Development of a Multiple Marker Test for Ectopic Pregnancy

Mary E. Rausch, MD, Mary D. Sammel, ScD, Peter Takacs, MD, PhD, Karine Chung, MD, MSCE, Alka Shaunik, MD, and Kurt T. Barnhart, MD, MSCE

OBJECTIVE: Many serum markers have been proposed to aid in the identification of an ectopic pregnancy, but few have been validated. Most studies have been limited by sample size and design. The goal of this study was to assess putative markers to identify which can be optimally combined.

METHODS: We conducted a case–control study using sera from 100 patients with ectopic pregnancy and 100 patients with intrauterine pregnancy who presented to three urban academic centers between September 2000 and April 2009 with first-trimester pain or bleeding. Samples were analyzed for 12 promising biomarkers. Classification tree analysis was used to examine markers simultaneously with the goal of optimizing the accuracy of ectopic pregnancy diagnosis, and validation was performed using bootstrapping.

RESULTS: Six of the 12 markers were differentially expressed between those with ectopic pregnancy and intrauterine pregnancy (P < .001) with fair diagnostic properties (area under the curve greater than 0.6) when examined individually (inhibin A, progesterone, activin A, vascular endothelial growth factor [VEGF], pregnancy-specific β-1-glycoprotein, and pregnancy-associated plasma protein-A). Six additional markers were found to have limited value. Using a two-step diagnostic algorithm with four markers (progesterone, VEGF, inhibin A, activin A), we diagnosed 42% of the sample with perfect specificity and 98% (93–100%) sensitivity. Overall, a single ectopic pregnancy was misclassified, achieving 99% (96–100%) accuracy.

CONCLUSION: Evaluating a large number of biomarkers simultaneously demonstrates that most of the putative markers of ectopic pregnancy are not useful. However, a select few can distinguish ectopic pregnancy from intrauterine pregnancy with superior accuracy as part of a multiple marker test.


LEVEL OF EVIDENCE: II

Ectopic pregnancy is a major cause of maternal morbidity and responsible for 6% of pregnancy deaths. Distinguishing normal from abnormal pregnancies is a clinical challenge because there is no definitive noninvasive diagnostic test available before visualization on ultrasonography. Clinicians must therefore follow patients over the course of several days to weeks for diagnosis, a time in which there is some potential for the ectopic pregnancy to rupture and result in life-threatening intra-abdominal hemorrhage. Early treatment may also allow for tubal-conserving procedures to be used, which is important for a patient’s future fertility. Therefore, development of a serum test to diagnose an ectopic pregnancy with high accuracy would be of great clinical significance.

The diagnosis of ectopic pregnancy in early pregnancy requires both superior sensitivity and specificity given that a false-negative could lead to serious morbidity and mortality and a false-positive could result in interruption of a potentially desired normal pregnancy. Multiple markers have been examined in the literature, and previous studies are often limited...
by small numbers of patients, conflicting results, and lack of validation. No one marker has been consistently demonstrated to have high discrimination. The aim of this study was to concomitantly evaluate a large number of markers to estimate the use of each, singly and in combination, to accurately identify ectopic pregnancy.

MATERIALS AND METHODS
The study was approved by the Institutional Review Boards of the University of Pennsylvania, University of Southern California, and University of Miami.

Potential markers were chosen after extensive review of the literature and a search for commercially available assays. Biomarkers with a variety of different biologic functions were considered, including markers of trophoblast function (activin A, pregnancy-specific β1-glycoprotein, pregnancy-associated plasma protein-A, human placental lactogen, and inhibin A), corpus luteum function (progesterone, inhibin A), endometrial function (glycodelin), inflammation (interleukin 8 [IL-8], IL-6, tumor necrosis factor-α [TNF-α]), muscle cell damage (creatine kinase [CK]), and angiogenesis (vascular endothelial growth factor [VEGF]).

Cases and controls were selected from among serum collected as part of the Ectopic Pregnancy Biomarkers Bank protocol between September 2000 and April 2009. A total of 100 cases and 100 control participants were selected that had enough volume to compete planned assays, had a minimum of missing clinical information, and were similar in gestational age. With 100 samples per group, this study was powered to examine sensitivity for the diagnosis of ectopic pregnancy of 50% or higher with precision ±10%. Sensitivity of 50% was chosen to conservatively estimate the sample size requirements for the study.

