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COMBINING β-CORE FRAGMENT AND TOTAL OESTRIOL MEASUREMENTS TO TEST FOR DOWN SYNDROME PREGNANCIES

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SUMMARY

Recent articles by Cuckle *et al.*, Canick *et al.*, and Isozaki *et al.* have evaluated urine β-core fragment as a screening test for Down syndrome in second-trimester pregnancies. They found over four-fold elevation of β-core fragment levels in Down syndrome pregnancies, and between 62 and 88 per cent detection of this trisomy at a 5 per cent false-positive rate. Urine β-core fragment may be a superior screening test for Down syndrome pregnancies. In the present study, urinary total oestriol has been evaluated as a marker to use in combination with β-core fragment in screening for Down syndrome pregnancies. The two markers were evaluated separately in relation to the urine creatinine concentration. To amplify screening performance, we evaluated the ratio of β-core fragment to total oestriol levels (creatinine-independent). β-core fragment and total oestriol levels were determined (normalized to creatinine, ng/mg creatinine) in urine samples from 480 unaffected and 12 Down syndrome pregnancies, collected consecutively at a single prenatal diagnosis centre. The median β-core fragment level in Down syndrome cases was 4·5 MOM. Fifty-eight per cent of Down syndrome cases had β-core fragment levels exceeding the 95th centile of unaffected pregnancies. The median total oestriol level in Down syndrome cases was 0·33 MOM. Forty-two per cent of Down syndrome cases had total oestriol levels exceeding the 95th centile of unaffected pregnancies. We investigated the ratio of the two determinants (β-core fragment, ng/ml ÷ total oestriol, ng/ml) in our sample set. The median β-core fragment:total oestriol ratio in Down syndrome cases was 13 MOM. Seventy-five per cent of Down syndrome cases had a ratio exceeding the 95th and the 99·5th centile of unaffected pregnancies. Total oestriol complements β-core fragment in urine screening for Down syndrome pregnancies. A test measuring the ratio of the two urine determinants may be a significant improvement over current serum methods for detecting Down syndrome.

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INTRODUCTION

Human chorionic gonadotropin (hCG) is a glycoprotein hormone composed of two dissimilar subunits, α- and β-, joined non-covalently. In addition to hCG dimer, free α- and free β-subunits can be detected in serum samples. The same molecules plus β-core fragment, a metabolite of β-subunit comprising residues 6 to 40 and 55 to 92 held together by disulphide bonds, are detectable in urine samples (Birken *et al.*, 1988).

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Down syndrome (trisomy 21) is the most common chromosomal cause of mental retardation. Abnormalities in the levels of certain maternal serum biochemicals are known to correlate with risk for Down syndrome. In the late 1980s, a triple test was developed for detecting Down syndrome in second-trimester pregnancies. It comprised hCG, α-fetoprotein (AFP), and unconjugated oestriol immunoassays (Bogart *et al.*, 1987; Wald *et al.*, 1988; Canick *et al.*, 1988). More recently, serum free β-subunit tests and free β-subunit–AFP combinations have been introduced as alternative tests for screening second-trimester pregnancies (Macri *et al.*, 1990; Spencer *et al.*, 1992, 1993; Wald *et al.*, 1993). The free β-subunit combination and the triple test, however, detect only 60–65 per cent of Down syndrome cases, with a 5 per cent false-positive rate. At these detection and false-positive rates many unnecessary amniocenteses are performed, and a significant minority of Down syndrome fetuses go undetected.

Recently, Cuckle *et al.* (1994) measured urine β-core fragment in seven Down syndrome and 67 unaffected second-trimester pregnancies. They found higher detection rates than the triple test. They indicated that urine β-core fragment may be a better discriminator of Down syndrome pregnancies than serum tests. A year later, the same group published the results of a larger retrospective study with 24 Down syndrome and 294 unaffected 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) reported similar results in a retrospective study of 14 Down syndrome and 91 unaffected cases: 88 per cent detection at a 5 per cent false-positive rate. Recently, we published the results of a prospective study of β-core fragment in Down syndrome pregnancies, testing over a 9-month period 726 consecutive urine samples collected at a single prenatal diagnosis centre (Isozaki *et al.*, 1997). Seven of thirteen (62 per cent) Down syndrome pregnancies had elevated β-core fragment levels (>95th centile). While this was a lower detection rate than had been suggested in the earlier papers, it was still very notable. It was similar to that of the serum triple test and superior to any other single assay for Down syndrome screening. A survey of published Down syndrome screening results using urine markers is presented in Table I.

