Incidence of chromosomal abnormalities in fetuses with first trimester ultrasound anomalies and a low-risk cell-free DNA test for common trisomies

Nicola Persico[1,2], Simona Boito[1], Paolo Volpe[3], Benedetta Ischia[1], Mattia Gentile[4], Luisa Ronzoni[5], Valentina De Robertis[3], Isabella Fabietti[1], Claudiana Olivieri[3], Enrico Periti[6], Romina Ficarella[4], Rosamaria Silipigni[7], Georgios Rembouskos[3]

1. Fetal Medicine and Surgery Service, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy
2. Department of Clinical Science and Community Health, University of Milan, Italy
3. Fetal Medicine Unit, Di Venere and Sarcone Hospitals, ASL BA, Bari, Italy
4. Medical Genetics Unit, Di Venere Hospital, ASL BA, Bari, Italy
5. Medical Genetics Unit, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy
6. Fetal Medicine Unit, Palagi Hospital, Florence, Italy
7. Laboratory of Medical Genetics, Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy

Funding statement: The costs of cell-free DNA analysis were covered by Natera Inc. (San Carlos, CA, USA).

Conflict of interest: The authors report no conflict of interest.

Short title: Cell-free DNA testing and ultrasound

Correspondence:
Nicola Persico
Fetal Medicine and Surgery Service
Fondazione IRCCS 'Ca' Granda' - Ospedale Maggiore Policlinico
Via della Commenda 12, 20122, Milan, Italy
Tel: +39 02 55032482
Email: nicola.persico@gmail.com

Manuscript word count: 2696
Figures: 3
Tables: 4

What's already known about this topic?

Fetuses with ultrasound anomalies are at increased risk for chromosomal abnormalities and genetic disorders. Since the introduction of cfDNA testing in prenatal screening for trisomy 21, 18 and 13, few studies have examined the incidence and type of chromosomal abnormalities in fetuses with ultrasound anomalies and a low-risk cfDNA test using data from actual clinical experience, with a wide variation between 3% and 27%.

What does this study add?

In a cohort of high-risk fetuses undergoing a detailed first trimester ultrasound examination, the incidence of chromosomal abnormalities in cases with ultrasound anomalies and a low-risk cfDNA result was about 19%, including abnormalities different from trisomy 21, 18 and 13 in all but one cases. Therefore, diagnostic testing in these patients should be offered with the intended aim to detect chromosomal abnormalities beyond common trisomies.

Key words

combined screening, ultrasound, nuchal translucency, fetal structural defect, cfDNA testing, NIPT, chorionic villous sampling, amniocentesis, chromosomal abnormalities

Abstract

Objective: To examine the incidence and type of chromosomal abnormalities in fetuses with first trimester ultrasound anomalies and a low-risk cfDNA test for common trisomies.

Mehods: In 486 singleton pregnancies undergoing invasive testing after combined screening, a detailed first trimester ultrasound assessment was carried out and a maternal blood sample was sent for cfDNA analysis. Ultrasound and cfDNA data were analysed in relation to fetal karyotype.

Results: Invasive testing demonstrated a chromosomal abnormality in 157 (32.3%) of 486 fetuses. In 348 cases with a low-risk cfDNA test for common trisomies, NT ≥ 3.5 mm and/or a major structural defect were observed in 92 (26.4%) fetuses. A chromosomal abnormality was found in 17 (18.5%; 95%CI 10.55-26.41) of these pregnancies, including 1 (1.1%) case of trisomy 21 and 16 (17.4%) fetuses with abnormalities different from common trisomies. The respective incidence in the 256 cases with a low-risk cfDNA test result and no ultrasound anomalies was 2.3% (95% CI 0.49-4.20; n=6).

Conclusions: In fetuses with first trimester ultrasound anomalies and a low-risk cfDNA result for trisomy 21, 18 and 13, diagnostic testing should be offered with the main objective to detect chromosomal abnormalities beyond common trisomies.

