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Original Article

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The Diagnostic Accuracy of Galectin-9 for Diagnosis of Endometriosis in Comparison with Laparoscopy

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Abstract

Background: Endometriosis is a common devastating gynecological disease with severe complications. Researches on noninvasive diagnostic tests with acceptable accuracy are still ongoing. The purpose of the present study was to evaluate the diagnostic value of serum Galectin-9 (Gal-9) level in comparison with laparoscopic results in endometriosis patients.

Methods: Sixty-one patients, referred to Booali, Rasool-e-Akram, and Pars Hospitals affiliated to Islamic Azad University of Medical Sciences, were recruited. Patients laparoscopically diagnosed with endometriosis were assigned to the case (n=32) and who diagnosed with other diseases were assigned to the control group (n=29). In general, 56 patients (30 in case and 26 in control group) completed the study. The serum level of Galectin-9 was measured using ELISA method before laparoscopy and was compared between the groups. Next, categorical variables were compared using Chi square and quantitative variables using independent samples t-test or Mann-Whitney U test. The Gal-9 cut-off was calculated using the Youden's index and ROC curve; then, sensitivity, specificity, positive and negative predictive value, and positive and negative likelihood ratio of Gal-9 were reported. The $p<0.05$ were considered statistically significant.

Results: Mean serum level of Galectin-9 was 669.3±416.50 *pg/ml* in the case group and 265.42±492.30 *pg/ml* in the control group (p=0.001). Considering a cut-off value of 138 *pg/ml*, Galectin-9 had a sensitivity of 100% and specificity of 88.46% for diagnosis of endometriosis ($p<0.001$).

Conclusion: Galectin-9 measurement is helpful in diagnosis of endometriosis. Future studies are recommended for investigating the generalizability of these results.

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Introduction

Endometriosis is a debilitating gynecologic disease, affecting about 10-15% of women of reproductive age in the world (1). However, diagnosis is delayed in most cases, because of the silent presence of endometriosis and chronicity of symptoms with mild to moderate severity, such as pain during periods (dysmenorrhea), intercourse (dyspareunia), defecation (dyschezia), and urination (dysuria) (2), which can also result in impaired quality of life for the women (3, 4). Despite the significant advances in medical technology, endometriosis is still a major health challenge (5)

and the rate of incidence, prevalence, and years of life lived with disability remains steady between the years 1990 to 2017, with only a negligible decrease (about 0.2%) (6). One of the underlying causes is the exact mechanism of endometriosis which is still undiscovered although several factors, especially the role of inflammatory hormones, have been confirmed (7).

Another important challenge of endometriosis is the diagnosis, as the gold standard diagnostic method is direct visualization using laparoscopic examination, while surgery, though minimally invasive, is not practical as the first line diagnostic test, due to its costs and risks (8, 9). Therefore, the research is ongoing on the alternative noninvasive diagnostic tests for endometriosis (10, 11). Galectin (Gal) is a member of β-galactoside binding lectins, homologous with amino acid sequence of the carbohydrate-binding site, a group of proteins highly prone to glycosylation. In humans, 15 subgroups of Gal (1, -2, -3, -4, -7, -7B, -8, -9, -9B, 9C, -10, -12, -13, -14, and -16) have been identified. Gals are synthesized in cytoplasm, but unlike most of the lectin members, Gals are not bound to membrane and they interact with cell surface; in addition, the solubility of this protein enables intra- and extra-cellular functions and it is detectable in cytosol, nucleus, extracellular matrix, and circulation. Gals play a role in many physiological and cellular functions, including immunologic reactions, inflammation, proliferation, differentiation, cell migration, autophagy, and signaling (12). Considering the multiple cellular functions, Gals are also involved in several diseases, such as asthma, atherosclerosis, atopic dermatitis, cardiac diseases, cerebral infarction, chronic obstructive pulmonary disease, diabetes mellitus, and cancer (13). The role of Gals has also been confirmed in gynecological and obstetric diseases, such as polycystic ovarian syndrome (14), endometrial cancer (15), and miscarriage (16).

