

## Serum interleukin-17 & nitric oxide levels in patients with primary Sjögren`s syndrome

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Received June 1, 2010

**Background & objectives:** The interleukin (IL)-17 producing T-helper cells have been linked to pathogenesis of autoimmunity and mostly investigated in rheumatoid arthritis (RA). In this study we tested the IL-17 levels, as well as the levels of nitric oxide (NO) as possible IL-17-induced product, in patients with primary Sjögren`s syndrome (pSS), an intricate and complex chronic autoimmune disorder of exocrine glands.

**Methods:** Serum IL-17 levels and nitrite concentrations determined in patients with pSS (n=30) were compared with the values obtained in patients with RA (n=10) and healthy controls (n=15). The values obtained for IL-17 in pSS patients were also associated with the patients` clinical characteristics, particularly the rheumatoid factor (RF) and total antinuclear antibodies (tANA) levels.

**Results:** Serum concentrations of IL-17 were significantly ( $P<0.01$ ) higher in patients with pSS ( $12.9 \pm 28.0$  pg/ml) as compared to those obtained in healthy individuals ( $0.2 \pm 0.6$  pg/ml), but not as high as the values obtained for the patients with RA ( $34.5 \pm 56.2$  pg/ml). The mean IL-17 levels were significantly ( $P<0.05$ ) higher in the pSS patients positive for rheumatoid factor ( $20.3 \pm 33.3$  pg/ml) than in RF-negatives ( $0.3 \pm 0.6$  pg/ml). Mean serum concentrations of IL-17 were also higher in antinuclear antibody (ANA)-positive samples ( $19.8 \pm 33.5$  pg/ml) in comparison to ANA-negative sera ( $1.1 \pm 3.1$  pg/ml) ( $P<0.05$ ). The NO levels also showed elevated values in both pSS and RA patients, as compared to the healthy controls, since mean nitrite levels in patients with pSS and RA were  $38.2 \pm 29.2$   $\mu$ M and  $41.7 \pm 21.1$   $\mu$ M, respectively, while those in healthy controls were significantly lower, at  $19.2 \pm 10.5$   $\mu$ M.

**Interpretation & conclusions:** The findings of this study showed that there was increased IL-17 and NO production in patients with primary SS, especially if they had associated elevated rheumatoid factor and antinuclear antibody values.

**Key words** Interleukin-17 - nitric oxide - primary Sjögren`s syndrome - rheumatoid arthritis

Interleukin-17 (IL-17) and its receptor are members of emerging cytokine family involved in pathogenesis of inflammation and host defense<sup>1</sup>, as IL-17 exhibits proinflammatory activity and appears to have a role in

regulation of granulopoiesis and erythropoiesis<sup>2,3</sup>. Also, newly recognized subset of IL-17-secreting T (Th17) cells has been identified and linked to pathogenesis of autoimmunity<sup>4</sup>. The significance of IL-17 was

mostly investigated in the pathology of rheumatoid arthritis and other T cell dependent tissue damages<sup>5</sup>. A hallmark feature of IL-17 is its ability to induce the production of a number of inflammatory mediators and in particular nitric oxide (NO) by stimulated chondrocytes, osteoblasts, fibroblast, endothelial cells and astrocytes<sup>6</sup>. NO itself is well known free radical implicated in cytotoxic tissue injury in a variety of diseases and its increased production has been linked to pathogenesis of autoimmune and inflammatory diseases<sup>7,8</sup>.

Sjögren's syndrome (SS) is a chronic autoimmune disorder of exocrine glands characterized as an autoimmune exocrinopathy, and there are limited reports concerning the levels, as well as the role, of both IL-17 and NO in the in pathogenesis of primary Sjögren's syndrome. Increased NO production has been previously reported in patients with SS, especially if they had associated arthritis<sup>9</sup>. Recently, the possible role of systemic and local IL-17 was associated with primary Sjögren's syndrome (pSS)<sup>10</sup>. Cytokines as important component in pathogenesis are considered as potential targets in pSS<sup>11,12</sup>. Due to complex pathogenesis of pSS, heterogeneity of disease and for determination of new potential therapeutic targets the role of IL-17 should be further elucidated<sup>13</sup>. In order to gain more data on the presence and the influence of IL-17 in the pathology of pSS, we investigated the levels of IL-17 and NO in patients with pSS and compared the values with those obtained in patients with rheumatoid arthritis (RA) and in healthy controls. Additionally, the IL-17 levels in the pSS patients were associated with their clinical characteristics, particularly the rheumatoid factors (RF) and total antinuclear antibodies (tANA) levels.

