Circulating miR-200–family micro-RNAs have altered plasma levels in patients with endometriosis and vary with blood collection time

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Objective: To determine whether circulating micro-RNA (miR) 200a, miR-200b, and miR-141 have altered levels in patients with endometriosis compared with control individuals.

Design: Experimental laboratory study.

Setting: University.

Patient(s): Patients with endometriosis (n = 61), laparoscopically confirmed endometriosis-free women (n = 35), and self-reported healthy women (n = 30) were included in the study.

Intervention(s): None.

Main Outcome Measure(s): Plasma miRNA levels in endometriosis patients and control subjects.

Result(s): We found that the levels of studied miRNAs varied with blood collection time, being lower in the morning than in the evening. When blood collection time was taken into account, the results revealed significantly lower levels of miR-200a and miR-141 in the evening plasma samples of women with endometriosis compared with surgically confirmed disease-free patients. However, the evening-sample levels of all three miRNAs were significantly lower in patients with stage I–II endometriosis than in endometriosis-free control subjects. In cases of stage III–IV endometriosis, only miR-200a levels were significantly lower compared with patients without endometriosis. Circulating miR-200a showed the best discriminative power to differentiate women with endometriosis from patients with similar complaints but without the disease.

Conclusion(s): Our findings suggest that miR-200a and miR-141 have a potential as novel noninvasive biomarkers for endometriosis. In addition, we found that the plasma miR-200a, miR-200b and miR-141 levels vary with blood sampling time, so it is important to take the sample collection time into account when studying miRNAs as biomarkers. (Fertil Steril 2015; )

Key Words: miRNA, miR-200 family, endometriosis, biomarker, circadian rhythm

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/rekker-circulating-mirnas-endometriosis/
to protein complexes [3, 4]. Alterations in miRNA levels in blood may reflect the changes during normal physiologic processes [5, 6] and have been related to several pathologic conditions, including gynecologic diseases [7, 8].

Endometriosis is a common gynecologic disease currently diagnosed mainly by a laparoscopy. The symptoms of the disease are mostly nonspecific, including pelvic pain and infertility, which are also related to a number of other disorders [9]. There are no reliable noninvasive diagnostic markers for endometriosis, so numerous women with uncertain complaints are directed to diagnostic laparoscopy. Although several noninvasive techniques can be applied to aid the diagnosis (e.g., imaging with ultrasound, magnetic resonance imaging), these are mainly useful for detecting the presence of ovarian endometriomas or deep infiltrating endometriosis [10]. Thus, there is a need for objective and reliable noninvasive diagnostic biomarkers, particularly for ultrasound-negative endometriosis [11–13].

However, regardless of extensive studies, only slight progress has been made in the development of noninvasive diagnostic tests for endometriosis. Recent studies have focused on different proteomic, transcriptomic, and metabolomic approaches for discovering endometriosis biomarkers [11], and during the past few years miRNAs have emerged as novel candidate markers for the disease. Several potential circulating blood miRNA biomarkers for endometriosis have already been proposed [14–18], but the results are inconsistent and no single miRNA alteration has been confirmed by other studies.

The miR-200 family miRNAs are differently expressed in endometriotic lesions compared with eutopic endometria [19–21]. Also, we have previously described higher levels of miR-200 in ectopic peritoneal lesions compared with surrounding tissues [22]. Although the relationship between the miR-200 family and endometriosis has been established, only one study has demonstrated altered levels of a single circulating miR-200 family member—miR-141* (miR-141–5p)—in endometriosis patients [16]. Therefore, the aim of the current study was to determine whether three miR-200–family miRNAs (miR-200a–3p, miR-200b–3p, and miR-141–3p) are detectable in the blood plasma of women with and without endometriosis, and to reveal the potential power of these circulating miRNAs as noninvasive diagnostic markers for endometriosis.

