

Analysis of changes in serum levels and gene expression profiles of novel adipocytokines (Omentin, Vaspin, Irisin and Visfatin) and their correlation with serum C-reactive protein levels in women diagnosed with endometriosis

Endometriozis tanısı alan kadınlarda yeni adipositokinlerin (Omentin, Vaspin, Irisin ve Visfatin) serum seviyelerindeki ve gen ekspresyon profillerindeki değişimlerinin analizi ve bu adipositokinlerin serum C-reaktif protein düzeyleri ile korelasyonu

Running head: Novel adipocytokines in endometriosis

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INTRODUCTION

Endometriosis is a painful disorder identified by the growth of endometrium-like tissue such as endometrial glands and stroma, outside the uterus ¹. Endometriosis reduces the quality of life among women due to dysmenorrhea, chronic pelvic pain, irregular uterine bleeding, infertility and affects about 5-10% of women of reproductive age ^{2,3}. Previous studies have reported that 30–40% of infertile women have endometriosis, and they are more likely to have endometriosis than fertile women ^{4,5}.

A standardised approach for the definite diagnosis of endometriosis is laparoscopic visualization of lesions together with histological examination ². Besides, transvaginal ultrasound is an alternative technique to diagnose pelvic endometriosis ⁶. The current gold standard for definitive treatment of endometriosis involves surgical removal of ectopic lesions and/or hormonal suppression ¹. The identification of mechanisms in the pathogenesis of

endometriosis remains critical for reducing various side effects of these treatment options and a high incidence of relapses ⁷.

Chronic inflammation plays a considerable role in the development and progression of endometriosis in the peritoneal cavity ⁸. Adipocytokines are proteins secreted from white adipose tissue and play a role in metabolism, immunity, endocrine system and inflammation regulation and play different and even opposing roles ^{9,10}. Accumulating evidence suggests that pro-or anti-inflammatory actions of adipocytokines and inflammatory markers are partly responsible for the pathogenesis of endometriosis ^{11,12}.

Omentin-1 is a circulating novel hydrophilic adipokine with extensive protective effects in various diseases ¹³. Vaspin known as visceral adipose tissue-derived serine protease inhibitor, is a member of the serine protease inhibitor family and was first identified as an adipokine that is expressed mainly in the visceral adipose tissue of Zucker fatty rats ¹⁴. Irisin has been identified as a novel myokine which is involved in white adipose tissue browning and anti-inflammatory pathways ¹⁵. A ubiquitous intracellular enzyme, visfatin is known also as nicotinamide phosphoribosyltransferase and pre-B-cell colony-enhancing factor ¹⁶.

This study was conducted to investigate a possible role of "omentin, vaspine, irisin and visfatin" adipocytokines called "new adipocytokines" and their association with the inflammatory marker C-reactive protein (CRP) levels in endometriosis.

MATERIALS AND METHODS

Study subjects

Thirty women attending Dr. Zekai Tahir Burak Women's Health Care Education and Research Hospital were enrolled in this study. The women were divided into two groups as control (n=14) and endometriosis (n=16) via ultrasound examination. Blood samples of all participants were collected and divided into test tubes containing K3EDTA and serum-separating tubes after overnight fasting to perform biochemical analysis. Serum was obtained by centrifugation at 400×g for 10 min and both whole blood and serum samples were stored at -70°C until further analysis. This study was conducted with the approval of Ankara University Faculty of Medicine Clinical Research Ethics Committee (19-1296-18) and standard informed consent was obtained from women involved in this study.

Measurement of adipocytokines (Omentin, Vaspin, Irisin) and inflammation marker (CRP) concentrations in serum

The circulating levels of omentin in the serum were assayed using commercially available ELISA kits (Catalog No. E-EL-H2028, Elabscience, Houston, Texas, United States). Serum vaspin and irisin concentrations in the serum were detected by a commercially available ELISA kit [Catalog No. CSB-E09771h (vaspin) and CSB-EQ027943HU (irisin), Cusabio Biotechnology, Wuhan, China]. The minimum detectable concentration of omentin, vaspin, and irisin was 0.33 ng/mL, 7.8 pg/ml and 0.78 ng/ml, respectively. For the omentin ELISA, the intra-assay coefficient of variation (CV) was <4.05%; the inter-assay CV was <3.12%. For the vaspin and irisin ELISA kits, the intra-assay CV was <8%; the inter-assay CV was <10%. A biomarker of inflammation, CRP levels were measured by Beckman Coulter AU680 clinical chemistry analyzer (Beckman Coulter Inc., Brea CA, USA).

