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RESEARCH

Levels of oxidative stress and apoptosis-related biomarkers in endometriosis

Endometrioziste oksidatif stres ve apoptozla ilişkili biyobelirteçlerin düzeyleri

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Abstract

Purpose: In this study, the apoptosis marker M30, the oxidative stress markers malondialdehyde, (MDA) and asymmetric dimethyl arginine (ADMA) have been studied in the context of endometriosis.
Materials and Methods: This prospective case-control study comprises 31 patients diagnosed with endometriosis and 31 controls. ADMA and M30 levels in blood serum were measured by the enzyme-linked immunosorbent assay (ELISA) method, and MDA levels were measured by the spectrophotometric method. In addition, some biochemical parameters and cancer antigen-125 (CA-125) levels were also measured.
Results: M30 levels were statistically lower in endometriosis patients (271.5 IU/L) than in controls (371.3 IU/L). ADMA levels were higher in endometriosis patients (19.3 ng/L) compared to controls (12.7 ng/L). CA-125 levels were statistically higher in the endometriosis patients (65.1 U/mL) compared to the controls (19.0 U/mL). There was no significant difference between the two groups in MDA levels. The results regarding dyspareunia, pelvic pain, AST, and ALP were statistically significant.
Conclusion: In our study, decreased M30 levels in the patient group were associated with reduced apoptosis in endometriosis. ADMA levels, elevated with the increase of oxidative stress, were higher in the patients. MDA levels, an indicator of increased oxidative stress, were also higher in the patient group. This study constitutes the first data regarding endometriosis patients' ADMA, M30, and MDA levels.
Keywords: Apoptosis, ADMA, endometriosis, MDA, M30

Öz

Amaç: Bu çalışmada apoptoz belirteci M30 ve oksidatif stres belirteçleri malondialdehit (MDA) ve asimetrik dimetil arjinin (ADMA) endometriozis de düzeyleri incelenmiştir.
Gereç ve Yöntem: Bu prospektif vaka-kontrol çalışması, endometriozis tanısı alan 31 hasta ve 31sağlıklı kontrol grubundan oluşmaktadır. Kan serumundaki ADMA ve M30 düzeyleri enzim bağlı immünosorbent assay (ELISA) yöntemiyle, MDA düzeyleri ise spektrofotometrik yöntemle ölçüldü. Ayrıca bazı biyokimyasal parametreler ve kanser antijen (CA-125) düzeyleri de ölçüldü.
Bulgular: M30 düzeyleri endometriozis grubunda (271,5 IU/L), kontrol grubuna (371,3 IU/L) göre istatistiksel olarak daha düşük bulundu. ADMA seviyeleri endometriozis grubunda (19,3 ng/L) kontrol grubuna (12,7 ng/L) göre daha yüksekti. CA-125 düzeyleri endometriozis grubunda (65,1 U/mL) kontrol grubuna göre (19,0 U/mL) istatistiksel olarak yüksekti. MDA düzeylerinde iki grup arasında anlamlı fark yoktu. Sonuçlar disparoni, pelvik ağrı, AST ve ALP açısından istatistiksel olarak anlamlıydı.
Sonuç: Çalışmamızda hasta grubunda azalmış M30 seviyeleri endometrioziste azalmış apoptoz ile ilişkilendirildi. Oksidatif stresin artmasıyla yükselen ADMA düzeyleri hastalarda daha yüksekti. Artmış oksidatif stresin bir göstergesi olan MDA düzeyleri de hasta grubunda daha yüksek bulundu. Bu çalışma, endometriozis hastalarında ADMA, M30 ve MDA düzeylerinin bir arada yer alması açısından ilk verileri oluşturmaktadır.
Anahtar kelimeler: Apoptoz, ADMA, endometriozis, MDA, M30

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INTRODUCTION

Endometriosis is a gynecological disease generally found in women of regenerative age and associated with pelvic agony and barrenness. Endometriosis is described by endometrial tissue outside the uterus, particularly in the pelvic peritoneum. It is estimated that 10-15% of women of reproductive age are affected by endometriosis, and 70% of women presenting with chronic pelvic pain are diagnosed with endometriosis[1]. The gold standard for diagnosing endometriosis requires visual evidence that can be obtained by laparoscopy and biopsy for pathological confirmation[2]. It has been determined that CA-125 and CA-19-9 levels are elevated in endometriosis, but their reliability in diagnosis is limited. Although there is controversy about using CA-125, it is the most studied among noninvasive diagnostic tests[3].

