Single Serum Activin A Testing to Predict Ectopic Pregnancy

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ECTOPIC PREGNANCY (EP) is an important cause of maternal deaths in early pregnancy because most fatal cases result from delayed diagnosis and inappropriate investigation (1). The diagnosis of EP should be based on the positive visualization of an extraterine pregnancy outside the uterus, rather than the absence of an intrauterine pregnancy (IUP), and the use of transvaginal sonography (TVS) is the method of choice for the initial assessment of women with suspected EP (1, 2). Indeed, with the use of TVS a normal IUP can be identified at 4 wk and 3 d in a woman with a regular 28-d cycle (3). However, in 8–31% of women with suspected abnormal early pregnancies who are referred for assessment, the diagnosis cannot be made by TVS at the initial visit (1). In these women, either surgical or biochemical assessment is used to reach the correct diagnosis (1, 4, 5). With respect to the biochemical assessment, it is usually based on the measurement of serum levels of human chorionic gonadotropin (β-hCG) (6, 7) and progesterone (8–10).

However, because none of the current methods combines accuracy, reproducibility, and simplicity to become a universal predictive marker of EP (1), there continues to be a compelling demand for new markers and also for new algorithms that extract the best information of current screening methods.

In the present study, we have evaluated whether activin A measurement at the initial visit may be useful in the diagnosis of EP in early pregnancies of unknown location (UPL). Activin A is a dimeric glycoprotein belonging to the TGF-β superfamily, a group of structurally similar but functionally diverse growth factors involved in cellular proliferation, differentiation, and cell fate determination (11). During pregnancy, the placenta is the main source of activin A (12); activin A serum levels in maternal circulation are higher than in nonpregnant women and increase throughout pregnancy until delivery (13). Moreover, the addition of activin A to primary cultures of human placental cells increases progesterone production and potentiated the GnRH-induced release of hCG (14), supporting the hypothesis that activin A plays a pivotal role in the endocrine physiology of human pregnancy.
Subjects and Methods

We did a prospective observational study of pregnant women with UPL (n = 536) who were referred for assessment by either general practitioners or hospital consultants at our tertiary referral center for obstetrics, presenting with complaints of bleeding, pain, or cramping. Patients were enrolled from November 2004 to September 2005. All women included in the study conceived spontaneously and were not taking exogenous progesterogens. A full medical history was documented, and clinical examination was carried out by the attending physician. A transvaginal ultrasound scan was then performed using a 4.5–7.0 MHz probe (Real Time Ultrasound Scan Equipment, Siemens Sonoline ELEGRA Millenium Edition; Siemens, Erlangen, Germany).

The diagnosis of UPL was made at the initial visit in all cases where there was no clear ultrasound evidence of an IUP, retained products of conception, or an EP. The definition of UPL did not include women with an early pregnancy-like structure within the uterine cavity, which required follow-up for verification, the identification of an adnexal mass that was believed to be an EP, clinically unstable patients, and patients with indirect signs of a specific pregnancy location such as the presence of hCG, and ultrasound evidence of conception, growth, or visualization on speculum examination. Ultrasound examinations were performed by either general practitioners or hospital consultants that visited the patients, but all cases were reviewed and reanalyzed by one physician (F.M.S.). In any case, no differences were noted among several operators.

Patients were advised not to travel, to avoid sexual intercourse, and to return immediately if they experienced significant increase in abdominal pain. The women were reviewed every 3 d until a final diagnosis was reached. Follow-up included clinical assessment, serial measurement of the serum hCG levels, and repeated transvaginal ultrasound scans. Surgical intervention was indicated in women on the basis of hCG ratio (rate of change in hCG over 48 h) (15) or because of worsening clinical symptoms.

The diagnosis of a normal IUP was made on follow-up by the demonstration of an intrauterine gestational sac on the scan. In these cases, another ultrasound scan was performed 2 wk later and demonstrated the presence of a live embryo. The diagnosis of a miscarriage was made histologically after surgical evacuation of the uterine contents. EP was diagnosed at laparoscopy and on histological examination of the surgical specimens. Spontaneous resolution of pregnancy was defined as a decrease of hCG levels to less than 5 IU/liter with the disappearance of symptoms.

