The Evaluation of Cardiovascular Risk Parameters Using Multiplex Immunoassay

Primer Hiperlipidemik ve Obez Hiperlipidemik Bayanlarda Multiplex İmmunoassay Yöntemi Kullanılarak Kardiyovasküler Risk Parametrelerinin Değerlendirilmesi

ABSTRACT
Objective: In order to detect the damage caused by obesity and hyperlipidemia to the cardiovascular system in the early period, parameters that will be used for follow up of these diseases and provide information about the changes are needed. It is of course valuable to measure multiple parameters simultaneously with fast, high efficiency and less sample quantity in the measurement of these parameters. Material and Methods: We have studied serum levels of cardiovascular risk parameters (E-Selectin, follistatin, PAPP-A, sPECAM-1, pentraxin-3 and tissue factor) using the multiplex immunoassay method in women primarily hyperlipidemic and obese hyperlipidemic. In addition, lipid profile, liver enzymes and blood glucose in the serum of the participants were measured by routine methods. We have studied these parameters in serum of 30 healthy women, 28 primary hyperlipidemic and 32 obese hyperlipidemic women. Results: We found that E-selectin (p<0.01) obese hyperlipidemic group were significantly higher than control group. Serum follistatin levels were significantly higher in obese hyperlipidemic group (p<0.001) and primary hyperlipidemic group (p<0.01) than control group. Moreover serum sPECAM-1 (p<0.001) levels were significantly higher in obese hyperlipidemic group and primary hyperlipidemic group than control group. We are not found significantly variance in the serum PAPP-A, pentraxin-3 and tissue factor levels of the groups. There were no significant differences between serum PAPP-A, pentraxin-3 and tissue factor levels of the groups. Conclusion: Our findings suggest that hyperlipidemia associated with obesity may be the cause of cardiovascular disease rather than hyperlipidemia.

Keywords: E-Selectin; follistatin; PAPP-A; sPECAM-1; pentraxin-3; tissue factor

ÖZET
Amaç: Öbezite ve hiperlipideminin kardiyovasküler sistemde verdiği hasarı erken dönemde ortaya koyabilmek için bu hastaların takibi altında kullanılacak ve değişiklikler hakkında bilgi verecek parametreler gerekşinidir. Bu parametrelerin ölçüldüğü ve eşzamanlı olarak birden fazla parametre hakkında hızlı, yüksek verimli ve daha az numane miktarı ile saptanması elbette değerli olacaktır. Gereç ve Yöntemler: Bu çalışmamızda obez hiperlipidemik kadınlarda multiplex immunoassay yöntemi kullanılarak kardiyovasküler risk parametrelerinin (E-Selektin, follistatin, PAPP-A, sPECAM-1, pentraxin-3, doku faktörü) serumda seviyeleri araştırılmıştır. İletken, Katılımcıların serumlarına lipid profili, karaciğer enzimleri ve kan glucose rutin yöntemler ile ölçülmüştür. 28 primer hiperlipidemik, 32 obez hiperlipidemik ve 30 sağlıklı kadınların serumda hedeflenen parametreler ölçülmüştür. Bulgular: Çalışmamızda obez hiperlipidemik grupa ait E-Selektin düzeyleri kontrol grubuna göre önemli derecede yüksek bulunmuş (p<0.01). Obez hiperlipidemik gruba ait serum follistatin (p<0.001) ve hiperlipidemik gruba ait serum follistatin düzeyleri (p<0.01) kontrol grubuna göre önemli derecede yüksek bulunmuş. Bunun yanı sıra obez hiperlipidemik ve hiperlipidemik gruba ait serum sPECAM-1 (p<0.001) düzeyleri kontrol grubuna göre önemli derecede yüksek bulunmuştur. Gruplara ait serum PAPP-A, pentraxin-3 ve doku faktörü düzeylerinde önemli bir istatistik fark bulunmamıştır. Sonuç: Bulgularımız, obeziteye birlikte ortaya çıkan hiperlipideminin sadece hiperlipidemi varlığından daha çok kardiyovasküler hastalıkların oluşmasında etken olabileceğini göstermektedir.

