

DIMERIC INHIBIN A AS A MARKER FOR DOWN'S SYNDROME IN EARLY PREGNANCY

DAVID A. AITKEN, PH.D., EUAN M. WALLACE, M.R.C.O.G., JENNIFER A. CROSSLEY, PH.D.,
IAN A. SWANSTON, B.SC., YVONNE VAN PAREREN, M.SC., MEREL VAN MAARLE, M.SC.,
NIGEL P. GROOME, PH.D., JAMES N. MACRI, PH.D., AND J. MICHAEL CONNOR, M.D.

Abstract Background. In screening for Down's syndrome in the second trimester of pregnancy, the concentrations of alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and intact human chorionic gonadotropin in maternal serum are widely used markers. We investigated a new marker, dimeric inhibin A, and compared its predictive value with that of the established markers.

Methods. Serum samples were obtained at 7 to 18 weeks of gestation from 58 women whose fetuses were known to be affected by Down's syndrome, 32 whose fetuses were affected by trisomy 18, and 438 whose fetuses were normal, and the samples were analyzed for each marker. Individual serum concentrations of each marker were converted to multiples of the median value at the appropriate length of gestation in the women with normal pregnancies, and rates of detection of Down's syndrome by screening for inhibin A in various combinations with the other markers were estimated by multivariate analysis.

Results. In the women with fetuses affected by Down's syndrome, the serum inhibin A concentrations were 2.06 times the median value in the women with nor-

mal pregnancies ($P < 0.001$). This compared with 2.00 times the median for the β subunit of human chorionic gonadotropin, 1.82 times the median for intact human chorionic gonadotropin, and 0.72 times the median for alpha-fetoprotein. The serum concentrations of inhibin A in the women with fetuses affected by Down's syndrome did not appear to be significantly elevated above normal until the end of the first trimester and were not significantly different from normal in the women with fetuses affected by trisomy 18 ($P = 0.17$). The rate of detection of Down's syndrome was 53 percent and the false positive rate was 5 percent when alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age were used together as predictors. The detection rate increased to 75 percent when inhibin A was added ($P = 0.002$).

Conclusions. In the second trimester of pregnancy, measuring inhibin A in maternal serum, in combination with measurements of alpha-fetoprotein and the β subunit of human chorionic gonadotropin, significantly improved the rate of detection of Down's syndrome. (N Engl J Med 1996;334:1231-6.)

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THE sensitivity and specificity of screening for Down's syndrome have improved in recent years with the identification of new biochemical markers of this disorder. Since the observation that serum levels of alpha-fetoprotein were reduced in women with fetuses affected by chromosomal abnormalities,¹ numerous other fetoplacental markers in maternal serum have been found to have altered levels in pregnancies with fetuses affected by aneuploidy. The most useful markers for screening in the second trimester are intact human chorionic gonadotropin,² the β subunit of human chorionic gonadotropin,^{3,4} unconjugated estriol,^{5,6} and alpha-fetoprotein.^{7,8} The serum levels of all these markers overlap in affected and unaffected populations, however, and so the odds that a particular value is associated with an affected pregnancy are used to modify the a priori risk specific to maternal age.⁹ The most effective approach to screening is to use combinations of markers (taking into account correlations between markers), and most protocols use alpha-fetoprotein and either intact human chorionic gonadotropin or its β subunit, with¹⁰ or without^{11,12} unconjugated estriol. In clinical practice, approximately two thirds of pregnancies in which the

fetuses are affected by Down's syndrome can be detected in the 3 to 5 percent of the screened population with the highest risk.¹³⁻¹⁷ Fetuses affected by trisomy 18, which is associated with very low levels of intact human chorionic gonadotropin and its β subunit, may also be identified with the use of a separate protocol.^{18,19}

Efforts to improve biochemical screening further have centered on the investigation of screening in the first trimester and on the search for better markers. The performance of certain markers varies between the first and the second trimesters. For example, intact human chorionic gonadotropin, a widely used marker in the second trimester, is a poor marker in the first,^{20,21} whereas the β subunit of human chorionic gonadotropin is a useful marker during both stages of pregnancy.^{21,22} Recent investigations of inhibin, a dimeric glycoprotein of placental origin composed of one α and one βA or βB subunit, found elevated serum levels in women in the second trimester of pregnancy whose fetuses were affected by Down's syndrome²³⁻²⁵ but no differences between women with such pregnancies and those with unaffected pregnancies during the first trimester.^{26,27} These studies used an enzyme immunoassay with antibodies directed against epitopes on the inhibin α subunit. Further studies using a new assay specific for dimeric inhibin A (α - βA) have suggested a possible role for dimeric inhibin A as a screening marker, since elevated serum levels have been found in women with fetuses affected by Down's syndrome in both the first and the second trimesters.^{28,29}

