Evaluation of Cell-free Fetal DNA as a Second-Trimester Maternal Serum Marker of Down Syndrome Pregnancy

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Background: Second-trimester cell-free fetal DNA (studied only in pregnancies with male fetuses) is higher in maternal serum samples from women carrying Down syndrome fetuses than in unaffected pregnancies. In this study we evaluated the potential performance of fetal DNA as a screening marker for Down syndrome.

Methods: Data on maternal serum fetal DNA concentrations and the corresponding concentrations of the quadruple serum markers were available from 15 Down syndrome cases, each matched for gestational age and length of freezer storage, with 5 control samples. Analyte values were expressed as multiple(s) of the control or population median. Screening performance of fetal DNA, both alone and when added to estimates of quadruple marker performance, was determined after modeling using univariate and multivariate gaussian distribution analysis.

Results: The median fetal DNA concentration in Down syndrome cases was 1.7 times higher than in controls. In univariate analysis, fetal DNA gave a 21% detection rate at a 5% false-positive rate. When added to quadruple marker screening, fetal DNA increased the estimated detection rate from 81% to 86% at a 5% false-positive rate.

Conclusions: Cell-free fetal DNA, measured in maternal serum, can modestly increase screening performance above what is currently available in the second trimester. If and when maternal serum fetal DNA can be measured in pregnancies with both male and female fetuses, the utility and cost-effectiveness of adding it as a Down syndrome screening marker should be assessed.

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In 1997, Lo et al. (1) reported that cell-free DNA from male fetuses was measurable in the plasma of pregnant women. Male fetuses were studied because they have unique DNA markers, sequences on the Y chromosome. Soon afterward, fetal DNA concentrations in the maternal circulation were found to be higher in women carrying fetuses with Down syndrome than in unaffected pregnancies. Increased fetal DNA was originally reported when measured in fresh maternal plasma samples (2, 3) but not in serum (4). However, we recently reported that cell-free fetal DNA is also increased when measured in archived maternal serum samples from affected pregnancies (5). The three studies that showed an increase in fetal DNA found the extent of the increase to be approximately twofold.

Presumably, the source of fetal DNA measured in the maternal circulation is, at least in part, the placenta (6). If this is true, fetal DNA in the maternal circulation resembles the major second-trimester serum markers of Down syndrome pregnancy, human chorionic gonadotropin (hCG)6 and inhibin A. Both are of placental origin and have concentrations approximately twofold higher in Down syndrome compared with unaffected pregnancies (7).

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6 Nonstandard abbreviations: hCG, human chorionic gonadotropin; AFP, α-fetoprotein; αE3, unconjugated estriol; and MoM, multiple(s) of the median.
In our recent study (5), the maternal serum samples used to measure cell-free fetal DNA had been previously used in routine clinical quadruple marker screening for Down syndrome. Because we already had the analyte measurements for a-fetoprotein (AFP), unconjugated estriol (uE3), hCG, and inhibin A, it was possible to compare the performance of fetal DNA as a marker and to assess the potential contribution of cell-free fetal DNA to multiple marker screening in the second trimester.

Materials and Methods

Cell-free fetal DNA was measured in archived residual maternal serum samples from the second-trimester quadruple marker screening program at Women and Infants Hospital for a study comparing Down syndrome and unaffected pregnancies; the results of this study, which analyzed 11 case-control sets, have been published (5). In the present study, data from the original 11 case-control sets and 4 previously unpublished additional case-control sets were used. A total of 15 samples from confirmed Down syndrome pregnancies with male fetuses were retrieved, each matched for fetal sex, length of freezer storage (within 1 month), and same completed week of gestation with five samples from pregnancies with presumed euploid male fetuses. All samples studied were necessarily from pregnancies with confirmed male fetuses because quantification of fetal DNA in maternal blood is at present accomplished using real-time quantitative PCR of Y-specific sequences [described in detail in Ref. (5)]. However, additional samples from women carrying female fetuses were included in the assay for quality-control purposes. All samples were analyzed blindly without knowledge of gender or case-control status. Median fetal DNA concentrations as a function of increasing gestational age were initially measured in genome-equivalents/mL and calculated using weighted log-linear regression for the 75 control samples, and all fetal DNA values, in both cases and controls, were expressed as multiples of the median (MoM).

