APRIL Level as a Marker of Disease Activity in Treated Rheumatoid Arthritis Patients: Association with Disease Activity and Anti-CCP Antibody

[1]Ghada Boghdadi, [1]Rehab H. El-Sokkary, [2]Enass A. Elewa, [2]Samah Fathi Abbas

Departments of [1]Immunology Research Lab., Medical Microbiology & Immunology and [2]Rheumatology & Rehabilitation, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Rheumatoid arthritis (RA) is a chronic autoimmune disease with joint inflammation and autoantibody production. Cytokines play an important role in the pathogenesis of RA. Among the cytokines that regulate B cell homeostasis is the "A Proliferation-Inducing Ligand" (APRIL). To determine the differences in APRIL in response to treatment in anti- cyclic citrllinated peptides (anti-CCP) positive versus anti-CCP negative patients with established RA. Concentrations of APRIL in sera of 10 anti-CCP positive RA patients, 18 anti-CCP negative RA patients, and 12 healthy controls were measured by enzyme-linked immunosorbent assay (ELISA) at treatment initiation and after 6 months of treatment with methotrexate and hydroxychloroquine. Correlations between serum anti-CCP, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), 28-joint Disease Activity Score (DAS28), and serum level of APRIL were analyzed. Serum APRIL levels were increased in rheumatoid arthritis patients in comparison with healthy volunteers. APRIL correlated positively with disease activity; swollen joint count, visual analog score and simplified disease activity index (all *P* <0.05). In addition, APRIL was significantly higher in patients with positive anti-CCP. After treatment, APRIL levels significantly decreased in the anti-CCP positive RA patients than in anti-CCP negative RA patients. In conclusions, serum APRIL may be a good predictor marker for joint injury and therapeutic response in patients with RA.

Rheumatoid arthritis is a systemic, autoimmune disease of uncertain etiology, which is characterized primarily by synovial inflammation with secondary skeletal destruction (Charles, 2007). An early diagnosis of RA patients is of utmost importance for early therapy establishment which is essential for improving disease outcome in the long term (Van Dongen *et al.*, 2007). Clinically, rheumatoid arthritis is diagnosed by the presence of four of the seven diagnostic criteria: morning stiffness, symmetrical arthritis, arthritis of hands joints, arthritis of three or more joints, rheumatoid nodules, serum rheumatoid factor positive and typical radiological changes in the hands and feet according to the American college of Rheumatology (ACR) (Arnett *et al.,* 1988). This has recently been revised by a joint ACR and EULAR committee. In the revised criteria, objective serological testing for the presence of 2 RA disease markers, rheumatoid factor (RF) and antibodies directed against cyclic citrllinated peptides (Anti-CCP), is included as an important criterion (Aletaha *et al.,* 2010). However one third of established RA patients are sreonegative for these 2 diagnostically applied disease markers (Nishimura *et al.*, 2007). These findings signify the need for additional disease markers, especially for early RA and for seronegative RA patients (RF-ve-Anti-CCP -ve) (Somers *et al.,* 2011). RA has long been considered a T cell/macrophage-driven pathology. The success of B cell targeted therapies, such as rituximab, in the treatment of RA patients has led investigators to reassess the critical role of B cells in RA pathogenesis (Edwards *et al.,* 2004). Several

reports have revealed that B cells not only release inflammatory or immunomodulatory cytokines (Fillatreau *et al.,* 2002), a proliferation-induced ligand (APRIL) is produced by a range of innate cells, including monocytes, macrophages, DCs, neutrophils, mast cells, activated T cells and B cells in adaptive immune response (Litinsky *et al.*, 2002) and follicular DCs in secondary lymphoid tissues (Zhang *et al.,* 2005). Several reports observed that APRIL might be a primary factor that act directly on the B cells to promote the production of autoantibodies, including anti-double-stranded DNA antibodies in systemic lupus erythromatosis (SLE), anti-Ro (SSA) antibodies in Sjogren disease and rheumatoid factors in RA (Pres *et al.,* 2005).

Data from clinical trials suggested that APRIL and other factors may have the potential as disease biomarker, although this remains to be confirmed (Dorner *et al.,* 2010). Owing to suggested role of APRIL in adult autoimmune diseases and its relation to activity and autoantibodies production, we aimed to determine differences in APRIL level in response to treatment in anti-CCP positive versus anti-CCP negative patients with established RA.

