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IL-17 patterns in synovium, serum and synovial fluid from treatment-naïve, early rheumatoid arthritis patients

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Abstract

Background: There are actually becoming controversial data regarding the profiles of interleukin-17 (IL-17) in different pathogenical stages of rheumatoid arthritis (RA). *Objectives*: To assessing the IL-17 patterns in synovium, serum and synovial fluid from treatment-naïve early RA patients and to identifying potential correlations with disease activity markers and with synovial histopathological profile. *Materials and Methods*: Serum samples from 30 treatment-naïve early RA patients were evaluated for C-reactive protein (CRP), erythrocytes sedimentation rate (ESR), rheumatoid factor (RF), anti-cyclic citrullinated peptide antibodies (anti-CCP). IL-17A levels were also assessed in serum and synovial fluid (SF). Disease activity score (DAS28) calculation was done for all patients. Control serum and SF samples were obtained from 29 patients with osteoarthritis (OA); control synovium specimens were obtained from eight patients with OA and during surgery for knee tear ligaments. Histopathological (Hp) score, immunohistochemical reactivity for IL-17 were also assessed in synovium of early RA patients and controls. Dependencies between serum and synovial profile of IL-17A and the other parameters were statistically tested. *Results*: In early RA patients, strong correlations of serum and SF IL-17A levels were found with ESR, CRP, RF, anti-CCP, Hp score and IL-17 synovial immunoreactivity; a good correlation was noted with DAS28 score. Also, strong correlation was noted between serum and SF IL-17A levels. *Conclusions*: In early stages of untreated RA, simultaneous IL-17 assessment of serum, SF and synovium might be valuable in defining activity and predictive patterns, given that synovium is highly suggestive for an disease aggressivity and might express specific therapeutically targets.

Keywords: interleukin-17, early rheumatoid arthritis.

Introduction

Rheumatoid arthritis (RA) is a complex, chronic autoimmune disease characterized by an inflammatory infiltrate of immune cells, in particular T-cells, which represent approximately 40% of the synovial cellular infiltration and participate in a number of inflammatory and destructive events, such as synovial hyperplasia, pannus setting, cartilage and bone erosion, and joint destruction [1–3]. Various cells from immune system and from synovium are involved and a panel of cytokines are produced, expressed and become functionally active even in early stages of RA [4–6].

RA was firstly recognized as a T-helper (Th)1-driven disease with a relative prevalence of interferon γ (IFNγ) and lack of Th2 cytokines, resulting in disease onset and perpetuation [7]. Both human RA and animal models of experimental arthritis demonstrates the critical role of pro-inflammatory CD4+ T-helper cells, in particular through cytokinic synthesis of IFNγ (Th1 cells), IL-17A (Th17 cells) and tumor necrosis factor alpha (TNF-α) (Th1 and Th17 cells, monocytes/ macrophages) [6, 8].

More evidences have proved the pathogenical role of Th17 cells and IL-17A in the structural damage of RA joints [9–11] by binding of IL-17 specific receptor, expressed on fibroblasts, endothelial and epithelial cells, IL-17 leads synthesis of several key-factors as TNF-α, IL-1β, IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), prostaglandin E2 and promotes neutrophil chemotaxis and granulocyte production [12, 13]. IL-17 also contributes to cartilage and bone destruction [11] by acting as potent inductor of the expression of matrix metalloproteinases, and receptor activator of nuclear factor kB ligand in rheumatoid synoviocytes and osteoblasts [14, 15], being an important promoter of osteoclastogenesis [16]. It is already demonstrated that in human RA, IL-17 plays a key role in the synergistic or additive effects expressed together with TNF-α and IL-1 [17, 18].

IL-17+ CD4+ T-cells were demonstrated in RA synovial tissue [19, 20], cells from RA bone and synovium can produce high levels of IL-17, in contrast with OA cells [21].

It is currently unclear why the observations regarding Th17 cell frequencies in RA are conflicting. A possible

explanation could be the different treatment regimes in the established RA patients, which could influence the profile of Th17 cells and IL-17.

Raza K *et al.* [22] detected increased levels of the Th2 cytokines interleukin-4 (IL-4) and IL-13, but not IFNγ, in synovial fluid samples from early RA patients. Recently, Colin EM *et al.* [23] demonstrated a high percentage of IL-17A-producing CCR6+ memory T-cells in peripheral blood mononuclear cell (PBMC) as well as the coexpression of the Th1 IFNγ and the Th17 IL-17 cytokines in human memory CD4+CD45+RO+ T-cells from treatment-naïve early RA patients.