MATERIALS AND METHODS

Study Participants

Ninety-six patients undergoing laparoscopy at Tartu University Hospital Women’s Clinic (Tartu, Estonia) or Elite Clinic (Tartu) with a suspicion of endometriosis and/or complaints of severe dysmenorrhea or infertility were enrolled in the study from April 2010 to March 2014. The diagnosis of endometriosis was confirmed by surgical and histologic findings for 61 women. The stage of endometriosis was assessed according to the revised American Society for Reproductive Medicine classification system [23]. The severity of the disease was classified as minimal–mild (stage I–II) and moderate–severe (stage III–IV) for 33 and 28 women, respectively. All patients with stage III–IV endometriosis had ovarian endometriomas, and 23 out of 28 had also peritoneal lesions.

Two different control groups were used: (1) The first group consisted of patients who were confirmed with the use of laparoscopy to be endometriosis free (n = 35); and (2) the second group consisted of self-reportedly healthy women recruited via advertisement (n = 30). A questionnaire was administered to all participants to obtain thorough information regarding general health characteristics, including menstrual history, use of medications, presence of systemic diseases, and other health conditions. Among the surgically confirmed endometriosis-free women, the indications for laparoscopy were as follows: primary infertility (n = 10), secondary infertility (n = 15), suspicion of endometriosis (n = 5), polycystic ovaries (n = 3), and pelvic pain (n = 2). The results of surgery revealed pelvic adhesions (n = 19) and occlusion of the fallopian tubes (n = 1); in 12 cases no detectable pathology was found, and in 3 cases ovarian drilling was necessary. Among the self-reportedly healthy women, no history of endometriosis or autoimmune disorders was reported and none of the individuals suffered from gynecologic, inflammatory, or any chronic diseases at the time of participation. None of the endometriosis patients or control individuals had used hormonal medications for ≥3 months before the study. The clinical characteristics of study participants are presented in Table 1.

An additional study group of eight healthy women (aged 23–53 years, body mass index [BMI] 24 ± 4.3 kg/m², non-smokers) was recruited to determine the effect of blood collection time on the studied miRNA levels. Two out of eight of these individuals were using hormonal medications at the time of participation. The individuals of this study group were not included in the above-mentioned control group of self-reportedly healthy women.

The study was approved by the Research Ethics Committee of the University of Tartu, and a written informed consent was obtained from every participant.

Blood Collection and Processing

Blood samples from patients attending the hospital for scheduled surgery were collected in the evening or in the morning before anesthesia for the laparoscopy. For healthy control subjects, the time of blood collection was not determined, but most samples were collected in the afternoon. Peripheral blood samples were collected into EDTA (9 mL) collection tubes and processed within 1 hour after collection.

To evaluate the impact of blood collection time on miRNA levels, paired samples from healthy participants (n = 8) were drawn into EDTA tubes in the evening (~4 p.m.) and in the subsequent morning (~8–9 a.m.).

Plasma was isolated by means of two centrifugations at 1,600g for 10 minutes and at 16,000g for 10 minutes. Centrifugations were performed at 4°C. Samples were stored at −80°C until further use.

RNA Isolation and Taqman Quantitative Real-time Polymerase Chain Reaction

RNA was isolated from 500 μL plasma with the use of miRNAeasy Mini Kit (Qiagen) according to the manufacturer’s instructions.
The expression levels of hsa-miR-200a-3p (assay ID 000502, hereafter called miR-200a), hsa-miR-200b-3p (ID 0002251, miR-200b), and hsa-miR-141 (ID 000463, miR-141) were determined by means of quantitative real-time polymerase chain reaction (PCR) with the use of Taqman microRNA assays (Applied Biosystems). For normalization, two miRNAs, hsa-miR–30e–5p (ID 0002223) and hsa-miR–99a–5p (ID 000435), showing stable expression within plasma samples according to our previous experiments (data not shown) were used as references for data normalization. cDNA synthesis was conducted with the use of Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems) and real-time PCR was performed with the use of Taqman Universal PCR Master Mix–No AmpErase UNG (Applied Biosystems).