Introduction

Cell-free DNA (cfDNA) testing in the maternal blood is by far the most effective method of prenatal screening for trisomy 21, 18 and 13, with a detection rate of 99,7%, 98% and 99%, respectively, and a cumulative false positive rate of about 0.3%[1]. At present, the main limiting factor for universal implementation of cfDNA testing into national healthcare policies is the high production cost, which is still in the range 200-300 euros across Europe. Irrespective of the cost, which is likely to progressively decrease over time, new screening strategies are under investigation to define whether cfDNA analysis should be used as a stand-alone test, contingent to the results of first trimester combined screening or in combination with ultrasound only[2-4]. Based on previous evidence that fetuses with ultrasound anomalies have an increased incidence of chromosomal abnormalities, there is general agreement that in cases with nuchal translucency (NT) thickness $\geq$ 3.5 mm and/or a major structural anomaly on ultrasound, patients should be offered a diagnostic invasive procedure[5,6]. However, only few studies used cfDNA data from actual clinical experience to examine in this group the incidence and type of chromosomal abnormalities after a low-risk cfDNA test for trisomy 21, 18 and 13. In addition, the reported incidence of chromosomal abnormalities beyond common trisomies ranged between 3% and 27%, possibly due to the different criteria used to define the presence of an ultrasound anomaly and the wide gestational age range of the examined populations (10-34 weeks)[7-10].

The aim of this study was to examine the incidence and type of chromosomal abnormalities in first trimester fetuses with ultrasound anomalies and a low-risk cfDNA test for common trisomies.

Methods

Study population

This was a multicentre prospective cohort study involving four fetal medicine centres in Italy (Ospedale Maggiore Policlinico in Milan, Ospedali Di Venere and Sarcone in Bari, Ospedale Palagi in Florence). During a three-year period (2014-2016), we examined 486 singleton pregnancies attending our centres for invasive testing because the estimated risk for trisomies 21, 18 or 13 after first-trimester combined screening was ≥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 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. In all cases, fetal karyotyping was carried out by chorionic villous sampling or amniocentesis. Chromosomal microarray analysis was offered in cases with NT thickness ≥ 3.5 mm and/or evidence of a major structural defect on ultrasound. In addition, microarray was also offered in selected cases, based on the geneticist's assessment, for further characterization of abnormal karyotype results.

Ultrasound assessment at 11-13 weeks

In each case, before invasive testing, a transabdominal ultrasound examination was carried out (RAB 4-8 and RM 6C probes, Voluson E8 and E10, GE Medical Systems, Milwaukee, WI, USA) to assess fetal anatomy through the demonstration of the following views (Fig. 1):

- mid-sagittal view of the face to assess the fetal profile, the maxilla, the posterior fossa and to measure nuchal translucency thickness;
- transverse sections of the head to demonstrate the skull, midline echo, the choroid plexuses, orbits and primary palate;
- transverse sections of the thorax to demonstrate, using a combination of B-mode and Color- or Power-Doppler modalities, the position of the heart within the chest, the four-chamber view with atrio-ventricular valve offsetting, equal ventricular filling, arterial crossing (X sign) and normal flow, size and course of the aortic arch and the ductus arteriosus at the level of the three vessel-trachea view (V sign);
- transverse and sagittal sections of the trunk and extremities to demonstrate the stomach, bladder, abdominal insertion of the umbilical cord, spine, all long bones, hands and feet.

Cell-free DNA analysis in the maternal blood

On the same day and before the invasive procedure, a maternal venous blood sample (20mL in Streck cell-free DNA BCTTM tubes) was obtained and shipped overnight to the laboratory for cfDNA testing using a SNP-based methodology (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[11]. 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[12]. 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. For the purpose of this study, only results regarding trisomy 21, 18 and 13 were considered. The risk cut-off used to define the screen positive group with the algorithm was ≥1 in 100. The results from invasive testing and cfDNA analysis were exchanged between the participating centres and the laboratory on two occasions, after the first 259 cases and 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, the median fetal crown-rump length was 63.0 (range 45-84) mm and the median NT was 2.9 (range 1-13) mm. On the total of 486 cases, invasive testing demonstrated chromosomal abnormalities in 157 (32.3%) fetuses. Of these, 122 (77.7%) were

trisomy 21, 18 and 13. The remaining 35 cases were classified into genetic defects at high (n=25; 15.9%) and low risk (n=10; 6.4%) of adverse outcome based on the assessment by a clinical geneticist (Table 1). The assessment was carried out by examining, for each of these defects, the association with ultrasound anomalies and the risk of death or significant physical and intellectual disability. Full karyotype details of chromosomal abnormalities beyond trisomy 21, 18 and 13 are provided in the Supplemental Tables 1 and 2.

