PRENATAL DIAGNOSIS
Prenat Diagn 2007; **27**: 808–813.
Published online 25 June 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/pd.1778

Hyperglycosylated-hCG (h-hCG) and Down syndrome screening in the first and second trimesters of pregnancy

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Objective To validate Down syndrome screening protocols that include hyperglycosylated-hCG (h-hCG) measurements.

Methods Measuring h-hCG in 21 641 fresh first- and second-trimester maternal serum samples, but not for clinical interpretation. Nuchal translucency (NT) and pregnancy associated plasma protein-A (PAPP-A) measurements were available in the first trimester; alpha-fetoprotein (AFP), unconjugated estriol (uE3), and human chorionic gonadotropin (hCG) measurements in the second trimester.

Results Of the 23 first- and 26 second-trimester Down syndrome pregnancies identified, 52 and 65% of h-hCG measurements were above the 95th centile, respectively. At a 3% false positive rate, maternal age, NT, PAPP-A and h-hCG detected 78% of cases (95% CI, 56–93%). Other combinations were consistent with previous modeling utilizing stored samples. A literature summary indicates h-hCG is as strong a marker as free-β between 10 and 13 weeks' gestation.

Conclusions Down syndrome screening performance of h-hCG using fresh samples meets published expectations based on stored samples. h-hCG could replace free β measurements, at gestational ages as early as 10 weeks. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: hyperglycosylated-hCG (h-hCG); Down syndrome; prenatal screening; first trimester; second trimester

INTRODUCTION

The most common Down syndrome screening test panel in the mid-1990s included maternal age in combination with second-trimester serum alpha-fetoprotein (AFP), unconjugated estriol (uE3) and human chorionic gonadotropin (hCG) measurements (Haddow *et al*., 1992; Palomaki *et al*., 1997; Wald *et al*., 1988). This 'triple' test was then expanded into the current 'quadruple' test by the addition of dimeric inhibin-A (DIA) measurement in the same sample (Wald *et al*., 1996a; Haddow *et al*., 1998). During the 1990s, the search for new serum markers was extended to the first trimester and focused on the free β subunit of hCG (free-β) (Wald *et al*., 1996b; Palomaki *et al*., 2007) or hCG (Brock *et al*., 1990; Haddow *et al*., 1998; Palomaki *et al*., 2007) and pregnancy associated plasma protein-A (PAPP-A) (Wald *et al*., 1992; Palomaki *et al*., 2007). The aim was to provide an earlier diagnosis. However, first-trimester screening became practical only with the inclusion of ultrasound measurements of nuchal translucency (NT) (Szabo and Gellen, 1990; Hyett *et al*., 1996), usually between 11 and 13 completed weeks' gestation. This 'combined' test includes maternal age, maternal serum measurement of PAPP-A and free-β (or, in some programs, hCG), and NT measurement (ACOG, 2004).

Rather than choosing either the first-trimester or the second-trimester screening protocol, 'integrated' screening utilizes both sets of markers and provides results only in the second trimester, when measurements of all of the markers are available for interpretation (Wald *et al*., 1999; Wald *et al*., 2003). The integrated test usually combines NT and PAPP-A measurements in the first trimester with the triple or quadruple test in the second trimester. When NT measurements are not included, this test is called the 'serum integrated' test. Further refinements of integrated screening include the sequential and contingent strategies (Wright *et al*., 2004; Benn *et al*., 2005; Palomaki *et al*., 2006; Wald *et al*., 2006; Wright *et al*., 2006).

This hyperglycosylated form of hCG (sometimes called invasive trophoblast antigen or ITA) (Cole *et al*., 1999) may improve one or more of the screening strategies described above (Palomaki *et al*., 2004; Pandian *et al*., 2004; Palomaki *et al*., 2005). In order to examine the relationship between hyperglycosylated-hCG (h-hCG) and Down syndrome in both first- and second-trimester maternal sera, we studied a cohort of women opting for routine prenatal screening in Ontario, Canada. Fresh serum samples were tested for h-hCG, but blinding ensured that the results were not used clinically. Once the pregnancy outcomes were known, the h-hCG test results were linked with existing test results and pregnancy outcome. The current analysis focuses on first- or second-trimester screening tests separately, rather than together, in the integrated format.

