Circulating MicroRNA miR-323-3p as a Biomarker of Ectopic Pregnancy

Zhen Zhao,1 Qiuhong Zhao,2 Joshua Warrick,1 Christina M. Lockwood,1 Alison Woodworth,3 Kelle H. Moley,2 and Ann M. Gronowski1,2*

BACKGROUND: The use of serum human chorionic gonadotropin (hCG) and progesterone to identify patients with ectopic pregnancy (EP) has been shown to have poor clinical utility. Pregnancy-associated circulating microRNAs (miRNAs) have been proposed as potential biomarkers for the diagnosis of pregnancy-associated complications. This proof-of-concept study examined the diagnostic accuracy of various miRNAs to detect EP in an emergency department (ED) setting.

METHODS: This study was a retrospective case–control analysis of 89 women who presented to the ED with vaginal bleeding and/or abdominal pain/cramping and received a diagnosis of viable intrauterine pregnancy (VIP), spontaneous abortion (SA), or EP. Serum hCG and progesterone concentrations were measured by immunoassays. The serum concentrations of miRNAs miR-323-3p, miR-517a, miR-519d, and miR-525-3p were measured with TaqMan real-time PCR. Statistical analysis was performed to determine the clinical utility of these biomarkers, both as single markers and as multimarker panels for EP.

RESULTS: Concentrations of serum hCG, progesterone, miR-517a, miR-519d, and miR-525-3p were significantly lower in EP and SA cases than in VIP cases (P < 0.01). In contrast, the concentration of miR-323-3p was significantly increased in EP cases, compared with SA and VIP cases (P < 0.01). As a single marker, miR-323-3p had the highest sensitivity of 37.0% (at a fixed specificity of 90%). In comparison, the combined panel of hCG, progesterone, and miR-323-3p yielded the highest sensitivity (77.8%, at a fixed specificity of 90%). A stepwise analysis that used hCG first, added progesterone, and then added miR-323-3p yielded a 96.3% sensitivity and a 72.6% specificity.

CONCLUSIONS: Pregnancy-associated miRNAs, especially miR-323-3p, added substantial diagnostic accuracy to a panel including hCG and progesterone for the diagnosis of EP.

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Ectopic pregnancy (EP)4 occurs when a conceptus implants outside the endometrial cavity. Although only 1.3%–2% of all pregnancies are ectopic, the prevalence of EP is 6%–16% among pregnant patients presenting to the emergency department (ED) with vaginal bleeding and/or abdominal pain (1). Patients are at risk of tubal rupture and death. This condition contributes to 9%–13% of pregnancy deaths in developed countries and 10%–30% of such deaths in African developing countries (2). The diagnosis of EP is made by transvaginal ultrasonographic identification of an extraterine gestational sac containing a yolk sac; however, surgical or biochemical assessment is necessary when ultrasound is not definitive, as occurs in approximately 8%–31% of patients seen in specialty centers (3).

Serum human chorionic gonadotropin (hCG) and progesterone have been the most intensively investigated biochemical markers for the diagnosis of EP. A single measurement of hCG or progesterone is primarily an indication of pregnancy viability rather than location, however, and is thus insufficient to diagnose EP. Alternatively, the approach of serial hCG monitoring is often used for patients with bleeding and/or pain in early pregnancy (4). The American College of Obstetricians and Gynecologists suggests that viable intrauterine pregnancy (VIP) is associated with an increase in the hCG concentration of ≥53% in 48 h (5). As with a single measurement, however, serial serum hCG measurements cannot identify the location of the gestational sac and are useful only to prove fetal viability, rather than to identify EP. With the use of serial serum hCG measurements, patients are at risk of tubal rupture during the wait for the next hCG measurement.

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Considering these 2 biomarkers together, the use of serum hCG and/or progesterone to identify EP is limited not only by high false-positive and false-negative rates but also by difficulties in distinguishing between EP and spontaneous abortion (SA). Therefore, there is a compelling demand for the development of new, noninvasive serum tests to diagnose EP with high sensitivity and high specificity to prevent not only sudden, life-threatening complications but also unnecessary surgical or medical management that may interrupt a potentially viable pregnancy.

