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Urinary Screening Tests for Fetal Down Syndrome: I. Fresh β-core Fragment

Laurence A. Cole[1]*, Kirsi M. Rinne[1], Smita M. Mahajan[1], Utku A. Oz[1], Shohreh Shahabi[1], Maurice J. Mahoney[1,2] and Ray O. Bahado-Singh[1]

[1]*Department of Obstetrics and Gynecology, Yale University School of Medicine, New Haven, CT 06520, U.S.A.*
[2]*Department of Genetics, Yale University School of Medicine, New Haven, CT 06520, U.S.A.*

Variable results have been reported using urine β-core fragment as a marker for fetal Down syndrome. Initial studies by Cuckle *et al.* (1994) and Canick *et al.* (1995) indicated that β-core fragment was an outstanding marker, detecting >80 per cent of Down syndrome cases. Since these reports, widely varying results have been published, indicating between 20 per cent and 66 per cent detection of cases at 5 per cent false-positive rate. The wide variation in the reported data has led to a loss of enthusiasm for this marker as a useful test for Down syndrome screening.

Here we report the results of a three-year prospective study in which urine samples were collected daily from women undergoing fetal karyotype analysis for advanced maternal age. Samples were tested within one week of collection and then frozen. We also investigated the likely causes of the variability observed in β-core fragment data.

We collected 1157 urine samples over 955 days. β-core fragment levels were measured. A regression line was calculated for the weekly medians of the 1134 control samples and multiples of the control median (MoM) were determined. The median MoM for the controls was 1.0 and the logarithmic standard deviation (log SD) was 0.41. The median MoM for the 23 Down syndrome cases was 5.44 and the log SD was 0.45. Over the study period, 65 per cent of Down syndrome cases exceeded the 95th centile of the control group. The median MoM of control samples and the proportion of Down syndrome cases detected by the test was relatively constant during the study period. The unaffected cases were divided into three equal divisions, corresponding to approximately the first, second and third year of sample collection. No trend was found in the median control MoM values in three sample collection periods ($r^2=0.04$). A similar number of cases exceeded the 95th centile of control samples in the three sample collection periods, 63 per cent, 66 per cent and 66 per cent. Consistent results were indicated during the three years of sample testing.

Levels of total oestriol were determined in urine samples and MoM statistics derived. The median oestriol level in Down syndrome cases was 0.59 MoM. Only 12 per cent of cases had MoM levels below the fifth centile. Gaussian models were prepared combining biochemical data and maternal age distribution. While β-core fragment by itself detected 65 per cent of Down syndrome cases, β-core fragment modelled with maternal age detected 66 per cent, and modelled with age and total oestriol levels detected 82 per cent of cases at 5 per cent false-positive rate.

At the completion of the study, we thawed and reassayed 20 random urine samples (10 control and 10 Down syndrome) collected at different times during the study period. While the control samples (74–1700 ng/ml) had slightly increased values when reassayed (mean value 137 per cent of original prospective value), the Down syndrome samples (360–20 500 ng/ml) all had decreased values when reassayed (mean=53 per cent, *t*-test, controls versus cases, $p=0.0003$). The Down syndrome samples were decreased to between 93 per cent and 12 per cent of the original value. A relationship was identified between the magnitude of the original β-core fragment value and the change in immunoreactivity when reassayed ($r^2=0.998$). The higher the initial β-core fragment value the greater the loss of immunoreactivity. We considered the possibility that the β-core fragment molecules aggregate upon storage in the freezer. We repeated the assay of the 20 samples after treatment with a high salt buffer. Down syndrome samples recovered half of the lost β-core fragment immunoreactivity (mean increase in β-core fragment levels 56 per cent, *t*-test, controls versus cases, $p=0.004$). We infer that aggregation of β-core fragment upon storage interferes with β-core fragment measurements. This may be the cause of the poor β-core fragment screening performance reported using stored/frozen urine samples.

We conclude that urine β-core fragment, or combinations of β-core fragment and total oestriol need to be considered as viable alternatives to current serum protocols for screening for Down syndrome. We also conclude that urine β-core fragment has no consistency or variability limitations, provided samples are tested within one week of collection, as occurs in clinical practice.

KEY WORDS: Down syndrome; β-core fragment; human chorionic gonadotrophin; hCG; screening; prenatal diagnosis; storage

*Correspondence to: L. A. Cole, Department of Obstetrics and Gynecology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, U.S.A. E-mail: laurence.cole@yale.edu.
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INTRODUCTION

hCG is a glycoprotein hormone composed of an α- and β-subunit, joined non-covalently. Trophoblast cells of the placenta produce hCG in pregnancy and in patients with trophoblastic diseases. β-core fragment is the urinary degradation product of hCG. The molecular weight of β-core fragment is approximately 9700, or one-quarter the size of the intact hormone. hCG is cleaved or nicked after secretion by trophoblast cells. Nicked hCG dissociates releasing a nicked free β-subunit, which may be degraded to β-core fragment in the maternal kidney (Birken *et al.*, 1988; Cole *et al.*, 1993).

In 1994, Cuckle and colleagues tested β-core fragment levels in second-trimester urine samples (Cuckle *et al.*, 1994). They suggested that β-core fragment might be a useful marker for fetal Down syndrome. One year later the same group published the results of a larger study with 24 Down syndrome cases and 294 unaffected second-trimester pregnancies (Cuckle *et al.*, 1995). They reported an 80 per cent detection rate for Down syndrome pregnancies with a 5 per cent false-positive rate. In the same year, Canick *et al.* (1995) examined 14 Down syndrome and 91 unaffected cases. They found that 93 per cent of Down syndrome cases exceeded the 95th centile of control samples (Table 1). In the following years, Hayashi and Kozu (1995) observed 20 per cent, Spencer *et al.* (1996) found 41 per cent, Isozaki *et al.* (1997) reported 62 per cent, Kellner *et al.* (1997) reported 66 per cent, Cole *et al.* (1997a) found 58 per cent detection, and Hallahan *et al.* (1998) found 31 per cent of Down syndrome samples exceeded the 95th centile of control samples (Table 1). In 1996 and 1997 studies were carried out in the United Kingdom and the United States using 6731 frozen or otherwise stored urine samples (Chiron Collaborative UGP Study). Disappointing results were recorded, only 35 per cent of 40 Down syndrome cases exceeded the 90th centile of controls (Canick *et al.*, 1999; Cuckle, Canick and Kellner, paper submitted). Extreme variability is reported in the results, 20 per cent to 93 per cent detection at 5 per cent false positive rate. The earlier reports (80 to 93 per cent detection) generated much excitement over the potential usefulness of this urine test. The finding in the later reports of variable and inconsistent results has led to greatly reduced expectations, and to the premature rejection of this antigen as a clinically viable marker for Down syndrome screening.