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Received: 29 March 2007
Revised: 27 April 2007
Accepted: 29 April 2007
Published online: 25 June 2007

MATERIALS AND METHODS

Down syndrome screening in Ontario

In July 1993, the province of Ontario funded screening for Down syndrome utilizing the 'triple' marker strategy. Laboratory testing was restricted to seven sites, which assumed responsibility for testing quality. At the same time, funding was provided for linking serum screening results with selected birth outcomes. The aim was to enable documentation of screening program performance. Data were collected either electronically through the provincial birth records or via hard copy search of local individual records. This data collection system identified outcomes in about 95% of all screened pregnancies. By April 2002, the province of Ontario provided funding for incorporation of first-trimester measurements of NT and PAPP-A. Since 2002, women have had access to multiple options including first-trimester screening, second-trimester screening, and integrated screening (both with and without NT measurements).

Study cohort and protocol

The study cohort included all 21 641 pregnant women opting for routine prenatal screening at North York General Hospital between October 2003 and November 2004. A fresh aliquot of serum from each woman was set aside to be tested for h-hCG. Each aliquot was assigned a unique h-hCG study number and analyzed separately from the other serum markers, to prevent h-hCG results from being used in routine patient care. Pregnancy outcomes were collected for routine screening as per the established protocol, which included electronic record searches of local patient records and cytogenetic records, along with case finding of local and regional birth records. Cases of Down syndrome (true positives and false negatives) were identified. After testing was completed, clinical outcome records were stripped of personal identifiers and linked by the h-hCG study numbers to other demographic/pregnancy-related information, as well as existing biochemical test results. The study protocols were reviewed and approved by the local Research and Ethics Board (equivalent to an IRB).

Biochemical testing

Routine prenatal screening utilized various combinations of NT, PAPP-A, AFP, uE3, hCG, and DIA as specified by the referring physician/midwife and assay availability. The AutoDELFIA (PerkinElmer) was used for AFP, uE3, hCG, and PAPP-A measurements. An automated Nichols Advantage Specialty System (Nichols Institute Diagnostics, San Clemente, CA) was used for h-hCG measurements (Pandian *et al*., 2003). Specimens were analyzed according to package insert protocols. Long-term coefficients of variation for high and low controls were less than 10% for all five assays. Long-term stability was assessed using three control samples (values of 26.9, 76.9, and 177.5 ng/mL). After storage for 22 months at −20 °C, the results were not systematically different (values of 26.6, 75.2, and 155.7 ng/mL).

Nuchal translucency measurements

Regularly scheduled courses of training and certification were offered through Fetal Medicine Foundation (FMF), Canada (later Fetal Medicine International). Laboratories throughout Ontario were encouraged to accept NT measurement only from operators who had completed the certification process of FMF and were participating in ongoing proficiency testing. Laboratories performed quality assessment (not assurance) of sonographers to determine whether they met international published standards.

Assigning Down syndrome risk

For research purposes, multiple Down syndrome risks were assigned to each pregnancy, according to various combinations of markers. None of the data from this study were used in the Down syndrome risk algorithm. The algorithm used to assign Down syndrome risks is based on a single set of published population parameters (Palomaki *et al*., 2004; Palomaki *et al*., 2005), except for the h-hCG parameters, which were obtained from two other reports (Palomaki *et al*., 2007). These reports also contained modeled performance estimates and describe the methodology used. Briefly, the population parameters (logarithmic means, standard deviations, and pairwise correlation coefficients) are used to generate a large number of simulated biochemical test results (e.g. 30 000) that exactly match these parameters for case and for control pregnancies. A Down syndrome risk is then assigned to cases and controls by randomly selecting a maternal age appropriate for each group, and interpreting the results as if it were an actual patient. Selecting a risk cutoff level allows for the computation of a detection rate (proportion of case pregnancies with risks at or above the cutoff risk) and associated false positive rate (proportion of control pregnancies with elevated risks).