In this study, we extended the investigation of inhibin A to a larger group of women with fetuses affected by Down's syndrome or trisomy 18 and compared its

From the Duncan Guthrie Institute of Medical Genetics, Glasgow (D.A.A., J.A.C., Y.P., M.M., J.M.C.); the University of Edinburgh Department of Obstetrics and Gynaecology, Centre for Reproductive Biology, Edinburgh (E.M.W.); the Medical Research Council Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh (I.A.S.) — all in Scotland; the School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, England (N.P.G.); and NTD Laboratories, Huntington Station, New York (J.N.M.). Address reprint requests to Dr. Aitken at the Duncan Guthrie Institute of Medical Genetics, Yorkhill, Glasgow G3 8SJ, United Kingdom.

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performance in screening with that of the established markers alpha-fetoprotein, intact human chorionic gonadotropin, and the β subunit of human chorionic gonadotropin.

METHODS

Maternal Serum Samples

All aliquots of maternal serum used in this study came from samples collected between 1987 and 1994 by the prenatal screening service in western Scotland. To avert repeated freeze-and-thaw cycles, which might affect marker levels in future studies, frozen serum samples from women whose fetuses were confirmed as being chromosomally abnormal were routinely retrieved from storage, thawed, divided into aliquots, and returned to a freezer bank for long-term storage pending further analyses. A total of 528 samples were analyzed, including 58 samples from women whose fetuses had Down's syndrome, 32 samples from women whose fetuses had trisomy 18, and 438 control samples from women with unaffected pregnancies at 7 to 18 weeks' gestation. For each trisomic sample, three to six control samples were selected and matched for the same completed week of gestation and length of time in frozen storage (within six months). Additional controls, selected on the basis of length of gestation, were added to supplement the small number of matched serum samples collected earlier in gestation. Fourteen of the Down's syndrome samples, which were collected at 7 to 14 weeks' gestation, were part of a series previously investigated for alpha-fetoprotein, unconjugated estradiol, the β subunit of human chorionic gonadotropin, and pregnancy-associated plasma protein A.^{21,30} The remaining 44 Down's syndrome samples were collected between 15 and 18 weeks' gestation; 35 of these were part of a previous series of 37 reported in a prospective study.¹⁶ Four of the trisomy-18 samples collected at 8 to 12 weeks' gestation were also included in previous studies.^{21,30} The remaining 28 trisomy-18 samples were collected at 15 to 18 weeks' gestation. The length of gestation was estimated by calculating the number of completed weeks from the date of the last menstrual period or by ultrasound scanning. If two different estimates were obtained for the same pregnancy, reliable information on the menstrual cycle was used as the primary determinant, unless the ultrasound estimate was one or more completed weeks greater than or two or more completed weeks less than the length of gestation as determined from the last menstrual period, in which case the ultrasound estimate was used.¹⁶

Marker Analyses

Serum dimeric inhibin A was measured with a two-site enzyme-linked immunosorbent assay (ELISA) that uses an immobilized monoclonal antibody (E4) against the β A subunit of inhibin as the capture antibody. This was coupled covalently through Fc carbohydrate residues to treated hydrazide microplates (Avidplate-HZ, UniSyn Technologies, Tustin, Calif.).^{31,32} The Fab fraction of a mouse monoclonal antibody (R1) against the α subunit of inhibin, conjugated to alkaline phosphatase, was then used to bind the α subunit of the captured dimeric inhibin. A sensitive amplified-enzyme assay (Ampak, Dako, High Wycombe, United Kingdom) allowed detection of the bound second antibody. Recombinant 32-kd human inhibin A (Genentech, South San Francisco) was used for standards, with results expressed in picograms per milliliter. The intraplate and interassay coefficients of variation were 2.5 percent and 7.0 percent, respectively, and assay sensitivity was 8 pg per milliliter.

