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Urinary Screening Tests for Fetal Down Syndrome: II. Hyperglycosylated hCG

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Hyperglycosylated hCG is a form of hCG with more complex oligosaccharide side chains. A specific immunoassay was developed to measure hyperglycosylated hCG. Levels were measured in urine samples from 1157 women between 11 to 22 weeks of gestation, undergoing genetic analysis because of advanced maternal age. Values were normalized to urine creatinine concentration and plotted against gestational age, median values were determined and multiples of the control median (MoM) calculated. The median MoM and log standard deviation (log SD) of the 1134 control samples was 1.0 and 0.47, and of the 23 Down syndrome cases was 7.8 and 0.48, respectively. This indicated a 7.8-fold increase in hyperglycosylated hCG levels in Down syndrome cases.

In the accompanying article, a stability problem was found with β-core fragment measurements in frozen urine samples. In anticipation of similar problems, nine urine samples were tested for hyperglycosylated hCG fresh and after storage in the freezer. No clear difference was found in hyperglycosylated hCG values. In addition, no trend was found in hyperglycosylated hCG MoM values or in Down syndrome detection rates in urine samples stored for one, two or three years in the freezer.

Samples were split into five equal groups according to creatinine concentration. A trend was observed, hyperglycosylated hCG MoM values decreasing with advancing creatinine concentration (1.77, 1.08, 1.01, 0.73 and 0.60 at 0.25, 0.50, 0.79, 1.11 and 1.73 mg/ml, respectively). An error was noted. This was corrected with a regression equation. After correction, the median MoM and log SD of the control samples was 1.0 and 0.44, and of Down syndrome samples was 7.3 and 0.42, respectively. Correction of this error, while reducing the elevation of Down syndrome cases, tightened the spread of samples.

Samples were ranked and centiles determined. 18 of 23 Down syndrome cases (78 per cent) exceeded the 95th centile of the control population. ROC analysis indicated 79 per cent detection at 5 per cent false-positive rate. Urine samples were collected during two periods of gestation, an early period (11th to 14th completed week) and the period when chemical screening is normally performed (15th to 21st week). ROC analysis indicated 80 per cent and 78 per cent detection rates, respectively, at 5 per cent false-positive rate, in the two gestational periods. Hyperglycosylated hCG values were modelled with β-core fragment values, total oestriol values and maternal age. ROC analysis indicated 97 per cent detection rate at 5 per cent false-positive rate. This detection rate and this level of Down syndrome and control patient discrimination surpasses that of any other serum, urine or ultrasound screening protocol.

Hyperglycosylated hCG should be considered as a new screening test for aneuploid pregnancies, with the potential of detecting almost all cases of Down syndrome. Evaluation is needed by other centres in order to bring hyperglycosylated hCG into clinical practice. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS: Down syndrome; hyperglycosylated hCG; glycosylation; oligosaccharide; human chorionic gonadotrophin; hCG; screening; prenatal diagnosis

INTRODUCTION

In the 1980s three serum antigens were identified as markers of Down syndrome fetuses in the second trimester of pregnancy, hCG (Bogart *et al*., 1987), AFP (Merkatz *et al*., 1984) and unconjugated oestriol (Wald *et al*., 1988a). Tests for the three antigens were combined to optimize detection (Haddow *et al*., 1992; Wald *et al*., 1988b), and are now used widely for screening for Down syndrome. This triple test is far from perfect. Its use is limited to second trimester pregnancies (Haddow *et al*., 1992; Wald *et al*., 1998b, Canick, 1990). Together, the three tests only detect about 60 per cent of Down syndrome cases at 5 per cent false-positive rate (Haddow *et al*., 1992; Canick, 1990). This detection rate can be boosted by 10 per cent by adding inhibin A to the triple-test markers (quadruple test) (Wald *et al*., 1996). Costs have also become a limitation on these tests (Auxter, 1997; Zoler, 1997), with high licence fees levied directly on laboratories running hCG-based screening tests. A new generation of high performance tests is needed to replace hCG and the triple and quadruple test.

During the past 10 years, we purified hCG from urine from women with normal and Down syndrome pregnancies, and with gestational choriocarcinoma. Peptide structures were examined, and no differences were found in the N-terminal sequences or in the amino acid compositions of the various preparations

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(Elliott *et al.*, 1997; Cole *et al.*, 1997a). We examined the oligosaccharide side chains on the various hCG preparations. Six normal pregnancy hCG preparations had primarily mono- and biantennary N-linked oligosaccharides, and tri- and tetrasaccharide O-linked sugar units. In contrast, five choriocarcinoma hCG preparations had primarily larger triantennary N-linked oligosaccharides and hexasaccharide O-linked oligosaccharides (hyperglycosylated oligosaccharides) (Elliott *et al.*, 1997).

In 1997, lectin affinity chromatography was used to identify hyperglycosylated oligosaccharides on hCG β-subunit in pregnancy urine. Hyperglycosylated oligosaccharides were identified in 18 of 109 normal and 9 of 15 (60 per cent) Down syndrome pregnancy urine samples (Cole *et al.*, 1997a). We inferred that hyperglycosylated oligosaccharides might also be more abundant on hCG-related molecules in Down syndrome pregnancies.

