DE GRUYTER
OPEN

IL-17, IL-6 and IFN-γ in systemic sclerosis patients

P. BĂLĂNESCU[1,2,3*], ANCA LĂDARU [2,4], EUGENIA BĂLĂNESCU[1],
ADRIANA NICOLAU[5], C. BĂICUŞ[2,3,5], GH.A. DAN[2,3,5]

[1]Clinical Immunology Department, "Colentina" Clinical Hospital, Bucharest , Romania
[2]"Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania
[3]Clinical Research Unit RECIF (Réseau d' Epidémiologie Clinique International Francophone), Bucharest, Romania
[4]"Alfred Rusescu" Institute for Mother and Child Protection, Bucharest, Romania
[5]Department of Internal Medicine, "Colentina" Clinical Hospital, Bucharest, Romania

Background. Systemic sclerosis (Ssc) is an autoimmune disease characterized by cutaneous and visceral fibrosis and its pathogenesis is incompletely understood. T helper cells are key regulators of the immune response and they seem to be involved in Ssc clinical manifestations. The aim of the study is to determine key cytokines secreted by Th1 (IFN-γ), Th2 (IL-6) and Th17 (IL-17) in Ssc patients and correlate them with specific manifestations of Ssc patients.

Material and methods. 35 consecutive Ssc patients and 20 age and sex matched controls were recruited. Serum IL-17, IFN-γ and IL-6 were determined using ELISA method.

Results. Serum IL-17 and IL-6 levels were not significantly different in Ssc patients and controls. Serum IFN-γ levels were higher in Ssc patients when compared to controls. Higher serum IFN-γ levels associated with pulmonary hypertension. After adjusting for gender and age, IL-17 levels remained independently associated with some clinical manifestations of Ssc patients (telangiectasia and high activity score of Ssc).

Conclusion. Th17 and Th1 cell responses are active in Ssc patients as their cytokines associated with higher disease activity scores and pulmonary manifestations. Th17 and Th1 specific activity and homing within Ssc patients still needs to be defined and determined in order to target them as potential future therapeutic targets in Ssc patients.

Key words: IL-17, systemic sclerosis, IFN-γ, T helper 17, T helper 1.

1. INTRODUCTION

Systemic sclerosis (Ssc) is an autoimmune disease characterized by cutaneous and visceral fibrosis. Its pathogenesis is incompletely understood and efforts are made in order to better define implication of the immune system. T helper (Th) cells are key regulators of the immune response and they seem to be involved in Ssc clinical manifestations [1]. There are mainly three sub-classes of Th cells that were demonstrated to be involved in autoimmunity: Th1, Th2 and Th17. Th1 secretes mainly IFN-γ .Th2 secretes IL-4, IL-5, IL-6 and IL-13. Th17 secretes mainly IL-17A and IL-17F [2]. Th 17 cells have already been demonstrated to be involved in various autoimmune diseases like rheumatoid arthritis [3] and systemic lupus erythematosus [4, 5]. Th1 and Th2 have already been demonstrated to be involved in Ssc pathogenesis *via* dermal fibroblasts [6].

Therefore, the aim of the study is to determine key cytokines secreted by Th1 (IFN-γ), Th2 (IL-6) and Th17 (IL-17) in Ssc patients and correlate them with specific manifestations of Ssc patients.

2. MATERIALS AND METHODS

SYSTEMIC SCLEROSIS PATIENTS AND CONTROLS

35 consecutive patients (32 females, 3 males, 55.60 ± 11.6 years) and 20 age and sex matched controls (18 females, 2 males, 52 ± 14.5 years) from "Colentina" Clinical Hospital (Internal Medicine Clinic) between January 2013 and December 2014 were enrolled. Ssc patients were diagnosed according to the classification criteria proposed by the American College of Rheumatology (ACR) [7]. All patients were clinically examined and evaluated for visceral involvement. Interstitial lung disease was evaluated with chest radiography and high resolution CT. Respiratory function tests (flow spirometry and diffusing capacity for carbon monoxide-DLCO) were performed. Pulmonary hypertension was determined indirectly by trans-