The β subunit of human chorionic gonadotropin was measured by ELISA as previously described.³³ Intact human chorionic gonadotropin was measured with the use of a commercial immunoradiometric assay (MAIA Clone, Serono, Biodata, Rome, Italy), after a 1:500 dilution of samples.¹⁶ Alpha-fetoprotein was measured with the use of a sensitive immunoradiometric assay developed in our institution.²¹

Aliquots of serum samples from all the selected women were obtained from frozen storage in Glasgow, coded, randomized, and shipped in dry ice to the Centre for Reproductive Biology in Edinburgh to be assayed for inhibin A. All other analyses of markers were performed in Glasgow. Alpha-fetoprotein and intact human chorionic gonadotropin were measured prospectively as part of the routine screening program in the second-trimester samples¹⁶ and retrospec-

Table 1. Serum Concentrations of Inhibin A, Intact Human Chorionic Gonadotropin, the β Subunit of Human Chorionic Gonadotropin, and Alpha-Fetoprotein in Control Women and Women with Pregnancies Affected by Down's Syndrome at Various Lengths of Gestation.*

MARKER	GESTATIONAL RANGE (WEEKS)	multiples of the median (no. of women)			P VALUE‡
		CONTROLS†	DOWN'S SYNDROME		
Inhibin A	7-11	1.00 (148)	0.98 (8)	0.68	
	13-14	1.00 (58)	2.60 (6)	0.002	
	15-18	1.00 (202)	2.24 (44)	<0.001	
Combined	7-18	1.00 (438)	2.06 (58)	<0.001	
Intact human chorionic gonadotropin	7-11	1.08 (148)	0.95 (8)	0.62	
	13-14	0.93 (58)	1.19 (6)	0.09	
	15-18	1.03 (202)	2.04 (44)	<0.001	
Combined	7-18	1.03 (438)	1.82 (58)	<0.001	
β Subunit of human chorionic gonadotropin	7-11	1.00 (140)	1.79 (8)	0.02	
	13-14	0.94 (58)	2.15 (6)	0.002	
	15-18	1.03 (112)	2.05 (44)	<0.001	
Combined	7-18	1.00 (340)	2.00 (58)	<0.001	
Alpha-fetoprotein	7-11	0.99 (148)	0.70 (8)	0.10	
	13-14	0.96 (58)	0.59 (6)	0.02	
	15-18	0.95 (202)	0.74 (44)	<0.001	
Combined	7-18	0.95 (438)	0.72 (58)	<0.001	

*Values are expressed in multiples of the median among the women with normal pregnancies.

†Combined numbers include 30 controls studied at 12 weeks' gestation. The controls studied for the β subunit of human chorionic gonadotropin were a subgroup of the 438 controls.

‡P values were determined by the Mann-Whitney test.

tively in the first-trimester samples.²¹ The β subunit of human chorionic gonadotropin was measured in all samples retrospectively. All data were collated in Glasgow, and sample decoding and data analysis were performed only after all assays had been completed.

Statistical Analysis

For inhibin A, the median level at each completed week of gestation was calculated from the results of the analyses in the control women's serum samples and used to convert individual marker values to multiples of the normal gestational median. Multiples of the median for alpha-fetoprotein, intact human chorionic gonadotropin, and the β subunit of human chorionic gonadotropin were calculated with the use of medians previously determined in a larger series of women with normal pregnancies from which the 438 controls were selected (Table 1). Goodness of fit to log gaussian distributions for the marker values in the women with normal fetuses and in those with chromosomally abnormal fetuses was assessed by probability plot and the Kolmogorov-Smirnov test. The measures of the distribution (means and standard deviations) for each marker in the control women and in women whose fetuses were affected by Down's syndrome were calculated by taking the \log_{10} of the median as the mean and the difference between the 10th and 90th percentiles in logs divided by 2.56 as the standard deviation.⁷ Correlations between pairs of markers were estimated with the use of log-transformed values.

Detection rates for Down's syndrome and corresponding false positive rates were calculated with gaussian models of the distribution of likelihood ratios and the age distribution of pregnancies in western Scotland. First, the proportion of fetuses affected by Down's syndrome that would be expected at each individual maternal age was calculated from the age-related risk⁸ and the proportion of pregnancies at each age in the population. Next, likelihood ratios were calculated for each marker and combination of markers for all samples from women with fetuses affected by Down's syndrome and from the control women with the use of the variables derived in this study.⁹ A specific risk threshold was then selected to define a high-risk group, and cutoff likelihood ratios were calculated from the age-specific risks. The distribution of likelihood ratios for the samples from women whose fetuses had Down's syndrome was then used to calculate the predicted detection rate at each maternal age. This detection rate was

multiplied by the proportion of fetuses affected by Down's syndrome expected for that age, and the overall detection rate was obtained by summation. Similarly, the corresponding false positive rates at each threshold risk were obtained with the use of the distribution of likelihood ratios for the control samples and the proportion of pregnancies at each individual maternal age in the population of pregnant women in western Scotland.