Subjects and Methods

Methods

This study was carried out at Department of Rheumatology and Rehabilitation and the Immunology Research Lab, Department of Microbiology and Immunology, Faculty of Medicine, Zagazig, Egypt. Twenty eight patients were enrolled in this study, divided into 2 groups: (Group A): 10 anti-CCP positive RA patients, (Group B): 18 anti-CCP negative RA patients, and 12 healthy age- and gender-matched volunteers served as controls. Patients were diagnosed based on fulfillment of the ACR criteria (2010). The study was approved by the ethical committee of the hospital and written informed consent was obtained from each patient. A detailed clinical history and a complete physical examination including locomotor system, skin, cardiovascular, chest, neurological and vascular examinations were carried out for each patient.

Investigations

In the study groups, the following laboratory parameters were analyzed: erythrocyte sedimentation rate (ESR), C reactive protein (CRP), IgM rheumatoid factor (RF), Antinuclear antibodie (ANA), APRIL, and anti-CCP.

-Rheumatoid factor was determined with the Latex agglutination method (RF-turbilatex, Spinreact, Spain). RF level of >8 IU/mL was considered as significant.

-Antinuclear antibodies (ANA) were determined with indirect immunoflorescent method. Glass slides with adherent Hep-2 cells (Kallestad ANA kit [Bio-Rad) were exposed to patient sera, which was initially diluted 1:40 with 1X phosphate buffered saline. If antinuclear antibodies were present they attached to the nucleus of cells on the slide, forming an antigen-antibody complex. Fluorescein labeled anti-human globulin (prediluted from the kit) was added to the slide, highlighting any Ag-Ab complexes when viewed with a fluorescent microscope using 40X objectives. Positive and negative controls were used in every evaluation. When the screening dilution (1:40) is positive, the pattern is noted and serial titrations are run to a maximum dilution of 1:1280 (Xu *et al.,* 2007).

-Determination of APRIL and anti-CCP levels: Blood samples taken from the patients, before and after 6 months of treatment with methotrexate and hydroxychloroquine, and healthy controls were kept at room temperature for 30 min till they coagulated. They were then centrifuged at 2,000 rpm for 10 min and serums were obtained. The serum samples were kept at -70 °C until the study.

-APRIL and anti-CCP were measured in serum samples by ELISA according to the kit manufacturer’s instructions. The paragraph of APRIL method should be moved up before Anti-CCP

-APRIL level was measured by ELISA using a commercial kit (human APRIL ELISA BMS2008 Affymetrix eBioscience, USA) according to manufacturer's instructions. In this assay, an anti-human APRIL coating antibody was adsorbed onto microwells. Human APRIL present in the sample or standard binded to antibodies adsorbed to the microwells. A biotin-conjugated anti-human APRIL antibody was added and binded to human APRIL captured by the first antibody. Following incubation unbound biotin-conjugated anti-human APRIL antibody was removed during a wash step. Streptavidin-HRP was added and bound to the biotin-conjugated anti-human APRIL antibody. Following incubation unbound Streptavidin-HRP was removed during a wash step, and substrate solution

reactived with HRP was added to the wells. A coloured product was formed in proportion to the amount of human APRIL present in the sample or standard. The reaction was terminated by addition of acid and absorbance was measured at 450 nm. The analytic sensitivity was APRIL= 0.4 ng/ml.

-Measurment of Anti-CCP level was done by ELISA using a commercial kit (Anti-CCP hs High sensitive", ORGENTEC Diagnostika GmbH, Germany) following manufacturer's instructions. This assay employs the quantitative enzyme immunoassay technique. The microtiter plate provided in this kit has been pre-coated with CCP antigen. Samples were pipetted into the wells with anti-human IgG conjugated Horseradish Peroxidase (HRP). Any antibodies specific for CCP present will bind to the pre-coated antigen. Following a wash to remove any unbound reagent, a substrate solution was added to the wells and color developed in proportion to the amount of human anti-CCP antibody bound in the initial step. The color development was stopped and the intensity of the color was measured. The analytic sensitivity was 5 U/ml.