In the present study, urinary total oestriol has been evaluated as a possible marker to use in combination with β-core fragment in screening prenatally for Down syndrome. β-Core fragment levels increase and total oestriol levels decline in Down syndrome cases. In an effort to amplify Down syndrome screening performance, we evaluated the ratio of these diverging determinants.

MATERIALS AND METHODS

Urine samples were collected on a continuous basis from women with singleton pregnancies at 12–24 weeks of gestation, coming for amniocentesis at the Maternal–Fetal Medicine Unit at Yale-New Haven Hospital. Between August 1995 and May 1996, urine samples were volunteered (whilst waiting or during preparation for amniocentesis). Oral consent was sought, using a protocol approved by the Yale University Human Investigation Committee. Urine samples were collected from 726 pregnancies as described in a previous paper (Isozaki *et al.*, 1997). Seventy-one per cent of the 726 women sought amniocentesis because of advanced maternal age concerns. These non-biased samples were evaluated in this study (480 normal karyotype and 12 Down syndrome pregnancies). No selection occurred, and no additional Down syndrome samples were added.

Urine samples were refrigerated immediately after collection and were carried to the laboratory twice each week for β-core fragment assay and creatinine determination. The gestational age of all cases was determined by ultrasound and was obtained prospectively from the ultrasound database. Two to three weeks after urine collection, the karyotype was obtained from the records at the Prenatal Diagnosis Section in the Department of Genetics at Yale University. Samples were stored in a freezer at -20°C. Total oestriol was determined on all samples (frozen) at the completion of the collection period.

β-Core fragment levels were determined by the B210 two-step sandwich assay, as described previously (Cole *et al.*, 1994). Briefly, microtitre plates were coated with monoclonal antibody B210 (a gift from O'Connor and Canfield at Columbia University, New York, NY, U.S.A.); urine samples were added; and β-core fragment was extracted. Plates were washed and peroxidase-labelled hCGβ antisera (Bios Specific, Emmeryville, CA, U.S.A.) were added to quantitate the bound β-core fragment. After a further

Table I—Reports of urinary markers for second-trimester Down syndrome pregnancies

Reference	Test	Control samples (No.)	Down syndrome (No.)	Detection rate, % (false-positive rate, %)
Reports of urinary markers (all)				
Cuckle *et al.* (1994)	β-Core fragment	67	7	>80 (5)
Cuckle *et al.* (1995)	β-Core fragment	294	24	80 (5)
Canick *et al.* (1995)	β-Core fragment	91	14	88 (5)
Spencer *et al.* (1996)	β-Core fragment	400	29	41 (5)
Kellner *et al.* (1996)	β-Core fragment	206	32	72 (5)
Isozaki *et al.* (1997)	β-Core fragment	709	13	62 (5)
Cole *et al.* (1997a)	β-Core fragment	91	14	93 (10)
Hyashi and Kozu (1995)	Free β with β-core	150	5	20 (5)
Spencer *et al.* (1996)	Free β-subunit	400	29	58 (5)
Cole *et al.* (1997a)	Free β-subunit	91	14	47 (10)
Cole *et al.* (1997b)	Free β-subunit	709	13	54 (5)
Cole *et al.* (1997c)	hCG only	91	14	43 (10)
Cole *et al.* (1997a)	hCG with free β	91	14	50 (10)
Kellner *et al.* (1996)	Total oestriol	206	32	22 (5)
Cuckle *et al.* (1995)	Total oestrogens	294	24	34 (5)
Reports of serum markers (examples)				
Spencer *et al.* (1992)	Free β-subunit	2400	90	54 (5)
Ryall *et al.* (1992)	Free β-subunit	1400	57	47 (5)
Wald *et al.* (1993)	Free β-subunit	367	75	55 (5)
Spencer *et al.* (1992)	hCG with free β	2400	90	48 (5)
Ryall *et al.* (1992)	hCG with free β	1400	57	42 (5)
Wald *et al.* (1993)	hCG with free β	367	75	46 (5)
Cuckle *et al.* (1984)	AFP	36 652	61	35 (5·3)
Zeitune *et al.* (1991)	AFP	113 000	114	37 (6·7)
Wald *et al.* (1988)	Unconj. oestriol	385	77	40 (5)