### Material & Methods

**Patients:** The study was conducted at the Institute of Rheumatology, Belgrade, Serbia, and 40 patients [30 with primary Sjögren's syndrome (pSS) and 10 with rheumatoid arthritis (RA)] were included with a written consent, during one year period (January to December 2004). The diagnosis of pSS was made according to the European classification criteria<sup>14</sup> since the average duration of disease of patients was 10 years. The clinical samples were formed in a such manner that each patient who met the entry criteria of the study during the research period was included in the corresponding group. Randomization of the samples was a result of indeterminable patients reporting to the clinic. All patients were women, mean age  $55.2 \pm 10.1$

yr with the mean duration of the disease  $10.6 \pm 5.6$  yr. The patients with pSS were on substitution therapy for dry eyes and mouth, four of them only on substitution, 25 were treated with glycocorticoids, in addition, 11 of them with chloroquine, five with azathioprine, one with cyclophosphamide. Seven patients with RA were treated with nonsteroid anti inflammatory drugs and monotherapy with disease modifying anti-rheumatic drug (Methotrexate, Salazopyrine, Hloroquine, Azathioprine), and three were on low doses of prednisolone (5-7.5 mg/day). The blood samples (10 ml) were drawn from patients and serum was separated and investigated by routine clinical analyses for the presence of rheumatoid factor (RF) by the nephelometric test (The Binding site, Birmingham, UK), total antinuclear antibodies (tANA) by an indirect immunofluorescent test<sup>15</sup> and antibodies against SS-A and SS-B by ELISA test (Varelisa, Pharmaciadiagnostics, Freiburg, Germany). Extraglandular manifestations occurred in all pSS patients studied.

RA patients fulfilling the revised criteria of the American College of Rheumatology were included<sup>16</sup>. The mean age of RA patients (all women) was  $60 \pm 13.8$  yr and the mean duration of the disease  $8.4 \pm 7.7$  yr; 80 per cent of RA patients had extra-articular manifestations.

Fifteen healthy volunteers (13 female and 2 male), after written consent obtained, were studied as controls and their mean age was  $49 \pm 11.1$  yr. Blood samples were drawn at the Institute of Rheumatology, Belgrade. Serum was separated immediately and stored at  $-20^{\circ}\text{C}$  until analysis. The study was approved by the Committee of Ethics, Institute of Rheumatology, Belgrade.

**Enzyme linked immunosorbent assay (ELISA) for human IL-17:** Serum IL-17 levels were determined in duplicate by ELISA kit for human IL-17 (sensitivity-2 pg/ml) purchased from BioSource Europe S.A. (BioSource IL-17 cytoscreen kit, Belgium), following manufacturer's instruction.

**Nitric oxide determination:** Production of NO was determined by measuring accumulation of nitrite in serum using the Griess reaction with sodium nitrate as the standard. Briefly, 50  $\mu\text{L}$  samples of serum were mixed with equal volumes of 1 per cent sulphanilamide and 0, 1 per cent N-1-naphthylethylene diamine-dihydrochloride in 0.5 per cent  $\text{H}_3\text{PO}_4$ . After 10 min at room temperature, the absorbance at 540 nm was measured in a microplate reader.

**Statistical analyses:** Descriptive statistical methods were used. Data were not normally distributed, as tested by one-sample Kolmogorov-Smirnov test (KSZ). Therefore, non-parametric tests were used, Kruskal-Wallis chi-squared (KW  $\chi^2$ ) for differences between three groups, and Mann-Whitney test (U) for inter-group differences.  $P < 0.05$  was considered significant.

