Cell-free DNA testing in the maternal blood in high-risk pregnancies after first trimester combined screening

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- **What's already known about this topic?** Cell-free DNA testing in the maternal blood is a highly efficient method of screening for common fetal trisomies.

- **What does this study add?** In high-risk patients after first-trimester combined screening, a policy of selecting a subgroup for invasive testing and another for cfDNA testing ensures that most trisomies are detected and allows to diagnose more than 60% of abnormalities that are not currently investigated by the cfDNA test.

Abstract

Objective: To investigate a strategy for clinical implementation of cfDNA testing in high-risk pregnancies after first-trimester combined screening.

Methods: In 259 singleton pregnancies undergoing invasive testing after first trimester combined screening, a maternal blood sample was sent to the laboratory Natera for cfDNA testing using a SNP-based methodology.

Results: The cfDNA test provided a result in 249 (96.1%) pregnancies and, among these, identified as being at high risk 35 of 36 cases of trisomy 21, 13 of 13 with trisomy 18, 5 of 5 with trisomy 13 and 3 of 4 with sex chromosome aneuploidies. A policy of performing an invasive test in women with a combined risk of >1 in 10 or NT ≥4 mm and offering cfDNA testing to the remaining cases, would detect all cases of trisomy 21, 18 or 13, 80% of sex aneuploidies and 62.5% of other defects, and would avoid an invasive procedure in 82.4% of euploid fetuses.

Conclusion: In high-risk pregnancies after combined screening, a policy of selecting a subgroup for invasive testing and another for cfDNA testing would substantially reduce the number of invasive procedures and retain the ability to diagnose most of the observed aneuploidies.

Key words: combined test, nuchal translucency, ultrasound, cfDNA testing

Introduction

One of the most widely used methods of screening for trisomy 21, 18 and 13 is based on the combination of maternal age, sonographic measurement of fetal nuchal translucency (NT) thickness and maternal serum free ß-hCG and PAPP-A at 11-13 weeks' gestation. It has been demonstrated that the combined test can identify about 90% of fetuses with trisomy 21 and approximately 95% of those with trisomy 18 and 13, for an overall invasive testing rate of 3-5%[1].

Recent studies have shown that cell-free DNA (cfDNA) testing in the maternal blood can detect more than 99% of fetuses with trisomy 21, 96% of trisomy 18, 92% of trisomy 13 and 90% of monosomy X, for an overall false positive rate of about 0.5%[2,3]. In the screen positive group of combined testing, the use of cfDNA analysis has the main objectives of firstly, retain the ability to diagnose the vast majority of the chromosomal defects included in this population and secondly, substantially reduce the overall invasive testing rate of first trimester combined screening by re-assessing the risk for common trisomies in pregnancies with a screen positive result. In this respect, the components of combined testing, such as the risk score and NT thickness, should be taken into account to identify those patients that should be offered a diagnostic invasive procedure independently from the results of the cfDNA test. In addition, first trimester combined screening incidentally detects chromosomal abnormalities which are different from those targeted by the test and that cannot currently be identified by cfDNA analysis[4].

The objective of this study was to examine the impact of cfDNA testing on the diagnosis of chromosomal defects in pregnancies with a screen positive result from the combined test and to assess the clinical value of using the combined risk score

and the measurement of NT thickness to select a subgroup that should be offered invasive testing and another that could have the cfDNA test.

Methods

This was a multicentre study involving four fetal medicine centres in Italy (Mangiagalli Hospital in Milan, Di Venere and Sarcone Hospitals in Bari, Palagi Hospital in Florence). During a 10 month period (March to December 2014), we examined 259 consecutive singleton pregnancies attending our centres for invasive testing because the estimated risk for trisomies 21, 18 or 13 after first-trimester combined screening was $\geq$1 in 250, which is the recommended risk cut-off for invasive testing according to local guidelines. Screening was undertaken at the participating centres or, in a minority of cases, women were referred from other hospitals because of a high risk after combined testing, which was based on the combination of maternal age, fetal NT thickness, fetal heart rate and maternal serum free ß-hCG and PAPP-A at 11-13 weeks' gestation. The ultrasound scans were carried out by trained sonographers accredited by the Fetal Medicine Foundation (FMF London, UK – www.fetalmedicine.org). In all cases fetal karyotyping was carried out by chorionic villous sampling or amniocentesis. Additional genetic testing, such as microarray-CGH analysis and others, was carried out in selected cases based on the clinician's decision, including NT thickness ≥ 3.5 mm and/or evidence of a major structural defect on ultrasound.

