Hyperglycosylated Human Chorionic Gonadotropin (Invasive Trophoblast Antigen) Immunoassay: A New Basis for Gestational Down Syndrome Screening

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Background: Serum human chorionic gonadotropin (hCG) and hCG free β-subunit tests are used in combination with unconjugated estriol and α-fetoprotein in the triple screen test, and with the addition of inhibin-A in the quadruple marker test for detecting Down syndrome in the second trimester of pregnancy. These tests have a limited detection rate for Down syndrome: ~40% for hCG or free β-subunit alone, ~60% for the triple screen test, and ~70% for the quadruple marker test, all at 5%, or a relatively high, false-positive rate. New tests are needed with higher detection and lower false rates. Hyperglycosylated hCG (also known as invasive trophoblast antigen or ITA) is a new test. It specifically detects a unique oligosaccharide variant of hCG associated with Down syndrome pregnancies. We evaluated this new Down syndrome-directed test in prenatal diagnosis.

Methods: Hyperglycosylated hCG was measured in urine samples from women undergoing amniocentesis for advanced maternal age concerns at 14–22 weeks of gestation, 1448 with normal karyotype and 39 with Down syndrome fetuses.

Results: The median hyperglycosylated hCG value was 9.5-fold higher in Down syndrome cases (9.5 multiples of the normal karyotype median). The single test detected 80% of Down syndrome cases at a 5% false-positive rate. Urine hyperglycosylated hCG was combined with urine β-core fragment (urine breakdown product of serum hCG free β-subunit), serum α-fetoprotein, and maternal age-related risk. This urine-serum combination detected 96% of Down syndrome cases at a 5% false-positive rate, 94% of cases at a 3% false-positive rate, and 71% of cases at a 1% false-positive rate. These detection rates exceed those of any previously reported combination of biochemical markers.

Conclusions: Hyperglycosylated hCG is a new base marker for Down syndrome screening in the second trimester of pregnancy. The measurement of hyperglycosylated hCG can fundamentally improve the performance of Down syndrome screening protocols.
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Human chorionic gonadotropin (hCG)[3] is a glycoprotein hormone composed of two subunits, α and β, joined noncovalently. The α-subunit is composed of 92 amino acids with two N-linked oligosaccharide side chains. The β-subunit comprises 145 amino acids with two N-linked oligosaccharides in the core of the subunit and four O-linked sugar structures on the C-terminal extension. hCG usually is produced by villous syncytiotrophoblast cells during pregnancy. It is also produced by villous syncytiotrophoblast cells in hydatidiform mole or molar pregnancy, and by non-villous intermediate cytotrophoblast and intermediate syncytiotrophoblast cells in choriocarcinoma, cancer of trophoblast cells. Recently, we examined the peptide and N-linked and O-linked sugar structures of the separated α- and β-subunits of purified hCG from normal pregnancies, molar pregnancies, and choriocarcinoma *(1)*. Although no significant difference was observed in the peptide structure of the α-subunit of hCG, variable nicking or peptide bond cleavage at β43–

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[3] Nonstandard abbreviations: hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; and MoM, multiple(s) of the median.

44, β44–45, and β47–48 was demonstrated on the β-subunit of hCG. Significantly greater nicking was observed in choriocarcinoma, compared with normal pregnancy and molar pregnancy hCG preparations (1). Primarily mono- and biantennary N-linked oligosaccharides, and tri- and tetrasaccharide-type O-linked sugar units were found in the β-subunit of hCG in normal and molar pregnancy samples. A small proportion of more complex triantennary N-linked oligosaccharides (0–30%) and larger hexasaccharide-type O-linked sugar units (0–20%) were also found in normal and molar pregnancy preparations. In contrast, primarily triantennary N-linked oligosaccharides (up to 100%) and hexasaccharide-type O-linked oligosaccharides (up to 100%) were found in choriocarcinoma hCG (1). hCG with these larger N- and O-linked oligosaccharides is called hyperglycosylated hCG (also known as invasive trophoblast antigen, or ITA).

One choriocarcinoma hCG preparation, hCG batch C5, had 100% hexasaccharide-type O-linked sugar units. In collaboration with Drs. S. Birken, A. Krichevsky, J. O'Connor, and R. Canfield of Columbia University, New York, NY, a monoclonal antibody was generated against C5 hCG (2). Using this antibody (monoclonal B152) an assay for hyperglycosylated hCG was developed (3). With this assay, O'Connor et al. (3) were able to show that primarily hyperglycosylated hCG was produced by trophoblast cells in the week after implantation in normal pregnancies. Using a similar assay, we showed that the proportion of hyperglycosylated hCG molecules declines as pregnancy advances. Using this assay, we found that the proportion of hyperglycosylated hCG detected was 26% of hCG molecules at 4–6 weeks, 11% at 6–8 weeks, and then 2.9% (third month) to 2.0% (third trimester) through the rest of pregnancy (Shahabi and Cole, unpublished data). Hyperglycosylated hCG accounted for virtually all hCG produced in five individuals with choriocarcinoma. The finding that hyperglycosylated hCG was produced by invasive or cancerous trophoblast cells in choriocarcinoma and by invasive normal pregnancy cells during aggressive implantation in the first week of gestation suggested that hyperglycosylated hCG is a product of separate trophoblast cells to normal hCG, and that it may be synthesized by invasive trophoblast cells (intermediate cytotrophoblast or fused cytotrophoblast cells). Furthermore, it was postulated that such cells are responsible for the small proportion of hyperglycosylated hCG molecules that are hyperglycosylated during most of the course of gestation.

In 1997, Cole et al. (4) used lectin affinity chromatography to demonstrate increased proportions of hyperglycosylated hCG molecules in Down syndrome (trisomy 21) pregnancies. A large prospective study was carried out in which urine samples from women undergoing amniocentesis or chorionic villous sampling for maternal age concerns were collected. Over a 31-month period, 1157 normal karyotype and 23 Down syndrome urine samples were collected and tested in the hyperglycosylated hCG assay (5). Cases were from 11 to 22 weeks of gestation. The test detected 79% of Down syndrome cases, with a 5% false-positive rate (5). This is an extremely high detection rate for a single analyte test for Down syndrome pregnancies. The reported detection rate is close to double that of other individual analytes such as normal hCG, hCG free β-subunit (free β-subunit), or inhibin-A (6–8).

The prospective urine study included only 2 Down syndrome and 98 normal karyotype cases from the first trimester of pregnancy (11–14 weeks). There thus are too few first-trimester samples to validate the performance of this test during early pregnancy. Here, we briefly reexamine the second-trimester cases used in this study (14–22 weeks of gestation) and show that the regression curve was adversely skewed by the first-trimester cases. A completely new study is presented with 389 normal karyotype and 18 Down syndrome cases to validate the prospective study. The combined set of second-trimester cases is examined (1448 normal and 39 Down syndrome cases). For the first time, trisomy 18 pregnancies are also investigated, as are combinations of urine hyperglycosylated hCG and serum markers.

Commercial kits are now being produced for detecting hyperglycosylated hCG, to replace or compliment the current triple and quadruple tests used for Down syndrome screening. We examine the needed conditions for shipping urine samples to laboratories for hyperglycosylated hCG measurements and for storage of urine samples in freezers.