DOI: 10.1515/rjim-2015-0006

thoracic echocardiography (values above 35 mm Hg) [8]. Cutaneous involvement was determined in all patients by assessing presence/absence and frequency of Raynaud's phenomenon, calculation of modified Rodnan skin score [9], presence/absence assessment of telangiectasias, presence/absence of ischemic digital ulcers (defined by loss in ephitelialisation and tissue that involved subcutaneous tissue), presence/absence of calcinosis (defined by calcium deposits in soft tissues that were visible and confirmed by the x-ray) [10]. Frequency of Raynaud's phenomenon was defined as daily or less frequent. Disease activity score was assessed using European League Against Rheumatism (EULAR) Scleroderma Trial and Research group criteria (EUSTAR criteria) [11]. In Ssc patients, erythrocyte sedimentation rate and C reactive protein levels were determined. Also therapy that had already been initiated in Ssc patients was recorded at the moment of blood sampling. All patients and controls signed an informed consent according to the Declaration of Helsinki and the study was approved by the local ethics committee.

SERUM CYTOKINE ANALYSIS IN PATIENTS AND CONTROLS

Blood samples were centrifuged for 20 minutes at 4000 rpm and the serum stored at -80°C for further analysis. Antinuclear antibodies (ANA) were detected by indirect immunofluorescence. Commercial enzyme linked immunosorbent assay (ELISA) kits were used according to manufacurer's instruction to determine anti-Scl70 and anti-centromere antibodies (Euro Diagnostica, Malmo, Sweden). IL-17, IL-6 and IFN-γ levels were determined using commercial ELISA kits according to manufacturers' instructions (R&D Systems, Minneapolis, USA). All samples were evaluated in duplicate. Mean optical density at 450 nm read on a plate reader (BioRad Hercules, CA, SUA) was determined for each sample.

STATISTICAL ANALYSIS

Continuous variables were presented as mean ± standard deviation if they were normally distributed or as median and minimum and maximum value if they were non-normally distributed. To test normality of the continuous variables, Kolmogorov-Smirnov tests were applied. Fischer's exact tests were used to determine whether two categorical variables are associated. Difference in serum concentration markers among groups was performed using Mann-Whitney U test. Spearman's rank correlation coefficient was used in order to assess a linear relationship among the continuous variables. Logistic regression was used to compare serum cytokine levels after adjustment for gender and age. A p value ≤ 0.05 was considered significant. Statistical analysis was performed using the Statistical Software for Social Sciences (SPSS), Version 16 for Windows (Chicago, USA).

3. RESULTS

35 Ssc patients and 20 age and sex-matched controls were recruited. Data about patients are summarized in Table 1. Serum IL-17 and IL-6 levels were not significantly different in Ssc patients and controls (IL-17 median value for SSc patients: 0 pg/mL minimum 0 pg/ml-maximum 18.4 pg/mL *versus* controls median value 0 pg/mL minimum 0 pg/mL maximum 1.41 pg/mL, Figure 1 and IL-6 median value for Ssc patients 1.15 pg/mL, minimum 0.73 pg/mL-maximum 2.70 pg/mL *versus* median value for serum IL-6 in controls 1.25 pg/mL, minimum 1.11 pg/mL maximum 1.46 pg/mL, Figure 2). However, serum IFN-γ levels were higher in Ssc patients when compared to controls (IFN-γ for SSc patients: 3.68 pg/mL minimum 0 pg/ml-maximum 17.60 pg/mL *versus* controls median value 0.64 pg/mL minimum 0 pg/mL maximum 4.76 pg/mL, Figure 3).