The frequency distribution of ultrasound findings according to fetal karyotype is shown in Table 2. NT $\geq$ 3.0 mm, $\geq$ 3.5 mm and major structural defects were observed in 77.7%, 69.4% and 34.4% of all fetuses with chromosomal abnormalities and in 34.0%, 20.1% and 5.5% of those with a normal karyotype, respectively. On the total of 486 examined cases, the most frequently diagnosed structural defects at 11-13 weeks' gestation were cardiac defects (9.1%), exomphalos (4.3%), facial defects (2.7%) and brain abnormalities (1.9%; Fig. 2).

CfDNA testing provided a result after a single blood draw in 462 (95.1%) of 486 cases. In this group, the test correctly classified as being at high risk 113 (99.1%) of 114 cases with common trisomies, including 71 (98.6%) of 72 fetuses with trisomy 21, 33 (100%) of 33 with trisomy 18 and 9 (100%) of 9 with trisomy 13. In one case with a high-risk result for trisomy 13, invasive testing by CVS and amniocentesis showed a confined placental mosaicism. There was no significant difference between cases with and without ultrasound anomalies in the detection rate of cfDNA screening for trisomy 21 (55/56 vs 16/16) and trisomy 18 (29/29 vs 4/4). No cases of trisomy 13 without ultrasound defects were observed in our population.

In the 348 cases with a low-risk cfDNA test result for common trisomies, NT ≥ 3.5 mm and/or a major structural defect were observed in 92 (26.4%) fetuses. In this group, a clinically significant chromosomal abnormality was found in 17 (18.5%; 95%CI 10.55-26.41) pregnancies, including 1 (1.1%) case of trisomy 21 and 16 (17.4%) fetuses with abnormalities at risk of adverse outcome but different from trisomy 21, 18 and 13. The respective incidence of such abnormalities in the 256 cases with a low-risk cfDNA test result and no ultrasound anomalies was 2.3% (95%CI 0.49-4.20; 6 cases; Fig.3).

In 24 (4.9%) of 486 cases, the cfDNA test did not provide results because of low fetal fraction in 7, uninformative DNA pattern in 6 and failure to pass quality metrics in 11. In the no result group, a major ultrasound anomaly was reported in 16 (66.7%) cases and 11 (68.8%) of these had a clinically significant chromosomal abnormality, including 2 cases of trisomy 21, 4 of trisomy 18, 2 of trisomy 13, 2 of triploidy and 1 of chromosome 8 microduplication. No cases with a clinically significant chromosomal abnormality were found among the 8 cases with a no result from cfDNA testing and no ultrasound anomalies (Fig. 3).

Discussion

Principal findings

In a cohort of high-risk fetuses undergoing a detailed first trimester ultrasound examination, the incidence of chromosomal abnormalities in cases with ultrasound anomalies and a low-risk cfDNA result was about 19%, including abnormalities different from trisomy 21, 18 and 13 in all but one cases. Therefore, diagnostic testing

in these patients should be offered with the intended aim to detect chromosomal abnormalities beyond common trisomies.

Clinical implications

This study confirmed that cfDNA testing is a highly efficient method of screening for trisomy 21, 18 and 13. In cases with a result, the test correctly classified as being at high risk all but one fetuses affected by one of these trisomies, with only one false negative case of trisomy 21 and a very low false positive rate (Fig. 3). In addition, the performance of cfDNA testing did not significantly differ between fetuses with and without major ultrasound findings. Therefore, if the main objective of screening is to identify fetuses with trisomy 21, 18 or 13 and cfDNA analysis returns a low-risk result, parents should be reassured that it is very unlikely that the fetus is affected, even in the presence of a major anomaly on ultrasound. However, it is well-established that cfDNA analysis is a screening rather than a diagnostic test and therefore, false negative cases might be encountered. In our one false negative 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%[13]. Although this finding could partially explain a false negative result, further clinical studies would be required to assess the relationship between the fetal fraction and the performance of cfDNA testing.

It was previously reported that 10-30% of fetal chromosomal abnormalities diagnosed prenatally are different from trisomy 21, 18 and 13[14-17]. These include sex aneuploidies, other trisomies, triploidy, deletions, duplications, mosaicisms and others. The prevalence for each of these conditions is currently uncertain because of the lack of

large studies in low-risk pregnancies with postnatal genetic ascertainment of cases that did not undergo prenatal invasive testing, which is required to diagnose abnormalities that may not be associated with obvious phenotypic features. However, studies examining prenatal samples from patients undergoing 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, reported an overall prevalence of 0.5-1%[18,19]. Under the assumption that the costs of cfDNA testing will continue to decrease over time and that the test may be offered to all pregnant women by national healthcare systems, the main objectives of first trimester fetal ultrasound, including measurement of NT thickness, would be firstly, to identify fetuses with major structural defects and secondly, to screen for other rare chromosomal abnormalities. Our data suggest that the positive predictive value of such screening, derived from the cumulative incidence of these conditions in cases with ultrasound anomalies and a low-risk cfDNA result, would be about 17%. From an individual patient perspective, this information should be made available in the decision-making process regarding prenatal invasive testing.