Materials and Methods

Urine samples were collected from pregnant women between 14 and 22 weeks of gestation, who were presenting for amniocentesis at Yale University (Yale-New Haven Hospital). In addition, samples were also collected through the Prenatal Diagnosis Service, Department of Genetics, at 14–22 weeks of gestation, from individuals with confirmed Down syndrome pregnancies who failed to provide urine samples at the time of amniocentesis. Oral consent was obtained using a protocol approved by the institutional review board. All samples were refrigerated immediately after collection. Samples were coded, and hCG β-core fragment and creatinine concentrations were determined. Within 1 week, samples were transferred to 15-mL tubes (~12-mL urine volume) and stored in a $-20\,°C$ freezer until tested for hyperglycosylated hCG. Personal data were collected from patients at the time of urine collection. Gestational age was recorded as determined by ultrasound at the time of amniocentesis. Karyotype was later documented from the records of the Prenatal Diagnosis Service. Coded personal information (dates, age, and date of last menstrual period), clinical data, karyotype, and immunoassay results were all recorded in a Microsoft Excel 98 spreadsheet. All studies were limited to singleton pregnancies with normal, Down syndrome, or trisomy 18 karyotypes and to women undergoing genetic analysis for advanced maternal age

reasons only. Cases selected for amniocentesis by the triple screen or other chemical methods were excluded.

Three groups of normal and Down syndrome cases are described here. All were tested in the hyperglycosylated hCG assay. The first group consisted of the second-trimester cases (1059 normal and 21 Down syndrome cases) from our original (1134 normal and 23 Down syndrome cases) Down syndrome study population. These samples were collected prospectively over a 31-month period between 1996 and 1998 *(5)* and included six Down cases collected by the Prenatal Diagnosis Service postamniocentesis. The screening performance for this group of samples was corrected, reanalyzed, and accumulated here. The second group consisted of new second-trimester cases collected between July 1998 and April 1999. There were 389 normal karyotype and 18 Down syndrome second-trimester samples, which constituted the confirmation study. This included three Down cases collected by the Prenatal Diagnosis Service postamniocentesis. The third group combined the original and confirmation studies, with 1448 normal and 39 Down syndrome cases. In the third group, a total of six trisomy 18 cases were also identified (cases compiled 1996–1999).

In the majority of our cases undergoing genetic amniocentesis for advanced maternal age concerns, blood for α-fetoprotein was drawn at the time of the procedure. This is useful as a marker of pregnancy outcome. We identified 692 subjects (21 with Down syndrome and 671 with normal karyotype) in the combined group that had blood taken at the time of amniocentesis for α-fetoprotein for determination. α-Fetoprotein concentrations were retrieved from the α-fetoprotein laboratory and added to our spreadsheet.

Samples (12 mL) were thawed overnight in the refrigerator and tested for hyperglycosylated hCG. For the original study, samples were tested sequentially in the spring of 1998. For the confirmation study, they were tested sequentially in the spring of 1999. In both studies, testing, calculation, and recording of results were carried out in a blind fashion, without knowledge of the karyotype.

The hyperglycosylated hCG test is a two-step sandwich-type ELISA. In brief, 96-well microtiter plates (Nunc Immulon-1; Fisher Scientific) are coated by incubation 16–24 h at 4 °C with capture antibody (0.2 mL per well of a solution containing 2.5 mg/L antibody B152 in 0.25 mol/L $NaHCO_3$ and 0.1 mol/L NaCl). Plates are then washed three times with water and blotted dry, and wells are blocked with phosphate-buffered saline, pH 7.4 (Life Technologies), containing 10 g/L bovine serum albumin and 0.4 g/L sodium azide (both from Sigma). After incubation for 1 h at ambient temperature, plates are again washed three times with water, blotted dry, and used for the assay. The total assay volume is 0.2 mL: 0.1 mL of sample or calibrator and 0.1 mL of phosphate-buffered saline containing 1 g/L bovine serum albumin and 0.4 g/L sodium azide. C5 hCG (100% hexasaccharide-type O-linked oligosaccharides), the immunogen for an-

tibody B152, that has been calibrated by amino acid analysis is used as the calibrator. C5 hCG at concentrations of 0, 60, 12, and 2.4 μg/L is added to quadruplicate wells of the plate. Urine samples are added at two- and fivefold dilutions. Buffer is added, and the plates are incubated 4 h at ambient temperature on an orbital plate shaker. Plates are again washed three times with water and blotted dry. Tracer antibody [0.2 mL of peroxidase-labeled anti-hCGβ batch 4001 (Medix Biotech), 1:5000 titer in Tris, pH 7.3 (Sigma) containing 1 g/L bovine serum albumin and 1.9 g/L $CaCl_2 \cdot 2 H_2O$] is added to each well. After an additional 2-h incubation at ambient temperature on the plate shaker, plates are again washed three times with water and blotted dry. Finally, 0.2 mL of substrate [TMB reagent (cat. no. T8665; Sigma) diluted 1:1 with water] is added to each well. After a 15-min incubation at ambient temperature, the reaction is stopped by the addition of 0.050 mL of 2 mol/L HCl. The plates are read on a microtiter plate reader at 450 nm, and the calibrators are plotted. The points best fit a cubic function, which was used to calculate sample values.

Plates included a quality control. The concentration was 21 μg/L or approximately in the middle of the calibration curve. The interplate/interassay variance was calculated. The mean result was 21 ± 1.8 μg/L, indicating an interassay variance (CV) of 8.9%.

The specificity of the hyperglycosylated hCG assay was investigated. Eight antigens were tested at multiple dilutions (Table 1 and Fig. 1). These included five preparations of pure intact hCG (non-nicked, nicked, or hyperglycosylated) and samples of pure hyperglycosylated hCG free β-subunit, pure free β-subunit missing the C-terminal extension, and pure human luteinizing hormone (hLH). All preparations were calibrated by amino acid analysis. The peptide and carbohydrate structures of the five intact hCG preparations have been determined (Table 1) *(1)*. The assay clearly discriminates the two hyperglycosylated hCG preparations (C7 hCG and C5 hCG from choriocarcinoma) from all other hCG-related antigens (Fig. 1). These hCG preparations have 57% and 48% triantennary N-linked oligosaccharides, and 68% and 100% hexasaccharide-type O-linked oligosaccharides (Table 1). The relative immunoreactivities of these two hCG preparation (93% and 100%, respectively) are much greater than those of the nonhyperglycosylated hCG or nicked hCG preparations (P8 and P3 hCG from normal pregnancies, 10% and 12%, respectively). We correlated the structural features of the 5 hCG preparations with the relative immunoreactivities. The most significant relationship was between immunoreactivity and the percentage of hexasaccharide type O-linked oligosaccharides ($r^2 = 0.94$). The low activities of P8 and P3 hCG correlated with the low hexasaccharide content in these preparations (13% and 12%, respectively). Minimal activity was detected with C5 hCG free β-subunit (10%), and no measurable activity was detected with hLH. It is inferred that the assay is specific for hyperglycosylated hCG, possibly for

Table 1. Specificity of the assay using anti-hyperglycosylated hCG coating antibody (monoclonal antibody B152) with peroxidase-labeled anti-hCGβ (monoclonal antibody 4001) as tracer.[a]

Antigen	Individual hCG preparations					hCG-related molecules		
	P8 hCG	P3 hCG	M4 hCG	C7 hCG	C5 hCG	C5β[b]	β-CTP[c]	hLH
Structure of antigen								
Nicking, %[d]	0	100	98	0	100			
Sialic acid, %[e]	80	87	73	58	70			
Triantennary, %[f]	22	10	30	57	48			
Hexsaccharide, %[g]	13	12	20	68	100			
Relative immunoreactivity, %	10	12	30	93	100	10	0.3	<0.3

[a] The activities of eight pure hCG, hCG free β-subunit, or hLH antigens were compared (Fig. 1). Relative immunoreactivity was determined relative to C5 hCG (the antibody B152 immunogen). The relative immunoreactivities were then equated with known structural features of the hCG preparation: percentage of nicking, percentage of sialic acid, percentage of triantennary oligosaccharides, and percentage of hexasaccharide-type oligosaccharides (1).