More recent, Lubberts E [24] identified IL-17+ TNF+ Th17 cells in PBMC of treatment-naïve early RA patients. In addition, also the IL-17A expression was increased, which could be a valuable argue for the induction of a proinflammatory feedback loop as an essential mechanism for arthritis persistence.

In this study, we analyzed the profile of IL-17A in serum, synovial fluid and synovial tissue samples, obtained from treatment-naïve early RA patients. A consecutive analysis of IL-17 profile contribution in disease activity and prediction was done, through establishing correlations with disease activity score (DAS28), serum levels of anti-CCP (anti-cyclic citrullinated peptide antibodies), RF (rheumatoid factor), C-reactive protein (CRP) and with synovial histo-pathological pattern.

Materials and Methods

Patients and controls

Thirty patients (26 females and four males) with early rheumatoid arthritis (RA) (less than 12 months from the onset) diagnosed in the Department of Rheumatology from Clinical Railway Hospital Craiova were enrolled. All patients fulfilled the inclusion criteria – early rheumatoid arthritis (<12 months from onset), naïve for any synthetic and/or biological DMARDs (disease modifying anti-rheumatic drugs), and the exclusion criteria (obesity, diabetes mellitus, metabolic syndrome, endocrinopathies, glucocorticoid therapy). Another 29 age- and sex-matched patients with osteoarthritis (OA) were enrolled as controls, all of them fulfilling the same exclusion criteria.

Standard demographical – age, sex, BMI (body mass index) and clinical rheumatological evaluation was performed – morning stiffness, number of painful joints, number of swollen joints, global disease activity measured on a Visual Analogue Scale (VAS) of 100 mm, with data integration in DAS28 score. ESR, CRP, anti-CCP, rheumatoid factor, as well as standard biochemical assays (complete blood count, serum glucose, creatinine, lipidic profile) were assessed in serum samples collected from RA patients.

In order to assess the synovitis and to select the optimal regions for performing synovial biopsy, the power Doppler ultrasound (PDUS) exam were performed on selected joints of all RA patients using multi-frequency linear array transducers (7–13 MHz). The ultrasonographer was blinded to clinical data at the time of PDUS examination.

Synovial fluid and tissue samples from RA patients were collected through US-guide minimal biopsy, using a Parker Pearson Synovial Biopsy Needle (Dixons Surgical Instruments Ltd., UK), in aseptic conditions. Knee, MCPII (metacarpophalangeal of the second finger) and PIPII (proximal interphalangeal of the second finger) joints were more often chosen for biopsy. Synovial fluid specimens from OA patients were collected through knee arthrocentesis, performed under strict aseptic conditions. Control synovial tissue specimens were obtained during joint replacement surgery from four patients with OA, and from four patients that underwent surgery for knee tear ligaments.

All the procedures were followed in accordance with the ethical standards of the institutional responsible committees for human studies and with the Helsinki Declaration of 1975, as revised in 2008. All RA patients and controls gave their written informed consent prior to the study. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova.

Assessing chosen parameters from serum and synovial fluid samples

Blood samples from all RA patients and controls were collected into tubes without additives. Synovial fluid samples were obtained all the RA patients during minimal invasive biopsy; for OA controls, synovial fluid specimens were collected through knee arthrocentesis, performed under strict aseptic conditions. Serum samples from early RA patients and from OA controls were stocked at -70^{0}C and before testing frozen probes were brought to room temperature, avoiding freezing–unfreezing cycles. Synovial fluid was collected into non-heparinized tubes and spun within 30 minutes of collection at 1000 g for 10 minutes with selection of the acellular sections, which were stored at -70^{0}C before subsequent analysis.

Anti-CCP antibody and rheumatoid factor assay

IgG anti-CCP antibodies were detected using the anti-CCP assay (FEIA assay, Unicap 100 Phadia), seropositivity was defined as a titer ≥10 UI/mL. RF was measured using immunoturbidimetric assay, ROCHE COBAS 6000, Japan (seropositivity was defined as a titer ≥14 UI/mL).

