Clinical Chemistry 49:12
2045–2049 (2003)

Endocrinology and Metabolism

Human Chorionic Gonadotropin Isoforms in the Diagnosis of Ectopic Pregnancy

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Background: **Early diagnosis of ectopic pregnancy uses ultrasound with serial measurements of total human chorionic gonadotropin (hCG). The objective of this study was to explore the possibility that an isolated measurement of hCG isoforms/subunits rather than total hCG could be used as a single test for ectopic pregnancy.**

Methods: **Total and intact hCG, free hCG β- and α-subunits (hCGβ and -α), and hCG β-core fragment were measured by RIA and IRMA in the serum and urine of 76 women presenting at outpatient emergency departments with a positive pregnancy test, lower abdominal pain, and/or vaginal bleeding. Final diagnoses were based on outcomes of pregnancies and tissue histology.**

Results: **Twenty-seven of the 76 women were subsequently diagnosed with viable pregnancies, 37 with spontaneous miscarriage, and 12 with ectopic pregnancy. Concentrations of all forms of hCG were lower in cases of ectopic pregnancy and spontaneous miscarriage than in viable pregnancies. Serum samples gave better results than urine samples. The free hCGβ isoform ($P < 0.0001$) had 100% sensitivity at a specificity of 79% at a 281 pmol/L (6.5 μg/L) cutoff. Total hCG ($P = 0.005$) had comparable ROC characteristics with a 100% sensitivity and 68% specificity at a cutoff value of 1053 pmol/L (375 IU/L). Neither hCGβ ($P = 0.7$) nor total hCG ($P = 0.4$) could distinguish ectopic pregnancies from spontaneous miscarriage.**

Conclusion: **Measurement of serum free hCGβ at the time of presentation can identify women with a high probability of ectopic pregnancy who may benefit from closer surveillance, reducing the risk of tubal rupture.**

Ectopic pregnancy is the major cause of morbidity and mortality in women of reproductive age, accounting for ~9% of all deaths associated with first-trimester pregnancies and affecting ~2% of all pregnancies *(1, 2)*. Since the introduction of laparoscopy in the late 1960s *(3)*, considerable effort has been made to define more sensitive and specific tests for its early diagnosis because >80% of ectopic pregnancies had been diagnosed after tubal rupture.

Several biochemical and imaging markers have been studied as single or combined (algorithm) tests for occult ectopic pregnancy *(4–6)*. The most common of these involves the combination of transvaginal ultrasound and serial measurements of human chorionic gonadotropin (hCG)[5] *(7, 8)*. The rate of increase of serum hCG indicates the presence or absence of viable uterine pregnancies: hCG doubles every 1.5 days up to 5 weeks after the last menstrual period, and then every 3.5 days from the 7th week (or when hCG is >10 000 IU/L) *(9)*. Although hCG concentrations do not increase at this rate in most ectopic pregnancies, they do in approximately one-third of cases, and anomalies in the dynamics of the hCG increase are also related to spontaneous miscarriage *(10)*. Despite these problems, the detection of hCG remains an indispensable tool in the diagnosis of ectopic pregnancy.

In women with inconclusive ultrasonographic findings, a "discriminatory serum hCG zone" has been used, where a diagnosis of ectopic pregnancy is most likely in

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Received May 16, 2003; accepted September 17, 2003.
DOI: 10.1373/clinchem.2003.022095

[5] Nonstandard abbreviations: hCG, human chorionic gonadotropin; hCGβ and hCGα, hCG β- and α-subunits, respectively; and hCGβcf, hCG β-core fragment.

the absence of demonstrable intrauterine pregnancy but with an adnexal mass or free fluid in the pouch of Douglas and serum hCG >6500 IU/L *(11)*. With the advent of transvaginal ultrasound, the hCG cutoff has decreased to 1000–2000 IU/L *(12)*. Although the introduction of this hCG discriminatory zone appears effective, a particular hCG concentration has yet to be defined as a good diagnostic indicator for ectopic pregnancy *(13)*.

hCG is a glycoprotein heterodimer *(14, 15)*. Only intact hCG, i.e., the α, β heterodimer, has biological activity, but other forms of hCG can be found in the placenta, urine, and serum *(16)*. hCG isoforms and subunits are generally found as metabolic breakdown products of the intact hormone, but occasionally free subunits can be expressed and released directly into the circulation. The most commonly assayed hCG fragments are the free β- and α-subunits of hCG (hCGβ and hCGα, respectively) and the terminal degradation product of hCG, hCG β-core fragment (hCGβcf), which is found in urine *(17)*. hCG assays currently used in the diagnosis of ectopic pregnancy measure so-called total hCG, i.e., intact hCG plus free hCGβ.

