ORIGINAL ARTICLE

DNA sequencing of maternal plasma to identify Down syndrome and other trisomies in multiple gestations†

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ABSTRACT

Objective Studies on prenatal testing for Down syndrome (trisomy 21), trisomy 18, and trisomy 13 by massively parallel shotgun sequencing (MPSS) of circulating cell free DNA have been, for the most part, limited to singleton pregnancies. If MPSS testing is offered clinically, it is important to know if these trisomies will also be identified in multiple pregnancies.

Method Among a cohort of 4664 high-risk pregnancies, maternal plasma samples were tested from 25 twin pregnancies (17 euploid, five discordant and two concordant for Down syndrome; one discordant for trisomy 13) and two euploid triplet pregnancies [Correction made here after initial online publication.]. Results were corrected for GC content bias. For each target chromosome (21, 18, and 13), *z*-scores of 3 or higher were considered consistent with trisomy.

Results Seven twin pregnancies with Down syndrome, one with trisomy 13, and all 17 twin euploid pregnancies were correctly classified [detection rate 100%, 95% confidence interval (CI) 59%–100%, false positive rate 0%, 95% CI 0%–19.5%], as were the two triplet euploid pregnancies.

Conclusion Although study size is limited, the underlying biology combined with the present data provide evidence that MPSS testing can be reliably used as a secondary screening test for Down syndrome in women with high-risk twin gestations. © 2012 John Wiley & Sons, Ltd.

Funding sources: Sequenom, Inc. fully funded the project through a grant to Women & Infants Hospital of Rhode Island. Sequenom Center for Molecular Medicine (SCMM) was responsible for developing an internally validated laboratory developed test (LDT) for detecting Down syndrome in maternal plasma using massively parallel shotgun sequencing and for providing clinical interpretation of the test results. SCMM also identified, equipped and trained an independent laboratory to test a subset of samples through a separate contract with UCLA. The sponsor did not control study design, identify or communicate with Enrollment Sites, thaw or test samples prior to the formal testing period, have access to patient information prior to all testing being completed, analyze study results, prepare drafts of the manuscript, or have final decisions on manuscript content.
Conflicts of interest: Palomaki and Canick were members of the Sequenom Clinical Advisory Board for 6 months, and resigned when the study was funded in 2008. Van den Boom, Ehrich, and Bombard are employees and shareholders of Sequenom, Inc. Deciu is an employee of Sequenom Center for Molecular Medicine and a shareholder of Sequenom, Inc.

INTRODUCTION

The identification of Down syndrome pregnancy by massively parallel shotgun sequencing (MPSS) of circulating cell free DNA in maternal plasma is highly effective. To date, studies using MPSS have provided results in more than 300 singleton pregnancies in which Down syndrome was caused by an extra copy of the number 21 chromosome in all cells examined (so-called 'classical' Down syndrome; karyotype: 47,XX,+21 or 47,XY,+21),[1–5] and in one case, due to an unbalanced Robertsonian translocation (46,XY,der(14;21)(q10;q10),+21).[6] Recent studies have also shown that singleton pregnancies affected by trisomies 18 and 13 can be reliably identified by MPSS.[1,7,8]

Twin pregnancies are considerably less common than singleton, with a live birth rate in the United States in 2009 of 1 in 30 deliveries (http://www.cdc.gov/nchs/fastats/multiple.htm). Multiple gestations are becoming more prevalent due to the increased use of assisted reproductive technologies. Among affected monozygous twins, discordance for a trisomy is rare, but among affected dizygous twins, the fetuses are nearly always discordant. Given that identification of Down syndrome by

MPSS of maternal plasma relies on a small incremental increase in the proportion of DNA fragments mapped to chromosome 21, it is uncertain whether Down syndrome (or other trisomies) in twins or other multiples will be reliably detected. A previously published study, by Sehnert *et al.*[8], included five twin pregnancies, four in a training set, and one in a test set for a new massively parallel testing method. One was presumably monozygotic and one dizygotic for Down syndrome, and three others were presumably euploid; all five were apparently correctly categorized by massively parallel sequencing.

As part of an international collaborative validation study for MPSS testing in which maternal plasma samples were collected prior to diagnostic testing from over 4000 women with pregnancies at high risk for trisomy, multiple gestations were included. The current study explores the results of testing in those multiple pregnancies.