[b] C5β is the separated β-subunit of C5 hCG (hCG free β-subunit).

[c] β-CTP was copurified with hCG preparation M1; it is free β-subunit missing the C-terminal extension and the attached O-linked oligosaccharides (1).

[d] The percentage of nicking is the proportion of hCG molecules with a cleavage on the β-subunit between amino acids 47 and 48 (1). When the percentage of nicking of the five hCG preparations is correlated with relative immunoreactivity, $r^2 = 0.002$, indicating no relationship.

[e] The percentage of silalic acid is the proportion of hCG β-subunit oligosaccharide side chains with nonreducing terminal sialic acid residues (1). When the percentage of sialic acid of the five hCG preparations is correlated with relative immunoreactivity, $r^2 = 0.69$.

[f] The percentage of triantennary oligosaccharides is the proportion of hCG β-subunit N-linked oligosaccharide side chains with larger or triantennary structures (1). When the percentage of triantennary oligosaccharides of the five hCG preparations is correlated with relative immunoreactivity, $r^2 = 0.88$.

[g] The percentage of hexasaccharides is the proportion of hCG β-subunit O-linked oligosaccharide side chains with larger or hexasaccharide structures (1). When the percentage of hexasaccharides of the five hCG preparations is correlated with relative immunoreactivity, $r^2 = 0.94$.

molecules with hexasaccharide-type O-linked oligosaccharides. It is by this means that it has poor recognition of pregnancy hCG (P3 and P8 hCG) and near total recognition of choriocarcinoma hCG molecules (C7 and C5 hCG). A partial response was detected with molar pregnancy hCG (M4 hCG).

hCG β-core fragment concentrations were determined by a method similar to that for the hyperglycosylated hCG assay. The only difference was the use of a different coating antibody, B210 (gift from Drs. S. Birken and R. Canfield, Columbia University, NY), and a different calibrator (P13 β-core fragment). The β-core fragment assay detected hCG β-core fragment. Although this assay had 100% activity with the hLH β-core fragment calibrator, no measurable activity was found with hCG free β-subunit or any of the intact-hCG calibrators.

Results were normalized to spot urine creatinine concentrations. Creatinine was determined using a commercial kit, cat. no. 555A (Sigma), and a microtiter plate adaptation of the protocol. Calibrators (0, 2.5, 1.5, 0.5, and 0.2 g/L creatinine) and urine samples (0.053 mL per well, in triplicate) were added to a 96-well microtiter plate. Alkaline picrate reagent was prepared fresh (5 parts of solution plus 1 part sodium hydroxide) and added (250 μL) to the wells. The plates were incubated 15 min at ambient temperature. The absorbance was measured at 492 nm by a plate reader, and the calibrators were plotted. The points best fit a cubic equation, which was used to calculate sample concentrations (g/L).

In the original study with 1134 normal karyotype samples, hyperglycosylated hCG results were first normalized for urine concentration. The hyperglycosylated hCG concentration (μg/L) was divided by the spot creat-

inine concentration (μg/g creatinine). A relationship was observed between the normalized hyperglycosylated values and the creatinine concentration. Whereas samples with low creatinine concentrations were giving unduly high creatinine-normalized values, those with high creatinine concentration were giving unduly low creatinine-normalized values (5). An equation was derived to correct this error: $c' = (0.877c) + 0.107$, where c is the actual creatinine concentration and c' is the corrected value. A correction algorithm was not needed with creatinine-normalized intact hCG and β-core fragment concentrations.

Results were analyzed using the multiple of the median methods of Royston and Thompson (9). Creatinine-normalized hyperglycosylated hCG and β-core fragment concentrations were each plotted against gestational age. Weekly median values were determined for normal karyotype samples, and a regression equation was calculated that best fit the median values. Using the equation, multiples of the calculated median (MoM) were determined for all samples. In all three groups of cases, median values best fit a simple logarithmic equation. In all three groups, probability plots with lines defined by log-gaussian distribution were used to show that MoM values fit a log-gaussian distribution for both normal pregnancy and Down syndrome data.

To assess screening performance, MoM values, log MoM values, median values, and log mean and 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 normal pregnancies. The detection rates were determined from the proportion of Down syndrome pregnancies exceeding a specific centile of the
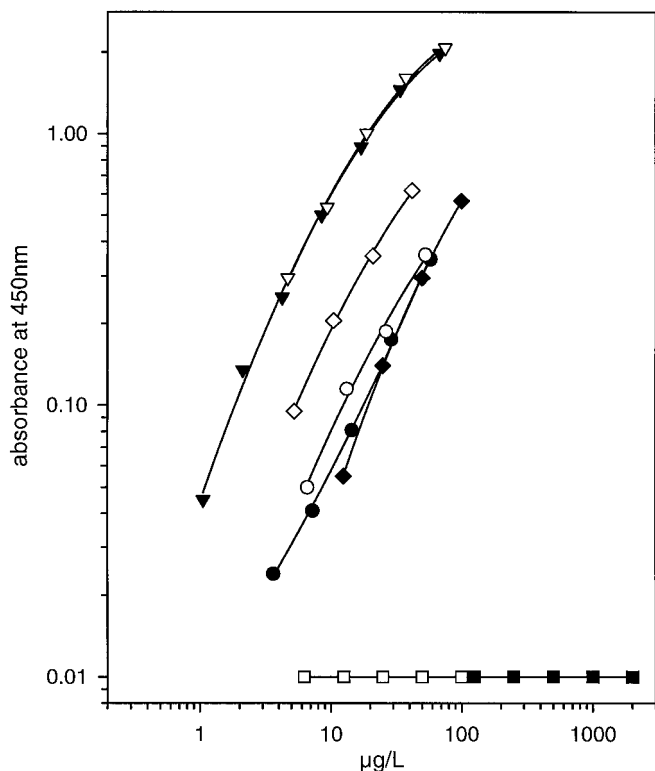
Fig. 1. Dose–response study testing eight antigens in the assay using an anti-hyperglycosylated hCG coating antibody (monoclonal antibody B152) with peroxidase-labeled anti-hCGβ (monoclonal antibody 4001) as tracer.