RESULTS

Serum concentrations of inhibin A in women with normal pregnancies rise to a median of about 550 pg per milliliter at 8 to 9 weeks' gestation, followed by a decline that levels out at about 180 pg per milliliter at 15 weeks' gestation. The serum levels of inhibin A were elevated in the women whose fetuses were affected by Down's syndrome, with an overall median value 2.06 times the median among the women with normal pregnancies (by the Mann-Whitney test, $P < 0.001$). The distribution of results according to length of gestation (Fig. 1) shows a rising trend, from a median value 0.98 times the median among the women with normal pregnancies at 7 to 11 weeks' gestation ($P = 0.68$) to 2.24 times the median among the women with normal pregnancies at 15 to 18 weeks' gestation ($P < 0.001$) (Table 1). This trend appears similar to that for intact human chorionic gonadotropin but contrasts with that for the β subunit of human chorionic gonadotropin, which is associated with elevated levels in women with fetuses affected by Down's syndrome both early and later in gestation (Table 1). Alpha-fetoprotein levels are reduced in the first and second trimesters in women with fetuses affected by Down's syndrome.

The means and standard deviations of the log gaussian distributions for inhibin A, intact human chorionic gonadotropin, the β subunit of human chorionic gonadotropin, and alpha-fetoprotein are summarized in Table 2. The goodness of fit for inhibin A in women with fetuses affected by Down's syndrome and in control women at 15 to 18 weeks of gestation was examined with the use of probability plots. Neither log-transformed nor untransformed values gave a perfectly straight line, but the fit to the log gaussian distributions was better, as assessed by the Kolmogorov-Smirnov test ($P = 0.51$ for Down's syndrome; $P = 0.06$ for normal pregnancies), and subsequent statistical analyses were based on the assumption of log normality. Previous studies of alpha-fetoprotein, intact human chorionic gonadotropin, and the β subunit of human chorionic gonadotropin have shown that these markers fit log gaussian distributions.^{8,11,12}

Correlation coefficients for inhibin A with the β subunit of chorionic gonadotropin, intact human chorionic gonadotropin, alpha-fetoprotein, and maternal age both in women with normal pregnancies and in those with fetuses affected by Down's syndrome are summarized in Table 3. The strongest correlation was with intact human chorionic gonadotropin; there was no significant correlation between inhibin A and the β subunit of human chorionic gonadotropin in the second trimester in either the control women ($P = 0.11$) or those with fetuses affected by Down's syndrome

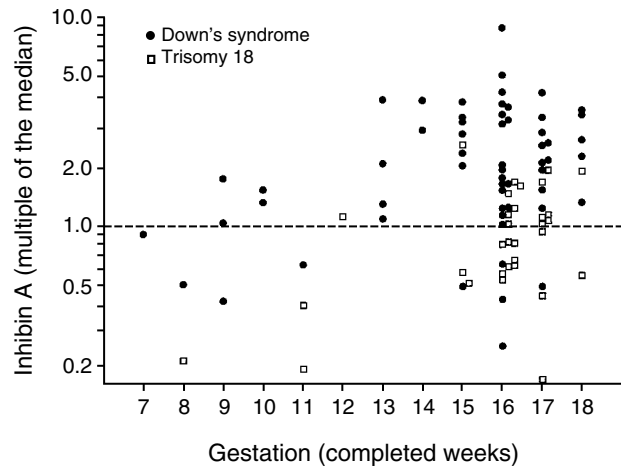


Figure 1. Serum Concentrations of Inhibin A, Expressed in Multiples of the Median Value in Women with Normal Pregnancies, in 58 Pregnant Women with Fetuses Affected by Down's Syndrome and 32 with Fetuses Affected by Trisomy 18 at 7 to 18 Weeks' Gestation.

($P = 0.13$). Intact human chorionic gonadotropin and the β subunit of human chorionic gonadotropin were strongly correlated.

Using the statistical characteristics of the distributions of each marker in the control women and in those whose fetuses were affected by Down's syndrome at 15 to 18 weeks' gestation, we calculated detection rates at a fixed 5 percent false positive rate for each marker in combination with maternal age, and for various groups of markers in combination with maternal age, in a typical screened population (Table 4). We obtained the highest predicted detection rate (75 percent at a 5 percent false positive rate) by adding inhibin A to the combination of alpha-fetoprotein and the β subunit of human chorionic gonadotropin. This protocol resulted in a 22 percent increase in the detection of Down's syndrome when tested against both the population model and the actual study cases (two-tailed $P = 0.002$ by McNemar's test).