Here we present the results of a three-year prospective study, in which urine samples were collected from women undergoing fetal karyotypic analysis. This is the largest study reported to date, and unique in that the urine samples were collected daily, refrigerated and tested fresh within one week of obtaining the specimen. We report the results of this prospective study. We also investigate the stability of samples and seek a plausible explanation for the variability reported in β-core fragment data.

MATERIALS AND METHODS

Urine samples were collected prospectively over a three-year period (May 1995–March 1998) from women at 11 to 22 weeks of gestation, who were having amniocentesis or chorionic villus sampling at the Maternal–Fetal Medicine Units at Yale University-affiliated hospitals (Yale–New Haven Hospital, Bridgeport Hospital and Norwalk Hospital). Verbal consent was obtained using the protocols approved by the institutional review boards.

Urine samples were refrigerated immediately after collection. Twice weekly, urine samples were carried to the laboratory. Samples were tested fresh without freezing, at least once weekly, without knowledge of the karyotype. Results were entered into a Microsoft

Table 1—Reports of urinary β-core fragment as a screening test for Down syndrome in the second-trimester of pregnancy

Reference	Control samples (*n*)	Down syndrome cases (*n*)	Down syndrome cases (median MoM)	Ranking of Down syndrome cases[c]
Canick *et al.* (1995)	91	14	5.3	93% >95th centile
Cuckle *et al.* (1995)	294	24	6.0	88% >90th centile
Cuckle *et al.* (1994)	67	7	6.1	71% >95th centile
Kellner *et al.* (1997)	206	32	5.4	66% >95th centile
This study	1134	23	5.4	65% >95th centile
Isozaki *et al.* (1997)	709	13	4.1	62% >95th centile
Cole *et al.* (1997a)	480	12	4.5	58% >95th centile
Spencer *et al.* (1996)	400	29	2.4	41% >95th centile
Chiron Collaborative Study[a]	6691	40	1.7	35% >90th centile
Hallahan *et al.* (1998)	163	13	2.4	31% >95th centile
Hayashi and Kozo (1995)[b]	150	5	2.8	20% >95th centile

[a]Canick *et al.* (1999); Cuckle, Canick and Kellner (paper submitted).
[b]Urine β-core fragment+free β-subunit.
[c]Proportion of Down syndrome samples exceeding the 95th centile of controls.

Excel-7 computer spreadsheet. Biographical data were collected on a multiple-choice form at the time or urine collection. Date of collection, maternal age, maternal weight, race, date of last menstrual period, history of smoking or of diabetes, and reason for karyotype analysis were all recorded, coded and entered into the computer spreadsheet. Gestational age of subjects, determined by ultrasound, was obtained from the computer databases in the three Maternal–Fetal Medicine Units. Two to three weeks after urine collection, fetal karyotype was obtained from the Genetics Department and entered into our computer spreadsheet.

Over the three-year period, a total of 1842 women volunteered urine. Patients volunteered urine samples while waiting to have genetic sampling procedures. 60 per cent of women who underwent karyotype testing provided a urine sample. The main reasons for not collecting urine specimens were failure to ask patients to volunteer during busy periods, and the inability of the patient to provide urine. Less than 3 per cent of women declined to volunteer a urine specimen. We limited this study to those undergoing genetic sampling procedures because of advanced maternal age concerns (those pre-selected for genetic analysis by ultrasound or triple screen test were excluded), and to singleton pregnancies with fetuses with normal and Down syndrome karyotype. This reduced the number of patents to 1151 (1134 normal karyotype and 17 Down syndrome cases). As a back-up procedure, the Genetics Department checked whether those women who received a Down syndrome karyotype had volunteered urine samples at the time of the procedures. If they had not, urine samples were requested at the time of counselling. These urine samples were added to the regular urine collection trays in the refrigerator, and tested and entered into the computer spreadsheet in an almost equally blind fashion (laboratory staff had no knowledge that the sample was collected differently, or of the karyotype). This brought the total number of Down syndrome case to 23. This back-up procedure explains the collection of six of the eight Down syndrome urine samples at 18 to 22 weeks of gestation, two to four weeks after the collection of the majority of unaffected urine samples.

In a previous publication (Isozaki *et al*., 1997) we measured β-core fragment levels in 709 unaffected and 13 Down syndrome pregnancies. These included individuals undergoing genetic sampling procedures for advanced maternal age, positive triple screen and abnormal ultrasound reasons. This included 540 of the unaffected and 7 of the Down syndrome cases tested in this study (those tested for advanced maternal age reason only).

β-core fragment levels were determined by the B210 assay, as described previously (Cole *et al*., 1994; Isozaki *et al*., 1997). This is a two-step sandwich assay. Briefly, microtitre plates are coated with monoclonal antibody B210 (gift from O'Connor and Canfield at Columbia University, New York, NY, U.S.A.), urine samples are added and β-core fragment extracted. Plates are washed and peroxidase-labelled hCG β-subunit antibody is added to quantitate bound β-core fragment. After a further wash, substrate is added and peroxidase enzyme activity measured spectrometrically. Urine samples were diluted with phosphate-buffered saline containing 0.1 per cent (w/v) bovine serum albumin (normal dilution buffer) for this assay. Samples were assayed at one or more dilutions, as needed (between 50 × and 10 000 ×). The B210 assay was standardized with pure β-core fragment, purified from pregnancy urine and calibrated by amino acid analysis. The B210 assay detects only β-core fragment, with <0.1 per cent cross-reactivity with free β-subunit and hCG. Plates included a high and a low quality control. The mean value of the high quality control (middle of range of detection) was 1.30 ng/ml, with 0.11 ng/ml standard deviation and 8.1 per cent coefficient of variation or inter-assay variation. The mean value for the low quality control (low end of the limit of detection) was 0.32 ng/ml, with 0.031 ng/ml standard deviation and 9.7 per cent coefficient of variation or inter-assay variation.

Total oestriol was determined by radioimmunoassay, using the kit sold by Diagnostic Products Corporation (Los Angeles, CA, U.S.A.). The kit utilized antibody-coated tubes, oestriol-releasing enzyme, radio-iodine-labelled tracer, and a set of six standards (catalogue number TKE35). Procedures were those described in the instruction booklet. Urine samples were initially diluted in the range 1 × to 100 × as needed. The ratio of β-core fragment to total oestriol levels (β-core fragment (ng/ml) ÷ total oestriol (ng/ml)) was also determined.