Endometriosis, the clinical significance of which has been explained above, is also an immune-mediated disorder and dysregulation of inflammatory and vascular signaling has been suggested as its pathophysiology. Therefore, it is important to know about the role of this protein in this disease, which may help identification of the disease pathology and open new windows to novel therapeutic strategies. As suggested, Gals-1, -3, -9, and -15 are expressed in human endometrium, decidua, and stroma and overexpressed in endometriotic tissue; they are also found in the peritoneal fluid of women with endometriosis, which suggests the role of galectins in progression of endometriosis (15, 17, 18). It has been suggested that Gal-3 may also be involved in development of endometriosis-related pain and nerve degeneration by influencing myelin phagocytosis and triggering neuronal apoptosis after nerve injury (19). Gal-3 has also been attributed to (progesterone resistance) infertility in women with endometriosis (20).

Cellular model suggests that Gal-1 is associated with the pathophysiology of endometriotic lesions and it has the potential to be used for reducing the size and vascularized area of endometriotic lesions within the peritoneal compartment of mice (21), which confirms this lectin as a novel therapeutic target in endometriosis. Although the increased level of Gal-9 protein in human endometrial epithelial cells (during the mid- and late-secretory phases) and in the decidua (15, 22, 23) besides its possible therapeutic role (21) have been confirmed previously, the use of serum level of Gal-9 for detection of endometriosis in the clinic has been investigated only in one study (24), as per our knowledge. Since approving this biomarker as a clinically useful agent for endometriosis diagnosis can be a great advancement, the purpose of the current study was to evaluate the diagnostic value of serum level of Gal-9 in comparison with laparoscopic results in patients who were referred for diagnosis of endometriosis.

Methods

Study design: Patients referred to Booali, Rasool-e-Akram, and Pars Hospitals affiliated to Islamic Azad University of Medical Sciences between 2018-19 as candidates for laparoscopy were included in this diagnostic test study. Those who were suspected of endometriosis by the gynecologist (including patients with symptoms of dysmenorrhea, dyspareunia, dyschezia, and dysuria) and recommended for laparoscopic examination for definite diagnosis of endometriosis were considered as the case group (n=32) and women who underwent laparoscopy for any reason, other than endometriosis were considered as the control group (n=29). The protocol of the study was approved by the Ethics Committee of Islamic Azad University of Medical Sciences (code: IR.IAU.TMU.REC.1397.047). The minimum sample size of the study was calculated as 24 individuals in each group, based on the results of the study by Brubel et al., who reported a sensitivity of 94% for Gal-9 and considered the overall incidence of

endometriosis as 10% in general population (24). Sample size calculation was done given a confidence interval of 95% and accuracy of 0.1.

The inclusion criteria for both groups consisted of not being currently pregnant, no history of hormone therapy during the past three months, no cancer of any type, and no history of hysterectomy and/or oophorectomy. The patients who had the above-mentioned inclusion criteria were asked to read and sign the written informed consent, after the researcher explained the study objectives to them. No additional costs were imposed on the patients and the extra cost of serum test of Gal-9 was provided by Pars Advanced and Minimally Invasive Medical Manners Research Center (PAMIM). The eligible patients who gave consent for participation were enrolled into the study using census method.

The demographic and obstetric/gynecologic characteristics of the participants, including age, body mass index (BMI), age at menarche, age at the first pregnancy, history of pregnancy, gravidity, and history and number of abortion(s) were recorded in the study's checklist. These variables were collected during the medical history taking from the patients by the researcher. BMI was calculated based on patients' weight and height, measured by the researcher; values within the range of 18.5 to 24.9 kg/m^2 were considered normal. The clinical characteristics of endometriosis were also recorded in the case group by taking history, which included clinical signs and symptoms of endometriosis.