Although many theories exist, endometriosis pathogenesis is still not elucidated. The retrograde menstruation pathway is known as the most plausible mode of spread of endometriosis[4]. Another potential cause is the localized response to estrogen receptor stimulation in the pelvis area[5]. Progesterone resistance is now considered a part of the pathogenesis of endometriosis[6]. In addition, studies show that immunological changes, genetic factors, and environmental factors play an essential role in the development of endometriosis[7].

Oxidative stress (OS) and apoptosis are two of the most important research topics in the study of endometriosis pathogenesis. Apoptosis also plays a vital role in the physiology of endometrial cells[8]. Since estrogen is a mitogen secreted closely and locally from the ovaries, it increases the proliferation of these cells, which blunts the progesterone resistance of these cells, the anti-mitogenic and apoptotic effect of progesterone[9]. Ectopic endometriosis cells are known to display a genetic profile of increased proliferation markers and decreased apoptotic markers[10]. These features explain the aggressive behavior of these cells in women with endometriosis compared to women without the disease[11].

The M30 antigen, one of the apoptosis-specific serum markers, is caspase-cleaved cytokeratin 18 (CK-18)[12]. Located in the cytoskeleton, CK-18 is one of the major substrates of caspases. CK-18 is cleaved in an apoptosis-specific position, resulting in the formation of a new antigenic site in cells that die by apoptosis. The new antigen formed is called the caspase-cleaved CK-18 or M30 antigen. The levels of M30 antigen, which can be released from apoptotic cells and released into the serum, can be easily measured by the ELISA method[13,14]. It has been reported that apoptosis is significantly reduced in tissues with endometriosis compared to normal endometrial tissues, and there may be an inverse relationship between apoptosis and endometriosis[15]. For this purpose, we measured M30 levels, one of the apoptosis markers, and analyzed the relationship between endometriosis and apoptosis.

Reactive oxygen species (ROS), which contribute to the development and growth of endometrial invasion, can promote the development of angiogenesis and neovascularization, endometriosis, and infertility[16]. Endothelial dysfunction is one of the first events in the process of atherosclerosis[17]. Nitric oxide (NO), produced during the oxidation of arginine by nitric oxide synthase (NOS) in endothelial cells, has anti-atherosclerotic effects[18]. Asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of NO synthesis, also suppresses vascular NO production, while symmetrical dimethyl arginine (SDMA), a stereoisomer of ADMA, lacks NO synthesis inhibitory activity[19]. Plasma ADMA levels are associated with elevated C-reactive protein (CRP) levels, a marker of systemic inflammation[20]. In addition, when OS is elevated, ADMA levels also increase[21]. In this study, we measured ADMA levels and analyzed their relationship with OS and endothelial function.

Lipid peroxidation is a well-established mechanism of cellular damage in humans and is used as an indicator of oxidative stress in cells and tissues. Malondialdehyde (MDA) is the most important end product of non-enzymatic lipid peroxidation with harmful effects. MDA measurement is widely used as an indicator of lipid peroxidation, and increased levels of peroxidation products have been associated with various acute, and chronic pathophysiological processes in human and animal models[22]. In this study, MDA levels, an indicator of OS, were measured, and their concentrations were compared with endometriosis patients and the control group.

In this study, we investigate the relationship between apoptosis biomarkers M30, thought to contribute to the pathophysiology of endometriosis, ADMA, and MDA levels, which increase in the presence of oxidative stress, and their relationship with the course of the disease. We showed for the first time that levels of M30 are decreased in endometriosis patients. In

addition, it will contribute to the literature as the first study in which M30, MDA, and ADMA levels are measured simultaneously in endometriosis patients. The relationship of M30 levels, which is a marker of apoptosis, with endometriosis disease and other measured molecules will contribute to the pathophysiology of endometriosis.

