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Detection of maternal serum hCG glycoform variants in the second trimester of pregnancies affected by Down syndrome using a lectin immunoassay

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Aim To assess whether glycoform variants of human chorionic gonadotrophin (hCG) are present in altered concentrations in the maternal serum in pregnancies affected by Down syndrome.

Methods In a series of 50 cases of pregnancies complicated by Down syndrome and 278 unaffected pregnancies, we have examined maternal serum levels of hCG glycoforms (GlyhCG) in samples collected in the second trimester (14 to 21 weeks) using a sialic acid binding lectin immunoassay. We have compared these levels with those of other second trimester serum markers (Free β-hCG, alpha fetaprotein (AFP) and Total hCG) and modelled detection rates and false positive rates of various biochemical markers in conjunction with maternal age using a maternal age standardized population.

Results Maternal serum GlyhCG in cases of Down syndrome was significantly elevated (Median MoM 1.81) with 15 of 50 (30%) cases above the 95th centile for unaffected pregnancies. Free β-hCG was also elevated (Median MoM 2.16) with 18 of 50 (36%) cases above the 95th centile. AFP levels were reduced (Median MoM 0.75) with 9 of 50 (18%) cases below the 5th centile. Total hCG levels whilst elevated (Median MoM 1.88) had only 15 of 50 (30%) cases above the 95th centile. Maternal serum GlyhCG levels showed significant correlation with total hCG and free β-hCG (r = 0.6880 and 0.6922) in the Down group but not with AFP ($r = 0.1237$).

When GlyhCG was combined together with AFP and maternal age, at a 5% false positive rate, the modelled detection rate was 53%, some 13% lower than when free β-hCG was used and some 7% lower than when total hCG was used.

Conclusion Maternal serum GlyhCG, as measured by the sialic acid–binding lectin immunoassay is unlikely to be of additional value when screening for Down syndrome in the second trimester. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS: free β-hCG; total hCG; AFP; trisomy 21; prenatal screening

INTRODUCTION

hCG exists in maternal pregnancy serum in many different forms, varying from the intact hCG (alpha and beta dimer), the free hCG subunits [free alpha (hCGα) and free beta (hCGβ)], various nicked forms of intact (hCGn) or free β-hCG (hCGβn) and the more recently identified hyperglycosylated forms of intact hCG (HhCG) (and presumably free β-hCG) in which there are additional sialyl N-acetyllactosamine antennae (Cole, 1997; Elliott *et al*., 1997; Birken *et al*., 1999).

In the second trimester of pregnancy, measurement of intact, total or free β-hCG, in conjunction with maternal age and maternal serum alpha-fetoprotein, have found clinical utility when screening for pregnancies complicated by Down syndrome. Retrospective and prospective studies over the past decade have achieved detection rates of 65 to 75% for a 5% false positive rate (Wald *et al*., 1997; Spencer, 1999).

Attempts to improve Down syndrome detection rates have been ongoing in recent years, and one avenue has focused on the measurement of urine metabolites of hCG. Initial studies with urine β-core (hCGβcf) (Cuckle *et al*., 1994, 1995) showed considerable promise with a median in cases with Down syndrome of around six Multiples of the Median (MoM); however this initial optimism has not been sustained, with more extensive studies showing much poorer discrimination (Hsu *et al*., 1999; Cuckle *et al*., 1999a). Cole *et al*. (1997) initially reported increased levels of hyperglycosylated variants of hCG in urine from women carrying a fetus affected by Down syndrome. Subsequent studies (Cole *et al*., 1998; Cuckle *et al*., 1999b; Cole *et al*., 1999a,b) using a specific immunoassay to HhCG have confirmed elevated levels in Down syndrome with a median MoM of 9.5. These data suggest potential detection rates of 80% at a 5% false positive rate in urine (Cole *et al*., 1998; Cole *et al*., 1999a,b). However, concerns exist over both sample stability and the ability of urine creatinine to normalize for renal concentrating ability (Cole *et al*., 1999a). When examined in maternal serum, a pilot study (Shahabi *et al*., 1999) showed elevated levels in 10 cases (Median MoM 3.9) with 60% of cases above the 95th centile. However, unpublished evidence (Cole *et al*.,

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1999b) suggests that gel separator tubes can interfere with hyperglycosylated hCG detection in serum.