Diagnostic algorithms for ectopic pregnancies that require serial measurements of hCG over several days necessitate delays in diagnosis because hCG test are often done on an outpatient basis, and the risk of tubal rupture is increased. The aim of our study was to explore whether measurement of the concentrations of a specific hCG isoform and/or its subunits in serum and/or urine at a single point in time could be used to test for ectopic pregnancy.

Materials and Methods

WOMEN AND SAMPLES

Between September 1998 and September 2000, matched urine and serum samples were collected at the Homerton and Whipps Cross Hospitals, London, emergency outpatient departments. Samples were collected from 97 women who presented at the Accident and Emergency Department with abdominal pain, vaginal bleeding, and a previous positive hCG test. Twenty-one were excluded for having no or insufficient matched urine or serum sample for all tests. Of the remaining 76 women, 27 (36%) were subsequently diagnosed with viable pregnancies, 37 (48%) with spontaneous miscarriage (11 missed, 6 complete, and 20 incomplete), and 12 (16%) with ectopic pregnancies. The 12 cases of ectopic pregnancy were confirmed by surgical intervention and histology.

Serum and urine samples were frozen at −20 °C and then assayed at the Williamson Laboratory at St. Bartholomew's Hospital. Samples were free of contamination and contained no hemolyzed blood. Sample concentrations were determined over several assays when run undiluted and at multiple dilutions of up to 1 in 10 000 (in phosphate-buffered saline containing 100 mL/L horse serum). The CV for all assays were ≤5% for the midrange quality control. At the extremes of the assay range, results for quality-control samples varied up to 20%. However, sample results were recorded as the mean analyte concentration from multiple, parallel dilution, determinations.

A laboratory technician and a junior faculty member of staff in laboratory medicine conducted the index tests. Data interpretation and quality-control audits were conducted by the Laboratory Director. Clinical data were collected by a House officer blind to the index test results and audited by a specialist registrar in Obstetrics and Gynecology. The North East Thames Region Health Authority Ethic Committee approved the study.

HORMONE ASSAYS

In accordance with IFCC recommendations *(18)*, we have calibrated all assays in terms of picomolar concentration of the immunoreactive analyte [for a review, see Iles and Chard *(16)*]. For discussion purposes, concentrations in the commonly used units for the hCG analyte have been added in parentheses where appropriate.

Total hCG was measured by an in-house RIA with sheep anti-hCGβ antiserum (S424) that binds both free hCGβ and the intact hormone. Standards were obtained from the National Institute of Biological Standards (Potters Bar, UK). Free hCGβ, labeled with ^{125}I by the chloramine-T method, was used as a tracer (NIH preparation CR123). The detection limit of this assay is 42 pmol/L (15 IU/L) *(19)*.

Intact hCG was measured with an in-house IRMA with a polyclonal anti-hCGα capture antibody conjugated to 1,1′-carbonyldiimidazole-activated cellulose and a ^{125}I-radiolabeled monoclonal antibody (1/07; Quantum Bioscience) to epitopes on the hCGβ C-terminal peptide. The calibration curve ranged from 2.6 to 813 pmol/L (0.93–289 IU/L) *(19)*.

Free hCGα was measured by use of an in-house RIA with an antiserum (S781) raised against free hCGα and affinity-adsorbed on a column of intact hCG conjugated to CNBr-activated Sepharose. The tracer was ^{125}I-radiolabeled purified free hCGα (NIH preparation CR123 α-subunit). The calibration curve ranged from 62.5 to 6250 pmol/L (0.9–90.6 μg/L) *(19)*.