MicroRNAs (miRNAs) may serve as improved biomarkers for numerous pathologic conditions, including cancer, autoimmune diseases, sepsis, acute myocardial infarction, and others (6). In contrast to many biomarkers, miRNAs appear to be highly stable. They are robust enough to tolerate enzymatic degradation, freeze–thaw cycles, and extreme pH conditions (7, 8). Most recently, the identification of pregnancy-associated circulating miRNAs has generated much interest in investigating their potential roles as biomarkers for the diagnosis of pregnancy-associated complications (9–12). The altered concentrations of pregnancy-associated miRNAs may reflect tissue-specific physiological or pathologic states during pregnancy, such as preeclampsia (13–16), preterm labor (17), exposure to toxins (18, 19), and fetal growth restriction (20, 21).

The objectives of this proof-of-concept study were to (a) identify pregnancy-associated miRNAs with altered serum concentrations in EP and (b) investigate their diagnostic accuracy for the detection of EP in patients exhibiting symptoms of vaginal bleeding and/or abdominal pain/cramping in an ED setting.

Materials and Methods

PARTICIPANT RECRUITMENT AND SAMPLE COLLECTION
This investigation was a multicenter, retrospective cohort study between Vanderbilt University, Nashville, Tennessee, and Washington University, St. Louis, Missouri. Remnant serum samples sent to the laboratory from the ED for physician-ordered hCG testing between November 14, 2007, and November 12, 2009, were used. Samples were included if they met the following criteria: serum hCG > 5 IU/L; women exhibiting symptoms of vaginal bleeding, abdominal pain, and/or abdominal cramping; an available volume of serum sufficient to perform progesterone and miRNA testing; patient age ≥18 years; singleton gestation; and gestational age (GA) ≤10 weeks. All serum samples were sent to the laboratory for quantitative hCG testing by the ED were retained and frozen at −70 °C for <42 months before progesterone and miRNA testing was performed. Charts were reviewed to determine patient symptoms, ultrasound findings, and pregnancy outcome. All diagnoses had been confirmed by ultrasound, histopathology, serial hCG measurements, and/or clinical follow-up. Samples from Vanderbilt University were shipped frozen to Washington University for hCG, progesterone, and miRNA testing. Human studies committee approval was obtained from both institutions for this study. Thirty-four patients with VIP, 28 patients with SA, and 27 patients with EP were included after they met all of the inclusion criteria mentioned above. At the time of serum collection, the location of the pregnancy was unknown in 7 of the VIP cases, 17 of the SA cases, and 11 of the EP cases. In this study, 13 samples were from Vanderbilt University, and 76 samples were from Washington University. This investigation was a substudy of a study examining activin A as a marker of EP (22). Forty-nine patients from the previous study were included in this study. The rest of the samples included in the previous study were not included in the present study owing to insufficient serum volume. An additional 40 samples were randomly selected from the cohort of hCG samples collected consecutively from the ED between December 27, 2007, and November 12, 2009.

RNA EXTRACTION
Total RNA containing miRNAs was extracted from 250 µL serum with TRIzol LS (Invitrogen) according to the manufacturer’s protocol, with the modification that the samples were extracted twice with chloroform. Total RNA was dissolved in 10 µL of RNase-free water (Mediatech).

