Chromosome-selective sequencing of maternal plasma cell–free DNA for first-trimester detection of trisomy 21 and trisomy 18

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OBJECTIVE: The purpose of this study was to assess the prenatal detection rate of trisomy 21 and 18 and the false-positive rate by chromosome-selective sequencing of maternal plasma cell–free DNA.

STUDY DESIGN: Nested case-control study of cell-free DNA was examined in plasma that was obtained at 11-13 weeks before chorionic villous sampling from 300 euploid pregnancies, 50 pregnancies with trisomy 21, and 50 pregnancies with trisomy 18. Laboratory personnel were blinded to fetal karyotype.

RESULTS: Risk scores for trisomy 21 and 18 were given for 397 of the 400 samples that were analyzed. In all 50 cases of trisomy 21, the risk score for trisomy 21 was ≥99%, and the risk score for trisomy 18 was ≤0.01%. In all 50 cases of trisomy 18, the risk score for trisomy 21 was ≤0.01%, and the risk score for trisomy 18 was ≥99% in 47

cases, 98.8% in 1 case, 88.5% in 1 case, and 0.11% in 1 case. In 3 of the 300 euploid pregnancies (1%), no risk score was provided, because there was failed amplification and sequencing. In the remaining 297 cases, the risk score for trisomy 21 was ≤0.01%, and the risk score for trisomy 18 was ≤0.01% in 295 cases, 0.04% in 1 case, and 0.23% in 1 case. Therefore, the sensitivity for detecting trisomy 21 was 100% (50/50 cases); the sensitivity for trisomy 18 was 98% (49/50 cases), and the specificity was 100% (297/297 cases).

CONCLUSION: In this study, chromosome-selective sequencing of cell-free DNA separated all cases of trisomy 21 and 98% of trisomy 18 from euploid pregnancies.

Key words: first trimester, trisomy 18, trisomy 21

Diagnosis of fetal aneuploidies relies on invasive testing by chorionic villous sampling or amniocentesis in pregnancies that are identified by screening to be at high risk for such aneuploidies.[1] In

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★ **EDITORS' CHOICE** ★

the 1970s and 1980s, the main method of screening for aneuploidies was by maternal age, with a cutoff of 35 years to define the high-risk group. This was associated with a 5% screen-positive rate and a detection rate of trisomy 21 of 30%. In the late 1980s and 1990s, screening was provided by a combination of maternal age and serum biochemistry in the second trimester, which resulted in improvement of the detection rate to 50-70%, with the same 5% screen-positive rate. In the last 15 years, the emphasis of screening shifted to the first trimester, where a combination of maternal age, fetal nuchal translucency (NT) thickness, maternal serum-free β-human chorionic gonadotropin (β-hCG), and pregnancy-associated plasma protein-A (PAPP-A) could identify approximately 90% of fetuses with trisomy 21, 18, and 13.[2,3] In specialist fetal medicine centers, the addition of other first-trimester sonographic markers, which include the nasal bone and Doppler blood flow in the ductus venosus, hepatic artery, and across the tricuspid valve, could improve the detection

rate of aneuploidies >95% and could reduce the screen-positive rate to <3%.[1,4]

Recently, noninvasive prenatal detection of fetal aneuploidies has been achieved by exploitation of the presence of cell-free DNA (cfDNA) in maternal plasma.[5] In trisomy 21, compared with euploid pregnancies, the amount of chromosome 21 in maternal plasma is slightly higher than that of other chromosomes, because there are 3, rather than 2, copies of fetal chromosome 21. Massively parallel shotgun sequencing (MPSS), which can identify and quantify millions of DNA fragments, has now made it possible to detect the increment in chromosome 21 in the plasma of affected pregnancies.[6,7] With this approach, trisomy 21 (and to a lesser extent trisomy 18) has been detected successfully noninvasively.[8-13] Essentially, maternal plasma DNA molecules are sequenced, and the chromosomal origin of each molecule is identified by a comparison with the human genome. In trisomy 21 pregnancies, the number of molecules that are derived from chromosome 21, as a proportion of all sequenced molecules,