Urine samples were collected at random times during the working day. β-core fragment levels were normalized to adjust for variations in urine concentration by dividing by the creatinine level. The creatinine concentration was determined using the Sigma Chemical Co. spectrometric creatinine kit, catalogue 555A (St. Louis, MO, U.S.A.).

Samples were frozen at − 20°C after assay (within approximately one week of collection). In February 1998, 10 frozen control and 10 frozen Down syndrome samples were thawed and reassayed. Samples were either tested by the normal procedures as described above (at titres of 250 × , 1000 × and 4000 × in normal dilution buffer), or by modified procedures designed to break up β-core fragment aggregates. In the modified procedures, samples were diluted 10 × in a dissociation buffer (0.2 M sodium acetate, 1 M sodium chloride, 1 per cent triton × 100, pH 5), and then incubated for 1 hour at 37°C. The treated materials were titred to 250 × , 1000 × and 4000 × in normal dilution buffer before assay by normal procedures.

Results were analysed using standard methods (Royston and Thompson, 1992; Palomaki *et al.*, 1995). Gestational age was determined by ultrasound. Concentrations were plotted against gestational age. A regression line was calculated to fit weekly median values. Using the regression equation, medians were predicted and multiples of the unaffected pregnancy median (MoM) were determined. To assess screening

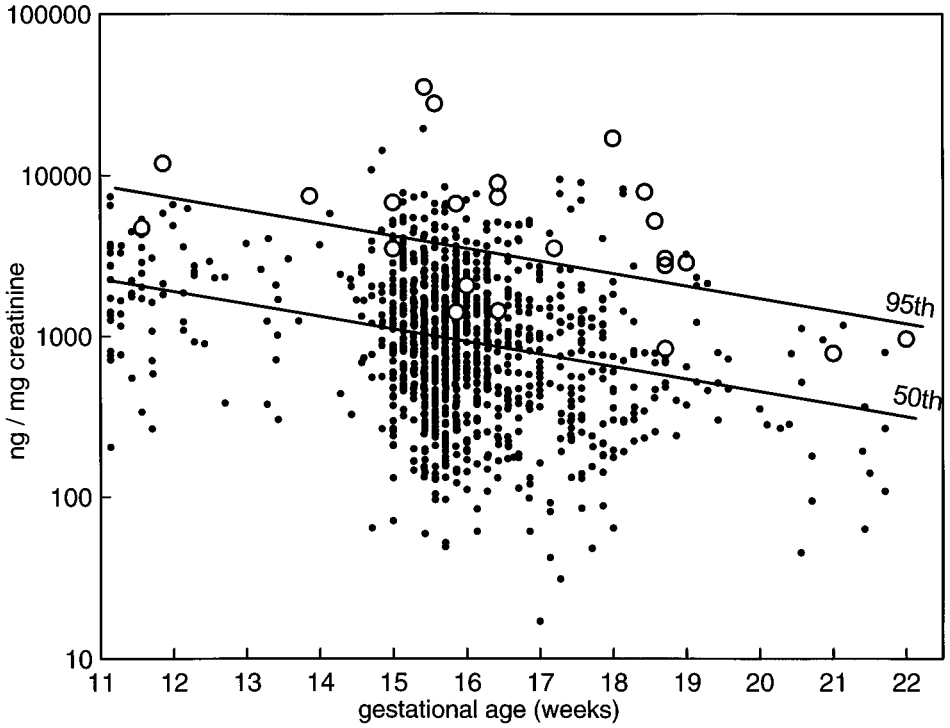

Fig. 1—β-core fragment levels in urine samples from 23 Down syndrome pregnancies (○) and 1134 unaffected pregnancies (●). β-core fragment levels (ng/ml) were normalized to spot urine creatinine level (mg/ml). The lines are the 50th centile (1.0 MoM, described by the regression equation give in the Results section), and 95th centile (4.3 MoM, determined from the rank of MoM values) of the unaffected pregnancies

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. Observed detection rates were determined from the proportion of aneuploid pregnancies above or below a specific centile of the control population using ROC (receiver operating characteristics) analysis. Univariate and multivariate Gaussian models were used to predict detection rates considering the general age distribution of the population of the United States (Palomaki *et al.*, 1993) and combination of markers. Models were used to calculate risk and applied to the observed distribution of values.

RESULTS

β-core fragment levels were determined in urine samples from 1134 unaffected (control) and 23 Down syndrome cases from 11 to 22 weeks of gestation. Concentrations were plotted against gestational age (Fig. 1). The weekly median values of control samples (1860, 2368, 1469, 1677, 916, 969, 670, 476, 683, 317 and 266 ng/mg creatinine, for the 11th to 21st completed week of gestation, respectively) fit a logarithmic regression line, $y = 16\,730 \times (0.834^{ga})$, where y is the median value corresponding to a specific gestational age (ga). The equation was used to predict median values for specific gestational ages. MoM values were determined for all samples, and median MoM values and log SD were calculated. The median MoM of the control samples was 1.00 and the log SD was 0.41. The median MoM of the Down syndrome samples was 5.44 and the log SD was 0.45. A probability plot showed that the MoM values for the controls and Down syndrome cases fit a log-Gaussian distribution reasonably well (Fig. 2). Control samples were ranked according to MoM value. The 95th centile corresponded to 4.3 MoM. 65 per cent of the Down syndrome samples exceeded this centile of control samples.

We divided samples into five equal sized groups according to maternal age and into five equal sized groups according maternal weight. The median MoM values were 0.91, 0.94, 1.20, 0.90 and 1.02 at median maternal ages of 34, 36, 37, 38 and 40, respectively. The median MoM values were 1.10, 1.09, 0.94, 1.04 and 1.01 at median maternal weights of 119, 132, 142, 156 and 185, respectively. No obvious relationship was indicated between β-core fragment MoM values and either maternal age or weight. No difference was observed in β-core fragment values in white individuals (1.05 MoM, 91 per cent of patients) compared with six other races (1.05 MoM, 9 per cent of patients).