In all cases, before invasive testing, a transabdominal ultrasound examination was carried out (RAB 4-8 probe, Voluson E8 Expert, GE Medical Systems, Milwaukee, WI, USA) and a maternal venous blood sample (20mL in Streck cell-free DNA BCTTM tubes) was obtained and shipped overnight to the laboratory to the USA for

cfDNA testing (Natera Inc., San Carlos, CA). The data provided to the laboratory were: patient unique identifier, maternal age, gestational age, racial origin and date of blood collection.

In the laboratory, cfDNA was amplified using a massively multiplexed PCR methodology targeting 20,000 SNPs, sequenced, and analyzed with Bayesian-based algorithm to determine fetal ploidy status, as previously described[5]. The algorithm analyses a number of quality control metrics to identify laboratory or sequencing failure, estimate the amount of total starting DNA, determine the fetal fraction, and calculate the extent to which the measured cfDNA data fit expected case-specific distributions. In this study, a determination of the ploidy state of the fetus was not made if the fetal fraction was <4.0%, if the amount of input DNA was below 1500 genome equivalents, or if off-allele contamination was >0.2%. Maternal genotypic information was incorporated into the analysis as previously described[6]. Results were provided in the form of the probability for a copy number of the five chromosomes (21, 18, 13, X and Y) interrogated in each sample, along with a sample-specific calculated accuracy for each chromosome. The risk cut-off used to define the screen positive group with the algorithm was $\geq$1 in 100. The results from invasive testing and cfDNA analysis were exchanged between the participating centers and the laboratory only upon study closure. The study was approved by the Institutional Review Board of Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy (reference n. 124, approved on 22nd January 2014).

Statistical analysis

The data were explored and analysed using the statistical software SPSS 12.0 (Chicago, IL, USA) and Excel for Windows 2000 (Microsoft Corp., Redmond, WA,

USA). Fisher's exact test was used to assess the differences in frequency distribution of categorical variables.

Results

The median maternal age of the study population was 36 (range 20-46) years, median fetal crown-rump length was 63.0 (range 45-84) mm and median NT thickness was 2.7 (range 1.0-12.7) mm. Invasive testing demonstrated chromosomal abnormalities in 71 (27.4%) of the 259 cases (Table 1). The abnormalities included 8 (11.3%) that were not targeted by cfDNA testing and these are described in detail in Table 2. Microarray-CGH analysis was carried out in 32 cases and it was abnormal in 3 fetuses (Table 2).

On the total of 259 examined pregnancies, the cfDNA test provided a result in 249 (96.1%) cases and, among these, correctly identified as being at high risk 35 of 36 cases of trisomy 21, 13 of 13 with trisomy 18, 5 of 5 with trisomy 13 and 3 of 4 with sex chromosome aneuploidies (Table 1). The test also correctly identified as being low-risk all 184 euploid cases. In 10 cases the cfDNA test did not provide results because the fetal fraction was <4% in 8 and the sample failed internal quality control in 2. The no result rate was significantly higher in the group of aneuploidies, mosaicisms and structural re-arrangements compared to chromosomally normal fetuses (8.5% vs 2.1%, $p<0.05$). The no result group included 1 case of trisomy 21, 2 of trisomy 18, 1 of trisomy 13, 1 of 47 XXX and 1 case with duplication in chromosome 8 (Table 1).

The frequency distribution of different fetal karyotypes according to various cut-off values of combined test risk and NT thickness is shown in Figure 1. The group of fetuses with a risk $\geq$ 1 in 10 and / or NT $\geq$ 4 mm included the majority of the cases

with common trisomies and sex aneuploidies, more than 50% of fetuses with other defects and less than 20% of euploid pregnancies (Table 3).

We examined the performance of a policy in which women with a risk from the combined test of ≥1 in 250 are subdivided into group A containing those with a risk of ≥1 in 10 or NT ≥4 mm and group B containing the remainder of cases (Figure 2). In group A, invasive testing is carried out and group B have cfDNA testing and subsequently invasive testing is carried out if the result is positive. Such policy would detect all cases of trisomy 21, 18 or 13, 80% of sex aneuploidies and 62.5% of cases with other chromosomal defects, and would avoid an invasive procedure in 82.4% of euploid fetuses.