is higher than in euploid pregnancies. However, this approach requires a significant amount of DNA sequencing, which can be costly and has a limited throughput. Because MPSS is not selective in the chromosomal origin of the sequenced DNA fragments, and chromosome 21 represents only approximately 1.5% of the human genome, it is necessary to sequence many millions of fragments to ensure sufficient chromosome 21 counts. An alternative to MPSS that may overcome these limitations is selective sequencing of loci from only chromosomes under investigation. Such chromosome-selective sequencing, referred to as digital analysis of selected regions (DANSR), has been applied successfully to the noninvasive detection of trisomy 21 and 18.[14] Sparks et al[14] have introduced the fetal-fraction optimized risk of trisomy evaluation (FORTE) by extending the process of chromosome-selective sequencing to assay nonpolymorphic and polymorphic loci, where fetal alleles differ from maternal alleles, which enables the simultaneous determination of chromosome proportion and fetal fraction.

The objective of this study was to assess the prenatal detection rate of trisomy 21 and 18 and false-positive rate at 11-13 weeks' gestation by the DANSR assay and the FORTE algorithm.

MATERIALS AND METHODS
Study population
This was a nested case-control study of stored maternal plasma from 400 singleton pregnancies at 11-13 weeks' gestation, including 300 pregnancies with euploid fetuses, 50 pregnancies with trisomy 21, and 50 pregnancies with trisomy 18. In all cases fetal karyotyping was carried out by chorionic villous sampling in our tertiary referral center, because screening by the combined test in the patients' hospitals demonstrated that the risk for aneuploidies was >1 in 300. Gestational age was determined from the measurement of the fetal crown-rump length.[15] The measured NT was transformed into likelihood ratio for each trisomy with the use of the mixture model of NT distributions.[16] The measured free β-hCG and PAPP-A were converted into a multiple of the median

(MoM) for gestational age that was adjusted for maternal weight, racial origin, smoking status, method of conception, parity, and machine for the assays.[17] The nasal bone was assessed as being present or absent; blood flow across the tricuspid valve was classified as normal or regurgitant, and blood flow in the ductus venosus was classified according to the a-wave as normal or reversed.[1]

Maternal venous blood (10 mL) that was collected before chorionic villous sampling in ethylenediaminetetraacetic acid vacutainer tubes (Becton Dickinson UK Limited, Oxfordshire, UK) was processed within 15 minutes of collection and centrifuged at 2000g for 10 minutes to separate plasma from packed cells and buffy coat (plasma 1) and subsequently at 16,000g for 10 minutes to further separate cell debris (plasma 2). Plasma 1 and 2 (2 mL each) were divided into 0.5-mL aliquots in separate Eppendorf tubes that were labeled with a unique patient identifier and stored at −80°C until subsequent analysis. Written informed consent was obtained from the women who agreed to participate in the study, which was approved by the King's College Hospital Ethics Committee.

We searched our database and selected 50 consecutive cases of trisomy 21 and 50 cases with trisomy 18 with 2 mL of available stored plasma 2, corresponding to 4 tubes of 0.5-mL aliquots per case. Each 1 of these 100 aneuploid cases was matched with 3 euploid control subjects for length of storage of their blood samples; none of the samples were previously thawed and refrozen. Maternal blood was collected between March 2006 and August 2011. We excluded pregnancies that were conceived by in vitro fertilization.

Laboratory analysis
Plasma samples (4 tubes of 0.5 mL per patient) from selected cases were sent overnight on dry ice from London to the laboratory of Aria Diagnostics, Inc, in San Jose, CA. The following information was provided to Aria Diagnostics for each case: patient-unique identifier, maternal age, gestational age, date of blood collection, and fetal sex but not fetal karyotype. Before evaluation for fetal trisomy, Aria Diagnostics, Inc, assessed

each sample for volume, adequacy of labeling, and risk of contamination or sample mixing and informed us that 25 samples did not meet their acceptance criteria (in 8 cases, the total plasma volume after pooling of individual tubes was <2 mL; in 5 cases, the labels on the tubes did not match the patient identifier on the file that was provided to the laboratory, and in 12 cases, there were potential issues of sample mixing or cross contamination after pooling of the individual tubes by laboratory personnel). In 11 cases, we had stored samples of plasma 1 (4 tubes of 0.5 mL per patient); however, in 14 cases, there was either no or insufficient plasma 1, which were replaced with the next available cases. The samples from these 25 cases were sent to Aria Diagnostics, Inc, and we were informed that all cases fulfilled the acceptance criteria of the laboratory. The 400 samples that fulfilled the acceptance criteria were then analyzed with their previously published technique of the DANSR assay with the FORTE algorithm.[14]

Results were provided for the risk of trisomy 21 and 18 on each of the 400 cases that fulfilled the acceptance criteria, and the correlation was determined between the assay results with the fetal karyotype.