wash, substrate was added and peroxidase enzyme activity was measured in a spectrometric plate reader. For this assay, urine samples were diluted in the range 1 to 50, to 1 to 10 000 with buffer for this assay. Initially, to ensure the integrity of the assay, all samples were tested at two dilutions (1 to 100 and 1 to 1000). Further dilutions were made as needed. The B210 assay was standardized with β-core fragment batch SB455 (Birken, Columbia University, New York, NY, U.S.A.), which was calibrated by amino acid analysis. The B210 assay detects only β-core fragment, with less than 0·3 per cent cross-reactivity with free β-subunit and hCG.

β-Core fragment levels were normalized to adjust for variations in the urine concentration by dividing by the creatinine concentration. The creatinine concentration was determined using the Sigma Chemical Co. spectrometric creatinine kit (St Louis, MO, U.S.A.) and the recommended procedures. Urine samples were diluted 1 to 10 for creatinine determination. Immunoassay results (ng/ml) were normalized to the urine creatinine concentration (ng/mg creatinine).

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, radioiodine-labelled tracer, and a set of six standards (catalogue number TKE35). The procedures were those described in the instruction booklet. Urine samples were initially diluted 1 to 31 for immunoassay. Further dilutions, 1 to 1, 1 to

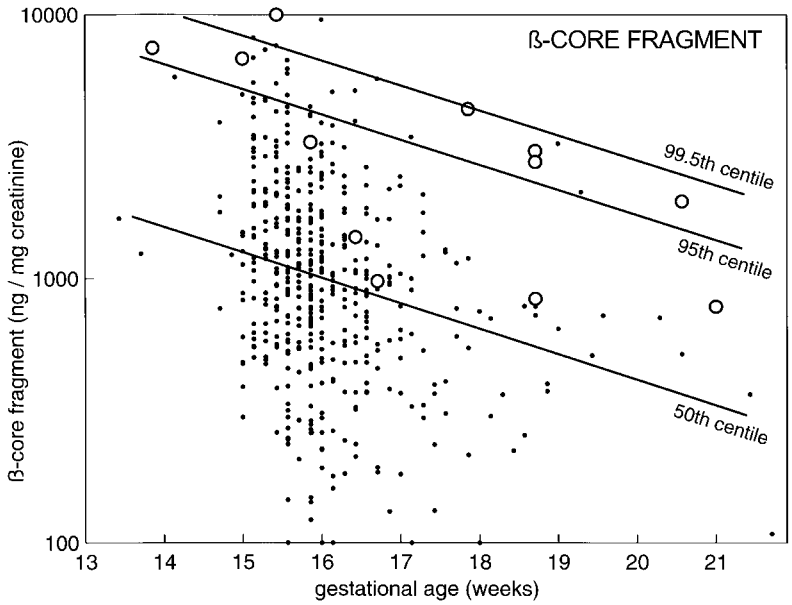

Fig. 1—Levels of *β*-core fragment in urine samples from 492 second-trimester pregnancies, 12 Down syndrome cases (○), and 480 controls (•). Concentrations are normalized to creatinine (ng *β*-core fragment/mg creatinine). Data are expressed as MOM, using the equation $\text{MOM} = x/10^{4{\cdot}5716 - 0{\cdot}1005y}$, where x is the value of the determinant and y is the gestational age (weeks)

10, and 1 to 100, were made as needed. The total oestriol levels (ng/ml) were also normalized to adjust for variations in the urine concentration by dividing by the creatinine concentration (mg/ml).

