Fetal Fraction Estimate in Twin Pregnancies Using Directed Cell-Free DNA Analysis

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\textbf{Abstract}

\textbf{Objective:} To estimate fetal fraction (FF) in monozygotic and dizygotic twin pregnancies. \textbf{Methods:} Maternal plasma samples were obtained from 35 monochorionic twin pregnancies with male fetuses (monozygotic) and 35 dichorionic pregnancies discordant for fetal sex (dizygotic) at 11–13 weeks’ gestation. Cell-free DNA was extracted and chromosome-selective sequencing with digital analysis of selected regions (DANSR\textsuperscript{TM}) was carried out. The fetal-fraction optimized risk of trisomy evaluation (FORTE\textsuperscript{TM}) algorithm was used to estimate FF using polymorphic alleles. In dizygotic twins the FORTE algorithm was modified to estimate the smallest FF contribution of the 2 fetuses. In both types of twins, FF was also determined by analysis of Y-chromosome sequences. \textbf{Results:} In monozygotic twins, the median total FF was 14.0\% (range 8.2–27.0\%) and in dizygotic twins the median smallest FF was 7.9\% (4.9–14.0\%). There were significant associations in FF between the methods using polymorphic alleles and Y-chromosome sequences for both monozygotic (r = 0.951, p < 0.0001) and dizygotic (r = 0.743, p < 0.0001) twins. \textbf{Conclusions:} The study demonstrates the feasibility of an approach for cfDNA testing in twin pregnancies. This involves estimation of total FF in monozygotic twins and estimation of the lower FF of the 2 fetuses in dizygotic twins.

\textbf{Introduction}

Cell-free DNA (cfDNA) testing in maternal blood has been successfully applied in singleton pregnancies and shown to provide effective screening for trisomy 21 with sensitivity of more than 99\% and a false positive rate of less than 0.1\% [1]. Evaluation of trisomies 18 and 13 and sex chromosome aneuploidies has also been done but with lower performance. The accuracy of cfDNA testing depends on the proportion of the fetal to maternal origin of cfDNA in maternal blood and the minimum required fetal fraction (FF) for aneuploidy assessment with current methods is 4\%; a lower FF could lead to a false negative result [2–6].

In twin pregnancies, cfDNA testing is more complex than in singleton pregnancies because the 2 fetuses could be either monozygotic, which are therefore genetically...
identical, or dizygotic, in which case only 1 fetus is likely
to have aneuploidy when present. In prenatal diagnosis
by ultrasound it is possible to accurately determine cho-
ionicity but not zygosity [7]. In monochorionic twins the
two monzygotic genetically identical fetuses contribute
the same alleles of cfDNA into the maternal circulation
and cfDNA analysis for assessment of aneuploidy risk can
be carried out in the same manner as in singleton preg-
nancies. In dichorionic twins the majority of cases are di-
zygotic. Previous studies using ultra-deep sequencing of
maternal plasma cfDNA in dizygotic twins reported that
each fetus can contribute different amounts of cfDNA
into the maternal circulation and this could vary by near-
ly 2-fold [8]. It is therefore possible that in a dizygotic
twin pregnancy discordant for aneuploidy, the FF of the
affected fetus is below the threshold of 4% for successful
cfDNA testing. This could lead to an erroneous result of
low risk for aneuploidy because a high contribution from
the disomic co-twin could result in a satisfactory total FF.
For example, if the total FF in a dizygotic twin pregnancy
discordant for trisomy 21 is 9% and it is assumed that
each fetus contributes equal amounts of cfDNA into the
maternal circulation, but in reality there is a contribution
of 6% from the euploid fetus and 3% from the trisomic
one, cfDNA testing could wrongly classify the fetuses as
euploid.

We propose that in monochorionic twins the approach
to cfDNA testing for aneuploidies could be the same as in
singleton pregnancies, but in dichorionic twins the ap-
proach should be modified to take into account the FF of
the twin with the lower contribution to cfDNA in mater-
nal blood. This study describes a directed cfDNA analysis
approach to estimate FF in monozygotic and dizygotic
twin pregnancies.

Methods

Study Population

Maternal venous blood samples were obtained at 11–13 weeks’
gestation from twin pregnancies undergoing first-trimester
screening for trisomies by a combination of fetal nuchal translu-
cency thickness and maternal serum-free β-human chorionic go-
nadotropin (β-hCG) and pregnancy-associated plasma protein-A
(PAPP-A) [9–11]. All patients gave written informed consent to
provide samples for research which was approved by the NHS Re-
search Ethics Committee. Gestational age was determined from
the measurement of the crown-rump length (CRL) of the larger
twin and chorionicity was determined by examining the inter-twin
membrane at its junction with the placenta [7, 12].