MATERIALS AND METHODS

Sample

This study was conducted at Sivas Cumhuriyet University, Gynecology and Obstetrics Clinic and Biochemistry Department between June 2014 and June 2016. The study was designed as a prospective case control. A total of 62 women were included. Ethical approval of the study was obtained from the Sivas Cumhuriyet University Faculty of Medicine Clinical Research Ethics Committee (dated 30.04.2014 and decision number 2014-04/40). Written informed consent was obtained from all individual participants before enrollment. Approximately 31 patients and 31 healthy controls were included, and precisely these volunteers were included in the study. The patient group had 31 women with chronic pelvic pain, severe dysmenorrhea, and infertility, whose endometriosis was confirmed by laparoscopic or laparotomy. The control group consisted of 31 healthy women without endometriosis and any complaints. Study groups were randomly selected in terms of age.

Procedure

Patients and control groups were compared in terms of serum MDA, M30 ADMA, and CA-125 levels. Detailed medical anamnesis was taken, including dysmenorrhea, dyspareunia, and pelvic pain of the participants. Sociodemographic characteristics, clinical findings, and hormone levels were recorded.

The blood samples required for the study were taken by Savaş KARAKUŞ, a specialist doctor from Sivas Cumhuriyet University Faculty of Medicine, Department of Obstetrics and Gynecology. Preoperative samples from the patient and control groups were taken into gel biochemistry tubes. Obtaining and storing serum from blood and experimental protocol was carried out by Dr. Dilara ÜLGER ÖZBEK in Sivas Cumhuriyet University, Faculty of Medicine, Department of Biochemistry. In order to obtain serums from the blood, they were centrifuged at 3000 g for 15 minutes. Serum samples were stored in microcentrifuge tubes in portions at -80°C until the study day.

Measurement of serum ADMA, M30, and MDA concentrations

MDA levels were determined with thiobarbituric acid according to the spectrophotometric assay defined by 'Jain SK' [23]. Serum ADMA and M30 levels were determined with commercially available Human ELISA kits (YH biosearch Laboratory, China). The detection ranges for ADMA (Catalog Number: YHB0416Hu) was 200-60000 ng/L, while the sensitivity was 100.21 ng/L. The detection ranges for M30 (Catalog Number: YHB3501Hu) was 2-600 IU/L, while its sensitivity was 1.01 IU/L. Routine biochemical parameters and hormones were measured by (Beckman Coulter AU5800, USA) instrument.

Statistical analysis

The statistical analysis was performed with the 'Statistical Package for the Social Sciences (SPSS) software (Version 16.0). The number of samples and the power of the study were calculated using G*Power software version 3.1 (Düsseldorf University, Germany). Using the data of another study, when $\alpha=0.05$ $ß=0.20$ $(1-ß)=0.80$, it was decided to include 31 individuals in each group, and the power of the test was determined as ($p=0.80846$). The Shapiro-Wilk test was used to determine the distribution characteristics of the variables and express them as a percentage. Since the data were not normally distributed, the Mann-Whitney U test was used to compare the differences between groups of non-parametric variables. It was expressed as mean, standard deviation (±). The chi-Square test and Fisher Exact Chi-Square test were used in 2x2 layouts. Spearman's Rho correlation coefficients were calculated to evaluate the relationship between our test parameters. Obtained results were assessed at a 95% ($p<0.05$) significance level.

RESULTS

While the age distribution of the study groups was 30.3±7.41 in the control group, it was 34.3±8.59 in the endometriosis group. There was no difference between the groups in terms of age. The groups were also compared in Table 1 in terms of dysmenorrhea, dyspareunia, pelvic pain, alcohol, and cigarette use.

The distinction between the two groups as far as dyspareunia and pelvic pain was found to be significant. While the incidence of dyspareunia in endometriosis patients was 45.2%, the incidence of pelvic pain was 71.0%.

Table 1. Comparison of the characteristics of the groups included in the study.