Cytokinic assay

Serum and synovial fluid IL-17A levels of all RA patients and controls were determined using DRG Interleukin-17A (human) ELISA EIA–4840 (DRG International Inc, USA), a solid phase enzyme-linked immunosorbent assays based on the sandwich principle. ELISA kit was used according to the manufacturers' instructions.

Histopathological features assessment

The tissue specimens were fixed with 10% buffered formalin and embedded in paraffin wax. Subsequently were stained with Hematoxylin and Eosin for examination by light microscopy. Histopathological parameters of synovitis were evaluated in accordance

with established criteria (Koizumi F *et al.*, 1999) [25] and a histological score for each lesion was established according to references [26, 27].

The evaluation of the histological pattern has been achieved using a hybrid composite index defined as the sum of the scores corresponding to each analyzed parameter. Sections were analyzed and the histological features scored in blinded fashion (A.S.). This included blinding to the sequence of biopsies as well as patient identification. Inter-individual variation was checked with a second, blinded scorer (C.M.).

IL-17 immunohistochemistry

Sections from 30 RA patients and from eight controls were analyzed for IL-17A synovium expression. Serial sections of 5-μm thickness were obtained from each paraffin-embedded block previously prepared. The sections were dewaxed in xylene and rehydrated in graded alcohols. Heat induced epitope retrieval was performed by microwave at 650 W for 20 minutes and 30 minutes cooling. Then the sections were treated with 5% hydrogen peroxide in PBS for 30 minutes and the nonspecific binding was blocked by incubating the slides for one hour with 5% BSA in PBS. As primary antibody was used rabbit polyclonal IL-17 (H-132; Santa Cruz Biotechnology, Redox, Bucharest) in dilution 1:500 and 18 hours incubation at 4^0C. The visualization system that we used was a Streptavidin–Biotin-peroxidase complex kit (LSAB kit, DAKO, Redox, Bucharest, Romania) in accordance with the protocols recommended by the manufacturer. The signal was detected with 3,3'-diaminobenzidine (DAB – DAKO, Redox, Bucharest, Romania) and counterstaining was done with Hematoxylin. As negative control, we omitted the primary antibody and as positive external controls, we used tonsil samples. No quantitative immunohistochemical assessment was targeted, we were just especially interested in the presence of the IL-17 reactivity in the synovial tissue.

Images acquisition was done with 20× and 40× objectives – Nikon Eclipse 90i microscope (Nikon, Apidrag, Bucharest) equipped with a 5 megapixels CCD camera and Nikon NIS-Elements software.

Statistical analysis

The data were analyzed using the SPSS Statistics software. First, the descriptive statistics parameters (mean, median, mode, range, standard deviation) have been calculated on the database containing records for patients and control subjects. For each random variable were used the calculated parameters, histograms and Shapiro–Wilk test to evaluate the probability distributions. Then, the correlations between different variables have been computed by finding the correlation coefficient and the associated significance level *p* (considering 0.01 as high significance level); also, at this step, we have calculated the difference in the RA and OA data distributions using two independent samples tests. Because the normality test has given the information that the variables are not normal distributed it were used non-parametric tests (Mann–Whitney test) for testing the difference in data distributions and correlation measure (Spearman's rank correlations – denoted with r_S in the present document) for finding the connections between variables.

Results

Demographical data and descriptive statistics of the early RA patients were illustrated in Table 1.

Table 1 – ***Demographical data and descriptive statistics of the early RA patients***

		Age [years]	Disease history [months]	ESR [mm/h]	CRP [mg/dL]	RF [UI/mL]	Anti-CCP [U/mL]	DAS28	IL-17A serum [pg/mL]	IL-17A syn. fluid [pg/mL]	Hp score
N	*Valid*	30	30	30	30	30	30	30	30	29	30
	Missing	0	0	0	0	0	0	0	0	1	0
Mean		42.77	6.03	39.43	1.65	41.90	92.04	5.79	11.80	18.77	11.63
Standard deviation		7.39	2.27	13.24	0.98	46.99	110.90	0.94	5.07237	12.188	3.95
Minimum		28	2.00	22.00	0.60	5.00	10.00	3.93	5.00	4.40	5.00
Maximum		58	10.00	82.00	4.40	180.00	400.00	7.79	22.50	44.10	17.00

Features of IL-17A in the early RA serum and synovial fluid

Serum levels of IL-17A were detectable in all early RA cases; these were higher in RA patients compared with OA patients (11.80±5.07 pg/mL *vs.* 5.18±1.49 pg/mL) (Figure 1A). Also, SF IL-17A levels were elevated in RA patients (18.77±12.18 pg/mL) than in controls (4.16±1.20 pg/mL) (Figure 1B). Serum and SF IL-17A levels in RA and OA controls were illustrated in Table 2.