The serum concentrations of inhibin A in women whose fetuses were affected by trisomy 18 were slightly reduced at 0.84 times the median in the women with normal pregnancies ($P = 0.17$). There were too few

Table 2. Measures of the Log Gaussian Distributions of Each Serum Marker, Expressed in Multiples of the Median Values in Samples from Control Women and Women with Pregnancies Affected by Down's Syndrome at 15 to 18 Weeks' Gestation.

MARKER	CONTROLS		DOWN'S SYNDROME	
	MEAN	SD	MEAN	SD
Inhibin A	0.0000	0.2967	0.3502	0.3521
Intact human chorionic gonadotropin	0.0128	0.2196	0.3086	0.2064
β Subunit of human chorionic gonadotropin	0.0128	0.2609	0.3188	0.3061
Alpha-fetoprotein	-0.0223	0.1609	-0.1337	0.1805

measurements at earlier gestational ages to permit examination of the values in relation to the length of gestation (Fig. 1), but for this chromosomal trisomy, inhibin A was clearly different from the other markers. The median values of intact human chorionic gonadotropin and its β subunit were 0.30 and 0.14 times the medians in the women with normal pregnancies, respectively ($P < 0.001$), and for alpha-fetoprotein the median value was 0.53 times the median in the women with normal pregnancies ($P < 0.001$).

DISCUSSION

Inhibin is a heterodimeric glycoprotein with a molecular weight of 32,000, composed of one α subunit and one of two related β subunits (βA or βB). Inhibin A (α - βA) and inhibin B (α - βB) are synthesized by the gonads and regulate the secretion by the pituitary of follicle-stimulating hormone.³⁴ In addition to these mature dimers, forms of inhibin with greater molecular weights, representing partially processed or unprocessed dimers and processed or unprocessed free α subunits, circulate in the peripheral blood.³⁵ In pregnancy, the main source of inhibin secretion switches from the corpus luteum to the placenta,³⁶ and the level of immunoreactive and bioactive inhibin is significantly higher than that in nonpregnant women.^{37,38}

Until recently, studies of immunoreactive inhibin in pregnancy have depended on assays using antibodies directed against epitopes only on the α subunit.^{37,39} Such assays detect the mature dimers and all unprocessed or partially processed molecules containing the α subunit.³⁹ A commercially available assay (Medgenix, Brussels, Belgium) with this type of inhibin specificity has been used in three separate studies to measure in-

Table 4. Detection Rates and 95 Percent Confidence Intervals (CI) for Down's Syndrome at a Constant 5 Percent False Positive Rate for Various Combinations of Serum Markers and Maternal Age.

VARIABLES	DETECTION RATE (%)	95% CI
Alpha-fetoprotein and age	33	19-48
Intact human chorionic gonadotropin and age	41	26-57
β Subunit of human chorionic gonadotropin and age	47	32-63
Inhibin A and age	48	32-63
Alpha-fetoprotein, intact human chorionic gonadotropin, and age	54	38-69
Alpha-fetoprotein, β subunit of human chorionic gonadotropin, and age	53	37-68
Alpha-fetoprotein, inhibin A, and age	57	41-72
Intact human chorionic gonadotropin, inhibin A, and age	57	41-72
β Subunit of human chorionic gonadotropin, inhibin A, and age	68	52-81
Intact human chorionic gonadotropin, β subunit of human chorionic gonadotropin, and age	40	25-56
Alpha-fetoprotein, intact human chorionic gonadotropin, inhibin A, and age	72	57-84
Alpha-fetoprotein, β subunit of human chorionic gonadotropin, inhibin A, and age	75	60-87
Alpha-fetoprotein, intact human chorionic gonadotropin, β subunit of human chorionic gonadotropin, and age	52	36-67

hibin levels in maternal serum from second-trimester pregnancies affected by Down's syndrome.²³⁻²⁵ Inhibin values that were 1.9,²³ 3.6,²⁴ and 1.3²⁵ times the median values in the women with normal pregnancies were reported.