Anahtar Kelimeler: E-Selektin; follistatin; PAPP-A; sPECAM-1; pentraxin-3; doku faktörü

Hyperlipidemia means that the lipid levels present in the plasma are higher than expected normal values.1 Hyperlipidemia, as a result of lipid accumulation under the intima layer, causes vascular disorder called atherosclerosis which causes cellular-humoral reactions.1,2 Obesity

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increases cardiovascular risk through risk factors such as increased fasting plasma triglycerides, high low density lipoprotein cholesterol (LDL-cholesterol), low high density lipoprotein cholesterol (HDL-cholesterol), elevated blood glucose and insulin levels and high blood pressure. Novel lipid dependent, metabolic risk factors associated to obesity are the presence of the small dense LDL phenotype, postprandial hyperlipidemia with accumulation of atherogenic remnants and hepatic overproduction of apoB containing lipoproteins. The prevalence of obesity in men and women in Turkey has substantially increased over the past 20 years. Among adults obesity is more common among men and more prevalent among women in Turkey.4 Cardiovascular diseases occur 10 years later in women than men. Despite this time advantage, cardiovascular diseases are among the most common diseases in women.5

Follistatin is a single chain glycosylated protein and is expressed from various tissues. The main task of follistatin is binding and neutralizing transforming growth factor β superfamily, including activin, myostatin, and bone morphogenetic protein. Emerging evidence indicates that follistatin also serves as a stress responsive protein, which plays a protective role under a variety of stresses. It has been reported to play an important role in the atherosclerosis process where it regulates the foam cell formation.7 Pentraxin-3 is an acute phase protein that has recently been shown to play pleiotropic activities in cardiovascular diseases. Tumor necrosis factor and interleukins up-regulates pentraxin-3 transcription in different cell types involved in atherogenesis. Pentraxin-3 acts as a modulator molecule of the complement system, inflammatory response, angiogenesis and vascular/tissue remodeling by interacting with a large number of ligands.8 Studies have shown that pentraxin-3 has a useful role in atherosclerotic plaque development and fragility.8 It is a biomarker of atherosclerosis and correlates with the risk of vascular events.9,10 Soluble E-selectin (sE-selectin) is a protein expressed from the surface of stimulated endothelial cells during the inflammatory process. This phenomenon is strongly induced by inflammatory mediators such as interleukin-1, tumor necrosis factor α or interferon γ.9 It is reported that high sE-selectin levels may be used as a new marker of inflammation and vascular damage.11 Soluble platelet-endothelial cell adhesion molecule-1 (sPECAM-1) is localized on the membranes of activated platelets and leukocytes and on the vascular endothelium. It has been thought to play an important role in the inflammation process and leukocyte-endothelial interaction.12 Pregnancy-associated plasma protein-A (PAPP-A) is a zinc-binding metalloproteinase protein and its peripheral blood levels have recently been suggested as a biological marker for acute coronary syndrome.13,14 Tissue factor is an upstream component of the cascade and a high-expressing factor under pathological conditions. Pathological release of the tissue factor has been reported to occur in macrophage derived foam cells, smooth muscle cells in atherosclerotic cells.15

In order to detect the damage caused by obesity and hyperlipidemia to the cardiovascular system in the early period, parameters that will be used for follow up of these diseases and provide information about the changes are needed. It is of course valuable to measure multiple parameters simultaneously with fast, high efficiency and less sample quantity in the measurement of these parameters. In this context, we have studied serum levels of cardiovascular risk parameters (sE-selectin, follistatin, PAPP-A, sPECAM-1, pentraxin-3 and tissue factor) using the multiplex immunoassay method in women primary hyperlipidemic and obese hyperlipidemic.