Table 2 – ***IL-17A profile in OA controls***

	No.	Minimum	Maximum	Mean	Standard deviation
Serum IL-17A [pg/mL]	29	2.80	8.10	5.1828	1.49787
SF IL-17A [pg/mL]	29	2.20	7.00	4.1690	1.20389

Furthermore, IL-17A SF levels were higher than serum levels of the cytokine in early RA cases, with strong correlation ($p<0.001$, $r_s=0.730$) (Figure 2).

Also, there were strong correlations of serum and SF IL-17A levels with inflammatory markers as ESR ($p<0.001$, $r_s=0.651$; respectively $p<0.001$, $r_s=0.616$), CRP ($p=0.001$, $r_s=0.555$, respectively $p=0.001$, $r_s=0.589$) (Figure 3), and with specific immune markers – anti-CCP ($p<0.001$, $r_s=0.778$; $p<0.001$, $r_s=0.853$) (Figure 4) and RF (p=0.004, $r_s=0.506$, respectively $p=0.001$, $r_s=0.573$) (Figure 5).

Also, a good correlation was noted with DAS28 score ($p=0.03$, $r_s=0.397$, respectively $p=0.027$, $r_s=0.411$) (Figure 6).

No correlation was found between serum, SF levels of IL-17A and, respectively, duration of the RA.

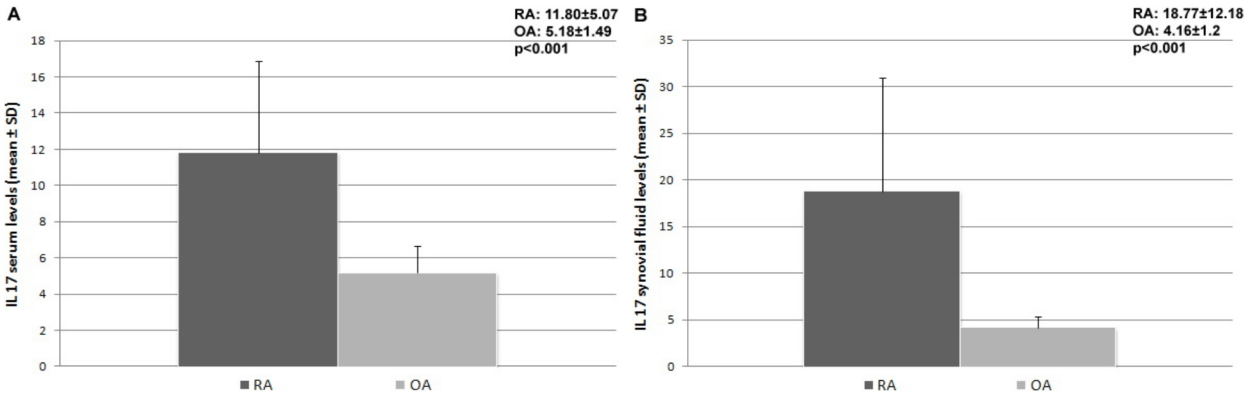

Figure 1 – ***Correlation of serum (A) and synovial fluid IL-17A (B) levels in RA and OA patients.***

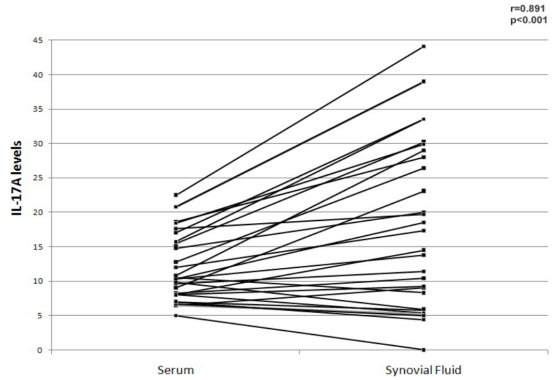

Figure 2 – ***Distribution of serum and SF IL-17A in RA cases showing strong correlation (p<0.001).***