Informed consent was obtained from all patients before inclusion in the study, for which local Human Investigation Committee approval was obtained.

Commercially available assays were used to analyze serum samples for quantitative hCG (EURO/DPC Ltd., Glyn Rhonwy, Llanberis, Gwynedd, UK), progesterone (DRG International, Minneapolis, MN), and activin A (Serotec, Kidlington, Oxford, UK), according to manufacturers’ instructions. Assays were done in samples collected at the initial visit, without knowledge of pregnancy outcomes, and results did not influence treatment.

Measurements were performed in duplicate. hCG assay had a sensitivity of 1.1 IU/liter, with a within-assay coefficient of variation (CV) of 2.1%, and an interassay CV of 3.1%. No cross-reactivity to LH, FSH, and TSH was shown. The progesterone assay detection limit was 0 be ng/ml, with a CV less than 3.6% for intraassay and less than 3.5% for the interassay, and the cross-reaction with 17-β estradiol, progesterone, estriol, 17α estradiol, testosterone, dehydroepiandrosterone sulfate, cortisol, and pregnenolone was less than 0.3%. Activin A assay had a limit of detection less than 100 pg/ml, with intraassay and interassay CV for quality control samples of 2.0 and 4.5%, respectively. Cross-reaction with the various inhibin-related proteins was less than 0.5%.

Statistical analysis

All data were analyzed with Prism software (GraphPad Software Inc., San Diego, CA) and were expressed as mean ± sd. The Kolmogorov-Smirnov test confirmed that the distributions of hCG, progesterone, and activin A concentrations were not Gaussian. Therefore, we used Kruskal-Wallis test followed by Dunn’s post hoc test to compute statistical significance, and χ² and Fisher exact test to analyze differences between proportions.

The sensitivity, specificity, predictive value, and likelihood ratios (LR) of hCG, progesterone, and activin A as diagnostic tests for the detection of EP in women with UPL were assessed by using the receiver operating curve (ROC) test (16). Therefore, the probability of EP after having none or one test positive (higher than the cutoff point) was estimated and compared with the pretest probability, defined as the prevalence of EP in the whole group of patients (17).

ROC analysis was performed by using MedCalc (version 8.21.0; MedCalc Software, 9030 Mariakerke, Belgium), and statistical significance was set at P < 0.05.

Results

Clinical findings

Pregnancy outcomes included 155 (28.9%) viable IUP, 305 (56.9%) first-trimester spontaneous abortion (SAB), and 76 (14.2%) EP. Seventy-two EP (94.7%) were tubal, three (4.0%) were ovarian, and one (1.3%) was interstitial.

Demographic information and clinical characteristics are given in Table 1. There were no significant differences in race, gravidity, parity, and symptoms prevalence. However, the mean age of subjects with EP was significantly higher than that of patients with IUP (P < 0.001) and SAB (P < 0.05) (Table 1), and the estimated length of gestation (days) was significantly higher in EP than SAB (P < 0.001) and IUP (P < 0.05) women (Table 1).

hCG, progesterone, and activin A levels

hCG, progesterone, and activin A levels were measurable in all samples. In details, hCG concentrations were the lowest (P < 0.0001) in women with SAB at follow-up, being significantly lower than in patients with EP (P < 0.001) and IUP (P < 0.001). In addition, levels in EP were significantly (P < 0.001) lower than those in IUP (Table 1 and Fig. 1).

Changes in progesterone levels showed the same trend of hCG, because they were lowest in patients with SAB (P < 0.0001), and lower than in those with EP (P < 0.001) and IUP (P < 0.001). In addition, progesterone concentrations in EP were significantly (P < 0.001) lower than those in IUP (Table 1 and Fig. 1).

