CLINICAL IMPLEMENTATION OF CELL-FREE DNA ANALYSIS OF MATERNAL BLOOD

Submitted by

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I confirm that Dr Maria Soledad Quezada Rojas has carried out under my supervision the studies presented in the Thesis: Clinical implementation of cell-free DNA analysis of maternal blood.

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Professor Jesus Florido Navio
Granada September 2015

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English

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1.1.1 Prevalence of trisomies 21, 18 and 13

The risk for trisomies increases with maternal age. Additionally, because chromosomally abnormal fetuses are more likely to die in utero than euploid fetuses, the risk decreases with gestational age (Tables 1.1-1.3). The rate of fetal death between 12 weeks (when first trimester screening is performed) and term is about 30% for trisomy 21 and 80% for trisomies 18 and 13 (Snijders *et al*., 1994, 1995, 1999).

Table 1.1: Estimated risk for trisomy 21 in relation to maternal age and gestation

Maternal age (years)	Gestational age					
	10 weeks	12 weeks	14 weeks	16 weeks	20 weeks	40 weeks
20	1/983	1/1068	1/1140	1/1200	1/1295	1/1527
25	1/870	1/946	1/1009	1/1062	1/1147	1/1352
30	1/576	1/626	1/668	1/703	1/759	1/895
31	1/500	1/543	1/580	1/610	1/658	1/776
32	1/424	1/461	1/492	1/518	1/559	1/659
33	1/352	1/383	1/409	1/430	1/464	1/547
34	1/287	1/312	1/333	1/350	1/378	1/446
35	1/229	1/249	1/266	1/280	1/302	1/356
36	1/180	1/196	1/209	1/220	1/238	1/280
37	1/140	1/152	1/163	1/171	1/185	1/218
38	1/108	1/117	1/125	1/131	1/142	1/167
39	1/82	1/89	1/95	1/100	1/108	1/128
40	1/62	1/68	1/72	1/76	1/82	1/97
41	1/47	1/51	1/54	1/57	1/62	1/73
42	1/35	1/38	1/41	1/43	1/46	1/55
43	1/26	1/29	1/30	1/32	1/35	1/41
44	1/20	1/21	1/23	1/24	1/26	1/30
45	1/15	1/16	1/17	1/18	1/19	1/23

Table 1.2: Estimated risk for trisomy 18 in relation to maternal age and gestation

Maternal age (years)	Gestational age					
	10 weeks	12 weeks	14 weeks	16 weeks	20 weeks	40 weeks
20	1/1993	1/2484	1/3015	1/3590	1/4897	1/18013
25	1/1765	1/2200	1/2670	1/3179	1/4336	1/15951
30	1/1168	1/1456	1/1766	1/2103	1/2869	1/10554
31	1/1014	1/1263	1/1533	1/1825	1/2490	1/9160
32	1/860	1/1072	1/1301	1/1549	1/2490	1/7775
33	1/715	1/891	1/1081	1/1287	1/1755	1/6458
34	1/582	1/725	1/880	1/1047	1/1429	1/5256
35	1/465	1/580	1/703	1/837	1/1142	1/4202
36	1/366	1/456	1/553	1/659	1/899	1/3307
37	1/284	1/354	1/430	1/512	1/698	1/2569
38	1/218	1/272	1/330	1/393	1/537	1/1974
39	1/167	1/208	1/252	1/300	1/409	1/1505
40	1/126	1/157	1/191	1/227	1/310	1/1139
41	1/95	1/118	1/144	1/171	1/233	1/858
42	1/71	1/89	1/108	1/128	1/175	1/644
43	1/53	1/66	1/81	1/96	1/131	1/481
44	1/40	1/50	1/60	1/72	1/98	1/359

Table 1.3: Estimated risk for trisomy 13 in relation to maternal age and gestation

Maternal age (years)	Gestational age					
	10 weeks	12 weeks	14 weeks	16 weeks	20 weeks	40 weeks
20	1/6347	1/7826	1/9389	1/11042	1/14656	1/42423
25	1/5621	1/6930	1/8314	1/9778	1/12978	1/37567
30	1/3719	1/4585	1/5501	1/6470	1/8587	1/24856
31	1/3228	1/3980	1/4774	1/5615	1/7453	1/21573
32	1/2740	1/3378	1/4052	1/4766	1/6326	1/18311
33	1/2275	1/2806	1/3366	1/3959	1/5254	1/15209
34	1/1852	1/2284	1/2740	1/3222	1/4277	1/12380
35	1/1481	1/1826	1/2190	1/2576	1/3419	1/9876
36	1/1165	1/1437	1/1724	1/2027	1/2691	1/7788
37	1/905	1/1116	1/1339	1/1575	1/2090	1/6050
38	1/696	1/858	1/1029	1/1210	1/1606	1/4650
39	1/530	1/654	1/784	1/922	1/1224	1/3544
40	1/401	1/495	1/594	1/698	1/927	1/2683
41	1/302	1/373	1/447	1/526	1/698	1/2020
42	1/227	1/280	1/335	1/395	1/524	1/1516
43	1/170	1/209	1/251	1/295	1/392	1/1134
44	1/127	1/156	1/187	1/220	1/292	1/846

1.1.2 Evolution in screening from maternal age to the first-trimester combined test

Trisomy 21

Chromosomal abnormalities are major causes of perinatal death and childhood handicap. Prenatal diagnosis of these abnormalities requires invasive testing, by amniocentesis or chorionic villus sampling (CVS). However, such testing is associated with a risk of miscarriage and therefore amniocentesis or CVS are carried out only in pregnancies considered to be at high-risk for chromosomal abnormalities.

The phenotypic features of Down syndrome were first described in 1866 by Langdon Down (Down LJ,1866). In 1959 a French paediatrician/ geneticist Professor Jerome Lejeune discovered that individuals with Down syndrome have an extra chromosome 21 (Lejeune *et al*,1959). The first prenatal diagnosis of trisomy 21 by amniocentesis and fetal karyotyping was in 1968 (Valenti *et al* 1968).

In the last 45 years a series of different methods have been used to identify the pregnancies at high-risk of fetal trisomy 21 that could be offered invasive diagnostic testing (Nicolaides 2011). During the 1970s and the early 1980s, advanced maternal age, defined in most countries as over 35 years, was the method of screening. At that time about 5% of the pregnant women were >35 years old and this population contained about 30% of the affected pregnancies. Since most of the screen positive group were actually normal the word screen positive became synonymous with false positive. Therefore, in screening by maternal age the detection rate (DR) was 30% and the false positive rate (FPR) was 5%. In recent years the trend to delayed childbearing has resulted in a significant increase in the number of pregnant women ≥ 35 years (20%). If all these women were to undergo invasive testing, the DR would be 50% and FPR 20%.

In the late 1980s and early 1990s, it was realised that in pregnancies with fetal trisomy 21 there are altered maternal serum concentrations of various feto-placental products, including increased free ß-hCG and Inhibin A and decreased AFP and unconjugated

estriol (uE3) (Merkatz *et al*., 1984; Canick *et al*., 1988; Macri *et al*., 1990; Van Lith *et al*., 1993; Brambati *et al*., 1993; Aitken *et al*., 1996). These biochemical changes were combined with maternal age to develop the double test (hCG and AFP), triple test (hCG, AFP and uE3) and the quad test (hCG, inhibin A, AFP and uE3). Screening by this approach was superior to that of maternal age alone with DR of 50-70% at FPR of 5% (Cuckle *et al*., 2005).

In the 1990s, aneuploidy screening shifted to the first trimester with the 'Combined' test which uses ultrasound measurement of fetal nuchal translucency (NT) together with maternal serum concentration of the placental proteins, free ß-human chorionic gonadotropin (free β-hCG) and pregnancy-associated plasma protein (PAPP-A) (Nicolaides *et al*., 1992; Snijders *et al*., 1998; Wald *et al*., 2003a; Nicolaides, 2004; Malone *et al*., 2005; Spencer *et al*., 2003a; Kagan *et al*., 2008a; Wright *et al*., 2010 Brizot *et al*., 1994, 1995; Noble *et al*., 1995; Spencer *et al*., 1999; Bindra *et al*., 2002; Spencer *et al*., 2003c; Wapner *et al*., 2003; Nicolaides *et al*., 2005; Ekelund *et al*., 2008; Kagan *et al*., 2009a). This combined test has a DR of 90% with FPR of 5%. Research suggests that screening using a combination of NT with other ultrasound markers (nasal bone, tricuspid regurgitation and ductus venosus) and serum biochemistry with free ß-hCG, PAPP-A and placental growth factor (PLGF) can have a DR of 97% at FPR of 3% (Matias *et al*., 1998; Cicero *et al*., 2001, 2006; Huggon *et al*., 2003; Nicolaides, 2004; Faiola *et al*., 2005; Falcon *et al*., 2006; Kagan *et al*., 2009b, 2009c; 2010; Maiz *et al*., 2009). However, in most hospitals these additional ultrasound and biochemical markers are not used.