Statistical Analysis

The collected data were encoded and analyzed using SPSS version 16. The power of the study is 80% i.e., the propability of error is 20%, confidence interval is 95% and level of significance < 0.05. Quantitative variables were given as mean ± SD, and range for summarization and Student t test, Anova test, Mann-Whitney U test, paired t test and Pearson's correlation for data analysis. For qualitative data, number and percentage of observation at each category were used for summarization and chi-square for analysis.

Results

Patient characteristics

Twenty eight patients with RA at the time of inclusion divided into 2 groups: (Group A): 10 anti-CCP positive RA patients, (Group B): 18 anti-CCP negative RA patients and 12 healthy controls were studied. The mean age for group A was 41.6±4.7 years; there were 8 females and 2 males and the mean age was 43.8±6.1 years for group B; there were 15 females and 3 male. The control group consisted of 12 females and 4 males with mean age 42.1± 5.6 years. The mean duration of disease was a 5.1 ± 4.9 years ranging from 1 to 20 years for group A. and a 7.3±2.8 years ranging from 2 to 12 years in group B. Characteristics of the enrolled patients are summarized in Table 1. There was no significant difference with regard to age, sex, morning stiffness (MS) Duration, tender joints, swollen joints, VAS and CRP between the groups (P>0.05). On the other hand, a statistical significant difference was observed between groups as regards disease duration, ESR and 28 DAS (P < 0.05).

Serum levels of APRIL in the studied groups and controls

Comparison of serum levels of APRIL between both groups of patients and between patients and controls revealed that serum levels of APRIL was significantly lower in the control group than patients (P<0.05) (Table 2). The correlation between APRIL level and parameters of activity was studied with a statistically significant correlation as follows APRIL and VAS: r=0.67 P=0.01* results with other studied parameters (number of tender joints, number of swollen joints and diseases score activity) are shown in figure 1.

The percentage of change in APRIL level combined with other parameters of activity after treatment (Figure 2) shows a statistical significant correlation considering APRIL/ CRP and APRIL/ ESR levels. Table 3 shows that levels of both APRIL and Anti-CCP decreased after treatment. However, APRIL levels decreased with a significantly twofold more in the anti-CCP positive RA patients than in anti-CCP negative RA patients after treatment.

Table 1. Demographic and clinical characteristics of the rheumatoid arthritis patients.

	Group A	Group B	*P value*
Age (years)			
Range	(36-52)	(32-59)	NS
Mean ± SD	41.6± 4.7	43.8±61	
Sex			
M/F	2/8	3/15	NS
Disease duration (months)			
Range	(2-10)	(2-72)	0.04
Mean ± SD	4.9±2.8	7.3±2.8	
Ms Duration (min)			
Range	(20-180)	(15-120)	NS
Mean ± SD	67.5±49.8	65.6±35.1	
Number of tender joints			
Range	(7-26)	(4-24)	NS
Mean ± SD	15.8±6.4	12.8±6.6	
Number of swollen joints			
Range	(5-22)	(4-24)	NS
Mean ± SD	11.9±4.9	12.8±6.7	
VAS			
Range	(30-90)	(20-90)	NS
Mean ± SD	55±19.6	48.9±21.7	
(DAS-28)			
Range	(4.2- 6.7)	(1.9-5.8)	0.00
Mean ± SD	5.3±0.7	3.7±1.2	
ESR before treatment			
Range	(22-60)	(17-43)	0.01
Mean ± SD	41.7± 10.9	29.8±7.7	
CRP before treatment			
Range	(9-21)	(8-16)	NS
Mean ± SD	12.5± 3.9	11.1±2.3	

M=male. F= female. SD=standard deviation. ESR= erythrocyte sedimentation rate. CRP= C-reactive protein, VAS= visual analogue scale. DAS 28 =28 –joint disease activity score. **P>0.05*= is Not Significant (NS).

Table 2. APRIL levels (ng/ml) of the studied groups and the control.