Participants were women who presented to emergency departments at one of the three participating sites with pain, bleeding, or both and a positive pregnancy test of less than 12 weeks of gestation. Serum samples were collected at the initial visit before treatment by peripheral venous puncture. Per the Institutional Review Board protocol, informed consent was obtained by all patients whom the study staff could reach before the clinical venipuncture. If the clinician was unable to make a diagnosis on this first visit, the patient was followed until a diagnosis of a viable intrauterine pregnancy or ectopic pregnancy was confirmed. The serum was separated into aliquot tubes and stored at −70°C to −80°C until assays were performed. Serum samples from the University of Southern California and University of Miami were shipped to the University of Pennsylvania for storage.

The diagnosis of intrauterine pregnancy was confirmed with ultrasonographic detection of fetal cardiac activity. The diagnosis of ectopic pregnancy was confirmed by visualization of an ectopic gestation using ultrasonography or laparoscopy or with an increased in human chorionic gonadotropin (hCG) after uterine evacuation when an ectopic gestation was not visualized. Information on last menstrual period, race, ethnicity, and maternal age was collected at the time of the initial visit.

Assays were conducted at the Penn Clinical and Translational Research Center by qualified experts.

Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Intrauterine Pregnancy (n)</th>
<th>Intrauterine Pregnancy Value</th>
<th>Ectopic Pregnancy (n)</th>
<th>Ectopic Pregnancy Value</th>
<th>P</th>
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<tr>
<td>Age (y)</td>
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<td>27.51±6.70</td>
<td>99</td>
<td>28.96±6.11</td>
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<tr>
<td>GA (d)</td>
<td>100</td>
<td>48.80±12.34</td>
<td>81</td>
<td>45.15±18.95</td>
<td>.137</td>
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<tr>
<td>Beta-hCG (milli-international units/mL)</td>
<td>98</td>
<td>7,586 (47–36,589)</td>
<td>100</td>
<td>1,199 (22–29,323)</td>
<td>&lt;.001</td>
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<tr>
<td>Race</td>
<td></td>
<td></td>
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<td>65</td>
<td>.941</td>
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<td>.18</td>
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<tr>
<td>Other</td>
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<td>1</td>
<td>0</td>
<td>0</td>
<td>.20</td>
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<td>University of Southern California</td>
<td>36</td>
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<td>35</td>
<td>35</td>
<td>.78</td>
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</tbody>
</table>

GA, gestational age; hCG, human chorionic gonadotropin.
Data are mean±standard deviation, median (range), or % unless otherwise specified.
Protein assays were performed in duplicate; average values were analyzed in a blinded fashion. Inhibin A was assayed using an enzyme-linked immunosorbent assay (ELISA) kit. The minimum detectable limit for inhibin A was 5 pg/mL, and the interassay coefficient of variation (CV) was 4.0%. Activin A, VEGF, pregnancy-associated plasma protein-A, IL-6, IL-8, and TNF-α were assayed using Quantikine Immunoassay kits. The minimum detectable limit for activin A was 10 pg/mL, and the CV was 4.9%. The minimum detectable limit for VEGF was 20 pg/mL, and the CV was 8.5%. The minimum detectable limit for pregnancy-associated plasma protein-A was 0.25 ng/mL, and the CV was 9.6%. The minimum detectable limit for IL-6 was 1 pg/mL, and the CV was 9.7%. The minimum detectable limit for IL-8 was 5 pg/mL, and the CV was 4.7%. The minimum detectable limit for TNF-α was 1 pg/mL, and the CV was 6.6%. Human placen-
Pregnancy-specific β1-glycoprotein and glycodelin were assayed using Cusabio ELISA kits. The minimum detectable limit for human placental lactogen was 0.4 mg/L, and the CV was 7.0%. Progesterone was analyzed using hormonal chemiluminescence technology. The minimum detectable limit for progesterone was 0.5 ng/mL, and the CV was 9.5%. CK was measured with a CK colorimetric assay kit. The minimum detectable limit for CK was 2.0 μg/mL, and the CV was 9.5%. Pregnancy-specific β1-glycoprotein and glycodelin were assayed using Cusabio ELISA kits. The minimum detectable limit for pregnancy-specific β1-glycoprotein was 0.02 ng/mL, and the CV was 3.7%. The minimum detectable limit for glycodelin was 0.3 pg/mL, and the CV was 5.0%. Beta-hCG was assayed using an ELISA kit. The minimum detectable limit for hCG was 20 milli-international units/mL, and the CV was 3.8%. Values below detection thresholds were given the threshold value in analyses.