We purified hCG from the urine of a patient with choriocarcinoma (hCG C5). This hCG was unique in having 100 per cent hexasaccharide (hyperglycosylated) O-linked oligosaccharides (Elliott *et al.*, 1997). In collaboration with Drs Birken, O'Connor and Canfield at Columbia University, monoclonal antibodies were made against hCG C5. A clone was found that bound hyperglycosylated hCG, and a specific assay was established to measure this unique antigen.

In 1997 a preliminary study was carried out with this new assay. Urine samples were tested from 142 unaffected and 10 Down syndrome cases from 11 to 24 weeks of gestation (Cole *et al.*, 1998). Multiples of the unaffected sample median (MoM) were determined and centiles calculated. 9 of 10 Down syndrome cases exceeded the 95th centile of unaffected cases. An application was indicated for hyperglycosylated hCG measurements in the detection of fetal Down syndrome. As described here, the assay was subsequently improved (new standard, longer incubation times and new substrate), and a more comprehensive study was carried out to fully assess the utility of urine hyperglycosylated hCG measurements in detecting Down syndrome pregnancies.

MATERIALS AND METHODS

Urine samples were accumulated over approximately a three-year period (May 1995–March 1998) from pregnant women between 11 and 22 weeks of gestation, who were presenting for amniocentesis or chorionic villus sampling at Yale University-affiliated hospitals (Yale–New Haven Hospital, Bridgeport Hospital and Norwalk Hospital). Oral consent was obtained using the protocol approved by the institutional review boards. Samples were stored in a -20°C freezer until tested. Sample and biographical data collection is described in detail in the preceding article (Cole *et al.*, 1999). The study was limited to singleton pregnancies with normal or Down syndrome karyotypes, and to women undergoing genetic analysis for advanced maternal age reasons only. A total of 1157 urine samples were collected, 1134 from unaffected and 23 from Down syndrome cases.

Between March and May 1998 the urine samples were thawed and tested in sequential batches for hyperglycosylated hCG. The test is a two-step immunoassay involving a four-hour incubation with capture antibody and a two-hour incubation with a tracer antibody, using our previously described microtitre plate methods (Cole *et al.*, 1993). The capture antibody used was antibody B152 (developed jointly with Columbia University), directed against a unique carbohydrate determinant on choriocarcinoma hCG β-subunit. The tracer antibody used was peroxidase-labelled monoclonal anti-hCGβ (batch 4001, Genzyme, San Carlos, CA, U.S.A). Pure hyperglycosylated hCG (choriocarcinoma hCG batch C5) (Elliott *et al.*, 1997) was calibrated by amino acid analysis and used to standardize this assay. Plates included a high and a low quality control. The mean value of the high quality control (middle of standard curve) was 21 ng/ml, with 1.8 ng/ml standard deviation and 8.9 per cent coefficient of variation or inter-assay variation. The mean value for the low quality control (close to the limit of detection) was 5.6 ng/ml, with 0.48 ng/ml standard deviation and 8.5 per cent coefficient of variation or inter-assay variation.

Intact hCG (normal plus hyperglycosylated hCG) levels were determined by a similar immunoassay, using parallel methods. This assay used monoclonal antibody 2119 (anti-hCGα) as capture antibody, and the same peroxidase-labelled monoclonal anti-hCGβ (batch 4001, Genzyme) as tracer antibody. hCG batch CR127 (gift from National Hormone and Pituitary Program, NIH, Bethesda, MD, U.S.A.) was calibrated by amino acid analysis and used to standardize this assay. As described in the accompanying article (Cole *et al.*, 1999), creatinine, β-core fragment and total oestriol levels were also determined on these same samples.

Results were analysed using standard methods (Royston and Thompson, 1992; Palomaki *et al.*, 1995). Gestational age was determined by ultrasound. MoM values were determined. The gestational age-specific MoM values fit log-Gaussian distributions, between the 5th and 95th centiles, for both unaffected pregnancy and Down syndrome data. To assess screening performance, medians and log standard deviation (log SD, estimated by the 10th–90th centile difference of the log MoM values, divided by 2.56) were determined for both Down syndrome and unaffected pregnancies. The detection rates were determined from the proportion of Down syndrome pregnancies exceeding a specific centile of the control population. ROC (receiver operating characteristics) curves were used to compare sensitivity and false-positive rates, and to determine the ability of the test to discriminate between affected and unaffected pregnancies. Univariate and multivariate Gaussian models were used to predict detection rates for hyperglycosylated hCG and combinations of this and other markers considering the general age distribution of the population of the United States (Palomaki *et al.*, 1993).