Data on the concentrations of the four serum markers (AFP, uE3, hCG, and inhibin A), also expressed as MoM, for the cases and controls had been calculated as part of our routine serum screening program and were obtained from our records. The normality of the distributions of fetal DNA concentrations in cases and controls was assessed by a probability plot of log_{10} fetal DNA MoM values (on a log scale) vs the expected gaussian centile scale. The relative correlation of fetal DNA with each of the serum markers was assessed by regression analysis of the affected and unaffected values for each marker pair after log_{10} transformation of the MoM values. To reliably estimate Down syndrome screening performance for fetal DNA, both alone and in combination with other serum markers, we used a published model (8) that incorporates each analyte’s distribution variables (and the correlations between them) along with the maternal age distribution in the United States in 2000 (9). The quadruple marker distribution variables were from Wald et al. (10, 11) (for modified AFP values).

Results

The median MoM for controls and cases was 1.00 and 1.71, respectively. The distributions of fetal DNA MoM values in both Down syndrome and control pregnancies followed a log-gaussian pattern at least between the 10th and 90th centiles of unaffected, as judged by a probability plot (Fig. 1). Distribution means and SDs (of the log MoM values) were 0.000 and 0.2925 for controls and 0.2324 and 0.3032 for controls.

Detection rates at three different false-positive rates were modeled for fetal DNA alone and compared with published data on the univariate performance of the other four serum markers (Table 1). Univariate performance of fetal DNA was the lowest of the five markers examined, with a detection rate of 21% at a 5% false-positive rate.

Correlations between fetal DNA and each of the other

<table>
<thead>
<tr>
<th>Marker</th>
<th>Detection rate (%) at a false-positive rate of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3%</td>
</tr>
<tr>
<td>AFP</td>
<td>19</td>
</tr>
<tr>
<td>uE3</td>
<td>29</td>
</tr>
<tr>
<td>hCG</td>
<td>32</td>
</tr>
<tr>
<td>Inhibin A</td>
<td>22</td>
</tr>
<tr>
<td>Fetal DNA</td>
<td>15</td>
</tr>
</tbody>
</table>

Detection rates for AFP, uE3, hCG, and inhibin A were calculated using univariate gaussian distribution analysis from coefficients in Wald et al. (10, 11), assuming ultrasound dating of pregnancies. Detection rates for fetal DNA were calculated the same way from coefficients given in the text.
markers were assessed (Table 2). The only significant correlation was between the log_{10} fetal DNA and the log_{10} inhibin A MoM, although there were low to moderate correlations noted throughout.

We assessed the impact of adding fetal DNA to quadruple serum markers, using all of the correlations obtained and previously published correlations, means, and SDs for the quadruple serum markers (Table 3). At a fixed 5% false-positive rate, adding fetal DNA to the quadruple marker test (AFP, uE3, hCG, and inhibin A) increased the detection rate from 81% to 86%.

### Discussion

Three studies have now shown that concentrations of cell-free fetal DNA, measured in the maternal circulation, are increased in Down syndrome pregnancy samples (2, 3, 5). In the present study, we have modeled the potential performance of fetal DNA in screening, both univariately and when combined with a current second-trimester screening method, the quadruple (or four-marker) test. The performance of fetal DNA as a screening marker, based on only 15 case-control sets studied, was less than current estimates for the four established markers. Its performance appears to be of the same order as older estimates for AFP, with an almost identical univariate detection rate as AFP when it was initially studied (20% for AFP vs 21% for fetal DNA, both at a 5% false-positive rate) (12). In recent years, the screening performance of AFP has marginally improved (to a univariate detection rate of 25% at a 5% false-positive rate) because of improvements in assay methods, leading to tighter distributions of AFP values (11). The same might be expected of fetal DNA; as methods of measurement improve, screening performance should improve.

It must be stressed, however, that the possibility of clinical implementation of fetal DNA as a screening tool will have to await the discovery of a reliable gender-independent fetal DNA marker that can be assayed by real-time PCR. Until that time, Y-chromosomal DNA sequences can be used as a theoretical model, so that additional and larger studies on the potential impact of fetal DNA measurement on screening performance can be performed.