RNA Isolation and and cDNA synthesis

Total RNA was isolated from whole blood samples using the RNA isolation kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The concentration of each total RNA was determined by spectrometry (NanoDrop ND-1000 spectrophotometer; Thermo Scientific, Wilmington, DE, USA). Equal amounts of total RNA (200 ng) from each sample were used for the production of complementary DNA (cDNA) using the ProtoScript II

First Strand cDNA synthesis kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol.

Each transcription was performed in reaction buffer containing 200 ng of total RNA (2 μ L for each sample), 50 μ M d(T)₂₃VN (2 μ L), 10 μ L reaction mix (dNTPs and optimized buffer), 2 μ L enzyme mix (reverse transcriptase and RNase inhibitor) and 4 μ L RNase-free water for a total volume of 20 μ L. After brief centrifugation, the reaction mixture was incubated at 42°C for 10 min, and then heated at 80°C for 5 min.

Quantitative real-time RT-PCR assay

For analysis of mRNA level of visfatin and vaspin, the newly synthesized cDNA was quantified on RT-PCR using CFX384 Touch Real-Time PCR System (Bio-rad, Hercules, CA, USA) and Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Each qRT-PCR reaction was carried out in 20 μ L buffer, containing the same volume of the newly synthesized cDNA (2 μ L) from each sample, 10 μ L Master Mix, 1 μ L forward and reverse primer (0,25 μ M), and 7 μ L DNA-free water. The oligonucleotide sequences of the primer pairs which were designed using the Ensemble genome browser and Primer 3 input programs are summarized in Table 1. The thermocycling protocol was performed as follows in duplicate: initial denaturation at 95°C for 1 min, followed by 40 cycles of PCR at 95°C for 15s and 60°C for 30s. After 40 cycles, a melting step was performed at 60°C for 15 s. Relative mRNA expression was quantified by comparison with β -actin as a housekeeping gene from the same sample as an internal control. The relative gene expressions of visfatin and vaspin were computed using the $2^{-\Delta\Delta CT}$ as fold changes relative to those in controls¹⁷. At the end of the RT-PCR, product specificity was verified by using a melting curve.

Statistical analyses

The statistical analyses were conducted using the SPSS software version 18.0. Data were presented as mean \pm SD. Mean values between the groups were compared by Student's t test. Pearson's correlation coefficient was used to identify the relationship between parameters. A $p < 0.05$ was considered statistically significant.

RESULTS

A total of 30 women between the ages of 25-40 years old participated in this study, including sixteen diagnosed with endometriosis and fourteen healthy women. Among the patients in the endometriosis group, the presence of dysmenorrhea was documented in 10 (35.8%) patients, while rest of 6 (64.2%) patients with endometriosis did not have dysmenorrhea.

There were no statistically significant differences in the serum omentin and vaspin concentrations between control and endometriosis groups (p values 0.861; 0.213, respectively; Figure 1). However, serum irisin levels were statistically higher in the endometriotic group than in the control group ($p=0.024$, Figure 2A) and significantly positively correlated with both BMI ($r=0.65$, $p=0.021$) and CRP levels ($r=0.714$, $p=0.023$) in women with endometriosis (Table 2). Serum CRP level was significantly increased in women with endometriosis compared to the control group ($p=0.022$, Figure 2B). In addition, we measured the gene expression of vaspin and visfatin in whole blood samples of women with and without endometriosis. Our results revealed that the mRNA expression levels of vaspin and visfatin in whole blood from endometriosis patients were significantly lower than the control group (p values 0.042; $p=0.00097$, respectively; Figure 3). While no significant association between the levels of vaspin, visfatin and clinical parameters was found in the endometriosis, the visfatin mRNA expression tended to positively correlate with BMI in patients with endometriosis, though without statistical significance ($r=0.5$, $p=0.059$, Table 2).

