








The Evaluation of Serum Tumor Necrosis Factor-Like Weak Inducer of Apoptosis, Interleukin-6, Fetuin-A, Homeostatic Model Assessment-Insulin Resistance, and Insulin Levels in Rheumatoid Arthritis Patients in Clinical Remission

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ABSTRACT

Objectives: This study aims to examine the relationship of serum tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) levels with interleukin (IL)-6, tumor necrosis factor-alpha (TNF- α), fetuin-A, insulin, homeostatic model assessment (HOMA)-insulin resistance (IR), and disease activity in patients with rheumatoid arthritis (RA) who are in remission or have low disease activity.

Patients and methods: Fifty-four patients with RA (8 males, 46 females; mean age 52.7 \pm 12.3 years; range 40 to 64 years) and 34 healthy controls (6 males, 28 females; mean age 53.2 \pm 11.3 years; range 41 to 65 years) were included in the study. The sTWEAK, fetuin-A, insulin, lipid profile and IL-6 concentrations were determined. The HOMA-IR levels were calculated using a calculator. Disease activity score 28 was used to assess the disease activity.

Results: The erythrocyte sedimentation rate, C-reactive protein, fetuin-A, and IL-6 levels were higher in the RA group than in the control group (p=0.004, 0.001, 0.001, and 0.003, respectively). sTWEAK levels were lower in the RA group than in the control group (p=0.007). There were no differences in the TNF- α , HOMA-IR, insulin, and lipid profile levels of the two groups (p>0.05). sTWEAK had a negative correlation with body mass index and fetuin-A (r=-0.261 and r=-0.287, respectively).

Conclusion: We found that RA patients had lower sTWEAK levels and higher fetuin-A levels than the control group subjects. Furthermore, these two molecules were associated with each other. This study demonstrated that in RA patients, even if the disease is controlled with treatment, some molecules associated with an increased metabolic and cardiovascular risk continue to function. Follow-up studies on larger populations are warranted to confirm these findings.

Keywords: Fetuin-A; insulin resistance; interleukin-6; rheumatoid arthritis; serum tumor necrosis factor-like weak inducer of apoptosis.

Rheumatic arthritis (RA) is a chronic disease of unknown etiology that causes the activation of pro-inflammatory pathways, leading to joint and systemic inflammations.¹ The mortality in RA patients is increased by 0.9 to 3% compared to that in healthy individuals.² Known metabolic and cardiovascular (CV) risk factors such as smoking

and hyperlipidemia may have an influence in the development of CV diseases (CVDs), which are the main cause of mortality in patients with RA; however, lesser-known risk factors such as high inflammatory activity and autoimmune inflammatory factors may also play a role in these patients.²

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Tumor necrosis factor-alpha (TNF- α) is the first cytokine that was reported to be related to obesity and insulin resistance.³ Recently, TNF-like weak inducer of apoptosis (TWEAK) has drawn attention as a potential regulator of the inflammatory/anti-inflammatory balance in the developmental mechanism of insulin resistance.³ It has been reported to cause various biological effects through ligation (binding) with its receptor, fibroblast growth factor-inducible 14 (Fn14). The biological effects of TWEAK also include the induction of pro-inflammatory cytokines, modulation of immune response and angiogenesis, stimulation of apoptosis, as well as tissue repair and regeneration.^{4,5} TWEAK induces the production of several pro-inflammatory molecules such as matrix metalloproteinase-1, interleukin (IL)-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and regulated upon activation of normal T cell expressed and secreted by the synoviocytes and fibroblasts, as well as intracellular adhesion molecule-1, E-selectin, IL-8, and MCP-1 by the endothelial cells.⁶ The pro-inflammatory effects of TWEAK are observed in various cell types, including glomerular mesangial cells, human umbilical vascular endothelial cells, human gingival fibroblasts, human dermal fibroblasts, synoviocytes, chondrocytes, and fibroblasts.^{3,7-11} Some authors have recommended the use of serum TWEAK (sTWEAK) as a potential biomarker in human metabolic and CV disorders.¹¹ Few studies that have assessed the sTWEAK levels in RA have reported varying results.¹²⁻¹⁴ Fetuin-A (Alpha 2-HS glycoprotein [g/L]) may qualify as a novel marker for the early detection of atherosclerosis. Recent data support the theory that the fatty liver/fetuin-A pathway plays an important role in regulating insulin sensitivity and may influence atherosclerosis in humans.^{15,16}

According to our hypothesis, we speculate that the sTWEAK pathway is important in the pathogenesis of RA, and the metabolic disorders may continue to rise because of this pathway even if the RA disease activity decreases.