It was consistently shown that cfDNA testing fails to produce a result in a percentage of cases that varied across the different studies between 0% and 12%[1]. The possible explanations for a no test result include low fetal fraction, problems with sample handling and laboratory assay failure for a variety of reasons, such as sub-optimal DNA extraction, amplification or sequencing. Our data, based on a single blood draw, are consistent with previous evidence that the incidence of chromosomal abnormalities is higher in the no result group[20], especially trisomy 18 or 13. In all of our cases with trisomy 21, 18 or 13 and a no result after cfDNA analysis, first trimester ultrasound showed major fetal anomalies, highlighting the importance of ultrasound in the clinical

management of cases with a no result. In the presence of high NT or a major structural defect, an invasive diagnostic procedure should be strongly recommended in these cases and a blood re-draw may not be appropriate.

Strengths and limitations

Only few previous studies have reported clinical data on fetuses with ultrasound anomalies and a low risk cfDNA result based on actual clinical experience, with a wide variation in the incidence of chromosomal abnormalities between 3% and 27%[7-10]. This difference may be related to several factors, such as sample size, definition of ultrasound anomaly, gestational age at inclusion and others. For example, in one study on 80 fetuses with NT $\geq$ 3.0 mm undergoing cfDNA testing, the reported incidence of chromosomal abnormalities beyond common trisomies was 27%[9]. In contrast, in a large study on 892 patients at medium/high risk for fetal aneuploidies, the rate of uncommon chromosomal abnormalities among the 258 fetuses with a negative cfDNA result and ultrasound anomalies was significantly lower (about 8%)[8]. However, the inclusion of fetuses with 'soft markers' into the definition of ultrasound anomaly and the wide gestational age range (more than 60% in the second and third trimesters) may have 'diluted' the number of other chromosomal abnormalities in a larger proportion of fetuses with normal karyotype. In this respect, the strengths of our study are that we only included first trimester fetuses, all undergoing both cfDNA and invasive testing. In addition, we used a standardized protocol for ultrasound assessment of fetal anatomy, including a detailed evaluation of the fetal heart by experienced operators, and we reported on a wide range of chromosomal and ultrasound abnormalities, the latter strictly defined by the presence of NT $\geq$ 3.5 mm and/or a major structural defect identified at 11-13 weeks' gestation. The higher than expected incidence of such

defects highlights the very high-risk nature of our population, with a likely selection bias towards referral for invasive testing of cases with major ultrasound anomalies. In addition, we did not perform microarray analysis in all cases with a normal standard karyotype but mostly in cases with NT > 3.5 mm and/or a major structural defect. Therefore, we cannot exclude that some sub-chromosomal abnormalities may have been missed in cases with normal ultrasound.

Research implications

Previous studies have reported that cfDNA testing could potentially identify chromosomal abnormalities different from common trisomies[21-23]. However, the number of examined cases was either small or data on prenatal / postnatal genetic verification of a screen-negative cfDNA result was reported only in a small fraction of the examined populations, making it difficult to provide reliable estimates on the detection rate for these abnormalities. Therefore, the performance of cfDNA testing beyond trisomy 21, 18 and 13 should be examined in large studies with postnatal genetic ascertainment. Future studies should also investigate whether the combination of biochemical or cfDNA testing and first trimester ultrasound may improve the detection of chromosomal abnormalities beyond common trisomies.