Table I
Demographic and clinical data of Ssc patients. N/A-not applicable

	Controls (n = 20)	**Systemic sclerosis patients (n = 35)**
Age (years)	52 ± 14.5	55.60 ± 11.6
Gender: Female/Male	18/2	32/3
Limited subtype (n, %)	N/A	15 (42.9%)
Diffuse subtype (n, %)	N/A	20 (57.1%)
Disease duration (median and min-max values, months)	N/A	78 (5-240)
EUSTAR score (median and min-max values)	N/A	3 (0.5-8)
Digital ulcers (n, %)	N/A	9 (25.7%)
Calcinosis (n, %)	N/A	8 (22.9%)
Telangiectasias (n, %)	N/A	23 (65.7%)
Pulmonary hypertension (n, %)	N/A	6 (17.1%)

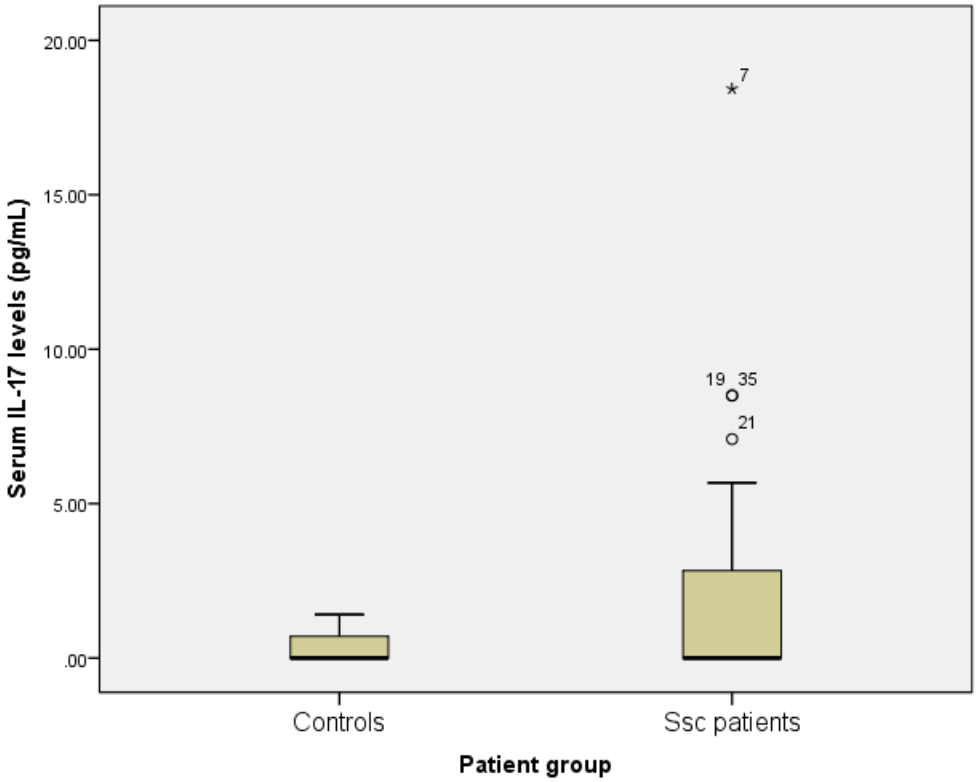

Figure 1. Serum IL-17 levels in SSc patients and controls. There were no statistically significant differences (p = 0.24, Mann Whitney U test).