Therefore, in this study, we examined the relationship of serum sTWEAK levels with IL-6, TNF- α , fetuin-A, insulin, homeostatic model assessment (HOMA)-insulin resistance (IR), and disease activity in patients with RA who are in remission or have low disease activity.

PATIENTS AND METHODS

The study was designed as a single center, prospective, cross-sectional study at the rheumatology outpatient clinic of Sakarya University School of Medicine between December 2016 and March 2017. Blood samples were analyzed at the physiology laboratory by the department of physiology of our hospital. The study protocol was approved by the Sakarya University School of Medicine Ethics Committee. A written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Fifty four patients diagnosed with RA (8 males, 46 females; mean age 52.7 ± 12.3 years; range 40 to 64 years) according to the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria and 34 healthy controls (6 males, 28 females; mean age 53.2 ± 11.3 years; range 41 to 65 years) were included in the study. The patients were sequentially selected from the RA patients followed-up by the outpatient clinic. The subjects in the control group were selected from patient accompanists and patient visitors who do not have any diseases affecting the musculoskeletal system, and the two groups were matched with respect to age and sex. Age, sex, body mass index (BMI), educational status, disease duration, occupation, comorbidities, used drugs and demographic data were recorded for each patient.

Disease activity of the patients was assessed using the disease activity score 28 (DAS28). A score of DAS28 between 2.6-3.2 indicates low disease activity, 3.2-5.1 indicates moderate and >5.1 indicates high disease activity.¹⁶

Patients with diabetes mellitus, CVD, malignant hypertension, acute or chronic pancreatitis, with an inflammatory-auto-inflammatory disease other than RA, with hepatic and renal failure, diagnosed with a malignancy, who are pregnant or lactating, or below 18 years of age were excluded from the study.

Blood samples from the patients and the control group were drawn into tubes without anticoagulant, allowed to completely clot at room temperature, then sera were separated by centrifuging at 4°C at $5000 \times g$ for five minutes, and stored at -80°C until the day of biochemical analyses.

After the completion of the study groups, the sera were allowed to reduce to room temperature (20-25°C) to prepare them for biochemical analyses. To determine the serum IL-6, TNF- α , insulin, fetuin-A and TWEAK levels, commercial kits following the kit protocols were used, and solid phase sandwich enzyme linked immunosorbent assay (ELISA) method (Biotek ELX50 Reader Instruments, Winooski, VT, USA and Biotek ELX-800 Washer, USA) was applied. For serum IL-6 (eBioscience Platinum ELISA, Catalog No: BMS213/2; Bender Medsystems GmbH, Vienna, Austria), IL-6 standards with known concentration and serum samples were placed into ELISA plates coated with human IL-6 specific monoclonal antibodies. Following the incubation, elimination and color reaction phases, color change was observed, evaluated by measurement and expressed in pg/mL. Similar procedures were applied for serum insulin levels (DRG insulin ELISA Enzyme Immunosorbent Test Kit, EIA-2935, DRG Instruments GmbH, Global Heda quarters, Marburg, Germany), serum TWEAK levels (Human TWEAK Instant ELISA kit, BMS2006 INST, eBioscience Bender MedSystems GmbH, Vienna, Austria),

serum TNF- α levels (eBioscience, TNF-Alpha Platinum ELISA, Catalog No: (BMS223/4TEN, eBioscience Bender MedSystems GmbH, Vienna, Austria) and serum fetuin-A levels (AssayPro, Catalog No: EG3501-1, Assaypro LLC, USA). All biomarker samples were read on the ELISA reader (ELISA instrument brand model, (Biotek ELX50 Reader Instruments, Winooski, VT, USA and Biotek ELX-800 Washer, USA) at 450 nm wavelength. The results were expressed in pg/mL taking into consideration the given sensitivity values and calculating from the standard curve.

Statistical analysis

Expert support was obtained for the statistical analysis of the article. The Number Cruncher Statistical System 2007 (Kaysville, Utah, USA) software was used for the statistical analyses. Between-group comparisons were performed using the Student's t-test, Mann-Whitney U test, and Pearson's chi-square test. Spearman correlation coefficient was used for correlation analysis. Values of $p < 0.01$ and $p < 0.05$ were considered statistically significant.