Discussion

This study shows that in patients identified by the first-trimester combined test as being at high-risk of trisomies, a policy of selecting a subgroup for invasive testing and another for cfDNA testing can substantially reduce the number of invasive procedures, retain the ability to diagnose most trisomies and also diagnose more than 60% of abnormalities that are not currently detectable by the cfDNA test.

Our data confirm previous evidence that sequencing of the SNP regions of cfDNA in the maternal blood is a highly efficient method of screening for common fetal trisomies[5-7]. Among cases with a result, a high risk score was provided in the vast majority of fetuses with trisomy 21, 18, 13 and monosomy X and all euploid fetuses were correctly classified as being at low risk. There were two false negative results, one each of trisomy 21 and monosomy X. In the case of trisomy 21 the fetal fraction was 4.4% and a recent study suggested, through statistical modelling, that the performance of cfDNA testing may be affected by the relative percentage of fetal

DNA in the maternal circulation, with a significantly lower detection rate for trisomy 21 when the fetal fraction is below 6%[8]. Although this finding could partially explain our false negative result, further clinical studies are required to assess the relationship between the fetal fraction and the performance of cfDNA testing. The false negative case of monosomy X could be explained by the presence of a feto-placental mosaicism with an under-represented monosomic cell line in the cytotrophoblast, which is the source of cfDNA. The frequent occurrence of feto-placental mosaicism in cases with monosomy X could also explain the lower reported detection rate of cfDNA testing for this abnormality compared to the detection rate for trisomy 21[2].

Analysis of cfDNA in the maternal blood did not provide a result in about 4% of the samples and this was mostly due to a low fetal fraction. A previous study showed that a repeated maternal blood sample would produce a result in about 60% of these cases[9]. However, our data confirm emerging evidence that the no result rate may be higher in fetuses with aneuploidies compared to those with normal karyotype[3,7]. Therefore, the importance of a low fetal fraction in re-assessing the risk for common trisomies after combined testing should be evaluated in large prospective clinical studies.

It has been previously reported that the screen positive group of combined testing includes cases with abnormalities which were not initially targeted and that are incidentally detected, such as other trisomies, sex aneuploidies, deletions, duplications, mosaicisms and others[4], and these defects accounted for about 10% of all aneuploidies in our study. Two recent studies analysed data on microarray-CGH results in a large number of pregnancies undergoing prenatal invasive testing for a variety of reasons, such as maternal age $\geq$ 35 years, high risk from combined testing or second trimester serum screening, maternal request and others[10,11]. In the group

of pregnancies with a high risk from aneuploidy screening, the authors reported an overall incidence of copy number variations with known clinical significance or potential clinical significance of about 1%, which is similar to what was observed in our population (Table 2). However, we cannot exclude that some sub-chromosomal defects may have been missed because we performed microarray-CGH analysis only in 12% of cases in our cohort. There is some evidence that cfDNA testing can identify a proportion of fetuses with sub-chromosomal abnormalities[12]. However, there are insufficient data on reliable estimates of the detection rate and false positive rate of cfDNA testing for each of these defects in the general obstetric population. Therefore, in order to reduce the overall invasive testing rate of combined screening by using cfDNA analysis, it is inevitable that a proportion of abnormalities different from trisomy 21, 18 and 13 will not be diagnosed.

In this study, the incidence of aneuploidies in fetuses with a combined risk $\geq$ 1 in 10 and NT $\geq$ 4 mm was very high and a recent large study reported that this population includes only about 0.7% of the total of euploid fetuses[4]. Therefore, a policy of offering direct access to a diagnostic procedure in these cases would have a small impact on the overall invasive testing rate. In addition, it is well established that fetuses with high NT and a normal karyotype should undergo additional genetic testing that require chorionic villi or amniotic fluid samples[13-14].

The prevalence of common trisomies in our cohort was higher than what would have been expected in the screen-positive group of combined testing on the general obstetric population[15] and this may be due to the fact that a minority of patients were referred from other centres, with a trend towards referral of very high-risk cases. Our results are unlikely to change significantly if a contingent strategy is applied to a screen-positive population with a relatively lower proportion of cases with common

trisomies and a higher number of non-trisomic fetuses, but larger clinical studies would be required to confirm our findings.

