Serum galectin-9 as a noninvasive biomarker for the detection of endometriosis and pelvic pain or infertility-related gynecologic disorders

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Objective: To investigate the usefulness of soluble galectin-9 (Gal-9) in the noninvasive laboratory diagnosis of endometriosis and various gynecologic disorders.
Design: Prospective case-control study.
Setting: University medical centers.
Patient(s): A total of 135 women of reproductive age were involved in the study, 77 endometriosis patients, 28 gynecologic controls, and 30 healthy women.
Intervention(s): Diagnostic laparoscopy and collection of tissue biopsies, peritoneal cells, and native peripheral blood from different case groups of gynecology patients and healthy women.
Main Outcome Measure(s): The expression of mRNA and serum concentration of Gal-9.
Result(s): Semiquantitative reverse transcription-polymerase chain reaction analysis and serum soluble Gal-9 ELISA were performed on three different cohorts of patients: those with endometriosis, those with benign gynecologic disorders, and healthy controls. Differences in the Gal-9 concentrations between the investigated groups and the stability of Gal-9 in the serum and diagnostic characteristics of Gal-9 ELISA were determined by statistical evaluation and receiver operating characteristic (ROC) curve analysis. Significantly elevated Gal-9 levels were found in both minimal-mild (I–II) and moderate-severe (III–IV) stages of endometriosis in comparison with healthy controls. At a cutoff of 132 pg/mL, ROC analysis revealed an excellent diagnostic value of Gal-9 ELISA in endometriosis (area under the curve = 0.973) with a sensitivity of 94% and specificity of 93.75%, indicating better diagnostic potential than that of other endometriosis biomarkers. Furthermore, various pelvic pain or infertility-associated benign gynecologic conditions were also associated with increased serum Gal-9 levels.
Conclusion(s): Our results suggest that Gal-9 could be a promising noninvasive biomarker of endometriosis and a predictor of various infertility or pelvic pain-related gynecologic disorders. (Fertil Steril® 2017;108:1016–25. ©2017 by American Society for Reproductive Medicine.)
Key Words: Endometriosis, biomarker, galectin-9, serum, ELISA

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Endometriosis is one of the most common infertility-related gynecologic disorders that affects approximately 10%–15% of women of reproductive age (1). Histologically it is defined as the presence of endometrial-like tissue implants or lesions outside the uterine cavity. The main symptoms of this disease are chronic pelvic pain (CPP), infertility, dysmenorrhea, and dyspareunia (2). The most widely used method for the clinical classification of endometriosis is the rAFS score system presented by the American Fertility Society. According to this system, the disease is classified into four grades of severity based on American Society for Reproductive Medicine

Received May 4, 2017; revised September 5, 2017; accepted September 6, 2017.
R.B. and A.B. should be considered similar in author order.
R.B. has nothing to disclose. A.B. has nothing to disclose. A.P. has nothing to disclose. G.K.S. has nothing to disclose. L.S. has nothing to disclose. R.B.-S. has nothing to disclose. J.R. has nothing to disclose. B.P. has nothing to disclose.
Supported by a grant from the National Research Development and Innovation Office (NKFIH K119529) and an Internal Scientific Grant of Pecs University Medical School (AOK-KA-2013/21, to B.P.).
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Fertility and Sterility® Vol. 108, No. 6, December 2017 0015-0282/$36.00

https://doi.org/10.1016/j.fertnstert.2017.09.008

(ASRM) scores: as stage I (minimal), stage II (mild), stage III (moderate), and stage IV (severe) endometriosis (3).

The etiology and pathogenesis of endometriosis have been widely investigated over the past 30 years. Although there have been several attempts to explain its development, none of them can be applied for all types of the disease. Among them the most accepted theory is Sampson's retrograde menstruation hypothesis (4–6). Other well-known ideas are Meyer's coelomic metaplasia (7), endometrial stem cell (6, 8), Halban's vascular and lymphatic metastasis (9), endometrial disease (10), embryonic rest (11), and composite theory (12). In addition, the invagination and ovarian cyst theories are also accepted in ovarian endometriosis (13).

The evolution of endometriosis involves both genetic and immunologic factors (14, 15). There is evidence for a familiar inheritance pattern of endometriosis with a seven-fold increased incidence in first-degree relatives, suggesting a polygenic/multifactorial pattern. It was found that women with affected first-degree relatives had increased probability to develop severe endometriosis (61%) compared with women without affected relatives (24%) (16). Later studies have demonstrated concordance in monozygotic twins-pairs, further underlining its genetic basis (17). From an immunological point of view, it is hypothesized that endometriosis will develop in those women who are genetically programmed not to respond to endometrial antigens (18) or who have defective immune effector functions. In these patients, refluxing autologous endometrial cells are either not destroyed by the immune system or their endometrial reflux is too abundant and therefore overload the scavenging capacity of the peritoneal immune cells (19).

During the course of lesion development, exfoliated endometrial fragments entering the peritoneal cavity can induce local inflammation by recruiting macrophages, neutrophils, eosinophils, and mast cells to the site of the attachment (20). Following activation, these cells secrete proinflammatory cytokines (21, 22), chemotactic and angiogenic proteins, and the macrophage migration inhibition factor (MIF) (23). Among them, TNF-α, CD74, and MIF are able to initiate signaling cascades that lead to the proliferation and survival of endometriotic implants (24). Since the cytotoxicity of the natural killer (NK) cells (25, 26) and the T-cell-mediated adaptive immune response is also defective in endometriosis, it further aids the survival of ectopic implants (15, 19, 27). The latter dysfunctionality mainly results from the low number and uneven antigen presentation of mature dendritic cells, the defective T helper (Th) mechanisms, and the enrichment of suppressive regulatory T (Treg) cells at the site of lesion development (18, 25). In addition, local production of immunosuppressive cytokines (IL-6, IL-10, TGF-β), the molecule RANTES, and autoantibodies are also increased in endometriosis (22, 28). At present, most evidence suggests that the chronic pelvic inflammation caused by these immunological alterations are a consequence rather than a cause of endometriosis (29).

From a clinical point of view there is no specific sign or sensitive laboratory test that allows the early, adequate recognition of endometriosis. Therefore, the estimated average delay between the onset of the symptoms and the definitive diagnosis is about 6.7–9.3 years. As of now, the only reliable "gold standard" in the diagnosis of endometriosis is still laparoscopy. Since laparoscopy is a surgical procedure with potential risks, the development of a noninvasive laboratory test would be of great benefit in the early, clinical management of this disease (29). Several molecules involved in the pathogenesis of endometriosis have been investigated as potential biomarkers, but the majority of them have proven inadequate for the diagnosis.