QUANTIFICATION OF miRNAs BY REAL-TIME QUANTITATIVE REVERSE-TRANSCRIPTION PCR ANALYSIS
Quantitative real-time PCR was performed according to a previously described protocol, with modifications (23, 24). The sequences for the primers and probes were obtained from previously validated assays (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol58/issue5) (23, 25). Primers and probes were purchased from Integrated DNA Technologies. In brief, for each sample we added 4 µL of total RNA, 5 nmol/L of each miRNA-specific reverse stem-loop primer and the 18S rRNA reverse primer into a 10-µL multiplex reverse-transcription reaction (High-Capacity cDNA Archive Kit; Applied Biosystems). We used 5 µL of the reverse-transcription product as template for a 25-µL multiplex pre-PCR reaction (18 cycles), which contained 5 µmol/L of the universal reverse primer, 50 nmol/L of each forward primer for the miRNAs, and 50 nmol/L of forward and reverse primers for 18S rRNA. One volume of pre-PCR product was diluted in 499 volumes of water and used as template in a singleplex TaqMan real-time PCR reaction,
chosen by fixing the sensitivity at the highest level. All optimize sensitivity. The cutoff point of each step was appropriate. A stepwise analysis that used hCG first, added pro-

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Table 2 presents the AUCs for all single markers and for various multimarker combinations. The ROC curves were constructed by comparing serum measurements in patients with EP to patients with VIP and SA; this comparison realistically represents the performance of

Results

CONCENTRATIONS OF SERUM hCG, PROGESTERONE, miR-323-3p, miR-517a, miR-519d, AND miR-525-3p

We included 89 patients in the study, 27 patients with EP, 28 patients with SA, and 34 patients with VIP. Patient characteristics are summarized in Table 1. There were no significant differences in maternal age or GA. Overall concentrations of hCG and progesterone in the VIP group were significantly higher than those in the EP and SA groups ($P < 0.001$). The EP and SA groups were not significantly different with respect to either progesterone or hCG. Thirty-one previously reported pregnancy-associated miRNAs (see Table 1 in the online Data Supplement) were initially screened by real-time PCR to identify candidate miRNAs with the capability to discriminate EP and intrauterine pregnancy (data not shown). Four miRNAs (miR-323-3p, miR-517a, miR-519d, and miR-525-3p) were confirmed to have significantly different serum concentrations in women with EP, SA, or VIP (Table 1). The serum concentrations of miR-517a, miR-519d, and miR-525-3p were significantly lower in the EP and SA groups than in the VIP group. In contrast, women with EP had significantly increased serum miR-323-3p concentrations compared with both the SA patients and the VIP patients, but the miR-323-3p concentrations for the SA and VIP groups were not significantly different. miR-323-3p was the only marker tested in this study that showed a significant difference between the EP and SA groups (Table 1; Fig. 1). The EP, SA, and VIP groups were evaluated for any correlations between the concentrations of serum markers and GA. Increased hCG concentrations were significantly correlated with increased GA in the VIP and SA groups ($P < 0.05$, and $P < 0.01$, respectively), and increased miR-517a and miR-519d concentrations were significantly correlated with increased GA in the VIP group ($P < 0.05$). In contrast, hCG concentrations in the EP group, miR-517a and miR-519d concentrations in the EP and SA groups, and progesterone, miR-323-3p, and miR-525-3p concentrations in the EP, SA, and VIP groups showed no correlation with GA (Fig. 2).

DIAGNOSTIC PERFORMANCE OF SERUM hCG, PROGESTERONE, miR-323-3p, miR-517a, miR-519d, AND miR-525-3p IN DIFFERENTIATING VIP, SA, AND EP

Table 2 presents the AUCs for all single markers and for various multimarker combinations. The ROC curves were constructed by comparing serum measurements in patients with EP to patients with VIP and SA; this comparison realistically represents the performance of
Circulating MicroRNAs as Biomarkers for Ectopic Pregnancy

The diagnostic performance of single markers to detect EP was evaluated first, and the new markers were compared with hCG alone and progesterone alone. As single markers, hCG and miR-323-3p had the highest AUCs (0.73 for both; Table 2 and Fig. 3A). At the fixed specificities of 90% and 95%, miR-323-3p had the highest sensitivities for detecting EP (37.0% and 33.3%, respectively). miR-323-3p demonstrated a sensitivity that was 22.2% greater than for hCG alone and 33.3% greater than for progesterone alone, at both the 90% and 95% specificities. These results represent statistically significant gains in sensitivities compared with progesterone (P < 0.001), but not compared with hCG. Each of the other miRNA markers had a sensitivity below that of miR-323-3p when they were analyzed as single markers. Additionally, at the fixed sensitivities of 90% and 95%, hCG had the highest single-marker specificity, 51.6%.