Pregnancies with a risk from the combined test of < 1 in 250 were not examined as per our study design. It has been shown that this group includes about 10% of fetuses with trisomy 21 and a proportion of euploid fetuses which increases with decreasing combined risk cut-offs[15]. A recent study showed that there was no difference in the performance of cfDNA analysis by the SNPs method between high and low risk pregnancies[7]. Therefore, extending the use of cfDNA testing to lower risk categories, with the main objective of increasing the detection rate for common trisomies, will depend upon local health policies and economic resources necessary to offer the test to larger proportions of the obstetric population.

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Figure legends

Figure 1. Frequency distribution of different fetal karyotypes according to various cut-offs of combined risk (a) and NT thickness (b).

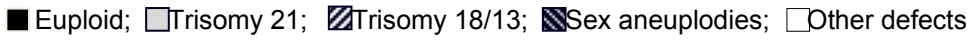

Figure 2. Flow chart illustrating the contingent strategy of offering direct access to invasive testing if the risk from combined screening is $\geq$ 1 in 10 and/or NT thickness is $\geq$ 4.0 mm and re-assessing the risk for common trisomies by cfDNA testing in the lower risk group.

Table 1. Results from cfDNA testing according to fetal karyotype.

Fetal karyotype	**n**	**Cell free DNA test**			
		No result	**Result**	**Screen +ve**	**Screen -ve**
Euploid	188	4 (2.1)	184	-	184 (100)
Trisomy 21	37	1 (2.7)	36	35 (97.2)	1 (2.8)
Trisomy 18	15	2 (13.3)	13	13 (100)	-
Trisomy 13	6	1 (16.7)	5	5 (100)	-
Monosomy X	3	0 (0.0)	3	2 (66.7)	1 (33.3)
47,XXX or 47,XXY	2	1 (50.0)	1	1 (100)	-
Other	8	1 (12.5)	7	-	7 (100)
Total	259	10 (3.9)	249	56 (22.5)	193 (77.5)

Other = one case each of trisomy 4, trisomy 22, deletions in chromosomes 2, 4, 16, duplication in chromosome 8, Beckwith-Wiedemann syndrome, mosaicism 45X0/46XY

Table 2. Details on karyotype and microarray-CGH results in the 8 cases with chromosomal defects that were not targeted by cfDNA testing.

Sample	Standard karyotype	Microarray-CGH	Details
CVS	47,XX+4	Not performed	Homogeneous form
CVS	47,XX+22	Not performed	Homogeneous form
Amnio	46,XY	arr[hg19]2p16.3(50,892,877-51,083,440)x1	Deletion of the region 2p16.3 (size 190 Kb)
CVS	46,XY,del(4)(q?)	arr[hg19]4q13.1q24(64,247,237-102,510.250)x1dn	Deletion of the region 4q13.1q24 (size 38 Mb)
CVS	46,XY	arr[hg19]16p11.2(29,673,984-30,190,539)x1	Deletion of the region 16p11.2 (size 516 Mb)
CVS	46,XX	arr[hg19]8p23.1(8,130,660-11,805,931)x3mat	Duplication of the region 8p23.1 (size 3.7 Mb)
CVS	46,XY	Not performed	Beckwith-Wiedemann (imprinting)
CVS	Mos45,X0/46,XY	Not performed	Mos45,X(10)/46,XY(36)

CVS = chorionic villous sampling; Amnio = amniocentesis

Table 3. Frequency distribution of cases with a combined risk ≥ 1 in 10 and NT ≥ 4.0 mm in relation to fetal karyotype.

Fetal karyotype	n	Screen +ve result from combined test		
		Risk ≥1 in 10	Fetal NT ≥4 mm	Either
Euploid	188	26 (13.8)	12 (6.4)	33 (17.6)
Trisomy 21	37	33 (89.2)	25 (67.6)	35 (94.6)
Trisomy 18	15	14 (93.3)	11 (73.3)	14 (93.3)
Trisomy 13	6	5 (83.3)	5 (83.3)	5 (83.3)
Monosomy X	3	2 (66.7)	3 (100)	3 (100)
47,XXX or 47,XXY	2	1 (50.0)	1 (50.0)	1 (50.0)
Other	8	4 (50.0)	3 (37.5)	5* (62.5)
Total	259	85 (32.8)	60 (23.2)	96 (37.1)

Other = one case each of trisomy 4 and trisomy 22, deletions in chromosomes 2, 4, 16, duplication in chromosomes 8, Beckwith-Wiedemann syndrome, mosaicism 45X0/46XY

*Trisomy 4, deletion in chromosomes 16, duplication in chromosomes 8, Beckwith-Wiedemann syndrome, mosaicism 45X0/46XY