Results were analysed at the Foundation for Blood Research, Maine, using standard statistical methods (Royston and Thompson, 1992; Palomaki *et al.*, 1995). The gestational age-specific medians for the 480 control samples (*β*-core fragment, total oestriol, and *β*-core fragment:total oestriol ratio) all best fit log Gaussian distributions, between the fifth and 95th centiles, for both unaffected pregnancy and Down syndrome data (expressed as MOM).

To assess screening performance, medians and log standard deviations (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 recorded (proportion of Down syndrome cases with levels exceeding the 95th centile of unaffected pregnancies). A univariate Gaussian model (*β*-core fragment:total oestriol ratio) and a bivariate Gaussian model (*β*-core fragment and total oestriol concentrations) were used to predict detection rates and false-positive rates, considering the general age distribution of the population of the United States (Palomaki *et al.*, 1993).

RESULTS

Figures 1–3 show the levels of urine *β*-core fragment (ng/mg creatinine), urine total oestriol (ng/mg creatinine), and the ratio of the two determinants (*β*-core fragment ng/ml ÷ total oestriol ng/ml) in 480 unaffected and 12 Down syndrome pregnancies.

The *β*-core fragment measurements in unaffected pregnancies best fit a log Gaussian distribution. The log median for unaffected samples was 0·01 and the log standard deviation was 0·37. Table II shows the karyotype, ages, and MOM values for the Down syndrome cases. The *β*-core fragment levels in all 12 Down syndrome cases exceeded 1·0 MOM, with a median value of 4·5 MOM (log median 0·65, log SD 0·31). As shown in Fig. 1, seven of 12 Down syndrome cases had *β*-core fragment levels exceeding the 95th centile of unaffected pregnancies.

Total oestriol measurements also best fit a log Gaussian distribution. The log median for

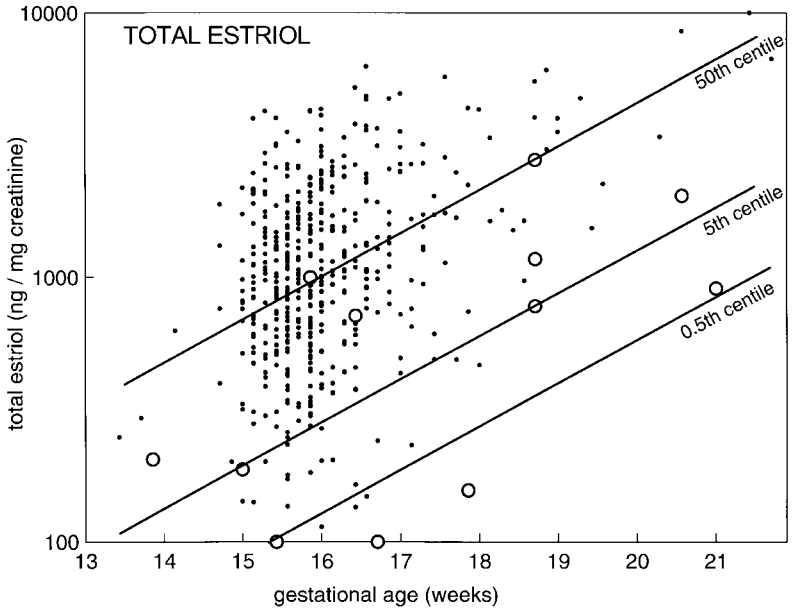

Fig. 2—Total oestriol levels in urine samples from 492 second-trimester pregnancies, 12 Down syndrome cases (○), and 480 controls (•). Concentrations are normalized to creatinine (ng total oestriol/mg creatinine). Data are expressed as MOM, using the equation $MOM = x/10^{0.4627+0.1641y}$, where x is the value of the determinant and y is the gestational age (weeks)

unaffected samples was 0·01 and the log standard deviation was 0·32. Total oestriol levels in ten of 12 Down syndrome cases were less than 1·0, with a median value of 0·33 MOM (log median −0·48, log SD 0·41) (Table II). As shown in Fig. 2, five of 12 Down syndrome cases had total oestriol levels below the fifth centile of unaffected pregnancies.