Summarizing the discriminatory power of first-trimester maternal serum markers

An existing literature summary was used to obtain information for intact (or total) hCG, as well as for the free β subunit of hCG (Palomaki *et al*., 2007) by individual week between 9 and 14 completed weeks. The literature was then searched for publications providing week-specific results for h-hCG measurements in the first trimester. In some instances, median MoM levels in Down syndrome pregnancies were estimated from a figure. The methods used to combine data from several studies are the same as used in our earlier publication (Palomaki *et al*., 2005). The composite standard deviations for unaffected and Down syndrome pregnancies were pooled (without weighting) in order to convert the median analyte value to a z-score (i.e. logarithm of median MoM/pooled SD). The 95% confidence interval

DOI: 10.1002/pd

of the z-score was estimated by computing a standard error equal to the composite standard deviation for Down syndrome pregnancies across all weeks, divided by the square root of the number of Down syndrome samples at each week minus one. The smoothed estimates were derived by fitting a quadratic equation on the log of the analyte MoM versus the gestational age in completed weeks, weighted by the square root of the number of samples at each week. Data at 9 and 14 weeks' gestation were included to help stabilize the regression analysis.

RESULTS

Table 1 shows demographic and pregnancy-related information for the cohort of 21 641 pregnant women included in our study. Of these, 8042 provided both a first- and second-trimester sample as part of integrated screening. The remaining women received stand-alone, first-trimester or second-trimester screening. Among Down syndrome pregnancies, 23 samples were collected between 10 and 13 weeks' gestation, along with 26 samples between 14 and 20 weeks' gestation. Thirteen women with a Down syndrome pregnancy provided both a first- and second-trimester sample. Among the 10 women providing only a first-trimester sample (total of 23 minus the 13 having integrated testing), only two requested first-trimester testing and had free-β measured. The remaining eight requested integrated testing, but did not provide a second-trimester sample. As expected, the average maternal age was higher for women with a Down syndrome pregnancy ($t = 4.8, p < 0.001$), but the average maternal weight, gestational age at screening and racial distributions were not significantly different between the first or second trimesters.

Figure 1 shows a scatterplot of h-hCG measurements in Down syndrome with the median (50th centile) and 95th centile of unaffected pregnancies versus gestational age. Among the samples collected 12 of 23 (52%, 95% CI 31–73%) were from women in the first trimester carrying a Down syndrome fetus and had an h-hCG level above the 95th centile (5% false positive rate). Similarly, levels in 17 of 26 samples from affected pregnancies (65%, 95% CI 44–83%) collected in the second trimester were at or above the 95th centile. All but one of the observations in the second trimester are above the median.

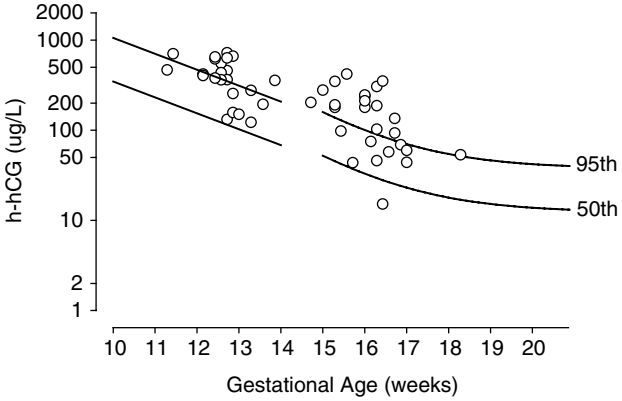

Figure 1—Scatterplot of maternal serum h-hCG results versus gestational age of sampling in Down syndrome (○) pregnancies. The completed week of gestation is shown on the horizontal axis, while the h-hCG results are shown on the logarithmic vertical axis. The lower line indicates the median level for unaffected pregnancies (h-hCG results in the first- and second-trimester samples are fitted separately). The upper line indicates the 95th centile, above which it is equivalent to a 5% false positive rate

Table 2 provides Down syndrome screening performance for different combinations of first-trimester markers. The upper half shows Down syndrome detection rates at false positive rates of 3 and 5% for three combinations of biochemical markers, both with and without NT measurements. For example, at a 3% false positive rate, the combination of maternal age, PAPP-A and h-hCG has an observed detection rate in this prospective cohort study of 57% (95% CI 34–77%), compared to the expected 59% based on modeling using stored samples