β-core fragment levels were normalized to spot creatinine levels. To assess the ability of creatinine to correctly normalize β-core fragment values, β-core fragment MoM values were plotted against creatinine concentration (Fig. 3). Samples were divided into five equal sized groups according to creatinine concentration. Median MoM values were determined and a logarithmic regression line plotted, $y = 1.25 \times (0.78^{cr})$,

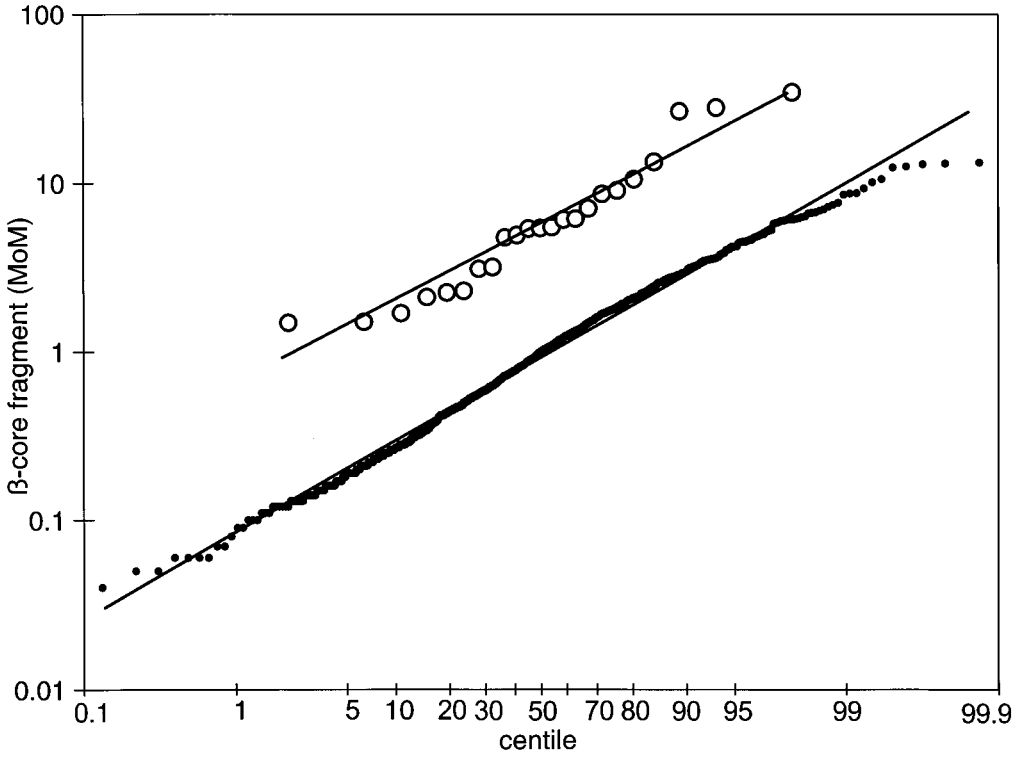

Fig. 2—Probability plot of the MoM values for β-core fragment from 23 Down syndrome pregnancies (○) and 1134 unaffected pregnancies (●). The two solid lines are defined by log Gaussian distributions

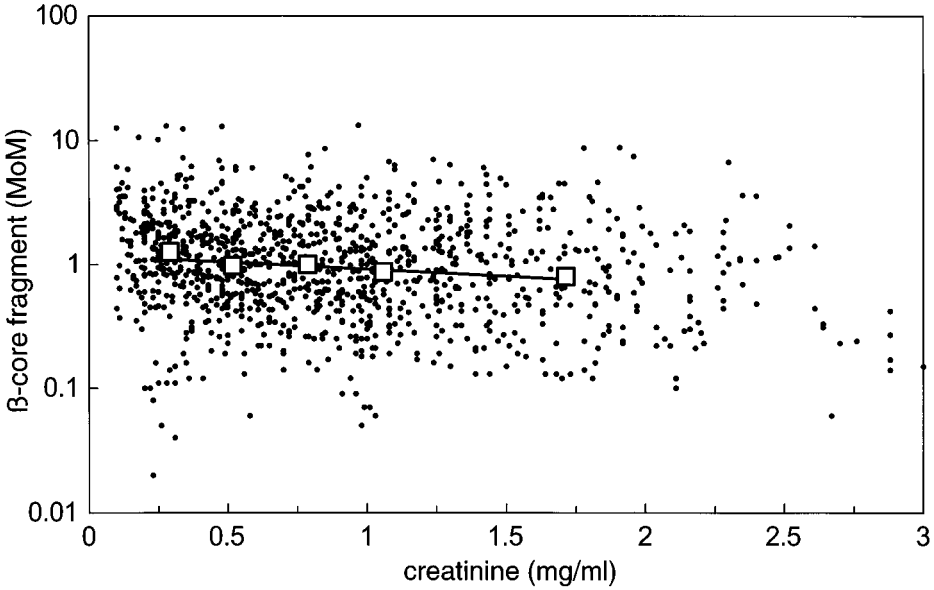

Fig. 3—Effect of creatinine concentration on β-core fragment MoM statistics. β-core fragment MoM values for 1134 unaffected pregnancies (●) were plotted against creatinine concentration. Median MoM values were determined for 5 equal sized divisions of the creatinine concentration values (□). A logarithmic regression line is shown fitting these median values. The equation of line was $y=1.25 \times (0.78^{cr})$, where y is the calculated MoM value and cr is the creatinine concentration

where y is MoM and 'cr' is creatinine concentration. The line had a small logarithmic slope. Thus, creatinine normalization might exaggerate MoM values in samples with lower creatinine levels (median MoM at 0.25 mg/ml creatinine=1.27), and underestimate MoM values in those with higher creatinine (median MoM at 1.73 mg/ml=0.80). The mean creatinine concentrations of the control (0.92 mg/ml) and Down syndrome (0.91 mg/ml) samples were very similar. We attempted to correct creatinine values using the logarithmic regression equation. This was not done, since it made no change to the control or Down syndrome median