MATERIAL AND METHODS

PARTICIPANTS

This study was made on 28 primary hyperlipidemic women, 32 obese women and 30 healthy normal weighed women aged 18–70. Obese and hyperlipidemic patients who came to our hospital for routine controls were included in the study. In our general inquiries, both obese and hyperlipidemic individuals have been diagnosed for at least 5 years or more. All measurements were made by the same physician in the Konya Training and Research Hos-
hospita Internal Diseases Clinic. Body mass index (BMI) and waist circumference measurements were used as obesity criteria. BMI was calculated with the weight (kg)/height (m²) criteria and the obese group included those with a BMI higher than 30 kg/m². Classification based on waist circumference was done as follows; in order to make a complete distinction in our cases, women with waist circumference shorter than 80 cm was included in the normal group and those with waist circumference longer than 88 cm were included in the obese group.

For the patients with hyperlipidemia, lipid profiles were studied after 12 hours of fasting, and women with total cholesterol higher than 200 mg/dL, LDL-cholesterol higher than 150 mg/dL and with no cardiovascular events, no antilipemic treatment within the last 6 months were included in our study. In addition, cases with situations which can cause secondary dyslipidemia (such as diabetes, obesity, uncontrolled hypothyroidism, nephritic syndrome, renal failure, liver failure, hepatobiliary diseases, oral contraceptive/hormone replacement treatment, glucocorticoid and use of alcohol at addiction level) were left out of the study.

Control cases were selected among voluntary healthy women who have no clinical complaints and no clinical findings and those with BMI values lower than 25 kg/m² were included in the study. The weight and height measurements of women in the primary hyperlipidemic, obese and control groups were made with “weight height scale” and waist and hip circumference measurements were made using tape measure. The blood pressure measurements of the women participating in the study were made after a rest for at least 10 minutes with a calibrated sphygmomanometer. All persons participating in the study were given written consent and informed verbally before the blood was taken. Approval for the study was obtained from Necmettin Erbakan University, Meram Medical Faculty Research and Application Hospital Ethics Board (14567952-050).

Adequate blood samples were taken to flat tubes from the patients after 12-14 hours of fasting in the morning hours. Blood samples in the flat tubes were centrifuged immediately after coagulation and serum was separated. Serum samples were stored at -80°C until the day of study. In serum samples, parameters of sE-Selectin, follistatin, PAPP-A, sPECAM-1, pentraxin-3, tissue factor, glucose and lipid panels were studied.

MEASUREMENT OF CARDIOVASCULAR RISK PARAMETERS USING THE MULTIPLEX IMMUNOASSAY METHOD

Multiplex immunoassays, or MILLIPLEX® MAP (multiple analyte profiling): A technique that enables analysis of more than 50 proteins in each well with very few samples (multiple ELISA). MILLIPLEX® MAP analysis panels contain microscopic, polystyrene beads (magnetic or non-magnetic) conjugated antibodies and work with Luminex® xMAP® technology.

Measurements were made similar to the ELISA technique following the protocol in the brochure that came in kit. Briefly, magnetic beads were incubated with beads in 96-well plates with samples, standards and quality control, antibody was incubated with Streptavidin-PE, and washing was made between incubation steps. Different beads have different spectral addresses. These beads can be mixed with a single test sample to form antibody-antigen complex, and the analyte due to bead color code, the PE signal obtained when the analyte was found and quantitation of that analyte was read with a Luminex device (MAGPIX®) equipped with xPONENT software.

MEASUREMENT OF OTHER ANALYTES

Serum total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and blood glucose was measured by commercially available kits based on routine methods by the Abbott Architect C16000 auto-analyzer (Architect C16000 auto-analyzer; Abbott Laboratory, Abbott Park, IL, USA).

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 16.0 program. Study groups were tested by one-
way analysis of variance (ANOVA). Tukey’s HSD (Tukey’s honestly significant difference) test was used among the multiple-comparison (post-hoc) tests among the significant groups. The mean values of the data were given combined with the ± standard deviation (SD). All of the tests were considered p <0.05 significant.

RESULTS

Demographic characteristics of primary hyperlipidemic, obese hyperlipidemic and control group are given in (Table 1). As seen in table 1, BMI (p<0.01), waist circumference (p<0.001) and systolic blood pressure (p<0.05) of the hyperlipidemic group were significantly higher than the control group. In addition, BMI (p<0.001), hip circumference (p<0.001), systolic and diastolic blood pressures (p<0.01) were significantly lower in the hyperlipidemic group than in the obese hyperlipidemic group. Also, the weight, BMI, waist circumference, hip circumference, systolic and diastolic blood pressure of the obese hyperlipidemic group were significantly higher than in the control group, and height was significantly lower than the control group (p<0.01 for the height, p<0.001 for the rest). There was no significant statistical difference in the age of primary hyperlipidemic, obese hyperlipidemic and control cases.