Table 1. Comparison of patient and control groups according to study parameters

	Rheumatoid arthritis (n=54)		Healthy subjects (n=34)		p value
	n	Mean \pm SD	n	Mean \pm SD	
Age (year)		52.7 \pm 12.3		53.2 \pm 11.3	0.839
Sex					0.472
Female	46		28		
Male	8		6		
Fasting glucose (mg/dL)		98.4 \pm 20.0		94.2 \pm 6.2	0.166
Triglyceride (mg/dL)		129.0 \pm 72.4		110.4 \pm 24.9	0.131
Total cholesterol (mg/dL)		206.7 \pm 32.5		212.2 \pm 25.5	0.476
High-density lipoprotein cholesterol (mg/dL)		54.3 \pm 11.1		50.9 \pm 3.7	0.071
Low-density lipoprotein cholesterol (mg/dL)		120.1 \pm 25.3		111.9 \pm 11.8	0.080
C-reactive protein (mg/L)		10.6 \pm 11.5		4.0 \pm 1.2	0.001*
Erythrocyte sedimentation rate (mm/hour)		30.4 \pm 18.2		22.6 \pm 4.2	0.004*
sTWEAK (pg/mL)		1051.4 \pm 439.2		1277.1 \pm 323.1	0.007*
Tumor necrosis factor-alpha (pg/mL)		17.9 \pm 7.5		17.1 \pm 2.1	0.471
Insulin (μ U/mL)		22.4 \pm 19.1		19.1 \pm 15.4	0.378
Homeostasis model assessment of insulin resistance		6.0 \pm 6.7		4.4 \pm 3.3	0.124
Fetuin-A (g/L)		939 \pm 7.7		1.5 \pm 4.3	0.001*
Interleukin-6 (pg/mL)		6.7 \pm 10.7		2.1 \pm 1.0	0.003*

SD: Standard deviation; * $p < 0.05$ was considered to be statistically significant; sTWEAK: Serum tumor necrosis factor-like weak inducer of apoptosis; Fetuin-A: Alpha 2-HS glycoprotein.

Table 2. Comparison of biochemical parameters between groups in patients with rheumatoid arthritis according to disease activity score 28

	Low disease activity group (n=38)	Group in remission group (n=16)	p value
	Mean±SD	Mean±SD	
Tumor necrosis factor-alpha (pg/mL)	18.4±9.0	16.6±1.7	0.245
Insulin (µU/mL)	12.8±4.0	11.7±3.2	0.644
Homeostasis model assessment of insulin resistance	6.1±7.4	5.9±5.2	0.894
sTWEAK (pg/mL)	1039.5±387.8	1077.4±547.3	0.799
Fetuin-A (g/L)	986.6±7.7	836.1±7.9	0.514
Interleukin-6 (pg/mL)	5.4±7.6	9.6±15.4	0.291

SD: Standard deviation; sTWEAK: Serum TNF-like weak inducer of apoptosis; Fetuin-A: Alpha 2-HS glycoprotein.

Table 3. Correlation of laboratory parameters with clinical parameters

	CRP	ESR	Insulin	HOMA-IR	sTWEAK	IL-6	Fetuin-A
Diagnosis duration	0.031	-0.096	-0.167	-0.126	-0.051	0.006	0.302
Body mass index	0.123	0.015	0.112	0.177	-0.261	0.047	0.053
Total cholesterol	0.203	-0.034	0.286*	0.343*	0.043	0.187	0.249*
Tumor necrosis factor-alpha	0.090	0.169	0.030	0.037	0.024	0.453*	0.138
Disease activity score 28 scores	0.517**	0.329*	0.229	0.163	0.006	0.157	0.026
sTWEAK	0.158	0.181	0.104	-0.241	-	-0.108	-0.287*

CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; HOMA-IR: Homeostasis model assessment of insulin resistance; sTWEAK: Serum tumor necrosis factor-like weak inducer of apoptosis; IL-6: Interleukin-6; Fetuin-A: Alpha 2-HS glycoprotein; * Spearman correlation. Values are shown as correlation coefficients (r); bold values indicate statistically significant values. Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed).

RESULTS

There were no statistically significant differences between the patient and control groups in terms of sex, age and BMI ($p>0.05$). The main demographic, clinical and laboratory characteristics of the groups are summarized in Table 1.