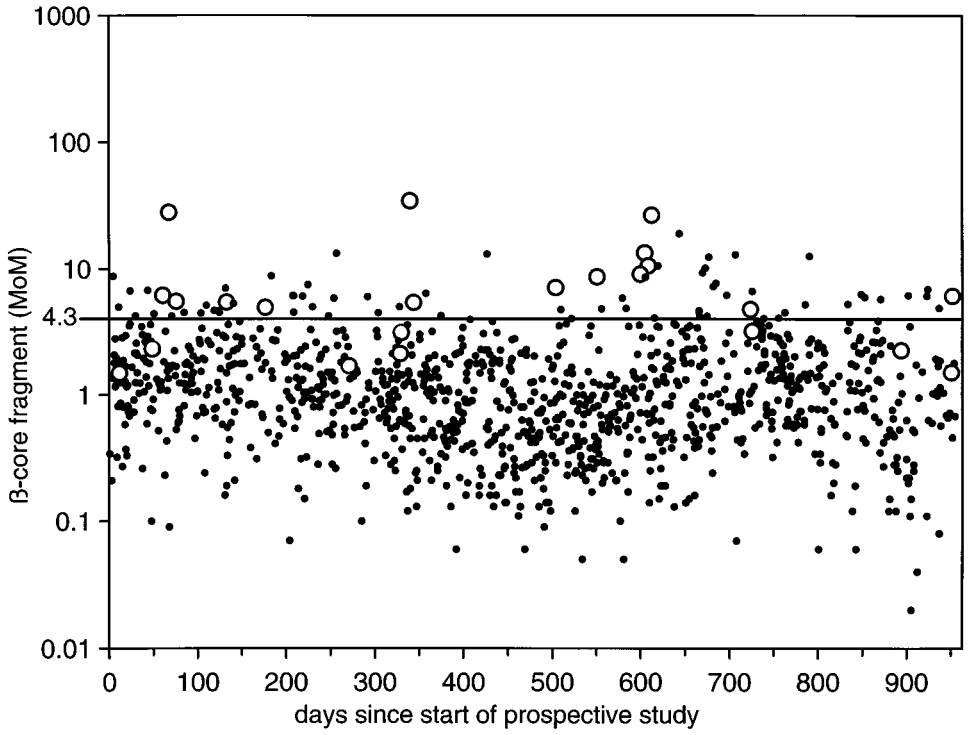

Fig. 4—Constancy of β-core fragment MoM statistics during the study period. β-core fragment MoM values for 23 Down syndrome pregnancies (○) and 1134 unaffected pregnancies (●) were plotted against the time of collection during the project. While β-core fragment and creatinine levels were determined weekly throughout the project, MoM values were calculated at the end of the study. The line indicates the 95th centile (4.3 MoM), calculated from the rank of MoM values at the end of the project

Table 2—Regression line and distribution parameters for urine β-core fragment, urine total oestriol and β-core fragment: oestriol ratio

	Control samples n=1134 (552—46,XX; 582—46,XY)				Down syndrome samples n=23 (9—47,XX; 14—47,XY)		
Test	Regression line	Median MoM	Log SD[a]	95th centile	Median MoM	Log SD[a]	Ranking[c]
β-core fragment	$y=16\,731 \times (0.834^{ga})$	1.00	0.41	4.3 MoM	5.4	0.45	65% >95th centile
Oestriol	$y=7.861 \times (1.352^{ga})$	1.00	0.41	0.12 MoM[b]	0.59	0.43	12% <5th centile
β-core fragment:oestriol ratio	$y=2380 \times (0.616^{ga})$	1.01	0.49	6.5 MoM	9.0	0.46	72% >95th centile

[a]Log SD, calculated from the difference between the log MoMs of the 10th and 90th centiles.
[b]5th centile, oestriol level decreases in Down syndrome cases.
[c]Proportion of Down syndrome samples exceeding the 95th centile (or below the 5th centile) of controls.

MoM or log SD values, or to detection or false-positive rates for Down syndrome. We considered the possibility that creatinine values may change with gestational age. We divided gestational age into five equal sized groups. The median creatinine concentrations were 0.74, 0.78. 0.89. 0.79 and 0.85 mg/ml at median gestation ages of 15, 15.43, 15.71, 16.28 and 17.57 weeks, respectively. No obvious relationship was indicated between gestational age and creatinine concentration.

The samples had been collected and tested continuously over a 955 day period. Fig. 4 shows the constancy of control and Down syndrome values during this period. The samples were divided into three equal sized groups, each corresponding to approximately one year of the prospective study (1–325, 326–583 and 584–955 days). No trend was noted in the control sample MoM values in the three periods (logarithmic regression, $r^2=0.04$). A similar proportion of Down syndrome samples was detected (exceeded 95th centile) in each of the three periods, five of eight cases (63 per cent) in the first, four of six cases (67 per cent) in the second and six of nine cases (67 per cent) in the third period.

As shown in Table 2, we also measured total oestriol levels in the urine samples. A regression line was determined for weekly medians, and MoM statistics and centiles were calculated. The median MoM and log SD of the control samples was 1.00 and 0.41. While β-core fragment levels were increased, total oestriol

Table 3—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]
Oestriol	0.63	0.066	12%	nd[b]	nd[b]
β-core fragment	0.92	0.023	65%	57%	31%
+Age	0.92	0.024	66%	50%	29%
+Oestriol+age	0.93	0.035	82%	74%	39%
β-core fragment:oestriol ratio	0.92	0.031	72%	50%	23%
+Age	0.93	0.035	77%	56%	23%

[a]Detection rate and false positive rate (fpr) from ROC curve.
[b]Not determined.

levels were decreased in Down syndrome cases (median 0.59 MoM, log SD 0.43). 12 per cent of the Down syndrome samples had MoM values below the 5th centile of controls (Table 2).

We also calculated the β-core fragment:total oestriol ratio (ng/ml÷ng/ml) in urine samples. A regression line was determined for weekly medians, and MoM statistics and centiles were calculated (Table 2). The median MoM and log SD of the control samples was 1.01 and 0.49. The median MoM for Down syndrome cases was 9.0 and the log SD was 0.46. 72 per cent of Down syndrome cases had MoM values exceeding the 95th centile of controls.

Gaussian models were prepared using the β-core fragment, total oestriol and β-core fragment: total oestriol ratio screening statistics and the general age distribution in the United States (Table 3). ROC analysis (detection rate versus false-positive rate) was used to analyse the modelled data. While β-core fragment alone detected 65 per cent of Down syndrome cases (area under ROC curve=0.92, or 92 per cent discrimination), β-core fragment modelled with age detected 66 per cent of cases (area=0.92), and that modelled with age and total oestriol levels detected 82 per cent of cases (area=0.93) at 5 per cent false-positive rate (Table 3). While β-core fragment:total oestriol ratio alone detected 72 per cent of Down syndrome cases (area=0.92), the ratio modelled with age detected 77 per cent of cases (area=0.93). Either the combination of β-core fragment/total oestriol/creatinine and age, or β-core fragment:total oestriol ratio and age may be useful in detecting Down syndrome cases.