One venous blood sample (5 *ml*) was taken from the participants before laparoscopy during the follicular phase, in a tube containing clot activator and separating gel and after 30 *min*, the blood samples were centrifuged at 300 *g* for 10 *min* and the serum was stored at -20 ℃. The serum level of Gal-9 was measured by human Gal-9 ELISA (Cat. No. E01G0073, BlueGene Biotech, China) and reported as *pg/ml*. According to the test principles, 50 *µl/well* of recombinant human Gal-9 standards and serum sample, pre-coated with Gal-9 monoclonal antibody, were added to the wells and incubated. The anti-Gal-9 antibodies, labeled with biotin, were added to unite with streptavidin-HRP, forming immune complex. Unbound enzymes were removed after incubation and washing the wells. Then, substrate A and substrate B were added to each well and incubated for 15 *min*. The solution turned blue and changed to yellow by the effect of acid. The solution shades and the concentration of human Gal-9 were positively correlated. The levels >132 *pg/ml* were considered positive, based on the results reported previously (24).

All patients underwent a similar laparoscopy procedure by a single surgical team; the procedure was performed by a 1-*cm* incision on the umbilicus to fill the peritoneal cavity with CO_2 and then the laparoscope was inserted for direct observation of the abdominal cavity. Two 5-*mm* incisions were made on the right and left side of the abdomen to allow the surgeon to insert the ports. By using these tools, the surgeon searched the abdomen for any pathologies and took a sample tissue for pathologic examination. Final diagnosis was made by the surgeon's gross observation during laparoscopy and the report of two pathologists. The disease stage was determined by the revised American Society for Reproductive Medicine (rASRM) score (25) and the pathologic reports were categorized into endometrioma and deep infiltrating endometriosis. Two patients in the case and three patients in the control group were excluded from the study analysis. The reason is included in the flow diagram.

Statistical analysis: The results of categorical variables were presented by frequency (percentage) and their comparison between the study groups was done by Chi square test. Numeric variables were described by mean±standard deviation (SD) or median and interquartile range (IQR) and compared using independent samples t-test or Mann-Whitney U test, according to the results of normality test (One–sample Kolmogorov–Smirnov test). Association of variables was tested by Pearson's or Spearman's correlation coefficient. The best Gal-9 cut-off of was calculated using the Youden's index and receiver operating characteristic (ROC) curve; accordingly, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) of Gal-9 were reported for diagnosis of endometriosis with 95% confidence interval (CI). For the statistical analysis, SPSS Statistics software version 21.0 (IBM, USA) was used and $p<0.05$ were considered statistically significant.

Results

A total of 56 patients including 30 in the case group and 26 in the control group (Figure 1) consisted the subjects of the study. The minimum age of the participants was 17 and maximum was 48