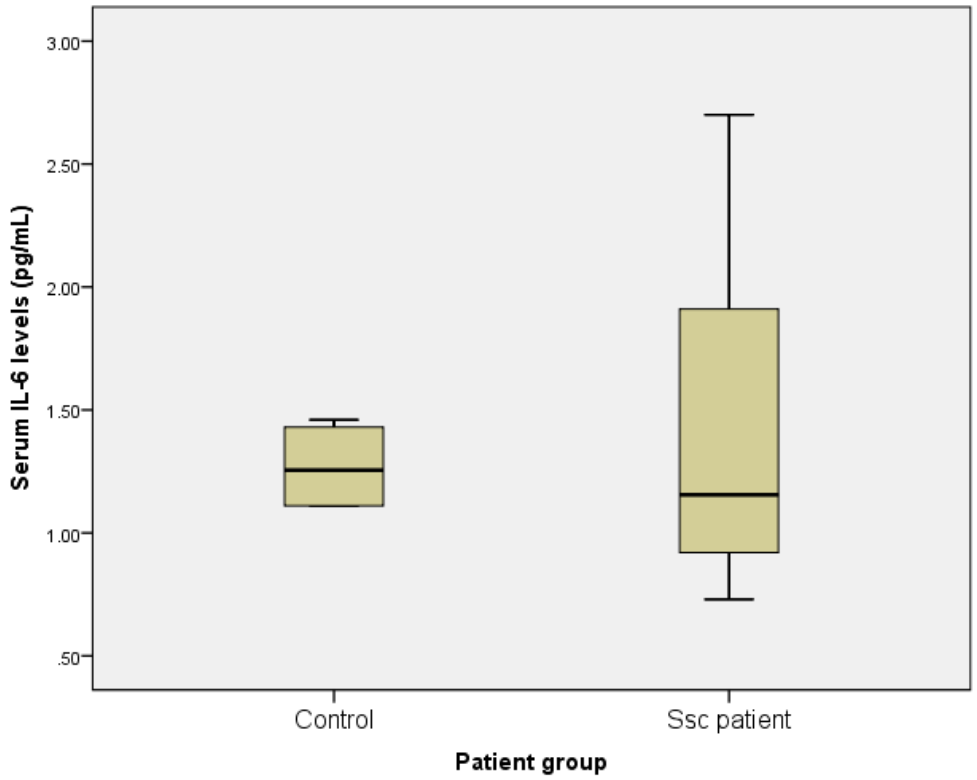

Figure 2. Serum IL-6 levels in Ssc patients and controls. There were no statistically significant differences (p = 0.70, Mann Whitney U test).

