Human Reproduction Vol.20, No.11 pp. 3163–3166, 2005 doi:10.1093/humrep/dei218
Advance Access publication July 29, 2005.

The value of a single combined measurement of VEGF, glycodelin, progesterone, PAPP-A, HPL and LIF for differentiating between ectopic and abnormal intrauterine pregnancy

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BACKGROUND: To evaluate whether serum concentrations of the non-placental markers vascular endothelial growth factor (VEGF), glycodelin (GLY) and progesterone (P) and the novel placental markers pregnancy-associated plasmaprotein A (PAPP-A), human placental lactogen (HPL) and leukaemia inhibiting factor (LIF) differ in ectopic pregnancy (EP) when compared with abnormal intrauterine pregnancy (aIUP). METHODS: A prospective clinical study was conducted at the University Hospital of Larissa, Greece. The study included 50 patients admitted with failed pregnancy and suspected ectopic pregnancy that were treated with curettage or laparoscopy and classified as histologically confirmed EPs ($n = 27$) or histologically confirmed aIUPs ($n = 21$) (mean gestational age of 7.15 and 7.3 weeks, respectively). Two suspected EPs proved to be normal IUPs and were excluded. VEGF, GLY, P, β-HCG, PAPP-A, HPL and LIF were measured by enxyme-linked immunosorbent assay (ELISA) methods in a single pre-operative blood sample. RESULTS: The median VEGF concentration was 227.2 pg/ml in the EP group versus 107.2 pg/ml in the aIUP group ($P < 0.001$), with a suggested threshold value of 174 pg/ml for their differential diagnosis. LIF, P, PAPP-A, HPL and GLY serum measurements did not differ significantly between EP and aIUP. CONCLUSION: VEGF serum levels might be a useful marker in differentiating between EPs and aIUPs.

Key words: ectopic pregnancy/incomplete abortion/non-placental markers/placental markers

Introduction

Ectopic pregnancy (EP) is still a major cause of maternal morbidity and mortality, accounting for 9% of first trimester pregnancy related deaths (Mueller *et al.*, 2004). Transvaginal ultrasound and the measurement of serial β-HCG values assist the diagnosis whereby an abnormal gestation exists when serial β-HCG values fall or do not rise appropriately but can not accurately separate an abnormal intrauterine pregnancy (aIUP) from an EP (Letterie and Hibbert, 2000).

Increased serum concentrations of vascular endothelial growth factor (VEGF) (Daniel *et al.*, 1999; Felemban *et al.*, 2002; Fasouliotis *et al.*, 2004; Mueller *et al.*, 2004) and decreased serum levels of leukaemia-inhibiting factor (LIF) (Wegner and Mershon, 2001), progesterone (P) (Dart *et al.*, 2002), pregnancy-associated plasmaprotein A (PAPP-A) (Sjoberg, 1987; Mueller *et al.*, 2004), human placental lactogen (HPL) (Tornehave *et al.*, 1987; Mueller *et al.*, 2004) and glycodelin (GLY) (Stabile *et al.*, 1994) have been previously associated with failed pregnancy.

In an emergency set-up, it would be extremely valuable if a single combined measurement of these new markers could differentiate not only between an EP and a healthy intrauterine pregnancy (IUP), but also between EP and an aIUP. Such a correlation would decrease the time to diagnosis and reduce the possibility of tubal rupture and its sequelae. In a recent publication, it has been stated that the 'triple marker analysis' [VEGF/(PAPP-A×P)] allows a clear discrimination between IUP and EP (Mueller *et al.*, 2004) and that elevated maternal serum levels of VEGF, as early as 11 days after embryo transfer, are associated with EPs (Fasouliotis *et al.*, 2004).

The aim of the present study was to evaluate whether a single combined serum measurement of all these markers will contribute to the differential diagnosis of patients presenting as failed pregnancies.