Trisomies 18 and 13

A beneficial consequence of screening for trisomy 21 is the early diagnosis of trisomies 18 and 13, which are the second and third most common chromosomal abnormalities. At 11-13 weeks, the relative prevalence of trisomies 18 and 13 to trisomy 21 are one to three and one to seven, respectively. All three trisomies are associated with increased maternal age, increased fetal NT and decreased maternal serum PAPP-A, but in trisomy 21 serum free ß-hCG is increased whereas in trisomies 18 and 13 this is

decreased. In addition, trisomy 13, unlike trisomies 21 and 18, is associated with fetal tachycardia, with the heart rate being above the 95th centile of euploid fetuses in 85% of fetuses with trisomy 13 (Hyett *et al*., 1996b; Liao *et al*., 2000; Papageorghiou *et al*., 2006).

Use of the algorithm for trisomy 21 identifies about 75% of fetuses with trisomies 18 and 13. The combined use of the algorithm for trisomy 21 with specific algorithms for trisomies 18 and 13 improves the detection of these aneuploidies to 95% with a small increase in false positive rate by about 0.1% (Kagan *et al*., 2008b).

Screening in twin pregnancies

In twin pregnancies, effective screening for chromosomal abnormalities is provided by a combination of maternal age and fetal NT thickness (Pandya *et al*., 1995; Sebire *et al*., 1996a, 1996b; Maymon *et al*., 2001). The performance of screening can be improved by the addition of maternal serum biochemistry, but appropriate adjustments are needed for chorionicity (Sepulveda, *et al*., 1996). In dichorionic twins at 11 to 13 weeks, the levels of maternal serum free *β*-hCG and PAPP-A are about twice as high as in singleton pregnancies, but in monochorionic twins the levels are lower than in dichorionic twins (Spencer and Nicolaides, 2000, 2003; Spencer *et al*., 2008; Linskens *et al*., 2009).

In dichorionic twins, patient-specific risks for trisomy 21 are calculated for each fetus based on maternal age and fetal NT, and the detection rate (75–80%) and falsepositive rate (5% per fetus or 10% per pregnancy) are similar to those in singleton pregnancies (Sebire *et al*., 1996a). In the calculation of risk for trisomies, it has been assumed that in each pregnancy the measurements of NT for CRL between the two fetuses were independent of each other. However, recent evidence indicates that in euploid dichorionic twins, the measurements of NT in each twin pair are correlated and this correlation is not a simple reflection of the common effect of sonographers (Wøjdemann *et al*., 2006; Cuckle and Maymon, 2010; Wright *et al*., 2011). In screening

in twins it is therefore necessary to take this correlation into account because it has a substantial impact on the estimated patient-specific risk for trisomies. In dichorionic twins the DR of trisomy 21 from the first-trimester combined test is about 90%, at FPR of 6% which is only mildly higher than in singleton pregnancies (Madsen *et al.,* 2011).

First-trimester screening allows the possibility of earlier and therefore safer selective fetocide in cases where one fetus is euploid and the other is abnormal (Sebire *et al*., 1996b). An important advantage of screening by fetal NT is that when there is discordance for a chromosomal abnormality, the presence of a sonographically detectable marker helps to ensure the correct identification of the abnormal twin should the parents choose selective termination.

In monochorionic twin pregnancies, the FPR of NT screening is higher than in dichorionic twins, because increased NT in at least one of the fetuses is an early manifestation of twin-to-twin-transfusion syndrome, as well as a marker of chromosomal abnormalities (Sebire *et al*., 1997, 2000; Kagan *et al*., 2007). In the calculation of risk of trisomy 21, the NT of both fetuses should be measured and the average of the two should be considered (Vandecruys *et al*., 2005).

Measurement of fetal nuchal translucency thickness

Nuchal translucency is the consequence of subcutaneous accumulation of fluid behind the neck (Figures 1.1 and 1.2).

It is essential that the same criteria are used to achieve uniformity of results among different operators for the measurement of fetal NT. The criteria, established by the Fetal Medicine Foundation (FMF) UK which have become the internationally accepted standard are described in Table 1.4.

It should be noted that fetal NT increases with crown–rump length and therefore it is essential to take gestation into account when determining whether a given translucency thickness is increased.

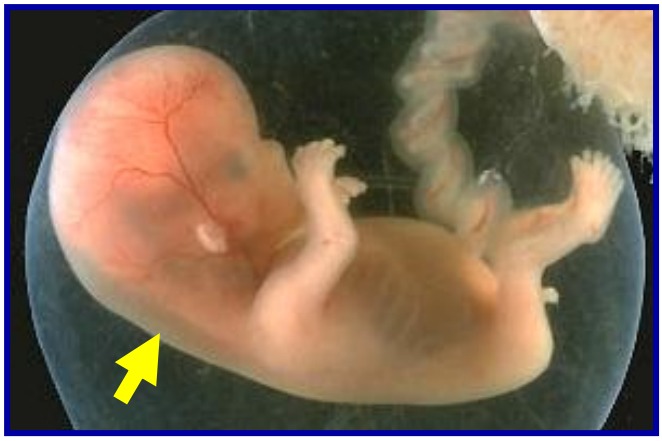


Figure 1.1 Picture of 11-weeks fetus demonstrating the subcutaneous accumulation of fluid behind the neck.

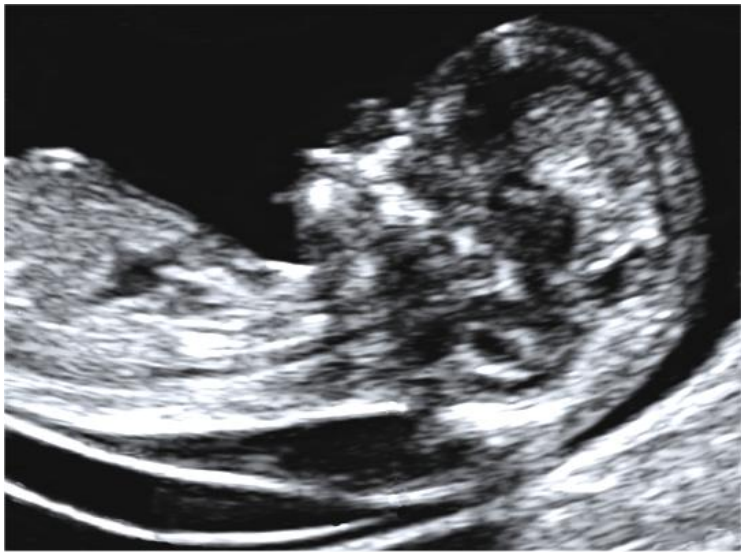
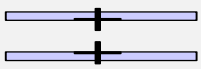


Figure 1.2 Ultrasound picture of 12-weeks fetus with trisomy 21 demonstrating increased subcutaneous accumulation of fluid behind the neck.

Table 1.6. Fetal Medicine Foundation criteria for measurement of fetal nuchal translucency

- The gestation should be between 11 weeks and one day and 13 weeks and six days.
- The fetal crown-rump length should be 45 to 84mm.
- A good sagittal section of the fetus, as for measurement of fetal crown–rump length, should be obtained and the NT should be measured with the fetus in the neutral position The fetal head should be in line with the spine and it should not be hyperextended or flexed.
- Care must be taken to distinguish between fetal skin and amnion. This is achieved by waiting for spontaneous fetal movement away from the amniotic membrane; alternatively the fetus is bounced off the amnion by asking the mother to cough and/or by tapping the maternal abdomen.
- The magnification should be such that the fetus occupies the entire image. Essentially, the aim is that each movement of the callipers produces a 0.1mm change in the measurement.
- The maximum thickness of the black space between the soft tissue overlying the cervical spine and the skin should be measured. During the scan more than one measurement must be taken and the maximum one should be recorded. Do not average the measurements.
- Measurements should be taken with the horizontal lines of the callipers placed ON the lines that define the nuchal translucency thickness (not in the line and not in the nuchal fluid), as shown in the diagram.



Correct

Incorrect

1.2 CELL-FREE DNA IN MATERNAL BOOD AND FETAL TRISOMIES

1.2.1 Historical perspective

Nucleic acids (DNA and RNA) in plasma were first observed 50 years ago. In the early 1970s increased quantities of DNA were verified in the plasma of cancer patients

(Leon *et al.*, 1977). In the late 1980s and 1990s several groups demonstrated that plasma DNA derived from cancer patients displayed tumour-specific characteristics,

including decreased strand stability, Ras and p53 mutations, mircrosatellite alterations, abnormal promoter hypermethylation of selected genes, mitocondrial DNA mutations and tumour-related viral DNA (Stroun *et al.*, 1989; Sorenson *et al.*, 1994; Vasioukhin *et al.*, 1994; Chen *et al.*, 1996; Nawroz *et al.*, 1996; Anker *et al.*, 1999; Chan *et al.*, 2002).

