Accurate Diagnosis of Endometriosis Using Serum MicroRNAs

Sarah Moustafa, MD, Martina Burn, MD, Ramanaiah Mamillapalli, PhD, Sepide Nematian, MD, Valerie Flores, MD, Hugh S. Taylor, MD

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Accurate Diagnosis of Endometriosis Using Serum MicroRNAs

Sarah Moustafa, MD; Martina Burn, MD*; Ramanaiah Mamillapalli, PhD*; Sepide Nematian, MD; Valerie Flores, MD; Hugh S. Taylor, MD

Department of Obstetrics, Gynecology and Reproductive Sciences, Yale School of Medicine, New Haven, CT 06520, USA

*Equally contributed

Corresponding authors:
Hugh S Taylor, MD
Email: taylor.hugh@yale.edu
Ramanaiah Mamillapalli, PhD
Email: ramana.mamillapalli@yale.edu

Department of Obstetrics, Gynecology & Reproductive Sciences
Yale School of Medicine, 310 Cedar Street, New Haven, CT 06510
Email: taylor.hugh@yale.edu
Phone: 203-785-4001, Fax: 203-785-4713

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Word count: 3656
This study describes the use of a combination of serum microRNAs for diagnosis of endometriosis with accuracy comparable to the current gold standard, laparoscopy.

**Short title:** Endometriosis diagnosis by MicroRNA biomarkers

**AJOG at a Glance:**

**A. Why was this study conducted?**

The current gold standard for the diagnosis of endometriosis is laparoscopy, which incurs costs, patient risk, and often delays diagnosis. An accurate, non-invasive test could mitigate these issues.

**B. What are the key findings?**

- Six miRNAs were evaluated to predict endometriosis in patients undergoing laparoscopy (41 cases, 59 controls).

- Serum levels of the miRNAs were measured by quantitative real-time polymerase chain reaction (qRT-PCR).

- A Random Forest algorithm incorporating the miRNA levels demonstrated an AUC of 0.939 in an independent set of patients.

**C. What does this study add to what is already known?**

This study demonstrates the independent validation of a set of miRNAs that can non-invasively differentiate endometriosis from other gynecologic pathologies in a clinical setting, thus avoiding laparoscopy and reducing barriers to diagnosis.

**Keywords:** endometriosis, microRNA, non-invasive diagnosis, biomarker, miR
ABSTRACT

BACKGROUND: Endometriosis, a chronic disease that afflicts millions of women worldwide, has traditionally been diagnosed by laparoscopic surgery. This diagnostic barrier delays identification and treatment by years, resulting in prolonged pain and disease progression. Development of a non-invasive diagnostic test could significantly improve timely disease detection. We tested the feasibility of serum microRNAs as diagnostic biomarkers of endometriosis in women with gynecologic disease symptoms.

OBJECTIVE: To validate the use of a microRNA panel as a non-invasive diagnostic method for detecting endometriosis.

STUDY DESIGN: This was a prospective study evaluating subjects with a clinical indication for gynecologic surgery in an academic medical center. Serum samples were collected prior to surgery from 100 subjects. Women were selected based on the presence of symptoms and laparoscopy was performed to determine the presence or absence of endometriosis. The control group was categorized based on absence of visual disease at the time of surgery. Circulating miRNAs miR-125b-5p, miR-150-5p, miR-342-3p, miR-451a, miR-3613-5p and let-7b were measured in serum by qRT-PCR in a blinded fashion, without knowledge of disease status. Receiver operating characteristic (ROC) analysis was performed on individual miRNAs, as well as combinations of miRNAs. An algorithm combining the expression values of these miRNAs, built using machine learning with a Random Forest classifier, was generated to predict the presence or absence of endometriosis on operative findings. This algorithm was then tested in an independent dataset of 48 previously identified subjects not included in the training set (24 endometriosis and 24 controls) to validate its diagnostic performance.