The levels of biochemical parameters of the primary hyperlipidemic, obese hyperlipidemic and control group are given in (Table 2). As seen from table 2, total cholesterol (p<0.001) and LDL-cholesterol (p<0.001) of the primary hyperlipidemic group were significantly higher than the control group. In addition, the glucose value of the primary hyperlipidemic group (p<0.01) was significantly lower than the obese hyperlipidemic group. Also, (p<0.001), AST (p<0.05), ALT (p<0.05), total cholesterol (p<0.001), triglyceride (p<0.01) and LDL-cholesterol (p<0.001) of the obese hyperlipidemic group were significantly higher than the control group. No significant statistical difference was found in HDL-cholesterol levels of primary hyperlipidemic, obese hyperlipidemic and control cases.

The cardiovascular risk parameters of the primary hyperlipidemic, obese hyperlipidemic and control group measured using group multiplex immunoassay method are given in (Table 3). As seen in Table 3, sE-Selectin (p<0.01) levels of the obese hyperlipidemic group were significantly higher than the control group. Serum follistatin (p<0.001) of the obese hyperlipidemic group and serum follistatin (p<0.01) levels of the hyperlipidemic group were significantly higher than the control group. In addition, serum sPECAM-1 (p<0.001) levels of obese hyperlipidemic and hyperlipidemic group were significantly higher than control group. There was no significant statistical difference in serum PAPP-A, pentraxin-3 and tissue factor levels of primary hyperlipidemic, obese hyperlipidemic and control cases.

### TABLE 1: Demographical characteristics of the primary hyperlipidemic, obese hyperlipidemic, and control group.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=30)</th>
<th>Primary Hyperlipidemic (n=28)</th>
<th>Obese Hyperlipidemic (n=32)</th>
<th>Mean comparison</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>38.30±12.5</td>
<td>40.64±12.4</td>
<td>36.21± 2.4</td>
<td>0.395</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>59.46±5.9</td>
<td>68.14±9.3</td>
<td>93.55±18.9</td>
<td>p&lt;0.001</td>
<td></td>
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<tr>
<td>Height (cm)</td>
<td>162.70±6.2</td>
<td>158.71±6.8</td>
<td>156.62±13.4</td>
<td>p&lt;0.047</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.26±1.9</td>
<td>25.95±3.9</td>
<td>36.18±5.6</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>77.33±4.9</td>
<td>87.03±11.7</td>
<td>108.50±11.5</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>100.01±8.8</td>
<td>104.18±8.7</td>
<td>124.19±10.4</td>
<td>p&lt;0.001</td>
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<tr>
<td>Systolic blood pressure</td>
<td>120.6±7.0</td>
<td>126.9±12.0</td>
<td>138.1±11.0</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>77.3±8.0</td>
<td>79.6±11.0</td>
<td>88.1±10.0</td>
<td>p&lt;0.001</td>
<td></td>
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</tbody>
</table>

All values are mean±standard deviations. One-way ANOVA for means comparison, *p<0.001, *p<0.001, *p<0.05 when compared with the control group, *p<0.001, *p<0.01 when compared with the obese hyperlipidemic group.
DISCUSSION

Hyperlipidemia causes vascular disorder called atherosclerosis that causes cellular-humoral reactions, as a result of lipid accumulation under the intima layer of the vessels. Inflammation plays an important role in the development and progression of atherosclerosis and therefore in the situation of cardiovascular disease. Various inflammatory markers have been found to be associated with serum lipid levels and atherosclerosis process. These include interleukin 1β, C-reactive protein, pentraxin-3, serum amyloid A and PAPP-A. In addition to being an inflammatory marker, PAPP-A is also effective in the workplace, and it also holds a different place compared to other markers particularly in macrophage activation and colonization.