In the past few years lectins have become the focus of reproductive immunology, inflammation, and autoimmunity (30, 31). Galectins (Gal) are β-galactoside binding lectins that play a key role in the regulation of the immune system, cell growth, adhesion, apoptosis, and angiogenesis (32, 33). As of now, 13 different types of galectins have been found in the human, among them Gal-1–4, 7–9, and 12 were detected in the normal endometrium. So far only Gal-1 and Gal-3 have been studied in relation to endometriosis. It is poorly understood whether other galectins might play a role in the pathogenesis of endometriosis. Earlier publications reported that Gal -9, a bidirectional immunomodulator, is exclusively expressed by the epithelial cells of the normal endometrium (34) and might be a marker of endometrial receptivity before implantation (35). Because our recent studies showed an increased Gal-9 staining in ectopic endometriosis lesions, our main goal was to examine the diagnostic potential of serum soluble Gal-9 measurement in the noninvasive diagnosis of endometriosis.

MATERIALS AND METHODS

Patients and Sample Collection

In accord with the Declaration of Helsinki, our prospective case-control study protocol was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics, Budapest, Hungary (registration no. 143/2008) for the protection of human subjects. Participants were chosen consecutively between March 1, 2012, and March 31, 2013, by clinical presentation and laparoscopic findings at the First Department of Obstetrics and Gynaecology, Endometriosis Outpatient Clinic, Budapest, Hungary. Previous or current hormone therapy was an exclusion criterion. Informed consent was obtained from all patients before entry into this study. Diagnostic or operative laparoscopy for investigation of pelvic pain and/or infertility was performed in 105 women of reproductive age (range, 28–49 years). Their demographic data are presented in Supplemental Table 1. Normal pelvis was observed in 28 patients. All of them had patent tubes. Endometriosis was found in 77 women and classified according to the ASRM scoring system as minimal-mild (stage I–II; n = 20) and moderate-severe (stage III–IV; n = 57) disease (3). The presence of endometriosis was confirmed histologically in their surgical samples. Tissue biopsies were collected by laparoscopy from two healthy control women and 15 patients with endometriosis, snap-frozen in liquid N_2, and stored at −80°C. Peritoneal fluid (PF) was aspirated from four women with endometriosis to collect peritoneal cells from the pouch of Douglas before any surgical manipulation. Special precaution was taken to avoid blood or other fluid contamination (saline, methylene blue dye). Peritoneal

washing was not performed. After collection, PF specimens containing peritoneal cells were aliquoted and frozen at –80°C until further analysis. For Gal-9 ELISA, native peripheral venous blood was collected before surgery from all patients who underwent laparoscopy (n = 105). Control blood samples (n = 30) were taken anonymously from age-matched, healthy female blood donors with the permission of the National Blood Bank Regional Centre, Pecs, Hungary, after receiving their written informed consent. The donor's health status was identified before venipuncture by case history, and all of those women who reported gynecologic problems were not included in the study. Their detailed demographic data were not available. The collected blood samples were allowed to coagulate at room temperature and centrifuged for 10 minutes at 3,000 rpm to separate cellular elements. The serum was decanted, aliquoted, and stored at –80°C until analysis. The flowchart in Supplemental Figure 1 summarizes the enrollment of study participants and the basis of the final data sheet for analysis.

RNA Purification and cDNA Synthesis

In a reverse transcription-polymerase chain reaction (RT-PCR) assay a total of 18 tissue biopsies obtained from two healthy women and 15 patients with endometriosis and four peritoneal fluid samples were processed: (a) control tissue samples: eutopic endometrium from healthy women (n = 2); nonaffected tissue controls, surrounding the implantation site of the ectopic lesions (n = 3) (control ovarium [n = 2], control intestine [n = 1]); (b) tissue biopsies obtained from patients with endometriosis: eutopic endometrium (n = 6), ectopic endometriotic lesions at different localization (n = 7) (ovarium [n = 3], intestine [n = 1], spatium rectovaginale [n = 1], urinary bladder [n = 1], tuba uterina [n = 1]). For RNA purification from tissue biopsies, frozen specimens were melted on ice, and then 30–60 mg of tissues were cut into fine pieces with scissors and immediately disrupted in TRIzol reagent (Invitrogen) with the aid of a Pellet Pestle tissue grinder (Merck Life Science). After homogenization, samples were incubated for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Then the suspensions were centrifuged at 12,000 *g* for 10 minutes at 4°C to remove insoluble materials and high molecular weight DNA. Supernatants were transferred to clean microcentrifuge tubes, and total RNA was extracted with Direct-ZOL RNA MiniPrep kit (ZymoResearch Irvine) by strictly following the manufacturer's instructions. For RNA extraction from peritoneal cells (n = 4) 1 mL of frozen PF specimens were melted on ice, and then 3 mL TRIzol/1 mL PF was added to them and mixed thoroughly by pipette. The mixtures were incubated for 5 minutes at room temperature to promote full disruption of peritoneal cells. Cellular debris was removed by centrifugation (12,000 *g*, 10 minutes, 4°C). Supernatants were aspirated into new tubes, and 200 μL chloroform for every 1 mL of TRIzol was added to them. The suspensions were mixed vigorously for 15 seconds and incubated for an additional 2–3 minutes at room temperature to precipitate proteins. After centrifugation (12,000 *g*, 15 minutes, 4°C) the upper, aqueous phase was collected and transferred into clean tubes. Again total RNA was extracted from them with Direct-ZOL RNA MiniPrep kit according to the manufacturer's instructions. During RNA purification "on-column" DNase treatment was also included to remove any genomic DNA contaminations. The concentration and purity of the obtained RNA solutions were determined by measuring the absorbance ratios at 260/280 and 260/230 nm with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Before RT, 500 ng of total RNA was preheated at 65°C for 10 minutes to disrupt any secondary structures in the RNA. First-strand cDNA was synthesized with a High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the supplier's protocol by M-MuLV RT and a mixture of random octamers and oligodT-16 to maximize RT efficiency. The successfulness of RT was confirmed by amplifying the sequence for the 40S ribosomal protein S9 (RibS9) as indicated below. The transcribed cDNA samples were stored at –80°C until further use.