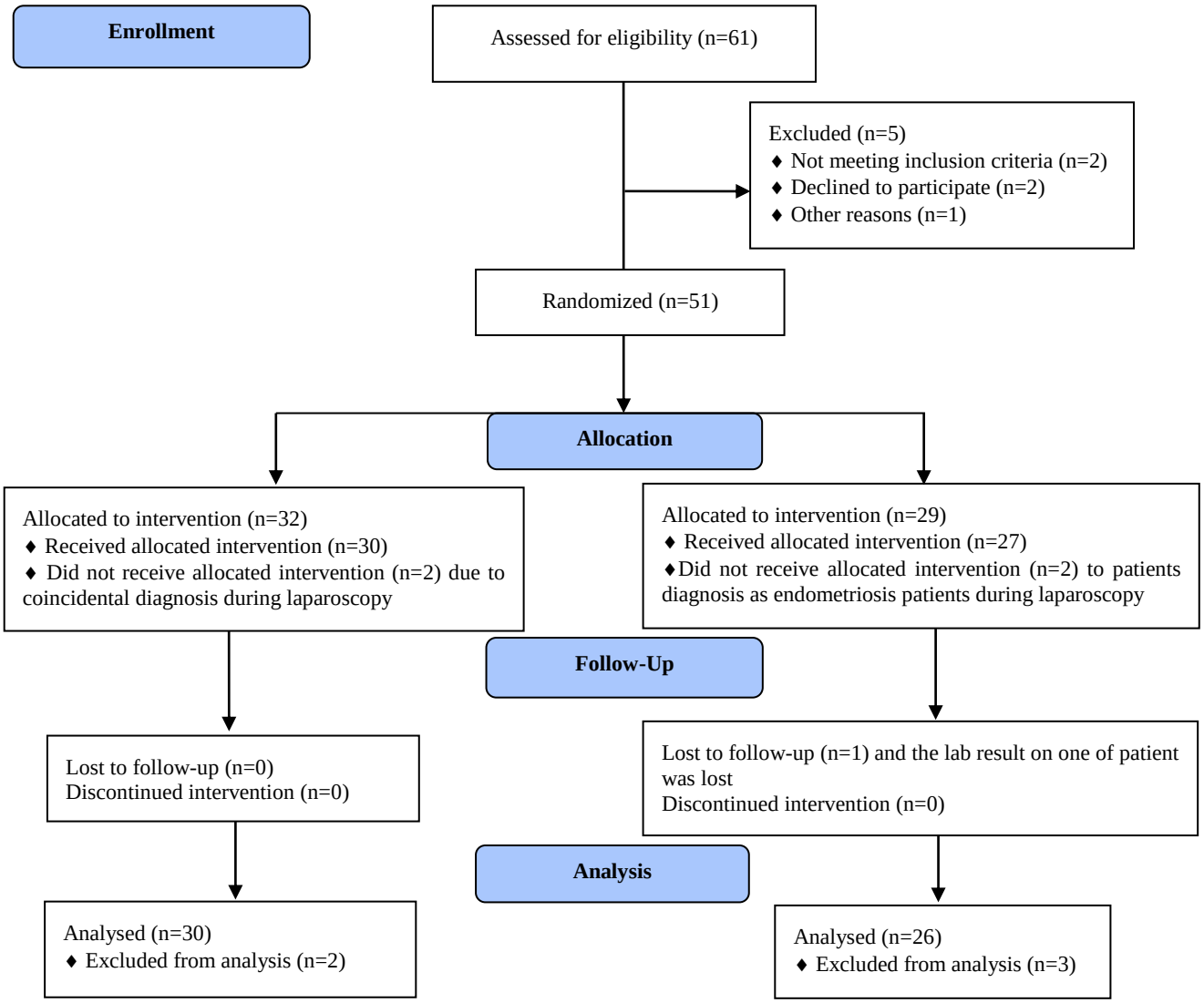

Figure 1. Flow diagram for participant screening and enrollment

years. The age of menarche was within the range of 11-13 years in about 64% of the patients. The range of BMI was 18 to 32 kg/m^2 and 53.5% had normal BMI values. The complete demographic and obstetric/gynecologic characteristics of the participants and their comparison based on the two study groups are shown in table 1. As shown, none of the variables was different between the groups (Table 1). The clinical signs and symptoms of endometriosis in the case group was as follows: dysmenorrhea (90%), abnormal uterine bleeding (80%), dyspareunia (56.6%), dyschezia (30%), and infertility (13.33%). Considering the menstrual blood volume, 40% had high, 40% moderate, and 20% low volume.

Laparoscopic examination showed stages I-II in 10 patients (33.3%) and stages III-IV in the other 20 patients (66.6%) in the case group (n=30); the pathology report showed nodule in 60%, deep infiltrating endometriosis in 73.3%, and endometrioma in 93.3% of the patients with endometriosis (the case group). The mean serum level of Gal-9 was 481.78±492.89 *pg/ml* in all patients, 669.3±416.50 *pg/ml* in the case group, and 265.42±49.30 *pg/ml* in the control group (p=0.001). The median serum level of Gal-9 was 450 *pg/ml* (*min*: 151, max: 588) and 89 *pg/ml* (*min*: 50, max: 1600) in patients with stages I-II and control group (p= 0.274), respectively. The median serum level of Gal-9 was 599 *pg/ml* (*min*: 236, max: 1600) and 89 *pg/ml* (*min*: 50, max: 1600) (p=0.001) in patients with stages III-IV and control group, respectively. Evaluating the association of serum level of Gal-9 with the study variables showed no

Table 1. The demographic characteristics of the participants and the comparison between the two study groups