RESULTS: The mean age of women in the study population was 34.1 and 36.9 for the endometriosis and control groups, respectively. Control group subjects displayed varying pathologies, with leiomyoma occurring the most often (n=39). Subjects with endometriosis had significantly higher expression levels of four serum miRNAs: miR-125b-5p, miR-150-5p, miR-342-3p, and miR-451a. Two serum miRNAs
showed significantly lower levels in the endometriosis group: miR-3613-5p and let-7b. Individual miRNAs had ROC areas under the curve (AUC) ranging from 0.68 to 0.92. A classifier combining these miRNAs yielded an AUC of 0.94 when validated in the independent set of subjects not included in the training set. Analysis of the expression levels of each miRNA based on rASRM staging revealed that all miRNAs could distinguish Stage I/II from control, and Stage III/IV from control, but that the difference between Stage I/II and Stage III/IV was not significant. Subgroup analysis revealed that neither phase of menstrual cycle or use of hormonal medication significantly impacted expression levels in the miRNAs used in our algorithm.

CONCLUSIONS: This is the first report showing that miRNA biomarkers can reliably differentiate between endometriosis and other gynecologic pathologies with an AUC > 0.9 across two independent studies. We validated the performance of an algorithm based on previously identified miRNA biomarkers, demonstrating their potential to detect endometriosis in a clinical setting, allowing earlier identification and treatment. The ability to diagnose endometriosis non-invasively could reduce the time to diagnosis, surgical risk, years of discomfort, disease progression, associated co-morbidities and healthcare costs.
Introduction

Endometriosis, an inflammatory disorder of endometrial cell proliferation outside the uterus, affects nearly 10% of reproductive age women, causing pain and infertility. Despite its prevalence, the average time from symptom onset to a correct diagnosis is 5-10 years.\textsuperscript{1,2} This disease can be difficult to recognize based on patients’ non-specific descriptions of symptoms, especially at early stages.\textsuperscript{3}

Definitive diagnosis of the condition presently requires laparoscopic examination\textsuperscript{4}, a surgical procedure that bears annual direct and indirect costs of $119 billion.\textsuperscript{5} Thus, further delay is incurred in the effort to avoid an invasive procedure, resulting in further disease progression and prolonged suffering.

The social, psychological, and economic impacts of endometriosis are manifold. Studies have described the negative effects of endometriosis on quality of life, extending to work, education, social and intimate relationships, as well as mental and emotional well-being.\textsuperscript{6-8} Identifying and treating the disease sooner would potentially prevent complications of advanced disease such as infertility, while decreasing the economic burden of untreated endometriosis. Laparoscopy is rarely undertaken early in the disease, due to the associated risk to the patient and reluctance to undergo surgery without severe symptoms.\textsuperscript{9} Other methods for detecting disease such as imaging and protein biomarkers have proven ineffective.

Serum microRNAs (miRNAs) are emerging as potential molecular indicators to non-invasively identify endometriosis. MiRNAs are short, non-coding RNAs that may be released into the circulation and are protected from endogenous RNase degradation due to inclusion within exosomes or association with specific protein complexes.\textsuperscript{10,11} 2500 miRNAs have been identified in the human genome\textsuperscript{12}, and unique expression profiles of miRNAs have been reported in multiple diseases.\textsuperscript{13,14} Many studies have investigated aberrant expression of miRNAs in endometrial lesions\textsuperscript{15} and circulating miRNAs in serum\textsuperscript{16-18} and plasma.\textsuperscript{19,20}
Previously, in a retrospective study we identified a panel of serum miRNAs by microarray analysis that showed at least 10-fold increased or decreased expression in serum of women with endometriosis. Using a logistic regression model and Receiver operating characteristic (ROC) analysis, an excellent area under the curve (AUC) was obtained for the combination of three miRNAs (miR-125b-5p, miR-451a, and miR-3613-5p). As this previous study evaluated miRNA expression profiles only in patients with moderate/severe (Stage III/IV) endometriosis, we sought to expand the clinical utility by testing a more diverse set of patients. This study design enabled us to test whether pre-operative evaluation of these miRNA biomarkers could distinguish endometriosis from other benign gynecological conditions, in a diverse patient population.