Although the present study was relatively small, with 15 cases studied, it was well controlled, with 5 controls carefully matched to each case. This was important because we have shown that both length of freezer storage and gestational age at time of serum sampling affect marker concentrations (5). Both of these covariates were accounted for in this study.

The results of this study indicate that the fetal DNA is not as informative as hCG or inhibin A. This is despite the fact that the average difference between fetal DNA concentrations in cases and controls is almost on the order of twofold. The apparent reason for the somewhat poorer performance is that the width of the distributions of fetal DNA concentrations in both cases and controls is larger than for either hCG or inhibin A, attenuating the benefit of the average separation between cases and controls.

Fetal DNA concentrations were only weakly or moderately correlated with the concentrations of the other four serum markers, but these correlations were taken into account in modeling screening performance. In one instance, the moderate correlation between fetal DNA and inhibin A concentrations in control samples, a correlation achieved significance. Although there is no clear explanation for such a correlation, it is not uncommon to see moderate correlations among pairs of the established screening markers, such as between inhibin A and hCG (13) and between estriol and hCG (14). All of the screening markers are believed to enter the maternal circulation through placental maternal transfer, so that secretion patterns of various markers may be linked, leading to correlations between marker pairs.

Like hCG, inhibin A, and a range of other secretory products of the placenta, including human placental lactogen, SP1 (β1-glycoprotein of pregnancy), and progesterone, cell-free fetal DNA in the maternal circulation is increased in Down syndrome pregnancies relative to unaffected pregnancies (15). Also like hCG and inhibin A, fetal DNA appears to be increased in other pregnancy

### Table 2. Correlations between maternal serum fetal DNA and each of the quadruple markers in Down syndrome and control pregnancies.a

<table>
<thead>
<tr>
<th>Marker pair</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n = 75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal DNA–AFP</td>
<td>0.178</td>
<td>0.13</td>
</tr>
<tr>
<td>Fetal DNA–uE3</td>
<td>0.156</td>
<td>0.18</td>
</tr>
<tr>
<td>Fetal DNA–hCG</td>
<td>0.195</td>
<td>0.09</td>
</tr>
<tr>
<td>Fetal DNA–inhibin A</td>
<td>0.303</td>
<td>0.01</td>
</tr>
<tr>
<td>Down syndrome (n = 15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal DNA–AFP</td>
<td>0.144</td>
<td>0.61</td>
</tr>
<tr>
<td>Fetal DNA–uE3</td>
<td>0.332</td>
<td>0.23</td>
</tr>
<tr>
<td>Fetal DNA–hCG</td>
<td>−0.318</td>
<td>0.25</td>
</tr>
<tr>
<td>Fetal DNA–inhibin A</td>
<td>0.449</td>
<td>0.09</td>
</tr>
</tbody>
</table>

a For all analytes, correlations used log_{10}-transformed MoM values.

### Table 3. Impact of the addition of maternal serum fetal DNA to the Down syndrome screening performance of the second-trimester quadruple marker test (AFP + uE3 + hCG + inhibin A).a

<table>
<thead>
<tr>
<th>Detection rate (%) at a false-positive rate of</th>
<th>3%</th>
<th>5%</th>
<th>7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quadruple test</td>
<td>75</td>
<td>81</td>
<td>85</td>
</tr>
<tr>
<td>Quadruple test + fetal DNA</td>
<td>80</td>
<td>86</td>
<td>90</td>
</tr>
</tbody>
</table>

a Detection rates for the quadruple test were calculated using multivariate Gaussian distribution analysis from coefficients in Wald et al. (10, 11), assuming ultrasound dating of pregnancies. Detection rates in which fetal DNA was included were calculated the same way from additional coefficients given in the text.
abnormalities that involve the placenta, such as hydrops and preeclampsia (16–19). This may indicate a generalized phenomenon in which abnormal placental function causes the increased trafficking of many cellular components, not just secretory proteins. Further study of the pathophysiologic basis of these observations may provide explanations as well as other screening marker candidates.

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References