To evaluate the value added by inclusion of the miRNAs, we conducted a multivariate logistic regression analysis to compare the performance of different combinations of biomarkers with that of the dual markers of hCG plus progesterone as a standard reference. The AUCs of different multimarker combinations ranged from 0.81 to 0.88 for detecting EP and were not significantly different from each other (Table 2 and Fig. 3B). The combination of hCG, progesterone, and miR-323-3p produced the highest sensitivities of 77.8% and 33.3%, which added 44.5% and 25.9% to the sensitivities of hCG plus progesterone at specificities of 90% and 95%, respectively (P < 0.01, and P < 0.05, respectively). The addition of other miRNAs to the combination of hCG and progesterone did not increase the sensitivity significantly. Furthermore, the addition of miR-323-3p to the dual-marker combination of hCG plus progesterone increased the specificity from 58.1% to 71.0% at a sensitivity of 90% but slightly reduced it from 41.9% to 40.3% at a sensitivity of 95%. Combining hCG, progesterone, and all 4 miRNAs yielded the highest specificities of 80.7% and 72.6% at the sensitivities of 90% and 95%, respectively. Thus, the “all marker” combination significantly increased the specificities over those for the combination of hCG and progesterone (P < 0.05, and P < 0.01 at sensitivities of 90% and 95%, respectively). The “all marker” sensitivities were decreased by 22.2% and 11.1%, however, compared with those for the panel of hCG, progesterone, and miR-323-3p when specificities were fixed at 90% and 95%, respectively.

To optimize sensitivity, we also performed a stepwise analysis (Fig. 4), which incorporated an hCG cutoff of $\geq 44$ 780 IU/L (the first step, 100% sensitivity and 45.2% specificity), a progesterone cutoff of $\geq 23$ ng/mL ($\geq 73$ nmol/L) (the second step, 100% sensitivity and 50.0% specificity), and an miR-323-3p cutoff of $\geq 0.2$ (the third step, 96.3% sensitivity and 72.6% specificity).

Table 1. Patient age, GA, and serum concentrations of hCG, progesterone, and miRNAs in women with EP, SA, or VIP.

<table>
<thead>
<tr>
<th></th>
<th>EP (n = 27)*</th>
<th>SA (n = 28)*</th>
<th>VIP (n = 34)*</th>
<th>Kruskal-Wallis test</th>
<th>EP vs SA*</th>
<th>EP vs VIP*</th>
<th>SA vs VIP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age, years</td>
<td>25.8 (22.3–30.3)</td>
<td>24.6 (20.7–30.9)</td>
<td>22.9 (20.3–29.3)</td>
<td>NS c</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>GA, weeks</td>
<td>7.0 (5.8–7.8)</td>
<td>7.7 (5.9–8.5)</td>
<td>7.0 (5.7–9.0)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>hCG, IU/L</td>
<td>1725.2 (231.6–10 405.7)</td>
<td>1537.0 (236.5–8049.1)</td>
<td>71 632.2 (47 837.1–124 537.7)</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Progesterone, ng/mL</td>
<td>8.5 (4.2–12.5)</td>
<td>3.2 (1.5–5.8)</td>
<td>23.1 (17.0–32.4)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>miR-323-3p</td>
<td>1.248 (0.525–4.752)</td>
<td>0.327 (0–1.796)</td>
<td>0.351 (0.022–1.244)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>miR-517a*</td>
<td>0.003 (0.002–0.007)</td>
<td>0 (0–0.010)</td>
<td>0.014 (0.005–0.070)</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR-519d*</td>
<td>0.005 (0–0.019)</td>
<td>0.004 (0–0.032)</td>
<td>0.066 (0.020–0.256)</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>miR-525-3p*</td>
<td>0.003 (0–0.012)</td>
<td>0 (0–0.017)</td>
<td>0.052 (0.012–0.215)</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Data are expressed as the median (interquartile range).