### Results

The clinical and laboratory characteristics of the patients with pSS and RA are shown in the Table. The most common extraglandular manifestations in patients with pSS were dermal changes (70%), Raynaud's phenomenon (63.3%), sensitive neuropathy (53.3%), arthralgia and arthritis (50%), and haematological manifestations (40%). In these patients RF and ANA were present in 19 (around 63%) patients, while anti-SS-A and anti-SS-B in 18 (60%) and 12 (40%) patients, respectively. In two patients with RA, rheumatic nodules as extra-articular manifestations were found.

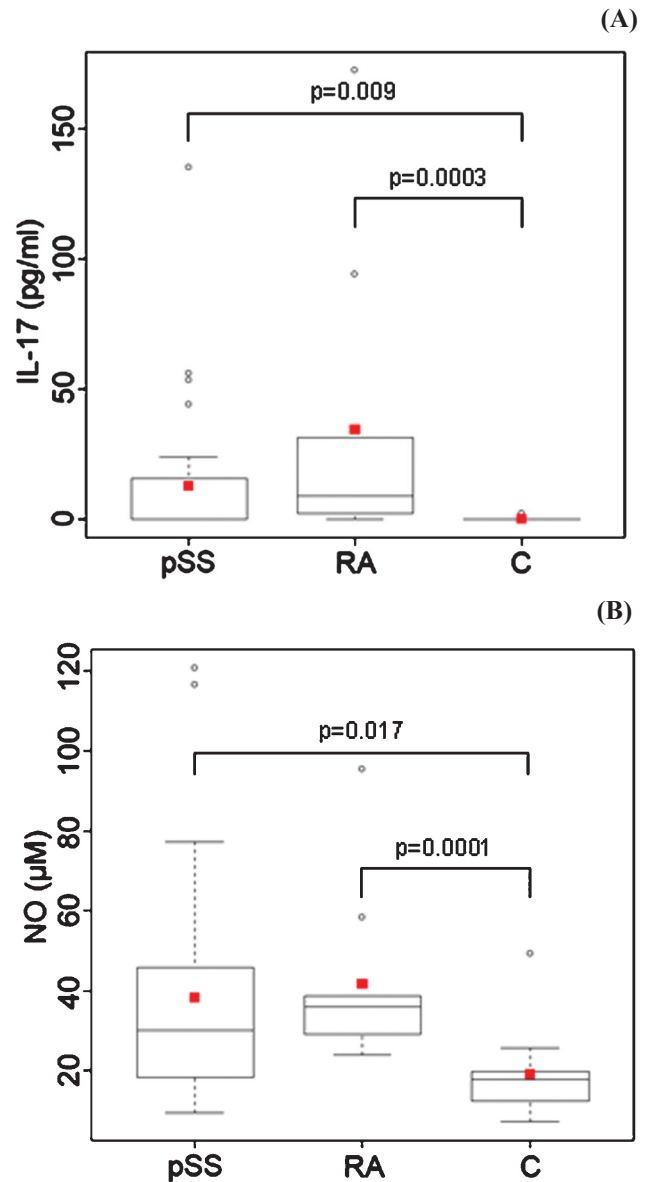
Serum IL-17 levels were significantly elevated in patients with pSS as compared to the healthy individuals ( $12.9 \pm 28.0$  vs.  $0.2 \pm 0.6$  pg/ml,  $P < 0.01$ ). Patients with RA also had significantly elevated serum levels of IL-17 when compared to the controls ( $34.5 \pm 56.2$  vs.  $0.2 \pm 0.6$  pg/ml,  $P < 0.001$ ) (Fig. 1A). Though the mean serum IL-17 levels were higher in patients with RA than in the patients with pSS, the difference was not statistically significant.