Intact hCG preparations were purified from individual urine samples, and structures were determined *(1)*. P3 hCG (○) and P8 hCG (●) were from normal first-trimester pregnancies, M4 hCG (◇) was from a case of hydatidiform mole, and C5 hCG (▼) and C7 hCG (▽) were from patients with choriocarcinoma. The characteristics of the hCG preparations are described in Table 1. We also tested an hCG free β-subunit from the dissociation of C5 hCG (◆), a free β-subunit missing the C-terminal peptide (□), and hLH, NIH batch I-1 (■).

normal karyotype population. ROC curves were used to compare detection rates (percentage of Down syndrome hyperglycosylated hCG results exceeding a specified centile of normals) and false-positive (100 − specified centile of normals) rates, and to determine the extent of discrimination between affected and unaffected pregnancies. Univariate and multivariate gaussian models were used to predict detection rates for hyperglycosylated hCG and combinations of hyperglycosylated hCG and other biochemical markers, and hyperglycosylated hCG and maternal age-related risk, considering the general age distribution of the population of the United States *(10)*.

Results

HYPERGLYCOSYLATED hCG CONCENTRATION IN NORMAL AND DOWN SYNDROME PREGNANCIES

Recently, we published a prospective study in which we tested the hyperglycosylated hCG concentration in urine samples from 11 to 22 weeks of gestation *(5)*. A single, somewhat irregular, exponential curve was identified to optimally fit both the first- and second-trimester median

values: median = $(294\,000\,000)\ ga^{-5.80}$, where *ga* is gestational age. Using this equation, MoM values and centiles were calculated. The median Down syndrome case was 7.3-fold higher than normals (7.3 MoM). Eighteen of 23 (78%) Down syndrome cases had MoM values exceeding the 95th centile of normal karyotype cases. The detection rate was plotted against the false-positive rate (ROC curve). The area under the ROC curve was 0.95. In this study, we reevaluated these data, excluding the small number of first-trimester cases (1059 normals; Table 2). Second-trimester (14–22 weeks of gestation) results were plotted against gestational age (Fig. 2A). Second-trimester median values best fit a simple logarithmic equation: median = 6383×0.706^{ga}. We used this equation to calculate MoM values and centiles. MoM values for both normal and Down syndrome cases fit a log-gaussian distribution between the 5th and 95th centiles. With the new equation, the median of the Down syndrome cases was 8.4-fold higher than that of the normals (median MoM = 8.4). The Down syndrome case results (mean log MoM ± SD, 1.05 ± 0.51) were very significantly different from normals (-0.001 ± 0.43; *t*-test, $P = 3.5 \times 10^{-26}$). Seventeen of 21 (81%) Down syndrome cases exceeded the 95th centile of the normal karyotype cases. From the ROC curve, 81% detection was indicated at a 5% false-positive rate (Table 2). The area under the ROC curve was 0.96.

The second-trimester only results were superior to the combined first- and second-trimester data, The attempt to combine the two trimesters with one median equation may have diminished the observed screening performance of hyperglycosylated hCG (median MoM, detection rate, and area under the ROC curve). Therefore, the two trimesters should be evaluated separately.

The finding of a single analyte test detecting ≥80% of Down syndrome cases is exceptional. A blind repeat study was needed to confirm these finding. We collected urine samples from 389 normal karyotype and 18 Down syndrome cases undergoing amniocentesis at 14–22 weeks of gestation for advanced maternal age concerns (Table 2). Hyperglycosylated hCG results were plotted against gestational age (Fig. 2B). Weekly medians were determined. Weekly median values best fit a simple logarithmic equation: median = 6050×0.716^{ga}. We used this equation to calculate MoM values and centiles. MoM values fit a log-gaussian distribution between the 5th and 95th centiles. With this equation, the median of the Down syndrome cases was 9.9-fold higher than normals (median MoM = 9.9). Again, a very significant difference was observed between Down syndrome (mean log MoM ± SD, 0.96 ± 0.42) and normal cases (-0.046 ± 0.43; *t*-test, $P = 5.2 \times 10^{-16}$). Fourteen of 18 (78%) Down syndrome cases exceeded the 95th centile of normal karyotype cases. From the ROC curve, 82% detection was indicated at a 5% false-positive rate (Table 2). The area under the ROC curve was 0.96.

The results of the two studies (original study − second trimester only, and the confirmation second-trimester

Table 2. Hyperglycosylated hCG and normal hCG to detect Down syndrome at 14–22 weeks of gestation: regression equations, MoM statistics, centiles, and ROC analysis.

Original study (1996–1998)

Normal karyotype cases (n = 1059)

Regression equation	Median = 6383 × (0.706^{qa})
Median MoM (log median MoM)	1.00 (0.0001)
Log mean MoM ± SD	−0.001 ± 0.43

Down syndrome cases (n = 21)

Median MoM (log median MoM)	8.44 (0.93)^a
Log mean MoM ± SD	1.05 ± 0.51^b
Proportion exceeding 95th centile of normals	81% (>5.32 MoM)
ROC, area under curve	0.96
ROC, detection rate at 5% false-positive rate	81%

Confirmation study (1998–1999)

Normal karyotype cases (n = 389)

Regression equation	Median = 6050 × (0.71^{qa})
Median MoM (log median MoM)	1.00 (0.003)
Log mean MoM ± SD	−0.046 ± 0.43

Down syndrome cases (n = 18)

Median MoM (log median MoM)	9.94 (1.00)^a
Log mean MoM ± SD	0.96 ± 0.42^b
Proportion exceeding 95th centile of normals	78% (>3.87 MoM)
ROC, area under curve	0.96^a
ROC, detection rate at 5% false-positive rate	82%

Combination: original + confirmation studies

Normal karyotype cases (n = 1448)

Regression equation	Median = 6180 × (0.710^{qa})
Median MoM (log median MoM)	1.00 (0.0001)
Log mean MoM ± SD	−0.019 ± 0.43

Down syndrome cases (n = 39)

Median MoM (log median MoM)	9.50 (0.98)
Log mean MoM ± SD	1.02 ± 0.47^b
Proportion exceeding 95th centile of normals	79% (>4.73 MoM)
ROC, area under curve	0.96
ROC, detection rate at 5% false-positive rate	80%

^{a} No significant difference found in the statistics for the original and the confirmation studies, in the log MoM values (*t*-test, P = 0.78), in the distribution of log MoM values (*F*-test, P = 0.68), and in the ROC area under the curve (*t*-test, P >0.2).

^{b} Significant difference found between normal karyotype and Down syndrome case MoM values: *t*-test original study, P = 3.5×10^{-26}; confirmation test, P = 5.2×10^{-16}; combined test, P = 3.6×10^{-42}.

study) virtually overlaid each other. The regression equations, the variation of normal samples expressed as log SD values, the detection rates, and the areas under the ROC curves were all either the same or close to being the same (Table 2). We interchanged the samples, combining the confirmation study normal samples with the original

study Down syndrome samples, and vice versa. After substitution, the same median MoM values were noted for Down syndrome cases, and the same proportion of sample exceeded the 95th centile. We inferred that the confirmation study verified the original study and that it was appropriate to combine the two groups of samples.