Of the RA patients included in the study, 34 (62%) were receiving synthetic disease modifying antirheumatic drugs therapy and 43 (79.6%) were receiving low-dose steroid therapy. Sixteen patients (29%) were receiving TNF blocker therapy, two (3%) were receiving IL-6 inhibitor therapy, and two (3%) were receiving rituximab therapy. The mean steroid cumulative dose was 2.9 ± 25.65 for 50 patients.

The mean DAS28 score for the RA patients was 2.7 ± 0.94 . According to DAS28, 38 patients had low disease activity ($2.6<DAS28<3.2$) and

16 were in remission ($2.6<DAS28$). None of the patients had moderate or high disease activity. When the RA patients were grouped according to their DAS28 scores, no significant difference was found between the groups in terms of the laboratory parameters ($p>0.05$) (Table 2).

Correlations of the laboratory parameters with some demographic characteristics and clinical parameters are shown in Table 3. A significant positive correlation was found between the IL-6 concentrations and TNF- α ($r=0.453$, $p=0.001$). Significant correlations were detected of fetuin-A concentration with disease duration and total cholesterol (positive) and with sTWEAK (negative).

DISCUSSION

It is noteworthy that while the sTWEAK levels were lower in the RA group than in the

control group and the serum IL-6 and fetuin-A levels were significantly higher in the RA group, the TNF- α , insulin, and HOMA-IR levels were similar between the two groups. Moreover, sTWEAK levels had a poor correlation with BMI and fetuin-A levels.

Disturbance in the homeostasis of the pro-inflammatory and anti-inflammatory systems plays an important role in the etiology of metabolic diseases. This could be because pro-inflammatory cytokines and metabolic abnormalities associated with systemic inflammation play a vital role in the mechanism leading to inflammation in RA patients.^{17,18}

Serum tumor necrosis factor-like weak inducer of apoptosis is a molecule involved in the apoptosis, proliferation, and migration phases of cells. Studies have shown that sTWEAK treatment increases cell proliferation in some cell types such as vascular endothelial cells and smooth muscle cells, and this effect is similar to the effect of the vascular endothelial growth factor, a strong angiogenic-mitogenic factor.¹⁹ In chronic inflammatory conditions such as RA, TWEAK may be more harmful at the endothelial level due to this effect. While some studies show that increased TWEAK levels indicate vascular damage, indirect evidence suggests that TWEAK may contribute to the development of atherosclerosis in inflammatory or metabolic diseases by increasing endothelin synthesis.²⁰

Recent studies on the pathogenesis of RA have drawn attention to the sTWEAK/Fn14 pathway. TWEAK is a member of the TNF superfamily that is structurally related to cytokines and is a protein that exhibits cytotoxic and pro-inflammatory activities.¹⁰ Recent *in vivo* studies have demonstrated that the blockage of TWEAK activity by neutralization of the anti-TWEAK antibodies causes significant reductions in the clinical response and synovial inflammation in collagen-induced arthritis.^{21,22} Following the detection of increased levels of the sTWEAK molecule in the synovia and the serum in mice arthritis model studies, *in vitro* studies were conducted. TWEAK, which acts as a pro-inflammatory and pro-angiogenic mediator, was found to have a strong relationship with rheumatoid synovitis. Chicheportiche et al.¹⁰ demonstrated that TWEAK induces the

production of pro-inflammatory cytokines and chemokines by the normal human dermal fibroblasts and synoviocytes obtained from RA patients. Under physiological conditions, TWEAK may contribute to tissue repair and regeneration after acute damage by binding to its receptor Fn14. In chronic inflammatory conditions, the TWEAK/Fn14 pathway increases chronic inflammation, pathological hyperplasia, and angiogenesis.²³ It has been suggested that in order for TWEAK to induce angiogenesis, the TWEAK/Fn14 pathway should be blocked.²⁴

Park et al.¹³ found a correlation between significantly increased sTWEAK levels and disease activity in RA patients. In contrast, unlike other studies, the research by Karkucak et al.¹² showed no significant difference in the serum TWEAK levels of RA patients and healthy controls; this finding was attributed to the fact that about 50% of the RA patients in this study were receiving TNF blocker therapy, a type of therapy known to lower sTWEAK levels. Park et al.¹³ demonstrated that sTWEAK levels significantly reduce following TNF blocker therapy in RA patients. In our study, we investigated the molecules involved in RA and the metabolic damage they cause. We therefore enrolled RA patients who were in remission or had low disease activity. Majority of the patients in the study were being administered biological agents.