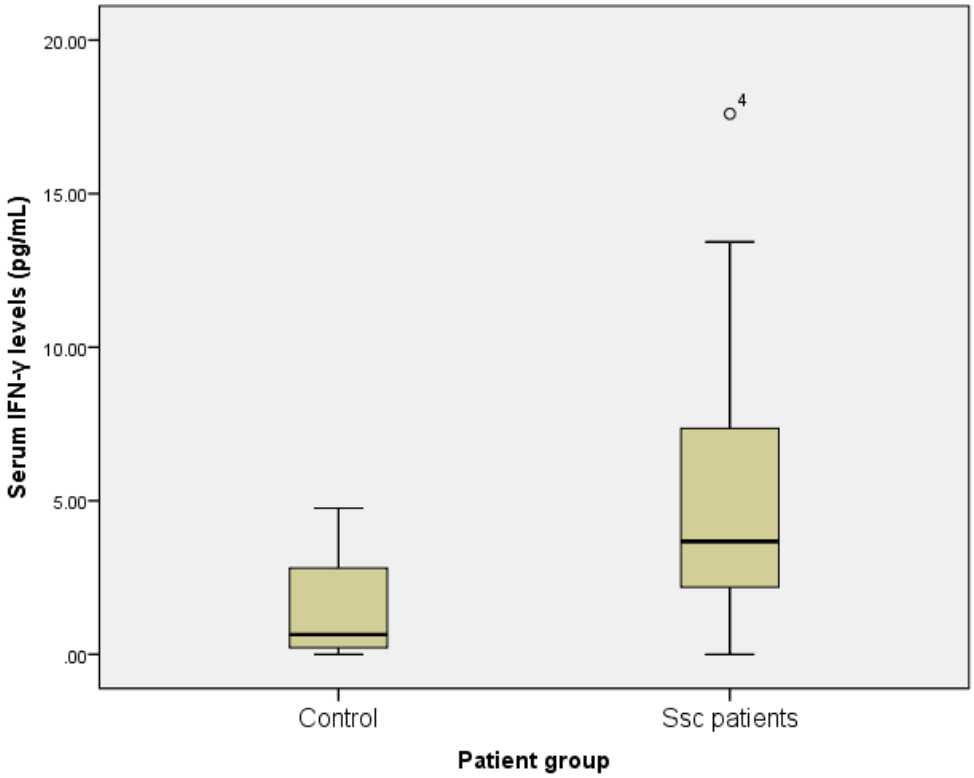

Figure 3. Serum IFN-γ levels in Ssc patients and controls. Higher levels were found in Ssc patients (p = 0.001, Mann Whitney U test).

When associating clinical manifestations within the Ssc group with serum cytokine levels, patients with digital ulcers had lower IL-6 levels compared to patients without digital ulcers (median IL-6 levels 0.92 pg/mL, minimum 0.76 pg/mL- maximum 1.71 pg/mL *versus* median 1.30 pg/mL minimum 0.73 pg/mL-maximum 2.70 pg/mL, p = 0.03, Mann Whitney U test). Patients with telangectasia had higher serum IL-17 levels compared to patients without telangiectasia (median 2.12 pg/mL minimum 0 pg/mL-maximum 18.43 pg/mL *versus* median 0 pg/mL minimum 0 pg/mL maximum 18.43 pg/mL, p = 0.023, Mann whitney U test). Patients with calcinosis also had lower serum IL-6 levels compared to patients without (median IL-6 levels 0.96 pg/mL minimum 0.73 pg/mL-maximum 1.71 pg/mL *versus* median 1.25 pg/mL minimum 0.76 pg/mL-maximum 2.70 pg/mL, p = 0.046, Mann Whitney U test). Patients with pulmonary hypertension also had higher IFN-γ levels compared to Ssc patients without pulmonary hypertension (median IFN-γ levels 8.80 pg/mL minimum 5.2 pg/mL-maximum 17.6 pg/mL *versus* median 3.03 pg/mL minimum 0 pg/mL maximum 9.96 pg/mL, p < 0.001, Mann Whitney U test). Interestingly, patients with active disease (EUSTAR score ≥3) had higher IL-17 serum levels compared to Ssc patients with inactive disease (median IL-17 levels 1.41 pg/mL minimum 0 pg/mL-maximum 18.43 pg/mL *versus* median 0 pg/mL minimum 0 pg/mL-maximum 5.67 pg/mL).

After performing logistic regression to control for age and gender of Ssc patients, IL-17 levels remained independently associated with clinical manifestations of Ssc patients (telangiectasia and active systemic sclerosis, Table 2).

DLCO values negatively correlated with IFN-γ levels (r = -0.57, p=0.001) and values of the mean pulmonary artery pressure positively correlated with levels of IFN-γ (r = 0.48, p = 0.006).

Table II
Results of logistic regression after adjusting for age and gender

Ssc clinical manifestation	Cytokine	Odds Ratio and 95% confidence interval	R^2	p-value
EUSTAR score ≥ 3	IL-17	2.06 (1.04-4.2)	0.51	0.043
Telangectasia	IL-17	4.53 (1.14-17.96)	0.67	0.034

4. DISCUSSION

Only serum levels of IFN-γ were higher in Ssc patients compared to controls. However, IL-17 serum levels independently associated with higher disease activity scores and presence of telangiectasia. Data about IL-17 levels in Ssc patients are conflicting in the literature. On the one hand there are studies that demonstrate that serum IL-17 were lower in Ssc patients compared to controls [12] and, on the other hand, there are reports about higher IL-17 levels in Ssc patients [13]. In this study there was no statistically significant difference between Ssc patients and controls. However, it was shown that IL-17 levels would differentiate subsets of disease (more active disease, patients with telangiectasia). In addition, recent reports suggested that Th17 cells are present in Ssc skin and associated with modified Rodnan skin score [14]. In this study there was no correlation between serum IL-17 levels and modified Rodnan skin score and therefore IL-17 is not a potential biomarker for skin score assessment. This is most probably due to the polymorphic aspects of Ssc disease and multiple visceral involvements, with multiple sources for IL-17 synthesis. There were no differences in serum IL-6, IFN-γ and IL-17 between diffuse and localized subsets of Ssc. Given all this growing body of evidence, it is more and more clear that Ssc is also a Th17 mediated disease as its main cytokine is implicated in more severe Ssc forms.