RT-PCR

RT-PCR was performed with primer pairs designed from cDNA sequences of human Gal-9 and RibS9 as a housekeeping gene control. The primer sequences (synthesized by IDT Inc.) were the following: Gal-9-F (5′-ACTATTCAAGGAGGTCTCCAG-3′), Gal-9-R (5′-GGATGGACTTGGATGGGTACA-3′), RibS9-F (5′-GATGAGAAGGACCCACGGCGTCTGTTCG-3′), RibS9-R (5′-AGACAATCCAGCAGCCCAGGAGGGACA-3′). PCR reactions were performed according to the manufacturer's protocol with HOT-FIREPol DNA polymerase (Solis BioDyne) using 1 μL cDNA as a template. Conditions for PCR reactions were 1 × (95°C, 15 minutes), 43 × (95°C, 30 seconds; 59°C, 1 minute; and 72°C, 1 minute for Gal-9) or 38 × (95°C, 30 seconds; 59°C, 30 seconds; and 72°C, 1 minute for RibS9), and 1 × (72°C, 5 minutes) for final extension. PCR reactions were performed in Eppendorf Mastercycler gradient thermal cycler. The amplicons were analyzed by electrophoresis on a 1.5% agarose gel containing 0.05% EtBr, and bands were visualized in a UV transilluminator (East Port). The expected amplicon sizes were 667 bp (transcript variant 1, full length or long), 571 bp (transcript variant 2, Gal-9Δ5 or medium) for Gal-9 and 431 bp for RibS9, respectively. Band intensities were determined with ImageJ software, and relative pixel density was calculated by normalizing band densities of Gal-9 to RibS9.

Galectin-9 ELISA

Native serum samples of a total of 105 gynecologic patients and 30 healthy blood donors were examined. Serum soluble Gal-9 levels were determined with sandwich ELISA by strictly following the manufacturer's protocol (human Gal-9 ELISA, cat. no. E01G0073, Shanghai BlueGene Biotech). Briefly, 50 μL/well of recombinant human Gal-9 standards and serum samples were added to a 96-well microplate precoated with monoclonal anti-Gal-9 antibody. Then 100 μL of HRP-conjugated polyclonal anti-Gal-9 was added to each well and incubated for 1 hour at 37°C. After washing the wells 5 times with 200 μL of wash buffer, 50 μL of substrate A and 50 μL of substrate B were added to each well and incubated for 10–15 minutes at 37°C in the dark. Finally, 50 μL/well of stop solution was added to the wells and mixed with gentle

tapping to terminate the reaction. Optical density of the wells was measured at 450 nm with FLUOstar Optima microplate reader (BMG Labtech). Serum Gal-9 concentrations were calculated with BMG Optima 2.10 R2 software, and test results were sent back to the clinical partners.

Statistical Analysis

Graphpad Prism version 3.00 for Windows (GraphPad Software, www.graphpad.com; 1999) and MedCalc version 16.8 software (MedCalc Software bvba, https://www.medcalc.org; 2016) were used for statistical analysis. Clinical and demographic data were provided to the research team after executing the ELISA test, and the established ASRM score (3) was used as a reference test of endometriosis. Study data were evaluated by descriptive statistical methods such as mean, SD, frequency, and distribution. Student's *t*-test was used to compare two groups of values demonstrating normal distribution, while groups of values without normal distribution were compared by the Mann-Whitney *U*-test. The nonparametric Kruskal-Wallis *H*-test with Dunn's comparison or the one-way analysis of variance (ANOVA) test with Bonferroni's correction was used for multiple comparison of the mean or median of Gal-9 measurements. $P \leq .05$ was considered statistically significant. The relationship between clinical parameters and Gal-9 concentrations was examined by Spearman's *r* correlation analysis. A receiver operating characteristic (ROC) curve was used to evaluate the diagnostic performance of Gal-9 ELISA in endometriosis. During ROC analysis, Gal-9 values of sera obtained from validated patients with endometriosis were used as positive samples, and the sera of healthy women as negative samples. Youden's index was used as a criterion for selecting the optimum cutoff point. The calculated area under the curve (AUC) was considered significantly different from the null hypothesis if its value was higher than 0.5.

RESULTS

Galectin-9 Is Overexpressed in the Eutopic Endometrium, Ectopic Lesions and Peritoneal Cells of Patients with Endometriosis

Previous publications revealed that Gal-9 is expressed in the human mid- and late secretory phase endometrium, in the uterodomes during the implantation window, and in the decidua (34, 35). To verify whether Gal-9 is also expressed in endometriosis, we performed a semiquantitative RT-PCR assay to examine the LGALS9 gene expression in the eutopic endometrium of healthy control women and patients with endometriosis, as well as in ectopic endometriotic implants at various localizations and in the peritoneal cells of affected women. The expression pattern of LGALS9 transcripts and comparative densitometry of Gal-9 mRNA expression were assessed by normalizing the expression density of Gal-9 mRNA to RibS9 as a reference gene. Because of the relatively small sample size involved in the study, statistical analysis was not doable.