Materials and methods

Patients and sera

Fifty patients admitted with failed pregnancy and suspected EP to the gynaecology department of the University Hospital of Larissa were recruited in the study. Women presented with either abdominal pain

or bleeding. All had a transvaginal ultrasound. Those who underwent a laparoscopy with the diagnosis of possible ectopic pregnancy had free fluid with no intrauterine pregnancy and a tubal mass (diameter: 2–6 cm). No tubal mass displayed a fetal heartbeat. Only patients with accurate assessments of their gestational age with a previous transvaginal ultrasound in the current pregnancy were included. The gestational age ranged from 5.5 to 10 weeks, with most pregnancies (n = 47, 94%) being between 6 and 9 weeks. Confirmed EPs were treated by laparoscopic salpingectomy, and histological diagnosis of tubal pregnancy was made on all excised specimens, while aIUPs had curettage. Patients were classified as histologically confirmed EPs or aIUPs, and analyzed accordingly. Two suspected EPs proved to be normal IUPs when re-examined by the consultant on call and were excluded.

Therefore, 48 patients were included in the analysis, namely the EP patients (n = 27) and the aIUP patients (n = 21). All blood samples (one per patient) were collected before treatment by peripheral venous puncture. The blood samples were allowed to coagulate at room temperature and serum was obtained by centrifugation. Twenty-five control early pregnancy blood samples were obtained from the antenatal clinic after ascertainment of the presence of a single and uneventful pregnancy with reliable gestational age. All sera were stored at –80°C until assays were performed in batches. The study was carried out in accordance with the 1975 Helsinki Declaration on Human Experimentation. It was approved by the local ethics committee and written informed consent was given.

Serum assays

The enzyme-linked immunosrobent assay (ELISA) methods applied in this study [enzymeimmunoassay (EIA)] were carried out using the fully automated analyzer Triturus (Grifols, Barcelona, Spain). Serum concentrations of VEGF, GLY, P, PAPP-A, HPL and LIF were estimated quantitatively in duplicate using an EIA method. VEGF was measured with reagents from Bender MedSystems (Burlingame, CA, USA) with a sensitivity of 11 pg/ml and a spectrum of 0–1000 pg/ml, an inter-assay coefficient of variation (CV) of 17.7% and intra-assay CV of 5.90%. P was measured with reagents from Diagnostic Systems Laboratories (DSL) (Webster, TX, USA) with a sensitivity 0.13 pg/ml, spectrum 0–80 pg/ml, an inter-assay CV of <4.1% and an intra-assay CV of <3.20%. PAPP-A was measured with reagents from DSL with a sensitivity of 0.013 mIU/ml, spectrum 0–75 mIU/ml, inter-assay CV of 3% and intra-assay CV of 2.90%. HPL was measured with reagents from Bioserv Diagnostics (Rostock, Germany) with a sensitivity of 0.05 mg/l, spectrum 0–20 mg/l, inter-assay CV of <4.97% and intra-assay CV of <4.06%. GLY was measured with reagents from Bioserv Diagnostics with a sensitivity of 6 ng/l, spectrum of 0–100 ng/l, inter-assay CV of 3.92% and intra-assay CV of 6.2%. LIF was measured with reagents from Bender MedSystems with a sensitivity of 3.3 ng/ml, spectrum of 0–200 ng/ml, inter-assay CV of 7% and intra-assay CV of 5.50%. β-HCG was measured with reagents from Bender MedSystems with a sensitivity of 0.8 mIUg/ml, inter-assay CV of <4.3% and intra-assay CV of <4.8%.

Statistical analysis

Data are presented as median and range (minimum, maximum). For all serum markers, the two groups were compared using the Kruskal–Wallis test and the Mann–Whitney U test with Bonferonni's correction.

The diagnostic performance of VEGF in aIUP was further evaluated using receiver operator characteristic (ROC) curve analysis.

Results were considered significant when $P < 0.05$. The statistical analysis was performed using SPSS r12.

Results

Table I shows the median values of the serum markers as well as their minimum and maximum values. There were no significant differences between EP and aIUP in the values of LIF, P, PAPP-A, HPL, GLY and β-HCG.

There was a statistically significant difference in the VEGF serum concentration between EP and aIUP ($P < 0.001$). The median VEGF was 227.2 pg/ml in the EP group (minimum = 34, maximum = 436) versus 107.2 pg/m (minimum = 0.84, maximum = 169.6) in aIUP (Table I, Fig. 1).

ROC analysis showed that VEGF can be used for the differential diagnosis of EP and incomplete abortion, with the area under the curve being 0.889 ($P < 0.001$). The threshold for the diagnosis of EP is 174.5 pg/ml (at this point sensitivity = 0.78 and specificity = 1.0) (Fig. 2).