Even if lower IL-6 levels did associate in univariate analysis with calcinosis and digital ulcers, IL-6 did not remain independently associated with these clinical features when adjusting to age and gender. Levels of IL-6 were relatively low, and one possible explanation is that most patients were already treated with steroids. Other studies demonstrated that serum levels of IL-6 were upregulated in Ssc patients [15] and correlated with pulmonary disease and pulmonary hypertension.

IFN-γ levels were higher in patients with arterial pulmonary hypertension and positively correlated with mean systolic pulmonary arterial pressure and negatively correlated with DLCO. These findings suggest that Th1 cell driven response is involved in pulmonary manifestations of Ssc. A previous study discussed about classification of Ssc diseases subset with the aid of Th17 cells and IFN-γ. The study suggested that limited subtype of disease is associated with high expression of IL-17 and IFN-γ [16]. In the present study, IL-17 and IFN-γ clustered a group of patients that had a higher disease activity score and pulmonary hypertension. Therefore, by simultaneously assessing IL-17 and IFN-γ, one could identify patients with high clinical score and pulmonary hypertension. It is more and more obvious that multiple cytokines need to be measured in Ssc patients in order to better define disease subgroups.

5. CONCLUSION

Th17 and Th1 cell responses are active in Ssc patients as their cytokines associated with higher disease activity scores and pulmonary manifestations. Th17 and Th1 specific activity and homing within Ssc patients still needs to be defined and determined in order to target them as potential future therapeutic targets.

Acknowledgement. This paper is supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/137390

Corresponding author: Paul Bălănescu, Colentina Clinical Hospital, CDPC Clinical Immunology Department,
19-21 Ştefan cel Mare street, Bucharest, Romania
Telephone/fax: 00403216259
E-mail plbalanescu@gmail.com