Rheumatoid arthritis not only causes the destruction of the cartilage and bone but also damages the metabolic and endocrine systems. This demonstrates a relationship between the inflammatory factors, insulin resistance, and metabolic syndrome.²⁵ Many different mechanisms related to increased CVD risk in RA were investigated. Physical inactivity, hypertension, diabetes, and obesity are associated with atherosclerosis in RA; however, the evidence for these risk factors is insufficient. Therefore, it is not possible to explain RA-associated atherosclerosis with only the known risk factors.²

Lower sTWEAK levels were observed in atherosclerosis patients and circulating sTWEAK levels were negatively correlated to the intima/media thickness in asymptomatic patients; therefore, it can be concluded that sTWEAK levels are low in many CVD indications.²⁶⁻²⁹ Consistent with these findings, low sTWEAK

levels were also observed in the peripheral blood samples of obese individuals.²⁹ sTWEAK levels were negatively correlated with glucose and glycosylated hemoglobin A1c levels as well as with HOMA-IR and abdominal obesity; this supports the hypothesis that low sTWEAK levels are associated with a poor CV profile. sTWEAK prevents TNF- α -induced insulin resistance by activating the protein phosphatase 2A pathway in human adipocytes.³⁰ All these molecules are among the known CV risk factors.³¹⁻³³ Moreover, the observed lower release of sTWEAK in carotid atheroma plaques compared to that in normal arteries supports an association between the lipotoxic effects of abnormal lipid accumulation and TWEAK synthesis.²⁴ In our study, we found that the sTWEAK levels of the RA group that comprised patients who were in remission or had low disease activity were lower than those of the control group. sTWEAK had a weak positive correlation with BMI and a weak negative correlation with fetuin-A levels. Previous studies have demonstrated an inverse correlation between increased CVD risk and sTWEAK levels.³¹⁻³³ The low sTWEAK levels found in RA patients suggest that because of the nature of the diseases, risk of CVD and subclinical atherosclerosis may exist even if RA subsides.

Lower levels of sTWEAK than those of other cytokines have a protective effect against the risk of CVDs associated with a high chronic inflammatory activity; however, the underlying mechanism for this is not clearly understood. Some hypotheses were suggested to explain this. One hypothesis suggests that serum sTWEAK levels decrease because of binding to the Fn14 receptor. An alternative hypothesis stated the cause as the increase in sCD163 and decrease in sTWEAK in carotid atheroma plaques.³⁴

Another molecule that has recently been demonstrated to be associated with atherosclerosis is fetuin-A. Fetuin-A, a protein secreted primarily by the liver, has been associated with subclinical markers of CVD, insulin resistance, type 2 diabetes, and incidence of total CVD, particularly ischemic stroke.³⁴⁻³⁹ However, the concentrations at which fetuin-A has a deleterious effect have not been identified. Several studies have reported contradictory results regarding the role of fetuin-A in rheumatologic diseases. Sato et al.³⁸

reported decreased levels of fetuin-A in patients with RA. Biswas et al.³⁹ reported that RA patients had significantly higher fetuin-A levels in the synovial fluid than the healthy controls. Tekeoglu et al.⁴⁰ found higher fetuin-A levels in RA patients than in healthy controls. Furthermore, in our study, the RA group had higher fetuin-A levels than the control group; however, the sTWEAK levels were lower in the RA group. A significant negative correlation was found between fetuin-A and sTWEAK levels. This result is in line with the established association of increased fetuin-A levels and decreased sTWEAK levels with CVD risk that is known to be increased in RA.

Our study has some limitations. First, we did not quantify the subclinical atherosclerosis. Moreover, data were collected using a cross-sectional research design. We excluded the RA patients with high disease activity from the study. We could have included such a group and investigated its relationship with the group of RA patients with low disease activity.

In conclusion, the main purpose of this study was to investigate, using some current molecules, whether metabolic risk persists in RA patients with low disease activity who are undergoing treatment. We found lower sTWEAK levels and higher fetuin-A levels in RA patients compared to those in the controls. We also observed that these two molecules are associated with each other. We demonstrated that in RA patients, some molecules associated with increased metabolic and cardiovascular risk continue to pose a risk even if the disease is controlled with treatment. Follow-up studies on larger populations are warranted to confirm our findings.

Declaration of conflicting interests

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