Tumour-specific DNA for a wide range of malignancies has been found: haematological, colorectal, pancreatic, skin, head-and-neck, lung, breast, kidney, ovarian, nasopharyngeal, liver, bladder, gastric, prostate and cervix. In aggregate, the above data show that tumour-derived DNA in plasma is ubiquitous in affected patients, and likely the result of a common biological process such as apoptosis (Bischof *et al.*, 2005).

Lo *et al*. in 1997, based on the previous knowledge that DNA of cancer patients can be detected in plasma, performed a study where he demonstrated the presence of fetal DNA in maternal blood. This discovery has revolutionized the field of fetal medicine. They used a rapid-boiling method to extract DNA from plasma and serum. DNA from plasma, serum, and nucleated blood cells from 43 pregnant women underwent a sensitive Y-PCR assay to detect circulating male fetal DNA from women bearing male fetuses. Fetus-derived Y sequences were detected in 24 (80%) of the 30 maternal plasma samples, and in 21 (70%) of the 30 maternal serum samples, from women bearing male fetuses. None of the 13 women bearing female fetuses,and none of the 10 non-pregnant control women, had positive results for plasma, serum or nucleated blood cells.

1.2.2 Techniques for analysis of cell-free DNA in maternal blood

Massively parallel sequencing

Many millions of molecules of cfDNA in maternal plasma are sequenced and both the chromosomal origin and quantity of each molecule are determined. In trisomic pregnancies the number of molecules derived from the extra chromosome, as a proportion of all sequenced molecules, is higher than in diploid pregnancies. The ability

to detect this difference necessitates that firstly, the number of counts for every chromosome is high, and secondly, the amount of cfDNA in maternal blood that is fetal in origin (fetal fraction) should be at least 4%. In MPSS, molecules from all chromosomes are examined with the potential to identify all aneuploidies. However, since chromosome 21 represents only approximately 1.5% of the human genome, it is necessary to sequence many millions of molecules from the complete genome to ensure sufficient chromosome 21 counts for differentiation between trisomy 21 and euploid pregnancies.

Selective sequencing

In this method selective amplification of specific regions on chromosomes 21, 18, 13, X and Y is carried out before sequencing analysis. The potential advantage of this approach is reduced cost because the number of regions that need to be sequenced is substantially lower than with whole genome sequencing in the detection of the specific aneuploidies of interest.

Single nucleotide polymorphism (SNP)-based approaches

In the SNP-based method, both maternal plasma cfDNA, which contains a mixture of maternal and fetal DNA, and buffy coat DNA, which is maternal in origin, are examined. Targeted amplification and sequencing of about 20,000 polymorphic loci on chromosomes 21, 18, 13, X, and Y is carried out.

A statistical method is then used to analyze allele distributions and determine the chromosomal count of the five chromosomes interrogated in each sample without the need to use a disomic reference chromosome. The method, also requires that the minimum fetal fraction is about 4%.

1.2.2 Importance of the fetal fraction

Cell-free DNA in maternal plasma is a mixture of DNA fragments belonging to both the mother and fetus. The proportion of fetal to total cfDNA, referred to as fetal fraction is about 10%. The ability to detect the small increase in the amount of a given chromosome in maternal plasma in a trisomic compared to a disomic pregnancy is directly related to the fetal fraction. For example, if the fetal fraction is 20% in 100 units of maternal cfDNA there would be 20 units coming from the fetus and 80 from the mother; in the presence of a fetus with trisomy 21, the maternal plasma cfDNA would contain 30 units of chromosome 21 coming from the fetus and 80 from the mother. Thus, there would be 110 units of chromosome 21 compared to 100 units of any other chromosome. This relative increase in chromosome 21 in maternal plasma cfDNA (110 vs. 100) is easier to detect than in a case with a fetal fraction of 4% where the relative increase would be 102 vs. 100.

Current methods of cfDNA testing necessitate that the minimum fetal fraction should be 4%. The greatest risk factor for low fetal fraction is obesity with a small contribution from Afro-Caribbean racial origin and early gestational age (Palomaki *et al.*, 2011; Ashoor *et al.*, 2012, 1013; Poon *et al.*, 2013). The estimated proportion of pregnancies with fetal fraction below 4% increases with maternal weight from <1% at 60 kg to >50% at 160 Kg (Ashoor *et al.*, 2012). The source of fetal cfDNA in maternal plasma is dying cells in the placenta (Faas *et al.*, 2012) and the inverse association between fetal fraction and maternal weight is likely to be due to a dilutional effect. A contributing factor is increase in maternal cfDNA levels because with increased weight there is active remodeling of adipose tissue with accelerated turnover of adipocytes (Poon *et al.*, 2013; Haghiac *et al.*, 2012).

The fetal fraction increases with increasing serum pregnancy-associated plasma protein-A and free β-human chorionic gonadotropin and is inversely related to maternal weight; the levels are not significantly altered in pregnancies with fetal trisomy 21 but they are reduced in those with trisomy 18 (Ashoor *et al.*, 2012; Ashoor *et al.*, 2013). It is therefore expected that, in trisomies 18 and 13, the failure rate of the cfDNA test would

be increased, thereby introducing bias if only the cases with results are included in the calculation of the performance of screening. One study has reported that the rate of failed results was considerably higher in aneuploid than in euploid pregnancies (Pergament *et al.*, 2014).

1.2.3 Performance of screening for trisomies 21, 18 and 13

Several studies in the last 4 years have reported the clinical validation and/or implementation of analyzing cfDNA in maternal blood in screening for trisomies 21, 18 and 13. A meta-analysis which included all such studies that were published up to January 2015, identified 37 relevant studies (Gil *et al.*, 2015). These studies reported cfDNA results in relation to fetal karyotype from invasive testing or clinical outcome. Most of the studies included in the meta-analysis were retrospective, using stored samples from pregnancies with known outcome, or prospective, using mainly samples from high-risk pregnancies undergoing invasive testing.

Performance in singleton pregnancies

Weighted pooled DR and FPR in singleton pregnancies were 99.2% (95% CI, 98.5–99.6%) and 0.09% (95% CI, 0.05–0.14%), respectively, for trisomy 21, 96.3% (95% CI, 94.3–97.9%) and 0.13% (95% CI, 0.07–0.20) for trisomy 18, 91.0% (95% CI, 85.0–95.6%) and 0.13% (95% CI, 0.05–0.26%) for trisomy 13, 90.3% (95% CI, 85.7–94.2%).

Screening in the general population

Only four studies reported on the clinical implementation of cfDNA testing in routine screening for trisomies in the general population. The first study, examined stored plasma samples from 2049 singleton pregnancies that underwent combined screening at 11–13 weeks' gestation and had known pregnancy outcome (Nicolaides *et al.*,

2012). Results were obtained from cfDNA testing in 1949 (95.1%) pregnancies and all 10 cases of trisomy 21 or 18 were correctly identified, with a FPR of 0.1%. In this study, the first-trimester combined test detected all cases of trisomy 21 with a FPR of 4.4%.

In the second study, cfDNA testing was performed prospectively in 1916 singleton pregnancies at a median gestational age of 16 (range, 11–21) weeks (Song *et al.*, 2013). The test did not provide a result in 3.8% of cases and there was loss to follow-up in 5.8% of cases. Of the 1741 pregnancies with cfDNA results and outcome data, the test correctly identified all 11 cases of trisomy 21, 18 or 13, with a FPR of 0.06%. In this study, second-trimester serum triple test detected only 55% of the trisomies, with a FPR of 14.1%.

In the third study, cfDNA testing was performed prospectively in 2042 singleton pregnancies at 17 (range, 8–39) weeks. The test did not provide a result in 0.9% of cases and there was loss to follow-up in 3.5% of cases (Bianchi *et al.*, 2014). Of the 1952 pregnancies with cfDNA results and outcome data, the test correctly identified all seven cases of trisomy 21 or 18, with a FPR of 0.5%. In this study, a range of first and/or second-trimester traditional tests detected all cases of trisomy 21, with a FPR of 3.6%.

In the fourth study, cfDNA testing was performed prospectively in 333 singleton pregnancies at 14 (range, 9–23) weeks (Comas *et al.*, 2014). The test did not provide a result in 1.2% of cases and there was no follow-up in 5.5% of cases. Of the 315 pregnancies with cfDNA results and outcome data, the test correctly identified all four cases of trisomy 21, with a FPR of 0.0%.

Screening in twin pregnancies

In twin pregnancies, while screening by cfDNA testing is feasible, the performance of screening may be worse than it is in singletons. In twins, cfDNA testing is more complex, because the two fetuses could be either monozygotic, and therefore

genetically identical, or dizygotic, in which case only one fetus is likely to have any aneuploidy identified. There is evidence that, in dizygotic twins, each fetus can contribute different amounts of cfDNA into the maternal circulation, and the difference can be nearly two-fold (Leung *et al.*, 2013; Qu *et al.*, 2013). It is therefore possible, in a dizygotic twin pregnancy discordant for aneuploidy, for the fetal fraction of the affected fetus to be below the threshold (4%) for successful cfDNA testing. This could lead to an erroneous result of low risk for aneuploidy, with a high contribution from the disomic cotwin resulting in a satisfactory total fetal fraction. To avoid this potential mistake, it was proposed that for cfDNA testing in twin pregnancies, the lower fetal fraction of the two fetuses, rather than the total fetal fraction, should be estimated in the assessment of risk for aneuploidies (Struble et al., 2014). However, an inevitable consequence of such a policy is that the no-result rate in twins is higher than that in singleton pregnancies (Bevilacqua *et al.*, 2015).