DISCUSSION

In the present study, we firstly demonstrated that serum irisin and CRP concentrations were significantly higher in women with endometriosis compared to those without the disease. A significant positive correlation between serum irisin levels and both BMI and CRP levels was observed. In particular, decreased visfatin and vaspin gene expression in whole blood samples was remarkable for women with endometriosis compared to healthy controls. There was a trend toward a positive correlation between BMI and visfatin mRNA expression, which was statistically non-significant.

Based on our results, serum omentin and vaspin concentrations did not differ between endometriosis and control groups and were not correlated with any of the parameters. However, vaspin mRNA expression levels were decreased in patients with endometriosis compared to healthy controls. Omentin and vaspin have been known as anti-inflammatory mediators¹⁸⁻²¹, and decreased expression of these adipocytokines was detected in a variety of chronic inflammatory diseases^{22,23}. In the current study, a non-significant partial decrease in vaspin protein level together with significantly decreased vaspin mRNA expression may be related to the small number of subjects and severity of endometriosis in patients. In accordance with the growing evidence suggesting anti-inflammatory effects of vaspin¹⁴, endometriosis occurrence may be associated with decreased vaspin mRNA levels and vaspin could serve as a novel biomarker of endometriosis.

The present study showed that circulating irisin levels were increased in patients with endometriosis compared to control subjects and were correlated with BMI and CRP. Some authors support our findings with reports of higher irisin levels in women with gestational diabetes and polycystic ovary syndrome^{24,25}. These findings are in contrast to other studies which indicated lower serum irisin levels compared to control in inflammation-related diseases^{15,26,27}. Similarly, there was a significant positive correlation between serum irisin concentrations and BMI^{25,28,29}, although other studies suggested an inverse association between irisin levels and BMI^{15,30}. Previous preclinical and clinical studies suggested that irisin may have anti-inflammatory properties, leading to the reduction of secretion of inflammatory cytokines like IL-6 and TNF- α ^{31,32}. It is well established that increased levels of various proinflammatory cytokines play an important role in the pathogenesis of endometriosis³³. The current results led us to speculate that increased circulating irisin may be an adaptive response to compensate for increased inflammation in endometriosis, which was also supported by a positive association between irisin and CRP and its anti-inflammatory properties. We can suggest an "irisin-proinflammatory/anti-inflammatory axis" to elucidate the role of irisin as a possible indicator in endometriosis.

CRP levels were increased in women with endometriosis in the present study. Results reporting CRP levels in peripheral blood of endometriosis patients compared to controls are relatively contradictory due to differences in the severity of endometriosis, different measurement techniques, the sample size of studies³⁴⁻³⁶. We suggest that CRP could reveal subclinical inflammation in serum of women with endometriosis and serve as a biomarker of endometriosis which is considered as a chronic inflammatory disease as reported previously^{37,38}.

Visfatin is considered as a new marker of inflammation, which is in line with reports suggesting that visfatin induces nuclear factor kappa B (NF- κ B) activity and the synthesis of other related pro-inflammatory molecules^{16,39,40}. Since higher relative visfatin mRNA levels in peripheral mononuclear cells from patients with a common inflammatory disease such as polycystic ovary syndrome and diabetes have been reported^{41,42}, we suggested that visfatin might contribute to endometriosis. Our findings demonstrated that visfatin mRNA expression in whole blood from the women with endometriosis was significantly lower than in the controls and tended to positively associate with the BMI, but not statistically significant. Similarly, a study by Seow et al.⁴² found a non-significant correlation between visfatin and BMI in patients with polycystic

ovary syndrome, which might be attributed to an insufficient sample size that might negatively affect the statistical power. Visfatin with its inflammatory properties might be involved in the pathogenesis of endometriosis. New insight into the role of visfatin may be attractive for novel therapeutic strategies targeting endometriosis-related chronic inflammation.