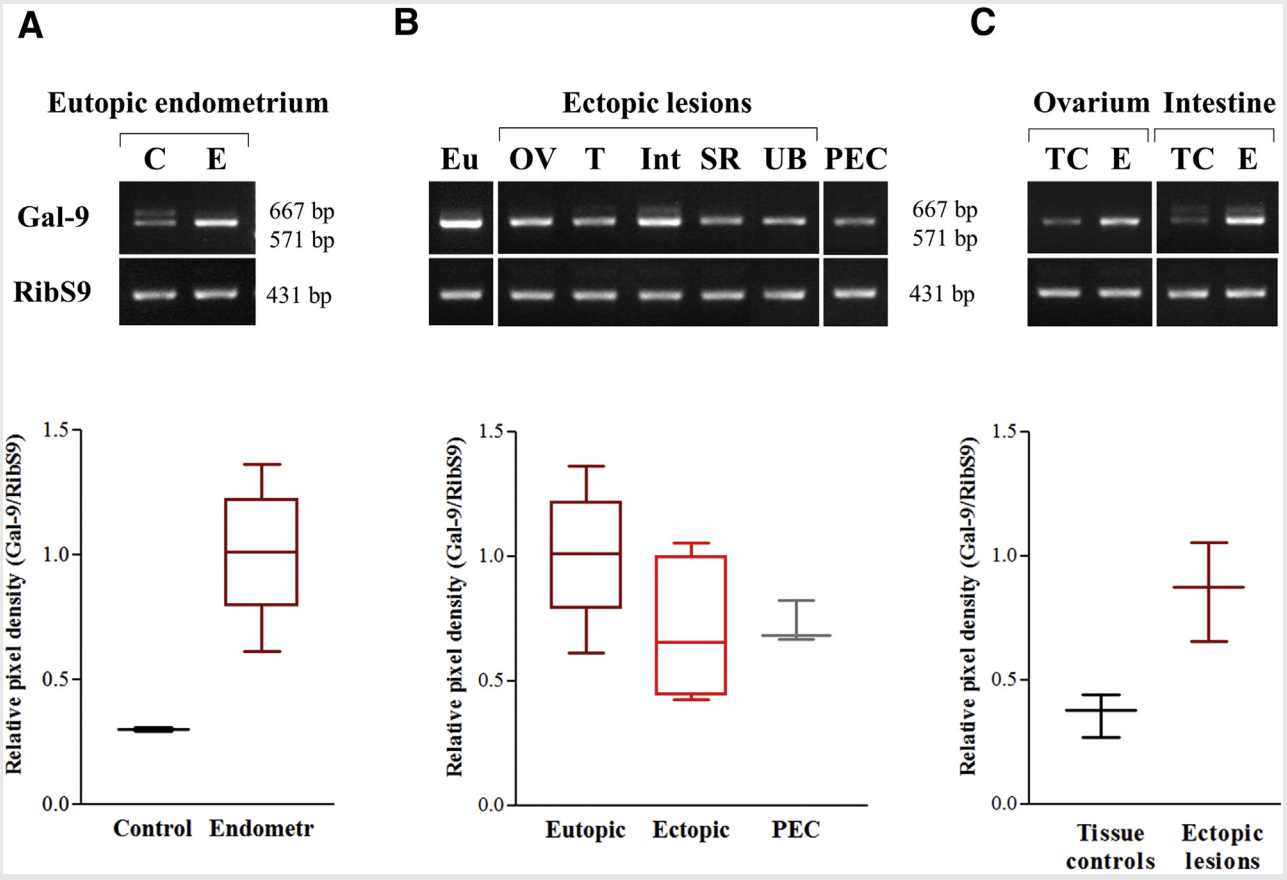
First, LGALS9 gen-expression in eutopic endometrium of healthy women was compared with matched eutopic tissue pairs of patients with endometriosis. As can be seen in Figure 1A, Gal-9 mRNA was markedly overexpressed in the eutopic endometrium of patients with endometriosis in comparison with healthy controls. In addition, our results showed that besides the eutopic endometrium, Gal-9 was also expressed in all types of tested ectopic implants regardless of the localization of the lesions. In these samples, the dominant Gal-9 mRNA variant was the 571 bp medium (Gal-9Δ5) variant, which differs in the linker peptide length from the long (full-length) transcript. Furthermore, we found increased Gal-9 expression in peritoneal cells of women with endometriosis, indicating that besides ectopic lesions, various cellular components of the PF might be also able to produce this lectin (Fig. 1B). Finally, we found, that ectopic implants revealed higher Gal-9 mRNA levels when compared with their surrounding tissue controls, indicating that the great majority of Gal-9 was expressed by the ectopic implant itself, instead of the cells surrounding the implantation area (Fig. 1C).

Serum Galectin-9 Level Is Elevated in Endometriosis

A total of 135 women of Caucasian ethnicity were enrolled in the study. The 105 patients who underwent diagnostic or operative laparoscopy were divided into two groups by the presence (n = 77) or absence (n = 28) of endometriosis. Within the endometriosis group, 20 patients had stages I–II of endometriosis and 57 of them had advanced (stages III–IV) disease. The demographic characteristics of the examined groups are summarized in Supplemental Table 1. Since healthy control women were anonymous blood donors, their epidemiological and clinical data were not available. There was no significant difference in age between the study groups. Patients with endometriosis had a higher rate of infertility than the gynecologic control group. In addition, a higher percentage of patients in advanced stages of endometriosis came through previous surgery and had CPP than that of women at stage I–II or women with normal pelvis. According to the demographic data, the presence of autoimmune disease or insulin resistance did not differ between the tested groups.

We found that patients with endometriosis (n = 50) had considerably higher serum Gal-9 levels when compared with healthy controls (n = 16). The means (SD) of Gal-9 levels in the endometriosis and control groups were 779.8 (623.7) pg/mL versus 97.98 (24.2) pg/mL, $P < .0001$ (Fig. 2A). In the case group, the maximum Gal-9 value was 2,100 pg/mL, whereas it was only 144 pg/mL in the control samples. Within the endometriosis group, the mean (SD) values of Gal-9 were 552.6 (460.6) pg/mL at ASRM I–II stages (n = 8) and 823 (645.5) pg/mL at III–IV stages (n = 42). Due to the low number of samples from the initial stages, only the advanced-stage groups displayed significantly higher Gal-9 levels than the healthy controls ($P < .001$). Although the level of soluble Gal-9 was approximately 1.5 times higher in the serum of III–IV stage groups, no statistically significant differences among minimal-mild and moderate-severe endometriosis were observed (Fig. 2B). This again might have resulted from the small sample size of the minimal-mild stages. There was no statistically significant correlation between serum Gal-9 levels and previous history of endometriosis-associated symptoms such as CPP (Spearman $r = 0.002873$, $P = .9852$) dysmenorrhea ($r = -0.2561$, $P = .0933$), dyspareunia ($r = 0.07692$, $P = .6197$),

FIGURE 1



Comparative RT-PCR analysis of Gal-9 mRNA expression in endometriosis. Gal-9 gene (LGALS9) expression was examined in different tissue samples obtained from healthy women and patients with endometriosis. The upper figures show representative Gal-9 and RibS9 RT-PCR results indicating the size of the amplified PCR products. RibS9 = 40S ribosomal protein S9. Note the presence of two Gal-9 PCR products representing the long (667 bp) and medium (557 bp) transcripts. The lower diagrams demonstrate the sum of comparative densitometry of the tested samples. The relative pixel density values of Gal-9 mRNA-specific bands were normalized to the expression of RibS9 mRNA in matched samples, and the calculated results were presented in box-and-whisker plots showing median (horizontal line), 25% and 75% percentile interquartile range (box), and maximum and minimum values (whiskers). Considering the small samples sizes, statistical analysis was not doable. (**A**) Comparison of LGALS9 gene expression in eutopic endometrium of healthy women and patients with endometriosis. Gal-9 mRNA expression was markedly elevated in the eutopic endometrium of patients with endometriosis (n = 6) in comparison with healthy controls (n = 2). C = healthy control; E = endometriosis patients. (**B**) Comparison of normalized Gal-9 mRNA expression in eutopic and ectopic tissue biopsies and in peritoneal cells of patients with endometriosis. The LGALS9 gene was overexpressed in both eutopic (n = 6) and ectopic endometrial biopsies (n = 7), as well as in peritoneal cells of women with endometriosis (n = 4). Eu = eutopic endometrium (n = 6); ectopic endometriotic lesions at different localization (n = 7): OV = ovarium; T = tuba uterina; Int = intestine; SR = spatium rectovaginale; UB = urinary bladder; PEC = peritoneal cells (n = 4). (**C**) Comparison of relative Gal-9 mRNA expression in ectopic endometriotic lesions with their nonaffected, normal tissue controls surrounding the site of implantation. The first figure pair shows a representative RT-PCR of Gal-9 expression in normal ovarium and ovarian endometriosis, and the second figure-pair presents the Gal-9 expression in a biopsy of nonaffected intestine and in ectopic lesion of bowel endometriosis. Densitometry analysis revealed that ectopic lesions (n = 7) have higher Gal-9 expression in comparison with the nonaffected tissue controls (n = 3), indicating that the majority of Gal-9 was expressed by the ectopic implant itself instead of the area surrounding the implantation site. TC = tissue control; E = ectopic endometriotic lesions.