Table 1—Demographic and pregnancy related information for the cohort of 21 641 women receiving prenatal screening for Down syndrome in either the first or second trimester

Characteristic	Unaffected[a]	Down syndrome[b]
First Trimester		
Number	10 775	23
Average maternal age (SD)	32.3 (4.6) years	34.6 (4.8) years
Average maternal weight (SD)	145 (32) pounds	142 (19) pounds
Average gestational age (range)	12.5 (10–13) weeks	12.7 (10–13) weeks
Race (Caucasian, Asian, Black, other)	56, 31, 5 and 8%	65, 26, 0 and 9%
Second Trimester		
Number	18 872	26
Average maternal age (SD)	31.3 (5.0) years	35.9 (5.1) years
Average maternal weight (SD)	145 (33) pounds	152 (33) pounds
Average gestational age (range)	16.6 (14–20) weeks	16.1 (14–20) weeks
Race (Caucasian, Asian, Black, other)	48, 35, 9 and 8%	58, 31, 8 and 3%

[a] Of the total number, 8042 women provided both first-trimester and second-trimester samples.
[b] Of the total number, 13 women provided both first- and second-trimester samples.
SD = standard deviation.

DOI: 10.1002/pd

Table 2—Observed first-trimester Down syndrome screening performance using measurements of hyperglycosylated-hCG (h-hCG) and other markers, along with previously modeled performance estimates

Maternal age and other markers	3% False positive rate	5% False positive rate
PAPP-A and h-hCG	57% (59%)[a]	74% (67%)
NT, PAPP-A and h-hCG	78% (80%)	83% (84%)
PAPP-A and free-β	(61%)	(67%)
NT, PAPP-A and free-β	(79%)	(84%)
PAPP-A and hCG	(56%)	(64%)
NT, PAPP-A and hCG	(79%)	(84%)
	Risk cutoff level	
	38 year old woman (1 : 100)	35 year old woman (1 : 200)
PAPP-A and h-hCG	6.5/74 (5.0/66)[b]	12/74 (9.5/78)
NT, PAPP-A and h-hCG	5.5/83 (2.7/78)	8.4/91 (5.2/84)
PAPP-A and free-β	(4.1/65)	(8.2/75)
NT, PAPP-A and free-β	(2.4/77)	(4.7/83)
PAPP-A and hCG	(4.3/61)	(8.7/73)
NT, PAPP-A and hCG	(2.5/77)	(4.8/82)

[a] Observed (previously modeled[19]) Down syndrome detection rate.
[b] Observed (previously modeled[19]) false positive rate/Down syndrome detection rate.

Table 3—Observed second-trimester Down syndrome screening performance using measurements of hyperglycosylated-hCG (h-hCG) and other markers, along with previously modeled performance estimates

Maternal age and markers	False positive rate (3%)	False positive rate (5%)
AFP, uE3 and h-hCG	85% (65%)[a]	85% (71%)
AFP, uE3 and hCG	81% (65%)	92% (72%)
AFP, uE3, hCG, h-hCG	85% (69%)	85% (76%)
	Risk Cutoff Level (1 : n)	
	38 year old woman (1 : 130)	35 year old woman (1 : 270)
AFP, uE3 and h-hCG	4.4/85 (3.1/64)[b]	9.0/88 (6.7/74)
AFP, uE3 and hCG	4.7/88 (3.7/67)	9.6/96 (7.6/77)
AFP, uE3, hCG, h-hCG	4.2/85 (3.1/68)	8.2/88 (6.3/78)

[a] Observed (previously modeled[18]) Down syndrome detection rate.
[b] Observed (previously modeled[18]) false positive rate/Down syndrome detection rate.

in a case/control study format (Palomaki *et al*., 2004). When NT measurements are included in the prospective cohort, detection improves to 78% (95% CI 56–93%). Because nearly all of these samples were collected for integrated screening, very few had routine measurements of first-trimester free-β or hCG. It is only possible, therefore, to provide the previously modeled detection rates for these combinations.