In the meta-analysis, on the performance of screening by cfDNA analysis for trisomies in twin pregnancies there were 31 trisomy-21 and 399 euploid pregnancies (Gil *et al.*, 2015). The DR was 93.7% (95% CI, 83.6–99.2%) and the FPR was 0.23% (95% CI, 0.00–0.92%). There were also nine trisomy-18 pregnancies and two trisomy-13 pregnancies and these were all classified correctly.

No-result rate from cfDNA testing

One issue with cfDNA testing as a method of screening for aneuploidies is failure to provide a result. There are essentially three reasons for such failure: first, problems with blood collection and transportation of the samples to the laboratory, including inadequate blood volume, hemolysis, incorrect labeling of tubes and delay in arrival to the laboratory; second, low fetal fraction (usually below 4%); and third, assay failure for a variety of reasons, including failed DNA extraction, amplification or sequencing. In the studies included in the meta-analysis (Gil *et al.*, 2015), the reported rates for problems of blood collection and transportation of the samples ranged from 0.03% to 11.1%. Failure to obtain results for samples that were analysed ranged from 0.0% to 12.2% and the reason for failure due to low fetal fraction ranged from 0.5% to 6.1%. On the

basis of the published data, it is not possible to offer an explanation for the wide range in failure rates between studies or to draw conclusions on the possible relationship between the no-result rate and the method used for the analysis of samples or gestational age at sampling.

1.3 CELL-FREE DNA IN MATERNAL BOOD AND PRETERM BIRTH

Preterm birth is responsible for more than 70% of all neonatal and infant deaths (Office for National Statistics 2012). Additionally, children born preterm, compared to those born at term, have a 10-fold increase in risk of cerebral palsy (Kodjebacheva and Sabo 2015). Mortality and morbidity are inversely related to gestational age at delivery and are therefore more common in cases with early preterm birth (Office for National Statistics 2012; Saigal et al., 2008; D'Onofrio et al., 2013).

Spontaneous delivery before 34 weeks occurs in about 1% of singleton pregnancies, and this is either due to spontaneous onset of labor or preterm pre-labor rupture of membranes (Celik *et al .,* 2008). Preterm births can also be subdivided according to gestational age: about 5% of preterm births occur at less than 28 weeks' (extreme prematurity), 15% at 28-31 week's (severe prematurity), 20% at 32-33 weeks' (moderate prematurity) and 60-70% at 34-36 weeks' (near term) (Goldenberg *et al.,* 2008).

Neonates born at <24 weeks' gestation rarely survive without serious handicaps. Among neonates born at $\geq$24 weeks, mortality and morbidity decline with advancing weeks of gestation. Serious neurodevelopmental complications are uncommon >32 weeks; however, neonates born before 36 weeks often have difficulties with respiration, thermoregulation, and feeding, as well as increased risks of health problems and death in childhood (Iams 2014).

Despite major improvements in medical care and socioeconomic status of the population in developed countries, there has not been a decrease in the incidence of preterm birth in the past 50 years.

Many studies have been performed with the aim to understand the mechanism that activates the pathway of parturition before term. Preterm labour is now thought to be a syndrome initiated by multiple mechanisms, including infection or inflammation, uteroplacental ischemia or hemorrhage, uterine overdistension, cervical disease, and other immunologically mediated processes (Romero *et al.*, 2006).

In contrast to traditional teaching that the placenta forms an impermeable barrier between the pregnant woman and her fetus, multiple studies reported that both intact fetal cells and cell-free nucleic acids circulate freely within the maternal circulation (Bianchi 2004). Several studies have examined the potential value of cfDNA in maternal blood as a marker of pregnancy complications.

Lo *et al.*, (1998) described increasing concentrations of cf fetal DNA in maternal plasma from 3.4% to 6.2% between early and late gestation in a group of 12 women sampled sequentially throughout pregnancy. Ariga *et al*, (2001) studied serial blood samples from 20 healthy pregnant women carrying male fetuses in order to quantify the fetal DNA; they found a gradually increased fetal DNA during pregnancy, which peaked at term and in the postpartum period the levels rapidly fell to almost undetectable. Majer *et al*., (2007) examined cfDNA in the third trimester in 96 women carrying male foetuses and reported that the level was significantly correlated with placental weight and pregnancy-associated complications.

Two studies examined women presenting with contractions and/or PPROM and reported that, in those with spontaneous preterm birth, maternal plasma fetal cfDNA was increased. Leung *et al.*, (1998) using real-time quantitative polymerase chain reaction (PCR) for the detection of the SRY gene, demonstrated that the median fetal cfDNA was higher in 13 pregnancies with male fetuses, presenting with threatened preterm labor and subsequent delivery at 26–34 weeks, when compared to 17 controls

that delivered at term. Farina *et al.*, (2005) assessed the DYS1 locus on the Y chromosome by real-time PCR to determine fetal cfDNA in 29 women with preterm labor delivering at <36 weeks, 21 with PPROM delivering at <36 weeks and 21 with preterm labor delivering at ≥36 weeks. They reported that cumulative rates of delivery at <30 weeks and delivery at <36 weeks were significantly higher for women with fetal cfDNA ≥1.82MoM than for those with fetal cfDNA concentrations below this cut-off (early preterm delivery: 45% vs 14%; preterm delivery: 73% vs 66%).

Some contradictory evidence suggests that, in cases of spontaneous preterm delivery, the increase in cfDNA precedes the clinical event. A cohort study of 876 women undergoing routine fetal rhesus D (RhD) genotyping, at 23–28 weeks' gestation, reported that if the fetal cfDNA level was above the 95th percentile there was a 6- and 16-fold increase in risk for spontaneous birth at <37 weeks (n=19) and at <34 weeks (n=8), respectively (Jakobsen *et al.*, 2012). In contrast, Stein *et al.*, (2013) reported that, in a cohort study of 611 women undergoing routine fetal RhD genotyping at 24–25 weeks' gestation, the levels of fetal cfDNA were not altered in pregnancies complicated by preterm birth.

A study examining 34 women with a short cervix and 22 women with normal cervical length at 22-24 weeks' gestation assessed the DYS14 locus on the Y chromosome by real-time PCR to determine fetal cfDNA levels and reported no significant difference in the level of fetal cfDNA between those that delivered before 37 weeks and those delivering at term (Illanes *et al.*, 2011). More recently, a study used chromosome-selective sequencing of non-polymorphic and polymorphic loci, in which fetal alleles differ from maternal alleles, to determine the cfDNA counts of fetal and maternal origin in maternal plasma at 11–13 weeks' gestation (Poon *et al.*, 2013). Both fetal and maternal cfDNA counts were affected by maternal characteristics, but the corrected values in 20 cases of spontaneous preterm birth were not significantly different from those of 1805 unaffected pregnancies.

1.4 CONCLUSIONS AND CHALLENGES

1.4.1 Screening for trisomies 21, 18 and 13 in singleton pregnancies

There is extensive evidence that effective screening for trisomies 21, 18 and 13 is provided by a combination of maternal age fetal NT thickness and maternal serum free ß-hCG and PAPP-A at 11-13 weeks' gestation. The DR is about 90% for trisomy 21 and 95% for trisomies 18 and 13, at combined FPR of 5%.

There is evidence, from clinical validation and a few clinical implementation studies, that the performance of screening for trisomies by cfDNA analysis of maternal blood is superior to that of the combined test; the reported DR is 99% for trisomy 21, 96% for trisomy 18 and 91% trisomy 13, at combined FPR of 0.35%. However, the test may fail to give a result and the exact rate of such failure is difficult to quantify from the existing studies. Additionally, only four studies, on a limited number of cases and a wide gestational age range, reported on the clinical implementation of cfDNA testing in routine screening for trisomies in the general population.

1.4.2 Screening for trisomies 21, 18 and 13 in twin pregnancies

In twin pregnancies, the performance of screening by the first-trimester combined test for trisomies is similar to that in singleton pregnancies but with a small increase in the FPR. Screening for trisomies in twins by cfDNA analysis of maternal blood is feasible. However, the number of cases reported is too small to allow accurate estimation of performance of screening.

1.4.3 cfDNA analysis of maternal blood in screening for preterm birth

Preterm birth is the leading cause of neonatal death and handicap in survivors. Accurate prediction of the high-risk group for preterm birth and prophylactic preventative

interventions are the major challenges in modern obstetrics. There is some limited contradictory evidence which suggests that quantification of cfDNA in maternal blood may be useful in predicting preterm birth.