The NO levels levels in patients with pSS and RA were significantly higher than those in healthy

**Table.** Clinical and laboratory characteristics of patients with primary Sjögren's syndrome (pSS) and rheumatoid arthritis (RA)

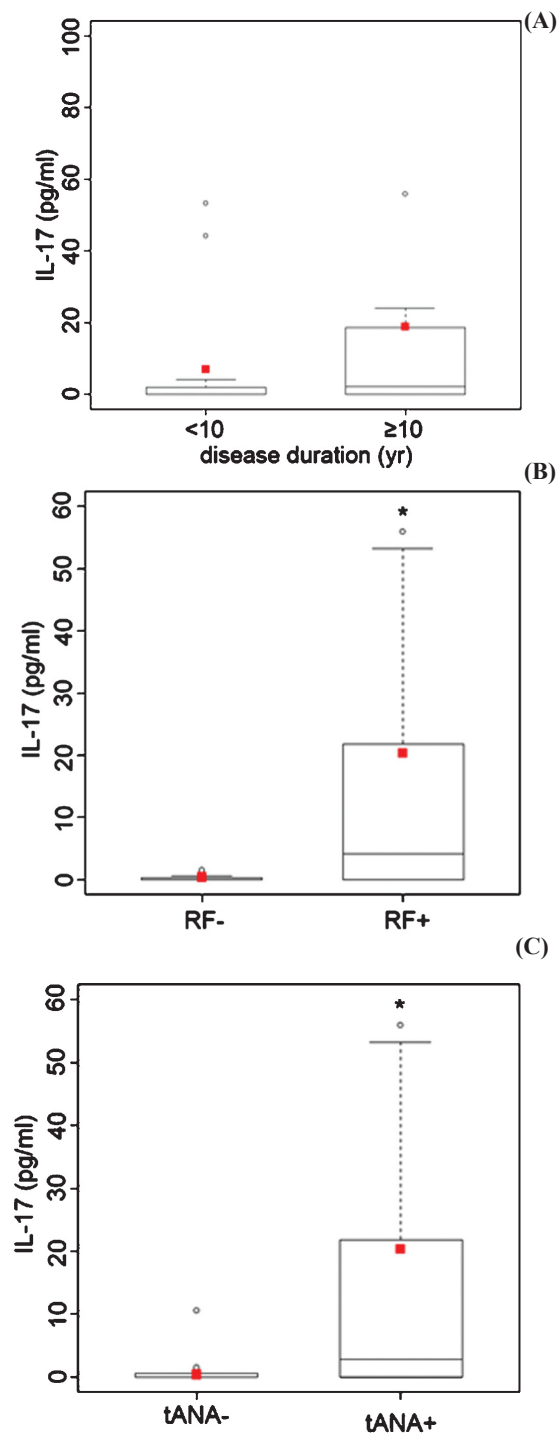
Parameter	pSS (n=30)	RA (n=10)
Leucocytes ( $\times 10^9$ )	$5.6 \pm 1.8$	$7.3 \pm 2.4$
Hemoglobin (g/l)	$119 \pm 1.4$	$10.1 \pm 1.3$
Thrombocytes ( $\times 10^9$ )	$223.3 \pm 76.9$	$294.8 \pm 98.1$
Erythrocyte sedimentation rate (mm/h)	$31.3 \pm 19.1$	$66 \pm 25.9$
Extraglandular manifestations	30 (100)	-
Extra-articular manifestations	-	2 (20)
RF positive	19 (63.3)	9 (90)
ANA positive	19 (63.3)	5 (50)
Anti-SS-A positive	18 (60.0)	-
Anti-SS-B positive	12 (40.0)	-

Values are expressed as mean  $\pm$  SD, values in parentheses are the percentages; RF, rheumatoid factor; ANA, antinuclear antibody; anti-SS-A (Ro) antibodies, anti-SS-B (La) antibodies



**Fig. 1.** IL-17 (A) and nitrite (B) serum concentrations in patients with pSS (n=30), patients with RA (n=10) and healthy controls - C (n=15). Squares (red) represent mean values, the median is shown as horizontal line within the box. The lower and upper margins of the box represent 25<sup>th</sup> and 75<sup>th</sup> percentiles, with the extended arms representing the highest and lowest values. Differences in serum levels between patients' groups and the control group were analyzed by Mann-Whitney U test.

individuals (Fig. 1B). Mean nitrite concentrations in patients with pSS were  $38.2 \pm 29.2$   $\mu$ M, while those in healthy controls were significantly lower, at  $19.2 \pm 10.5$   $\mu$ M ( $P < 0.05$ ). In patients with RA, mean NO levels were  $41.7 \pm 21.1$   $\mu$ M, which was also significantly higher in comparison to the control group ( $P < 0.001$ ).