Data Analysis
The potential confounding factors (age, BMI, menstrual cycle phase) and other variables characterizing the study participants were compared among five study groups (evening and morning samples from patients with and without endometriosis and from healthy individuals) with the use of one-way analysis of variance (ANOVA) or analogous logistic modeling. The normalized expression levels of studied miRNAs were characterized by relative cycles to threshold values (ΔCt) calculated according to the formula ΔCt = C_{miRNA} – C_{Ref}, with C_{miRNA} being the cycles to threshold value of corresponding target miRNA, and C_{Ref} being the arithmetic mean of cycles to threshold values of the two reference miRNAs. Normally distributed ΔCt values of different study groups were compared with the use of ANOVA. Also, models considering potential confounding factors, such as age, BMI and menstrual cycle phase, and their interactions with grouping factor were fitted. Because neither added factors nor their interactions proved to be statistically significant (all P > .05) and more complicated models also were not statistically significantly better compared with simple ANOVA (all P > .05; F test), only results of one-way ANOVA are presented. In post hoc pairwise comparisons of five study groups, after the modeling step the correction for multiple testing was applied with the use of the Tukey–Kramer method. Additional comparisons of evening and morning samples over study groups and study groups over sampling time comparisons were performed with the use of properly defined contrasts. The same methodology was used to compare the miRNA levels (ΔCt values) in patients without endometriosis, with stage I–II endometriosis, and with stage III–IV endometriosis; because there were no differences in miRNA levels between morning samples, only evening samples were used in these analyses. The miRNA levels in the evening and morning samples of additionally recruited healthy individuals were compared with the use of paired-samples t test. All results of P < .05 were considered to be statistically significant. To transform the values such that lower Ct value corresponds to higher miRNA level, the group means of ΔCt values were multiplied by −1. The group differences between ΔCt values (ΔΔCt) were expressed in the form of fold changes (FC) calculated by formula FC = 2−ΔΔCt (24). Receiver operating characteristic (ROC) curve analysis following the logistic regression modeling was used to evaluate the suitability of circulating miR-200a, miR-200b, and miR-141 levels to discriminate between evening samples of patients with and without endometriosis and to establish the sensitivity and specificity of each individual miRNA; because there were no differences in miRNA levels between morning samples, only evening samples were used in these analyses; also, healthy individuals were omitted. To determine the diagnostic applicability for the combination of miRNAs, the multivariate logistic regression model was used as described earlier (25). The statistical analyses were performed and figures

**TABLE 1**

Clinical characteristics of the study participants.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with endometriosis</th>
<th>Patients without endometriosis</th>
<th>Healthy participants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morning samples (n = 29)</td>
<td>Evening samples (n = 32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age, y</td>
<td>32 ± 5.3</td>
<td>33 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>BMI, kg/m²</td>
<td>23 ± 3.0</td>
<td>23 ± 3.8</td>
</tr>
<tr>
<td>Menstrual cycle phase, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>12 (41%)</td>
<td>12 (38%)</td>
<td>4 (36%)</td>
</tr>
<tr>
<td>Luteal</td>
<td>17 (59%)</td>
<td>19 (59%)</td>
<td>7 (64%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>0</td>
<td>1 (3%)</td>
<td>0</td>
</tr>
<tr>
<td>Sexually active during past year, n (%)</td>
<td>22 (76%)</td>
<td>25 (78%)</td>
<td>10 (91%)</td>
</tr>
<tr>
<td>Complaints of infertility, n (%)</td>
<td>17 (65%)</td>
<td>22 (71%)</td>
<td>8 (73%)</td>
</tr>
<tr>
<td>rASRM stage, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>14 (48%)</td>
<td>19 (59%)</td>
<td>NA</td>
</tr>
<tr>
<td>I–IV</td>
<td>15 (52%)</td>
<td>13 (41%)</td>
<td>NA</td>
</tr>
<tr>
<td>Menstrual cycle–related pain, b n (%)</td>
<td>18 (64%)</td>
<td>17 (53%)</td>
<td>6 (55%)</td>
</tr>
<tr>
<td>Nonmenstrual pelvic pain, n (%)</td>
<td>2 (7%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dyspareunia, n (%)</td>
<td>4 (14%)</td>
<td>1 (3%)</td>
<td>1 (9%)</td>
</tr>
</tbody>
</table>

Note: Data are presented as mean ± SD. BMI = body mass index; rASRM = revised American Society for Reproductive Medicine; NA = not applicable; follicular phase = menstrual cycle day 1–14; luteal phase = menstrual cycle day 15–28.