Variables	Categories	Total	Case group	Control group	p-value
Age (years)		32.46±7.85 ***	32.5±7.85 ***	29.23±6.50 ***	0.098 *
History of pregnancy, n (%)		36 (64.2)	20 (66.6)	16 (61.5)	0.690 †
Gravidity, n (%)	0	20 (35.7)	10 (33.3)	10 (38.5)	0.943 ††
	1	15 (26.8)	8 (26.7)	7 (26.9)	
	2	15 (26.8)	8 (26.7)	7 (26.9)	
	>2	6 (10.7)	4 (13.3)	2 (7.7)	
History of abortion, n (%)		7 (12.5)	5 (16.6)	3 (11.5)	
Abortions, n (%)	0	49 (87.5)	25 (83.4)	24 (92.3)	0.675 ††
	1	6 (10.7)	4 (13.3)	2 (7.7)	
	>1	1 (1.8)	1 (3.3)	0	
Age at menarche (years)		12.00 ** (11.00-13.00)	12.00 ** (11.17-13)	12.00 ** (11.00-13.00)	0.847 ‡
Age at the first pregnancy (years)		24.72±3.73 ***	25.50 ** (24.17-28.66)	23.00 ** (21.63-25.36)	0.057 ‡
Body mass index (kg/m^2)		24.05±2.88 ***	24.5 ** (23.17-25.00)	23.5 ** (22.00-25.00)	0.234 ‡

* The results of independent samples t test, † The results of Chi square test, †† The results of Fisher-exact test, ‡ The results of Mann Whitney U test. ** Median (IQR), *** Mean±SD

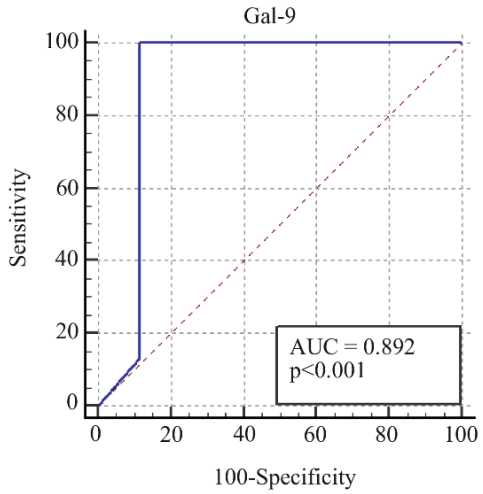

Figure 2. The ROC curve for the diagnostic accuracy of serum levels of Galectin-9 for endometriosis; the AUC was 0.885 at cut-off level of 138 *pg/ml* (p<0.001)

relation between serum level of Gal-9 and numeric variables (including age [p=0.388], gravidity [p=0.524], and pain severity [p=0.254]).

As shown in figure 2, the area under the curve (AUC) of serum level of Gal-9 for diagnosis of endometriosis was 0.892 at cut-off level of 138 *pg/ml* (p<0.001). Considering the fact that serum level of Gal-9 was positive in all patients of the case group, a sensitivity of 100% (88.43-100%), specificity of 88.46% (69.85-97.55%), PPV of 90.91% (77.53-96.67%), NPV of 100%, PLR of 8.67 (2.99-25.12), and NLR of 0.00 were indicated.

Discussion

The results of the present case-control study showed that Gal-9 had a high sensitivity (100%) for diagnosis of endometriosis and an acceptable specificity (88.46%), compared to the gold standard diagnostic method, laparoscopy, which suggests this measurement as an accurate and reliable diagnostic tool in women suspected of endometriosis. These results are in line with the ones reported by Brubel et al., as the only similar study in this regard, which reported a lower sensitivity (94%) and a higher specificity (93.75%) (24). The cut-off point estimated by Brubel et al. (132 *pg/ml*) was also close to that found in the present study (138 *pg/ml*). However, in their study, they considered two control groups for the patients with endometriosis, one with benign gynecologic diseases other than endometriosis, and the other as healthy control; the results showed that Gal-9 mRNA was overexpressed in eutopic and ectopic endometrium, as well as peritoneal cells of women with endometriosis, compared to the healthy control group (24). The overexpression of Gal-9 protein in human endometrial epithelial cells (during the mid- and late-secretory phases) and in the decidua have also been confirmed previously (15, 22, 23). However, despite this information, Gal-9 had not been used as a tool for endometriosis di-

agnosis, except in the study by Brubel et al. and the present study.