1.5 AIMS OF THIS THESIS

The aims of the studies in this thesis are:

1. To report the results of clinical implementation of cfDNA testing for trisomies 21, 18 and 13 in the general population at 10-11 weeks' gestation and compare its performance to that of the first trimester combined test.

2. To examine the clinical implementation of chromosome-selective sequencing in the assessment of risk for trisomies in twin pregnancies at 10-13 weeks' gestation.

3. To explore whether in pregnancies that are complicated by spontaneous preterm birth fetal fraction of cfDNA in maternal plasma at 11-13 weeks' gestation is altered and if this measurement is useful in the prediction of this pregnancy complication.

Chapter 2 PUBLISHED STUDIES

Study 1 Screening for trisomies 21, 18 and 13 by cell-free DNA analysis of maternal blood at 10-11 week's gestation and the combined test at 11-13 weeks.

Study 2 Cell-free DNA analysis for trisomy risk assessment in first trimester twin pregnancies

Study 3 Fetal fraction of cell-free DNA in maternal plasma in the prediction of spontaneous preterm delivery

Study 1

Quezada MS, Gil MM, Francisco C, Oròsz G, Nicolaides KH. Screening for trisomies 21, 18 and 13 cell-free DNA analysis of maternal blood at 10-11 weeks' gestation and the combined test at 11-13 weeks. Ultrasound Obstet Gynecol 2015; 45: 36-41.

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Screening for trisomies 21, 18 and 13 by cell-free DNA analysis of maternal blood at 10–11 weeks' gestation and the combined test at 11–13 weeks

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KEYWORDS: cell-free DNA; combined test; fetal fraction; first trimester; prenatal diagnosis; screening; trisomies

ABSTRACT

Objective *To examine in a general population the performance of cell-free DNA (cfDNA) testing for trisomies 21, 18 and 13 at 10–11 weeks' gestation and compare it to that of the combined test at 11–13 weeks.*

Methods *In 2905 singleton pregnancies, prospective screening for trisomies was performed by chromosome-selective sequencing of cfDNA in maternal blood at 10–11 weeks' gestation and by the combined test at 11–13 weeks' gestation.*

Results *Median maternal age of the study population was 36.9 (range, 20.4–51.9) years. Results from cfDNA analysis were provided for 2851 (98.1%) cases and these were available within 14 days from sampling in 2848 (98.0%) cases. The trisomic status of the pregnancies was determined by prenatal or postnatal karyotyping or clinical examination of the neonates. Of the 2785 pregnancies with a cfDNA result and known trisomic status, cfDNA testing correctly identified all 32 cases with trisomy 21, nine of 10 with trisomy 18 and two of five with trisomy 13, with false-positive rates of 0.04%, 0.19% and 0.07%, respectively. In cases with discordant results between cfDNA testing and fetal karyotype, the median fetal fraction was lower than in those with concordant results (6%* vs *11%). Using the combined test, the estimated risk for trisomy 21 was ≥ 1/100 in all trisomic cases and in 4.4% of the non-trisomic pregnancies.*

Conclusion *The performance of first-trimester cfDNA testing for trisomies 21 and 18 in the general population is similar to that in high-risk pregnancies. Most false-positive and false-negative results from cfDNA testing could be avoided if the* a priori *risk from the combined test is taken into account in the interpretation of individual risk.*

INTRODUCTION

Several studies have shown that cell-free DNA (cfDNA) analysis of maternal blood can detect about 99% of cases of trisomy 21, 97% of trisomy 18 and 92% of trisomy 13, with respective false-positive rates (FPR) of approximately 0.1%, 0.2% and 0.2%[1]. Most of these studies were retrospective, using stored samples from pregnancies with known outcome, or prospective, using samples from high-risk pregnancies undergoing invasive testing[1]. There are also some studies reporting on the clinical implementation of cfDNA testing in routine screening for trisomies in the general population, but most of these studies do not provide data on complete pregnancy outcome and they cannot be used for assessment of the screening performance.

Only three studies in the general population reported outcome data on nearly all cases examined[2–4]. The first study examined stored plasma samples from 2049 singleton pregnancies that underwent combined screening at 11–13 weeks' gestation. They obtained results from cfDNA testing in 95.1% of cases and correctly identified all eight cases of trisomy 21 and the two with trisomy 18, with an FPR of 0.1%[2]. The second study performed cfDNA testing prospectively at a median gestational age of 16 (range, 11–21) weeks in 1916 singleton pregnancies[3]. The test did not provide a result in 3.8% of cases and there was loss to follow-up in 5.8% of cases. Of the 1741 pregnancies with cfDNA results and outcome data, the test correctly identified all eight cases of trisomy 21, two with trisomy 18 and one with trisomy 13; there was only one false-positive result for trisomy 18, but in this case there was low-grade maternal mosaicism for trisomy 18. The third study performed cfDNA testing prospectively in 2042 singleton pregnancies at 17 (range, 8–39) weeks' gestation[4]. Outcome was based on prenatal or postnatal karyotyping or clinical examination of the neonate. The

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trisomic status of the fetus could not be ascertained in only 3.5% of cases, either because of loss to follow-up or because the patient miscarried and the products of conception were not karyotyped. Results from cfDNA testing were provided for 99.1% of cases, and of the 1952 with known outcome, the test correctly identified all five cases of trisomy 21 and the two with trisomy 18, with an FPR of 0.5%.

The aim of this study was to report the results of clinical implementation of cfDNA testing for trisomies 21, 18 and 13 at 10–11 weeks' gestation in the general population, and compare its performance to that of the first-trimester combined screening test.

METHODS

The data for this study were derived from the clinical implementation of cfDNA testing in screening for trisomies 21, 18 and 13 at 10–11 weeks' gestation in women with singleton pregnancies. The women attended the Fetal Medicine Centre in London, UK, between October 2012 and January 2014. In addition to cfDNA testing, all women underwent the combined test at 11–13 weeks' gestation. In the first visit to the center, we recorded maternal characteristics and medical history, carried out an ultrasound examination to determine if the pregnancy was singleton with a live fetus and to estimate gestational age from measurement of the fetal crown–rump length (CRL). Maternal serum pregnancy-associated plasma protein A (PAPP-A) and free β-human chorionic gonadotropin (β-hCG) levels were measured (Thermo Scientific, Berlin, Germany) and 20 mL of maternal blood was collected in Streck cell-free DNA BCT™ tubes and sent via courier to the USA for cfDNA testing (Harmony™ Prenatal Test, Ariosa Diagnostics, Inc., San Jose, CA, USA)[5–7]. At the second visit, we combined maternal age with the results of the ultrasound measurement of fetal CRL, nuchal translucency (NT) thickness and serum concentrations of PAPP-A and free β-hCG levels to estimate the patient-specific risk for trisomy 21[8]. Patients were classified as high risk if the estimated risk was ≥ 1/100, which is the cut-off recommended by the UK National Screening Committee for invasive testing.

The results from cfDNA testing were presented as risk scores for trisomy 21, 18 and 13, which in most cases were either > 99% or < 1/10 000. In cases in which the cfDNA test did not provide results, the parents were offered repeat testing or they had to rely on the results of the combined test. In cases with a high-risk result, the parents were advised to consider having invasive fetal karyotyping before deciding on further management of their pregnancy. During the first part of the study, women with a low-risk result from cfDNA testing were reassured that the fetus was unlikely to be affected by these trisomies, irrespective of the results of the combined test[9]. In the second half of the study, the results of the combined test were used to derive the *a priori* risk for each trisomy and this was reduced by a factor of 100 for trisomy 21, 31 for trisomy 18 and 13 for trisomy 13[10].

Patient characteristics and results of the investigations were recorded in a fetal database. Results from invasive testing (obtained from laboratories) and pregnancy outcome (obtained from obstetricians, general practitioners or the patient) were recorded in the same database. The outcomes were divided into (1) trisomy 21, 18 or 13, if the karyotype of chorionic villi, amniotic fluid or neonatal blood demonstrated the relevant trisomy; (2) no trisomy 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood was normal or the neonate was phenotypically normal; (3) no known karyotype because the pregnancies resulted in miscarriage or stillbirth and no karyotyping of fetal tissue was carried out; and (4) outcome unknown because the cases were lost to follow-up.

RESULTS

Study population

During the study period, we examined 2905 women with singleton pregnancies and a live fetus at 10 + 0 to 11 + 6 (median, 10 + 4) weeks' gestation. The median maternal age was 36.9 (range, 20.4–51.9) years and 1958 (67.4%) women were aged 35 years or older. The median maternal weight was 62.8 (range, 40.5–137.7) kg. The racial origin of the women was Caucasian in 2570 (88.5%), South Asian in 173 (6.0%), East Asian in 96 (3.3%), Afro-Caribbean in 21 (0.7%) and mixed in 45 (1.5%) women. 1555 (53.5%) women were parous and 1350 (46.5%) were nulliparous. Conception was spontaneous in 2438 (83.9%) of the pregnancies and 467 (16.1%) were the result of assisted reproduction techniques.