The two groups of samples were combined, for a total of 1448 normal karyotype and 39 Down syndrome cases from 14 to 22 weeks of gestation. Hyperglycosylated hCG results were again plotted against gestational age (Fig. 2C). Weekly medians were determined. Weekly median values again best fit a simple logarithmic equation, median = 6180 × 0.710^{ga}. We used this equation to calculate MoM values and centiles. MoM values fit a log-gaussian distribution between the 5th and 95th centiles. With this equation, the median of the Down syndrome cases was 9.5-fold higher than that for the normals (9.5 MoM). A very significant difference was observed between Down syndrome (mean log MoM ± SD, 1.02 ± 0.47) and normal karyotype cases (−0.019 ± 0.43; *t*-test, P <0.001). Thirty-one of 39 (79%) Down syndrome cases exceeded the 95th centile of the normals. From the ROC curve, 80% detection was indicated at a 5% false-positive rate (Table 2 and Fig. 3). The area under the ROC curve (0.96) again indicated 96% discrimination between normal and Down syndrome cases.

The true-positive Down syndrome cases included eight of the nine cases collected by the Prenatal Diagnosis Service postamniocentesis at 17–22 weeks of gestation. No significant difference was observed between the postamniocentesis (log MoM, 0.98 ± 0.43) and regularly collected (log MoM, 1.05 ± 0.52) cases.

CLINICAL UTILITY OF HYPERGLYCOSYLATED
hCG MEASUREMENTS
The original and confirmation studies described above examined mostly high-risk cases for Down syndrome, i.e., older individuals [mean age 36 ± 3.0 years (original group) and 36.9 ± 3.1 year (confirmation group)] undergoing amniocentesis for advanced age concerns. Gestational age was determined by ultrasound measurements. Down syndrome screening tests are most commonly used for low-risk or younger individuals. These tests use less precise last menstrual period-based measurements of gestational age. We investigated how maternal age and the accuracy of the measurement of gestational age influence hyperglycosylated hCG Down syndrome screening utility.

We used the combined group of samples to investigate the relationship between hyperglycosylated hCG values and maternal age. Linear regression indicated the significant absence of a relationship between maternal age and MoM values (r^2 = 0.0002). We divided the cases into two groups: low-risk/younger women (19–34 years; 169 normal and 7 Down syndrome cases) and high-risk/older women (35–49 years; 1279 normals and 32 Down syndrome cases). We examined the mean log MoM ± SD of
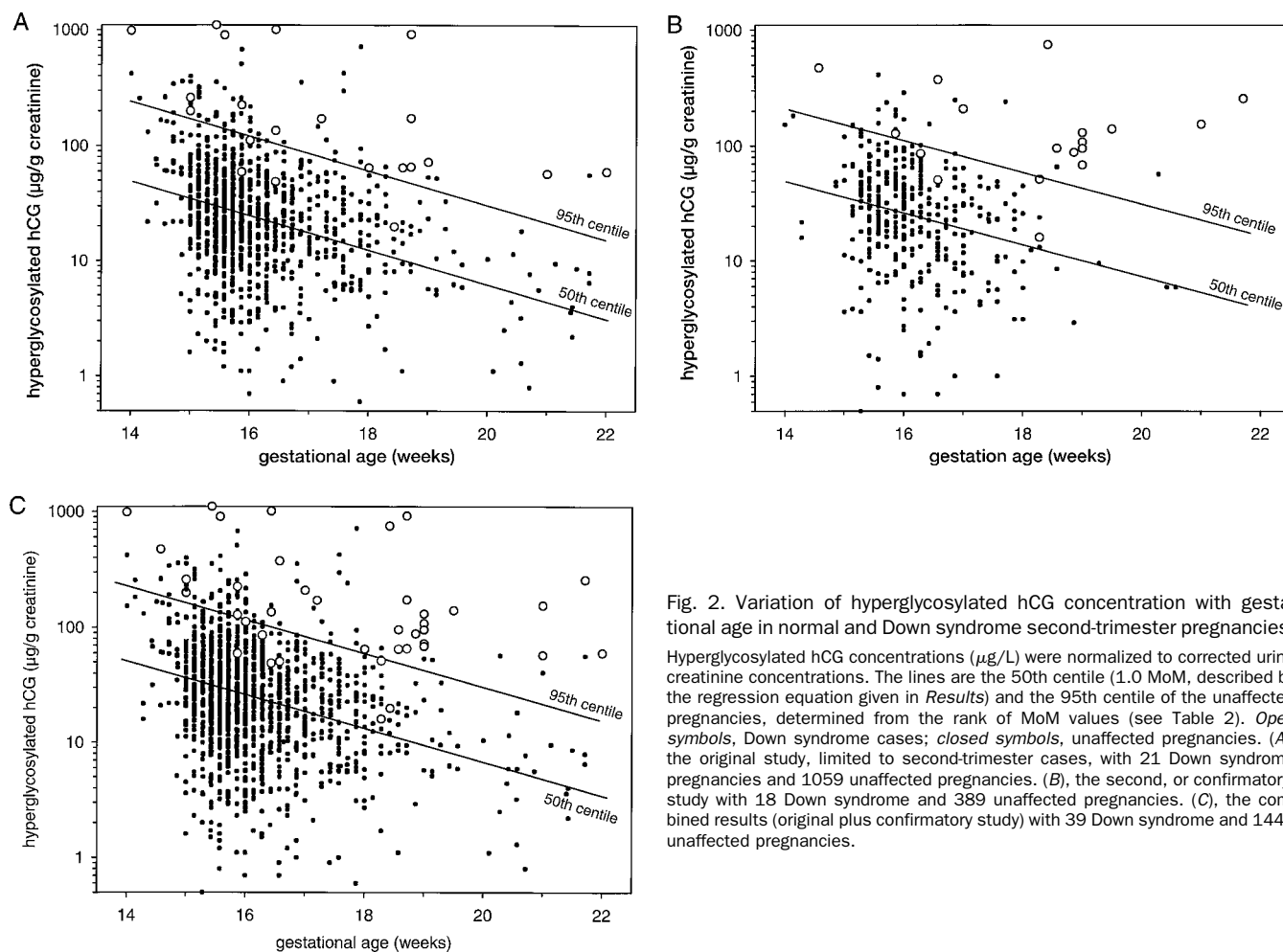
Fig. 2. Variation of hyperglycosylated hCG concentration with gestational age in normal and Down syndrome second-trimester pregnancies.

Hyperglycosylated hCG concentrations (μg/L) were normalized to corrected urine creatinine concentrations. The lines are the 50th centile (1.0 MoM, described by the regression equation given in *Results*) and the 95th centile of the unaffected pregnancies, determined from the rank of MoM values (see Table 2). *Open symbols*, Down syndrome cases; *closed symbols*, unaffected pregnancies. (*A*), the original study, limited to second-trimester cases, with 21 Down syndrome pregnancies and 1059 unaffected pregnancies. (*B*), the second, or confirmatory, study with 18 Down syndrome and 389 unaffected pregnancies. (*C*), the combined results (original plus confirmatory study) with 39 Down syndrome and 1446 unaffected pregnancies.

the two normal karyotype groups (-0.016 ± 0.43 and -0.011 ± 0.43, respectively). By *t*-test, the two groups were statistically indistinguishable ($P = 0.994$). We examined the mean log MoM $\pm$ SD of the two Down syndrome groups (0.922 ± 0.49 and 0.983 ± 0.47, respectively). By *t*-test, no clear difference was observed ($P = 0.77$). We examined the screening statistics of the two groups. Eighty-six percent of the low-risk/younger group and 78% of the high-risk/older group of Down syndrome cases exceeded the 95th centile of normal karyotype cases. It was concluded that hyperglycosylated hCG measurements are independent of maternal age.