In our study, serum PAPP-A levels were found to be higher in both the hyperlipidemic group and the obese hyperlipidemic group than the control group. However, this quantity statistic is not significant in terms of statistics. We believe that statistical significance can be achieved in a study with a larger patient population. When we review the literature, we saw results that support our findings. Bayes-Genis et al. found that circulating PAPP-A levels were increased in both the non-stable angina patients and the vulnerable atherosclerotic diseases in their study, and they expressed this as a determinant of atherosclerosis. Cosin-Sales et al. have found findings that support the results of Bayes-Genis et al. in also patients having stable angina pectoris diagnosed with diagnostic coroner angiography. Another study also suggested that PAPP-A might be a marker for future events in patients with acute coronary syndrome. Elesber et al. as a result of their studies, suggested that people with stable coronary artery diseases increased levels of PAPP-A may be a predictive marker in all-cause mortality.

In our study, serum sE-Selectin levels were significantly higher in the obese hyperlipidemic
group when compared with the control group. Literature findings also supported our results. Egert et al. found a statistically significant positive correlation between decreases in body fat mass and decrease in serum sE-Selectin levels in studies involving dietary restriction for 6 months on overweight and obese subjects with metabolic syndrome.\textsuperscript{21} Another study revealed gender differences in serum sE-Selectin levels.\textsuperscript{22} Szabova et al. found that serum sE-Selectin levels were higher in only obese women other than obese men in the study they performed compared to the control group.\textsuperscript{22} Ito et al. took obese women without diabetes and blood pressure into a diet program for 3 months and observed a significant decrease in serum sE-selectin level in parallel with significant weight loss after 3 months.\textsuperscript{23} In addition, they reported that serum sE-selectin levels were significantly positive correlated with total body fat. It has been reported that endothelial dysfunction may be important in metabolic syndrome. sE-selectin, which is regarded as an endothelial dysfunction marker, is associated with insulin resistance.\textsuperscript{11,24,25} This is accompanied by weakened carbohydrate oxidation and lipid oxidation. Adamska et al. have done a study that supports the above information.\textsuperscript{24} According to the results of the study, they found serum sE-selectin levels to be high in obese women who showed high propensity to metabolic syndrome and they stated that insulin sensitive had negative correlation with serum sE-selectin level. Johnston found a higher level of sE-selectin in mouse models of atherogenic dyslipidemia when compared to the control group.\textsuperscript{26} Nomura et al. found significantly increased serum sE-selectin levels in hyperlipidemic subjects compared to the control group.\textsuperscript{27}

In our study, no significant statistical difference was found in serum levels of pentraxin-3 in hyperlipidemic, obese hyperlipidemic and control cases. According to our findings, it can be said that the serum pentraxin-3 levels of the women in the control group were higher than the women in the other two groups. Witasp et al. also reported that serum pentraxin-3 levels were negatively correlated with BMI and waist circumference in obese subjects, and that the same correlation also occurred in non-obese individuals.\textsuperscript{28} In addition, they also reported that serum pentraxin-3 levels increased in parallel with weight loss. Miyaki et al. supporting this study, found that serum pentraxin-3 levels were higher in normal weight subjects compared to overweight and obese subjects.\textsuperscript{29} Sahin et al. have also achieved results that support the findings of the above researchers in obese women only.\textsuperscript{30} According to findings, serum pentraxin-3 levels showed negative correlation with BMI and insulin resistance.

Obesity level and energy balance modulate interactions between pentraxin-3 and systemic inflammation.\textsuperscript{31} The association between pro-inflammatory cytokines related potential adaptive changes and increased pentraxin-3 levels is a new finding and many studies have revealed pentraxin-3 as a new negative inflammatory marker for obesity.\textsuperscript{32} Jylhätä et al. reported a positive correlation between serum pentraxin-3 concentration and HDL-cholesterol in hypercholesterolemic subjects.\textsuperscript{32} We believe that the difference in pentraxin-3 levels among groups in a study with a higher number of patients may be statistically significant.