β-Core fragment and total oestriol levels were both normalized to the urine creatinine concentration. β-Core fragment levels were elevated and total oestriol levels were reduced in Down syndrome pregnancies. We considered the possibility of eliminating the need for creatinine determinations by measuring the ratio of β-core fragment to total oestriol levels (β-core fragment ng/ml ÷ total oestriol ng/ml). This ratio also best fits a log Gaussian distribution. The log median for unaffected samples was −0·04 and the log standard deviation was 0·42. The ratio was very high in Down syndrome pregnancies; all 12 cases exceeded 1·0 MOM, with a median of 13 MOM (log median 1·13, log SD 0·63) (Table II). Measuring the determinants as a ratio amplified the differences between Down syndrome and control samples. As shown in Fig. 3, the ratio in nine of 12 Down syndrome cases exceeded both the 95th and the 99·5th centile of unaffected pregnancies.

The results of our chemical tests are age-independent. Screening performance was predicted using univariate (ratio) and bivariate (β-core fragment and total oestriol) Gaussian models, considering the distribution parameters and the general age distribution in the United States (Fig. 4). Very similar performance was predicted using the ratio of the analytes (85, 78, and 71 per cent detection with false-positive rates of 10, 5, and 2·5 per cent, respectively), or by using β-core fragment and total oestriol as independent creatinine-adjusted variables (86, 79, and 71 per cent detection with false-positive rates of 10, 5, and 2·5 per cent, respectively).

DISCUSSION

Seven papers have been published using specific urine β-core fragment assays to evaluate Down syndrome pregnancies (Table I). Six of these papers (Cuckle *et al.*, 1994, 1995; Canick *et al.*, 1995; Cole *et al.*, 1997a; Kellner *et al.*, 1996;

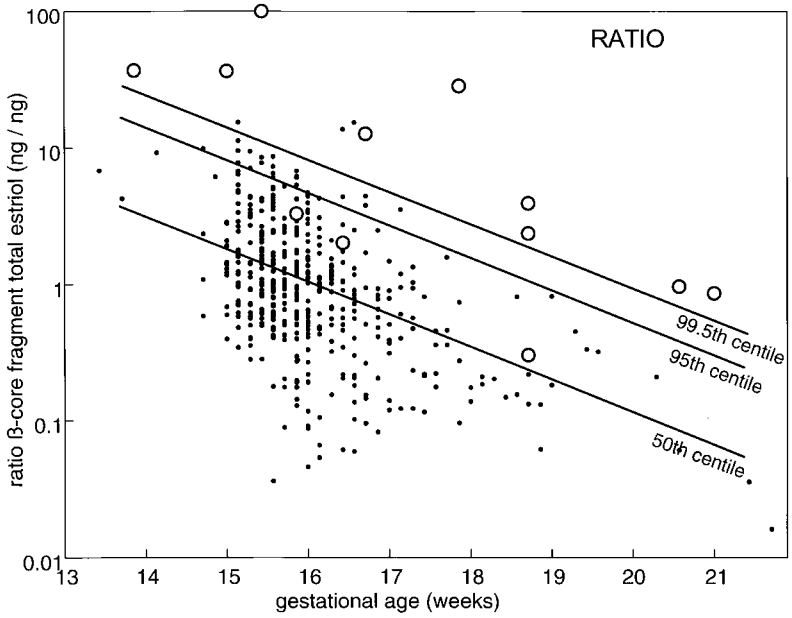

Fig. 3—*β*-Core fragment:total oestriol ratio (ng/ml *β*-core fragment ÷ ng/ml total oestriol) in urine samples from 492 second-trimester pregnancies, including 12 Down syndrome cases (○), and 480 controls (•). Data are expressed as MOM, using the equation $MOM = x/10^{3{\cdot}8449 - 0{\cdot}2463y}$, where x is the value of the determinant and y is the gestational age (weeks)