References

1. Gil MM, Accurti V, Santacruz B, et al. Analysis of cell-free DNA in maternal blood in screening for aneuploidies: updated meta-analysis. Ultrasound Obstet Gynecol 2017; 150:302-314.
2. Gil MM, Revello R, Poon LC, et al. Clinical implementation of routine screening for fetal trisomies in the UK NHS: cell-free DNA test contingent on results from first-trimester combined test. Ultrasound Obstet Gynecol. 2016; 47:45-52.
3. Kagan KO, Sroka F, Sonek J, et al. First-trimester risk assessment based on ultrasound and cell-free DNA vs combined screening: a randomized controlled trial. Ultrasound Obstet Gynecol. 2018; 51:437-444.
4. Le Bras A, Salomon LJ, Bussières L, et al. Cost-effectiveness of five screening strategies for trisomies and other unbalanced chromosomal abnormalities: a model-based analysis. Ultrasound Obstet Gynecol. 2019 Apr 22. doi: 10.1002/uog.20301. [Epub ahead of print]
5. Souka AP, Von Kaisenberg CS, Hyett JA, et al. Increased nuchal translucency with normal karyotype. Am J Obstet Gynecol 2005; 192:1005-1021.
6. Chitty LS, Kagan KO, Molina FS, et al. Fetal nuchal translucency scan and early prenatal diagnosis of chromosomal abnormalities by rapid aneuploidy screening: observational study. BMJ 2006; 332:452–455.
7. Beulen L, Faas BHW, Feenstra I, et al. Clinical utility of non-invasive prenatal testing in pregnancies with ultrasound anomalies. Ultrasound Obstet Gynecol 2017; 49:721-728.
8. Benachi A, Letourneau A, Kleinfinger P, et al; Collaborative SEquençage a Haut Debit et Aneuploidies (SEHDA) Study Group. Cell-free DNA analysis in maternal

plasma in cases of fetal abnormalities detected on ultrasound examination. Obstet Gynecol 2015; 125:1330-7.

9. Grossman TB, Bodenlos KL, Chasen ST. Abnormal nuchal translucency: residual risk with normal cell-free DNA screening. J Matern Fetal Neonatal Med 2019; 22:1-6.
10. Reiff ES, Little SE, Dobson L, et al. What is the role of the 11- to 14-week ultrasound in women with negative cell-free DNA screening for aneuploidy? Prenat Diagn 2016; 36:260-5.
11. Nicolaides KH, Syngelaki A, Gil M, et al. Validation of targeted sequencing of single-nucleotide polymorphisms for non-invasive prenatal detection of aneuploidy of chromosomes 13, 18, 21, X, and Y. Prenat Diagn 2013; 33:575-9.
12. Zimmermann B, Hill M, Gemelos G, et al. Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. Prenat Diagn 2012; 32:1233-41.
13. Wright D, Wright A, Nicolaides KH. A unified approach to risk assessment for fetal aneuploidies. Ultrasound Obstet Gynecol 2015; 45:48-54.
14. Alamillo CM, Krantz D, Evans M, et al. Nearly a third of abnormalities found after first-trimester screening are different than expected: 10-year experience from a single center. Prenat Diagn. 2013; 33:251-6.
15. Lindquist A, Poulton A, Halliday J, Hui L. Prenatal diagnostic testing and atypical chromosome abnormalities following combined first-trimester screening:implications for contingent models of non-invasive prenatal testing. Ultrasound Obstet Gynecol. 2018; 51:487-492.

16. Syngelaki A, Pergament E, Homfray, et al. Replacing the combined test by cell-free DNA testing in screening for trisomies 21, 18 and 13: impact on the diagnosis of other chromosomal abnormalities. Fetal Diagn Ther 2014; 35:174-84.
17. Vogel I, Petersen OB, Christensen R, et al. Chromosomal microarray as primary diagnostic genomic tool for pregnancies at increased risk within a population-based combined first-trimester screening program. Ultrasound Obstet Gynecol. 2018; 51:480-486.
18. Wapner RJ, Martin CL, Levy B, et al. Chromosomal microarray versus karyotyping for prenatal diagnosis. N Engl J Med 2012; 367:2175-84.
19. Papoulidis I, Sotiriadis A, Siomou E, et al. Routine use of array comparative genomic hybridization (aCGH) as standard approach for prenatal diagnosis of chromosomal abnormalities. Clinical experience of 1763 prenatal cases. Prenat Diagn 2015; 35:1269-77.
20. Revello R, Sarno L, Ispas A, et al. Screening for trisomies by cell-free DNA testing of maternal blood: consequences of a failed result. Ultrasound Obstet Gynecol 2016; 47:698-704.
21. Wapner RJ, Babiarz JE, Levy B, et al. Expanding the scope of noninvasive prenatal testing: detection of fetal microdeletion syndromes. Am J Obstet Gynecol 2015; 212: 332.e1-9.
22. Srinivasan A, Bianchi DW, Huang H, et al. Noninvasive detection of fetal subchromosome abnormalities via deep sequencing of maternal plasma. Am J Hum Genet 2013; 92:167-76.
23. Fiorentino F, Bono S, Pizzuti F, et al. The clinical utility of genome-wide non invasive prenatal screening. Prenat Diagn 2017; 37:593-601.