Materials and Methods

Study population

Institutional Review Board (IRB) approval was obtained from the Yale University School of Medicine (New Haven, CT). Written informed consent was obtained from patients undergoing surgery for suspected benign indications between September 2016 and October 2017. Women aged 18-49 years were included. Exclusion criteria consisted of post-menopausal state, pregnancy, critical anemia, hyperplasia, polyps, or malignancy. Serum samples were collected from women prior to undergoing laparoscopy for suspected benign gynecological conditions, and miRNA expression analysis was performed blinded to the surgical findings. Subjects were stratified into the disease group if visual or pathology findings from surgery confirmed the presence of endometriosis, or the control group if surgery revealed other benign pathology. Categorization was validated among three clinicians independently. Staging was done using the revised American Society of Reproductive Medicine (rASRM) classification and independently confirmed by evaluating the surgical reports and pathology results. A separate independent dataset used to validate our findings was obtained from our previously published cohort of 24 patients with surgically confirmed endometriosis and 24 controls.
Sample collection

Prior to surgery, blood (5-10 ml) was drawn from the subjects and collected in sterile tubes (BD, Franklin Lakes, NJ, USA). Serum was collected immediately by centrifuging at 2500 rpm for 15 min at 4°C and stored at -80°C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total miRNA was extracted using the miRNeasy mini Kit from Qiagen and reverse transcribed using Invitrogen NCode miRNA First-Strand cDNA Synthesis MIRC-50 kit (Life Technologies) according to the manufacturer's instructions. Primers (Supplemental Table 1) for miRNAs and human U6 small nuclear RNA were obtained from the W. M. Keck Oligonucleotide Synthesis Facility (Yale University, New Haven, CT), and universal reverse primer was obtained from Applied Biosystems. MicroRNA levels were quantified by qRT-PCR using SYBR Green and reaction conditions were followed as described. MicroRNA expression was normalized to U6 and experiments were carried out twice independently, each in duplicate. The miRNAs evaluated in this study were selected from a large screen that identified many miRNAs altered in endometriosis. For this study we specifically used only those that showed minimal or no alteration through the menstrual cycle or in response to sex steroid hormone treatment.

Statistical analysis

Based on a power of 0.8, alpha of 5%, incidence of 50% and the effect size observed in our previous study, a minimum of 52 subjects were needed to power the study. As we were initially blinded to the diagnosis, we collected 100 samples to assure an adequate number of subjects with endometriosis. A Student’s t-test was used to compare the clinical characteristics of subjects in the endometriosis and control groups. Mean expression levels of serum miRNAs between the groups were compared using the Mann-Whitney U-test. The Bonferroni correction was performed to adjust for multiple comparisons since six miRNAs were analyzed. To evaluate the diagnostic utility of each
miRNA biomarker, receiver operating characteristic (ROC) analysis was performed, and the area under the ROC curve (AUC) was calculated. To evaluate the performance of the prior 3-marker classifier formula developed from our previous study dataset\textsuperscript{16} in the current dataset (n=100), we normalized and rescaled the two datasets to account for differences in qRT-PCR methodology. Each miRNA of the two datasets was standardized using the z-score method, by setting the within dataset mean to 0, standard deviation (SD) of 1, with the following formula: $\text{miRNA standard} = \frac{\text{miRNA} - \text{mean (miRNA)}}{\text{SD (miRNA)}}$. Imputation was performed for missing values (representing <3% of the values in the current dataset), by applying the MICE (Multivariate imputation by chained equations) method. The prior classifier used the combination of three miRNAs (miR-125b, miR-451a, and miR-3613) and was derived using a logistic regression model\textsuperscript{16}. After adjusting coefficients for each variable according to the rescaled data, the performance of the 3-marker classifier was evaluated by comparing the predicted outcome values to the true outcome variables and calculating the AUC.

To build and test an optimal classifier using the current dataset (n=100), we compared two statistical approaches: penalized regression model and machine learning with Random Forest. The later was chosen due to the ability to yield an AUC of 1 in the training dataset. Given that all biomarkers assessed were strongly associated with disease status, using Random Forest to obtain importance measures was more beneficial in order to rank all the variables rather than obtaining non-zero coefficients from the penalized regression model. This dataset was split into training and testing sets to train and assess the performance of the classifier. To balance upward and downward bias of prediction accuracies and to simulate a larger sample size, we applied the 0.632 stratified bootstrapping method to generate 1000 replicates. For each replicate, Random Forest was applied to the training set to build decision trees (n=500) and predict the disease status in the testing set. In addition to validation of the algorithm with random subsampling, we evaluated the diagnostic performance of the classifier algorithm.
using an independent dataset, by testing the optimal Random Forest model in the rescaled dataset previously collected (n=48).\textsuperscript{16}