Literature shows an extensive research on different parameters for noninvasive diagnosis of endometriosis, while most serum biomarkers have failed to show a high diagnostic accuracy, perhaps due to the fact that the investigated parameters did not have an etiologic role in endometriosis pathogenesis (26). According to the review of biomarkers used for diagnosis of endometriosis, only four of them including CA-125, CA19-9, interleukin (IL)-6, and anti-endometrial antibody were suggested as reliable markers with sensitivity of >95% and specificity of >79% (27). Accordingly, as the present study suggests, serum levels of Gal-9, especially at a cut-off of >138 *pg/ml*, have a high accuracy for diagnosis of endometriosis. Various studies have focused on accuracy of CA-125 for diagnosis of endometriosis (28-30). However, comparing their results with that of ours shows that none has reported a sensitivity of 100%, not even in combination with other biomarkers (28-30). The high diagnostic accuracy of Gal-9, reported in the present study, can be attributed to the critical role of galectins, especially Gal-9, in endometrial tissue and in endometriosis. Other studies have also confirmed association of Gal-1 and -3 with the severity of endometriosis and infertility in women with endometriosis, respectively (20, 21, 31).

The immunoregulatory function of Gal-9, its substantial increase during pathologic conditions, and its association with disease severity have been shown previously (31, 32). Meggyes et al. have also shown an altered pathway for expression of Gal-9 on peripheral blood and fluid lymphocytes of women with endometriosis, compared to those without the disorder, which confirms the role of Gal-9 in the impaired immunologic mechanism of endometriosis (33). According to the fact that endometriosis is an inflammatory disease that stimulates immune response and inflammation in the body (34) with numerous immunologic and inflammatory changes (35), the response to immunologic conditions is mainly impaired in women with endometriosis. Similar to other diseases, Gal-9 may also have an immunoregulatory function in endometrium that fails to act properly in patients with endometriosis and results in the increased level of serum Gal-9 in these patients. Nevertheless, there is still insufficient evidence about the roles of Gal-9 in endometrium and endometriosis; therefore, there is a substantial need for cellular and molecular investigations in this regard in order to determine the pathologic role of Gal-9 in endometriosis.

In the current study, it was found that not only the serum level of Gal-9 can be a strong predictor of endometriosis, but it can also be used in determining the early or late stages of the disease. In the study by Brubel et al., categorizing mean serum level of Gal-9 according to the endometriosis stage showed that mean Gal-9 level was 1.5 times higher in women with endometriosis at stages III-IV, compared to the healthy control group (24), which is similar to the results of the present study. However, the limited number of patients in low grade endometriosis in the study by Brubel et al. resulted in no statistically significant difference between the women at stages I-II (8 women) and the control group (24). The regression analysis of the present study confirmed the significant association of Gal-9 with endometriosis stage, in addition to the significantly higher serum levels of Gal-9 in women at higher stages of endometriosis, compared to minimal stages. This unique finding of the present study suggests the usefulness of this biomarker for prediction of the endometriosis stage as well. On the contrary, the severity of patients' pain was not associated with serum level of Gal-9, which confirms the notion that the pain severity is not associated with the endometriosis severity (36). These results confirm the necessity of using biomarkers and other examinations for diagnosis of endometriosis, rather than relying on the clinical symptoms and their severity.

One of the limitations of the present study was subjective nature of some of the variables tested, such as pain, which varies based on the person's pain threshold and perception. Another limitation was the small sample size in the present study, which was mainly related to the exorbitant costs of Gal-9 measurement as a result of sanctions and restrictions imposed on various activities in our country.

Conclusion

Considering the high diagnostic accuracy of serum level of Gal-9, the results of the present study showed that this biomarker has a great clinical value for diagnosis of endometriosis. Also, our results showed the value of Gal-9 in prediction of the endometriosis disease stage. Considering the paucity of evidence in this regard, there is a need for further evaluations in order to determine the Gal-9 mechanism of action in endometriosis and

compare its sensitivity with other confirmed biomarkers. Also, future studies with larger statistical population are recommended for investigating the generalizability of these results in other gynecologic diseases.

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Conflict of Interest

There are no actual or potential conflicts of interest concerning this paper.

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