We investigated the stability of β-core fragment immunoreactivity in urine samples. In 1998, at the completion of this study, we thawed and retested 20 random urine samples (10 control and 10 Down syndrome), collected at different times during the 955 day collection period (Table 4). Samples were reassayed using the same test procedures as used during the prospective study. In unaffected cases the β-core fragment values (original values 74–1700 ng/ml) were slightly increased when reassayed (reassayed values 65–2300 ng/ml). The reassayed control values were 137 ± 19 per cent (mean ± SEM) of the original values. Analyte levels in the Down syndrome cases (original values 360–20 500 ng/ml) were decreased when reassayed (reassayed values 335–4290 ng/ml). The reassayed Down syndrome values were 53 ± 9.5 per cent of the original value. A significant difference was noted in the changes in values in control versus Down syndrome upon reassay (t-test, $p=0.0003$). A significant difference was also noted in the changes occurring in the five lower and five higher original concentration Down syndrome samples ($p=0.001$). This indicated a relationship between the original concentration and change in values when samples were reassayed. We plotted the 20 original values against the percentage changes in values when samples were reassayed (Fig. 5). An inverse logarithmic relationship was noted between the percentage change and the original values ($r^2=0.86$). Significant variance was noted in the data points. When they were divided into five equal groups of four samples according to percentage change, the five median β-core fragment concentrations fit an inverse logarithmic regression line ($r^2=0.998$, Fig. 5). A relationship exists between original assay concentration and level when reassayed after storage in a freezer. Low original β-core fragment concentrations were associated with gains in immunoreactivity, mid-range concentration with little change in immunoreactivity, and high β-core fragment levels with losses in immunoreactivity when reassayed.

We considered the possibility that aggregation of monomeric β-core fragment and disaggregation of endogenously formed β-core fragment complexes may be the cause of the concentration–immunoreactivity phenomena. The 20 random samples were reassayed after incubation for 1 hour with a dissociation buffer, and diluted to test concentrations with normal dilution buffer (Table 4). While the dissociation treatment had a small and variable effect on the reassayed control samples value (-10 ± 7.1 per cent, mean ± SEM), it increased 9 of 10 Down syndrome samples values (56 ± 14 per cent, t-test control versus Down syndrome, $p=0.004$). Concentration-related aggregation is probably a contributing factor to the variability of β-core fragment results after storage.

DISCUSSION

Consistency is essential in Down syndrome screening. Widely varying results have been reported using urine

Table 4—Repeat of β-core fragment assay in February 1998 after storage of samples for 3 to 30 months in a −20°C freezer. Samples were either thawed and tested by the same procedures used previously (final dilutions 250 ×, 1000 × and 4000 ×), or diluted 10-fold in dissociation buffer (0.2 M sodium acetate, 1 M sodium chloride, 1 per cent triton × 100, pH 5), incubated for 1 hour at 37°C, before assay at these same final dilutions

Time in freezer[a] (months)	Gestational age (weeks)	Original value[a] (ng/ml)	Repeat value 2/2/98 normal procedures		Repeat value 2/2/98 with dissociation buffer		Change due to dissociation buffer (%)
			(ng/ml)	(% change)	(ng/ml)	(% change)	
Control samples							
22	16.4	74	65	88%	56	76%	−14%
21	8.3	250	339	136%	290	116%	−15%
10	15	488	987	202%	880	180%	−11%
11	15	493	1400	284%	982	199%	−30%
3	13.6	548	495	90%	710	130%	43%
22	15.9	670	1210	181%	870	130%	−28%
12	14.7	734	1010	138%	1140	155%	13%
22	15.9	880	940	107%	1120	127%	19%
22	15.9	1440	2300	160%	2110	147%	−8.3%
29	15.7	1700	2000	118%	2000	118%	0%
			Mean	137%[b]		130%	−10%
			SEM	19%		11%	7.1%
Down syndrome cases							
24	21	360	335	93%	218	61%	−35%
30	18.7	950	847	89%	1513	159%	79%
21	16.4	1180	675	57%	896	76%	33%
6	15	1270	1170	92%	1860	147%	59%
26	15.9	1350	860	64%	1025	76%	19%
10	18.9	1800	680	38%	1107	61%	63%
26	18.7	2200	1070	49%	1710	78%	60%
13	15.9	4660	555	12%	850	18%	53%
28	17.1	7000	2080	30%	4720	67%	127%
10	16.4	20500	4290	21%	5070	25%	18%
			Mean	53%[c,d]		72%	56%[e]
			SEM	9.5%		14%	14%

[a]Samples were collected over a 955-day period and tested for β-core fragment within a week of collection (original value). Time in freezer is months since urine collected.
[b]*t*-test, percentage change, lower half versus higher half of original assay value (control samples), $p=0.64$.
[c]*t*-test, percentage change, lower half versus higher half of original assay value (Down syndrome cases), $p=0.001$.
[d]*t*-test, percentage change, Down syndrome cases versus control samples, $p=0.0003$.
[e]*t*-test, percentage change, Down syndrome cases versus control samples, $p=0.004$.

β-core fragment to detect Down syndrome pregnancies. Some studies have used fresh samples, others have used frozen or otherwise stored collections of urine samples (Cuckle *et al.*, 1994, 1995; Canick *et al.*, 1995; Hayashi and Kozu, 1995; Spencer *et al.*, 1996; Kellner *et al.*, 1997; Isozaki *et al.*, 1997). The Chiron Collaborative UGP Study collected over 6731 urine samples at a large number of different centres in the United States and Great Britain. In this study, interim freezing, refrigeration or other storage conditions were a necessity for accumulating and then testing such a large number of urine samples (Canick *et al.*, 1999; Cuckle, Canick and Kellner, paper submitted). The reported variability in immunoassay results has almost completely abandoned interest in urine β-core fragment as a useful screening marker. Here we report the results of a long prospective study. It was carried out exclusively with fresh samples collected within a single university hospital system. Good screening performance was observed. Here we investigated the potential causes of variability in β-core fragment screening data. We found that freezing or storage of samples may lead to aggregation of β-core fragment and to variability in immunoassay data. This study should rekindle interest in urine β-core fragment. It should also make testing laboratories aware that a study with fresh samples may be needed before abandoning or blackballing a marker.

In this study, urine β-core fragment levels were 5.5-fold elevated in Down syndrome pregnancies. As a single marker, urine β-core fragment detected 65 per cent of Down syndrome cases at 5 per cent false-positive rate. This is a screening performance similar to or better than the serum triple-screen test (approximately 60 per cent detection at 5 per cent false-positive rate (Knight *et al.*, 1998)).