* Analysis of variance for age and BMI logistic model for binary variables.
*b Severe pain symptoms before and at the time of menstruation. Pain was scored on a 5-point scale, and levels 4–5 with duration longer than 1 y were considered to be severe.

generated with the use of SAS 9.4, Medcalc version 13.0.4.0, and Graphpad Prism version 6.04.

RESULTS

Circulating miRNA Levels in Patients with and without Endometriosis and in Healthy Control Subjects

The levels of three miR-200 family miRNAs were determined from the plasma samples of patients with endometriosis (n = 61), endometriosis-free patients (n = 35), and healthy individuals (n = 30). The blood samples from all patients undergoing laparoscopic surgery (patients with and without endometriosis) were collected either early in the morning or in the evening; therefore we assumed that the sample collection time may have an impact on the miRNA levels and divided the patient groups further according to the blood sampling time. For healthy individuals, the exact blood sampling time was not recorded, but the majority of the samples were collected in the afternoon.

Accordingly, the plasma levels of miR-200a, miR-200b, and miR-141 were compared among five study groups: morning (n = 29) and evening (n = 32) samples of the patients with endometriosis, morning (n = 11) and evening (n = 24) samples of the patients without endometriosis, and samples of healthy women (n = 30). The post hoc pairwise comparisons revealed that miR-200a and miR-141 levels in the evening samples were significantly lower in women with endometriosis compared with endometriosis-free patients (P = 0.002 and P = 0.024, respectively, both FC = −1.7; Fig. 1) but the levels of miR-200b did not differ among these groups. The levels of miR-200a and miR-200b (but not miR-141) were significantly different between evening samples of the patients with endometriosis and healthy women (P = 0.003 and P < 0.001, respectively). No significant differences in miRNA levels were observed when evening samples from patients without endometriosis were compared with healthy individuals (all P > 0.05).

Furthermore, no alterations were detected when morning samples from patients with and without endometriosis were compared (all P > 0.05). However, the levels of all three studied miRNAs were statistically different between morning samples of endometriosis patients and healthy individuals (all P ≤ 0.001), and statistically significant differences were observed in miR-200a and miR-200b levels between morning samples from endometriosis-free patients and healthy individuals (both P < 0.001; Fig. 1), confirming the influence of the blood sampling time because the majority of samples from healthy individuals were collected toward the evening. The descriptive statistics (mean, standard deviation, minimum, maximum) of relative miRNA levels in study groups are also presented in Supplemental Table 1 (available online at www.fertstert.org).

The Impact of Blood Collection Time on miRNA Levels

Because the preceding analysis suggested that in addition to the disease status the differences in the studied plasma miRNA levels significantly vary according to the blood collection time, the miRNA levels in blood samples drawn in the evening (n = 56) and morning (n = 40) from all patients undergoing laparoscopic procedure were compared (patients with and without endometriosis were divided according to the blood sampling time without considering disease status). The data analysis showed that miRNA levels significantly varied with the blood collection time being 1.9-, 1.6-, and 1.6-fold lower in the morning for miR-200a, miR-200b, and miR-141, respectively (all P < 0.001). When miRNA levels were analyzed inside the study groups, post hoc tests showed that miR-200a levels were 1.5 and 2.5 times lower in morning samples of patients with and without endometriosis compared with the respective evening samples (P = 0.029 and P < 0.001, respectively; Fig. 1), miR-200b levels differed only between the morning and evening samples of the endometriosis-free patients (P = 0.025, FC 1.9), and miR-141 levels revealed no significant differences.