Baseline characteristics were compared using a two-sample t test for continuous variables and Pearson’s chi square analysis or Fisher’s exact test for categorical variables. Given the skewed distributions of the biomarkers, levels are presented as median and range, the Wilcoxon rank sum test was used to compare biomarker levels, and Spearman correlations were used for correlations of the biomarkers with hCG. Area under the receiver operating characteristic curves (AUC) was calculated to assess the discrimination for each biomarker. An optimal cut point that minimized misclassification of the groups was determined, and sensitivity and specificity of the marker at that cut point were calculated. Pearson’s correlation coefficient was calculated for the log-transformed hCG levels at initial draw and time of biomarker analysis to ensure good reliability.

Classification and Regression tree software was used to evaluate combinations of the biomarkers. Classification tree development is based on recursive partitioning with a binary split of the biomarkers at a cut point determined by the software. The choice of biomarker for each node is dependent on the variable that minimizes misclassification given user-specified costs for each type of error for that node before splitting the data set and then reanalyzing the subgroups to assess for the best next split. The root node, or first node of the tree, was then reassessed by manually inserting variables of similar diagnostic importance as determined by the Classification and Regression tree software and then rebuilding the tree to see the global effect of the change of variable on the diagnostic ability of the tree as a whole. The performance of the trees in Classification and Regression tree was assessed through replications of 10-fold crossvalidations. Trees optimizing sensitivity were created by adjusting the cost of misclassifying an ectopic pregnancy relative to an intrauterine pregnancy, whereas trees optimizing specificity were created by adjusting the cost of misclassifying an intrauterine pregnancy as an ectopic pregnancy before tree development. To create a final diagnostic test, a sensitivity tree and specificity tree were combined into a three-tiered algorithm by labeling any participant diagnosed with an ectopic pregnancy in both trees as an “EP,” any patient diagnosed with an intrauterine pregnancy in both trees as an “IUP,” and any participant diagnosed with an ectopic pregnancy in one tree and an intrauterine pregnancy in the other as “indeterminate.”

Sensitivity in the final model was defined as the number of true-positives (correctly diagnosed ectopic pregnancies) divided by the total number of ectopic pregnancies diagnosed as either “EP” or “IUP.” Specificity in the final model was defined as the number of true-negatives (correctly diagnosed intrauterine pregnancies) divided by the total number of intrauterine pregnancies diagnosed as either “IUP” or “EP.” All 95% confidence intervals for sensitivity, specificity, and accuracy were estimated from 1,000 bootstrap samples with each sample having the same size as the original data subset. Accuracy was defined as the number of true-positives and true-negatives divided...
by the total number of patients who were diagnosed and not labeled as “indeterminate.” Subgroup analyses were performed for participants with hCG levels less than or 1,500 milli-international units/mL or greater. All statistics were performed using either CART 6.0 or STATA 11.0. Statistical significance was defined \( P<.05 \).

**RESULTS**

We studied sera from 100 women with an intrauterine pregnancy and 100 women with an ectopic pregnancy. Participant characteristics are shown in Table 1. There were no significant differences in maternal age, gestational age, race, ethnicity, or site between the cases and control participants. Gestational age was missing in 19 of 100 women in the ectopic pregnancy group as a result of an unknown last menstrual period.

The median level of hCG was higher in the intrauterine group (7,586 milli-international units/mL) compared with the ectopic pregnancy group (1,199 milli-international units/mL, \( P<.001 \)). Scatterplots of all 12 markers are shown in Figure 1. There was considerable overlap of values for each group for all markers.