Blood samples were collected in ethylenediaminetetraacetic
acid BD Vacutainer™ tubes (Becton Dickinson UK Ltd, Oxford,
UK) and within 15 min of collection they were centrifuged at
2,000 g for 10 min to separate plasma from packed cells and sub-
sequently at 16,000 g for 10 min to further separate cell debris.
Plasma samples were divided into 0.5-ml aliquots in separate Ep-
pendorf tubes which were labeled with a unique patient identifier
and stored at –80°C until subsequent analysis.

For the present study we searched our database of stored sam-
ple and identified 35 cases of monochorionic twins and 35 cases
of dichorionic twins discordant for fetal sex. In all cases the preg-
nancies resulted in phenotypically normal neonates that were as-
sumed to be euploid. Plasma samples (4 tubes of 0.5 ml per patient)
were sent overnight on dry ice from London, UK, to the USA (Ar-
rosa Diagnostics, Inc., San Jose, Calif., USA) for cfDNA analysis.

Analysis of Samples

cfDNA was extracted from maternal plasma samples and chro-
mosome-selective sequencing, referred to as digital analysis of se-
lected regions (DANSR™), against loci in the human genome was
performed as previously described [13]. The DANSR assays in-
clude a set of 192 single nucleotide polymorphic (SNP) loci on
chromosomes 1–12, where two middle oligonucleotides differing
by one base were used to query each SNP. SNPs were optimized for
minor allele frequency in the HapMap-3 dataset (http://hapmap.
ncbi.nlm.nih.gov/). DANSR assay products were sequenced using
the Illumina HiSeq 2000 as previously described [13].

Informative polymorphic loci were defined as loci where fetal
alleles differ from maternal alleles. Because DANSR exhibits high
allele specificity, informative loci were readily identified when the
fetal allele proportion of a locus was measured to be between 1 and
25%. When the fetal contribution to the cfDNA in maternal blood
is at 4% or higher, corresponding to a 2% fetal allele proportion, a
truly informative locus has a greater than 95% chance of having a
measured fetal allele proportion greater than 1%.

The fetal-fraction optimized risk of trisomy evaluation
(FORTE™) algorithm was used to assay non-polymorphic and
polymorphic loci, where fetal alleles differ from maternal alleles,
allowing simultaneous determination of chromosome propor-
tion and FF [13]. In dizygotic pregnancies, the original FORTE
algorithm for singleton pregnancies was modified to estimate the
smallest FF contribution of the 2 fetuses and this was used to es-

tablish the expected change in chromosome proportion for triso-
mic samples. Estimation of the smallest FF was accomplished by
identifying the lower 45th percentile of polymorphic DANSR as-

sa with a non-maternal allele and using these assays as represen-
tative of FF.

In both monochorionic and dizygotic pregnancies FF was also
measured using 32 non-polymorphic loci on the Y-chromosome.
Median counts from these assays were compared with median
counts of non-polymorphic DANSR assays developed for chromo-
somes 13, 18 and 21 to estimate the FF.

Results

The characteristics of the monozygotic and dizygotic
twin pregnancies are outlined in Table 1. The median total
sequencing counts per sample was 2.89 million (range
2.52–3.30) with a median coverage of 874 counts (range
768–1,004) per genomic locus.

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In the monozygotic twin pregnancies, the median total FF, determined using polymorphic alleles, was 14.0% (range 8.2–27.0%) and there was a highly significant association with the fraction determined by Y-chromosome counts \((r = 0.951, p < 0.0001; \text{fig. } 1)\). In the dizygotic twin pregnancies, the median lower FF of the 2 fetuses, using a modified FORTE algorithm, was 7.9% (range 4.9–13.0%) and there was a significant association with the fraction determined by Y-chromosome counts \((r = 0.743, p < 0.0001; \text{fig. } 2)\).

### Discussion

This study demonstrates the use of chromosome-selective sequencing of cfDNA in maternal blood to estimate FF in twin pregnancies. In monochorionic twins, cfDNA analysis was carried out in a similar manner as in singleton pregnancies and the total FF was estimated because the 2 fetuses contribute the same alleles of cfDNA into the maternal circulation. In dichorionic dizygotic twins, the

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**Table 1.** Characteristics of the monozygotic and dizygotic twin pregnancies

| Characteristic                  | Monozygotic (n = 35) | Dizygotic (n = 35) |
|---------------------------------|----------------------|-------------------|
| Maternal age, years             | 31.8 (30.0–36.0)     | 33.0 (29.2–36.3)  |
| Maternal weight, kg             | 68.5 (62.0–79.0)     | 64.5 (59.4–74.0)  |
| Maternal height, cm             | 167.6 (162.6–171.9)  | 164.0 (159.0–167.6)|
| Racial origin                   |                      |                   |
| Caucasian                       | 23 (65.7)            | 23 (65.7)         |
| Afro-Caribbean                  | 6 (17.1)             | 9 (25.7)          |
| South Asian                     | 1 (2.9)              | 1 (2.9)           |
| East Asian                      | 1 (2.9)              | 1 (2.9)           |
| Mixed                           | 4 (11.4)             | 1 (2.9)           |
| Cigarette smoker                | 2 (5.7)              | 1 (2.9)           |
| Mode of conception              |                      |                   |
| Spontaneous                     | 16 (45.7)            | 26 (74.3)         |
| In-vitro fertilization          | 2 (5.7)              | 0                 |
| Ovulation drugs                 | 17 (48.6)            | 9 (25.7)          |
| Gestational age, days           | 92 (90–96)           | 91 (88–94)        |
| CRL of the larger fetus, mm     | 69.1 (63.7–77.2)     | 67.6 (62.4–74.1)  |
| PAPP-A, IU/ml                   | 6.44 (4.49–10.44)    | 7.41 (6.10–9.95)  |
| Free β-hCG, IU/ml               | 47.05 (39.25–86.75)  | 77.50 (50.20–98.80)|