Next, because all of the above comparisons were performed based on the blood samples collected in the hospital, these miRNAs were also measured from additional paired blood samples of healthy women collected in the evening (n = 8) and in the subsequent morning (n = 8). As in patients, the levels of studied miRNAs in healthy individuals were significantly lower in the morning (all P < 0.01; FCs for miR-200a, miR-200b, and miR-141 were −2.8, −2.7, and −2.5, respectively; Fig. 2).

Effect of Disease Severity on Plasma miRNA Levels

The patients were divided according to disease stage, and, owing to the effect of blood sampling time, only the samples collected in the evening were used for this analysis. miR-200a, miR-200b, and miR-141 levels were significantly lower in patients with stage I–II endometriosis (n = 19) than in endometriosis-free control subjects (FC −1.9, −1.6, and −2.1; P = 0.001, P = 0.021, and P = 0.002; respectively). However, no differences in miR-200b and miR-141 levels were found between patients with stage III–IV endometriosis (n = 13) and patients without endometriosis (P > 0.05), whereas miR-200a levels were 1.7 times lower in patients with stage III–IV endometriosis compared with patients without endometriosis (P = 0.010).

Suitability of Studied miRNAs as Potential Endometriosis Biomarkers

An ROC curve analysis was performed to evaluate the ability of plasma miRNAs to discriminate individuals with endometriosis (n = 32; evening samples only) from patients with similar complaints but without endometriosis (n = 24; evening samples only). The analysis showed that the studied miRNAs enable the differentiation of women with and without endometriosis. Area under the ROC curve (AUC) values for miR-200a, miR-200b, and miR-141 were 0.75 (95% confidence interval [CI] 0.62–0.86), 0.67 (95% CI 0.53–0.79), and 0.71 (95% CI 0.57–0.82), respectively. The sensitivity and specificity for miR-200a were 90.6% and 62.5%, respectively; for miR-200b 90.6% and 45.8%; and for miR-141 71.9% and 70.8%. The combined signature of three miRNAs showed a
Relative miRNA levels in plasma: (A) miR-200a; (B) miR-200b; (C) miR-141. Values are presented as mean ± SD; results of pairwise comparisons were corrected for multiple testing with the use of the Tukey-Kramer method, and only P values corresponding to statistically significant differences (P ≤ .05) are shown. For illustrative purposes, the group means of ΔCt values were multiplied by –1.

to determine, we can only hypothesize that the lower levels of circulating miR-200a and miR-141 in endometriosis patients makes the cells in these individuals more prone to EMT. However, with our study design it could not be determined whether the levels of miRNAs under investigation were altered earlier the disease occurrence or were caused by the presence of endometriosis.

Circulating miR-200a, miR-200b, and miR-141 have been proposed as candidate biomarkers for several other pathologic conditions as well. Elevated levels of cell-free miR-200a and miR-200b have been shown in various cancer patients, including ovarian (29) and pancreatic (30) cancer, and high levels of plasma miR-141 were found in advanced colon cancer (31). In contrast, lowered levels of particular circulating miRNAs, including miR-200a and miR-200b, have been reported in systemic lupus erythematosus patients’ serum and urine samples (32). Interestingly, whereas some researchers have found that the increased circulating miR-200 levels are consistent with elevated levels in cancer tissues (29, 30), others have suggested that elevated miRNA level in plasma is not a simple reflection of the expression in the corresponding tumor tissue (31). Aberrant miRNA expression has been observed in the endometria of women with endometriosis (33, 34), and these changes may also be reflected in circulating miRNA levels. For example, down-regulation of miR-9-5p has been demonstrated in the endometria (33) as well as serum samples (16) of women with endometriosis, but most of the reported endometriosis-associated dysregulated endometrial miRNAs have not been shown to be altered in plasma. Interestingly, miR-135a that was overexpressed in the endometrium of endometriosis patients (35) showed significantly lower levels in sera of patients with endometriosis compared with control subjects (18). We previously demonstrated that miR-200a, miR-200b, and miR-141 are not differentially expressed in the endometrium of women with and without endometriosis (22), so endometrium is likely not the tissue of origin for the miR-200 plasma alterations in endometriosis.