Table I. VEGF, PAPP-A, β-HCG, P, LIF, HPL and GLY serum measurements for ectopic pregnancy and abnormal intrauterine pregnancy

	Group	*n*	Median	Minimum	Maximum
VEGF (pg/ml)	EP	27	227.2	34	436
	aIUP	21	107.2	0.84	169.6
PAPP-A (mIU/ml)	EP	27	0.02	0.01	0.31
	aIUP	21	0.02	0.01	0.76
β-HCG (mIU/ml)	EP	27	2180	194	17486
	aIUP	21	2750	58.2	14970
P (ng/ml)	EP	27	13.6	2.31	80
	aIUP	20	11.85	2.29	75.4
LIF (ng/ml)	EP	27	45.8	29	180
	aIUP	21	42	27.6	57.2
HPL (mg/l)	EP	27	0.09	0.05	0.22
	aIUP	21	0.08	0.01	0.2
GLY (ng/ml)	EP	27	82.3	1.78	100
	aIUP	21	79	4.27	103
Gestational age (weeks)	EP	27	7.15	5.5	10
	aIUP	21	7.30	5.3	10

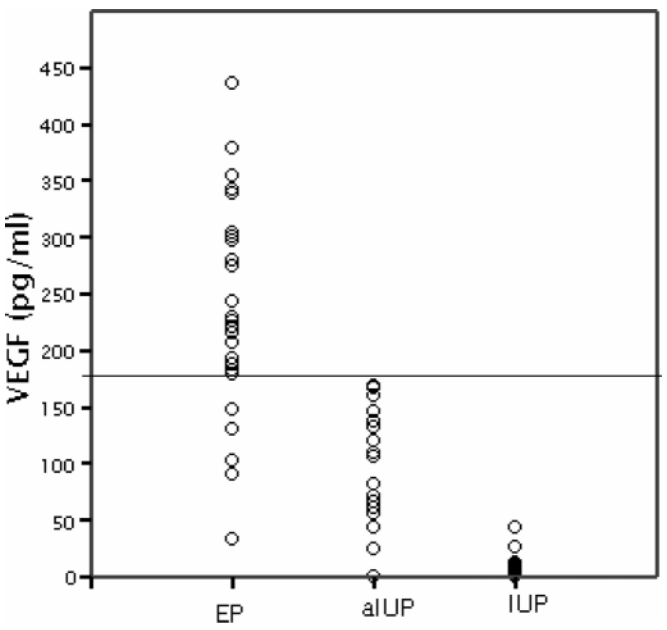

Figure 1. Scatter diagram of VEGF values per diagnostic category. The distribution of EP values above the suggested cut-off value of 174.5 pg/ml (sensitivity 1, specificity 0.78) is shown.

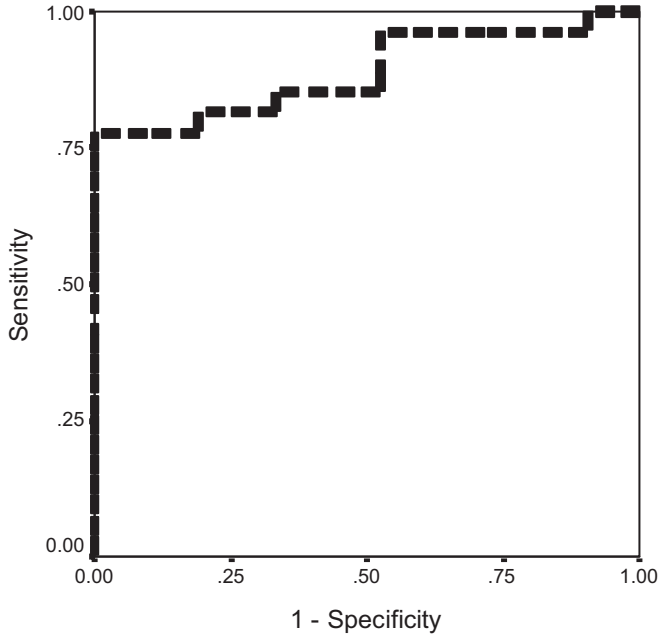

Figure 2. Receiver operating Characteristic (ROC) curve for VEGF for discriminating ectopic pregnancy from abnormal intrauterine pregnancy.