** Dunn multiple-comparison post hoc test.

† NS, not statistically significant (P ≥ 0.05).

‡ Factor for converting progesterone concentration to SI units: 1 ng/mL = 3.18 nmol/L.

§ miRNA concentrations are expressed relative to 18S rRNA.

the tests in an ED setting. The sensitivities at the predefined specificities of 90% and 95% and the specificities at the predefined sensitivities of 90% and 95% were calculated for all markers individually and in combination; these data are presented in Table 2.
Discussion

The diagnosis of EP currently relies on the combination of sonography and serial hCG measurements in women with suspicious clinical symptoms (28). Life-threatening risks are associated with this approach, because women may experience tubal rupture during the 48-h wait for the second hCG measurement. There is a great need for new markers and new algorithms with high sensitivity and specificity.
to permit earlier diagnosis and proper management of EP.

More than 20 serum biomarkers have been investigated to date in an attempt to allow an earlier and more accurate diagnosis of EP; however, their clinical utility is very limited at present (28). At least 2 intrinsic properties of some of these markers limit their use. First, their serum concentrations often change with GA. Second, a number of markers can discriminate EP from VIP but are unable to distinguish EP from SA (28). An ideal marker would be one that remains steady throughout pregnancy and is capable of discriminating EP from both SA and VIP. In the current study, miR-323-3p and miR-525-3p demonstrated steady serum concentrations across GA, whereas the concentrations of miR-517a and miR-519d increased with GA during normal pregnancy. These findings agree with previous studies, which have demonstrated no GA-dependent changes in serum miR-323-3p concentrations between the first and third trimesters; in contrast, the concentration of circulating miR-323-3p differentiated itself from all of the other markers tested in the current study by showing significantly higher serum concentrations in the EP group of patients than

**Fig. 2.** Graphs of serum concentrations of hCG, progesterone, miR-323-3p, miR-517a, miR-519d, and miR-525-3p in patients with EP, SA, and VIP, respectively, at the GA of the ED visit. Factor for converting progesterone concentration to SI units: 1 ng/mL = 3.18 nmol/L.
in the SA and VIP groups. These characteristics of miR-323-3p fulfill the criteria described above for the selection of a marker with improved potential for detecting EP.

The single-marker results for hCG and progesterone and the dual-marker results for hCG plus progesterone that we have obtained were similar to those of previously published studies (29, 30), indicating that these measurements are insufficient to diagnose EP. Nevertheless, when evaluating the diagnostic accuracy of new biomarkers for detecting EP, the sensitivity and specificity of any single or multiple serum biomarker panel would need to be significantly higher than that achieved by serum hCG alone, by progesterone alone, or by the hCG-plus-progesterone dual-marker panel in order to be incorporated into a diagnostic algorithm.

When considered as a single biomarker, miR-323-3p presented the highest sensitivity, 37% at 90% specificity, which was significantly higher than that of progesterone alone and also improved the sensitivity compared with that of hCG. This sensitivity for miR-323-3p as a single biomarker is not sufficient to be of clinical value, however. In addition, using any of the candidate miRNAs as single serum biomarkers yielded limited specificity. Given that no stand-alone markers for EP, including the markers examined in this study, have been consistently demonstrated to have superior diagnostic performance, analysis with a panel of multiple serum markers might be a solution to the problem of EP diagnosis. The combination of hCG, progesterone, and miR-323-3p significantly improved the diagnostic utility, and multivariate logistic regression analyses showed this panel to have the highest sensitivity of all the multimarker panels. Furthermore, the combination of hCG, progesterone, and miR-323-3p also improved the specificity, although the addition of other miRNA markers was necessary to achieve the highest specificity and statistical significance. Addition of all the miRNAs reduced the sensitivity, however, compared with that achieved by the combination of hCG, progesterone, and miR-323-3p. The maximum specificity gained by adding the other miRNAs to the combination of hCG, progesterone, and miR-323-3p could be due to the different diagnostic abilities of these miRNA markers. It could also be limited by the small number of patients analyzed in this study. A significant improvement in specificity without compromising the optimal sensitivity of the combination of hCG, progesterone, and miR-323-3p may be achieved by increasing the patient sample size. The alternative strategy is to use the stepwise analysis to optimize sensitivity, which incorporated hCG, progesterone, and miR-323-3p and achieved a sensitivity of 96.3% and a specificity of 72.6%. These observations suggest that miR-323-3p may be useful for improving the diagnostic perfor-