We investigated normalization of hyperglycosylated hCG results to last menstrual period-based gestational age calculations. Hyperglycosylated hCG results were plotted against gestational age, weekly medians determined, and a new regression equation determined. MoM statistics were calculated and centiles determined. As with ultrasound-based data, 31 of 39 (79%) Down syndrome cases exceeded the 95th centile. No loss of sensitivity was indicated. It is inferred that hyperglycosylated hCG is not maternal age specific and can be used with last menstrual period-base gestational age calculations, and so

may be suitable for general low-risk screening for Down syndrome.

Regular hCG is the principal or base analyte test for current Down syndrome screening protocols. Regular serum hCG detects ~40% of Down syndrome case at a 5% false-positive rate (*6, 7*). Regular serum hCG is combined with maternal age-related risk, serum α-fetoprotein, unconjugated estriol, and more recently, with a fifth variable, inhibin A, to screen for Down syndrome pregnancy. Taking all five screening markers together (the quadruple test), screening performance did not reach the sensitivity observed for hyperglycosylated hCG. We considered using hyperglycosylated hCG as the principal or base analyte test and combining it with other markers. Using ROC analysis, hyperglycosylated hCG alone detected 80% of Down syndrome cases at a 5% false-positive rate, 67% at a 3% false-positive rate, and 49% and a 1% false-positive rate (area under ROC curve, 0.97; Fig. 3). Previously, we have shown that urine β-core fragment complements hyperglycosylated hCG measurements (*5*). We combined hyperglycosylated hCG results with age-related risk and β-core fragment measurements. This combination detected 92% of Down syndrome cases at a 5% false-positive
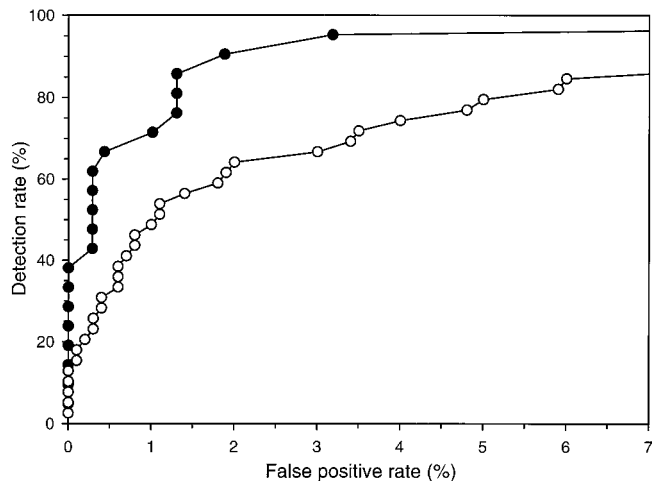
Fig. 3. ROC analysis of Down syndrome screening performance of urine hyperglycosylated hCG only (○), and for the combination of urine hyperglycosylated hCG with age-related risk, urine β-core fragment, and serum α-fetoprotein (●).

The detection rate was plotted against the false-positive rate, and the area under the ROC curve was calculated.

rate, 79% at a 3% false-positive rate, and 53% and a 1% false-positive rate (area under ROC curve, 0.97). Serum α-fetoprotein is routinely determined to evaluate risk for neural tube defect. Serum α-fetoprotein data were found for 671 of the normal and 21 of the Down syndrome cases. We combined the two urine tests with age-related risk and serum α-fetoprotein. This combination detected 96% of Down syndrome cases at a 5% false-positive rate, 94% at a 3% false-positive rate, and 71% at a 1% false-positive rate (Fig. 3). The area under the ROC curve was 0.98.

Trisomy 18 is a less common genetic abnormality in second-trimester pregnancies. Over 3.5 years, we accumulated six cases of trisomy 18. The median for the hyperglycosylated hCG values was 10-fold lower than that for normals (0.10 MoM of normal karyotype samples). All six cases had results below 0.6 MoM. Four of the six cases had values below both the fifth (0.14 MoM) and the third (0.11 MoM) centiles. Hyperglycosylated hCG may also be useful for identifying trisomy 18.

SHIPPING AND STORAGE CONDITIONS

We investigated the stability of hyperglycosylated hCG immunoreactivity in urine samples from normal and Down syndrome cases to asses the impact on shipping and storage conditions. Ten normal and 10 Down syndrome case urine samples refrigerated after collection and, once frozen, were rapidly thawed and tested. Aliquots (1 mL) were stored for 3 days in a refrigerator (4 °C), in a bench drawer (22 °C), or in a heating block (37 °C). As shown in Table 3, no significant difference was observed between the results of normal karyotype or Down syndrome samples after 3 days at room temperature (95% ± 8.7% and 96% ± 4.9% recovery of control immunoreactivity, respectively) or after 3 days in a refrigerator (95% ± 6.9% and 98% ± 2.3%, respectively). In addition, no significant difference was observed between the losses observed in normal karyotype and Down syndrome samples. These losses were within in the range of the interplate/interassay variance of the hyperglycosylated hCG assay (8.9%). More significant losses in immunoreactivity occurred after 3 days at 37 °C: 81% ± 19% of normal and 83% ± 10% of Down syndrome sample immunoreactivity was recovered (t-test, 22 °C vs 37 °C, $P = 0.05$ for normal and $P = 0.0017$ for Down syndrome samples). It is inferred that urine samples can be shipped or stored for 3 days at 4 or 22 °C before assay.

In the original and confirmatory Down syndrome screening studies, samples were frozen in −20 °C freezers, 12 mL of urine in 15-mL vials. Sample were later thawed overnight (slowly) in a refrigerator and tested for hyperglycosylated hCG. We examined the effect of these freezing and thawing procedures on hyperglycosylated hCG immunoreactivity (Table 4). When urine samples from 84 normal karyotype pregnancies were refrozen, thawed in the refrigerator, and assayed again, there was an overall loss in hyperglycosylated hCG immunoreactivity (86% ± 39% remained of prior hyperglycosylated hCG activity). When urine from six Down syndrome cases was similarly refrozen and assayed again, there was a much greater loss of immunoreactivity (48% ± 12% of the original activity remained; t-test, Down syndrome vs normal karyotype, $P < 0.0001$). We examined the normal

Table 3. The constancy of hyperglycosylated hCG immunoreactivity in second-trimester pregnancy urine samples.[a]

Source of urine sample	Immunoreactivity[b] after 3 days at:		
	4 °C	22 °C	37 °C
Normal karyotype pregnancy (n = 10)	95% ± 6.9%	95% ± 8.7%[c]	81% ± 19%[d]
Down syndrome pregnancy (n = 10)	98% ± 2.3%	96% ± 4.9%[c]	83% ± 10%[d]
t-statistic normal vs Down syndrome	P <0.2	P >0.2	P >0.2

[a] Samples were either stored at −80 °C or thawed rapidly (frozen only once) in a water bath and stored at 4, 22, or 37 °C. After 3 days, hyperglycosylated hCG immunoreactivity was determined and compared to samples maintained frozen at −80 °C during the incubation period (controls). Immunoreactivity is expressed relative to control values.

[b] Mean ± SD.