In an alternative immunoassay approach, Abushoufa *et al.* (2000) measured hCG glycoforms in serum from unaffected pregnancies and in cases with Down syndrome using a sialic acid–binding lectin immunoassay. In this study, in the second trimester the lectin immunoassay was shown to have greater discrimination than total hCG. When this assay was used to compare the performance in the first trimester levels of this lectin, reactive hCG (GlyhCG) were also elevated with a median MoM of 1.97 compared with 1.34 for Total hCG. However, compared with the use of free β-hCG, detection rate when combined with PAPP-A and fetal nuchal translucency (NT) were 6% lower when GlyhCG was used in place of free β-hCG (Spencer *et al.*, 2002).

In this present study, we extend the work of Abushoufa *et al.* (2000) by re-evaluating the value of measurement of hCG glycoforms with a sialic acid–binding lectin immunoassay in a further series of cases in the second trimester of pregnancy and compare the performance with that of free β-hCG and total hCG.

MATERIALS AND METHODS

Maternal serum samples from unaffected pregnancies and pregnancies affected by Down syndrome have been collected from women attending Harold Wood Hospital as part of a routine second trimester screening program (Spencer, 1999). Blood samples were collected into plain vacutainers with no clotting activator and with no gel separators. After clotting, the maternal serum was separated into aliquots and stored at $-20\,^{\circ}C$. From this archive of samples from unaffected pregnancies and those affected by Down syndrome, a series of 279 unaffected and 50 Down syndrome samples were retrieved for study. All pregnancies were dated either by crown rump length (CRL) prior to 14 weeks or by Biparietal Diameter after 14 weeks. Table 1 summarizes the two study populations.

Maternal serum free ß-hCG, AFP and Total hCG were measured using the Kryptor analyser—a rapid random access immunoassay analyser using Time Resolved Amplified Cryptate Emission (TRACE) technology and the automated immunofluorescent assays [Brahms GmbH, Berlin (formerly CIS)]. The precision and performance of these assays has been previously reported (Spencer *et al.*, 1999; 2000a).

Maternal serum hCG glycoforms were measured at two dilutions (1 in 500 and 1 in 1000) in singleton using the lectin immunoassay described by Abushoufa *et al.* (2000). The mean of the two results after correction for dilution was used in further statistical analysis. Analysis of samples was performed with outcomes blinded to the assayist. The specificity of wheat germ lectin for sialic acid has been previously published and shown to be an *N*-acetylglucosamine and sialic acid binding lectin (Bhavanandan and Katlia, 1979; Monsigny *et al.*, 1980; Ming-Chuan Shao, 1992).

Table 1—Median for maternal age, gestational age, fetal crown rump length, maternal weight and sample storage time in the Down syndrome group and the control group

	Down syndrome	Controls
Number	50	278
Maternal age (years)	35.0	30.4
Gestational age (days)	109	115
Maternal weight (kg)	63.6	62.6
Sample storage time (days)	840	851

Statistical analysis

To correct for marker variation with gestational age, each value was converted to MoM for unaffected pregnancies at the same gestational age using either previously established relationships or as established for hCG glycoforms in this study. Regression analysis was carried out to derive the relationship between marker levels and gestational age. Correction of each MoM for maternal weight was also performed using the reciprocal-linear regression weight correction procedure of Neveux *et al.* (1996).

Statistical analysis of data was performed using Microsoft Excel 97 and Analyse-It (Smart Software, Leeds, UK), a statistical software add-in.

The performance of various marker combinations as potential screening procedures was examined using standard statistical modelling techniques (Royston and Thompson, 1992). Using the observed population parameters for hCG glycoforms and those for total hCG, Free β-hCG and AFP from Spencer *et al.* (1992), a series of 15 000 random MoM values were selected for each marker from within the Gaussian distributions of the $\log_{10}$ MoM of affected and unaffected pregnancies. These values were then used to calculate likelihood ratios for the combinations. The likelihood ratios were then used together with the age-related risk of trisomy 21 at term (Cuckle *et al.*, 1987) to calculate the expected detection rate of affected pregnancies at a fixed false positive rate, in a population with the maternal age distribution of pregnancies in England and Wales (Office for National Statistics, 1997–1999).

RESULTS

hCG glycoform levels decreased progressively with gestational age. The data showed a best fit to a regression with the following form—Median MoM = 581 952 – 114 965 [Ln(gestational day)]. Median levels of GlyhCG fell by an average 1167 iu/L per day (2.1%) between the 98th and 130th day of gestation.

GlyhCG in both unaffected and Down syndrome pregnancies followed a Gaussian distribution after $\log_{10}$

transformation of MoM values, as determined by Kolmogorov and Anderson Darling tests with significance at the 0.01 level.

Previous studies have shown that AFP, Free β-hCG, and Total hCG follow a Gaussian distribution after $\log_{10}$ transformation in both unaffected and affected pregnancies (Spencer *et al.*, 1992).