To date, five reports have been published demonstrating circulating miRNAs as potential endometriosis biomarkers (Table 2). However, each study found a different set of possible markers (14–18), with miR-199a being the only marker reported twice (16, 17). Still, the direction of alteration in miR-199a levels among patients was opposite in these reports. The discrepancies are most likely due to the differences in the study design, because the groups, sample types, and normalization strategies varied among the reports. In addition, Wang et al. (36) reported altered levels of miR-141* (miRBase ID hsa-miR-141-5p) in endometriosis patients’ serum. Similarly to this, we showed lower levels of plasma miR-141 in endometriosis patients. miR-141* and miR-141 originate from the opposite arms of the same precursor miRNA, but miRNAs marked with an asterisk (*) are the less abundant forms in cells and tissues (36).

The blood samples of the patients directed to laparoscopic surgery were collected either in the previous evening or in the morning before surgery; therefore we hypothesized that the blood collection time may have an impact on miRNA levels
might be in (41). So far there are no data on whether the miR-200 family miR-142-5p levels in vitreous humor (40) but not in blood differ significantly, because day-night rhythmicity was observed in that the same miRNA may be regulated in distinct tissues (39), and data from humans are scarce. Moreover, it seems confirming that the blood sampling time is a factor having an apparent impact on the miR-200 levels.

If the blood sampling time was considered in the endometriosis analysis and only the samples taken in the morning were analyzed, no differences were detected in patients with endometriosis compared with patients without the disease. The probable cause is that the impact of circadian rhythm on the studied miRNA levels is stronger than the disease effect and as a result masks the endometriosis-associated alterations. However, clear differences in miR-200a and miR-141 levels were seen between these study groups when the evening samples were studied, and evening samples from endometriosis-free control subjects did not differ from healthy individuals, whose blood samples were also mostly collected in the afternoon. Additionally, analysis of patients’ age, BMI, and menstrual cycle phase and their interactions with miRNA levels revealed that the use of miR-200a, miR-200b, and miR-141 as potential endometriosis biomarkers

and divided the study groups accordingly. We discovered that miRNA levels varied significantly with the blood sampling time, and all three miRNAs studied had lower levels in the blood samples taken in the morning, suggesting a possible impact of the circadian rhythm on miR-200 levels. Recent publications have already provided evidence that miRNAs are involved in the regulation of the circadian rhythm and, more importantly, that the level of some miRNAs is probably regulated by the circadian clock (37, 38). Bioinformatics tools were used to predict miRNAs presenting putative sites for circadian transcription factors in their promoters, and the diurnal variation of miR-27 in human leukocytes was experimentally validated (37). However, most of the studies demonstrating rhythmic profiles of miRNA expression are conducted in model organisms, such as mice, Drosophila, and Arabidopsis (39), and data from humans are scarce. Moreover, it seems that the same miRNA may be regulated in distinct tissues differently, because day-night rhythmicity was observed in miR-142-5p levels in vitreous humor (40) but not in blood (41). So far there are no data on whether the miR-200 family might be influenced by the circadian rhythm.

Because we found fluctuations in miRNA levels in patients’ blood samples drawn in the hospital, additional paired plasma samples from healthy individuals were investigated to confirm that these evening-morning alterations were not caused by other confounding factors (e.g., drugs administered in the hospital, various levels of hospital stress in the evening vs. morning). Although the group of healthy individuals involved in this part of the study was heterogeneous (the use of hormonal medications by some individuals, age and BMI differences), similar effect of blood sampling time on miRNA levels was observed in healthy participants, confirming that the blood sampling time is a factor having an apparent impact on the miR-200 levels.