[c] No significant difference, 22 vs 4 °C: t-test, P >0.2.

[d] Significant difference, 3 vs 22 °C: t-test, P = 0.05 (normal karyotype) and P = 0.0017 (Down syndrome).

Table 4. The constancy of hyperglycosylated hCG immunoreactivity in second-trimester pregnancy urine samples after freezing and thawing.

Source of urine sample[a]	Immunoreactivity[b] after 3 days in −20 °C freezer, %	t-test
Freezing 12-mL urine aliquots in 15-mL vials, thawing overnight in refrigerator		
Normal karyotype pregnancy (n = 84)[a]	86 ± 39	
Normal karyotype pregnancy samples with lowest hyperglycosylated hCG result (8 of 84, <10th centile)	95 ± 7.5	$P = 0.02^c$
Normal karyotype pregnancy samples with highest hyperglycosylated hCG result (8 of 84, >90th centile)	62 ± 37	$P = 0.08^c$
Down syndrome pregnancy (n = 6)[a]	48 ± 12	$P <0.0001^d$
Down syndrome pregnancy (n = 4)[e]	47 ± 22	$P <0.01^d$
Freezing 1-mL urine aliquots in 1.5-mL vials, rapid thawing in 22 °C water bath		
Normal karyotype pregnancy (n = 10)[a]	97 ± 5.1	
Down syndrome pregnancy (n = 10)[a]	96 ± 4.5	$P <0.2$

[a] Urine samples were refrigerated after collection and frozen. Samples were thawed and assayed for hyperglycosylated hCG, and stability to freezing and thawing was investigated.

[b] Immunoreactivity after freezing and thawing is given as a percentage of prior values. Values are mean ± SD.

[c] Compared with balance of normal karyotype samples (n = 76).

[d] Compared with 84 normal karyotype samples.

karyotype samples with the lowest and with the highest concentration of hyperglycosylated hCG (Table 4). Those with lowest concentration had the better recovery (95% ± 7.5% of the original activity remained; $P = 0.02$), and those highest concentration had the poorer recover (62% ± 37% of the original activity remained; $P = 0.08$). The poor recovery found in large aliquots of urine from Down syndrome pregnancies that had been frozen and then thawed may be related to the very high concentration of hyperglycosylated hCG.

In a further experiment, urine samples were tested fresh (1–2 days in refrigerator) before any freezing and then after an initial freeze-thaw cycle. A similar loss in hyperglycosylated hCG immunoreactivity was detected (47% ± 22% of the original activity was recovered; *t*-test, Down syndrome vs normal karyotype, $P <0.01$). It was inferred that freezing and thawing 12-mL samples of urine led to a loss of hyperglycosylated hCG immunoreactivity, particularly in samples with a high concentration of hyperglycosylated hCG.

Further studies were carried out to confirm these findings and to develop a solution for the freeze-thaw problem. Experiments were repeated with 12-mL volumes of urine and similar procedures but with 200 mL/L glycerol, neutral pH buffer (50× concentrated Tris, pH 7.5), and antibiotics (50× concentrated penicillin-streptomycin-fungizone) added to the urine samples. None of the additives significantly changed or improved the freeze-thaw problem. Believing that the slow freezing of packed racks of 12-mL urine samples in a regular −20 °C freezer and slow thawing of the tubes overnight in a refrigerator may be responsible for the losses, we repeated the experiment with smaller urine aliquots. Ten urine samples from normal and 10 from Down syndrome cases were rapidly thawed, tested for hyperglycosylated hCG, and then refrozen again in 1-mL aliquots. Three days later, the small aliquots were rapidly thawed in a water bath and retested. No significant loss was noted in hyperglycosylated hCG immunoreactivity (97% ± 5.1% and 96% ± 4.5%). No difference was apparent between normal and Down syndrome cases. It was inferred that the freezing of racks of 12-mL samples, as was carried out in the Down syndrome screening studies described above, might have been detrimental to the results. The clear implication is that better screening data might have been obtained if the original measurements had been performed before freezing the samples. Conversely, if urine samples must be frozen, they should be stored in small aliquots (<1 mL) in noncrowded vials and thawed rapidly in a water bath before assay.

Discussion

In the 1980s, three serum tests were identified as markers of Down syndrome fetuses in the second trimester of pregnancy. These were hCG *(11)* and two less-discriminating tests: α-fetoprotein *(12)* and unconjugated estriol *(13)*. To these three tests were added maternal age-related risk, a fourth marker, to optimize Down syndrome screening performance *(14, 15)*. This triple biochemical marker test became widely used for screening for Down syndrome between 15 and 22 weeks of gestation. This triple test is far from perfect. It detects ~60%, or misses ~40%, of Down syndrome cases *(6, 15, 16)*. It has a 5% false-positive rate, so that a relatively large number of amniocentesis procedures must be performed to identify one case with a Down syndrome fetus. The triple-marker is criticized by physicians and patients because of its poor screening performance. Some laboratories are now adding a fifth marker (or a fourth biochemical test) to the hCG-based mixture, inhibin A. This test may, depending on the report, boost performance by an additional 10% *(7, 17, 18)*. It is still, however, an unsatisfactory screening

test. New tests are needed, not just to replace the triple-marker test or inhibin-containing quadruple-marker test, but also to supplant the hCG assay, the base test at the root of these combinations.

Recently, we described a large prospective study examining the screening performance of an independent marker, urine hyperglycosylated hCG, in the first and second trimesters of pregnancy *(5)*. In this report, we started by realizing the restraints of this data set and showing better performance when limiting it to the second trimester of pregnancy, 14–22 weeks of gestation. An 81% detection rate was indicated at a 5% false-positive rate. This is the highest detection rate ever reported for a single screening marker (without adding maternal age-related risk). We now describe a blind confirmation study. An 82% detection rate was indicated at a 5% false-positive rate. The original data set for 14-22 weeks of gestation and the new confirmatory data sets overlapped. Down syndrome and normal cases were interchangeable with no decline in screening performance. When the two data sets were combined, we had 1448 normals and 39 Down syndrome cases with 80% detection at a 5% false-positive rate. This one urine test outperforms all current individual analyte tests and all currently available combinations of four or five screening markers.

This study was limited to urine specimens. A preliminary study has now been completed indicating that hyperglycosylated hCG can also be measured in serum samples *(8)*. Unfortunately, gel separator tubes, like those used in tiger top or similar tubes, can interfere with hyperglycosylated hCG detection (Cole, unpublished observations). Most libraries of normal and Down syndrome serum samples have been accumulated using gel separator tubes. This has slowed down the evaluation of hyperglycosylated hCG as a serum marker. Thus, to date, our studies have been carried out with more easily obtainable urine samples. Urine hyperglycosylated hCG may be an effective replacement for serum analytes in Down syndrome screening. We considered the combination of urine hyperglycosylated hCG, urine β-core fragment (renal degradation product of serum hCG free β-subunit), and maternal age-related risk as a screening test for Down syndrome pregnancies. This combination detected 92% of Down syndrome cases at a 5% false-positive rate. The area under the ROC curve was 0.97. Although collection of urine is less invasive and may be preferred by patients, venipuncture must be carried out for serum α-fetoprotein determination, which is needed for detection of neural tube defects *(19)*. We considered the addition of serum α-fetoprotein to the package of age and two urine screening markers. This urine-serum triple marker combination detected 96% of Down syndrome cases at a 5% false-positive rate. The area under the ROC curve was 0.98.