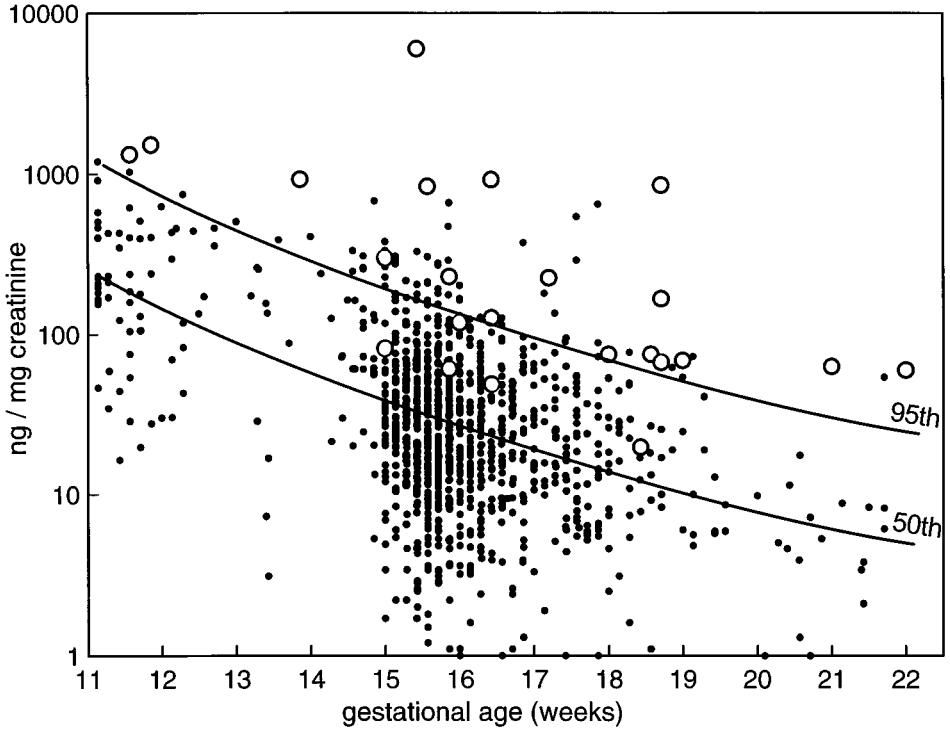

Fig. 1—Hyperglycosylated hCG in urine samples from 23 Down syndrome pregnancies (○) and 1134 unaffected pregnancies (●). Hyperglycosylated hCG levels (ng/ml) were normalized to spot urine creatinine level (mg/ml). The lines are the 50th centile (1.0 MoM, described by the regression equation give in the Results section) and 95th centile (5.3 MoM, determined from the rank of MoM values) of the unaffected pregnancies

Models were used to calculate risk and applied to the observed distribution of values.

RESULTS

Hyperglycosylated hCG levels were determined in urine samples from 1134 unaffected and 23 Down syndrome pregnancies from 11 to 22 weeks of gestation. Values were normalized to spot urine creatinine concentration (ng/ml÷mg/ml). Results were plotted against gestational age (Fig. 1). The weekly medians of control samples fit an exponential regression line, $y=2.94\times10^{8}\times(ga^{-5.80})$, where y is the predicted median value corresponding to a specific gestational age (ga). MoM values were determined and medians and log SDs were calculated. The median MoM of the control samples was 1.0 and the log SD was 0.47 (Table 1). The median MoM of the Down syndrome samples was 7.8, and the log SD was 0.48. Control samples were ranked. The 95th centile corresponded to 5.3 MoM. 18 of 23 (78 per cent) Down syndrome samples exceeded the 95th centile of control samples.

We investigated the relationship between hyperglycosylated hCG and normal hCG immunoreactivity. Intact hCG levels were measured in the urine samples (normal hCG+hyperglycosylated hCG) and the normal (non-hyperglycosylated) hCG content was calculated (intact hCG minus hyperglycosylated hCG). Values were adjusted to spot urine creatinine concentration. Intact hCG and normal hCG values were plotted against gestational age, regression lines were fitted to weekly median values and MoM statistics determined (Table 1). In unaffected pregnancies, the median hyperglycosylated hCG level was 3.8 per cent of the intact hCG level. In Down syndrome cases the median hyperglycosylated hCG level was 14 per cent of the intact hCG level (Table 1). Normal, non-hyperglycosylated hCG was assumed to account for the balance of intact hCG immunoreactivity (96 per cent and 86 per cent, respectively). Measuring intact hCG, the median MoM was 1.0 in controls (log SD=0.32) and 2.5 in Down syndrome cases (log SD=0.40). Measuring normal hCG, similar results were recorded, the median MoM was 1.0 in controls (log SD=0.34) and 2.1 in Down syndrome cases (log SD=0.42). No significant difference was found between normal hCG and intact hCG Down syndrome screening data in the log MoM values (t-test, $p>0.2$), in the distribution of log MoM values (f-test, $p=0.98$), and in the values for ROC area under the curve values (detection rate versus false-positive rate, t-test, $p>0.2$). A significant difference was found, however, between normal hCG and hyperglycosylated hCG values in the log MoM values (t-test, $p=0.00002$), distribution of the log MoM values (f-test, $p=0.00001$) and in the values for ROC area under the curve values (detection rate versus false-positive rate, t-test, $p=0.0055$). It is inferred that hyperglycosylated hCG is an independent variable to normal hCG for Down syndrome screening. It is also inferred that the use of hCG as a marker

Table 1—Occurrence, regression equation and distribution parameters for urine intact hCG immunoreactivity (normal and hyperglycosylated hCG), normal hGC (all except hyperglycosylated component) and hyperglycosylated hCG