Kellner *et al.* (1997) found that urine total oestriol measurements complement β-core fragment determinations. We concurred and found that modelling β-core fragment with maternal age and total oestriol levels raised the sensitivity to 82 per cent detection at 5 per cent false-positive rate. Similarly, modelling β-core fragment:total oestriol ratio with maternal age permits

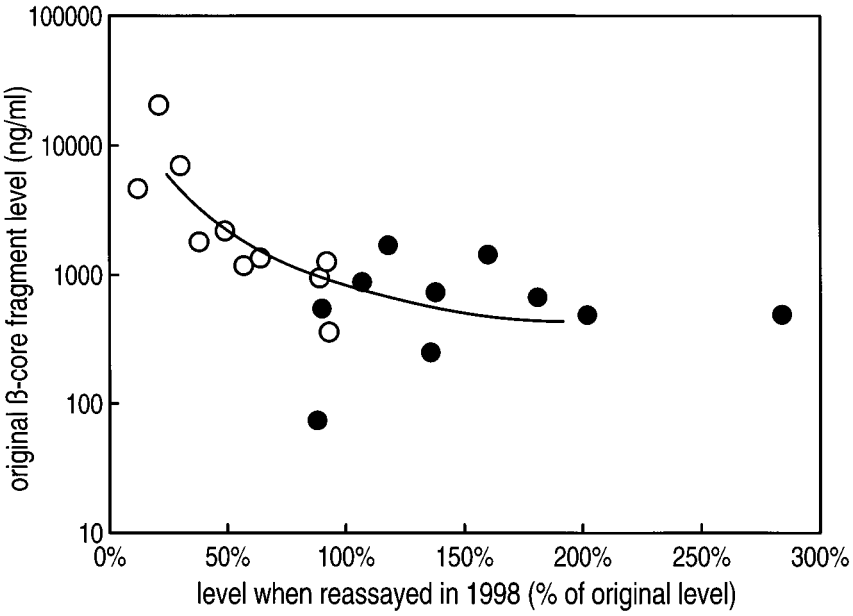

Fig. 5—The effect of β-core fragment concentration on the constancy of urine β-core fragment immunoreactivity after storage in a freezer. The original β-core fragment levels (ng/ml, determined with one week of collection) of 10 random Down syndrome (○) and 10 unaffected pregnancy samples (●), were plotted against changes in immunoreactivity when reassayed in 1998. Percent change values were divided into 5 equal groups. An inverse logarithmic regression line is shown fitting the median β-core fragment values for the 5 groups (r^2=0.998)

77 per cent detection at 5 per cent false-positive rate. Using either of these algorithms with two urine markers provides superior screening performance to serum screening protocols using as many as four different markers (Knight *et al.*, 1998). Urine collection is more convenient and more acceptable to patients than blood drawing, and does not require centrifugation and separation. There are also less handling restrictions (blood-born pathogens) than with blood-based samples. Urine screening with β-core fragment and total oestriol should be considered as an alternative to current serum protocols.

In this study, samples were collected and tested over approximately a three-year period. To accurately determine the consistency of our findings large sample numbers were needed. We divided the study into three equal sample collection periods (378 samples each). No trend was noted in the median control sample MoM values or significant change in the detection rates for Down syndrome in the initial, middle or final collection periods. It was therefore concluded that β-core fragment levels determined continuously on fresh urine samples yielded consistent results, as would be desirable for Down syndrome screening.

Values were normalized to spot creatinine levels. A correlation was noted with β-core fragment MoM values and creatinine concentration. β-core fragment values were slightly overexaggerated in samples with very low creatinine and somewhat under exaggerated in those with very high creatinine concentration. Correction of the creatinine normalization limitation did not change our results. The potential for error was noted with extremely concentrated or dilute spot urine samples. Canick *et al.* (1999) have found that β-core fragment levels are affected by time of urine collection, with values highest in the morning and lowest in the evening. Time of day was not recorded in this study. Future studies should consider time of day and correction of extremely dilute or concentrated samples in their final algorithms. This may further improve urine β-core fragment screening statistics.

β-core fragment is an unusual glycoprotein. It lacks the sialic acid residues found on hCG and free β-subunit (Birken *et al.*, 1988; Cole *et al.*, 1997c). This eliminates the acidic component of the glycoprotein, leaving it with a basic charge (Birken *et al.*, 1988). A mixture of single or monomeric β-core fragment molecules and dimeric and trimeric β-core fragment polymers is found in urine samples (Birken *et al.*, 1988). Aggregates between β-core fragment and other proteins are also common in urine samples (Kardana *et al.*, 1993).

At the end of the prospective study, we investigated the potential causes of the variable results reported with β-core fragment in Down syndrome screening. We thawed out and retested 20 of our samples. Significantly different results were found when samples were retested. In general, samples that initially had low β-core fragment levels (control samples) gained immunoreactivity when thawed and retested. Those with middle range levels (control and Down syndrome samples) had the least change in immunoreactivity, and those with initially high β-core fragment levels (Down syndrome samples) lost immunoreactivity when retested. A significant difference was found in the changes occurring in control and Down syndrome samples, and in high and low concentration Down syndrome samples. A trend was indicated linking

initial concentration (74 to 20 500 ng/ml) with change in assay value when retested (284 per cent to 12 per cent). Aggregates of β-core fragment, whether dimers, trimers or aggregates with other proteins will not be correctly detected by β-core fragment immunoassays. Most β-core fragment immunoassays use one antibody to immobilize β-core fragment and a second assay to detect and label the captured molecule (Cole *et al.*, 1997b). Such assays may detect a trimeric β-core fragment, for instance, as one rather than three molecules. Aggregation may interfere with β-core fragment determinations, particularly in frozen and thawed samples.

The law of mass action and equilibrium kinetics governs aggregation of molecules (X+X↔XX, X+XX↔XXX), with increasing concentrations of molecules driving the kinetics towards aggregation and decreasing levels forcing disaggregation (Kittsley, 1969). Aggregation may explain the appearance and disappearance of β-core fragment when retested and the observed concentration relationship. In this article we found a 5.4-fold increase in β-core fragment levels in Down syndrome pregnancies in freshly tested samples. If, after storage or freezing and thawing, aggregation hides the high concentration of Down syndrome samples, and disaggregation exposes additional molecules in low level control samples, the 5.4-fold difference between Down syndrome and control samples may be reduced. Aggregation may be an explanation of the variable findings reported with β-core fragment in Down syndrome screening.

We attempted to disaggregate the frozen and thawed samples with a dissociation buffer. After treatment, samples were diluted to test concentrations with normal buffer and assayed again. While the dissociation buffer made little change to control samples (already disaggregated from the storage process), it regained approximately half the immunoreactivity (+56 per cent) lost by the Down syndrome samples. This was consistent with the aggregation hypothesis. We do not know why it only recovered half the immunoreactivity. We must consider the possibility that the dissociation treatment was not completely effective, the possibility that samples partially re-aggregated when diluted out of the high salt dissociation buffer, or the likelihood that other reactions, beyond aggregation, may affect Down syndrome β-core fragment values.