MATERIALS AND METHODS

Women with a pregnancy at high risk for Down syndrome based on age, family history, or a positive serum and/or sonographic screening test provided consent, plasma samples, and demographic and pregnancy-related information under local institutional review board approval as part of a multicenter study (www.clinicaltrials.gov NCT00877292). Information regarding the use of assisted reproductive technologies was not collected. Samples were drawn immediately prior to invasive testing (chorionic villus sampling or amniocentesis), processed within 6 h, stored at −80 °C and shipped on dry ice to the Coordinating Center at Women and Infants Hospital, Rhode Island, where samples were kept frozen until tested at a Clinical Laboratory Improvement Amendments-certified laboratory (Sequenom Center for Molecular Medicine, San Diego, CA); now also accredited by the College of American Pathologists. Karyotype data on each pregnancy were obtained from the enrollment sites and kept at the coordinating center site in Maine. In 0.3% of all pregnancies, only quantitative fluorescent–polymerase chain reaction results were available due to diagnostic standards in place at four sites. Enrollment sites, site investigators, oversight committee, and other study personnel have been cited in the primary publication.[5] Samples used in this study were tested without knowledge of the karyotype, gestational age, or geographic origin of the sample. A subset of results for singleton pregnancies was validated at an independent academic laboratory at the University of California, Los Angeles.[5,7] Samples obtained from all multiple gestations with Down syndrome, trisomy 18, or trisomy 13 were submitted for analysis, along with all euploid triplet pregnancies and a random selection of euploid twin pregnancies (22% of a total of 77).

Testing was by MPSS, during a single 9 week time period as described previously.[3,5] In brief, circulating cell-free DNA fragments were isolated from 4 mL of maternal plasma and quantified with an assay that determines the fetal contribution (fetal fraction).[1] The remaining isolate was used to generate sequencing libraries, which were normalized and multiplexed to allow four samples to be run in a single flow cell lane (eight lanes per flow cell, allowing for up to 32 patient samples per flow cell). The DNA libraries were prepared and quality controlled using a microfluidics platform (Caliper Life Sciences, Hopkinton, MA) and clusters generated using the cBot platform (Illumina, Inc, San Diego, CA). Flow cells were sequenced on the Illumina HiSeq 2000; data were analyzed using Illumina software, and a robust estimate of the standard deviations above or below the central estimate (z-score) was calculated for chromosomes 21, 18, and 13. Z-scores at or above 3 were reported to be consistent with the presence of trisomy. All three chromosome results were corrected for the guanine−cytosine content bias, and the chromosome 21 results were also repeat-masked, post alignment. The MPSS results from the multiple gestations were compared with the reference range established for the interpretation of singleton pregnancies. The Sequenom Center for Molecular Medicine did not know whether the samples were from singleton or multiple gestations.

RESULTS

Among the 4644 pregnancies enrolled, seven twin pregnancies affected by Down syndrome were identified by their karyotypes; two in which both fetuses were affected and five in which just one fetus was affected. One of the concordant Down syndrome cases was due to an unbalanced 14;21 translocation. One twin pregnancy discordant for trisomy 13 was also identified. MPSS testing was performed on maternal plasma samples from all eight cases of trisomy, along with control samples from 17 euploid twin pregnancies (randomly selected from the 62 eligible twin pregnancies) and the only two eligible euploid triplet pregnancies (Table 1).

Figure 1 shows the chromosome 21, 18, and 13 results, expressed as robust z-scores versus the fetal fraction for the 27 multiple gestations. The upper limit of euploid is set at a z-score of +3. As a reference, the median chromosome 21 z-score (after alignment with a repeat-masked genome and accounting for guanine−cytosine content bias) for the euploid singleton pregnancies reported previously[5,7] was 0.013 with 99.8% of the samples below a z-score of 3. For chromosomes 18 and 13, the median z-scores and proportion of samples below a z-score of 3 for the euploid singleton pregnancies was 0.000, 99.0% and 0.000, 99.7%, respectively. For all sample groups, approximately half of the gestational ages were from the late first trimester (9–14 completed weeks) and half from the early second trimester (15–21 completed weeks).

Chromosome 21 z-scores were elevated in all seven twin pregnancies affected by Down syndrome (100% detection rate, 95% CI 59%–100%), with a median of 12.3 (range: 5.3–17.4) (Table 1). Among the five twin pregnancies discordant for Down syndrome, chromosome 21 z-scores (range 5.3–17.4) were similar to those from the two concordant twin pregnancies (12.3, 13.2). Chromosome 18 and 13 z-scores were under 3 for all seven of the twin pregnancies affected by Down syndrome. The twin pregnancy discordant for trisomy 13 had a chromosome 13 z-score of 5.1, with chromosome 21 and 18 z-scores of 0.3 and 0.1, respectively.