Moreover, there is no adipocytokine known as non invasive biomarkers of endometriosis.

Further studies with larger population will be required to determine whether these novel adipocytokines are involved in a particular developmental stage of the endometriosis and to clarify the mechanism of action of these inflammatory/antiinflammatory adipocytokines under endometriotic conditions. The imbalance between pro- and anti-inflammatory adipocytokines may be an important causative factor in the pathogenesis of endometriosis.

In conclusion, our study revealed, for the first time, decreased visfatin and vaspin gene expression together with increased serum irisin and CRP levels were observed in endometriosis patients compared to control subjects. Our findings suggest that these adipocytokines may have a potential role in the development of endometriosis. Although this study has provided helpful information, many unknown aspects regarding the mechanisms and markers of endometriosis warrant further investigation. Finally, all the data suggest that additional studies are needed to define the significance of novel adipocytokines as prognostic markers and therapeutic targets in etiology of endometriosis disease.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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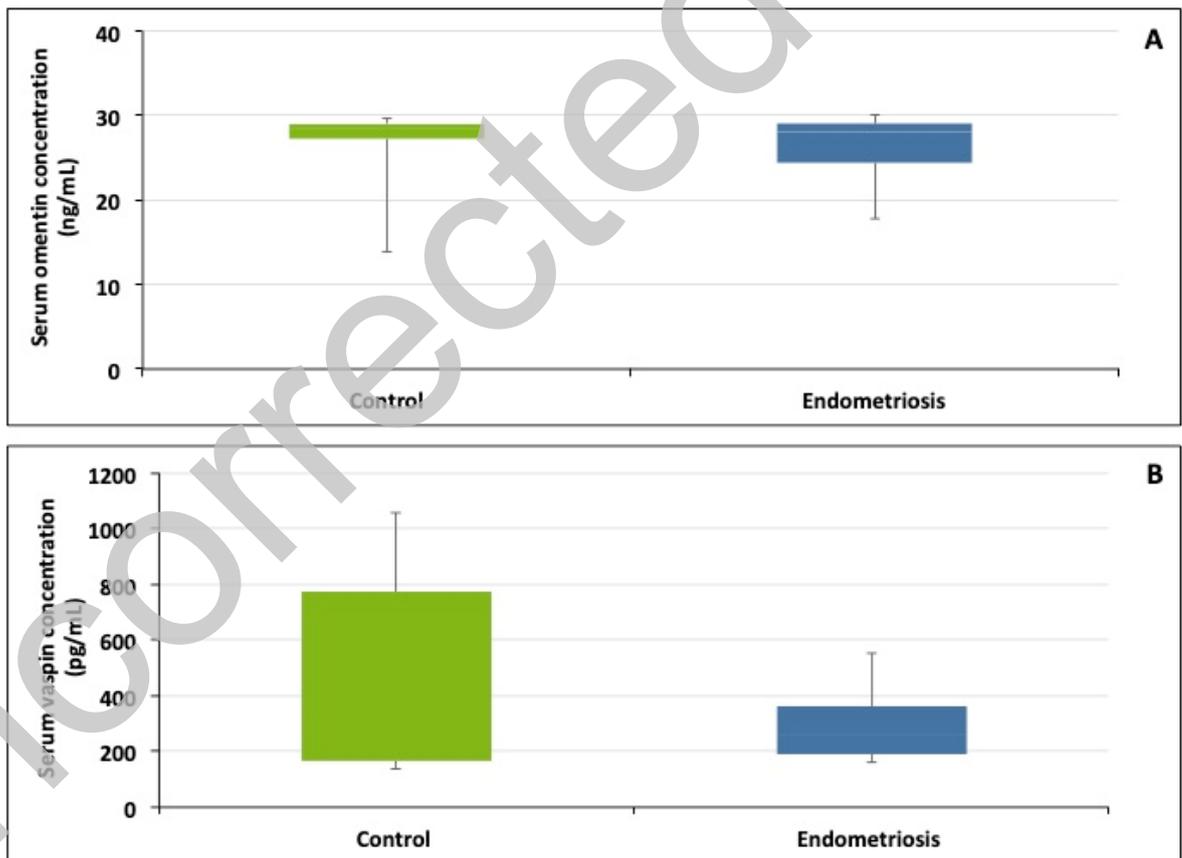
Table 1. Sequences of Primers Used in RT-PCR