In the control group of normal IUPs, which continued through to term, the VEGF median was 2.3 pg/ml (minimum = 0.55, maximum = 44 pg/ml). When correlated with EP (ROC curve not shown), the area under the curve was 0.999 ($P < 0.001$). The threshold for the differential diagnosis of EP versus IUP is 31 pg/ml (at this point sensitivity = 1 and specificity= 0.96).

Discussion

Pregnant patients presenting with vaginal bleeding as an emergency represent a diagnostic challenge. VEGF was selected as a possible marker since it has been observed that serum levels are significantly higher in women with EP than in those with IUP (Daniel *et al.*, 1999; Felemban *et al.*, 2002; Fasouliotis *et al.*, 2004; Mueller *et al*., 2004). Another study concluded that a VEGF level >200 pg/ml could not discriminate EP from aIUP at 6 weeks of gestation (Kucera-Sliutz *et al*., 2002). Our sample had a median gestational age of 7.15 weeks and the threshold for the differential diagnosis of EP versus aIUP was set at 174.5 pg/ml.

The diagnostic relevance of VEGF for differentiating EP versus IUP as early as 11 days after day 3 embryo transfer has been reported recently by Fasouliotis *et al*. (2004) at a much higher threshold concentration of >700 pg/ml for VEGF, which might be anticipated due to the preceded superovulation.

It has been previously suggested that the measurement of GLY, an endometrial protein with proposed immunomodulatory activity during human embryonic nidation (Mueller *et al.*, 2000; Vigne *et al.*, 2001), is useful in distinguishing between EP and other abnormal pregnancies such as missed and incomplete abortions (Stabile *et al.*, 1994). This was not confirmed by our results as the other two non-placental markers, GLY and P, did not differ signicantly between the EP and aIUP patients.

A single serum P measurement in our sample could not discriminate between aIUP and EP. Tthis supports the findings of a published meta-analysis (Mol *et al.*, 1998).

Furthermore, we were unable to reproduce the results of Wegner and Mershon (2001) for LIF. The placental proteins HPL and PAPP-A could be the method of choice, as they are independent from VEGF, but this was not confirmed in our sample.

Although one can argue that gestational age ranged substantially (5–10 weeks) in the present study, our VEGF median value was only 2.3 pg/ml in IUPs versus 227.2 pg/ml in the EP group and 107.2 pg/ml in the aIUP group. Therefore, this marked difference cannot be attributed to the relatively wide range of gestational ages of the samples. In addition, there are conflicting data in the literature regarding the effect of gestational age on VEGF serum measurements. In particular, one previous study showed that, in early IUPs, VEGF remains low up to 6 weeks and shows a progressive increase between 6 and 8 weeks, after which concentrations tend to plateau (Evans *et al.*, 1998), while a recent report in a sample with gestational age range of 5–10 weeks measured VEGF in IUPs to be ~5 pg/ml, decreasing with gestational age (Mueller *et al*., 2004).

In an attempt to explain what could affect the production and secretion of VEGF, we need to consider that the fact that extrauterine implantation environments are very different from those of the primed and well-vascularized endometrium. Hypoxia at the abnormal implantation site may trigger increased VEGF production (Ikeda *et al.*, 1995; Shore *et al.*, 1997). Recently, mRNA expression of VEGF and its receptors (KDR and flt-1) were measured in the implantation and nonimplantation sites of the human oviduct with EP. The mRNA expression was significantly higher in the implantation site of the human oviduct with ectopic gestation compared with the non-implantation site. It has been suggested that VEGF may be the angiogenic factor responsible for the implantation and placentation of an ectopic pregnancy in the oviduct (Lam *et al.*, 2004).

Our study continues the line of investigation of biomarkers for the early detection of EP. It reports that only VEGF serum measurement differed significantly between EP and aIUP. Although the measured VEGF values display a rather wide range that overlaps between EP and aIUP (Fig. 1), there was a statistically significant difference between the two groups ($P < 0.001$). This could be expected to contribute to an early and accurate differential diagnosis of EP, resulting in the timely institution of medical treatment, which might prevent tubal rupture. The clinical applicability of these findings remains to be evaluated in larger prospective studies.

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Submitted on April 8, 2005; resubmitted on June 13, 2005; accepted on July 1, 2005