	Control samples $n=1134$ (552—46,XX; 582—46,XY)				Down syndrome samples $n=23$ (9—47,XX; 14—47,XY)		
Test	Portion[a]	Regression line	Median MoM	Log SD[b]	Portion[a]	Median MoM	Log SD[b]
Intact HCG	100%	$y=14\,387\times(0.825^{ga})$	1.0	0.32	100%	2.5[c]	0.40
Normal hCG	96.2%	$y=9125\times(0.846^{ga})$	1.0	0.34	86%	2.1[c,d]	0.42
Hyperglycosylated hCG	3.8%	$y=2.94\times10^{8}\times(ga^{-5.80})$	1.0	0.47	14%	7.8[d]	0.48
Hyperglycosylated hCG[e]			1.0	0.44		7.3	0.42

[a]Normal hCG (hCG − hyperglycosylated hCG, ng/ml) and hyperglycosylated hCG (ng/ml) as portions (median %) of measurable hCG immunoreactivity (ng/ml).
[b]Log SD, calculated from the difference amid the log MoMs of the 10th and 90th centiles.
[c]No significant difference was noted between normal hCG and hCG immunoreactivity values, firstly in the log MoM values (*t*-test, $p>0.2$), secondly in the distribution of log MoM values (*f*-test, $p=0.98$) and finally in the values for ROC area under the curve (detection rate versus false-positive rate, *t*-test, $p>0.2$).
[d]A significant difference was noted between normal hCG and hyperglycosylated hCG values, firstly in the log MoM values (*t*-test, $p=0.00002$), secondly in the distribution of the log MoM values (*f*-test, $p=0.00001$) and finally in the values for FOC area under the curve (detection rate versus false-positive rate, *t*-test, $p=0.0055$).
[e]Hyperglycosylated hCG with MoM values corrected for creatinine error, using the error curve shown in Fig. 2.

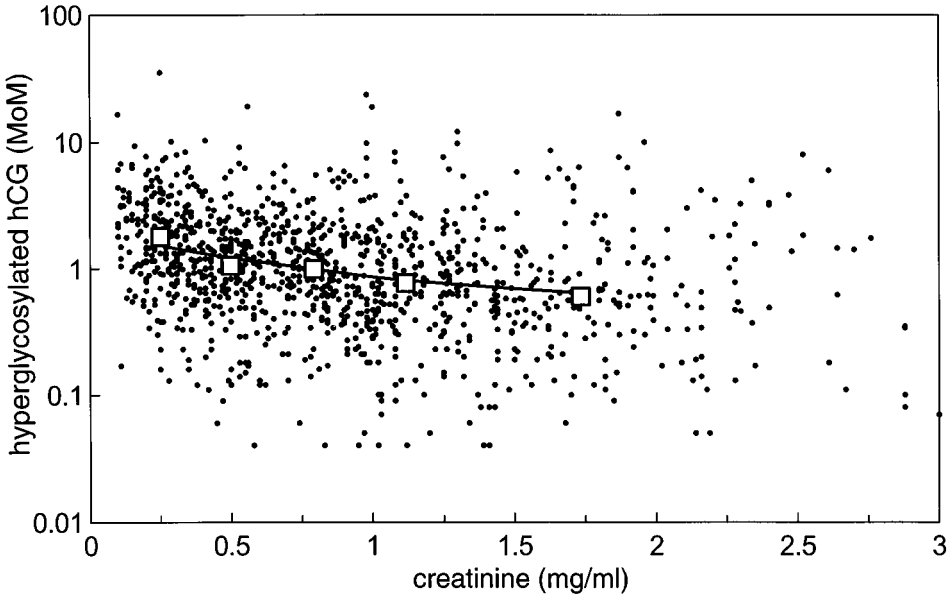

Fig. 2—Effect of creatinine concentration on hyperglycosylated hCG MoM statistics. Hyperglycosylated hCG MoM values for 1134 unaffected pregnancies (●) were plotted against creatinine concentration. Median MoM values were determined for five equal sized divisions of the creatinine concentration values (□). A complex exponential regression curve fit these median values, median=0.54+(1.61 × (0.18^{cr}), where 'cr' is the creatinine concentration

for Down syndrome cases is not dependent on the presence of hyperglycosylated hCG.

Hyperglycosylated hCG levels were normalized to spot creatinine levels. To determine whether creatinine concentration correctly normalized hyperglycosylated hCG levels, hyperglycosylated hCG MoM values were plotted against creatinine concentration (Fig. 2). Samples were divided into five equal sized groups according to creatinine concentration. The median MoM values in these groups were 1.77, 1.08, 1.01, 0.73 and 0.60 at creatinine concentration of 0.25, 0.50, 0.79, 1.11 and 1.73 mg/ml, respectively. Creatinine normalization exaggerated MoM values in samples with lower creatinine concentration, and underestimated values in those with higher creatinine concentration. A complex logarithmic regression curve, median=0.54+(1.61 × (0.18^{cr})), fit the creatinine concentration (cr) and median MoM values ($r^2=0.95$). We used the regression equation to correct our data (MoM values were divided by the calculated median value). After correction, the median MoM values in the five creatinine groups were 1.10, 0.86, 1.01, 0.98 and 0.91, respectively. The correction tightened the spread of the unaffected and Down syndrome case MoM values.