**Results**

From the original 103 subjects, three were excluded due to an unexpected co-morbidity including malignancy. Of the remaining 100 patients, 41 were categorized as endometriosis and 59 as controls. The demographics and clinical characteristics of the subjects are summarized in Table 1. There was no statistically significant difference between age and BMI in the study groups. Of those with endometriosis, approximately 90% of the surgeries were performed for pelvic pain and 10% for infertility. The endometriosis group consisted of varying degrees of disease as categorized by rASRM stage and divided into Stage I, II, III or IV, while the control subjects had varying benign pathologies as shown in Table 1. The menstrual cycle phase and presence of hormonal medications for all subjects are also recorded in Table 1. In nearly half of the study subjects, the phase of the menstrual cycle could not be accurately determined based on either use of hormonal medication or a history of irregular cycles.

In this case-control study, the presence of endometriosis was not known prior to surgery. We initially included miRNAs for evaluation that showed the largest difference between disease and control populations with little to no overlap in our prior study, as well as those that were menstrual cycle and sex steroid independent. Alterations of the algorithm were made with inclusion and exclusion of the previously evaluated cohort of miRNAs that demonstrated correlation with disease\textsuperscript{16, 22}, and miRNAs which did not significantly contribute to the model were ultimately excluded from the final algorithm. Figure 1 shows the expression levels of six miRNAs that were prospectively measured. Among these six miRNAs, miR-125b, miR-150-5p, miR-342-3p, and miR-451a were significantly increased in patients with endometriosis while miR-3613-5p and let-7b were significantly decreased. Subgroup analysis based on cycle phase for both control or endometriosis subjects showed no significant difference in miRNA expression levels between those sampled during the proliferative vs. secretory phase (Figure 2).
Hormonal therapies (Table 1) included predominantly combined oral contraceptives (10, 24%) and GnRH agonists (6, 14%). The presence of hormonal treatment did not significantly affect the average expression levels of the six target miRNAs tested (Supplemental Figure 1).

To evaluate whether the expression of these miRNAs correlates with the stage of endometriosis, we separated minimal/mild (Stage I/II) endometriosis from moderate/severe (Stage III/IV). Using a Kruskal-Wallis test, all six miRNAs were found to have significantly different variances (p<0.05) between the three groups: control, Stage I/II, and Stage III/IV (Figure 3). However, after using Dunn’s multiple comparisons test for the three pairwise comparisons, each subgroup of endometriosis had significantly different miRNA levels compared to the control group, but not between minimal/mild vs. moderate/severe.

ROC analysis of individual miRNAs showed AUC scores ranging between a low of 0.68 for miR-150-5p to 0.92 for miR-342-3p (Table 2). Using machine learning with Random Forest approach, a new classifier algorithm was developed using the six miRNAs. This algorithm was validated in two ways: by random subsampling dividing the total dataset into training and testing subsets, and by testing against our previous dataset, which was not used for model development (n=48, 24 endometriosis and 24 control subjects). The AUC scores for the model performance in the training and testing datasets are shown in Figure 4. An AUC of 0.939 for the 6-marker classifier algorithm was attained in the independent data set.

**Structured Discussion/Comment**

1. **Principal findings**

   This is the first study performed within a diverse population that demonstrates the ability of circulating miRNAs to reliably differentiate endometriosis from other gynecologic pathologies, with robust diagnostic performance in an independent test dataset. The clinical characteristics of the current study population was reflective of real-world patients with endometriosis, including diverse racial
demographics, early- and late-stage disease, varying phases of the menstrual cycle, and presence of hormonal treatments. Evaluation of these markers amongst a cohort of patients with varied pelvic pathologies supports the utility of using these markers in a general population to distinguish endometriosis from other conditions.

**Results**

ROC analysis again demonstrated the significant diagnostic value of combinations of these miRNAs. In contrast to our prior work\textsuperscript{16}, which excluded women undergoing hormonal treatments and only included women with moderate/severe (Stage III/IV) endometriosis, the current study included cases of minimal/mild (Stage I/II) disease and women using hormonal therapies. Here we sought to optimize our combination of miRNA biomarkers to reflect this more diverse and representative patient population.