Table 1. Maternal and fetal characteristics according to fetal karyotype in the study population of 486 pregnancies.

Fetal karyotype	**N=486**	**Age, years**	**CRL, mm**	**NT, mm**	**β-hCG, MoM**	**PAPP-A, MoM**
Normal	329 (67.7)	36 (21-45)	63.7 (46-84)	2.5 (1-10)	1.1 (0.1-10.2)	0.6 (0.1-3.2)
All abnormalities	157 (32.3)	38 (20-46)	61.3 (45-80)	4.7 (1.3-12.7)	0.9 (0.1-7.8)	0.4 (0.1-2.4)
Trisomy 21	74 (47.1)	38 (23-46)	63.8 (47-80)	4.5 (1.6-9.5)	1.4 (0.4-7.8)	0.4 (0.1-2.4)
Trisomy 18	37 (23.6)	39 (28-42)	55.7 (45-80)	6.3 (1.7-10.4)	0.2 (0.1-0.9)	0.2 (0.1-2.3)
Trisomy 13	11 (7.0)	37 (22-39)	54.1 (48-65)	5.8 (2.6-9.0)	0.7 (0.3-1.1)	0.4 (0.1-0.9)
Others at high risk of adverse outcome	25 (15.9)	33 (20-44)	58.5 (46-75)	4.5 (1.3-12.7)	0.7 (0.2-3.8)	0.8 (0.1-2.0)
Monosomy X	8	-	-	-	-	-
Trisomy 4	1	-	-	-	-	-
Trisomy 22	1	-	-	-	-	-
Triploidy (69, XXX)	2	-	-	-	-	-
Tetrasomy 12p	2	-	-	-	-	-
Pathogenic deletions	4	-	-	-	-	-
Pathogenic duplications	2	-	-	-	-	-
Unbalanced translocations	2	-	-	-	-	-
Placental mosaicism	2	-	-	-	-	-
Beckwith-Wiedemann syndrome	1	-	-	-	-	-
Others at low risk of adverse outcome	10 (6.4)	36 (28-44)	63.3 (51-74)	2.9 (1.3-8.7)	0.7 (0.1-2.2)	0.5 (0.2-1.8)
47,XXY	1	-	-	-	-	-
47,XXX	2	-	-	-	-	-
Non-pathogenic deletions	2	-	-	-	-	-
Placental mosacisim	5	-	-	-	-	-

CRL = crown-rump length; NT = nuchal translucency; β-hCG = β-human chorionic gonadotropin; PAPP-A = pregnancy associated plasma protein-A; MoM = multiple of median

Table 2. Ultrasound findings at the 11-13 weeks' scan and their relation to fetal karyotype.

	Fetal karyotype		**Common trisomies**			**Other chromosomal abnormalities**	
	Normal	**Abnormal**	**Trisomy 21**	**Trisomy 18**	**Trisomy 13**	**High risk of adverse outcome**	**Low risk of adverse outcome**
Number of cases	329	157	74	37	11	25	10
NT ≥ 3.0 mm	112 (34.0)	122 (77.7)	62 (83.8)	31 (83.8)	8 (72.7)	18 (72.0)	3 (30.0)
NT ≥ 3.5 mm	69 (20.1)	109 (69.4)	54 (73.0)	29 (78.4)	8 (72.7)	17 (68.0)	1 (10.0)
One or more major structural defects	18 (5.5)	54 (34.4)	12 (16.2)	20 (54.1)	11 (100)	11 (44.0)	0 (0.0)
NT ≥ 3.0 mm and /or major defect	120 (36.5)	133 (84.7)	65 (87.8)	34 (91.9)	11 (100)	20 (80.0)	3 (30.0)
NT ≥ 3.5 mm and /or major defect	79 (24.0)	122 (77.7)	58 (78.4)	33 (89.2)	11 (100)	19 (76.0)	1 (10.0)
All structural defects							
Cardiac	7 (2.1)	37 (23.6)	10 (13.5)	14 (37.8)	6 (54.5)	7 (28.0)	0
Atrio-ventricular septal defect	2	18	8	8	1	1	-
Hypoplastic left heart	-	5	-	1	2	2	-
Cardiomegaly	1	-	-	-	-	-	-
Transposition of great arteries	1	1	-	1	-	-	-
Tetralogy of Fallot	-	2	-	-	-	2	-
Pulmonary atresia	-	2	-	-	1	1	-
Common arterial trunk	1	1	-	1	-	-	-
Complex defect	2	8	2	3	2	1	-
Brain and neural tube	1 (0.3)	8 (5.1)	1 (1.4)	4 (10.8)	3 (27.3)	0	0
Acrania	1	1	-	1	-	-	-
Encephalocele	-	1	-	1	-	-	-
Holoprosencephaly	-	3	-	-	3	-	-
Spina bifida	-	3	1	2	-	-	-
Face	1 (0.3)	12 (7.6)	0	4 (10.8)	6 (54.5)	2 (8.0)	0
Facial cleft	-	10	-	4	5	1	-
Micrognathia	1	2	-	-	1	1	-
Abdominal wall	6 (1.8)	15 (9.6)	1 (1.4)	8 (21.6)	4 (36.4)	2 (8.0)	0
Exomphalos	6	15	1	8	4	2	-
Others	8 (2.4)	12 (7.6)	2 (2.7)	4 (10.8)	4 (36.4)	2 (8.0)	0
Diaphragmatic hernia	3	2	-	1	-	1	-
Skeletal defects	2	7	2	3	2	-	-
Hydrops	2	2	-	-	2	-	-
Other	1	1	-	-	-	1	-