**TABLE 2**

Comparison of endometriosis miRNA biomarker studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample type</th>
<th>Study participants</th>
<th>Methods</th>
<th>Endogenous reference</th>
<th>Detected miRNA markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang et al., 2013 (16)</td>
<td>Blood serum</td>
<td>Endometriosis patients (n = 60) and laparoscopically confirmed endometriosis-free patients (n = 25)</td>
<td>765 Taqman miRNA array; validation qRT-PCR with Sybr Green</td>
<td>U6</td>
<td>miR-199a↑ miR-122↑ miR-9*↓ miR-145*↓ miR-141↑ miR-542–3p↓ miR-17-5p↓ miR-20a↓ miR-22↓</td>
</tr>
<tr>
<td>Jia et al., 2013 (14)</td>
<td>Blood plasma</td>
<td>Stage III-IV endometriosis patients (n = 23) and laparoscopically confirmed endometriosis-free patients (n = 23)</td>
<td>Microarray with 1,205 miRNA probes, validation qRT-PCR with Sybr Green</td>
<td>miR-16</td>
<td></td>
</tr>
<tr>
<td>Suryawanshi et al., 2013 (15)</td>
<td>Blood plasma</td>
<td>Endometriosis patients (n = 33) and healthy women (n = 20)</td>
<td>qRT-PCR based kit for 1,113 miRNAs, validation qRT-PCR with Sybr Green</td>
<td>miR-132</td>
<td>miR-16↑ miR-191↑ miR-195↑ miR-199a-5p↓</td>
</tr>
<tr>
<td>Hsu et al., 2014 (17)</td>
<td>Blood serum</td>
<td>Endometriosis patients (n = 40) and surgically confirmed endometriosis-free patients (n = 25)</td>
<td>Seramir microarray with 380 oligonucleotides, validation Taqman qRT-PCR</td>
<td>18S RNA</td>
<td></td>
</tr>
<tr>
<td>Cho et al., 2015 (18)</td>
<td>Blood serum</td>
<td>Endometriosis patients (n = 24) and laparoscopically confirmed endometriosis-free patients (n = 25)</td>
<td>qRT-PCR with Sybr Green</td>
<td>U6</td>
<td>let7b↓ miR-135a↓</td>
</tr>
<tr>
<td>This study</td>
<td>Blood plasma</td>
<td>Endometriosis patients (n = 61), laparoscopically confirmed endometriosis-free patients (n = 35), and healthy women (n = 30)</td>
<td>Taqman qRT-PCR</td>
<td>miR-30e and miR-99a</td>
<td>miR-200a↓ miR-141↓</td>
</tr>
</tbody>
</table>

Note: ↓ = lower levels in endometriosis patients compared with control subjects; ↑ = higher levels in endometriosis patients compared with control subjects; qRT-PCR = quantitative real-time PCR.

does not depend on such features as age, BMI, or menstrual cycle phase (at least in the patients of the present study). These results suggest that if we avoid the bias caused by blood collection time, these miRNAs indeed enable distinguishing endometriosis patients from women with similar symptoms. Furthermore, the alterations in miR-200a and miR-200b levels were also present when the samples from endometriosis-free patients taken in the morning were compared with samples from healthy individuals, but miR-141 levels were similar between endometriosis-free control subjects and healthy women regardless of the blood collection time. On the one hand, this confirms the possible diurnal changes in miRNA levels, but on the other hand, it suggests that there are some differences in miRNA expression between these control groups.

Laparoscopic surgery with subsequent histologic confirmation of endometriotic lesions is the criterion standard for defining endometriosis patients. However, selecting an appropriate control group is more challenging, and therefore using multiple control groups in endometriosis studies is suggested [42, 43]. The best control group for discovering noninvasive endometriosis biomarkers is composed of symptomatic women who are laparoscopically proved to be disease free but that have other benign gynecologic conditions [43]. Generally, visual examination during laparoscopy ensures that women undergoing the procedure do not suffer from endometriosis, but even then some small lesions may remain unnoticed. Furthermore, because some of these gynecologic conditions may in turn influence the levels of studied markers, we used a second control group of self-reported healthy women. Similarity of the two control groups’ miRNA levels in plasma samples collected in the afternoon confirms the validity of the hospitalized patients as endometriosis control subjects in our study and suggests that the observed differences between patients with and without endometriosis are indeed related to the diagnosis of endometriosis. However, because the exact time of the blood collection was not recorded for healthy women, and this control group may also include some blood samples collected in the early afternoon, these data should be considered with caution.