Free hCGβ was measured with an in-house IRMA using antiserum (S752) raised in sheep against free hCGβ and conjugated to 1,1′-carbonyldiimidazole-activated cellulose. Captured hCGβ was detected by an ^{125}I-radiolabeled monoclonal antibody, 1/07, which recognizes epitopes on the hCGβ C-terminal peptide. The calibration curve used hCGβ (NIH preparation C123) and ranged from 22 to 1110 pmol/L (0.5–24.6 μg/L) *(19)*.

The in-house hCGβcf RIA used a polyclonal antibody to hCGβcf (S504) in a late-addition competition assay using ^{125}I-radiolabeled purified hCGβcf. The calibration curve ranged from 9 to 500 pmol/L (90–5000 μg/L) *(20)*.

Data were summarized using box-and-whisker plots and ROC curves. The Kruskal–Wallis test was used for comparisons of concentrations. A P value of 0.05 was

regarded as the upper limit of significance. Statistical analysis was performed with the Stats-Direct program, and graphs were plotted using Origin 6.0

Results

The mean gestational ages (weeks) in women with ectopic pregnancy, viable pregnancy, and spontaneous miscarriage were 7.0, 8.0, and 8.3 weeks, respectively; thus, no significant difference in gestational age was found among the groups. Table 1 shows the descriptive statistics for the concentrations of hCG and its isoforms in serum and urine sample for each patient group. The corresponding figure can be seen in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol49/issue12/. The Kruskal–Wallis test for nonparametric comparison of the patient groups showed that, generally, concentrations of hCG and its subunits were lower in ectopic pregnancy than in normal pregnancies to a statistically significant margin (see Table 2 in the online Data Supplement). The greatest difference was found in serum free hCGβ in ectopic pregnancy vs viable pregnancy ($P < 0.0001$). By way of contrast, the concentrations of serum and urinary free hCGα were not statistically different for ectopic pregnancies and viable pregnancies ($P = 0.07$ and 0.48, respectively; Table 1; also see Table 2 in the online Data Supplement). However, women with early pregnancy losses by spontaneous miscarriage also had dramatically lower concentrations of the hCG isoforms and consequently could not be distinguished from those with ectopic pregnancy ($P = 0.45$ for serum total hCG and $P = 0.70$ for serum free hCGβ; see Table 2 in the online Data Supplement).

The ROC curves for urinary and serum intact hCG, free hCGβ, total hCG, free hCGα, and hCGβcf are shown in Fig. 1. These curves show the likelihood of an ectopic pregnancy determined by the different analytes below optimal cutoff limits compared with a viable pregnancy (Table 3 in the online Data Supplement). Serum free hCGβ and serum total hCG are the best hCG-related analytes and biological fluid for this purpose. Estimated areas under the curves (95% confidence intervals) were 0.91 (0.72–1.0) and 0.86 (0.65–1.0) for serum free hCGβ and total hCG, respectively. The reason for this was not simply that they showed the greatest mean decrease in concentrations: When we compared the decrease in group median values, the serum analytes intact hCG and free hCGβ had the lowest values, 12% and 13%, respectively, of the values in women with viable pregnancies. However, it was the variability in the concentrations that determined which analyte had superior diagnostic characteristic (Table 1; see also Tables 2 and 3 in the online Data Supplement). The range of values for each analyte was large, and most data sets did not follow a gaussian distribution. Serum free hCGβ and total hCG had the

Table 1. Descriptive statistics for concentrations of hCG and related fragment molecules in serum and urine samples from women with viable pregnancies, ectopic pregnancies, or spontaneous miscarriage.