Table II—Breakdown of patients according to karyotype

Karyotype	Gestational age (weeks)	Maternal age (years)	*β*-Core fragment (MOM)	Total oestriol (MOM)	*β*-Core:oestriol ratio (MOM)
			Median 0·01	Median 1·01	Median 0·99
Normal ($n=480$)	Mean 16·0	Mean 36·7	Log median 0·01	Log median 0·01	Log median −0·04
	SD 0·09	SD 2·8	Log SD 0·37	Log SD 0·32	Log SD 0·42
			Median 4·5	Median 0·33	Median 13
Trisomy ($n=12$)	Mean 17·4	Mean 37·8	Log median 0·65	Log median −0·48	Log median 1·13
	SD 2·2	SD 3·4	Log SD 0·31	Log SD 0·41	Log SD 0·63
47,XX,+21	13·9	41	4·06	0·52	8·35
47,XX,+21	15·0	38	5·86	0·22	25·69
47,XX,+21	15·4	36	8·63	0·12	70·72
47,XX,+21	15·9	36	2·84	1·19	2·33
47,XX,+21	16·4	42	1·56	0·58	2·51
47,XX,+21	16·7	37	1·06	0·06	15·77
47,XX,+21	17·9	44	6·02	0·09	62·03
47,XX,+21	18·7	36	1·45	1·07	1·17
47,XX,+21	18·7	38	4·79	0·45	9·16
47,XX,+21	18·7	37	5·27	0·30	15·23
47,XX,+21	20·6	31	5·40	0·37	11·62
47,XX,+21	21·0	38	2·71	0·11	18·31

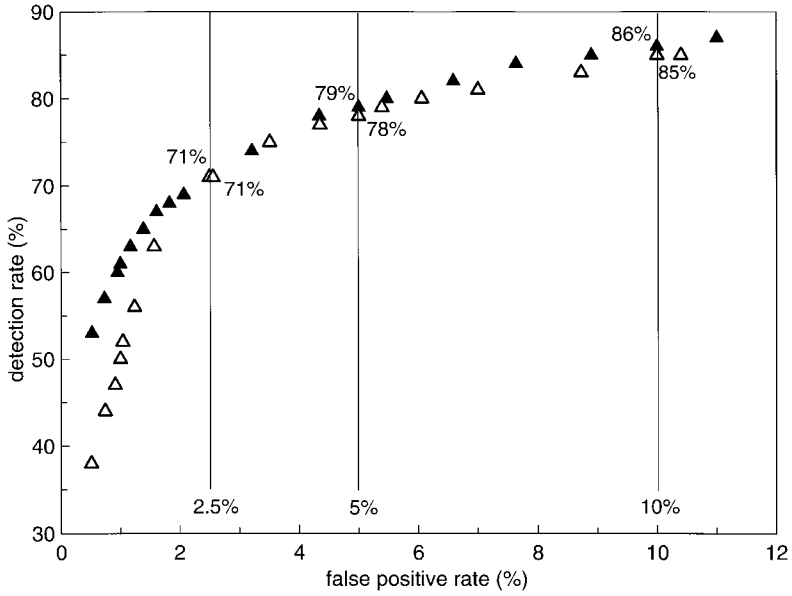

Fig. 4—ROC curve comparing the Down syndrome screening performances of β-core plus total oestriol (▲, bivariate Gaussian model) and β-core fragment:total oestriol ratio (△, univariate Gaussian model), adjusting for the maternal age distribution of the population of the United States

Isozaki *et al.*, 1997) report 4·1- to 6·0-fold elevation of β-core fragment levels in Down syndrome pregnancies and 62–88 per cent detection of Down syndrome cases at a 5 per cent false positive rate. Elevated levels and detection rates in these ranges exceed those of any other currently available single biochemical marker of Down syndrome pregnancies (Table I). The other paper reports distinctly less impressive results, 2·4-fold elevation of β-core fragment levels in Down syndrome pregnancies along with correspondingly low detection rates (Spencer *et al.*, 1996). The poor results may be explained by an aberrant control population with a high standard deviation and an irregular peak at 13 weeks' gestation.