On the basis of the results of fetal karyotyping or clinical examination of the neonates, there were 34 cases of trisomy 21, 10 of trisomy 18, five of trisomy 13, 2787 without trisomy 21, 18 or 13, 48 miscarriages or stillbirths with unknown karyotype, and 21 that were lost to follow-up (Table 1).

Results of cfDNA testing

Results of cfDNA testing were obtained after first sampling in 2782 (95.8%) of the 2905 cases. In 110 of the 123 cases with no result, a further blood sample was obtained and a result provided in 69 (62.7%) cases; consequently, cfDNA results were obtained for 2851 (98.1%) cases. The 54 cases with no result included one case for which the sample was not received by the laboratory, 38 cases with fetal fraction below the minimal requirement of 4% and 15 cases of assay failure. The median time interval between blood sampling and receiving results was 9 (range, 5–20) days, with 2848 (98.0%) results being available within 14 days of sampling. The median fetal fraction in the cases with a result was 11% (range, 4–40%).

Of the 54 pregnancies with no cfDNA result, there were 49 non-trisomic cases, two cases of trisomy 21 and three cases of miscarriage with no karyotype (Figure 1). Of the

Table 1 Results of cell-free DNA analysis of maternal blood and combined test in screening for trisomies 21, 18 and 13 in 2905 singleton pregnancies according to risk

		Cell-free DNA result			*Combined test*		
Trisomic status	n	*High-risk*	*Low-risk*	*No result*	*High-risk*	*Low-risk*	*No result*
Non-trisomic	2787	8	2730	49	124	2663	—
Trisomy 21	34	32	—	2	34	—	—
Trisomy 18	10	9	1	—	10	—	—
Trisomy 13	5	2	3	—	5	—	—
Not known	69	1	65	3	5	52	12

Data are presented as *n*.

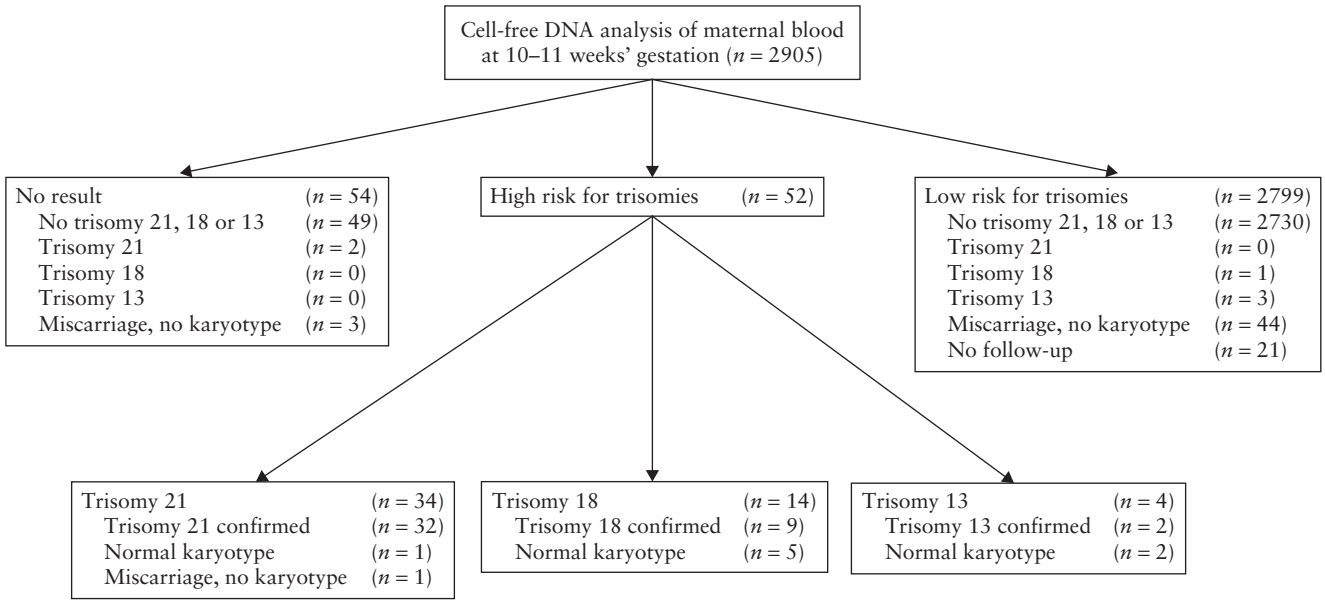

Figure 1 Flow-chart of pregnancy outcome according to results of cell-free DNA testing of maternal blood in 2905 singleton pregnancies.

34 cases with a high-risk result for trisomy 21, there were 32 cases in which invasive testing confirmed trisomy 21, one case of miscarriage before planned chorionic villus sampling (CVS) and one case with a normal karyotype. Of the 14 cases with a high-risk result for trisomy 18, there were nine cases in which invasive testing confirmed trisomy 18 and five cases with a normal karyotype. Of the four cases with a high-risk result for trisomy 13, there were two cases in which invasive testing confirmed trisomy 13 and two cases with a normal karyotype. Of the 2799 cases with a low-risk result for all three trisomies, there were 2730 pregnancies with no trisomy 21, 18 or 13, one case of trisomy 18, three of trisomy 13, 44 of miscarriage and no karyotype and 21 cases that were lost to follow-up.

Of the 2836 pregnancies with known trisomic status, cfDNA testing correctly identified 32 of the 34 cases of trisomy 21 but did not provide results in two, nine of the 10 cases of trisomy 18 and two of the five cases of trisomy 13 (Table 1). Of the 2787 non-trisomic pregnancies, cfDNA testing correctly provided a low-risk result for each of the three trisomies in 2730 cases, did not provide a result in 49 and gave a false-positive result for trisomy 21 in one (0.04%) case, for trisomy 18 in five cases (0.19%) and for trisomy 13 in two cases (0.07%).

Results of combined screening

In the 2836 pregnancies with known trisomic status, the estimated risk for trisomy 21 at 11–13 weeks' gestation was ≥ 1/100 in all 49 trisomic pregnancies and in 124 (4.4%) of the 2787 non-trisomic pregnancies (Table 1).

Discordant results between cfDNA testing and fetal karyotype

The discordant results between cfDNA testing and fetal karyotype are summarized in Table 2. The median fetal fraction in the 12 cases with discordant results (6.0% (range, 4.2–8.7%)) was significantly lower than in the 2730 cases with concordant normal results (11.1% (range, 4.1–40.2%)) and the 43 cases with concordant abnormal results (9.6% (range, 4.7–20.4%)) (Mann–Whitney *U*-test, $P < 0.0001$).

In the 32 cases of trisomy 21 with concordant results, the median fetal fraction was 10.1% (range, 5.4–20.4%) and the median estimated risk from the combined test was 1/2 (range, 1/2 to 1/81). In the case with discordant results, the fetal fraction was 4.7% and the risk from the combined test was 1/6966.

In the nine cases of trisomy 18 with concordant results, the median fetal fraction was 9.6% (range, 4.7–14.7%)

Table 2 Summary of the 12 cases with discordant results between cell-free DNA analysis of maternal blood and fetal karyotype among 2905 singleton pregnancies

Cell-free DNA analysis of maternal blood		*Combined test result*	*Fetal karyotype*	*Outcome of pregnancy*
Result	*Fetal fraction (%)*			
Risk for trisomy 21				
High	4.7	1:6966	CVS: normal	Healthy live birth
Risk for trisomy 18				
High	4.2	1:906	—	Healthy live birth
High	4.3	1:496	PM: normal	Miscarriage (20 weeks)
High	5.1	1:1120	CVS: normal	Healthy live birth
High	5.6	1:34 483	CVS: normal	Healthy live birth
High	6.3	1:210	Amnio: del 18(p11.1)	Termination
Low	8.7	1:6	Amnio: trisomy 18	Termination
Risk for trisomy 13				
High	5.9	1:1645	CVS: normal	Healthy live birth
High	7.2	1:6152	CVS: normal	Healthy live birth
Low	6.2	1:2	CVS: trisomy 13	Termination
Low	6.0	1:2	CVS: trisomy 13	Termination
Low	8.6	1:2	CVS: trisomy 13	Termination

Amnio, amniocentesis; CVS, chorionic villus sampling; del, deletion; PM, postmortem examination.

and the median estimated risk from the combined test was 1/4 (range, 1/2 to 1/14). In five cases with a positive result from cfDNA testing but disomy 18 on fetal karyotyping, the median fetal fraction was 5.1% (range, 4.2–6.3%) and the median estimated risk from the combined test was 1/906 (range, 1/210 to 1/34 483). In one of these five cases the first cfDNA test failed because of low fetal fraction, but after repeat testing 2 weeks later the result was > 99% for trisomy 18; amniocentesis at 16 weeks' gestation demonstrated a deletion in the short arm of chromosome 18. In one case cfDNA testing gave a low risk, but the risk from the combined test was 1/6; CVS was carried out and the quantitative fluorescence polymerase chain reaction (QF-PCR) result was reported as normal but the culture failed. Subsequently, amniocentesis was performed because ultrasound examination at 20 weeks' gestation demonstrated fetal growth restriction, choroid plexus cysts and flexion deformity of the hands, and both the QF-PCR and culture results indicated trisomy 18.