Gene	Primer sequences (5'-3')
Vaspin	Forward: TACTGGGGATGTGGGGAGAG
	Reverse: TGTAGGGCCGATGAGTCAGA
Visfatin	Forward: TCGGTTCTGGTGGAGGTTTG
	Reverse: CAAAATTCCTGCTGGCGTC
β-actin	Forward: ACCTGCATTCCTGGGAGTG

Table 2. Correlations between BMI, CRP and adipocytokines in patients with endometriosis

	Serum omentin concentration (ng/ml)		Serum vaspin concentration (pg/ml)		Serum irisin concentration (ng/ml)		Vaspin gene expression		Visfatin gene expression	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
BMI	-0.316		0.422		0.65		0.405		0.5	0.059
	0.101		0.086		0.021*		0.109			
CRP	0.175		0.005		0.714		-0.546		0.079	0.427
	0.266		0.495		0.023*		0.081			

* $p < 0.05$ demonstrates the significance of the correlation

**Figure 1****Figure 1.**

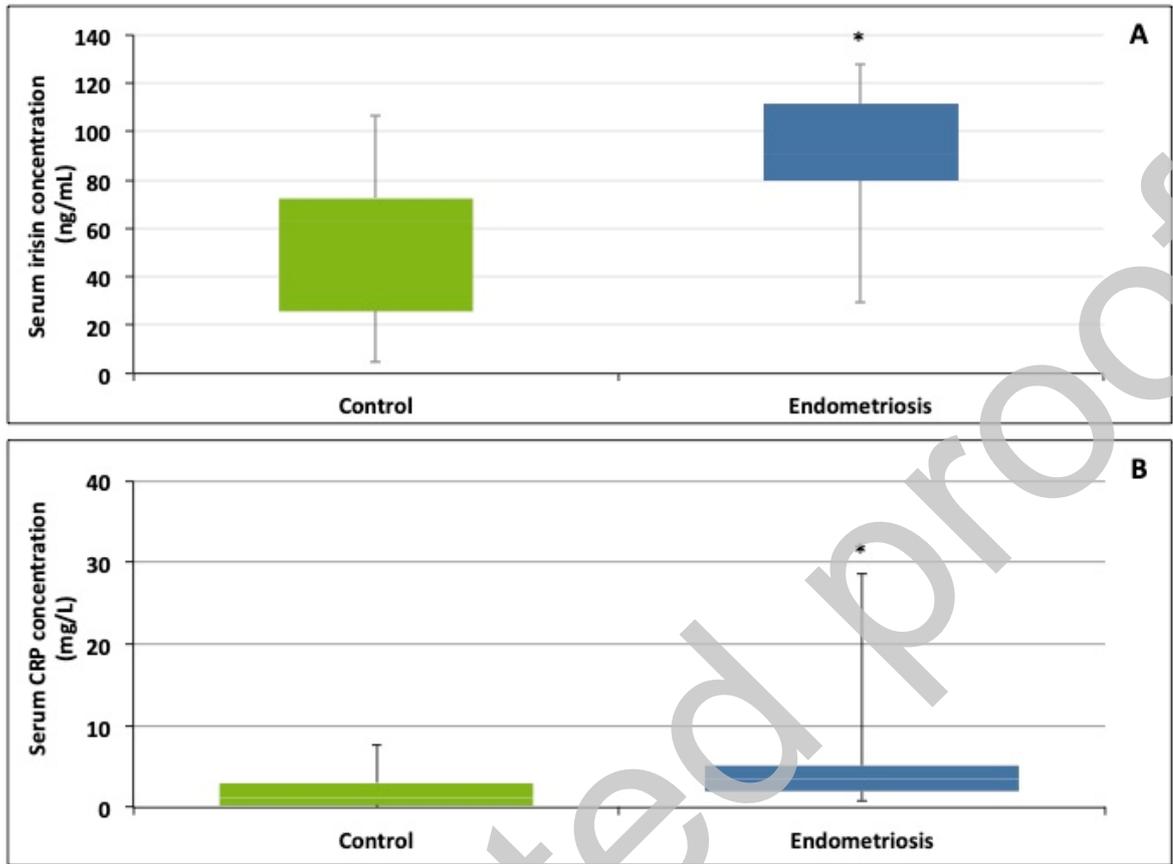


Figure 2.

Figure 2

Uncorrected proof

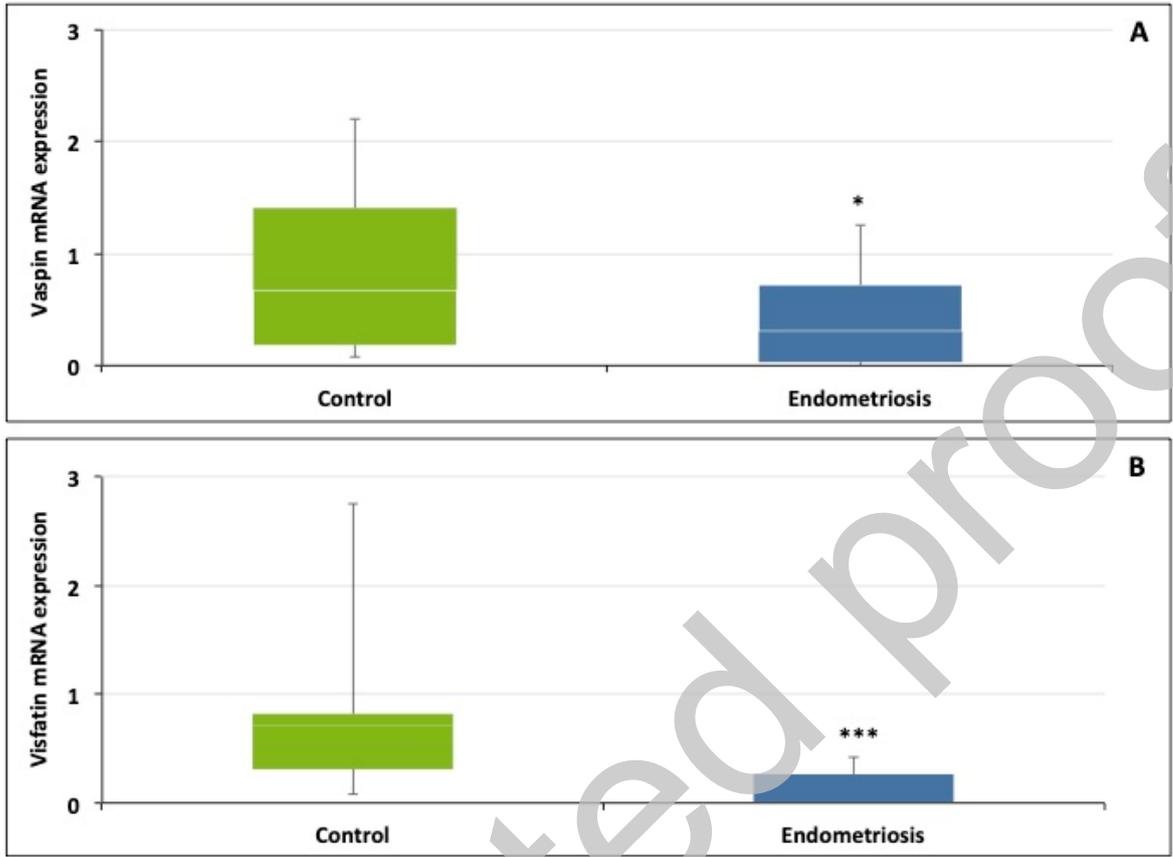


Figure 3.

Figure 3

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