The lower half of Table 2 provides a different view of the same data by using two selected risk cutoff levels, equivalent to the Down syndrome risk of a 38 and a 35-year-old woman. For example, using the risk cutoff level of 1 : 100 (the first-trimester risk in a 38-year-old woman), the combination of maternal age, PAPP-A and h-hCG results in an observed false positive rate of 6.5% and a detection rate of 74% (95% CI 52–90%). This is again similar to the expected false positive and detection rates of 5.0 and 66%, respectively, that are based on modeling from measurements obtained from stored samples.

Using the same structure, Table 3 focuses on various combinations of second-trimester markers. At a 3% false positive rate, maternal age in combination with AFP, uE3 and h-hCG has an observed detection rate of 85% (95% CI 65–96%), AFP, uE3 and hCG has a detection rate of 81% (95% CI 61–93%), and the quadruple combination that includes both hCG and h-hCG has a detection rate of 85%. All of these observed detection rates are higher than expected (65–69%), but not significantly so. At the two Down syndrome risk cutoff levels shown in the bottom half of Table 3, both the false positive rates and detection rates are also higher than expected. At least a portion of these differences might be explained by the study cohort being several years older than the maternal age distribution in the USA used in previously published modeling (Weinans *et al*.,

DOI: 10.1002/pd

2004; Palomaki *et al*., 2005; Weinans *et al*., 2005). Although not shown in Table 3, the additional detection gained by adding h-hCG to the quadruple test is likely to be less than that shown for the triple test in Table 3 (Palomaki *et al*., 2004).

The current study and two others collectively report h-hCG measurements between 9 and 14 weeks' gestation on 99 Down syndrome pregnancies. Similarly, 1010 and 755 observations from affected pregnancies are available over the same gestational age range for free-β and hCG, respectively. In order to make a fair comparison among the three, it is necessary to examine more than the median value in Down syndrome pregnancies, because the strength of the marker is also dependent on how spread out the values are in both affected and unaffected pregnancies (the logarithmic standard deviation). To account for this, the separation between the unaffected and Down syndrome populations can be divided by the pooled standard deviation from both cases and controls. This z-score measure takes account of both the separation and the spread of data; the higher the z-score, the stronger the association. Table 4 contains a listing of these data, by week, for all three markers. Figure 2 provides a visual summary of the literature comparing the ability of hCG, free-β and h-hCG to identify Down syndrome late in the first trimester by plotting the separation (as a z-score) by gestational age. All three markers are best at 13 weeks' gestation (farthest away from the horizontal dashed line), with the separation between the unaffected and Down syndrome populations (after regression) being 1.17 (hCG), 1.30 (free-β) and 1.44 (h-hCG). However, hCG measurements are less useful at earlier gestational weeks. Both h-hCG and free β measurements remain significantly higher. At 10 weeks' gestation, for example, the regressed separations are 0.26, 0.89, and 1.07, for hCG, free-β and h-hCG, respectively.

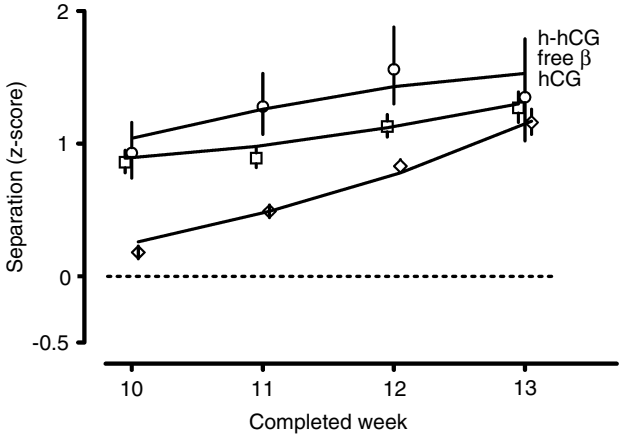

Figure 2—The relative screening effectiveness of three serum markers in the late first-trimester of pregnancy by week of gestation. The completed week of gestation is shown on the horizontal axis, while the relative separation between the populations of Down syndrome and unaffected pregnancies is shown on the vertical axis. The separation is represented by a z-score (separation between the two populations divided by the pooled standard deviation of the two populations). The vertical bars indicate an estimate of the 95% confidence interval for each observation. The horizontal dashed line is drawn at a z-score of 0, indicating the point at which there would be no separation between the two populations. Higher values indicate greater separation and are associated with higher screening efficiencies. Both h-hCG and free β measurements are similar and show modest reductions in screening effectiveness at earlier gestational ages. The hCG z-scores are associated with larger reductions in screening performance, especially at 10 and 11 weeks' gestation