In two cases with concordant results for trisomy 13, the respective fetal fractions were 5.6% and 6.1% and the estimated risks from the combined test were 1/21 and 1/43, respectively. In two cases with a positive cfDNA result but normal fetal karyotype, the respective fetal fractions were 5.9% and 7.2% and the risks from the combined test were 1/1645 and 1/6152, respectively. In three cases at low risk for trisomy 13 from cfDNA testing and an abnormal result from fetal karyotyping, the risk from the combined test was 1/2.

DISCUSSION

Main findings of the study

This prospective study in women undergoing routine first-trimester screening for the major trisomies, by cfDNA analysis of maternal blood and by the combined test, examined 2905 cases and provided outcome data for nearly 98% of cases, which makes it possible to assess accurately the performance of screening.

The combined test, at a risk cut-off of 1/100, identified all cases of trisomy 21, 18 and 13, with an FPR of 4.4%. Screening by cfDNA analysis of maternal blood provided results in 98% of pregnancies and these were available within 2 weeks of sampling in 98% of cases. In the pregnancies with a cfDNA result, all cases of fetal trisomy 21 were detected, with an FPR of 0.04%. The test also detected nine of the 10 cases of fetal trisomy 18, with an FPR of 0.19%; in the one false-negative case, QF-PCR of chorionic villi reported disomy 18. The performance of screening for trisomy 13 was poorer, with only two of five affected cases being detected.

In cases of discordant results between cfDNA testing and fetal karyotype, the fetal fraction was lower than in those with concordant normal or abnormal results.

Limitations of the study

The median maternal age of the study population was 36.9 years, which is higher than the median age of 31.7 years in our NHS hospital in London[11]. The patients were self-selected and, inevitably, a high proportion of women were of advanced age and had conceived by assisted reproduction techniques. Nevertheless, the women did not have prior screening for trisomies by other methods and their results are representative of the general population.

Another limitation of the study relates to the high performance of the combined test. The results of cfDNA analysis were commonly available at the time of the ultrasound examination for measurement of fetal NT, and could have potentially biased the measurements.

Comparison with results of previous studies

Our findings on the performance of maternal blood cfDNA analysis in screening for trisomies 21 and 18

in a general population are compatible with the results of previous studies in high-risk pregnancies[1], but also with those that examined general populations[2–4]. Our detection rate of trisomy 13 was lower, however the number of cases we examined was too small for valid conclusions to be drawn. A meta-analysis of clinical validation or implementation studies of cfDNA testing reported that the weighted pooled detection rates in 809 cases of trisomy 21, 301 of trisomy 18 and 85 of trisomy 13 were 99.0% (95% CI, 98.2–99.6%), 96.8% (95% CI, 94.5–98.4%) and 92.1% (95% CI, 85.9–96.7%), respectively, with FPRs of 0.08% (95% CI, 0.03–0.14%), 0.15% (95% CI, 0.08–0.25%) and 0.20% (95% CI, 0.04–0.46%), respectively[1].

In the two previous prospective screening studies in a general population, the median gestational age at cfDNA testing was 16 and 17 weeks, respectively[3,4]. Our study focused on the application of cfDNA testing at 10–11 weeks because first-trimester screening and diagnosis of aneuploidies leads to early reassurance, for the majority of parents, that their fetus is unlikely to be trisomic, and for the few with an affected fetus, the parents have the option of an earlier and safer termination of pregnancy. The two-stage strategy of cfDNA testing at 10–11 weeks followed by the combined test retains the benefits of early detection of many major fetal defects and the prediction and potential prevention of a wide range of pregnancy complications[12].

Implications for clinical practice

There are essentially two options in the clinical implementation of cfDNA testing in screening for the major trisomies: firstly, routine screening of the whole population and secondly, contingent screening based on the results of first-line screening by another method, preferably the first-trimester combined test[1]. In the first option, it would be best to undertake screening in the first trimester, and our results establish the feasibility of such an approach.

cfDNA testing is not a diagnostic test and in the interpretation of individual patient results it is necessary to know the *a priori* risk for the given trisomy. On the basis of the results of our meta-analysis of cfDNA testing, the positive likelihood ratios for trisomies 21, 18 and 13 are 1238, 645 and 461, respectively[1]. It is therefore not surprising that in most of our cases with false-positive results, the estimated risk from the combined test was very low. The negative likelihood ratios for trisomies 21, 18 and 13 are 0.01, 0.03 and 0.08, respectively[1], and therefore, with a negative cfDNA result for these trisomies, there is a 100-fold, 31-fold and 13-fold reduction in the *a priori* risk[10]. In our three cases with a false-negative result for trisomy 13 the estimated risk from the combined test was 1/2, consequently their individual risk was not $< 1/10\,000$ as reported by the cfDNA test, but approximately 1/25 when the results of both the cfDNA test and the combined test were taken into account.

The use of the *a priori* risk in the interpretation of results from cfDNA testing is particularly important in cases with a low fetal fraction. The ability to detect the small increase in the amount of a given chromosome in maternal plasma in a trisomic compared to a disomic pregnancy is directly related to the relative proportion of the fetal to maternal origin of the cfDNA in maternal plasma[5,13–15]. In our cases with false-positive and false-negative results the median fetal fraction was lower than in those with concordant results between the cfDNA test and fetal karyotype.

The most accurate method for defining the *a priori* risk for each patient is the combined test[8]. However, it is unrealistic to expect that universal screening for trisomies by cfDNA testing would necessitate that all women should also have the combined test. Nevertheless, it is desirable that all women should have a high-quality first-trimester ultrasound scan, including measurement of fetal NT, which is a marker not only for trisomies but also for other aneuploidies, cardiac defects and many genetic syndromes. The *a priori* risk for trisomies derived from a combination of maternal age and fetal NT is certainly more accurate than that obtained from maternal age alone. As for the additional measurement of serum biochemical markers, this will essentially depend on the extent to which there is widespread uptake of first-trimester screening for pregnancy complications, such as pre-eclampsia.

Conclusions

This study has shown that routine screening for trisomies by cfDNA testing at 10–11 weeks' gestation is feasible, allowing diagnosis of aneuploidies and the option of first-trimester termination of pregnancy. The study has highlighted that firstly, cfDNA testing has a substantially lower FPR than the combined test, secondly, in cases of discordant results between the cfDNA test and fetal karyotype, the fetal fraction is lower than in those with concordant results and thirdly, in the interpretation of cfDNA results, particularly in cases with a low fetal fraction, the *a priori* risk should be considered.

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A slide presentation, prepared by Dr Maddalena Morlando, one of UOG's Editors for Trainees, is available online.

Study 2

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Cell-Free DNA Analysis for Trisomy Risk Assessment in First-Trimester Twin Pregnancies

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Key Words
Non-invasive prenatal testing · Trisomy 21 · Fetal aneuploidy · Twin pregnancy · First-trimester screening

Abstract
Objective: To examine the clinical implementation of chromosome-selective sequencing of cell-free DNA (cfDNA) in maternal blood and an algorithm that relies on the lower fetal fraction contribution of the 2 fetuses in the assessment of risk for trisomies in twin pregnancies. ***Methods:*** Risk for trisomies 21, 18 and 13 by cfDNA testing were estimated in stored plasma samples obtained at 11–13 weeks' gestation from 207 pregnancies with known outcome and prospectively in 68 twin pregnancies undergoing screening at 10–13 weeks. ***Results:*** Risk scores for trisomies were provided for 192 (92.8%) of stored plasma and for 63 (92.6%) of the prospective cases. In the retrospective study, 10 of 11 trisomic pregnancies were correctly identified with no false positive results. In the prospective study, 3 trisomic pregnancies were correctly identified with no false positive results. The median of the lower fetal fraction in the prospective study of twins was 7.4% (IQR range 5.9–10.0%), which was lower than in our previous study in singletons (median 10.0%, IQR 7.8–13.0%). ***Conclusions:*** cfDNA testing in twins is feasible but the reporting rate of results is lower than in singletons due to a lower fetal fraction.