RESULTS
The characteristics of the euploid and aneuploid pregnancies are summarized in the Table. In the aneuploid pregnancies, compared with the euploid pregnancies, the median maternal age, median delta NT, prevalence of absent nasal bone, tricuspid regurgitation, and reversed a-wave in the ductus venosus were significantly higher. In trisomy 21, the serum-free β-hCG level was higher, and the PAPP-A level was lower; in trisomy 18 both free β-hCG and PAPP-A levels were lower.

Risk scores for trisomy 21 and 18 were given for 397 of the 400 samples that were analyzed. In all 50 cases of trisomy 21, the risk score for trisomy 21 was ≥99%, and the risk score for trisomy 18 was ≤0.01% (Figure). In all 50 cases of trisomy 18, the risk score for trisomy 21 was ≤0.01%; the risk score for trisomy 18 was ≥99% in 47 cases, 98.8% in 1

TABLE
Maternal and fetal characteristics in euploid and aneuploid pregnancies

Maternal characteristic	Euploid (n = 300)	Trisomy 21 (n = 50)	Trisomy 18 (n = 50)
Maternal age, y[a]	35.4 (29.9–38.5)	38.9 (34.7–41.2)[b]	38.0 (33.4–40.7)[b]
Maternal weight, kg[a]	66.7 (60.0–76.3)	62.9 (58.3–68.3)	69.3 (60.5–78.0)
Maternal height, m[a]	163.5 (160.0–167.6)	165.1 (160.0–167.6)	165.1 (162.6–170.2)
Racial origin, n (%)			
White	268 (89.3)	45 (90.0)	42 (84.0)
Afro Caribbean	15 (5.0)	0	5 (10.0)
South Asian	9 (3.0)	4 (8.0)	3 (6.0)
East Asian	6 (2.0)	2 (1.0)	0
Mixed	2 (0.7)	0	0
Nulliparous, n (%)	87 (29.0)	11 (22.0)	18 (36.0)
Cigarette smoker, n (%)	35 (11.7)	6 (12.0)	2 (4.0)
Crown-rump length, mm[a]	72.6 (64.9–77.7)	71.2 (65.7–76.4)	58.5 (55.0–63.5)[b]
Gestation, d[a]	93.6 (89.8–96.0)	92.9 (90.2–95.4)	86.4 (84.6–89.1)[b]
Delta nuchal translucency, mm[a]	0.66 (0.22–1.36)	1.80 (1.13–3.14)[b]	4.76 (2.20–6.14)[b]
Absent nasal bone, n (%)	18 (6.0)	29 (58.0)[b]	34 (68.0)[b]
Ductus venosus reversed a-wave, n (%)	22 (7.3)	29 (58.0)[b]	31 (62.0)[b]
Tricuspid regurgitation, n (%)	21 (7.0)	36 (72.0)[b]	23 (46.0)[b]
Pregnancy-associated plasma protein-A (multiple of the median)[a]	0.71 (0.47–1.06)	0.55 (0.42–0.77)[c]	0.19 (0.11–0.26)[b]
β-human chorionic gonadotropin (multiple of the median)[a]	1.51 (0.85–2.45)	2.54 (1.75–4.36)[b]	0.21 (0.12–0.38)[b]

Comparison between outcome groups by Mann-Whitney U test for continuous variables and χ^2 test or Fisher's exact test for categoric variables, both with post hoc Bonferroni correction.

[a] Data are given as median (interquartile range); [b] $P < .0001$; [c] $P < .05$.