Supplemental Table 1. Details of the 25 chromosomal abnormalities at high risk of adverse outcome different from trisomy 21, 18 and 13 in the study population of 486 fetuses.

Chromosomal abnormality	**N=25**	**Invasive test**	**Standard karyotype / Microarray / Genetic testing**	**Ultrasound findings at 11-13 weeks**	**Follow-up**
Monosomy X	8	CVS	45,X	All had NT ≥3.5 mm and 3 (37.5%) had a major structural defect (2 HLV and 1 CDH)	TOP
Trisomy 4	1	CVS	47,XX,+4	NT ≥3.5 and HLV	Miscarriage
Triploidy	2	CVS	69,XXX	One each with NT ≥3.5 and TOF	TOP
Chr 1 deletion	1	CVS	arr[GRCh37] 1q23.2q24.2(159127288_168609527)x1 dn	NT ≥3.5 mm, PA and cleft palate	TOP
Chr 16 deletion	1	CVS	arr[GRCh37] 16p11.2(29673984_30190539)x1 dn	NT ≥3.5 mm	TOP
Chr 16 deletion	1	CVS	arr[GRCh37] 16p13.11(15534320_16292304)x1 dn	NT ≥3.5 mm	TOP
Chr 1 duplication	1	CVS	arr[GRCh37] 1q31.3q41(196407478_224091349)x3 dn	NT ≥3.5 and complex cardiac defect	TOP
Chr 8 duplication	1	CVS	arr[GRCh37] 8p23.1(8130660_11805931)x3 mat	NT ≥3.5, AVSD and TOF	TOP
Unbalanced translocation	1	CVS	46,XY,der(15)t(3;15)(q23;p11.1)mat	NT ≥3.5 and exomphalos	TOP
Placental mosaicism Trisomy 2	1	CVS	mos 47,XX,+2[6]/46,XX[11]	NT ≥3.5 and body stalk anomaly	TOP
Beckwith-Wiedemann syndrome	1	CVS	46,XY- Positive molecular testing	Exomphalos	TOP
Trisomy 22	1	CVS	47,XX,+22	No ultrasound anomalies	TOP
Placental mosaicism Trisomy 22	1	CVS/Amnio	CVS: mos 47,XY,+22[7]/46,XY[13]; Amnio: 46,XY	No ultrasound anomalies	Intrauterine death

Tetrasomy 12p (Pallister-Killian syndrome)	1	CVS	arr[GRCh37] 12p13.33p11.1(189608_34756180)x4	No ultrasound anomalies	TOP
Tetrasomy 12p (Pallister-Killian syndrome)	1	CVS	arr[GRCh37] 12p13.33p11.1(148375_34760977)x4	No ultrasound anomalies	TOP
Chr 4 deletion	1	CVS	arr[GRCh37] 4q13.1q24(64247237_102510250)x1 dn	No ultrasound anomalies	TOP
Unbalanced translocation	1	Amnio	46,XY,der(21)t(8;21)(q23.3;p11)	No ultrasound anomalies	TOP

Chr = chromosome; CVS = chorionic villous sampling; NT = nuchal translucency; HLV = hypoplastic left ventricle; CDH = congenital diaphragmatic hernia; TOF = tetralogy of Fallot; PA = pulmonary atresia; AVSD = atrioventricular septal defect; TOP = termination of pregnancy

Supplemental Table 2. Details of the 10 chromosomal abnormalities at low risk of adverse outcome different from trisomy 21, 18 and 13 in the study population of 486 fetuses.