The median MoM levels for the various markers in the study population are shown in Table 2 along with the significance of the difference between cases and controls (Mann–Whitney U-test). For glyhCG, the 10th to 90th centile of controls was 0.54–2.04, and for Down syndrome cases it was 1.00–4.44. The respective 5th to 95th centiles were 0.47–2.66 and 0.83–4.96. GlyhCG levels were significantly elevated in cases of Down syndrome with 15 of 50 (30%) cases above the 95th centile and 22 of 50 (44%) cases above the 90th centile (see Figure 1). In comparison, 36% of cases of Free β-hCG were above the 95th centile and 18% of AFP were below the 5th centile. Similarly, Total hCG was above the 95th centile in 30% of cases. The median MoMs obtained for the markers Free β-hCG, AFP and Total hCG were consistent with those obtained in larger series (Spencer *et al.*, 1992) and from a meta analysis of published series (Wald *et al.*, 1997).

Correlation of GlyhCG was investigated with other second trimester markers. GlyhCG was significantly correlated with Total hCG and Free β-hCG in both populations (r(down syndrome) = 0.6880 and r(controls) = 0.6079 for total hCG; r(down syndrome) = 0.6922 and r(controls) = 0.6070 for Free β-hCG). For AFP a significant correlation was not found in the Down syndrome population ($r = 0.1237$) or in the control population ($r = 0.0371$). The statistical distribution of GlyhCG in the control and Down syndrome population is shown in Table 3.

Table 2—Median marker MoMs in the study populations and the significance of the difference

Marker	Down syndrome	Unaffected	Probability
GlyhCG	1.81	1.00	<0.001
Free β-hCG	2.16	1.00	<0.001
ThCG	1.88	1.00	<0.001
AFP	0.75	1.00	<0.001

When detection rates and false positive rates for various marker combinations were modelled against the most recent age distribution of pregnancies in England and Wales using previously derived population parameters for Free β-hCG, Total hCG and AFP (Spencer *et al.*, 1992) and the parameters for GlyhCG from this study, the results at a 5% fixed false positive rate showed a 53.1% detection rate with GlyhCG and AFP, whilst that

Table 3—Statistical parameters for the GlyhCG population

	Down syndrome cases	Controls
Mean log10 GlyhCG MoM	0.2958	0.0155
SD log10 GlyhCG MoM	0.2469	0.2244
5th–95th centile MoM	0.83–4.96	0.47–2.60
10th–90th centile MoM	1.00–4.44	0.54–2.04
r v free β-hCG	0.6922	0.6070
r v total hCG	0.6880	0.6079
r v AFP	0.1237	0.0371

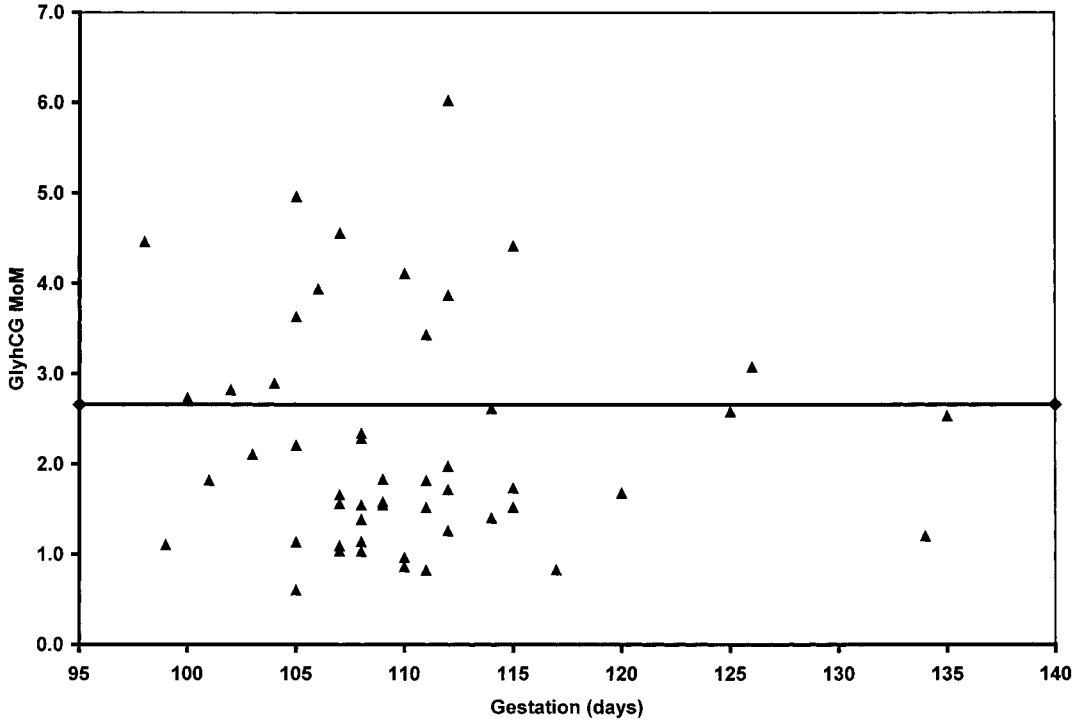

Figure 1—GlyhCG in 50 cases of Down syndrome in the second trimester. The solid line is the 95th centile in unaffected pregnancies