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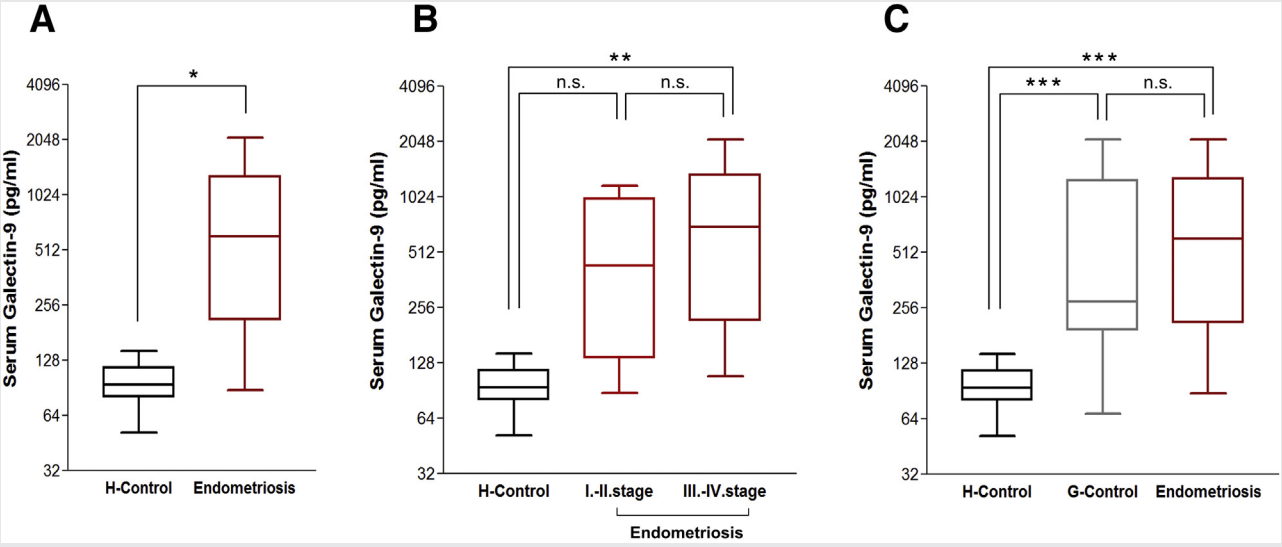
dyschesia ($r = 0.05211$, $P = .7369$), or dysuria ($r = -0.006639$, $P = .9659$). In addition, the number of previous pregnancies did not have a significant impact on serum Gal-9 levels of women with endometriosis ($r = 0.1409$, $P = .3289$).

Serum Galectin-9 Levels in Benign Gynecologic Disorders

To examine the diagnostic value of soluble Gal-9 in benign gynecologic conditions, its concentration was measured in serum samples of patients with various types of gynecologic disorders associated with CPP and/or infertility. These samples were divided into two subgroups according to the presence (n = 50) or absence (n = 28) of endometriosis. The latter cases were marked as gynecologic controls: leiomyoma (n = 12), ovarian cyst (n = 6), unexplained infertility (n = 4), Turner syndrome (n = 1), sterilization (n = 2), ectopic pregnancy (n = 2), and CPP without other symptom or pathology (n = 1).

Comparative, multiparametric statistical analysis of the obtained ELISA results revealed significantly elevated serum

FIGURE 2



Soluble Gal-9 concentration in serum of healthy controls, women with stages I–IV of endometriosis, and gynecologic controls. Serum Gal-9 values were represented on a log2 scale as a scatter dot plot, and medians of each group were marked. (**A**) Serum Gal-9 levels in healthy controls (H-Control, n = 16) and patients with endometriosis (n = 50). Gal-9 level was significantly elevated in the serum of the endometriosis group in comparison with the healthy controls by the Mann-Whitney *U*-test (*$P<.0001$). (**B**) Serum Gal-9 concentrations in healthy controls (n = 16) and patients with stages I–II (n = 8) and III–IV (n = 42) of endometriosis. The differences were significant between healthy controls and stages III–IV of endometriosis by the one-way ANOVA test with Bonferroni's correction (**$P<.001$). (**C**) Serum Gal-9 in healthy control (H-Control, n = 16), gynecologic control (G-Control, n = 28), and endometriosis (n = 50) case groups. Gal-9 level was significantly higher (***$P<.001$) in the gynecologic control samples when compared with healthy controls but did not differ from the endometriosis group according to the Kruskal-Wallis *H*-test with Dunn's multiple comparison. Box-and-whisker plots showing median (horizontal line), 25% and 75% percentile interquartile range (box), maximum and minimum values (whiskers), and significant differences.

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Gal-9 levels both in the endometriosis ($P<.001$) and gynecologic control cases ($P<.001$) when compared with the healthy controls. In contrast, no statistically significant difference was found between the endometriosis and gynecologic control groups regarding serum Gal-9 levels (Fig. 2C). In the gynecologic control cohort, 92.86% of patients showed higher Gal-9 levels than that of the previously determined 132 ng/mL threshold value. Within this group, mainly leiomyoma (mean [SD], 609.7 [631.3] pg/mL, n = 12), ovarian cyst (762 [922.5] pg/mL, n = 6), infertility with hypothyroidism (1,790 pg/mL, n = 2), and genetic disorders (Turner syndrome: 1,470 pg/mL, n = 1) were associated with increased serum Gal-9 levels. Among them, only leiomyoma cases were proven statistically different from healthy controls ($P<.001$; Supplemental Table 2). This may account from the low sample size of the other examined gynecologic control subgroups.