On the contrary, activin A levels were the lowest (P < 0.0001) in EP, significantly lower than in patients with SAB (P < 0.001) and IUP (P < 0.001) (Table 1 and Fig. 1). Finally, women with IUP had significantly (P < 0.001) lower activin A levels than those who underwent SAB (Fig. 1).

Diagnostic accuracy of hCG, progesterone, and activin A measurement for EP

The sensitivity/specificity, positive/negative predictive values, positive and negative LR and the area under the curve (AUC) of hCG, progesterone, and activin A as diagnostic tests were evaluated by the ROC curve.

In details, hCG at the cutoff of 688 IU/liter achieved a sensitivity of 75.0% [95% confidence interval (CI 95%), 63.7–84.2] and a specificity of 76.1% (CI 95%, 71.9–79.9) as a single marker for EP prediction in patients with UPL [ROC AUC: 0.806 (CI 95%, 0.77–0.839)], with a positive and negative LR of 3.14 and 0.33, respectively (Fig 2 and Table 2).

With respect to progesterone, at a cutoff of 17.2 ng/ml
combined a sensitivity of 85.5% (CI<sub>95%</sub>, 75.6–92.5) with a specificity of 66.1% (CI<sub>95%</sub>, 61.6–70.4) for prediction of EP, with a ROC AUC of 0.622 (CI<sub>95%</sub>, 0.579–0.663), and positive and negative LR of 2.52 and 0.22, respectively (Fig. 2 and Table 2).

On the contrary, activin A at the cutoff of 0.37 ng/ml combined a sensitivity of 100% (CI<sub>95%</sub>, 95.2–100) with a specificity of 99.6% (CI<sub>95%</sub>, 98.4–100) for prediction of EP, with a ROC AUC of 1.0 (CI<sub>95%</sub>, 0.993–1.00), and positive and negative LR of 230.0 and 0.0, respectively (Fig. 2 and Table 2). The ROC AUC of activin A was significantly higher than that of hCG (P = 0.000; difference between areas, 0.194; CI<sub>95%</sub>, 0.133–0.255) and of progesterone (P = 0.000; difference between areas, 0.378; CI<sub>95%</sub>, 0.307–0.449), as evaluated by pairwise comparison of ROC curves.

**Computation of the probability to have an EP**

The probability of EP after having none or one test positive was calculated in the whole group of patients and compared with the pretest probability of the disease in the population evaluated. Seventy-six of 536 patients had EP, giving an overall prevalence of the disease in the study population of 14.18% (CI<sub>95%</sub>, 11.2–17.1%). This was the predicted probability of developing EP before having performed hormones measurement (pretest probability).

With respect to the early prediction of EP, when hCG levels were found high (i.e. above the thresholds defined by the ROC curve analysis), the probability of having EP (positive predictive value) was as high as 34.13% (CI<sub>95%</sub>, 26.9–41.3%), whereas with hCG values below the cutoff, the probability of having an EP was 5.14% (CI<sub>95%</sub>, 2.1–7.4%) (Fig. 3).

Women with UPL and progesterone concentrations higher than 5.01 ng/ml (as indicated by the ROC curve analysis) had a probability of having EP as high as 29.41% (CI<sub>95%</sub>, 23.4–35.4%), whereas with progesterone values below the cutoff the probability of having an EP was 3.49% (CI<sub>95%</sub>, 1.5–5.5%) (Fig. 3).

On the contrary, if serum activin A concentrations were low (i.e. below the thresholds defined by the ROC curve analysis), its positive predictive value for EP was as high as 97.43% (CI<sub>95%</sub>, 93.9–100%) (Fig. 3), but if activin A concentrations were within reference intervals, that probability was as low as 0% (CI<sub>95%</sub>, 0–1.9%).