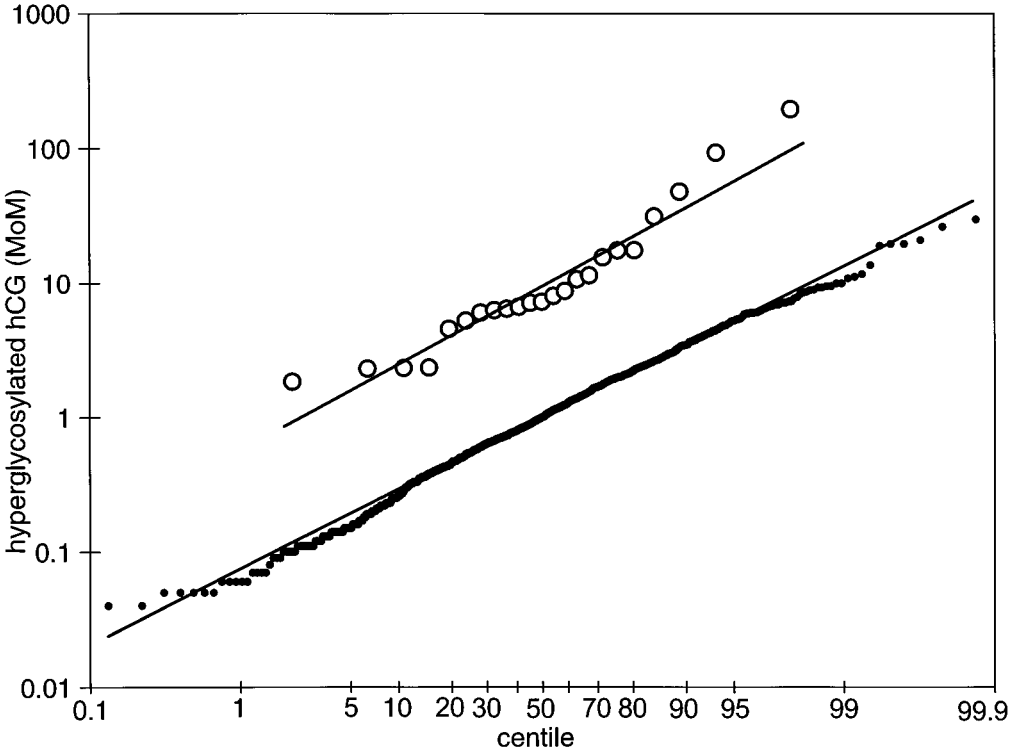

Fig. 3—Probability plot of the MoM values for hyperglycosylated hCG from 23 Down syndrome pregnancies (○) and 1134 unaffected pregnancies (●). The creatinine error was corrected using the error curve shown in Fig. 2. The two solid lines are defined by log-Gaussian distributions

After correction, the median MoM of the control samples was 1.0 and the log SD was 0.44 (Table 1). The median MoM of the Down syndrome samples was 7.3 and the log SD was 0.42. A probability plot showed that the corrected control and Down syndrome MoM values satisfactorily fit a log Gaussian distribution (Fig. 3).

Correction of the creatinine error had no obvious effect on the discrimination of control (mean creatinine concentration 0.92 mg/ml) and Down syndrome (mean creatinine concentration 0.91 mg/ml) samples. After correction, 18 of 23 Down syndrome cases (78 per cent) still exceeded the 95th centile (5.2 MoM) of the control population. Detection rates and false-positive rates were assessed by ROC analysis. 79 per cent detection was indicated at 5 per cent false-positive rate, 62 per cent at 3 per cent and 40 per cent at 1 per cent false-positive rate (Fig. 4). The area under the ROC curve was 0.95. Urine samples were collected during two gestational periods, an early period (11th to 14th completed week, 156 controls and 5 affected cases) and the regular period when biochemical screening is normally performed (15th to 21st week, 978 controls and 18 affected cases). ROC analysis indicated similar detection rates, 80 per cent and 78 per cent, respectively, at 5 per cent false-positive rate, during the early and regular gestational periods. The area under the ROC curve was 0.95 in both gestational intervals.

Gaussian models were prepared using the corrected hyperglycosylated hCG screening statistics, and those described for β-core fragment and total oestriol in the preceding article (Cole *et al.*, 1999). ROC analysis (detection rate versus false-positive rate) was used to analyse the modelled data (Table 2). Modelling of hyperglycosylated hCG with β-core fragment and maternal age distribution led to 92 per cent detection of Down syndrome at 5 per cent false-positive rate (53 per cent detection at 1 per cent false-positive rate). The area under the ROC curve for this combination of tests was 0.97. Modelling of hyperglycosylated hCG with β-core fragment, total oestriol and maternal age resulted in a 97 per cent detection at 5 per cent false-positive rate (67 per cent detection at 1 per cent false-positive rate). The area under the ROC curve for this triple urine test was 0.99.

Urine samples were accrued over a 955-day period, stored frozen, then thawed and tested at the end of this term. We examined the effect of storage in the freezer on hyperglycosylated hCG MoM values (Fig. 5). The unaffected pregnancies were divided into three equal sized groups (1–325, 326–583 and 584–955 days from introduction of urine collection for Down syndrome screening). The median MoM values for the three groups were 0.91, 0.85 and 1.2, respectively. Six of eight Down syndrome cases collected in the first period, six of six in the second period and six of nine in the third period exceeded the 95th centile of control samples or 5.2 MoM. No trend was observed between the period of collection (storage time) and MoM values or detection rates.