	Group A	Group B	Control group	*P value*
Mean ± SD	10.4± 2.5	7.8±2.6	0.69±0.5	0.00
Range	(6.2-15.3)	(3-12)	(0.7-4.3)	

P <0.001=Significant. *Annova test is used

Group A (Anti-CCP positive RA patients). Group B (Anti-CCP negative RA patients)

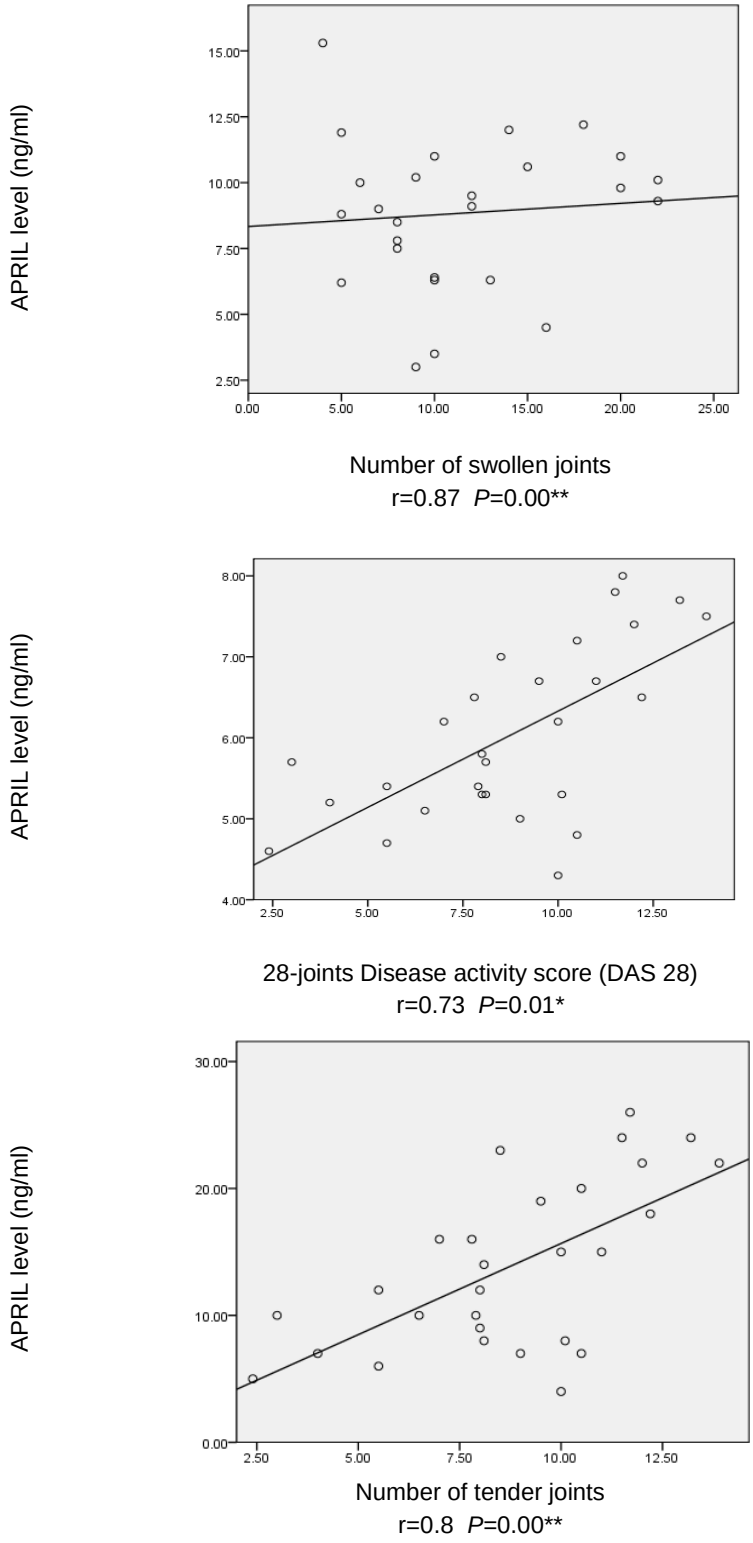

Figure 1. The positive correlation between APRIL level and parameters of activity in patients with rheumatoid arthritis.