There are many reports in the literature that follistatin is associated with obesity and diabetes, especially in experimental studies on rodents.\textsuperscript{33,34} In vitro studies have suggested that follistatin is associated with inflammation and has been reported to enhance secretion of tumor necrosis factor α.\textsuperscript{34} Brandt et al. found that plasma follistatin levels were higher in the obese than in the control group, regardless of glycemic status.\textsuperscript{33} In addition, researchers also noted that plasma follistatin levels correlate positively with fat mass and plasma leptin levels in their studies. It is reported that obesity is associated with chronic low grade inflammation. Follistatin is claimed to be a new proinflammatory cytokine expressed from adipose tissue. In addition, follistatin has been found to cause inflammation in both adipose tissue and in macrophages.\textsuperscript{34} In our study, follistatin levels were also found to be significantly increased in both the obese and hyperlipidemic groups when compared with the control group. Our results support the literature findings.
Unlike the literature, we have shown that high follicle-stimulating hormone (FSH) and insulin resistance. It has been reported that inflammation, hepatic steatosis, and inflammation are active in circulation, and some pathological conditions are associated with these conditions. In our study, no significant statistical difference was found in serum tissue factor levels of primary hyperlipidemic, obese hyperlipidemic, and control cases. When we examined the literature, we reached different conclusions from our findings. We think that this difference is caused mainly by the small number of our patients. Ay et al. found that their serum tissue factor levels were significantly higher in 74 morbidly obese patients. They also reported that serum tissue factor levels of morbid obese patients decreased after they lost weight in the same study. It is also stated that coagulation profiles of these patients improve after weight loss.

It has been reported that obese patients are frequently upregulated in the tissue factor following coagulation activation in adipose tissue and are associated with complications of diabetes in particular. Genetic and pharmacological evidence indicates that tissue factor provides important contributions to the development of the metabolic syndrome via G-protein linked protease-activated receptors (PARs). The adipocyte tissue factor-PAR2 signal contributes to diet-induced obesity by reducing metabolism and energy consumption. Whereas hematopoietic tissue factor-PAR2 signaling is an important cause for adipose tissue inflammation, hepatic steatosis, and inflammation as well as insulin resistance. It has been reported that in the liver of mice treated with high fat diets, PAR2 signals increase transcripts of important regulators of pathways such as gluconeogenesis and lipogenesis. Clarification of the prothrombotic universe originating from the tissue factor may help to clarify the metabolic syndrome complications and, at this point, some pathological conditions in obesity. It is stated that hematopoietic cell-derived tissue factor expression causes hyperlipidemia with coagulation activation and increased thrombosis.

In our study, we found that serum sPECAM-1 levels were increased in both our primary hyperlipidemic and obese hyperlipidemic women when compared to the control group. In the literature review, we found that there were few studies showing sPECAM-1 levels in these disease groups. In a study done on rats, Lin et al. found that plasma sPECAM-1 levels increased in a hyperlipidemic rat model. This study supports our findings. We think that we are enriching the literature with the present result.

In conclusion, our findings suggest that hyperlipidemia associated with obesity may be the cause of cardiovascular disease rather than hyperlipidemia. On the other hand, determining the changes in serum levels of these parameters explained and discussed above in primary hyperlipidemic and obese hyperlipidemic women will lead to new developments in the treatment and diagnosis of the disease.

**Source of Finance**

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

**Conflict of Interest**

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

**Authorship Contributions**

**Idea/Concept:** Ü. Betül Bacak, Fatma Hümeýra Yerlikaya; **Design:** Ü. Betül Bacak, Fatma Hümeýra Yerlikaya; **Control/Supervision:** Fatma Hümeýra Yerlikaya; **Data Collection and/or Processing:** Ümmügülüm Can, Murat Baş; **Analysis and/or Interpretation:** Ümmügülüm Can, Fatma Hümeýra Yerlikaya; **Literature Review:** Ü. Betül Bacak; **Writing the Article:** Ü. Betül Bacak, Fatma Hümeýra Yerlikaya; **Critical Review:** Ü. Betül Bacak, Ümmügülüm Can; **Materials:** Murat Baş.
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