### Table 2. Diagnostic accuracies of measurements of single markers (hCG, progesterone, miR-323-3p, miR-517a, miR-519d, and miR-525-3p) and of multimarker combinations for predicting EP in patients with abdominal pain/cramping and/or vaginal bleeding at predefined specificities and sensitivities.

<table>
<thead>
<tr>
<th></th>
<th>AUC(^a) (95% CI)</th>
<th>Sensitivity at:</th>
<th>Specificity at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>90% Specificity, %</td>
<td>95% Specificity, %</td>
</tr>
<tr>
<td>hCG</td>
<td>0.73 (0.63–0.83)</td>
<td>14.8</td>
<td>11.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.62 (0.51–0.73)</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td>miR-323-3p</td>
<td>0.73 (0.62–0.84)</td>
<td>37.0</td>
<td>33.3</td>
</tr>
<tr>
<td>miR-517a</td>
<td>0.62 (0.50–0.73)</td>
<td>11.1</td>
<td>0</td>
</tr>
<tr>
<td>miR-519d</td>
<td>0.64 (0.53–0.76)</td>
<td>11.1</td>
<td>7.4</td>
</tr>
<tr>
<td>miR-525-3p</td>
<td>0.63 (0.52–0.75)</td>
<td>11.1</td>
<td>0</td>
</tr>
<tr>
<td>hCG + progesterone(^b)</td>
<td>0.81 (0.72–0.91)</td>
<td>33.3</td>
<td>7.4</td>
</tr>
<tr>
<td>hCG + progesterone + miR-323-3p</td>
<td>0.87 (0.79–0.96)</td>
<td>77.8(^c)</td>
<td>33.3(^d)</td>
</tr>
<tr>
<td>hCG + progesterone + miR-517a</td>
<td>0.82 (0.73–0.91)</td>
<td>29.6</td>
<td>11.1</td>
</tr>
<tr>
<td>hCG + progesterone + miR-519d</td>
<td>0.82 (0.73–0.91)</td>
<td>33.3</td>
<td>11.1</td>
</tr>
<tr>
<td>hCG + progesterone + miR-525-3p</td>
<td>0.81 (0.72–0.90)</td>
<td>26.0</td>
<td>11.1</td>
</tr>
<tr>
<td>All</td>
<td>0.88 (0.79–0.96)</td>
<td>55.6</td>
<td>22.2</td>
</tr>
</tbody>
</table>

\(^a\) AUC, area under the curve.
\(^b\) AUCs, sensitivities, and specificities of each multimarker combination were compared with those of hCG + progesterone (boldface) in the same column.
\(^c\) \(p < 0.01\).
\(^d\) \(p < 0.05\).

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performance in a multiple marker panel designed for the early detection of EP.