DISCUSSION

The current study relies on measurements made on 21 641 fresh serum samples to confirm the earlier findings that h-hCG measurements are useful for Down syndrome screening in both the first and second trimester of pregnancy. This is important, because those earlier studies relied on stored sample banks and a case/control study design. It is possible that the true performance of h-hCG in fresh samples might be better, or worse, than those predictions. In order to help ensure an unbiased estimate of screening performance, we also relied on existing Down syndrome risk algorithms that already include h-hCG measurements to retrospectively assign risks to these pregnancies. In the first trimester, the observed screening performance closely matched expectations. Unfortunately, neither hCG nor the free β subunit of hCG was routinely measured in women who

Table 4—A comparison of discriminatory power for three first-trimester maternal serum markers between 9 and 14 completed weeks' gestation, based on the combined literature

Completed	Hyperglycosylated-hCG			Free-β subunit of hCG			Total (or intact) hCG		
Week	N[a]	Median[b]	Z-score[c]	N	Median	Z-score	N	Median	Z-score
9	2	1.77	0.85	35	1.71	0.88	32	1.08	0.16
10	19	1.79	1.04	164	1.69	0.86	140	1.09	0.18
11	30	2.22	1.26	241	1.82	0.89	181	1.26	0.49
12	27	2.65	1.43	301	1.99	1.13	226	1.48	0.78
13	12	2.32	1.53	205	2.17	1.27	134	1.73	1.16
14	9	2.80	1.59	64	2.52	1.52	42	2.13	1.60
Pooled SD[d]		0.2711			0.2649			0.2057	

[a] Number of Down syndrome pregnancies.
[b] Median analyte value (in MoM) in Down syndrome pregnancies.
[c] Log of the median MoM in Down syndrome pregnancies divided by the pooled logarithmic standard deviation (SD).
[d] Unweighted pooled logarithmic standard deviation for Down syndrome and unaffected pregnancies.

DOI: 10.1002/pd

opted for screening beginning in the first trimester because most of the women opted for integrated screening. Thus, it was not possible to compare observed and predicted rates for these other combinations. However, the predicted screening performance for these other combinations has already been validated in other prospective trials. In the second trimester, there is less agreement between the observed and predicted detection rates, but the differences are not statistically significant. There does, however, seem to be better agreement at selected risk cut-off levels when the NT measurements are not included.

CONCLUSIONS

Taken together, the present analysis provides strong evidence that h-hCG measurements made on either fresh or frozen samples provide consistent and reliable results for Down syndrome screening. These results confirm our previously published h-hCG parameters and modeling based on 45 second-trimester Down syndrome pregnancies (Palomaki *et al*., 2004) and 54 first-trimester Down syndrome pregnancies (Palomaki *et al*., 2005). Our study also has provided, for the first time, compelling evidence that h-hCG measurements are materially equivalent to free β subunit measurements in identifying Down syndrome pregnancies in the first trimester, even as early as 10 weeks' gestation. If reliable and cost effective testing and/or assays for h-hCG were to be readily available, programs could feel confident in the expected performance of Down syndrome screening protocols that involve h-hCG measurements in fresh samples.

ACKNOWLEDGEMENTS

We thank Christian Meier and Tianhua Huang for help in data and outcome collection at North York General Hospital. Quest Diagnostics Nichols Institute provided financial support and reagents to North York General Hospital for study activities and for performing h-hCG measurements. They also provided financial support for the analysis of the data. Employees of Quest Diagnostics Nichols Institute did not participate in the data analysis or in drafting the final report.

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