In this study, urine β-core fragment levels were determined in a uniform population which presented over a 9-month period to a single prenatal diagnosis centre for amniocentesis because of maternal age concerns. The level of β-core fragment in the 480 unaffected pregnancy samples declined continuously from 13 to 21 completed weeks of gestation. There was a 4·4-fold elevation of β-core fragment levels in 12 Down syndrome pregnancy urine samples. In seven of the 12 Down syndrome cases, β-core fragment levels exceeded the 95th centile of the unaffected pregnancy samples. These results are similar to those reported previously by our laboratory (4·1 MOM and eight of 12 cases exceeding the 95th centile) for a wider population (those coming for amniocentesis for maternal age concerns, positive triple test or other reason, $n = 726$) (Isozaki *et al.*, 1997). While these results suggest slightly lower detection rates than other papers, they are still encouraging and confirm the superiority of urine β-core fragment as a single marker for Down syndrome screening (Table I).

Kellner *et al.* (1996) showed that taking urine total oestriol levels into consideration may improve Down syndrome detection rates. We determined total oestriol levels in our set of Down syndrome and control urine samples. The kinetics of total oestriol levels were contrary to β-core fragment levels. While β-core fragment levels declined, total oestriol levels increased from 13 to 21 weeks of pregnancy. While β-core fragment levels increased (4·4 MOM), total oestriol levels decreased (0·33 MOM) in Down syndrome pregnancies. In five of 12 (42 per cent) Down syndrome cases, very low total oestriol levels, below the fifth centile for unaffected pregnancies, were detected. Our urine total oestriol detection rate (42 per cent) is significantly higher than the 22 per cent

detection rate indicated by Kellner *et al*. (1996) using a somewhat different immunoassay (Abbott Diagnostics IMX test). It is closer to the 34 per cent detection rate indicated by Cuckle *et al*. (1995) using a urine total oestrogen assay.

We considered the divergence in the kinetics of β-core fragment and total oestriol levels, and the possibility of amplifying the differences between unaffected and Down syndrome samples by measuring the ratio of β-core fragment:total oestriol levels. This ratio was very high in Down syndrome cases, 13-fold higher than unaffected pregnancies (13 MOM). Measuring the ratio expanded the separation of Down syndrome and unaffected pregnancy samples. Nine of 12 Down syndrome cases (75 per cent) exceeded both the 95th and the 99·5th centile of unaffected pregnancies. A seventy-five per cent detection rate at a 0·5 per cent false-positive rate was achieved. This screening efficiency is extraordinary and is without equal by any currently known combinations of serum or urine assays.

Screening performance was predicted for the ratio of β-core fragment:total oestriol, and for β-core fragment and total oestriol together, by modelling methods. Very similar performance was predicted measuring either ratio, 78 per cent at 5 per cent, and 71 per cent at 2·5 per cent false-positive rate, or the two independent determinants as creatinine-normalized variables, 79 per cent at 5 per cent, and 71 per cent at 2·5 per cent false-positive rate. The use of the two markers as independent determinants requires three urine assays: β-core fragment, total oestriol, and creatinine. Measurement of the ratio, however, needs only two of these tests. The more assays that are required to generate values and determine a risk factor, the greater the error. A two-assay approach determining risk may be preferable.

Urine β-core fragment and total oestriol, while superior markers of Down syndrome pregnancy, may not be able to replace serum screening methods entirely. Serum AFP determination may still be needed for detecting neural tube defects. It is possible that AFP-related molecules may be measurable in urine samples, in which case, a triple urine test may be indicated. Ultrasound might be used instead of serum AFP as a complementary test to the urine analytes for detecting neural tube defects and aneuploid pregnancies.

We conclude that it is worthwhile to combine urine β-core fragment and total oestriol measurements to screen for Down syndrome in second-trimester pregnancies. The introduction of β-core fragment:total oestriol ratio measurements could have a profound impact on biochemical screening for Down syndrome detection. It could reduce the large number of genetic amniocenteses performed in chromosomally normal pregnancies. It could also increase the detection of Down syndrome. Double and triple serum tests detect 60–65 per cent of Down syndrome cases with a 5 per cent false-positive rate. With this new technology, we have observed 75 per cent detection at a 0·5 per cent false-positive rate. Using modelling methods, 71 per cent detection was suggested at a 2·5 per cent false-positive rate. Measurement of the ratio could replace serum double- and triple-test technology. This study, by its nature, was limited to a relatively small number of Down syndrome cases. Much larger controlled studies are needed with four or more times the number of affected and unaffected samples to confirm these findings.

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