REFERENCES

1. BREMBILLA NC, CHIZZOLINI C. *T cell abnormalities in systemic sclerosis with a focus on Th17 cells*. Eur Cytokine Netw. 2012; 23(4):128-39.
2. ZHU J, PAUL WE. *CD4 T cells: fates, functions, and faults*. Blood. 2008; 112(5):1557-69.
3. PAVLOVIC V, DIMIC A, MILENKOVIC S, KRTINIC D. *Serum levels of IL-17, IL-4, and INFγ in Serbian patients with early rheumatoid arthritis*. J Res Med Sci. 2014; 19(1):18-22.
4. BĂLĂNESCU P, BĂLĂNESCU E, TĂNĂSESCU C, NICOLAU A, TĂNĂSESCU R, GRANCEA C, VAGU C, RUȚĂ S, BLEOȚU C. *T helper 17 cell population in lupus erythematosus*. Rom J Intern Med. 2010; 48(3):255-9.
5. TANASESCU C, BALANESCU E, BALANESCU P, OLTEANU R, BADEA C, GRANCEA C, VAGU C, BLEOTU C, ARDELEANU C, GEORGESCU A. *IL-17 in cutaneous lupus erythematosus*. Eur J Intern Med. 2010; 21(3):202-7.
6. CHIZZOLINI C, PAREL Y, SCHEJA A, DAYER JM. *Polarized subsets of human T-helper cells induce distinct patterns of chemokine production by normal and systemic sclerosis dermal fibroblasts*. Arthritis Res Ther. 2006; 8(1):R10.
7. Subcommittee for scleroderma criteria of the American Rheumatism Association diagnostic and therapeutic criteria committee: Preliminary criteria for the classification of systemic sclerosis (scleroderma). Arthritis Rheum 1980; 23:581-90.
8. GALIE N, HOEPER MM, HUMBERT M, TORBICKI A, VACHIERY JL, BARBERA JA, BEGHETTI
9. M, CORRIS P, GAINE S, GIBBS JS, GOMEZ-SANCHEZ MA, JONDEAU G, KLEPETKO W, OPITZ C, PEACOCK A, RUBIN L, ZELLWEGER M, SIMONNEAU G, ESC Committee for Practice. *Guidelines for the diagnosis and treatment of pulmonary hypertension: the task force for the diagnosis and treatment of pulmonary hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT)*. Eur Heart J 2009; 30:2493-2537.
10. CLEMENTS P , LACHENBRUCH P, SEIBOLD J, WHITE B, WEINER S, MARTIN R, WEINSTEIN A, WEISMAN M, MAYES M, COLLIER D. *Inter- and intraobserver variability of the total thickness score (modified Rodnan TSS) in systemic sclerosis*. J Rheumatol 1995; 22:1281-5.
11. AMANZI L, BRASCHI F, FIORI G, GALLUCCIO F, MINIATI I, GUIDUCCI S, CONFORTI ML, KALOUDI O, NACCI F, SACU O, CANDELIERI A, PIGNONE A, RASERO L, CONFORTI D,MATUCCI-CERINIC M. *Digital ulcers in scleroderma: staging, characteristics and sub-setting through observation of 1614 digital lesions*. Rheumatolgy (Oxford) 2010; 49:1374-82.
12. VALENTINI G, D'ANGELO S, DELLA ROSSA A, BENCIVELLI W, BOMBARDIERI S. *European Scleroderma Study Group to define disease activity criteria for systemic sclerosis. IV. Assessment of skin thickening by modified Rodnan skin score*. Ann Rheum Dis. 2003; 62(9):904-5.
13. OLEWICZ-GAWLIK A, DANCZAK-PAZDROWSKA A, KUZNAR-KAMINSKA B, GORNOWICZ-POROWSKA J, KATULSKA K, TRZYBULSKA D, BATURA-GABRYEL H, SILNY W, POPLAWSKI D, HRYCAJ P. *Interleukin-17 and interleukin-23: importance in the pathogenesis of lung impairment in patients with systemic sclerosis*. Int J Rheum Dis. 2014; 17(6):664-70.
14. MURATA M, FUJIMOTO M, MATSUSHITA T, HAMAGUCHI Y, HASEGAWA M, TAKEHARA K, KOMURA K, SATO S. *Clinical association of serum interleukin-17 levels in systemic sclerosis: is systemic sclerosis a Th17 disease?* J Dermatol Sci. 2008; 50(3):240-2.
15. ZHOU Y, HOU W, XU K, HAN D, JIANG C, MOU K, LI Y, MENG L, LU S. *The elevated expression of Th17-related cytokines and receptors is associated with skin lesion severity in early systemic sclerosis*. Hum Immunol. 2014 Dec 11. pii: S0198-8859(14)00508-4.
16. GOURH P, ARNETT FC, ASSASSI S, TAN FK, HUANG M, DIEKMAN L, MAYES MD, REVEILLE JD, AGARWAL SK. *Plasma cytokine profiles in systemic sclerosis: associations with autoantibody subsets and clinical manifestations*. Arthritis Res Ther. 2009; 11(5):R147.
17. RADSTAKE TR, VAN BON L, BROEN J, HUSSIANI A, HESSELSTRAND R, WUTTGE DM, DENG Y, SIMMS R, LUBBERTS E, LAFYATIS R. *The pronounced Th17 profile in systemic sclerosis (SSc) together with intracellular expression of TGFbeta and IFNgamma distinguishes SSc phenotypes*. PLoS One. 2009 Jun 17; 4(6):e5903.

Received February 10, 2015