Ninety-six percent is an extremely high detection rate for Down syndrome pregnancies. We examined the ROC curve for the urine-serum triple marker combination and the use of different false-positive rates. Ninety-four percent detection was indicated at a 3% false-positive rate and 71% detection at a 1% false-positive rate. The use of a 3% or even a 1% false-positive rate would lead to a major reduction in the number of amniocenteses that must be performed because of biochemical screening to identify a single Down syndrome case. This would produce major reductions in the miscarriages of normal fetuses as a result of the amniocentesis procedure and in the overall cost of prenatal screening. It could also renew the confidence of both physicians and patients in the screening process.

All studies were completed with women undergoing amniocentesis for advanced maternal age concerns. This is a mostly older group of women than that usually tested by biochemical screening methods. Furthermore, gestational age was determined by ultrasound, a more accurate method than extrapolation from the date of last menstrual period used in biochemical screening methods. We investigated the effects of maternal age and methods of determination of gestational age on hyperglycosylated hCG screening statistics. No loss of sensitivity was found when the combined group of samples was normalized to the last menstrual period-based gestational age. In the combined group of samples, no statistically significant relationship was present between hyperglycosylated hCG and maternal age. The mean MoM value was compared in normal cases under and over 35 years of age. The two groups were statistically indistinguishable. It is inferred that hyperglycosylated hCG is suitable for general low-risk biochemical screening for Down syndrome pregnancies.

The serum triple-marker test can also be used for detecting trisomy 18 *(20)*. Unduly low hCG concentrations (less than the fifth centile of normal cases) are detected in approximately two-thirds of second-trimester pregnancies with trisomy 18. We investigated the use of hyperglycosylated hCG as a marker of trisomy 18. In four of six cases, hyperglycosylated hCG MoM values were below the fifth centile. Thus, hyperglycosylated hCG may also be useful for identifying trisomy 18.

The effect of storage or shipping at different temperatures was investigated. Although no significant losses of immunoreactive hyperglycosylated hCG were noted after 3 days of storage at −4 or 22 °C, losses were noted after 3 days at 37 °C. Although no significant losses were noted when urine was frozen and thawed in 1-mL aliquots, losses were found after urine was frozen in large 12-mL aliquots. We conclude that urine samples should either be shipped to laboratories with refrigerant or shipped frozen in 1-mL or smaller vials. Samples should be stored at the testing center either in a refrigerator or at ambient temperature (up to 22 °C) for no more than 3 days if they have never been frozen, or kept in 1-mL or smaller aliquots if they are shipped frozen.

In conclusion, the combination of urine hyperglycosylated hCG, urine β-core fragment, serum α-fetoprotein, and maternal age-related risk can detect 96% of Down

syndrome cases at a 5% false-positive rate or 94% of cases at a 3% false-positive rate. Hyperglycosylated hCG-based tests and multiparameter algorithms may be able to entirely replace hCG-based tests (triple-marker test and quadruple-marker test incorporating inhibin A) and their algorithms in screening for Down syndrome.

References

1. Elliott M, Kardana A, Lustbader JW, Cole LA. Carbohydrate and peptide structure of the α- and β-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. Endocrine 1997;7:15–32.
2. Birken S, Krichevsky A, O'Connor J, Schlatterer J, Cole LA, Kardana A, Canfield, R. Development and characterization of antibodies to a nicked and hyperglycosylated form of hCG from a choriocarcinoma patient: Generation of antibodies that differentiate between pregnancy hCG and choriocarcinoma hCG. Endocr J 1999;10:137–44.
3. O'Connor JF, Ellish N, Kakuma T, Schlatterer J, Kobalebskaya G. Differential urinary gonadotropin profiles in early pregnancy and early pregnancy loss. Prenat Diagn 1998;18:1232–40.
4. Cole LA, Cermik D, Bahado-Singh RO. Oligosaccharide variants of hCG-related molecules: potential screening markers for Down syndrome. Prenat Diagn 1997;17:1188–90.
5. Cole LA, Shahabi S, Rinne KM, Oz UA, Bahado-Singh RO, Mahoney, MJ. Urinary screening tests for fetal Down syndrome. II. Hyperglycosylated hCG. Prenat Diagn 1999;19:351–9.
6. Haddow JE, Palomaki GE, Knight GJ, Williams J, Pulkkinen A, Canick JA, et al. Prenatal screening for Down's syndrome with use of maternal serum markers. N Engl J Med 1992;327:588–93.
7. Wald NJ, Densem J, George L, Muttukrishna S, Knight P. Prenatal screening for Down's syndrome using inhibin-A as a serum marker. Prenat Diagn 1996;16:143–53.
8. Shahabi S, Rinne K, Oz UA, Bahado-Singh RO, Mahoney MJ, Omrani A, et al. Serum hyperglycosylated hCG: a potential screening test for fetal Down syndrome. Prenat Diagn 1999;19:488–90.
9. Royston P, Thompson G. Model based screening by risk with application to Down's syndrome screening. Stat Med 1992;11:257–68.
10. Palomaki GE, Knight GJ, McCarthy J, Haddow JE, Eckfeldt JH. Maternal serum screening for fetal Down syndrome in the United States: a 1992 survey. Am J Obstet Gynecol 1993;169:1558–62.
11. Bogart MH, Pandian MR, Jones OW. Abnormal maternal serum hCG levels in pregnancies with fetal chromosome abnormalities. Prenat Diagn 1987;7:623–30.
12. Merkatz IR, Nitowsky HM, Macri N. An association between low maternal serum α-fetoprotein and fetal chromosome abnormalities. Am J Obstet Gynecol 1984;148:886–94.
13. Wald NJ, Cuckle HS, Densem JW, Nanchahal K, Canick JA, Haddow JE, et al. Maternal serum unconjugated estriol as an antenatal screening test for Down syndrome. Br J Obstet Gynecol 1988;95:334–41.
14. Wald NJ, Cuckle HS, Densem JW, Nanchahal K, Royston P, Chard T, et al. Maternal serum screening for Down syndrome in early pregnancy. Br Med J 1988;287:883–7.
15. Canick JA. Screening for Down syndrome using maternal serum α-fetoprotein, unconjugated estriol and hCG. J Clin Immunoassay 1990;13:30–3.
16. Knight GJ, Palomaki GE, Neveux LM, Fodor KK, Haddow JE. hCG and the free β-subunit as screening tests for Down syndrome. Prenat Diagn 1998;18:235–45.
17. Haddow JE, Palomaki GE, Knight GJ, Foster DL, Neveux LM. Second trimester screening for Down's syndrome using maternal serum dimeric inhibin A. J Med Screening 1998;5:115–9.
18. Lam YH, Hoi M, Tang, Y. Second-trimester maternal serum inhibin A screening for fetal Down syndrome in Asian women. Prenat Diagn 1999;19:463–7.
19. Ferguson-Smith MA. Prenatal diagnosis. Practitioner 1974;213:655–66.
20. Sinosich MJ, Cameron B, Robertson RD, Saunders DM. Triple test for prenatal detection of Edwards syndrome (trisomy 18). J Obstet Gynaecol Res 1996;22:57–60.