The single serum miRNA biomarker with the most reproducible diagnostic performance was miR-451a. This miRNA had an AUC of 0.84 in the current study, nearly identical to the AUC of 0.835 in our prior study\textsuperscript{16} and was found to have an AUC of 0.86 by a separate research group in a study of serum from 81 patients.\textsuperscript{23} Recently, other circulating miRNA biomarkers, including miR-122 and miR-199a in serum\textsuperscript{24} and miR-31 and miR-145 in plasma\textsuperscript{25} were also reported to have high sensitivity and specificity for identification of endometriosis.

Assessing the combination of three miRNAs that led to the highest AUC score in our previous retrospective analysis (miR-125b-5p, miR-451a, miR-3613-5p)\textsuperscript{16}, an AUC of 0.8 was obtained in the current study. This performance is expectedly lower than was previously achieved with this combination, as prior studies compared near-pristine controls with an advanced disease group. For every combination of miRNAs, we applied numerous non-machine learning algorithm approaches and machine learning approaches and analyzed the performance results for each. In an independent dataset, Random Forest applied to six of the miRNAs yielded an optimal classifier with an AUC of 0.939. Since
endometriosis is not a fatal condition, optimizing the classifier for specificity (avoiding false positives) would help prevent over-diagnosis, and women could be re-tested if symptoms persist. Using this strategy to define our thresholds, the current model yielded 96% specificity and 83% sensitivity. Alternatively, optimizing both values simultaneously, we achieved a sensitivity and specificity of approximately 90% each. A test with higher sensitivity and a low false negative rate could be appropriate for use of the biomarker panel as a screening test.\textsuperscript{16} We previously selected these miRNAs based on lack of significant change through the menstrual cycle and here we again observed no difference in the expression levels of these six miRNAs based on the phase of menstrual cycle (secretory vs. proliferative).\textsuperscript{16} This supports consistency of these markers in diagnosis irrespective of cycle timing\textsuperscript{16} with the exception of let-7b, which previously had shown a greater reduction compared to control levels during the proliferative phase.\textsuperscript{22} Further we observed that hormonal medications alone do not significantly alter expression levels of these miRNAs. The potential of these miRNAs to monitor response to therapy was demonstrated in our recent non-human primate study, in which miR-150-5p, miR-451a, and miR-3613-5p showed expression changes that correlated with the reduction of lesion volume after endometriosis treatment.\textsuperscript{26} Here, our study model did not allow for miRNA level evaluation at different durations of therapy; future longitudinal studies will measure how the biomarkers change over time with effective medical or surgical therapy.

A significant advantage over prior work\textsuperscript{16}, this study included patients with all stages of disease. Analysis of the expression levels of each miRNA based on rASRM staging revealed that all miRNAs could distinguish Stage I/II from control, and Stage III/IV from control, but that the difference between Stage I/II vs. Stage III/IV was not significant. This may reflect limitations of the rASRM staging system. The ASRM staging does not exclusively record active disease, it also reflects scarring, adhesions and reactive damage that often lead to Stage IV characterization. MiRNA changes may be a more accurate way to measure active disease. Alternatively, because miRNAs are produced by multiple organ systems,
the levels seen may represent an effect on organs outside of the pelvis. The mechanisms by which alterations in miRNAs occur are poorly understood. Some miRNAs are likely made directly by the lesions while others are altered due to the effects of endometriosis on other tissues. Endometriosis is a systemic inflammatory disease that may broadly affect miRNA production independent of stage.\textsuperscript{15, 16}

Finally, the inability to detect significant differences between endometriosis stages may also be due to distinct molecular profiles of different subtypes of endometriosis that are also independent of stage; this will be investigated in future studies. Despite the inability to distinguish stage of disease, in our test the ability to capture early stage disease may have significant clinical advantages.