Inhibin levels in women with first-trimester pregnancies affected by Down's syndrome have also been investigated in two studies with the commercial assay. No differences were found between women with fetuses affected by trisomy 21 and control women.^{26,27} In our study with a new assay specific for dimeric inhibin A,^{31,32} the increase in the concentration of inhibin A associated with affected pregnancies did not occur until the end of the first trimester. Recently, however, significant increases in inhibin A as early as 11 weeks' gestation have been reported,²⁸ although there was some evidence of an effect of the length of gestation; there was a better rate of detection of Down's syndrome at 13 weeks than at 11 or 12 weeks. It is clear that larger numbers of samples are required to determine more accurately the levels of inhibin A in women with fetuses affected by Down's syndrome before 15 weeks' gestation. The greater increase in the serum concentration of inhibin A from women with fetuses affected by Down's syndrome later in gestation is similar to the trend noted previously for other placental markers, including intact human chorionic gonadotropin,²¹ pregnancy-specific β_1 glycoprotein,^{40,41} and pregnancy-associated plasma protein A.³⁰ The exception to this rule appears to be the β subunit of human chorionic gonadotropin, which

Table 3. Coefficients of Correlation (r) between Serum Inhibin A and Other Variables in Control Women and Women with Fetuses Affected by Down's Syndrome at 15 to 18 Weeks' Gestation.*

VARIABLES	CONTROLS		DOWN'S SYNDROME	
	r	P VALUE	r	P VALUE
Inhibin A and intact human chorionic gonadotropin	0.27	<0.001	0.39	0.008
Inhibin A and β subunit of human chorionic gonadotropin	0.15	0.11	0.23	0.13
Inhibin A and alpha-fetoprotein	0.24	0.001	0.24	0.11
Inhibin A and maternal age	-0.03	0.69	0.25	0.10
Intact human chorionic gonadotropin and β subunit of human chorionic gonadotropin	0.87	<0.001	0.76	<0.001
Intact human chorionic gonadotropin and alpha-fetoprotein	0.23	0.001	0.25	0.11
β Subunit of human chorionic gonadotropin and alpha-fetoprotein	0.15	0.12	0.20	0.19

*Correlation coefficients were calculated with the log multiple of the median for each variable.

is elevated in women with fetuses affected by Down's syndrome in both the first and the second trimesters,^{21,42} although it is not known whether the increases are of the same magnitude throughout gestation.

Two studies of the same series of 19 women with second-trimester pregnancies affected by Down's syndrome^{25,29} provide a comparison of the performance of the commercial (Medgenix) assay with that of the specific assay for dimeric inhibin A used in the present investigation. Overall median values 1.3 times the median value for immunoreactive inhibin²⁵ and 1.6 times the median value for dimeric inhibin A in the women with normal pregnancies²⁹ were obtained, suggesting that the latter is more specific for Down's syndrome. One other study using an inhibin assay with the same antibody specificity as our own reported a median value 1.88 times the control median value in 20 women with second-trimester pregnancies affected by Down's syndrome and a strong correlation between inhibin and intact human chorionic gonadotropin.⁴³

In this study, we found a statistically significant 22 percent increase in the rate of detection of Down's syndrome when inhibin A was added to the established screening protocol including alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age. This compares with detection rates estimated at 54 percent for the combination of alpha-fetoprotein, intact human chorionic gonadotropin, and maternal age and 53 percent for the combination of alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age. These results are consistent with those of previous retrospective studies with respect to alpha-fetoprotein, intact human chorionic gonadotropin, and maternal age¹⁰⁻¹² but lower than those reported in some studies with respect to alpha-fetoprotein, the β subunit of human chorionic gonadotropin, and maternal age.^{3,12} The confidence limits of our estimates are wide, however, because of the small size of the sample.

The inclusion of inhibin A in multimarker screening protocols did not, however, contribute to the detection of trisomy 18. In contrast to the elevated levels found in women with second-trimester pregnancies affected by Down's syndrome, levels of inhibin A associated with trisomy 18 were not significantly different from normal, whereas levels of intact human chorionic gonadotropin and the β subunit of human chorionic gonadotropin were both significantly reduced.

A practical advantage of the use of inhibin A as a screening marker is the very small change in average levels of inhibin A with increasing lengths of gestation between 15 and 18 weeks in women with unaffected pregnancies. Inaccuracies in estimating the length of gestation will therefore have a much smaller effect on the calculation of risk estimates than would be the case with a marker such as unconjugated estriol, whose levels change rapidly during gestation.⁴⁴ If the improved detection rate provided by inhibin A is confirmed in a larger series, then screening for inhibin A, alpha-fetoprotein, and the β subunit of human chorionic gonado-

tropin, in combination with maternal age, will be a very effective method for detecting Down's syndrome in the second trimester.

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