	Statistics	Viable pregnancies	Ectopic pregnancies	Spontaneous miscarriage
LMP,[a] weeks	Mean (SD)	8.2 (2.3)	7 (1.2)	9 (2.8)
	Range	6.8–10	6.7–8.0	6.8–11
Urinary isoforms, pmol/mmol creatinine				
Total hCG	Mean (SD)	8109 (10 824)	1843 (3501)	1320 (2091)
	Median (interquartile range)[b]	2874 (10 982–1599)	716 (1591–178)	657 (1743–227)
Intact hCG	Mean (SD)	22 814 (35 875)	4499 (8041)	4675 (8447)
	Median (interquartile range)	8613 (26 246–3408)	1649 (4395–660)	890 (5651–188)
Free hCGβ	Mean (SD)	4007 (5958)	1698 (2020)	895 (1769)
	Median (interquartile range)	1481 (4233–563)	820 (3300–83)	299 (846–48)
Free hCGα	Mean (SD)	33 407 (144 020)	26 054 (70 693)	3457 (5657)
	Median (interquartile range)	3519 (6632–947)	1317 (5914–404)	850 (3110–251)
hCGβcf	Mean (SD)	17 477 (27 201)	4017 (4545)	2440 (3255)
	Median (interquartile range)	12 753 (20 336–1034)	2443 (7847–349)	920 (3137–398)
Serum isoforms, pmol/L				
Total hCG	Mean (SD)	8909 (23 299)	360 (271)	7988 (26 813)
	Median (interquartile range)	1548 (5891–688)	284 (624–153)	430 (1634–123)
Intact hCG	Mean (SD)	25 737 (200 813)	59 664 (85 175)	38 900 (67 473)
	Median (interquartile range)	203 993 (480 245–39 231)	24 719 (88 050–6134)	16 977 (39 953–1153)
Free hCGβ	Mean (SD)	936 (939)	115 (90)	188 (474)
	Median (interquartile range)	645 (989–357)	82 (195–35)	69 (129–34)
Free hCGα	Mean (SD)	3171 (3714)	1231 (1177)	1027 (1105)
	Median (interquartile range)	2664 (4329–1132)	793 (2314–167)	343 (1948–167)

[a] LMP, last menstrual period.
[b] Interquartile range is 75th–25th centile.

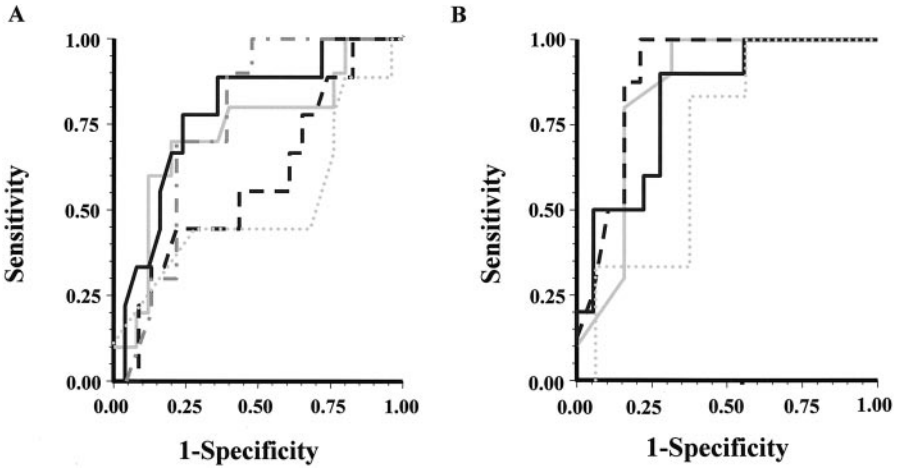

Fig. 1. ROC curves for the hCG isoforms in urine (*A*) and serum (*B*).

Solid light gray line, total hCG; *solid black line*, intact hCG, *dashed black line*, hCGβ; *dotted light gray line*, hCGα; *dot-dashed medium gray line*, hCGβcf.

tightest distribution of values around the median (and mean) values and consequently gave the best results.

Discussion

The validity of the current diagnostic algorithm for ectopic pregnancy, i.e., ultrasound and serial measurements of hCG, has already been demonstrated by a decrease in the risk of tubal rupture and morbidity of women whose diagnosis was achieved by this protocol *(21)*. Nevertheless, there are factors that reduce the sensitivity of ultrasound, such as lack of experience by the ultrasonographer, obesity of the patient, uterine myoma, or more commonly, "inconclusive ultrasound", which then must be repeated several times *(22)*. The subjective nature of scanning contributes to this uncertainty. When considering these drawbacks to a diagnostic algorithm, it is clear that a single blood test that would help to separate patients with high risk for an ectopic pregnancy from low-risk patients could possibly advance clinical practice and be less expensive. This study was designed to examine whether the answer lay within the isoforms of hCG produced by an ectopic pregnancy.