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case, 88.5% in 1 case, and 0.11% in 1 case. In 3 of the 300 euploid pregnancies (1%), no risk score was provided, because there was failed amplification and sequencing. In the remaining 297 cases, the risk score for trisomy 21 was ≤0.01%; the risk score for trisomy 18 was ≤0.01% in 295 cases, 0.04% in 1 case, and 0.23% in 1 case. Therefore, the sensitivity for the detection of trisomy 21 was 100% (50/50 cases); the sensitivity for trisomy 18 was 98% (49/50 cases), and the specificity was 100% (297/297 cases).

COMMENT

This nested case-control study has shown that, in pregnancies that were at high risk for aneuploidies, the chromosome-selective sequencing of cfDNA in maternal plasma that was obtained during the first trimester of pregnancy distinguished all cases of trisomy 21 and

98% cases of trisomy 18 from euploid pregnancies. The FORTE algorithm combined the risk that was computed from DANSR with the maternal age–related risks to estimate the patient-specific odds of trisomy vs disomy. In all cases of trisomy 21, the estimated risk for this aneuploidy was ≥99%, whereas in all euploid pregnancies and in those with trisomy 18 the risk score for trisomy 21 was ≤0.01%. In the case of trisomy 18, noninvasive testing correctly identified 98% of the cases in which the risk score for this aneuploidy was >88%; however, in all euploid pregnancies and in those pregnancies with trisomy 21, the risk score for trisomy 18 was <0.3%.

This study was based on small volumes of stored plasma samples. We provided 4 tubes of 0.5-mL aliquots per patient; 25 cases did not fulfill the acceptance criteria of the laboratory. In 11 of these cases, we had a further plasma sample of 2 mL

that was adequate for analysis. The remaining 14 cases with no additional stored samples were replaced with new patients. In prospective clinical studies with collection of larger volumes of blood, the likelihood is that most of these problems will be overcome.

In 1% of the samples that were considered to be adequate for analysis, there was failure to get a result. This is compatible with the 1.4% failure rate in the combined data from 3 previous studies that used MPSS.[7-9] In all these studies, plasma samples were obtained from high-risk pregnancies in which there is some evidence of impaired placental function that was reflected, for example, in low first-trimester serum PAPP-A levels. Because in pregnancies with impaired placentation, the maternal plasma concentration of cfDNA is increased,[18,19] the failure rate of noninvasive testing in low-risk pregnancies may be

increased. It is necessary therefore to evaluate the fetal DNA fraction in clinical studies in the general pregnant population.

Recent studies that have used MPSS have demonstrated that most cases of trisomy 21 can be detected from the analysis of maternal plasma cfDNA with a very low false-positive rate.[8-12] Maternal plasma was examined from a combined total of 350 pregnancies with trisomy 21 and 2061 euploid pregnancies at 6-38 gestational weeks (median, 15 gestational weeks), with reported detection and false-positive rates of 99% and 0.3%, respectively.[8-11] These results suggest that the testing of maternal plasma cfDNA by MPSS is a high-performance screening, rather than a diagnostic test for fetal trisomy 21.[20] Additionally, current MPSS-based approaches are costly and have low throughput. Each sequencing analysis that is run examines approximately 50 patient samples and takes several days to complete.

In this study, compared with previous publications on cfDNA, we have used samples that were obtained in the first trimester exclusively. This is important because, in the last decade, there has been a major shift from second- to first-trimester screening and diagnosis of aneuploidies. The use of chromosome-specific sequencing of polymorphic and nonpolymorphic loci that were described in this study requires 10 times less DNA sequencing than MPSS approaches and can analyze approximately 750 patient samples per sequencing analysis that is run. This opens the possibility to a more affordable noninvasive cfDNA test for fetal trisomy 21 and 18. Other targeted approaches in development could also lead to affordable cfDNA testing.[21]

Ultrasound examination at 11-13 weeks' gestation allows the accurate determination of gestational age, the early diagnosis of major fetal malformations, the diagnosis of multiple pregnancies, and the determination of chorionicity and ultrasound scanning in combination with maternal serum biochemical testing that provides effective screening for aneuploidies.[1] There is also increasing evidence that many pregnancy complications, which include preterm birth, preeclampsia,

