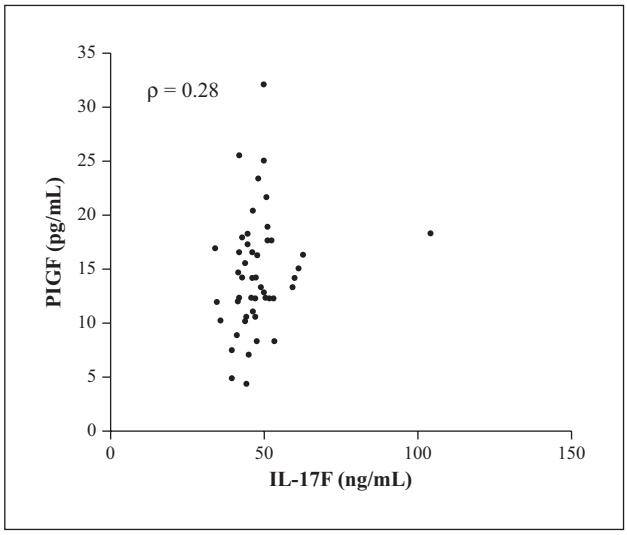

Figure 8
Correlation between levels of PIGF and IL-17F.

process such as T gamma/delta lymphocytes, NK and NKT cells, produce IL-17. Our results - IL-17 levels significantly higher in SLE patients - are in accordance with the results obtained by other authors [36-38]. Similarly to other authors, we have also found the correlation between IL-17 and VEGF levels to be significantly higher, which confirms the involvement of IL-17 in angiogenesis [39-41]. Most papers have evaluated only IL-17A levels in the serum of patients with different diseases, including SLE, as well as IL-17A, IL-17B, IL-17F expression in the affected tissues such as joints in rheumatoid arthritis or neoplastic tissues. We herein report the first study evaluating different IL-17 types in the serum of SLE patients.

Immune complex (IC) deposition in different organs and vascular walls, followed by development of inflammation, is a prominent feature of SLE, particularly in lupus nephritis. Destruction of blood vessels and EC release affected by the vascular immune process stimulate angiogenesis as a repair mechanism [42-44].

The cytokines investigated modulate both the inflammatory response and angiogenesis. Most studies focus on their proinflammatory activity, but only very few evaluate the role of these cytokines in angiogenesis, and then mostly in neoplastic diseases. Many authors have suggested that IL-17 could affect angiogenesis directly, or indirectly, by stimulating cancer cells to produce angiogenic factors through binding with IL-17R on endothelial cells [39, 40]. Numasaki *et al.* [41] found that IL-17 can promote angiogenesis and tumor growth by promoting chemotactic factors, endothelial cell migration, and neogenesis of blood vessels in non-small cell lung cancer. Our results indicate a similar role for them in the development of SLE. Some authors suggest that IL-17B and

IL-17F, despite proinflammatory activity, inhibit formation of new blood vessels in contrast to IL-17A [17, 45]. The proinflammatory effect of IL-17B was confirmed in the development of rheumatoid arthritis [46]. Such IL-17B activity has been suggested to be due to increased IL-6 and TNF-alpha release from macrophages. It has also been pointed out that neutralization of this cytokine significantly inhibits development of the disease (IL-17B and IL-17C may exacerbate arthritis owing to increased TNF-alpha and IL-6 production). Moreover, neutralization of IL-17B significantly suppresses progression of arthritis, which suggests that IL-17B acts in a similar way in SLE patients with joint symptoms. Results obtained by Yamaguchi and colleagues [46] indicate a relationship between joint pain in rheumatoid arthritis and IL-17B levels. So far there are no data on the role of IL-17B in the pathogenesis of joint pain or angiogenesis in SLE patients. The studies evaluated angiogenesis by investigating the influence of *in vitro* recombinant IL-17B on a human HECV endothelial cell line purchased from the European Collection of Animal Cell Cultures (ECACC). rIL-17B was found to inhibit HECV endothelial cell-matrix adhesion and cellular migration in neoplastic diseases [13]. IL-17 inhibition of angiogenesis may be a defense mechanism supporting the relative balance between angiogenesis and angiosuppression in favour of angiosuppression. In our studies, no correlation between IL-17B levels and the number of endothelial cells and proangiogenic cytokine levels has been found. Evaluation of IL-17B expression in the affected tissue in the group investigated may confirm local anti-angiogenic activity. However, the increased level of IL-17B in the serum indicates its involvement in the pathogenesis of the disease.

So far there are also few data regarding the angiogenic role of IL-17F, which is homologous to IL-17A. According to the literature IL-17F is a factor that strongly stimulates production of other cytokines and is involved in the regulation of angiogenesis [18, 47-49]. We found that IL-17F levels were higher in SLE patients and show a significant correlation with SLE activity and the angiogenic factors evaluated. Hedrich *et al.* [50] found diminished expression of IL-17F in T lymphocytes and an imbalance between IL-17A and IL-17F in SLE patients. Some authors point out that activated T lymphocytes are the main source of this cytokine. Interestingly, it was found that activation of T lymphocytes, including Th17, plays an important role in the pathogenesis of SLE [51, 52]. Our results show higher levels of all IL-17 members evaluated, which may be due to Th17 lymphocytes activation and is in accordance with other authors [47]. We have shown a significant correlation between IL-17 levels and the number of activated endothelial cells, VEGF and PIGF levels. Increased VEGF and PIGF levels correlated with the number of EC. This result suggests a special role for IL-17F in endothelial cell activation, and consequently in angiogenesis. There are no data evaluating IL-17 family levels and the number of aCEC or PIGF levels in SLE patients. Some papers suggest IL-17A and IL-17B stimulation of angiogenesis by a direct effect on EC. However, most studies focus on neoplastic diseases. Starnes *et al.* found that recombinant IL-17F had anti-angiogenic activity [15] and an inhibiting effect of IL-17F on endothelial cell capillary tubule formation which was dose-dependent. This activity may be due to increased release of TGF beta, another cytokine with anti-angiogenic activity. Tong *et al.* found diminished mRNA expression for IL-17F in colon cancer tissue [53] and suggested *in vivo* inhibition of tumor angiogenesis by IL-17F.

Our results do not correspond with the results from investigations on cancer tissue or experimental models. The divergence may be due to different disease mechanisms or different conditions *in vitro* or possibly to structural differences of the proteins investigated in SLE patients. Some authors have suggested that IL-17 has both pro- and anti-tumour functions in different cancers, which may be related to both type of cancer and lymph system function [54]. Conversely, immune dysregulation and impaired functioning of the lymphatic system in SLE may contribute to the disrupted balance of IL-17A, which is different from that observed in oncogenesis.

CONCLUSION

Increased levels of the selected IL-17 family members in SLE patients in our study indicate its role both in the inflammatory reaction and angiogenesis. Our findings reveal the special role of IL-17F in endothelial cell activation followed by angiogenesis and the relationship between the inflammatory reaction and angiogenesis in the development of SLE. Further research is needed to fully understand the involvement of IL-17 in angio-pathogenesis, and to formulate new therapeutic strategies. The conflicting results described by different authors may be due to the heterogeneity of the groups investigated – clinical group diversity, different disease activity, and the variety of management approaches.

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