Six markers had both a highly statistically significant difference between the groups (\( P<.001 \)) and an AUC of greater than 0.6: inhibin A, progesterone, activin A, VEGF, pregnancy-specific \( \beta \)-1-glycoprotein, and pregnancy-associated plasma protein-A (Table 2).

Three markers demonstrated a significant difference in median values between the ectopic pregnancy and intrauterine pregnancy (glycodelin, \( P=.02 \); IL-8, \( P=.02 \); and TNF-\( \alpha \), \( P=.03 \)), but with AUCs below our threshold (0.57 [0.49–0.65], 0.60 [0.52–0.68], and 0.59 [0.51–0.67], respectively). The remaining markers did not demonstrate a difference in median values and did not have an AUC of greater than 0.6. Levels of human placental lactogen and glycodelin were very low with 62 intrauterine pregnancy and 57 ectopic pregnancy samples for human placental lactogen and 36 ectopic pregnancy and 51 intrauterine pregnancy samples for glycodelin below the minimum detectable limit for each assay. Table 2 shows the sensitivity and specificity for the six best markers at a cut point minimizing misclassification.

Combining the markers through Classification and Regression tree, we developed a tree optimizing sensitivity (Fig. 2), which incorporated VEGF and progesterone. The sensitivity of this two-marker tree was 99% (95% confidence interval, 95–100%) with a specificity of 39% (29–49%). We developed two additional trees that maximized specificity (Fig. 3).

The two-marker tree incorporating progesterone and inhibin A had a specificity of 98% (93–100%) and sensitivity of 48% (40–58%). The addition of activin A to this tree enhanced the specificity to 100% (97–100%), whereas the sensitivity was 43% (33–53%).

The results of combining these trees into a three-marker test (VEGF, progesterone, and inhibin A) and four-marker test (VEGF, progesterone, inhibin A, and activin A) are shown in Table 3. The three-marker test diagnosed 45% of patients with 97% accuracy, misdiagnosing three participants: one with an ectopic pregnancy and two with intrauterine pregnancies. The four-marker test diagnosed fewer participants but with higher accuracy (99%), misdiagnosing only one participant with an ectopic pregnancy. Validation by bootstrapping demonstrated consistent results as shown in Table 3.

We also evaluated the performance of the tests for the subgroups of patients with hCG levels less than and 1,500 milli-international units/mL or greater (Table 3). The multiple marker profile resulted in perfect discrimination in women who presented with hCG values less than 1,500 milli-international units/mL at the time of presentation for care. Furthermore, more than two thirds of participants with ectopic pregnancy were classified in the low hCG group (70%) in the three-marker test compared with only 24% in the higher hCG group (\( P=.005 \)).

We assessed the correlation of each marker with hCG. Among the four markers, hCG was the most closely associated with inhibin A in both groups.
(intrauterine pregnancy: \(\rho=0.64, P<.001\) and ectopic pregnancy: \(\rho=0.54, P<.001\)). A weaker but significant association was also noted with activin in the intrauterine pregnancy group (0.42, \(P<.001\)), progesterone in the ectopic pregnancy group (\(\rho=0.52, P<.001\)), and VEGF in both groups (\(\rho=-0.48, P<.001\) in the intrauterine pregnancy group and \(\rho=-0.34, P=.005\) in the ectopic pregnancy group).

**DISCUSSION**

Given the clinical challenge of diagnosing ectopic pregnancy at an early gestational age, the identification of a biomarker to simplify and improve the diagnosis of ectopic pregnancy is a research priority.\(^1,5\) There are two general strategies to identify a biomarker, testing putative markers and discovery-driven screening of the proteome. In this study, we concomitantly examined a large set of biomarkers already demonstrated to have discriminative ability but not yet validated. We demonstrated that as single markers, none were adequate to discriminate ectopic pregnancy from intrauterine pregnancy. However, markers with limited ability when used together can often improve prediction,\(^7,8\) especially if they reflect different pathways. We developed a three-marker test combining progesterone, inhibin A, and VEGF that could diagnose almost half of the sample with 97% accuracy and the majority of women with a low hCG value with 100% accuracy. A four-marker test incorporating activin A diagnosed slightly fewer patients but with 99% accuracy.