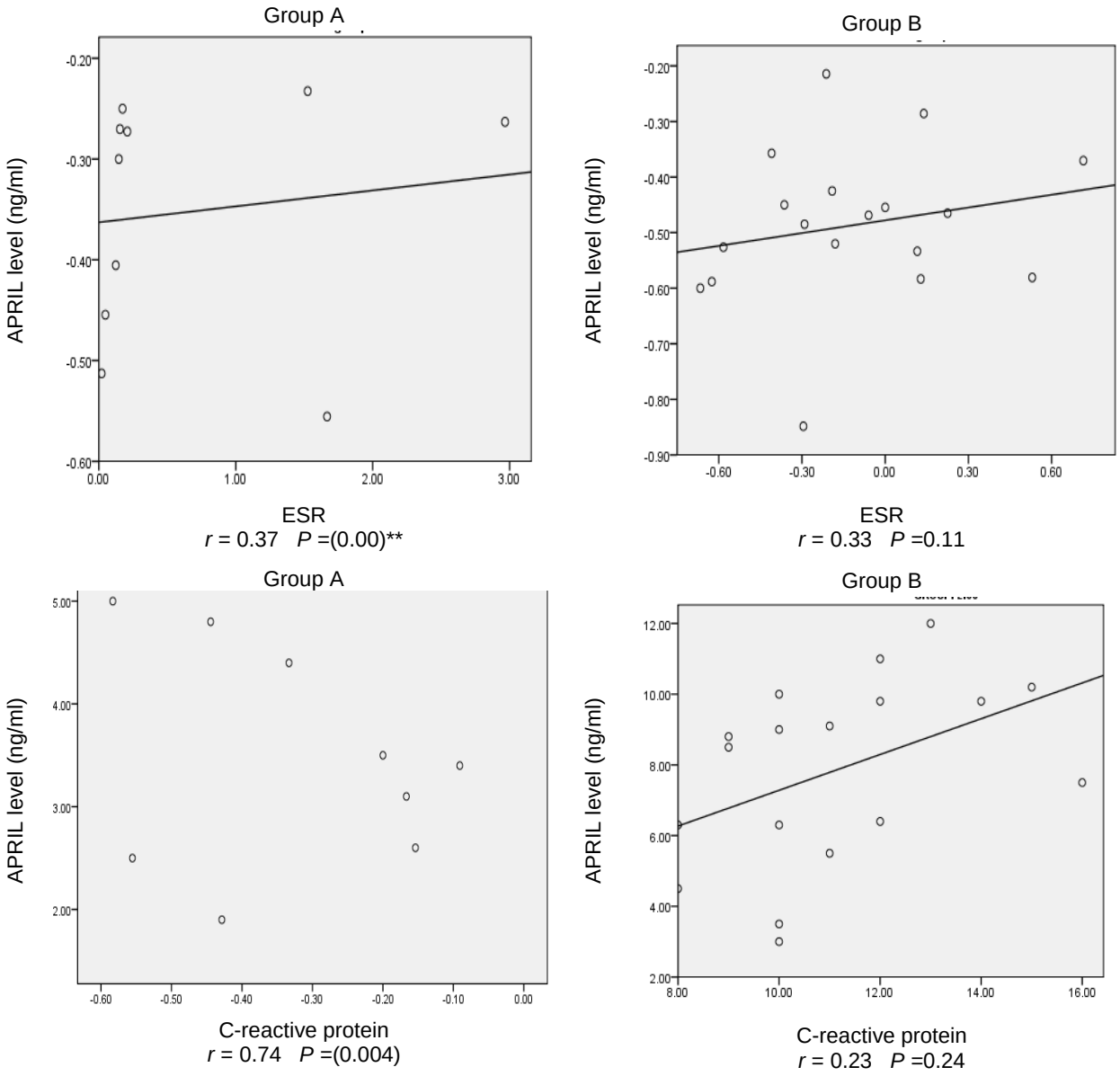

Figure 2. The relation between the percentage of change of APRIL before and after treatment with ESR and CRP.

Table 3. APRIL and Anti-CCP level before and after treatment in patients with rheumatoid arthritis.