Median z-scores for chromosomes 21, 18, and 13 in the 17 euploid twin pregnancies were −0.1, 0.1, and −0.2, respectively. No z-score in euploid twins was over 3 (0% false positive rate, 95% CI 0%–21%). The two euploid triplet pregnancies had unremarkable chromosomes 21, 18, and 13 z-scores (−0.1, −0.7,

Table 1 Gestational age and DNA test results in multiple pregnancies with and without a common autosomal trisomy

Number of fetuses and their karyotypes			Gestational age (weeks)	Fetal fraction (%)	Chromosome *z*-score		
Fetus 1	Fetus 2	Fetus 3			21	18	13
47,XX,+21	47,XX,+21	—	13.6	15	13.2	0.6	−2.9
46,XX,t(14;21)	46,XX,t(14;21)	—	12.7	11	12.3	−1.2	−0.5
47,XX,+21	46,XX	—	13.3	16	5.3	−0.9	0.6
47,XX,+21	46,XX	—	18.6	12	10.1	0.7	0.6
47,XY,+21	46,XX	—	15.7	41	14.5	−0.8	−1.5
47,XY,+21	46,XX	—	19.0	21	17.4	−0.1	−0.5
47,XY,+21	46,XX	—	12.1	21	5.3	1.0	2.99
		Mean	15.0	19.6	11.2	−0.1	−0.2
47,XY,+13	46,XX	—	16.6	7	0.3	0.1	5.1
46,XX	46,XX	—	11.7	42	−0.1	0.9	−1.2
46,XX	46,XX	—	12.0	10	0.0	0.9	−0.5
46,XX	46,XX	—	13.7	29	−0.8	−1.2	−0.9
46,XX	46,XX	—	12.1	18	0.6	1.8	2.3
46,XX	46,XX	—	16.0	14	0.1	1.5	1.9
46,XX	46,XX	—	15.0	18	−0.2	1.4	−0.6
46,XX	46,XY	—	10.9	39	1.5	0.3	0.3
46,XX	46,XY	—	12.9	22	−0.8	0.0	−0.1
46,XX	46,XY	—	15.6	23	−0.1	−0.7	−1.4
46,XX	46,XY	—	17.7	18	0.3	0.1	−0.1
46,XX	46,XY	—	16.9	9	0.9	−0.2	−0.9
46,XY	46,XY	—	18.0	15	−2.3	−0.6	−0.5
46,XY	46,XY	—	16.1	27	0.1	0.4	0.7
46,XY	46,XY	—	14.0	11	0.0	−0.7	0.0
46,XY	46,XY	—	16.1	21	−0.4	−1.7	−0.7
46,XY	46,XY	—	17.0	20	−0.6	−0.2	−0.5
46,XY	46,XY	—	15.2	7	0.1	0.1	−1.9
		Mean	14.8	20.2	−0.1	0.1	−0.2
46,XX	46,XX	46,XY	16.0	55	0.1	−0.7	−0.6
46,XX	46,XY	46,XY	16.0	18	−0.1	−0.6	0.4

−0.6 and −0.1, −0.6, 0.4, respectively). There was no obvious difference in *z*-scores based on sex chromosome concordance or discordance (Table 1). For each of the sets of chromosome test results, the case and control *z*-score results were plotted against fetal fraction (Figure 1). Figure 1A (chromosome 21 results) shows the expected relationship between increasing *z*-score in Down syndrome pregnancies versus increasing fetal fraction. There were insufficient cases available to explore this relationship for trisomy 18 (none) or trisomy 13 (one) in twins.

To help understand why *z*-scores among discordant Down syndrome twin pregnancies were in the same range as both concordant twin and classical singleton Down syndrome pregnancies, we compared the fetal fraction of DNA in the maternal plasma in twin pregnancies with the fetal fraction in singleton pregnancies. The fetal fraction in 17 euploid twin pregnancies [geometric mean 18.1%, average 20.2%, log standard deviation (SD) 0.208] was significantly higher than the geometric mean of 13.4% (log SD 0.205) for 1471 euploid singleton pregnancies ($t=2.59$, $p=0.0097$, after logarithmic transformation). The fetal fraction in the seven Down syndrome pregnancies was 17.8% (geometric mean), nearly the same as for all twin pregnancies (18.1%). The fetal fraction for the trisomy 13 twin case was 7%, the lowest fetal fraction for a twin pregnancy sample undergoing testing.