Median (IQR) or n (%) values are shown.
In both monozygotic and dizygotic twins there were significant associations in the FF estimated by polymorphic alleles and that using Y-chromosome sequences. The higher correlation in values by the two methods in monozygotic than dizygotic twins is because in the former, both fetuses were male and the total FF was considered. The lower correlation in dizygotic twins is likely to be because the method using Y-chromosome sequences reflected the contribution to maternal plasma cfDNA of the male fetus but the method using polymorphic alleles reflected the lower contribution between the 2 fetuses which could have been either male or female.

The finding that in monozygotic twins the median total FF was 14% and in dizygotic twins the median lower FF of the 2 fetuses was 8% is compatible with the 10% value in singleton pregnancies at 11–13 weeks' gestation [14, 15]. The likely source of fetal cfDNA in maternal plasma is dying cells in the placenta [16] and consequently the number of apoptotic cells would be proportional to the placental mass. We have previously reported significant associations between FF and serum-free β-hCG and PAPP-A whose concentration provides an indirect measure of placental mass [15, 16]. In monochorionic twins, the levels of serum-free β-hCG and PAPP-A, expressed as multiple of the median (MoM) in singletons, increase from about 1.0 MoM each at 9 weeks’ gestation to 2.0 MoM for free β-hCG and 1.5 MoM for PAPP-A at 13 weeks [17, 18]. In dichorionic twins the levels of these metabolites are higher and they both increase from about 1.5 MoM at 9 weeks to 2.0 MoM at 13 weeks. Consequently, the total FF in monochorionic twins would be expected, as found, to be about 1.5 times rather than twice as high as in singletons. Similarly, the lower FF in dizygotic twins would be expected, as found, to be less than the value in singletons.

In monochorionic twins, effective screening for aneuploidies by cfDNA testing in maternal blood can be applied as in singleton pregnancies provided the total FF contributed by both fetuses is at least 4%. In dizygotic twins, accurate assessment of risk necessitates that the minimum FF of 4% is reached by each of the 2 fetuses. In three previous studies reporting on the use of cfDNA testing in screening for trisomies in twin pregnancies it was assumed that each fetus had contributed adequate amounts of cfDNA into the maternal circulation [5, 19, 20]. Supportive evidence, that each of the fetuses in a twin pregnancy is producing a similar amount of cfDNA as in singleton pregnancies, was provided by the results of studies which used a Y-chromosome-associated gene as a marker for fetal DNA and reported that the fetal cfDNA concentration in maternal plasma in twin pregnancies with mixed fetal sex is the same as in singleton male pregnancies [21, 22].

In contrast to the results of the studies using the Y-chromosome-associated gene as a marker for fetal DNA, studies using massively parallel shotgun sequencing of cfDNA in maternal plasma, to look across multiple genomic regions for differences between the fetuses in dizygotic twins, reported that there are large differences in the contribution of the 2 fetuses to cfDNA in maternal blood [8, 23]. In the massively parallel shotgun sequencing studies it was necessary to carry out ultra-deep sequencing with a median of 431 million sequencing reads to achieve a median of 530-fold coverage at each genomic locus. An advantage of the directed cfDNA analysis with DANSR used in our study is the higher efficiency with DNA sequencing which requires only 2.89 million sequencing reads to achieve a median of 874-fold coverage at each genomic locus to provide a reasonable estimate for the lower FF of the 2 fetuses. We believe that estimation of the lower FF is what matters clinically, because the potential source of an erroneous result from cfDNA screening for aneuploidies in dizygotic twins is failure to recognize the cases with a satisfactory total FF but with an unsatisfactory lower FF for 1 fetus of less than 4%.

Fetal aneuploidy assessment with cfDNA analysis has proven to be clinically useful in singleton pregnancies. While twin pregnancies pose some additional complexity, it is likely that cfDNA analysis will also be clinically useful in this population. However, the proposed approach for cfDNA testing in monozygotic and dizygotic twins requires further investigation in clinical implementation studies.

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Disclosure Statement

A.S. and K.H.N. have no conflicts of interest to disclose. C.A.S. and A.O. are employees of Ariosa Diagnostics and K.S. is an employee and board member of the company.
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