**Fig. 2.** IL-17 concentration in serum of the patients with pSS (n=30). The IL-17 levels were associated with (A) the disease duration, (B) between the RF-negative and RF-positive patients, and (C) between the tANA-negative and tANA-positives. Squares represent mean values, the median is shown as horizontal line within the box. The lower and upper margins of the box represent 25<sup>th</sup> and 75<sup>th</sup> percentiles, with the extended arms representing the highest and lowest values. Differences in IL-17 levels between the tested groups were analyzed by Mann-Whitney U test (\* $P < 0.05$ ).

The possible association between the elevated IL-17 levels in pSS patients was studied with their clinical characteristics, especially the duration of the disease and the presence of rheumatoid factors (RF) and total antinuclear antibodies (tANA) levels. The mean serum IL-17 concentrations were higher in patients with pSS disease duration lasting longer than 10 years when compared to those with disease duration < 10 yr. The difference was not statistically significant. The results also demonstrated significantly higher IL-17 concentrations in RF-positive than in RF-negative patients ( $P < 0.05$ ) (Fig. 2B), as well as in ANA-positive in comparison to ANA-negative patients ( $P < 0.05$ ) (Fig. 2C).

No differences in the mean concentrations of IL-17 were found between the anti-SS-A positive and anti-SS-A negative patients ( $13.6 \pm 18.7$  vs.  $15.4 \pm 44.9$  pg/ml), nor between the anti-SS-B positive and anti-SS-B negatives ( $13.6 \pm 18.5$  vs.  $12.5 \pm 33.4$  pg/ml). Although no relation between presence of anti-SS-A, anti-SS-B antibodies and elevated IL-17 concentrations was found, the values of anti-SS-A antibodies in patients (n=11) with elevated IL-17 concentration were considerably higher than in those with normal (n=19) IL-17 values ( $72.3 \pm 54.7$  vs.  $37.7 \pm 37.5$  U/ml,  $P < 0.05$ ).

## Discussion

This study demonstrated elevated IL-17 and NO levels in the circulation of the patients with pSS, which is in agreement with reported data<sup>9,10,17,18</sup>. The elevated IL-17 levels in pSS patients are most probably a reflection of the systemic response to the inflammation, like often seen in autoimmune diseases. Proinflammatory effects of IL-17 were clearly demonstrated in various autoimmune diseases. Stimulation of its production and the release of inflammatory mediators from synovial fluid monocytes, synoviocytes and peripheral blood mononuclear cells<sup>5,19</sup>, as well as the additive and synergistic effects with interleukin-1 (IL-1) and tumour necrosis factor (TNF) in inducing joint pathology have been described in rheumatoid arthritis<sup>20</sup>. Similarly, the role of IL-17 as a crucial proinflammatory mediator was demonstrated in the pathogenesis of other autoimmune diseases, including multiple sclerosis<sup>21</sup>, systemic lupus erythematosus<sup>22</sup> and autoimmune encephalomyelitis<sup>23</sup>. IL-17 was, therefore, shown to be incorporated in cytokine network acting in tissue damage<sup>24</sup>. In patients with pSS, systemic levels of Th17-associated cytokines, including IL-17, significantly varied between different subgroups of