Because the results indicated that the lower levels of the studied miRNAs in the morning may mask or artificially enhance the differences between the study groups, only the data from evening samples were used for determining the suitability of miR-200a, miR-200b, and miR-141 as potential endometriosis biomarkers. Out of the three miR-200 family members investigated, miR-200a indicated the best discriminative power to distinguish women with endometriosis from patients with similar complaints but without the disease with relatively high sensitivity (90.6%) but moderate specificity (62.5%). The ROC curve analysis with the combination of three miRNAs (miR-200a, miR-200b, and miR-141) did not result in better sensitivity and specificity, which can be explained with the highly correlated expression pattern of miR-200 family miRNAs. The miR-200 family consists of five members, which form two clusters in different chromosomes and are transcribed from distinct epigenetically regulated promoters [44]. However, the members of the same cluster are regulated similarly; and moreover, several studies have demonstrated that miR-200 family members from different clusters can be repressed [45] or activated [46] coordinately. Also, the fact that the interactions of the three studied miRNAs did not influence their distinguishing ability between patients with and without endometriosis supports the assumption that there is no intensifying or suppressing effects of these miRNAs on each other.

Some limitations of our study should be specially mentioned. First, the numbers of studied individuals are rather small. Collection of blood samples from women in reproductive age and not using hormonal medication and undergoing laparoscopy is a rather time-consuming process. Therefore, the numbers of analyzed samples in studies using similar design have remained moderate (Table 2), and the solution for this shortcoming could possibly be in cooperation between study centers. Second, the study lacks validation with independent samples. To propose some marker as diagnostic tool for endometriosis, several requirements should be fulfilled, including a retrospective validation and a prospective validation phase; unfortunately, most endometriosis biomarker studies have remained at the discovery phase [47]. Third, it should be taken into account that the plasma miR-200 level differences between the studied groups are probably valid only with the use of the same endogenous references as those used in this study. There is no consensus about which approach should be used to normalize plasma miRNA levels, and the issue of normalization strategies for circulating miRNAs in endometriosis studies was recently discussed by Nothnick et al. [48] with the suggestion of using digital PCR to measure the absolute quantity of the amount of the target.

In conclusion, we identified lower levels of miR-200a and miR-141 in the plasma of patients with endometriosis compared with women with similar complaints but without the disease. Although the accuracy of the studied miRNAs as noninvasive endometriosis biomarkers is not sufficient enough to set a clinical diagnosis, it is probable that these miRNAs can be used in combination with other markers or imaging techniques to yield improved precision. We also discovered that the levels of all three studied plasma miRNAs varied with the blood sampling time, possibly due to the circadian fluctuations of these miRNAs. Our results indicate that it is crucial to take the blood sampling time into account when studying circulating miRNAs as biomarkers.

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REFERENCES


SUPPLEMENTAL FIGURE 1

Receiver operating characteristic (ROC) curve analysis of plasma miRNAs. Area under the ROC curve (AUC), sensitivity (SN), and specificity (SP) for (A) miR-200a, (B) miR-200b, (C) miR-141, and (D) the combination of these miRNAs were calculated based on the data from women with endometriosis (n = 32) and patients with similar complaints but without the disease (n = 24).

<table>
<thead>
<tr>
<th>miRNA</th>
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<th></th>
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<td>SD</td>
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<td>Max</td>
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<td>SD</td>
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<td>−7.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Patients with endometriosis

Patients without endometriosis

Healthy women (afternoon samples)

miRNA levels are presented as relative to reference miRNA levels: Ct (target miRNA) – Ct (the mean of reference miRNAs).