DISCUSSION

In the present study, MPSS testing correctly identified all seven twin pregnancies with Down syndrome and the one twin pregnancy with trisomy 13, indicating that this test can be clinically useful in testing twin pregnancies. On average, multiple pregnancies in the present study contributed 35% more fetal DNA than singleton pregnancies to the total free DNA in maternal plasma (18.1% vs 13.4%). Twin pregnancies, both monozygous and dizygous, have a higher placental mass than singleton pregnancies[9] and therefore would be expected to have a higher fetal fraction due to more fetal DNA entering

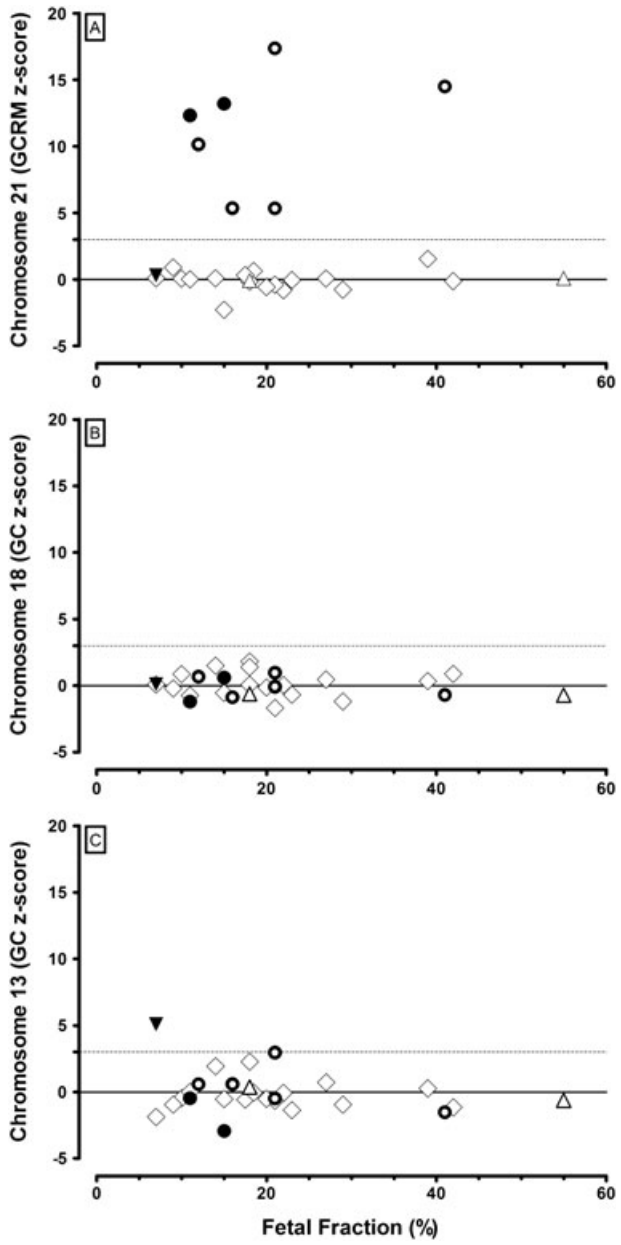

Figure 1 Chromosomes 21, 18, and 13 testing results in singleton and twin pregnancies. The proportion of circulating cell-free DNA derived from the fetus/placenta (fetal fraction) is shown on the horizontal axis. The massively parallel shotgun sequencing testing results are reported as flow-cell adjusted chromosome *z*-scores (vertical axis) for chromosomes (A) 21, (B) 18, and (C) 13. All three sets of results were adjusted for the guanine–cytosine (GC) content bias. The chromosome 21 results were filtered with respect to a repeat-masked genome and then adjusted for GC bias (GCRM). The dashed line at a *z*-score of 3 indicates the original test cut-off for indicating the demarcation between euploid and Down syndrome (or other aneuploid) fetuses. The solid line at a *z*-score of 0 indicates the average value in singleton euploid pregnancies. Five twin pregnancies discordant for Down syndrome are shown as open circles, whereas the two concordant affected twins are filled circles. The filled triangle indicates the one twin pregnancy discordant for trisomy 13. The 17 open diamonds are results from euploid twin pregnancies. The open triangles indicate results for the two euploid triplet pregnancies

the maternal circulation. An association between increasing fetal fraction and increasing chromosomes 21, 18, and 13 *z*-scores in singleton affected pregnancies has been clearly shown.[1,6,7]