FIGURE
Estimated risk for aneuploidies
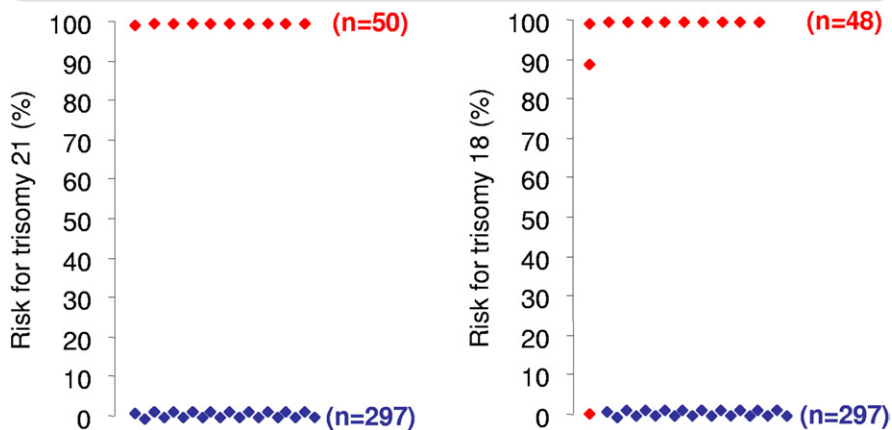
Risk scores for trisomy 21 (**left**) and trisomy 18 (**right**) in pregnancies with trisomic (*red diamonds*) and euploid (*blue diamonds*) fetuses. The risk score for trisomy 21 was ≥99% in all 50 cases of trisomy 21 and ≤0.01% in all 297 euploid cases (**left**). The risk score for trisomy 18 was ≥88.5% in 49 of the 50 cases of trisomy 18 and ≤0.23% in all 297 euploid cases (**right**).

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gestational diabetes mellitus, stillbirth, fetal growth restriction, and macrosomia, now can be predicted at an integrated first hospital visit at 11-13 weeks' gestation by the combination of data from maternal characteristics and history with findings of biophysical and biochemical tests.[22] It is therefore likely that in the future, such 11- to 13-week assessments will become more widespread rather than be replaced by a test for aneuploidies.

Fetal trisomy evaluation with cfDNA testing inevitably will be introduced into clinical practice. This would be useful as a secondary test contingent on the results of a more universally applicable primary method of screening. For example, for some parents, maternal plasma cfDNA testing may be preferable to invasive testing, when the first-trimester combined test results suggest that their pregnancies are at very high risk for trisomy 21. Such parents should be made fully aware that a positive result does not always imply that the fetus is affected and that a negative result does not always imply that the fetus is euploid.[20] Indeed, in approximately one-half of the aneuploid fetuses that are identified by the combined test as being at high risk for trisomy 21, the chromosomal abnormality is other than trisomy 21.[2,23]

Another population that may benefit from screening by maternal plasma cfDNA is the one identified by the combined test as being at intermediate risk for trisomy 21, because in these cases the risk will be revised to either very high or very low, thereby making their decision in favor or against invasive testing easier.[24,25]

The extent to which cfDNA analysis could also be applied as a universal screening tool for trisomy 21 in all pregnant women would depend on whether the cost becomes comparable with that of current methods of sonographic and biochemical testing. It would also be necessary to demonstrate that the observed accuracy with cfDNA testing that is obtained from the investigation of pregnancies at high risk for aneuploidies is applicable to the general population in which the prevalence of fetal trisomy 21 is much lower. This may well prove to be the case, because the ability to detect aneuploidy with cfDNA is dependent on assay precision and fetal DNA percentage in the sample, rather than the prevalence of the disease in the study population.

This nested case-control study has demonstrated that the DANSR assay with FORTE algorithm represents a promising method for accurate detection of fetal trisomy 21 and 18 from maternal blood cfDNA in the first trimester of pregnancy.

However, further research is needed to investigate the accuracy of the test in intermediate- and low-risk pregnancies, to improve the FORTE algorithm by the incorporation of risks that are derived from various biochemical and sonographic markers of aneuploidy (in addition to maternal age–related and DANSR risk), and to expand the spectrum of aneuploidies that could be detected from analysis of maternal plasma cfDNA. ■

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