We started testing for hyperglycosylated hCG in the final week of the collection period (March 1998). We had the opportunity to test the final seven samples (controls) fresh, less than one week old, before

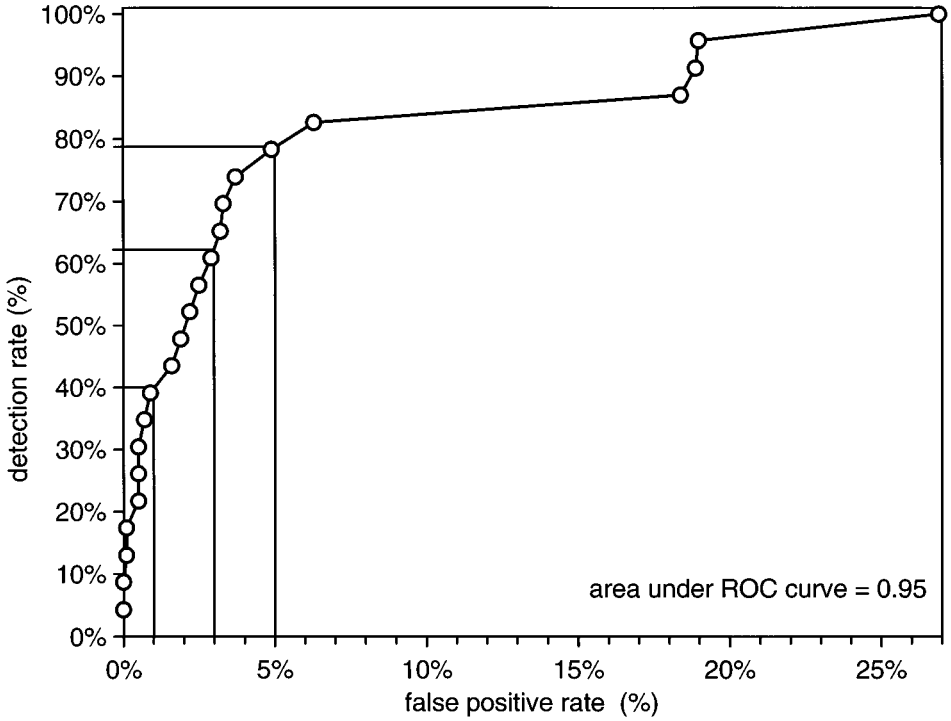

Fig. 4—ROC analysis of hyperglycosylated hCG Down syndrome screening statistics. Creatinine error was corrected using the error curve shown in Fig. 2. The false-positive rate was calculated from the ranked MoM values of unaffected pregnancies for detection rates of 0/23 (0 per cent detection) to 23/23 (100 per cent detection) Down syndrome cases (○). Detection rate was plotted against false-positive rate and area under the ROC cure was calculated

freezing. We tested the same samples again in August 1998 after $4\frac{1}{2}$ months in the −20°C freezer. The original values before freezing were 27, 28, 28, 38, 43, 45 and 82 ng/ml. The repeat values after storage in freezer were 30, 20, 18, 33, 62, 55 and 68 ng/ml, respectively. While no change was apparent in the overall values (change in values when reassayed, 98 ± 8.3 per cent, mean ± standard error), higher than normal variation was found in these measurements $4\frac{1}{2}$ months apart (mean variation 20 per cent).

DISCUSSION

Distribution and properties of hyperglycosylated hCG

Hyperglycosylated hCG accounts for 3.8 per cent of intact hCG molecules in the urine of normal pregnant women between 11 and 22 weeks of gestation. While normal hCG levels are 2.1-fold increased, hyperglycosylated hCG levels are 7.8-fold elevated in pregnancies with a Down syndrome fetus. Hyperglycosylated hCG is an independent screening marker from normal hCG, not making a significant contribution to the elevation of normal hCG in Down syndrome cases, or to that which forms the basis of the current triple and quadruple screening tests.

The proportion of hyperglycosylated hCG molecules produced by the placenta changes throughout gestation. O'Connor *et al.* (1998), using a B152-based hyperglycosylated hCG assay, found that hyperglycosylated molecules account for the large majority of hCG molecules produced in the beginning of pregnancy (third to fourth week after last menstrual period). The study described here, also using a B152-based assay, found a median of 12 per cent hyperglycosylated molecules at the end of the first trimester (11th–14th completed week) and 3.5 per cent hyperglycosylated molecules in the second trimester of pregnancy (15th–22nd completed week). In a further study using a B152-based assay, a median of 1.7 per cent hyperglycosylated hCG molecules was detected in the third trimester (27th–41st completed week) of pregnancy (Kingston, 1998). The antibody used in all of these studies, B152, was raised against 100 per cent hyperglycosylated hCG from a patient with choriocarcinoma. Such an hCG molecule would be produced by trophoblastic cancer or poorly differentiated trophoblast cells (Ashitaja *et al.*, 1980). The pattern of hyperglycosylated hCG production observed during pregnancy is consistent with production by early or poorly differentiated trophoblast cells. While normal hCG is produced by syncytiotrophoblast cells (Ashitaja *et al.*, 1980), hyperglycosylated hCG may be produced by less well-differentiated trophoblast cells, such as intermediate or cytotrophoblast cells.