Variables		Groups		
		Endometriosis (n= 31**)**	**Control (**n= 31**)**	p-value
Smoking	+	7 (21.9 %)	3 (9.7 %)	0.164
	-	25 (78.1 %)	28 (90.3 %)	
Alcohol	+	2 (6.45 %)	0 (0 %)	0.164
	-	29 (93.6 %)	31 (100 %)	
Dysmenorrhea	+	22 (71 %)	16 (51.6 %)	0.118
	-	9 (29 %)	15 (48.4 %)	
Dyspareunia	+	14 (45.2 %)	4 (12.9 %)	0.010 *
	-	17 (54.8 %)	27 (87.1 %)	
Pelvic Pain	+	22 (71.0 %)	12 (38.7 %)	0.011*
	-	9 (29.0 %)	19 (61.3 %)	

Data expressed as % (number), *p<0.05.

At the point when the groups were assessed as far as some hormones and blood parameters, the differentiation between the two groups was tracked down just in terms of AST (p=0.038) and ALP (p=0.038) (Table 2).

Table 2. Comparison of some hormones and blood parameters between groups

Parameters	**Endometriosis (n= 31)**	**Control (n= 31)**	***p*- value**
FSH (mIU/L)	8.04 ± 12.0	6.23 ± 5.54	0.907
E2 (pg/mL)	76.5 ± 53.0	70.9 ± 73.3	0.148
Prolactin (ng/mL)	28.8 ± 27.4	27.2 ± 37.8	0.164
LH (mIU/L)	4.78 ± 3.20	6.27 ± 7.50	0.796
Progesterone (ng/mL)	1.23 ± 1.70	0.86 ± 2.04	0.321
AST (U/L)	16.7 ± 3.41	18.9 ± 4.04	0.038 *
ALT (U/L)	13.7 ± 4.64	17.3 ± 8.41	0.054
ALP (U/L)	52.6 ± 17.2	78.4 ± 20.1	0.038 *

Results are expressed as mean ± Standard Deviation, *p<0.05 Man Whitney U test. (FSH: Follicle stimulating hormone, E2: Estradiol, LH: Luteinizing hormone, AST: aspartate aminotransferase, ALT: Alanine transaminase, ALP: Alkaline phosphatase.)

ADMA, M30, MDA, and CA-125 levels of the groups were compared in Table 3. A statistical difference was also observed in ADMA (p<0.001) levels and it was found to be higher in patients than in the control group (Figure 1). M30 (p=0.003) levels were found to be low in patients compared with the control group, and the difference was statistically significant (Figure 2). CA-125 levels were higher in endometriosis compared to the control group and the difference was statistically significant (p<0.001) (Figure 3). No evaluable distinction was found about MDA levels (p=0.607). Although MDA levels were slightly higher in patients, no statistically significant difference was found.

Table 3. Comparison of ADMA, M30, MDA, and CA-125 levels of the groups.

Parameters	Endometriosis (n= 31)	Control (n= 31)	*p-value*
ADMA (ng/L)	19.3 ± 7.84	12.7 ± 5.44	**0.000 ***
M30 (IU/L)	271.5 ± 99.9	371.3 ± 134.6	**0.003 ***
MDA (mM)	0.25 ± 0.06	0.24 ± 0.05	**0.607**
CA-125 (U/mL)	65.1 ± 56.9	19.0 ± 15.2	**0.000 ***

Results are expressed as mean ± Standard Deviation, *p<0.05 Man Whitney U test. (ADMA: Asymmetric dimethyl arginine, M30: caspase-cleaved cytokeratin 18 antigen, MDA: Malondialdehyde, CA-125: Cancer antigen-125).

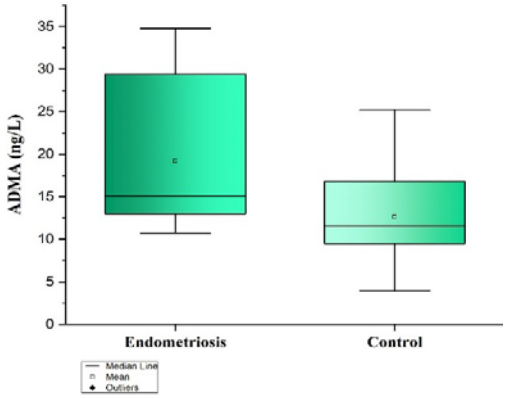

Figure 1. Comparison of the groups in terms of ADMA levels.