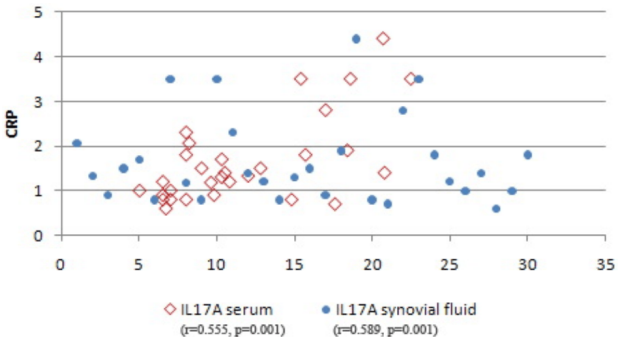

Figure 3 – ***Serum and SF IL-17A levels correlated with CRP (p=0.001) in RA cases.***

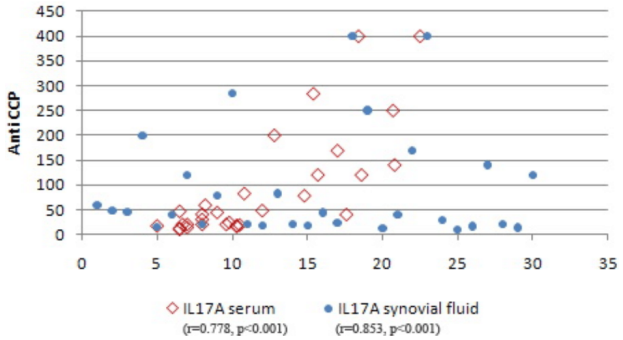

Figure 4 – ***Correlations of serum and SF IL-17A levels with anti-CCP (p<0.001) in RA patients.***

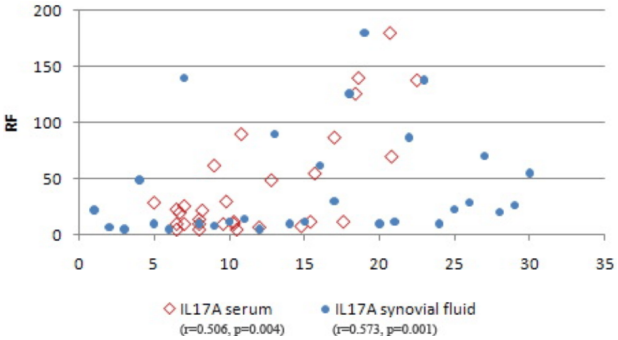

Figure 5 – ***Graphical distribution of serum and SF IL-17A levels depending on RF.***

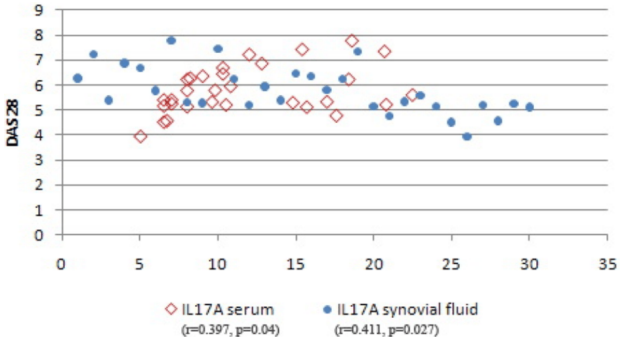

Figure 6 – ***Graphical distribution of serum and SF IL-17A levels depending on DAS28 score: showed good correlation (p<0.05).***

Histopathological and immunohistochemical features

As we stated in previous works [26, 28], the histology of the biopsy specimens had a heterogeneous pattern of lesions with synoviocytic proliferation, with more than 5–6 rows (score 2) and the neovascularization as the most encountered histopathological findings.

In the present study, the assessment of histopathological score showed maximum values p to 17 and a mean of 11.63±3.58 SD (95% CI).

Strong correlations were noted between Hp score and serum and SF levels of IL-17A (p<0.001, r=0.624, respectively p<0.001, r=0.669) (Figure 7).