Hyperglycosylated hCG has more complex oligosaccharide side chains than normal hCG, with triantennary N-linked oligosaccharides and hexasaccharide O-linked sugar structures (Elliott *et al.*, 1997). Hyperglycosylated hCG (molecular weight ≃ 41 000

Table 2—ROC analysis of monovariate and multivariate Down syndrome screening data

Data	ROC area under curve		Down syndrome detection rate		
	Area	Standard error	5% fpr[a]	3% fpr[a]	1% fpr[a]
Hyperglycosylated hCG (creatinine corrected)	0.95	0.017	79%	62%	40%
+Age	0.95	0.017	70%	58%	44%
+Oestriol+age	0.95	0.018	80%	73%	49%
+β-core fragment+age	0.97	0.014	92%	79%	53%
+Oestriol+β-core fragment+age	0.99	0.0052	97%	80%	67%

[a]Detection rate and false-positive rate (fpr) from ROC curve.

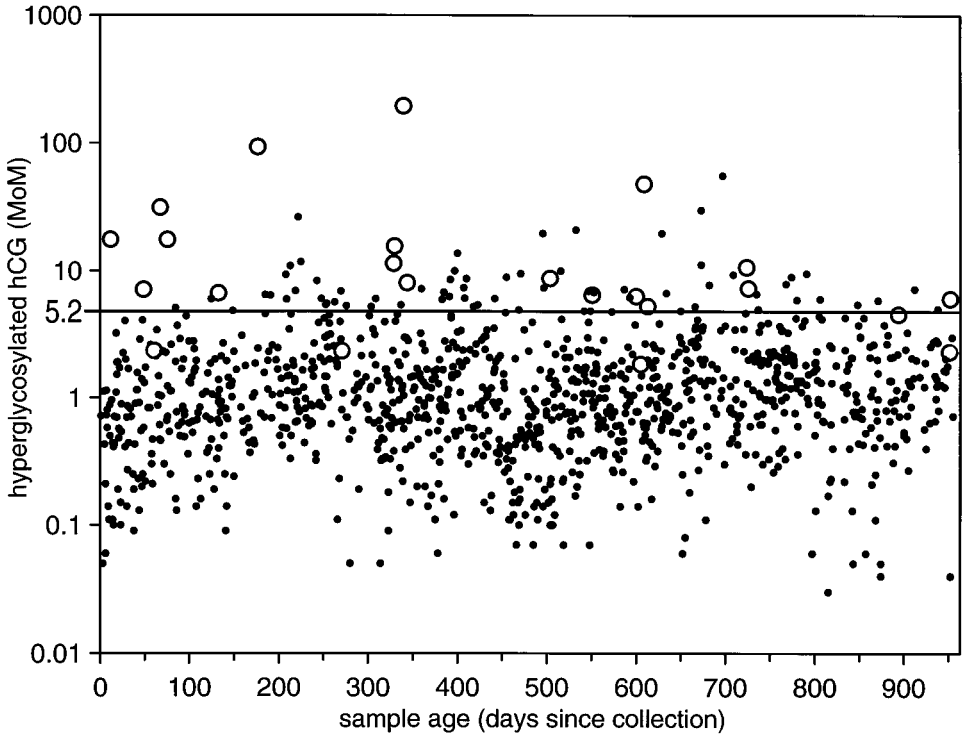

Fig. 5—Effect of storage in a −20°C freezer on hyperglycosylated hCG MoM statistics. Hyperglycosylated hCG MoM values for 23 Down syndrome pregnancies (○) and 1134 unaffected pregnancies (●) were plotted against the time of collection during the 955 day amassment period (values corrected for creatinine error using the curve shown in Fig. 2). Line indicates 95th centile (5.2 MoM)

daltons) migrates slower than normal hCG (molecular weight ≃ 36 700 daltons) on sodium dodecyl sulphate polyacrylamide electrophoresis gels (Elliott *et al.*, 1997). Hyperglycosylated hCG is also five-times less active in promoting progesterone production at the hCG/hLH receptor than normal hCG (Cole *et al.*, 1991). All told, while hyperglycosylated hCG may have a similar name to normal hCG, it is a very different molecule. The difference between hyperglycosylated hCG and hCG is analogous to that between hCG and hLH. They are about 20 per cent different in molecular structure and molecular weight, different in biological activity at the hCG/hLH receptor and different in cellular origin. Recently, high licence fees have been levied directly on laboratories in the United States running normal hCG-based Down syndrome screening tests (Auxter, 1997; Zoler, 1997). This has limited the profitability of these tests. Replacing normal hCG-based measurements with hyperglycosylated hCG determinations could resolve this problem.

Problems with urine hyperglycosylated hCG measurements

There were two potential problems with hyperglycosylated hCG measurements. The first was the stability of this molecule when stored in a freezer. In the preceding article (Cole *et al.*, 1999), a stability problem was found with urine β-core fragment measurements. Deleterious changes in immunoreactivity were detected after storage of samples in a freezer. In the current study, samples were stored for up to three years in a freezer. We considered the possibility that freezing may

affect hyperglycosylated hCG immunoreactivity. Seven control samples were tested fresh, and then again after $4\frac{1}{2}$ months in the freezer. While no clear difference was found in the average results of the fresh and frozen samples, a higher than normal variation was noted in these measurements. Storage in the freezer may cause small variations in hyperglycosylated hCG levels. Screening performance was compared in samples stored approximately 0–1, 1–2 and 2–3 years in the freezer. No promotive or deleterious trends were found in control MoM values or in detection rates within the three storage periods.