Multimarker approaches for the diagnosis of EP have been examined in previous studies. Most recently, Rausch et al. evaluated a large number of biomarkers and developed a multimarker algorithm combining 4 markers (progesterone, vascular endothelial growth factor, inhibin A, and activin A) that demonstrated improved diagnostic utility (31). That study did not evaluate the algorithm in a patient population that included SA, however. Our study more accurately reflects the diagnostic utility of the biomarkers in an ED setting, in that it includes not only EP and VIP patients but also SA patients.

miRNA-323-3p, miRNA-517a, miRNA-519d, and miRNA-525-3p are pregnancy-associated miRNAs that are found in the maternal circulation during pregnancy and are rapidly cleared from maternal circulation after delivery (11). Nucleic acids (DNA and RNA) of placental origin were originally proposed to be released into the maternal circulation in the form of apoptotic bodies (32). Luo et al. recently demonstrated that miRNAs are exported from human placental syncytiotrophobasts into the maternal circulation via exosomes and suggested that circulating trophoblast-derived miRNAs reflected the physiological status of the pregnancy and could be used diagnostically (10). Although the fundamental mechanisms that underlie miRNA physiology and their contribution to the clinical manifestation of EP are not completely understood and although the systemic targets of circulating pregnancy-associated miRNAs remain unknown, it is now clear that miRNAs are released into the circulation and that their concentrations likely reflect tissue phys-

Fig. 3. ROC curve analysis of serum hCG, progesterone, miR-323-3p, miR-517a, miR-519d, and miR-525-3p as single markers (A) and multiple markers (B) for predicting EP.
iological or pathologic states (21, 33). Recent studies have demonstrated that fetal growth restriction is associated with increased total concentrations of a set of trophoblastic miRNAs in the maternal circulation, a finding that highlights the need to explore circulating miRNAs as potential biomarkers of pregnancy-related diseases (20).

The gene encoding miR-323-3p occurs in an miRNA cluster in chromosomal region 14q32.31, and the genes encoding miRNA-517a, miRNA-519d, and miRNA-525-3p occur in another cluster on 19q13.42. These 2 chromosomal regions are critical for placental growth and embryonic development (11). In the case of tubal EP, trophoblasts invade and erode the tubal muscular wall, and maternal blood vessels are opened (34). During this process, it is possible that the miRNAs are released into the maternal circulation, as seen in the present study. The downregulation of all 3 miRNAs in the chromosome 19 cluster (miRNA-517a, miRNA-519d, and miRNA-525-3p) in both SA and EP may reflect abnormal embryo/trophoblast growth. In contrast, miR-323-3p was the only marker tested in the current study whose serum concentrations were increased in EP. Other serum components tend to be increased in EP, such as creatine kinase and vascular endothelial growth factor, which may be released from the damaged fallopian tube. Various cytokines, which may be associated with peritoneal irritation, may also be released. It is possible that the tissue origin of the circulating miR-323-3p in EP patients is not restricted to the trophoblast, as has been suggested by Miura et al. (11). Consequently, the increased serum concentration of this miRNA may be due to tubal damage, tubal implantation, or peritoneal inflammation. Obviously, this hypothesis warrants further investigation.

This proof-of-concept study is the first to examine the relationship between pregnancy-associated miRNAs and early pregnancy outcomes, and it is the first to explore the diagnostic value of miRNAs for EP. Given that miRNA molecules are highly stable in circulation, they are more robust and more suitable for serving as blood-based biomarkers than some protein-or peptide-based markers. There are several limitations to this study, however. First, the development of miRNAs as noninvasive diagnostic markers is in its infancy stage. miRNA assays are not routinely used in clinical practice, and no universally accepted standard procedure exists at the present time. Recent reports suggest, however, that miRNAs may serve as improved markers for numerous diseases, including cancer, autoimmune diseases, sepsis, acute myocardial infarction, and others (6). It appears promising that manufacturers will develop faster and cheaper miRNA assays suitable for clinical use in the near future. Second, in-

Fig. 4. A stepwise analysis using hCG, progesterone, and miR-323-3p to predict EP.

18S, 18S rRNA. Factor for converting progesterone concentration to SI units: 1 ng/mL = 3.18 nmol/L.
creased variances were seen in the test characteristic estimates, in part because of a relatively small sample size within any given patient group. A larger, randomized study is needed to validate this multimarker strategy.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the intellectual content of this paper.

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