**Clinical implications**

Prior attempts to establish markers for this disease have been focused on non-specific inflammatory markers, while the use of circulating miRNAs provides a disease-specific signature, unique to endometriosis. A highly sensitive and specific test will have great clinical significance in women with pelvic pain or unexplained infertility. The ability to diagnose endometriosis non-invasively could reduce the time to diagnosis, surgical risk, years of discomfort, hospitalizations and healthcare spending, and ultimately, disease progression and associated co-morbidities. Further studies are warranted to assess how these markers are altered by endometriosis treatment, or if unique marker profiles can provide insight into fertility or patient pain scores. Nonetheless, the combination of six miRNAs validated in this study yielded high AUC scores, supporting the excellent diagnostic potential of these biomarkers for endometriosis.

2. **Research Implications**

Our study model did not allow for evaluation of levels over time or in response to therapy, however ongoing longitudinal studies will measure how the biomarkers are affected by treatment. While our study is not powered to detect unique signatures for different disease phenotypes, these findings support the need for further studies to investigate this. Further larger prospective studies are required to evaluate
the relationships between microRNAs levels in serum and endometriosis and their impact on the
accuracy of diagnosis, management and outcome of the different stages of endometriosis.

3. Strengths and limitation of the study

Our study has many strengths compared to other contemporary studies. The study group was evaluated
prospectively allowing direct correlations between levels of microRNAs and presence of endometriosis.
In addition, our study includes patients with other pelvic pathologies in the control group, accurately
distinguishing between endometriosis and other potential sources for pelvic inflammation. This expands
the generalizability of this test and supports its use and validity in patients with concurrent pathology.
Furthermore, these results reveal that the levels of these six miRNAs is unaltered by hormonal therapy
or cycle phase, further broadening its potential use.

The weakness of the study rests on the limited sample size of both groups, which does not allow
for more detailed correlations between microRNA levels and endometriosis subtypes. This also limits
the ability to assess whether certain features of endometriosis such as severity, stage, presence of
endometrioma, deep infiltrating or rectovaginal disease (which was not a specified endpoint of this
study) drive particular shifts in miRNA expression. While we note above that this model detects both
mild and advanced disease, the study is not powered to detect differences between subgroups. Another
possible limitation to our study is the use of surgical cases across a large number of physicians, without
standardization beyond ASRM staging for documentation of endometriotic burden.

Conclusions

This is the first report showing that miRNA biomarkers can reliably differentiate between endometriosis
and other gynecologic pathologies with an AUC > 0.9 across two independent studies. Prior attempts to
establish markers for this disease have been focused on non-specific inflammatory markers, while the
use of circulating miRNAs provides an endometriosis specific signature. A highly sensitive and specific
test will have great clinical significance in women with pelvic pain or unexplained infertility. The ability
to diagnose endometriosis non-invasively could reduce the surgical risk, years of discomfort, hospitalizations and healthcare spending, and ultimately, disease progression and associated co-morbidities.

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References


### Table 1. Patient Demographics and Clinical Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Endometriosis (n=41)</th>
<th>Control (n=59)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
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<td>36.9 ± 8.2</td>
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<td><strong>Body Mass Index (BMI)</strong></td>
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<td>30.4 ± 7.5</td>
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<td><strong>Race</strong></td>
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<tr>
<td>Caucasian</td>
<td>28 (68)</td>
<td>24 (40)</td>
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<tr>
<td>Black/African American</td>
<td>4 (10)</td>
<td>18 (31)</td>
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<td>Hispanic</td>
<td>7 (17)</td>
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<tr>
<td>Asian</td>
<td>2 (5)</td>
<td>2 (3)</td>
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<tr>
<td>Other</td>
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<td>3 (5)</td>
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<td><strong>rASRM Endometriosis Stage</strong></td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>11 (27)</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>7 (17)</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>15 (36)</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>8 (19)</td>
<td>-</td>
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<tr>
<td><strong>Control Diagnoses</strong></td>
<td></td>
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<tr>
<td>No abnormality</td>
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<td>18 (31)</td>
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<tr>
<td>Leiomyoma</td>
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<tr>
<td>Cystadenoma</td>
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<tr>
<td>Chronic Infection</td>
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<tr>
<td>Teratoma</td>
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<tr>
<td>Paratubal Cyst</td>
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<td>6 (10)</td>
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<td><strong>Hormonal Treatment</strong></td>
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<tr>
<td>Combined OCP</td>
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<td>10 (17)</td>
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<td><strong>Phase of Menstrual Cycle</strong></td>
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<td>Secretory</td>
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<td>32 (54)</td>
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Table 2. ROC Analysis of Individual miRNAs