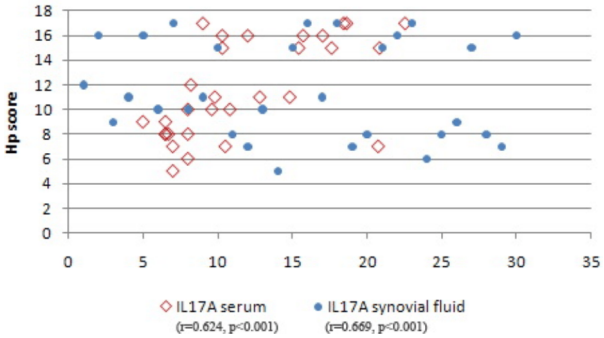

Figure 7 – ***Distribution of serum and SF IL-17A depending on Hp score – showed strong dependency (p<0.001).***

IL-17 reactivity was present in the synovium in 18 (60%) patients with early rheumatoid arthritis; it also important to mention that this was more intense at the level of inflammatory infiltrate. IL-17 expression tended to be scattered throughout the synoviocytes (Figure 8A). In contrast, the synovium samples from the control patients were negative to IL-17 (data not shown).

IL-17 synovial pattern was both cytoplasmic and nuclear (Figure 8, A and B) and was detected in all the layers of the synoviocytic proliferation. At the level of inflammatory infiltrate, the IL-17 reactivity was more intense in lymphocytes, plasmocytes and macrophage-like cells (Figure 8, C and D).

Only a part of lymphocyte population was IL-17 positive and we suspected that it was about T-cells (Figure 8D). A weaker reaction was also noticed at the level of endothelial cells, plasmocytes, neutrophils and fibroblasts (Figure 8, E and F).

To mention that no aggregates of IL-17 positive cells were found.