(ADMA: Asymmetric dimethyl arginine)

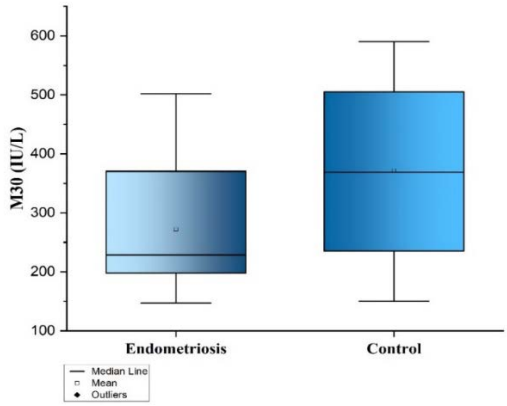

Figure 2. Comparison of the groups in terms of M30 levels.

(M30: caspase-cleaved cytokeratin 18 antigen)

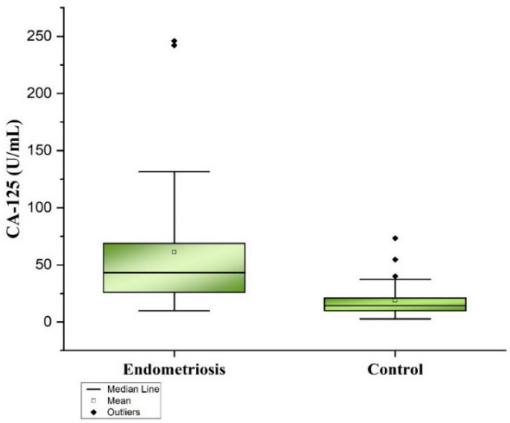

Figure 3. Comparison of the groups in terms of CA-125 levels

(CA-125: Cancer antigen-125).

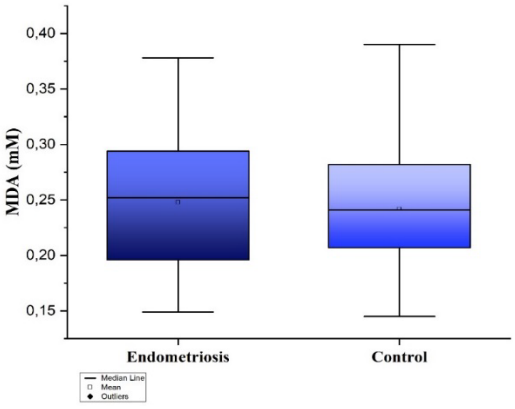

Figure 4. Comparison of the groups in terms of MDA levels

(MDA: Malondialdehyde)

According to the Spearman's Rho correlation test in Table 4, ADMA levels showed a positive low-grade correlation with M30 (r=0.268, p=0.035). M30 levels were moderately negatively correlated with CA-125 (r=-0.393, p=0.002) levels. CA-125 levels showed a positive and moderate correlation in terms of dyspareunia (r=0.418, p=0.003) and pelvic pain (r=0.530, p=0.000). The dyspareunia factor showed a positive low-strong correlation with pelvic pain (r=0.307, p=0.030

Table 4. Spearman's Rho Correlation Test

Variables	Correlation	r	*p*-value
M30	CA-125	-0.378 **	0.002
Dysmenorrhea	Pelvic Pain	0.530 **	0.000
	Dyspareunia	0.418 **	0.003
Dyspareunia	Pelvic Pain	0.307 *	0.030

*The correlation is significant at the 0.05 level (2-tailed). **The correlation is significant at the 0.01 level (2-tailed). (CA-125: Cancer antigen-125, M30: caspase-cleaved cytokeratin 18 antigen)