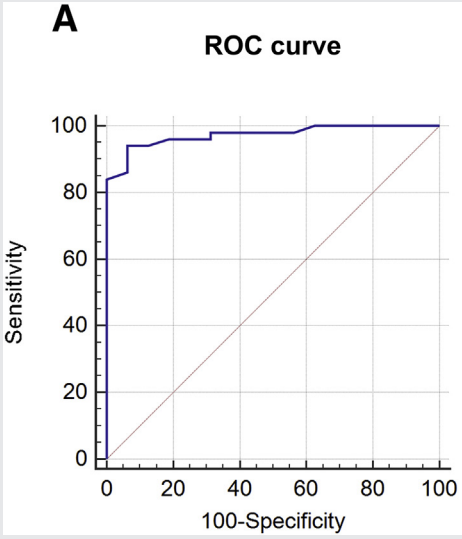
Diagnostic Performance of Serum Galectin-9 in Endometriosis

ROC curve analysis revealed a very high diagnostic value of serum Gal-9 levels with respect to the diagnosis of endometriosis ($P<.0001$). In this case group, the calculated AUC value was 0.973 (95% confidence interval, 0.900–0.997; Fig. 3A). The optimal cutoff point estimated from the Youden's index was 132 pg/mL, with a sensitivity of 94% and specificity of 93.75%. Using this threshold, the positive predictive value was 97.92% and the negative predictive value was 83.33%, with a prevalence of 75.76%. The diagnostic accuracy of this test was 93.94%, indicating better diagnostic potential than that of other serum biomarkers published by Foda et al. (36) (Fig. 3B).

Stability of Soluble Galectin-9 in the Serum

Previously Nishi et al. (37) demonstrated that tandem-repeat-type lectins including Gal-8 and Gal-9 are more susceptible to proteolysis than other type of galectins due to the presence of the relatively long linker peptide chain. To examine the stability of soluble Gal-9 in the serum, native blood samples from healthy women and patients with endometriosis were collected, and serum was decanted and aliquoted. The specimens were frozen and stored at –80°C under stable conditions until ELISA. Since the claimed stability for Gal-9 in the serum, plasma, and cell culture fluid was ≤6 months according to the ELISA kit manufacturer, we divided the collected samples into two groups according to the length of storage. In group 1 we selected those samples that were collected ≤6 months before the execution of test, whereas group 2 included those sera that were >6 months old (7–19 months). Measurement of soluble Gal-9 concentrations were carried out for each group, and the stability of serum Gal-9 was established by comparing the average of Gal-9 levels of group 1 with group 2. The samples were considered stable if the average Gal-9 values in group 2 were within ±25% of the group 1 values. After analysis, we observed that the concentration of Gal-9

FIGURE 3



B

	Gal-9	IL-6	CA-125	TNFa	Hs-CRP	VEGF
Cut off Level	>132	>12.2	>17.6	>12.45	>438	>236
Area under curve (AUC)	0.973	0.929	0.851	0.890	0.880	0.908
Sensitivity (%)	94.00	95.58	60.00	89.23	83.33	91.67
Specificity (%)	93.75	83.33	100.00	86.87	86.67	76.67
Diagnostic accuracy (%)	93.94	91.58	73.33	86.67	84.44	86.67
Positive predictive value (PPV)	97.92	91.44	100.00	92.86	92.59	88.71
Negative predictive value (NPV)	83.33	89.29	55.56	76.47	72.22	82.14

ROC curve analysis and comparison of diagnostic performance of Gal-9 ELISA with other serum biomarkers in endometriosis. (**A**) ROC curve (marked thick line) and AUC of the serum Gal-9 ELISA. (**B**) Comparison of ROC curve characteristics of Gal-9 and other serum biomarkers of endometriosis published by Foda et al. (2012) (36).

Brubel. Galectin-9 as a new endometriosis marker. Fertil Steril 2017.

was significantly lower in group 2 than in group 1 both in the control and endometriosis case groups, and it dropped 8.46%–39.71% to the original Gal-9 values of group 1 samples. Means (SD) of control group 2 versus group 1 were 38.87 (6.083) pg/mL versus 97.98 (24.2) pg/mL, $P<.01$, and means (SD) of the endometriosis group 2 versus group 1 were 65.97 (27.06) pg/mL versus 779.8 (623.7) pg/mL, $P<.001$. In addition, the statistically significant difference between healthy control and endometriosis groups was also lost after long-term storage (Fig. 4). These data suggest that soluble Gal-9 was relatively unstable in the serum and began to degrade after 6 months of storage. We concluded that handling and storage conditions can markedly influence the reliability of Gal-9 measurement.

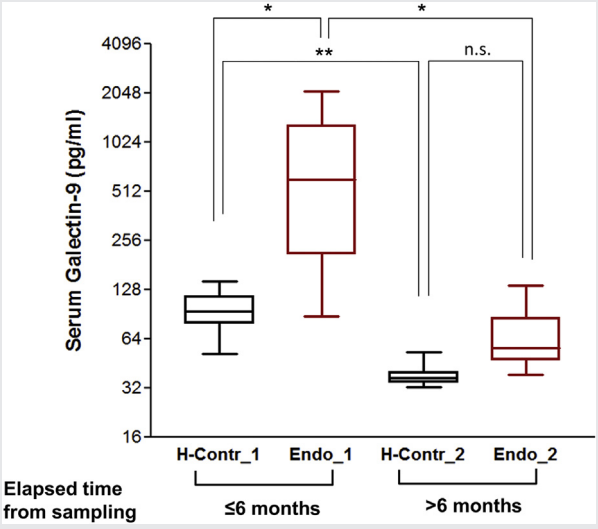
DISCUSSION

We report data on the serum Gal-9 levels in patients with endometriosis in comparison with healthy controls. In addition, the present study may represent an important step in the development of a potential noninvasive blood test for the diagnosis of endometriosis.