Recently, we examined the peptide and oligosaccharide structures of β-core fragment purified from normal pregnancy and Down syndrome pregnancy urine. Additional N-acetylglucosamine sugar residues were found on the N-linked oligosaccharide side chains of Down syndrome pregnancy molecules (Cole *et al.*, 1997c). These additional sugar residues may selectively promote aggregation of Down syndrome molecules, or in other ways destabilize or interfere with the detection of frozen and thawed Down syndrome pregnancy urine samples. It is concluded that aggregation and the law of mass action, and/or specific structural variations in the Down syndrome pregnancy molecule, are plausible explanations for the changeable β-core fragment screening statistics reported between fresh and variably stored and frozen urine samples.

We conclude that β-core fragment is a highly sensitive marker for Down syndrome pregnancy. It needs to be considered seriously as a potential replacement for serum screening protocols. β-core fragment can be used on its own, as a β-core fragment:total oestriol ratio, or modelled with maternal age and total oestriol for the detection of Down syndrome. Consistent results are reported over an approximately three-year period. Testing samples fresh within one week of collection, as one would in practice, and not stored, accumulated or frozen, may be essential for achieving good and consistent Down syndrome screening performance. Kits are now sold for specifically measuring β-core fragment by Toagosei Co. Ltd., Tokyo, Japan (UGF kit) and Novamed Ltd., Jerusalem, Israel, (β-core fragment kit), and will soon be available from Quest Diagnostics/Nichols Institute Diagnostics, U.S.A. These can be utilized to realize the full potential of fresh urine measurements.

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REFERENCES

Birken S, Armstrong EG, Kolks MAG, Cole LA, Agosto GM, Krichevsky A, Canfield RE. 1988. The structure of the human chorionic gonadotropin β-subunit core fragment from pregnancy urine. *Endocrinology* **123:** 572–583.

Canick JA, Kellner LH, Saller DN Jr, Palomaki GE, Walker RP, Osathanondh R. 1995. Second trimester levels of maternal urinary gonadotropin peptide in Down syndrome pregnancy. *Prenat Diagn* **15:** 752–759.

Cannick JA, Kellner LH, Cole LA, Cockle HS. 1999. Urinary analyte screening: a noninvasive detection method for Down syndrome. *Molec Med Today*, in press.

Cole LA, Kardana A, Park S-Y, Braunstein GD. 1993. The deactivation of hCG by nicking and dissociation. *J Clin Endocrinol Metab* **76:** 704–710.

Cole LA, Kardana A, Seifer DB, Bohler HC. 1994. Urine hCG β-subunit core fragment, a sensitive test for ectopic pregnancy. *J Clin Endocrinol Metab* **78:** 497–499.

Cole LA, Acuna E, Isozaki T, Palomaki GE, Bahado-Singh RO, Mahoney MO. 1997a. Combining β-core fragment and total oestriol measurements to test for Down syndrome pregnancies. *Prenat Diagn* **17:** 1125–1133.

Cole LA, Kellner LH, Isozaki T, Palomaki GE, Iles RK, Walker R, Ozaki M, Canick J. 1997b. Comparison of 12 assays for detecting hCG and related molecules in urine samples from Down syndrome pregnancies. *Prenat Diagn* **17:** 607–614.

Cole LA, Cermik D, Bahado-Singh R. 1997c. Oligosaccharide variants of hCG-related molecules: Potential screening markers for Down syndrome. *Prenat Diagn* **17:** 1188–1190.

Cuckle HS, Iles RK, Chard T. 1994. Urinary β-core human chorionic gonadotropin: a new approach to Down's syndrome screening. *Prenat Diagn* **14:** 953–958.

Cuckle HS, Iles RK, Sehmi IH, Chard T, Oakey RE, Davies S, Ind T. 1995. Urinary multiple marker screening for Down's syndrome. *Prenat Diagn* **15:** 745–751.

Hallahan TW, Krantz DA, Tului L, Alberti E, Buchanan PD, Orlandi F, Klein V, Larsen JW Jr, Macri JN. 1998. Comparison of urinary free beta (hCG) and beta-core (hCG) in prenatal screening for chromosomal abnormalities. *Prenat Diagn* **18:** 893–900.

Hayashi M, Kozu H. 1995. Maternal urinary β-core fragment of hCG/creatinine ratios and fetal chromosomal abnormalities in the second trimester of pregnancy. *Prenat Diagn* **15:** 11–16.

Isozaki T, Palomaki GE, Bahado-Singh RO, Cole LA. 1997. Screening for Down syndrome pregnancy using β-core fragment: prospective study. *Prenat Diagn* **17:** 407–413.

Kardana A, Bagshawe KD, Coles B, Read D, Taylor M. 1993. Characterization of urinary gonadotropin peptides (UGP) and its relationship with β-core fragment. *Br J Cancer* **67:** 686–692.

Kellner LH, Canick JA, Palomaki GE, Neveux LM, Saller DN, Walker RP, Osathanondh R, Bombard AT. 1997. Levels of urinary β-core fragment, total oestriol and the ratio of the two in second trimester screening for Down syndrome. *Prenat Diagn* **17:** 1135–1141.

Kittsley SL. 1969. Chemical equilibria and free energy. In: Kittsley SL (ed) *Physical Chemistry*, 3rd edition. New York: Harper and Row; 75–85.

Knight GJ, Palomaki GE, Neveux LM, Fodor KK, Haddow JE. 1998. hCG and the free β-subunit as screening test for Down syndrome. *Prenat Diagn* **18:** 235–245.

Palomaki GE, Knight GJ, McCarthy J, Haddow JE, Eckfeldt JH. 1993. Maternal serum screening for fetal Down syndrome in the United States: a 1992 survey. *Am J Obstet Gynecol* **169:** 1558–1562.

Palomaki GE, Neveux LM, Haddow JE. 1995. Are DADs (discriminant aneuploidy detection) as good as MoMs (multiples of the median)? *Am J Obstet Gynecol* **173:** 1895–1897.

Royston P, Thompson SG. 1992. Model based screening by risk with application to Down's syndrome screening. *Stat Med* **11:** 257–268.

Spencer K, Aitken DA, Macri JN, Buchanan PD. 1996. Urine free beta hCG and beta core in pregnancies affected by Down's syndrome. *Prenat Diagn* **16:** 605–613.