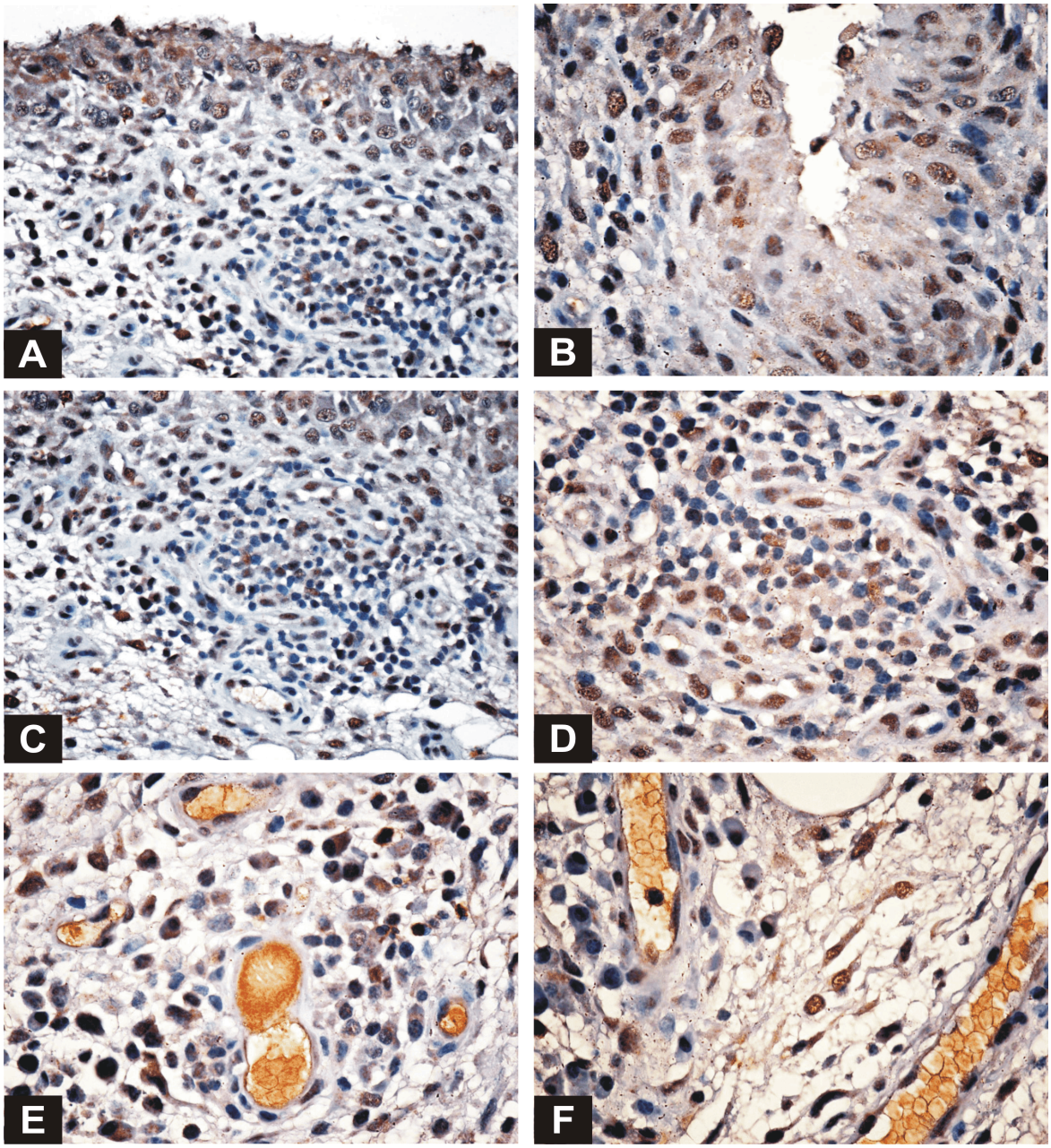

Figure 8 – ***IL-17 immunoreactivity in the synovial tissue of the early RA patients: (A and B) Cytoplasmic and nuclear reactivity in the synoviocytes: 400×, 600×. (C and D) Positive immunostaining in inflammatory cells and macrophage-like cells: 400×, 600×. (E and F) Positive reaction in plasmocytes, endothelial cells, neutrophils and fibroblasts: 600×.***

Regarding the IL-17 synovial reactivity in early RA cases, we have considered two groups: one with synovial reactivity (1) and one without synovial reactivity (0). We have tested the difference in these two groups for each of chosen variables using the Mann–Whitney U (non-parametric) test. There were founded significance correlation of IL-17 reactivity with ESR (p=0.002), CRP (p=0.019), RF (p=0.046), DAS28 (p=0.003), anti-CCP and Hp score (p<0.001).

Table 3 details the correlations between IL-17 profile and other analyzed parameters.

Table 3 – ***Correlations between IL-17A profiles and analyzed parameters***

		Serum IL-17A	SF IL-17A	IL17 reactivity
ESR	*r*	0.65	0.62	0.59
	p	<0.001	<0.001	0.001
CRP	*r*	0.56	0.59	0.44
	p	0.001	0.001	0.016
RF	*r*	0.506	0.573	0.371
	p	0.004	0.001	0.044
Anti-CCP	*r*	0.778	0.853	0.693
	p	<0.001	<0.001	<0.001
DAS28	*r*	0.397	0.411	0.546
	p	0.03	0.027	<0.001
Hp score	*r*	0.624	0.669	0.763
	p	<0.001	<0.001	<0.001

Discussion

In this study, we examined the expression of IL-17 in synovial tissue from treatment-naïve early RA patients, in the same time with serum and synovial fluid levels of IL-17A. We demonstrated that IL-17 expression was markedly higher in RA synovium compared with controls, results consistent with previous studies [21, 29]. Other report (Shahrara S *et al.*) [30] demonstrated that IL-17 levels in synovial homogenates were comparable in OA and normal synovial tissues, and these values were significantly increased in RA synovial tissue, but patients had established RA and were not naïve for treatment.

On the other side, in a recent study comparing individuals before the onset of symptoms (defined as pre-patients) and after the onset of RA with matched control subjects, Kokkonen H *et al.* [31], showed that IL-17 was present at its highest concentrations in pre-patients, and the level had already decreased within 7.7 months following the onset of disease, but these findings targets only serum level of IL-17, without any information about synovial tissue IL-17 behavior.

In the present study, we found only a weak IL-17 immunoreactivity, with a tendency of scattering in all layers of synoviocytic proliferation, in contrast with other report [29], which showed a prevalent distribution of IL-17 expression in sublining areas.

An interesting finding was the correlation existing between IL-17 synovial tissue immunoreactivity and Hp score, which was previously demonstrated as a predictor for disease activity and progression [26, 28]. This finding is especially relevant as previous data have already demonstrated that synovial tissue IL-17 mRNA expression have been independently associated with increased joint damage progression in RA [32].