DISCUSSION

Endometriosis is a chronic and common gynecological disease characterized by hormone-dependent localization of endometrial tissues outside the uterine cavity. The characteristic signs of endometriosis are severe dysmenorrhea, deep dyspareunia, chronic pelvic pain, and infertility. Pelvic pain may occur independently of menstruation. In addition, the problem of infertility, chronic fatigue, and constipation belongs to the clinical picture of endometriosis[24]. In the ongoing study, we assessed the patients by considering the complaints of dysmenorrhea, dyspareunia, and pelvic pain. While dysmenorrhea was observed in 71% of the patients, the presence of this complaint was determined in 51.6% of the controls; what's more, there was no important distinction between the two groups. In addition, a positive and moderately strong correlation was found between dysmenorrhea, dyspareunia and pelvic pain in the correlation test. The complaint of dysmenorrhea is not only related to endometriosis but may also be caused by an underlying cause such as leiomyoma, cervical stenosis, adenomyosis, ovarian cyst, pelvic adhesions, IUD use, and pelvic infections[25]. Therefore, although there was a higher frequency of dysmenorrhea in the patient group compared to the control group, the reason why no significant difference was observed may be the presence of these factors. When the groups were evaluated in terms of dyspareunia and pelvic pain, the difference was statistically significant. Dyspareunia was observed in 45.2% of endometriosis patients and 12.9% of healthy individuals. While 71.0% of patients with endometriosis have pelvic pain, this rate is 38.7% in healthy controls. However, a frail positive relationship was found between dyspareunia and pelvic pain. In line with these complaints, our findings are consistent with the literature.

Studies have reported that the non-invasive CA-125 test is highly expressed in endometriosis patients and can be used to predict recurrence and evaluate therapeutic effects, but does not have acceptable sensitivity and specificity for early diagnosis[26]. In our study, we evaluated CA-125 levels. The levels in the patient group were statistically significant compared to the control group, and their levels were relatively high. Our results are in concurrence with the literature data. In one study, the increase in serum CA-125 was substantially higher in all stages of endometriosis disease[27]. Although the sensitivity of the CA-125 level, which decreases during treatment and increases in case of recurrence, is low, it may be an important parameter in treatment and follow-up. Correlation with different parameters and studies should be supported to be used definitively in diagnosis.

In endometriosis, endometrial cells can show ectopic localization and proliferate in these places. The reason for ectopic settlement is; that it is based on a wide variety of pathological processes such as neoangiogenesis, fibrosis, adhesion formation, immune dysfunction, neuronal infiltration, and avoidance of apoptosis[28]. One of the pathophysiological mechanisms of endometriosis that has been emphasized in recent years is the changes in the regulation of apoptosis. Disruption in the normal functioning of apoptosis in endometrial tissue leads to abnormal implantation and growth of endometrial tissue in ectopic areas[29]. Apoptosis is

genetically programmed cell death that ensures the safe removal of cells that have lost their function, aged, irregularly developed, DNA damaged, or proliferated uncontrollably. There are three main factors involved in the mechanism of apoptosis, death receptors, adapter proteins, and caspases, which are proteolytic enzymes[30]. Broken cytokeratin's 18 (CK-18), formed as a result of the effect of apoptosis-specific caspase enzymes in cells that die by apoptosis, is an important apoptosis marker released from tumor cells and used to evaluate epithelial-derived malignant disease processes[31]. During apoptosis, CK-18 is cleaved by caspases at aspartate 238 and aspartate 396, the fragmented fragment of CK-18 in aspartate 396 is known as the M30 antigen biomarker, and this biomarker can be quantitatively measured[32]. Numerous investigations have been led on the regulation of apoptosis in endometriosis. In a meta-analysis by Harada et al., it was reported that apoptosis decreased in endometriosis patients[33]. Based on the hypothesis of reduced regulation of apoptosis in endometriosis patients, we measured M30 levels.

A statistically huge distinction was found between the patient and control groups. M30, detected at very low levels in the patient group compared to the control group, can be interpreted as an indicator of the presence of suppressed apoptosis in endometriosis patients. Our literature review has found no study in which M30 levels have been measured in endometriosis patients to date. Our study constitutes the first data. In the light of this information, supportive studies should be conducted that will contribute to the reliability and pathophysiology of the study.