patients as related to the histopathological features<sup>25</sup>. Other studies showed the presence of IL-17 and other factors fostering Th17 lineage polarization, such as IL-23, TGF- $\beta$ , IL-6, in the local salivary gland milieu<sup>10,17</sup>, that correlated with the degree of inflammation and objective clinical evidence. These data pointed towards the important role of IL-17 in the immunopathogenesis of pSS and identified this cytokine as a potential therapeutic target. In a mouse model of pSS, blood levels of IL-17 were detected at early time points of the disease and were decreasing further, indicating that early induction of a CD4+ Th1/Th17 pathway leads to systemic release of IL-17<sup>17</sup>. However, based on the current knowledge, the question of when Th17 cells become involved in the autoimmune response and whether these act directly through secretion of inflammatory IL-17 family cytokines or by activating autoimmune T and B cells remains still to be defined. In our study, IL-17 was detected in the one third of our patients with pSS, and showed tendency to be higher in those patients with long disease duration (more than 10 yr), implying that the blood levels of this cytokine might be associated with natural progression of the SS disease. Doreau *et al*<sup>26</sup> have shown that IL-17 can influence the survival and proliferation of B cells, and their differentiation into immunoglobulin-secreting cells. It has been assumed that the activation of B cells is an important event in the pathogenesis of the disease<sup>27</sup>. The presence of autoantibodies in the circulation is one of the evidence supporting B-cell activation in pSS, and presence of RF and ANA in the serum of more than 60 per cent of our patients is in line with this assumption. As in these pSS patients, either RF-positive or ANA-positive, the average level of IL-17 was increased, a role of IL-17 in maintenance of disease activity could be supposed. Therefore, the consideration of the disease heterogeneity might be important for finding the most adequate therapeutic strategy.

In addition to IL-17, the results of this study revealed increased NO levels in the circulation of pSS patients, thus confirming that NO may contribute to inflammatory damage in pSS<sup>9</sup>. Several studies have established that the proinflammatory action of IL-17 depends considerably on its ability to induce inducible NOS (iNOS) and stimulates significant NO production in various cell types, including chondrocytes, astrocytes, fibroblasts and endothelial cells<sup>6</sup>. The possible role of NO in the pathogenesis of different systemic autoimmune disorders has also been indicated, since

elevated NO levels have been shown in pSS, SLE and RA patients<sup>8,28,29</sup>.

The functional outcome of the increased NO production in patients with pSS, as well as the mechanisms by which NO contributes to pathophysiology, are still not resolved. Recent data presented a role of the NO-mediated nitrosylation in downregulation of salivary function in a mouse model for SS<sup>30</sup>. However, other possible mechanisms of NO action are not excluded; such is its effect in triggering apoptosis, since in low concentrations NO may protect cells from apoptosis<sup>31</sup>, but at high concentrations, resulting from the activation of iNOS, NO can induce apoptosis<sup>32</sup>. As concerning SS apoptosis of the acinar and ductal epithelial cells of the salivary and lacrimal glands has been suggested as a possible mechanism responsible for the impairment of secretory function<sup>33</sup>. In addition, in SS patients increased apoptosis and increased iNOS expression were detected in salivary epithelial cells, after the exposure to inflammatory cytokines, such as INF-gamma<sup>34</sup>.

In our study, there was no relation between the NO production and the other clinical characteristics, such as the disease duration, rheumatoid factors or antinuclear antibody positivity, as well as with the elevated IL-17 concentrations (data not shown). However, one cannot assert the objectivity of such findings, bearing in mind that the majority of patients were under appropriate therapy and that steroids are known to suppress NO production<sup>35</sup>. As the NO is also known to be implicated in the enhancement of Th1 cell proliferation and function<sup>36</sup>, it will be of great interest for future work to investigate the impact of NO on the development and function of Th17 effector's T cells.

In summary, the present data demonstrated increased IL-17 and NO production in patients with primary SS. The elevated IL-17 levels were associated with elevated rheumatoid factors and antinuclear antibody values, confirming that IL-17 is an important cytokine linked to the immunopathogenesis in patients with pSS. But, since SS is a very intricate and complex chronic autoimmune disease, further studies are needed to determine the exact role of both IL-17 and NO, as well as their mutual link, in pSS disease pathogenesis.

#### Acknowledgment

This work was supported by a grant (#175062) from the Ministry of Science and Technological Development, Republic of Serbia.

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