We found that concentrations of hCG and its subunits were significantly lower in women with an ectopic pregnancy than in those with a normal pregnancy. There was, however, considerable variability in individual patient results, and serum results were far better discriminators than urinary concentrations, even when corrected for creatinine concentration. Nevertheless, certain hCG-related isoforms were clearly better than others. Serum free hCGβ was <500 pmol/L (<10 ng/mL) in all of the study women presenting with ectopic pregnancies but >500 pmol/L in the vast majority of those women with viable pregnancies. This is consistent with the findings of Holman et al. *(23)*, who found that all women with ectopic pregnancies in their study had hCGβ concentrations <25 μg/L (1250 pmol/L). ROC analysis demonstrated up to 100% sensitivity for ectopic pregnancy with a 21% false-positive rate (viable pregnancy) for serum free hCGβ at a cutoff of 281 pmol/L (6.5 ng/mL). Total hCG performed well when measured in both serum and urine samples, but again serum measurements were best, giving 100% sensitivity with a 32% false-positive rate (viable pregnancy) at a cutoff of 1053 pmol/L (375 IU/L). The data would suggest that free hCGβ might have marginally better performance characteristics with respect to the diagnosis of ectopic pregnancy than total hCG. However, the confidence intervals of the areas under the ROC curves for these two measurements overlapped to such an extent that the difference in performance was not statistically significant. A much larger study is required to determine whether there is a significant difference in the diagnostic accuracy of free hCGβ and total hCG for ectopic pregnancy.

Measurement of intact hCG and free hCGα gave less significant results (Table 3 in the online Data Supplement), a finding inconsistent with that of Barnea et al. *(24)*, who reported that in combination with total hCG, free hCGα could be used as a marker of ectopic pregnancy. Our results are also in disagreement with those of Reuter et al. *(25)*, who found that free hCGα concentrations were unchanged in ectopic pregnancies and increased in spontaneous abortions. No urinary analyte apart from hCGβcf was found to be a good predictor of ectopic pregnancy. Cole et al. *(26)* proposed that hCGβcf could predict up to 84% of all ectopic pregnancies with only 1% false positives. In our study, although hCGβcf measurements reached 100% sensitivity at a very high cutoff of 14 201 pmol/L, this was at the expense of a very poor specificity of 48% (see Table 3 in the online Data Supplement).

Although serum free hCGβ and total hCG concentrations resolved ectopic pregnancy from viable pregnancy with a high degree of diagnostic efficiency, spontaneous miscarriage could not be distinguished. Because ~50% of women presenting with suspected ectopic pregnancy will subsequently be diagnosed with spontaneous miscarriage, the clinical utility of a single hCG measurement is limited. Other markers, such as serum progesterone measurements, have been used to distinguish viable from

nonviable (spontaneous miscarriage) pregnancies but alone are unable to resolve ectopic from viable intrauterine pregnancies *(22, 27)*.

To date, no single-point cutoff value for hCG has been proposed as a discriminator of ectopic pregnancy. This pilot study suggests that quantitative measurement of free hCGβ in serum may provide this single-point cutoff for resolving women at high risk from those with low risk of ectopic pregnancy. Serum total hCG gave diagnostic discriminator characteristics almost identical to those of serum free hCGβ. This may be because total hCG assays measure both free hCGβ and intact hCG, with the free hCGβ component being the discriminating factor. The study group in this report is too small to show any statistical difference between the ability of total hCG and free hCGβ to discriminate high-risk from low-risk suspected ectopic pregnancies.

In conclusion, based on our results, a single-point measure of total hCG or fee hCGβ could potentially improve clinical practice in the diagnosis of ectopic pregnancy by defining low risk of ectopic from high risk of ectopic pregnancy for those women presenting at emergency departments with lower abdominal pain and/or vaginal bleeding and with a positive pregnancy test. A much larger study is underway in a clinical setting, using hospital-based automated assay platforms to compare the abilities of total hCG and free hCGβ to differentiate ectopic from viable pregnancies. This, in combination with ultrasound and other serum markers that distinguish ectopic pregnancy from spontaneous miscarriage, may be a more useful test for ectopic pregnancy than current diagnostic algorithms.

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