**Discussion**

The present study first shows that, in spontaneously conceived UPL, activin A levels are lowest in women with EP, and lower than in those who will progress to term delivery (IUP) or who will have a SAB. The finding of low activin A concentrations in EP and SAB patients are novel, and lead us to suggest that an impaired secretion of activin A occurs in the presence of problems related to trophoblast invasion and implantation. Indeed, during pregnancy, the human placenta is the main source of activin A in maternal bloodstream (12) because serum levels in maternal circulation progressively increase throughout pregnancy until delivery (13). Furthermore, variations in maternal activin A levels occurring in trophoblast diseases are believed to be part of the
adaptive response of the placenta to adverse environmental conditions, probably representing a nonspecific functional counterpart of structural placental changes such as proliferation of trophoblast cells and/or placental dysfunction, that are part of the evolving diseases (18). Indeed, activin A levels are reduced in the presence of nonviable trophoblast, as happens in complete miscarriage (19–21); increase when disrupted placentation and placental ischemia occur, as in the case of preeclampsia (22), fetal growth restriction (23), and abruptio placentae (24); and are dramatically boosted in the presence of excessive trophoblast proliferation and implantation, as takes place in hydatidiform mole (25). This evidence suggests that a reduction of activin A secretion in EP may signal the difficulty of the trophoblast to correctly implant. Indeed, in vitro data on the role of activin A in facilitating endometrial decidualization (26, 27) and the fact that decidualization is a requisite for successful implantation collectively lead us to hypothesize that an impairment of activin A synthesis and release may take place in the placenta, causing the lack of a well-vascularized and appropriately constructed endometrium and, therefore, trophoblast implantation outside the uterus.

Finally, the role of the endometrium as a relevant source of activin A needs to be considered because the main difference between IUP/SAB and EP is essentially in the ground of implantation (28). During the secretory phase of the menstrual cycle (at the time of blastocyst implantation), activin A is present in the uterine fluid of cycling women in higher amounts than the proliferative phase, and levels correlate with endometrial thickness (29). It has been suggested (26) that such an increase of endometrial activin A and secretion at the time of blastocyst apposition may have an important role as a local endometrial phenomenon involved in embryo implantation, because activin A is a well-known regulator of the differentiation of proliferative cytotrophoblast into extravillous invasive trophoblast cells of the anchoring villi (30). These findings lead us to suggest that the lack of an adequate endometrial secretion of activin A may be related to the lack of appropriate messages to the placenta for a right implantation.

Whatever the source of reduced activin A concentrations in EP, the clinical usefulness of its measurement in the case of a UPL needs to be discussed. Indeed, the distinction between UPLs that are developing EP, IUP, and SAB based on the interpretation of hormonal markers is the most difficult
diagnostic problem. Although the vast majority will be SAB and early IUPs, it is the group of women with an EP too early to visualize that poses the greatest concern (1). Currently single progesterone measurement followed by hCG surveillance and/or ultrasound is used to detect EP with relatively high success (1). Indeed, serum progesterone concentrations are higher in women with viable IUP than in those with EP or pregnancies who will progress as SAB, as also confirmed by our findings.

In the present study the clinical usefulness of single serum hCG, progesterone, and activin A measurement for discriminating the presence of EP in a selected group of at risk pregnant women was evaluated. As expected (1, 7, 8), the discriminatory capacity of a single measurement both of hCG and progesterone is insufficient to diagnose EP with certainty. With respect to the potential advantage of using activin A as a single marker for identifying EP, we have found that, at the cutoff identified by the ROC curve analysis, its measurement reached a sensitivity of 100% and a specificity of 99.6% for recognition of EP. In other words, measuring activin A yields positive (97.4%) and negative (0%) predictive values that differ materially from the overall prevalence of EP (14.18%) in the study population. It means that the probability of having an EP may be estimated more precisely and more quickly if activin A measurement is performed. These findings are of relevance because a highly sensitive and specific test to identify EP would be useful in a clinical setting when women present with symptoms in an emergency setting.

In conclusion, we found that, in women with UPL, maternal serum activin A levels are lowest in those with an EP, and that single activin A measurement may identify patients at risk of EP with a high sensibility and specificity. These findings may open up a new cue for biochemical markers assessment in maternal bloodstream to select pregnancies at higher risk of EP earlier and possibly to prevent unnecessary interventions.

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