To date there are several research groups that are intensively involved in the development of a noninvasive diagnostic method for endometriosis. Over the last few years several potential endometriosis biomarkers have been described; nevertheless, the majority of them have proven inadequate. One of the best known and most widely used biomarkers for endometriosis is the glycoprotein CA-125. This molecule has been mainly used as a noninvasive laboratory marker of epithelial ovarian cancer, but its expression might be also elevated in ovarian endometriosis—especially at late stages. Although its diagnostic sensitivity is moderate, when CA-125 and PRL levels were assessed together, they allowed the diagnosis of peritoneal endometriosis with an acceptable sensitivity (77%) and specificity (88%) and high negative predictive value (97%) (38). Furthermore, a panel of six selected biomarkers (IL-6, IL-8, TNF-α, hsCRP, CA-125, CA-19-9) was also tested on serum samples collected from the secretory phase of the menstrual cycle or during menstruation and was found to help the diagnosis of both minimal-mild and moderate-severe endometriosis with a high sensitivity (87%–92%) and an acceptable specificity (60%–71%) (39). Recently in their fundamental study Vodolazkaia et al. analyzed plasma samples of women at the phase of menstruation and observed that the combination of five biomarkers (annexin-V, VEGF, CA-125, slCAM-1, glycodelin) enabled the diagnosis of endometriosis that was otherwise undetectable by ultrasonography (40) with 81%–90% sensitivity and 63%–81% specificity.

In the past few years lectins have become in the focus of research regarding the immunity of the female reproductive system, pregnancy, and infertility. Among them Gal-1 and Gal-3 are the most intensively examined β-galactoside-binding lectins in human. It was reported that Gal-1 had a cycle-dependent expression in normal endometrial stromal cells, and its production was significantly elevated in the early pregnancy decidua (41). In relation to endometriosis, Gal-1 was found to be more abundantly expressed in ectopic endometriotic lesions when compared with the eutopic tissues. Furthermore, the eutopic endometrium of the affected patients showed higher Gal-1 expression than its normal counterparts (31). In addition to this, Noël et al. have found that similar to Gal-1, Gal-3 was overexpressed in various

FIGURE 4



Stability of Gal-9 in the serum of healthy controls and patients with endometriosis. Serum soluble Gal-9 values were represented on a log2 scale as a scatter dot plot, and medians of each group was marked. The elapsed time from sampling was ≤6 months in group 1 (H-control_1: healthy controls, n = 16; Endo_1: endometriosis, n = 50), whereas samples in group 2 were >6 months old (H-control_2: healthy controls, n = 14; Endo_2: endometriosis, n = 27). Serum Gal-9 levels were significantly lower in group 2 samples by Kruskal-Wallis *H*-test with Dunn's multiple comparison than that of the group 1 samples (significance values: $^{**}P<.01$ between groups of healthy controls and $^{*}P<.001$ between groups with endometriosis). Box-and-whisker plots showing median (horizontal line), 25% and 75% percentile interquartile range (box), maximum and minimum values (whiskers), and significant differences.

Brubel. Galectin-9 as a new endometriosis marker. Fertil Steril 2017.

forms of endometriosis as well as in the eutopic endometrium of diseased women (30, 42). The angiogenetic property of these galectins was also proven in a rodent model of endometriosis and showed that they evoke the formation of vascular networks in vivo by facilitating the growth of ectopic lesions (43, 44). In contrast to Gal-1 and Gal-3, the expression of the tandem-repeat Gal-9 was examined only in the normal endometrium. It was found that this lectin is exclusively expressed by uterodomes during the implantation window and in the early pregnant decidua (35). Later, a distinct decidual Gal-9 mRNA splicing profile was reported in pathological pregnancies when compared with normal conditions and it was shown that the identified D5 isoform was able to down-regulate the IFN-γ production of decidual NK cells (45). These data supposed that Gal-9 might play a key role in the regulation of the fetomaternal immunotolerance by modulating NK cell cytotoxicity and that its altered expression might have adverse effects on pregnancy (46).

In the present study we detected a markedly increased serum Gal-9 level in both minimal-mild and moderate-severe endometriosis when compared with healthy controls. Our results showed a very good diagnostic value of soluble Gal-9 ELISA in endometriosis with a sensitivity of 94% and specificity of 93.75%. At a cutoff value of 132 pg/mL, the diagnostic accuracy turned out to be as high as 93.94%. In addition, we observed that besides endometriosis various benign gynecologic conditions related to CPP or infertility might be also associated with elevated serum Gal-9 levels. In these cases, further noninvasive diagnostic methods, such as ultrasonography or laboratory determination of serum (thyroid) hormone levels, may help in the differential diagnosis of endometriosis.

In our opinion, an ideal noninvasive test with clinical relevance should have a high sensitivity and acceptable specificity in a group of patients with subfertility and/or endometriosis symptoms. In cases of women of reproductive age who have an active or passive desire to become pregnant, the findings of this study hold great significance—especially regarding clinical practice. Gynecologists could be vastly helped by an early, noninvasive diagnosis of minimal and mild endometriosis to help women who try to conceive. Based on such a diagnosis, these women can be treated in time with laparoscopic excision of endometriotic implants, which could improve fertility (47) and might prevent the progression of endometriosis to moderate or severe stages (39).

It is known that the biological function of Gal-9 is pleiotropic and depend on its concentration and intra-/extracellular location (48). Although under physiological conditions Gal-9 has a bidirectional immunoregulatory (thermostat) function, its expression became abundant under pathological conditions, which might result in the loss of its homeostatic function and lead to the development of pathologic states. We propose that in the early phases of endometriosis Gal-9 might act as an alarm to trigger local inflammation and recruit immune cells (mainly eosinophils, neutrophils, dendritic cells, NK, and T cells) to the site of the lesion to promote tissue regeneration and repair. Since the scavenger mechanisms of the immune system are not effective enough in endometriosis, ectopic implants will survive, further activate the local proinflammatory cytokine production of monocytes and Th1 cells, and enhance neutrophil and macrophage-mediated tissue damage. As the local concentration of Gal-9 increases, it represses the original Th1-type immune response and shifts it to Th2 dominance. In addition, the rising Gal-9 level will inhibit the immune-effector mechanisms of CD4+ Th1, Th17, NK, and mast cells and promote the differentiation/function of suppressive Treg cells (49–52). All of the above mentioned effects will worsen the dysfunctionality of the immune system and aid the progression of the disease.

Based on our recent data (Supplemental Figure 2) we hypothesize that the elevated soluble Gal-9 levels detected in our experiments may originate from both the ectopic endometriotic implants and the local or circulating immune cells. However, we cannot determine to what extent each of the two sources contributed to the measured serum Gal-9 levels.