Two factors could potentially influence the reliability of testing in twin pregnancies. First, the higher fetal fraction in twin pregnancies (euploid or Down syndrome) would be expected to lead to better separation between Down syndrome and euploid *z*-scores in twin compared with singleton pregnancies. However, a second factor might reduce this advantage. Because most affected singleton pregnancies will be discordant for Down syndrome and assuming that each fetus contributes equally to the circulating cell-free DNA, then the ratio of fetal to maternal of 1 : 9 (10%) in a discordant twin pregnancy is effectively a ratio of trisomic to disomic material of 0.5 : 9.5 (1 : 19 or 5%). When these two factors are taken together, the effective fetal fraction in twins discordant for Down syndrome would be about 6.8%, roughly two-thirds that of euploid singleton pregnancies (10%). This is an especially important finding when the fetal fraction approaches the lower limit of acceptability for singleton pregnancies (4%). The equivalent limit for discordant twins would be at about 6%. For twin samples falling between 4% and 6%, interpretations would need to rely more on the laboratory director's judgment. In contrast, when both twins are affected by Down syndrome, the observed fetal fraction and effective fetal fraction are equivalent, and the interpretation can be made as though it were a singleton pregnancy. When MPSS test results are consistent with a fetal trisomy in twin pregnancies, however, amniocentesis of each fetal sac is necessary to conclusively determine concordance versus discordance. The 35% higher fetal fraction associated with twin pregnancies cannot, by itself, reliably identify multiple gestations.

In the present study, the chromosome 21 *z*-score was high in one twin pregnancy concordant for Down syndrome with a chromosome 14;21 translocation. This reinforces that translocation Down syndrome will be identified through MPSS testing in twin pregnancies, as was previously shown for the same karyotype in a singleton pregnancy.[6] A recent study[10] used *DYS14* Y-specific marker levels to conclude that fetal fractions in 29 twins with two male fetuses were higher than in the 36 twins with one male fetus. The majority of sampling occurred after 20 weeks' gestation. Our numbers were much smaller; we found that the six twins with two male fetuses had the lowest fetal fraction (geometric mean 15.4%) compared with the five with one male (20.0%) or the six with two female fetuses (19.5%).

The cohort of pregnancies used in this study was large enough to include only a modest number of aneuploid twin pregnancies. Because Down syndrome is more common than either trisomy 18 or trisomy 13, there were seven twin pregnancies with one or both fetuses having Down syndrome but only one twin pregnancy discordant for trisomy 13 and none with trisomy 18. For this reason, it is not possible to make definitive assessments of clinical sensitivity for those trisomies. In addition, the trisomy 13 twin pregnancy in this study had the lowest fetal fraction

of any twin pregnancy tested. This finding may be consistent with smaller fetal and placental size reported in trisomy 13 at mid-pregnancy (16 weeks, 3 days in this case). Although in our recent study of singleton pregnancies, the median fetal fraction for 12 cases of trisomy 13 was 13.5%, almost the same as the median for euploid singleton pregnancies (13.4%). Smaller placentas have been noted in trisomies 18 and 13 but not in trisomy 21.[11–13] Further studies of twin pregnancies with trisomies 18 and 13 are needed to reach any firm conclusions on test sensitivity for these pregnancies. Because there were no instances of trisomy among triplet pregnancies, no conclusions can be reached. On the basis of the two observations, however, euploid twin and triplet pregnancies may be associated with the same high specificity found for singleton pregnancies.

Based on the results of this study, the biology of twin pregnancies, and the rationale behind MPSS testing, it is reasonable to expect that at least Down syndrome can be identified in twin pregnancies. Test performance would be similar to that found for singleton pregnancies, with the caveat that the interpretation of fetal fraction at lower levels may differ in twin versus singleton pregnancies. This finding underscores the usefulness of routinely quantifying fetal fraction as part of the testing and interpretive process. As we and others have suggested for the clinical implementation of MPSS testing in women with singleton pregnancies, women with twin pregnancies identified as being at high risk for Down syndrome can be offered MPSS testing to refine their risk as part of the decision-making process. Performance of chromosomes 18 and 13 testing in twin pregnancies should be reliable but awaits further study.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Several studies, including two authored by this group and based on the cohort used in this report, have demonstrated that applying next-generation sequencing techniques to circulating cell-free DNA from maternal plasma can detect nearly all singleton pregnancies with Down syndrome, trisomies 18 and 13 in singleton pregnancies with few false positive results or failures.

WHAT DOES THIS STUDY ADD?

- One previous study reported on five twin pregnancies, two with Down syndrome; this report is the largest to date to examine test performance among twenty-five twin pregnancies, seven with Down syndrome and one with trisomy 13.

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