	APRIL (ng/ml) (group A)	APRIL (ng/ml) (group B)	Anti-CCP (U/ml) (group A)
Before treatment	10.4± 2.5 (6.2-15.3)	7.8±2.6 (3-12)	156.6±117.6 (32.9-369.6)
After treatment	6.3± 2.4 (5-13)	7.0±2.6 (5-12)	66.1±40.9 (12-15)
P value	0.00	0.00	0.01

Group A (anti-CCP positive RA patients). Group B (anti-CCP negative RA patients). *P* <0.05= Significant.

Discussion

Since APRIL was discovered, a great amount of evidence has been reported about the involvement of APRIL in autoimmune diseases including RA (Bosello *et al.,* 2006). APRIL is known to contribute to autoimmune responses and have been shown to play roles in the process of inflammation associated lymphoproliferation and germinal center formation in the rheumatoid synovium (Seyler, *et al.,* 2005).

Early detection of RA by serological tests is very important for better prognosis. Antibodies reactive against citrulline-containing proteins have been identified in the inflamed joints of RA patients. These anti-cyclic citrullinated peptide antibodies (anti-CCP) appear early in the disease process; furthermore, they are rarely found in healthy individuals and their presence is associated with a more severe disease prognosis (van Gaalen, *et al.,* 2005). To estimate the role of APRIL, we investigated the level of APRIL before and after treatment in order to determine the differences in APRIL in response to treatment in anti-CCP positive versus anti-CCP negative patients with established RA. Methotrexate (MTX) has been demonstrated to lower total B cell numbers in peripheral blood, but no detailed analysis regarding the effect of low-dose MTX on B cell subpopulations has been reported so far.

We found that serum APRIL levels were increased in rheumatoid arthritis patients as compared to healthy volunteers, this consists with the study done by Katsuya *et al.,* 2007 and Dillon *et al.,* 2010 who reported that the level of soluble APRIL in RA serum was significantly higher than that in normal serum. In concordance with our results, Mackay et al, 2003 observed that T cell independent class IgA class switching might also be favored by APRIL. In the study done by Tan *et al.,* 2003 they found that APRIL levels are elevated in synovial fluid (SF) from patients with established RA indicating increased local production of this complex in arthritic joints, furthermore, synovial expression of APRIL is reported to be highest in patients with germinal center-like structures in the synovium, a form of lymphoid organization known to enhance B-cell activation (Seyler, *et al.,* 2005). Actually, a highly positive association between the infiltration of plasma cells and SF levels of APRIL has been demonstrated in RA patients (Dong *et al.,* 2009).

Moreover, we observed that APRIL correlated positively with disease activity parameters; swollen joint count, tender joint count, visual analog scale and simplified disease activity index corroborating previous studies (Gotenberg *et al.,* 2009) which reported that markers of B-cell activation were associated with disease activity, RF and anti-CCP secretion in agreement with a previous report (Katsuya *et al.,* 2007) who concluded that APRIL might act as an upstream mediator of the cytokine network to facilitate inflammatory reactions, specifically in the RA synovium.

In the study done by Vallerskog *et al.,* 2006, patients with RA had APRIL levels 10-fold higher than normal, which did not change during depletion of B cell. At baseline, correlations between levels of B cells and APRIL, and DAS28 (disease activity score using 28 joint counts) were observed in patients with RA.

The mean serum APRIL level of seropositive RA patients was significantly higher than that of seronegative patients, similar results were observed by Zhao *et al.,* 2014 who concluded that the mean serum APRIL level of seropositive RA patients was significantly higher than that of seronegative patients and APRIL may participate in the

formation of seropositive RA. In addition, similar previous findings in a study done by Moura *et al.,* 2011 who found that APRIL and BAFF levels were significantly higher in patients as compared controls. In contrast to our findings, they observed no differences between treated or untreated patients after therapy with CSs and MTX.

Furthermore, they found no correlation between DAS-28, anti-CCP or RF autoantibodies and APRIL and BAFF serum concentrations. These differences might be due to different population included in our study since we investigated the level of APRIL before and after treatment in patients with established RA and they included only very early rheumatoid arthritis patients (disease duration <6 weeks).

In conclusion, serum APRIL showed elevated levels that correlated significantly with RA disease activity and serum APRIL had a good prediction performance to evaluate the joint injury status and therapeutic effect in patients with RA, accordingly anti-APRIL therapy might be of great benefit to offset disease flare.

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