Conclusion

Summarizing the importance of our results, we conclude that we identified Gal-9 as a new, potential biomarker for noninvasive, laboratory diagnosis of endometriosis that has a better

diagnostic performance than that of the other endometriosis biomarkers like CA-125, IL-6, Hs-CRP, TNF-α, or VEGF. We suggest that Gal-9 might be potentially used for the noninvasive monitoring of endometriosis. Since our study was limited to a small sample size for subgroup analysis of gynecologic controls with normal pelvis or patients with minimal and mild endometriosis, we could not address the issue of early-stage noninvasive diagnostic testing. However, the notable elevation of soluble Gal-9 level in the serum of stages I–II of endometriosis in comparison with healthy controls strongly suggests its potential usefulness in the sensitive, early laboratory diagnosis.

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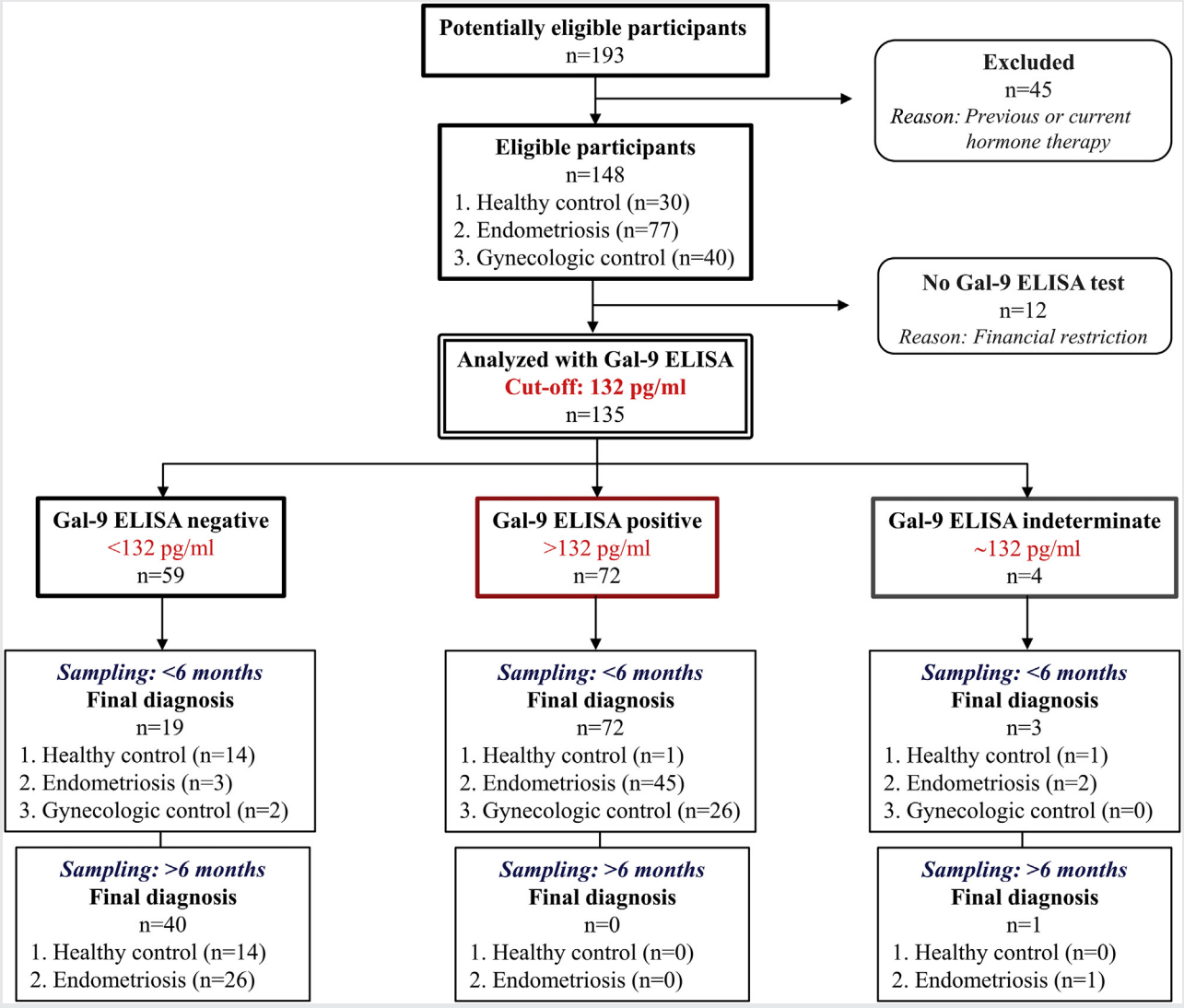
SUPPLEMENTAL FIGURE 1



Flowchart summarizing the enrollment and exclusion of study participants and basis of final data sheet analysis. This flowchart shows the mode of sectioning and the number of individuals selected during recruitment, during preselection, and after execution of the indicated Gal-9 ELISA test. The reasons for exclusion and the classification criteria used for the division of test groups are indicated in boxes. In the diagram, angled boxes mark case groups that were included in the assay, whereas rounded boxes show the patients excluded from the study. According to the cutoff value of 132 pg/mL (marked with red), the tested individuals were divided into three subgroups, and indicated as positive (Gal-9 level >132 pg/mL), negative (Gal-9 >132 pg/mL), and indeterminate (Gal-9 level ~132 pg/mL). Within these cohorts, specimens were further divided into two subclasses (<6 months or >6 months, marked with blue) by the elapsed time from sampling until the completion of the test.

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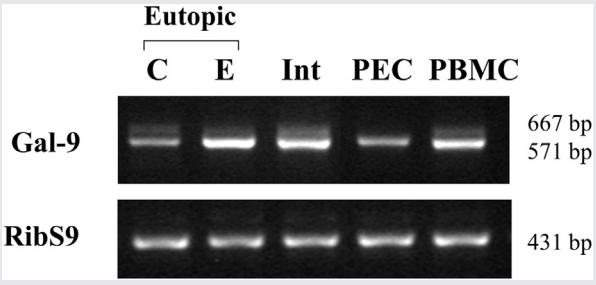
SUPPLEMENTAL FIGURE 2



Comparison of Gal-9 mRNA expression pattern in various tissue and cell samples collected from healthy controls and women with endometriosis. Representative Gal-9 and RibS9 RT-PCR assay indicating the differences in the splicing pattern of LGALS9 gene in different tissue samples obtained from healthy women and patients with endometriosis as well as in the peritoneal cell and peripheral blood mononuclear cells of affected patients. The sizes of the amplified PCR products are indicated on the right. RibS9 = 40S ribosomal protein S9; C = control eutopic endometrium of healthy women; E = eutopic endometrium of patients with endometriosis; Int = deep infiltrating endometriosis lesion from the intestine; PEC = peritoneal cells of a patient with endometriosis; PBMC = peripheral blood mononuclear cells of affected patient.

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