Gullick NJ *et al.* [33] reported significant levels of IL-17+ T-cells in synovial tissue, but only in patients with active RA, suggesting that IL-17+ T-cell numbers may vary with disease activity. In our study, we found IL-17 reactivity in 60% of early RA cases, results that are consistent with other studies [32, 34] but only a part of lymphocytic population was IL-17 positive and we suspected that it is about T-cells. A possible explanation might be the T-cell stimulation with ionomycin, used in the protocol of Gullick NJ *et al.* [33] especially since Stamp LK *et al.* [20] did not detect IL-17 production by other cell types.

Our finding could be taken into account through other recent published data, which confirmed that mast cells rather than T-cells are the major source of synovium IL-17, but synovial tissue had come from long history RA patients at the time of joint replacement [35].

It remains currently unclear why the data regarding IL-17 in RA synovium are so varied, but this may be due in part to different demographics and treatment regimes in the RA patients. Indeed, the most studies has been focused on established RA, previously treated with a wide variety of DMARDs regimens, which, in combination with their impact on disease activity, could influence both IL-17 behavior and the correlations with disease activity markers.

Another aim of our study was to assess the serum and SF profile of IL-17A in early RA patients. We noted higher serum and SF IL-17A levels in patients with seropositivity for RF and anti-CCP, especially in cases with elevated disease activity scores. It was known that IL-17A was detected at higher levels in early disease compared with late, established disease [22, 24, 31]. We also report significantly higher IL-17A levels in SF and serum from RA patients compared with OA. Furthermore, SF levels of IL-17A were significantly higher than matched serum suggesting that the cytokine is predominantly produced locally in the inflamed joint. Although previous findings showed higher serum and SF IL-17A levels in RA patients compared with healthy controls [29]. Interestingly, we noted a weaker correlation of synovial IL-17A levels with CRP, compared with ESR. This finding is somehow surprising because previous studies already have suggested that IL-17A is a potent inducer of CRP from human smooth muscle cells and hepatocytes [36] and, in addition, demonstrated a strong correlation between these parameters, but only in established RA [29]. In contrast, other data noted that despite the significant increases in Th17 and IL-17+IFNc+ CD4+ T-cells in the blood of RA patients, these subsets did not correlate with markers of disease such as ESR, CRP or DAS28 – suggesting that the presence of IL-17 producing CD4+ T-cells in the blood from patients with established RA is of limited use as a biomarker to indicate disease activity [33, 37].

In a most recent paper (Leipe J *et al.*) [38], it was demonstrated that levels of IL-17 production was strongly correlated with the magnitude of disease activity and systemic inflammation as assessed according to the

DAS28 scores and levels of CRP, in the treatment-naïve group of patients with both early RA and early psoriatic arthritis.

An interesting finding was the strong correlation of synovial fluid IL-17A levels with composite histopathological score of rheumatoid synovium. This is the first study, which analyses the relationships between almost validated disease activity and prediction markers (such as CRP, DAS28, RF, anti-CCP), IL-17 profile and histopathological score. Our findings could support the hypothesis already formulated [39–41] that in early stages of RA, synovium patterns are highly suggestive for an aggressive trend of disease and, in addition might express specific therapeutical targets.

We are aware of the statistical limitations of the present study, mainly in terms of power, due to the relatively low number of patients. Furthermore, another limitation of histopathological and immunohistochemistry assessment of IL-17 consist in the application of results derived from a limited number of samples collected from each RA patients, limit which was counterbalanced through US-guided minimal invasive synovial biopsies. In addition, IL-17 reactivity of the synovium was assessed only in a qualitative, descriptive manner. The study of the synovial tissue can be a valuable research tool, allowing for this tissue to be incorporated into future trial designs, which is critical for further understanding of the pathogenesis of RA and for as assessing marker changes in the early course of disease or in response to targeted therapies.

Conclusions

In early stages of untreated RA, simultaneous IL-17 assessment of serum, SF and synovium might be valuable in defining activity and predictive patterns, given that synovium is highly suggestive for an disease aggressivity and might express specific therapeutically targets.

Authors' contributions

A.R. – Conception of the study, acquisition of clinical data, analysis and interpretation of the data, final manuscript writing and final approval of the manuscript-publishing version. **C.M.** – Histopathological and immunohistochemistry performing, analysis and interpretation of the data, article drafting. **A.M.** – Ultrasonographic examination and synovial tissue minimal invasive biopsies performing. **A.S.** – Synovial tissue preparation, histopathological performing and images acquisition. **M.E.** – Analysis of data, statistical analysis, article drafting. All authors have approved the content of the manuscript.

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