Each of our markers is biologically plausible. Progesterone is secreted by the corpus luteum in early pregnancy before placental production and is a critical hormone for the establishment of normal pregnancy.\(^9\) A systematic review found that a single value of serum progesterone had insufficient capacity to diagnose ectopic pregnancy, although it was capable of identifying those at risk.\(^10\) In combination with other markers in our study, progesterone played a role in both optimizing sensitivity as well as specificity, likely as a result of its reflection of the viability of the pregnancy.

Inhibin A is also a major peptide product of the corpus luteum and also appears to have a fetoplacental source in early pregnancy.\(^11,12\) It is secreted by cytotrophoblasts\(^13\) and is involved in the fetomaternal communication necessary to maintain pregnancy.\(^14,15\) Previous studies have shown elevated levels of inhibin A to be a good predictor of intrauterine pregnancies but with varying discrimination of ectopic pregnancy among groups with abnormal intrauterine pregnancies.\(^16,17\)

Vascular endothelial growth factor was the only marker in our diagnostic tree that was elevated in ectopic pregnancies. VEGF rises during the first trimester of pregnancy.\(^18\) Vascular endothelial growth factor may also be involved in the implantation of pregnancy in the fallopian tube. There is increased expression of VEGF and its receptor at the implantation site of an ectopic pregnancy compared with the nonimplantation site of the same oviduct.\(^19\) Our val-
Fig. 3. Classification and Regression trees (CART) for optimizing specificity. **A.** Two-marker tree. **B.** Three-marker tree. The three-marker specificity tree is identical to the two-marker tree (progesterone and inhibin A) except for the addition of activin A. Each node is identified by node number, splitting variable name and criteria, and class histogram. Terminal nodes also display class assignment and breakdown as well as the number of cases in the node.

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uses are similar to other studies showing statistically significant elevations in serum VEGF levels in ectopic pregnancies compared with normal intrauterine pregnancy levels. 20–22

Activin A was a component of our four-marker test. Activin A is localized in placental trophoblast and Hofbauer cells in early pregnancy, 23,24 is secreted by cytotrophoblasts, and promotes their invasion in pregnancies to 10 weeks of gestation in vitro. 13,25 Activin A levels begin at prepregnancy levels in the first trimester and progressively rise throughout pregnancy. 17,26 There are conflicting data on the use of other markers of pregnancy development (pregnancy-specific β1-glycoprotein, pregnancy-associated plasma protein-A, human placental lactogen, and glycodelin) were less discriminatory. Levels of human placental lactogen and glycodelin were low in both groups. Pregnancy-associated plasma protein-A did discriminate significantly higher in intrauterine pregnancies than ectopic pregnancies with AUCs of 0.76 and 0.67, respectively, but did not add to discrimination beyond that of the markers in the model. Pregnancy-specific β1-glycoprotein 22,28 and pregnancy-associated plasma protein-A 22,29–31 are elevated in normal pregnancies, but their discriminatory ability increases as the pregnancy advances. 23,28–30 Other recently examined markers potentially involved in the progression of ectopic pregnancy such as inflammatory cytokines IL-6, IL-8, and TNF-α 31,32 and the smooth muscle enzyme CK 33–38 demonstrated poor discrimination with AUCs between 0.5 and 0.6.

The higher level of hCG in the intrauterine pregnancy group over the ectopic pregnancy group was expected. To aid in the diagnosis of women with an ectopic pregnancy, new markers have to improve discrimination above that of hCG alone. Although our identified markers do have some correlation with hCG, it is only modest and in some cases only noted in intrauterine pregnancy or ectopic pregnancy (and not the other). Of interest was that our new models demonstrated perfect discrimination with more than two thirds of ectopic pregnancies definitively classified in the subgroup with low hCG (less than 1,500 milli-international units/mL). Determination of pregnancy type in this subgroup would be impossible by current diagnostic methods and the improved results, both in terms of diagnostic ability and number diagnosed, in this subgroup are encouraging.