It is presently broadly acknowledged that OS, characterized as an awkwardness between ROS, and antioxidants, may assume a part in the pathophysiology of endometriosis, causing an overall inflammatory reaction in the peritoneal cavity[34]. Lipid metabolism and its associations with inflammation factors may play a role in the formation of OS. The increase in lipid peroxides can be considered a marker of oxidative stress. MDA has been evaluated as a lipid peroxide index. A limited number of studies examine the level of MDA in endometriosis, and the results could be more consistent and quite different. For example, while Nasiri et al. observed higher MDA levels in the serum of women with endometriosis compared to healthy controls[35], Andrade et al., on the other hand, compared oxidative stress markers in the blood serum of infertile women with/without endometriosis, and while some markers were significant, the contrast between the two groups with regards to MDA levels was found to be insignificant[36]. We wanted to evaluate the disease in terms of oxidative stress by measuring MDA levels in the serum samples of our study group. As a result, there was no statistically significant difference between the two groups in terms of MDA levels. In any case, it was seen that the MDA levels of the patient group were marginally higher than the control group. MDA is a lipid peroxidation product expected to increase under oxidative stress and inflammation. In contrast, MDA levels may remain unchanged in serum, although they increase in the peritoneal fluid due to unknown mechanisms or relatively local inflammatory presence in endometriosis.

Endothelial dysfunction characterizes OS, which can promote the production of ROS and the reduction of NO. There is a relationship between NO and ADMA, the first reducing NO production and the second functioning as an endogenous inhibitor[37]. The formation of ADMA occurs through two complex events. The first is the methylation of the arginine residue in proteins, and the second is the breakdown of these methylated proteins into free amino acids by proteolysis. This degradation produces free ADMA[38].

Moreover, clinical studies have shown that the endothelial-monocyte relationship is mediated by ADMA, which improves how endothelial cells adhere to monocytes[39]. In this study, we planned to examine the conceivable role of ADMA as an indicator of oxidative stress in endometriosis. We found a statistically significant difference between the serum ADMA levels of the patient and control groups. ADMA levels in endometriosis patients were found to be relatively high in contrast to the control group. In case of elevated OS in the body, an increase in ADMA levels occurs, and high ADMA levels may also be associated with impaired endothelial function in women with endometriosis. There are a number of predetermined studies measuring ADMA levels in endometriosis. While ADMA levels increased significantly between patients and controls[40], some did not[41]. It has been suggested that OS causes differences in ADMA levels by creating changes in the activities of some enzymes involved in the production and destruction of ADMA[21]. In this

ongoing study, it is envisioned that the increase in OS causes elevated ADMA levels.

The present study has some limitations that should be considered. Because the study had a small sample size, our results should be confirmed by prospective studies of a large sample group. Another is that measuring enzymatic activities such as SOD, CAT, and GPx against oxidative stress and free radicals will make the study more potent in showing oxidative stress, it is estimated that the increase in OS causes increased ADMA levels.

With this study, we present new findings to confirm the increased oxidative stress and decreased apoptosis levels in endometriosis, which were previously studied. M30 levels measured for the first time in endometriosis patients and seen to decrease may inspire further research. Studies of molecules such as M30, ADMA, and MDA, thought to contribute to pathophysiology, with additional biomolecules and larger sample groups, will contribute to our understanding of biochemical mechanisms. The combined study of molecules such as M30, ADMA, and MDA, which are thought to contribute to pathophysiology, with different biomolecules covering a larger sample group, will contribute to our understanding of biochemical mechanisms.

Author Contributions: Concept/Design : DÜÖ, SK, SB; Data acquisition: DÜÖ, SK; Data analysis and interpretation: DÜÖ, SK; Drafting manuscript: DÜÖ, SK, SB; Critical revision of manuscript: DÜÖ, Final approval and accountability: DÜÖ, SK, SB; Technical or material support: DÜÖ, SK, SB; Supervision: DÜÖ; Securing funding (if available): n/a.
Ethical Approval: Ethical approval of the study was obtained from the Sivas Cumhuriyet University Faculty of Medicine Clinical Research Ethics Committee (dated 30.04.2014 and decision number 2014-04/40). Written informed consent was obtained from all individual participants prior to enrollment.
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