The second potential problem was the inability of spot creatinine measurements to correctly normalize hyperglycosylated hCG levels. At first, we adjusted hyperglycosylated hCG levels for urine density by dividing values by spot creatinine concentration. While this correctly normalized samples in the mid-range of creatinine concentration, it exaggerated hyperglycosylated hCG MoM values in samples with lower creatinine levels and underestimated MoM values in those with higher creatinine levels. A regression equation was established to correct this error, and used successfully to amend the MoM values.

With correction of the creatinine error, the median control MoM value was 1.0, and the median Down syndrome MoM value was 7.3. This indicated a 7.3-fold elevation of hyperglycosylated hCG levels in Down syndrome cases. 18 of 23 (78 per cent) Down syndrome cases exceeded the 95th centile of control values. Using ROC analysis 79 per cent detection was indicated at 5 per cent false-positive rate. In a pilot study with hyperglycosylated hCG (Cole *et al.*, 1998), we tested 142 unaffected and 10 Down syndrome cases. 90 per cent of the Down syndrome cases had MoM values exceeding the 95th centile of unaffected pregnancies. This study did not include correction of the creatinine error. All but two of this group of samples were retested with no significant change in values and incorporated into the current study (two Down syndrome samples were not retested because of insufficient urine). When retested and corrected for creatinine error, only six of eight (75 per cent) exceeded the 95th centile. The unduly high sensitivity found in the pilot study was likely due to lack of correction of the creatinine error.

Screening performance of hyperglycosylated hCG

Urine samples were tested over a wide range of gestational ages, 11–22 weeks. This comprised an early period and a regular period when chemical screening is normally performed. ROC analysis indicated similar results for both gestational periods, 80 per cent detection in the early period and 78 per cent detection in the regular testing period, at 5 per cent false–positive rate. The area under the ROC curve indicated 95 per cent discrimination of normal and Down syndrome samples in both periods. 79 per cent detection of Down syndrome by a single screening test is exceptional. The screening performance of this single urine test surpasses the performance of serum hCG, the serum triple screen test (60 per cent detection at 5 per cent false-positive rate) (Haddow *et al.*, 1992; Wald *et al.*, 1998b; Canick, 1990), and the recently introduced quadruple serum screen test (70–76 per cent detection at 5 per cent false-positive rate) (Wald *et al.*, 1996). Furthermore, this single test may be useful from 11 to 22 weeks of gestation, or over a wider gestational age range than hCG or any serum marker.

We considered the possibility of combining this new highly sensitive marker with other urine tests to improve the screening performance. As described above, hyperglycosylated hCG levels are independent of normal hCG levels. We thought about combining hyperglycosylated hCG with the best normal hCG-derived marker. In our experience, normal hCG levels are increased 2.1-fold in Down syndrome urine samples (Table 1), free β-subunit levels are elevated 3.9-fold in Down syndrome urine samples (Cole *et al.*, 1997b), and β-core fragment levels are enhanced 5.4-fold in these samples (Cole *et al.*, 1999). β-core fragment was the most promising normal hCG-derived marker in urine samples. We combined hyperglycosylated hCG with β-core fragment values. Modelling these two markers together with maternal age achieved a 92 per cent detection of Down syndrome cases at 5 per cent false-positive rate. The area under the ROC curve indicated 97 per cent discrimination of control and Down syndrome samples. We added a third independent marker, total urine oestriol. This achieved 97 per cent detection of Down syndrome cases at 5 per cent false-positive rate. The area under the ROC curve indicated 99 per cent discrimination of normal and Down syndrome samples. While the serum triple test detected approximately 60 per cent of cases at 5 per cent false-positive rate, the urine triple marker combination detected 67 per cent of Down syndrome cases at 1 per cent false-positive rate. As such, the urine combination was approximately five-times more efficient than the triple test.

Urine versus serum samples

In this study hyperglycosylated hCG was measured in urine samples. Recently, we measured hyperglycosylated hCG in 66 control and 10 Down syndrome serum samples from the second trimester of pregnancy (Shahabi *et al.*, 1999). Multiples of the median were calculated and centiles determined. 60 per cent of the Down syndrome cases exceeded the 95th centile of unaffected cases. This pilot study indicated that serum hyperglycosylated hCG may also be useful in Down syndrome screening. Much larger studies are needed to establish the full utility of serum hyperglycosylated hCG measurements.

Testing urine may have several advantages over blood samples. Obtaining urine is less invasive, does not require a phlebotomy or use of hypodermic needles, and is more acceptable to patients. Urine does also not require centrifugation and separation of fluids.

Risk of HIV, hepatitis and other serum-borne infectious agents are minimized in urine testing. Currently, however, urine is not suitable for α-fetoprotein measurements. Blood collection may still be needed for α-fetoprotein measurements if they are desired.

CONCLUSIONS

We conclude that hyperglycosylated hCG is a highly sensitive marker for detecting fetal Down syndrome. The performance of this single urine test may be vastly superior to all currently used serum screening tests. Hyperglycosylated hCG may also be applicable to wider range of gestational ages than other screening methodologies. Evaluation is needed by other centres in order to bring hyperglycosylated hCG into clinical practice.

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