Table 4—Studies of hCG glycoforms in screening for Down syndrome

Study	hCG form	Gestation (weeks)	Cases/ controls	Median MoM	Cases > 95th Centile	Fluid	Assay
Cole *et al*., 1998	HhCG	11–21	10/142	5.7	90%	Urine	Immunoassay
Cole *et al*., 1999a	HhCG	11–22	23/1134	7.8	78%	Urine	Immunoassay
Shahabi *et al*., 1999	HhCG	15–22	10/66	3.9	60%	Serum	Immunoassay
Cuckle *et al*., 1999b	HhCG	10–19 >14	45/304	3.63 4.64	36%	Urine	Immunoassay
Cole *et al*., 1999b	HhCG	14–22	21/1059[a] 18/389 39/1448	8.44 9.94 9.50	81% 82% 80%	Urine	Immunoassay
Weinans *et al*., 2000	HhCG	10–12	8/55	3.6	63%	Urine	Immunoassay
Abushoufa *et al*., 2000	GlyhCG	16–18	39/105	2.2	74%	Serum	Lectin immunoassay
Spencer *et al*., 2002	GlyhCG	11–13	54/224	1.97	44%	Serum	Lectin immunoassay

[a] Repeat of samples from study by Cole *et al*., (1999a).

with total hCG and AFP was 59.4% and with free β-hCG and AFP was 66.4%.

DISCUSSION

hCG glycoform levels decrease across the second trimester of pregnancy (Abushoufa *et al*., 2000) as in the first trimester (Spencer *et al*., 2002). The observation in this present study is consistent with these previous observations.

Following the initial observation by Cole *et al*. (1997) of abnormal oligosaccharides on hCG from pregnancies affected by Down syndrome, there have been a handful of studies that have been investigating the potential use of altered carbohydrate forms of hCG as a screening tool. The studies that have measured the hyperglycosylated form of hCG, predominantly in urine in the second trimester are summarized in Table 4. The studies of Cole and colleagues consistently show high median MoM's with approaching 80% or more cases greater than the 95th centile. The study by Cuckle *et al*. (1999b) showed a much lower median MoM with only 38% above the 95th centile. When examined in the first trimester, Weinans *et al*. (2000) have shown a lower median MoM than in the second trimester and Cuckle *et al*. (1999b) in their study also showed lower MoM's in cases prior to 14 weeks. In serum, the one study thus far in the second trimester shows a median MoM of 3.9 (Shahabi *et al*., 1999), but with greater than 60% of cases above the 95th centile.

Abushoufa *et al*. (2000), using an alternative approach have examined altered carbohydrate forms of hCG by measuring hCG glycoforms. In the second trimester, they showed that in cases of Down syndrome the median MoM of 2.2 (Abushoufa *et al*., 2000) provided better clinical discrimination than total hCG. In the first trimester, serum GlyhCG levels are raised to similar levels with a median MoM of 1.97 (Spencer *et al*., 2002) but the clinical discrimination was not improved over that using free β-hCG, although glyhCG was a better marker than total hCG at this time.

In our present second trimester study, although the median MoM GlyhCG was elevated to similar levels that we had found in our previous study (Abushoufa *et al*., 2000), we could only find 30% cases of Down syndrome in which the GlyhCG was above the 95th centile. In our study, GlyhCG performed similarly to total hCG and was significantly poorer than Free β-hCG.

Our results suggest that GlyhCG does appear to have a different biological profile to total hCG or intact hCG in that it appears elevated across both trimesters in a similar way to free β-hCG. The data currently does not support the initial conclusion of (Abushoufa *et al*., 2000) of improved clinical discrimination measuring this altered hCG glycoform. Whether a more specific immunoassay could improve clinical discrimination needs further consideration. Certainly there is evidence that moving from a lectin affinity assay to a more specific monoclonal assay improves the clinical discrimination for hyperglycosylated hCG.

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† for hyperglycosylated hCG in the title read hCG glycoforms measured using a sialic acid binding lectin immunoassay.