Others have proposed a multiple marker for the diagnosis of ectopic pregnancy. One group in Switzerland developed a multiple marker test, the “triple marker analysis” comparing sera from 43 patients with ectopic pregnancy and 79 normal intrauterine pregnancy from an antenatal clinic in Switzerland. 23 Their test had a sensitivity of 97.7% with a specificity of 92.4% in diagnosing ectopic pregnancy. 23 We were able to achieve a sensitivity of 69% and specificity of 84% using their algorithm. Pregnancy-associated plasma protein-A did discriminate between the groups in our study, but not as well as inhibin A or activin A. The combination of VEGF and progesterone with these two markers may prove to be a more robust test.

Table 3. Three-Marker and Four-Marker Tests

<table>
<thead>
<tr>
<th>Tree</th>
<th>Total</th>
<th>Ectopic</th>
<th>Intrauterine</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Accuracy (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>3 marker*</td>
<td>90/200 (45)</td>
<td>49/100 (49)</td>
<td>41/100 (41)</td>
<td>0.98 (0.94–1.00)</td>
<td>0.95 (0.87–1.00)</td>
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<tr>
<td></td>
<td>4 marker†</td>
<td>83/200 (42)</td>
<td>44/100 (44)</td>
<td>39/100 (39)</td>
<td>0.98 (0.93–1.00)</td>
<td>1.00 (CI N/A)</td>
</tr>
<tr>
<td></td>
<td>hCG less than 1,500</td>
<td>3 marker*</td>
<td>42/67 (63)</td>
<td>38/54 (70)</td>
<td>4/13 (31)</td>
<td>1.00 (CI N/A)</td>
</tr>
<tr>
<td></td>
<td>4 marker†</td>
<td>38/67 (57)</td>
<td>34/54 (63)</td>
<td>4/13 (31)</td>
<td>1.00 (CI N/A)</td>
<td>1.00 (CI N/A)</td>
</tr>
<tr>
<td>hCG 1,500 or greater</td>
<td>3 marker*</td>
<td>46/131 (35)</td>
<td>11/46 (24)</td>
<td>35/85 (41)</td>
<td>0.91 (0.70–1.00)</td>
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<td>4 marker†</td>
<td>43/131 (33)</td>
<td>10/46 (22)</td>
<td>33/85 (39)</td>
<td>0.90 (0.67–1.00)</td>
<td>1.00 (CI N/A)</td>
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</table>

CI, confidence interval; hCG, human chorionic gonadotropin; VEGF, vascular endothelial growth factor.

Data are n/N (%) unless otherwise specified.

* “3 marker” is a test combining inhibin A, progesterone, and VEGF.
† “4 marker” is a test combining inhibin A, progesterone, VEGF, and activin A.
‡ Results and confidence intervals of sensitivity, specificity, and accuracy obtained by bootstrapping. Given that bootstrapping confidence intervals are obtained by repeated sampling within the data set, the confidence intervals of a data set with 1.00 results is always (1.00–1.00) and are reported here as not applicable (N/A).
Strengths of this study include the large sample size, the number of biomarkers studied concurrently, a study population including only symptomatic women at risk for ectopic pregnancy, and the combining of markers to create a test with superior accuracy. A limitation of this study is that results from Classification and Regression tree are “data-driven” with a potential for overstating positive results, although validation with bootstrapping was reassuring. Finally, we have not yet evaluated these markers in women with a failed intrauterine gestation to confirm that we are discriminating the location and not solely viability of an early gestation. However, a test to discriminate viability of an early gestation would still be of clinical value. These findings are considered preliminary, and many of the assays are not routinely used in clinical practice. Future planned external validation will be performed in a separate set of samples, collected over a shorter time span, and among patients with failed intrauterine pregnancies in addition to intrauterine pregnancies and ectopic pregnancies.

In summary, many of the markers previously examined in smaller groups of women were found to be poor discriminators although differences between the groups may be statistically significant. We have examined a large number of markers concurrently in women at risk for ectopic pregnancy and have combined them to create a test that diagnoses ectopic pregnancy with excellent accuracy.

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