Chromosomal abnormality	**N=10**	**Invasive test**	**Standard karyotype / Microarray / Genetic testing**	**Ultrasound findings at 11-13 weeks**	**Follow-up**
Klinefelter syndrome	1	CVS	47,XXY	NT ≥3.5 mm	Live birth
Triple X	2	CVS	47,XXX	No ultrasound anomalies	Live birth
Chr 2 deletion	1	Amnio	arr[GRCh37] 2p16.3(50892877_51083440)x1 mat	No ultrasound anomalies	Live birth
Chr X deletion	1	Amnio	arr[GRCh37] Xp21.3p11.4(27958276_41683549)x1 dn	No ultrasound anomalies	Live birth
Placental mosaicism Trisomy 9	1	CVS/Amnio	CVS: mos 47,XY,+9[10]/46,XY[4]; Amnio: 46,XY	No ultrasound anomalies	Live birth
Placental mosaicism Trisomy 13	1	CVS/Amnio	CVS: mos 47,XY,+13[1]/46,XY[7]; Amnio: 46,XY	No ultrasound anomalies	FGR, intrauterine death
Placental mosaicism Trisomy 13	1	CVS/Amnio	CVS: mos47,XX,+13[4]/46,XX[12]; Amnio: 46,XX	No ultrasound anomalies	Live birth
Placental mosaicism Trisomy 21	1	CVS/Amnio	CVS: mos 47,XY,+21[2]/46,XY[37]; Amnio: 46,XY	No ultrasound anomalies	Live birth
Placental mosaicism Tetraploidy	1	CVS/Amnio	CVS: mos 92,XXYY[4]/92,XXYY/46,XY[22]/46,XY[3]; Amnio: 46,XY	No ultrasound anomalies	Live birth

Chr = chromosome; CVS = chorionic villous sampling; NT = nuchal translucency; FGR = fetal growth restriction

Figure legends

Figure 1. Normal fetal anatomy at 11-13 weeks: **a.** mid-sagittal view of the face showing the forehead, nose, lips and chin superiorly, the intracranial spaces in the middle (brain stem, fourth ventricle and cisterna magna shown by the stars) and nuchal translucency inferiorly; **b.** transverse section of the head showing the midline echo (arrow) and the chorioid plexuses; **c.** transverse section of the face showing the primary palate (arrow); **d.** transverse section of the thorax showing the four chambers of the heart with equal filling of the ventricles on Color-Doppler; **e.** crossing of the aorta and the pulmonary artery on Color-Doppler (X sign); **f.** forward flow, equal size and normal course of the aortic arch and the ductus arteriosus at the level of the three vessel-trachea view (V sign); **g.** transverse section of the abdomen demonstrating the stomach filled with amniotic fluid (arrow); **h.** normal insertion of the umbilical cord into the fetal abdomen (arrow); **i.** sagittal section of the pelvis showing a visible bladder (arrow); **l.** parasagittal section showing upper and lower extremities.

Figure 2. Ultrasound images showing examples of major fetal defects detected at 11-13 weeks' gestation: **a.** severe micrognathia; **b.** alobar holoprosencephaly; **c.** bilateral cleft palate; **d.** complete atrioventricular septal defect shown by the loss of the normal valve off-setting on a four-chamber view and by the presence of a common atrioventricular valve (arrow); **e.** left diaphragmatic hernia demonstrated on a four-chamber view of the heart (H) showing a right cardiac shift and the herniation of the stomach (S), bowel and part of the liver (L) into the thorax; **f.** giant exomphalos with herniation of bowel and most of the liver (L).

Figure 3. Flow-chart showing the incidence and details of clinically significant chromosomal abnormalities according to the results of cfDNA testing and to the presence or absence of major ultrasound anomalies in our study population of 486 cases.

T = trisomy; Tetr = tetrasomy; Del = deletion; Dup = duplication; Unb trans = unbalanced translocation; BWS = Beckwith-Wiedemann syndrome