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<th>ROC Model</th>
<th>Area</th>
<th>Standard Error</th>
<th>95% Wald Confidence Limits</th>
<th>Optimal cut-off</th>
<th>Correct %</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
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<tbody>
<tr>
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<td>0.73</td>
<td>0.05</td>
<td>0.63 0.83</td>
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<td>68.0</td>
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<tr>
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<td>0.68</td>
<td>0.06</td>
<td>0.57 0.78</td>
<td>0.44</td>
<td>63.9</td>
<td>20.0</td>
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<td>miR_342</td>
<td>0.92</td>
<td>0.04</td>
<td>0.86 0.99</td>
<td>0.085</td>
<td>90.8</td>
<td>90.0</td>
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<tr>
<td>miR_451a</td>
<td>0.84</td>
<td>0.04</td>
<td>0.76 0.92</td>
<td>0.35</td>
<td>79.8</td>
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<tr>
<td>miR_3613</td>
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<td>0.66 0.85</td>
<td>0.014*</td>
<td>74.0</td>
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<td>let_7b</td>
<td>0.78</td>
<td>0.05</td>
<td>0.69 0.87</td>
<td>0.012*</td>
<td>73.7</td>
<td>82.5</td>
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*Greater than cut-off indicates lower odds of being in endometriosis group.

Supplemental Table 1. Primer sequences

<table>
<thead>
<tr>
<th>miRNA</th>
<th>primer</th>
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<td>Let-7b-5p</td>
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<td>UCUCACACAGAAAUCGCACCCGU</td>
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<tr>
<td>miR-451a</td>
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<td>AAACCGUUAACAUACUGAGU</td>
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<td>miR-3613-5p</td>
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<tr>
<td>U6</td>
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<td>CTCGCTTCGCGCAGCACA</td>
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FIGURE LEGENDS

**Figure 1.** miRNA expression in control vs. endometriosis subject serum. Expression levels of six miRNAs, normalized relative to levels of the small nuclear RNA gene U6. Data are plotted showing the median and the interquartile range (IQR). Whiskers and outliers are displayed according to the Tukey method, which plots whiskers at the points falling less than or equal to 1.5 times IQR (25th percentile minus IQR or 75th percentile plus IQR), with points falling outside this range plotted individually. *p<0.05, ***p<0.001, using the Mann-Whitney U test followed by the Bonferroni correction for multiple comparisons.

**Figure 2.** miRNA expression during proliferative or secretory phase. MiRNA expression levels in control subjects, separated by phase in menstrual cycle at the time of serum sampling, and normalized relative to levels of the small nuclear RNA gene U6. Data are plotted showing the median and the interquartile range (IQR). No significant differences were found (p>0.05, Mann-Whitney U test).

**Figure 3.** miRNA expression according to rASRM staging. MiRNA expression levels in endometriosis subjects, divided by stages of endometriosis: I/II, minimal/mild; III/IV, moderate/severe according to rASRM guidelines. Levels were normalized relative to levels of the small nuclear RNA gene U6. Data are plotted showing the median and the interquartile range (IQR). Groups were compared using the Kruskal-Wallis test (a non-parametric one-way ANOVA), and the Dunn’s multiple comparisons test was used to compare pairwise means of each subgroup. *p<0.05, **p<0.01, ***p<0.001

**Figure 4.** Performance of the classifier algorithm in the training and independent dataset. Receiver operating characteristic analysis of the Random Forest model using six miRNA biomarkers (miR-125b-
miR-150-5p, miR-342-3p, miR-451a, miR-3613-5p, let-7b). The model was derived in the current (n=100) dataset (“Train”) and tested in an independent cohort (n=48) dataset (“Test”).

Supplemental Figure 1. miRNA expression with or without hormonal treatment. MiRNA expression levels in endometriosis subjects, analyzed by presence or absence of hormonal treatment (HT). Levels were normalized relative to levels of the small nuclear RNA gene U6. Data are plotted showing the median and the interquartile range (IQR). No significant differences were found (p>0.05, Mann-Whitney U test).
Figure 1

miR-125b

miR-342

miR-3613

miR-150

miR-451a

Let-7b