Introduction

In singleton pregnancies, cell-free DNA (cfDNA) testing in maternal blood can detect about 99% of cases of trisomy 21, about 97% of trisomy 18 and 92% of trisomy 13, with respective false positive rates of <0.1, <0.2 and 0.2% [1]. In cfDNA testing the ability to detect the small increase in the amount of a given chromosome in maternal plasma in a trisomic compared to a disomic pregnancy is directly related to the relative proportion of the fetal to maternal origin of the cfDNA. When the fetal fraction is low, it is more difficult to discriminate between aneuploid and euploid pregnancies and a minimum fraction of about 4% is currently necessary for accurate cfDNA analysis [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. There is evidence that in dizygotic twins each fetus can contribute different amounts of cfDNA into the maternal circulation, which could vary by nearly twofold [7, 8]. It is therefore possible that in a dizygotic twin pregnancy discordant for aneuploidy, the fetal fraction of the affected fetus is below the threshold of 4% for successful cfDNA testing. This could lead to an erroneous result of

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low risk for aneuploidy because a high contribution from the disomic co-twin could result in a satisfactory total fetal fraction. To avoid this potential mistake it was proposed that in cfDNA testing in twin pregnancies the lower fetal fraction of the 2 fetuses, rather than the total, should be estimated in the assessment of risk for aneuploidies [9].

The objective of this study is to examine the clinical implementation of chromosome-selective sequencing and an algorithm that relies on the lower fetal fraction of the twins [9] in the assessment of risk for trisomies in twin pregnancies at 10–13 weeks' gestation.

Methods

Maternal blood cfDNA testing was undertaken in two groups of twin pregnancies: in the first group retrospectively and in the second prospectively. In both groups, 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 [10, 11]. Maternal characteristics recorded were age, racial origin, smoking status during pregnancy, method of conception and weight, which was measured at the time of screening.

Study on Stored Plasma Samples

We had 207 stored plasma samples obtained at 11–13 weeks' gestation from twin pregnancies undergoing first-trimester screening for trisomies by a combination of fetal nuchal translucency thickness (NT) and maternal serum-free β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) [12–14]. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) 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 subsequently at 16,000 *g* for 10 min to further separate cell debris. Plasma samples were divided into 0.5-ml aliquots in separate Eppendorf tubes which were labelled with a unique patient identifier and stored at –80°C until subsequent analysis. All patients were recruited at Kings' College Hospital in London and they gave written informed consent to provide samples for research which was approved by the NHS Research Ethics Committee.

Prospective Study

This consisted of 68 twin pregnancies undergoing prospective screening for trisomies 21, 18 and 13 by cfDNA testing at the Fetal Medicine Centre in London, between March and October 2013. The patients attended the clinic at 10–13 weeks' gestation, received pre-test counselling [15] and provided written consent for cfDNA testing. Maternal blood (20 ml) was collected in Streck cfDNA BCT™ tubes. In all cases the combined test was also carried out either in the same visit, if the gestational age was 11–13 weeks, or in a subsequent visit for those presenting during the 10th week.

Analysis of Samples

Stored plasma samples (2 ml/patient) and prospectively collected blood samples (20 ml/patient) were sent without any further processing via courier to the USA for cfDNA testing (Harmony™ Prenatal Test; Ariosa Diagnostics, Inc., San Jose, Calif., USA). The information given to the laboratory for each case was: patient-unique identifier, maternal age, maternal weight, method of conception and date of blood collection, but not fetal karyotype.

cfDNA was extracted from the maternal plasma or blood samples and chromosome-selective sequencing with digital analysis of selected regions (DANSR™) was carried out as in singleton pregnancies [16]. However, in the assessment of risk for trisomies the fetal-fraction optimized risk of trisomy evaluation (FORTE™) algorithm used for singletons was modified so that the smallest fetal fraction contribution of the 2 fetuses was considered [9]. Risk scores for trisomy 21, 18 and 13 were provided as a percentage with ranges capped at >99 and <0.01%.

Results

Study on Stored Plasma Samples

The characteristics of the study population are summarized in table 1. This group included 193 twin pregnancies with euploid fetuses (109 dichorionic and 84 monochorionic), 10 with trisomy 21 (8 dichorionic and discordant for aneuploidy, 1 dichorionic concordant for aneuploidy and 1 monochorionic), 1 with trisomy 18 and 3 with trisomy 13 (all 4 dichorionic and discordant for aneuploidy).

Risk scores for trisomies 21, 18 and 13 by cfDNA testing were provided for 192 (92.8%) of the 207 samples (table 2). In 15 dichorionic twin cases, including 1 case of trisomy 18 and 2 of trisomy 13, no result was provided either due to low fetal fraction (n = 11) or laboratory processing issues (n = 4).

In all 181 euploid cases with a result, the risk score for each trisomy was less than 1:10,000 (table 2). In all 10 cases of trisomy 21, the risk scores for trisomies 18 and 13 were less than 1:10,000 and the score for trisomy 21 was >99% in 8, 72% in 1 and 1:714 in 1; the last case had the lowest fetal fraction of all the trisomy 21 cases at 5.3%. In the 1 case of trisomy 13 with a result, the risk score for trisomy 13 was >99% and the scores for trisomies 21 and 18 were less than 1:10,000.

In the dichorionc twin pregnancy with 1 trisomy 21 and 1 euploid fetus where the cfDNA test risk score was 1:714, the maternal age was 38 years, serum-free β-hCG was 2.809 multiples of the median (MoM) and PAPP-A was 0.748 MoM, and the NT thickness was 8.6 mm for the trisomic and 2.5 mm for the euploid fetus. The risks from the combined test were 1:2 and 1:13, respectively.

Table 1. Characteristics of the study population for cfDNA testing of stored maternal plasma samples

Characteristic	Euploid (n = 193)	Trisomy 21 (n = 10)	Trisomy 18 (n = 1)	Trisomy 13 (n = 3)
Maternal age, years	33.6 (29.0–36.6)	36.7 (34.2–37.9)	41.0	28.3 (26.7–34.5)
Maternal weight, kg	67.0 (60.5–78.0)	69.5 (62.5–73.2)	65.0	71.0 (65.5–78.4)
Racial origin				
Caucasian	135 (69.9)	8 (80.0)	1 (100.0)	1 (33.3)
Afro-Caribbean	48 (24.9)	1 (10.0)	0	0
South Asian	0	1 (10.0)	0	0
East Asian	1 (0.5)	0	0	0
Mixed	9 (4.7)	0	0	2
Cigarette smoker	13 (6.7)	0	0	0
Mode of conception				
Spontaneous	105 (54.4)	5 (50.0)	0	1 (33.3)
Assisted	88 (45.6)	5 (50.0)	1 (100.0)	2 (66.7)
Gestational age, days	91 (88–94)	90 (87–93)	89	96 (94–97)
CRL of the larger fetus, mm	67.0 (60.5–74.3)	65.0 (60.0–72.2)	62.8	78.2 (74.3–81.0)
Nuchal translucency, mm	1.9 (1.7–2.2)	4.5 (3.4–6.4)	3.1	3.7 (3.0–4.5)
PAPP-A MoM	0.96 (0.71–1.38)	0.68 (0.31–0.88)	0.03	0.88 (0.81–0.95)
Free β-hCG MoM	1.09 (0.72–1.57)	2.32 (1.53–3.25)	0.04	0.83 (0.62–1.02)
Fetal fraction, %	9.8 (7.4–12.1)	10.8 (6.8–12.1)	–	7

Values are median (IQR) or n (%).

Table 2. Risk scores for trisomies by cfDNA testing of maternal plasma in mono- and dichorionic twin pregnancies

Fetal karyotype	Risk score from cfDNA testing			
	n	trisomy 21	trisomy 18	trisomy 13
Monochorionic euploid (n = 84)	84	<1:10,000	<1:10,000	<1:10,000
Dichorionic euploid (n = 109)	97	<1:10,000	<1:10,000	<1:10,000
Monochorionic trisomy 21 (n = 1)	1	>99%	<1:10,000	<1:10,000
Dichorionic concordant trisomy 21 (n = 1)	1	>99%	<1:10,000	<1:10,000
Dichorionic discordant trisomy 21 (n = 8)	6	>99%	<1:10,000	<1:10,000
	1	72%	<1:10,000	<1:10,000
	1	1:714	<1:10,000	<1:10,000
Dichorionic discordant trisomy 18 (n = 1)	0			
Dichorionic discordant trisomy 13 (n = 3)	1	<1:10,000	<1:10,000	>99%

Prospective Study

The median maternal age of the study population was 37.2 (range 30.2–47.7) years, and 53 (77.9%) were 35 years or older. The median maternal weight was 64.2 (47.0–95.7) kg. The racial origin of the women was Caucasian in 57 (83.8%), South Asian in 7 (10.3%), East Asian in 3 (4.4%) and Afro-Caribbean in 1 (1.5%). Conception was spontaneous in 22 (32.4%) and by in vitro fertilization in 46 (67.6%). There were 16 monochorionic and 52 dichorionic pregnancies and the median gestational age at cfDNA testing was 10.6 (range 10.0–13.9) weeks.

In the dichorionic twin pregnancies the combined test of maternal age, fetal NT and serum-free β-hCG and PAPP-A provided a risk for trisomy 21 and trisomies 18 or 13 for each fetus, whereas in monochorionic pregnancies the average risk for the 2 fetuses was provided [11–14]. The risk for the 3 trisomies (highest of the 2 fetuses in dichorionic twins and average in monochorionic twins) was 1:100 or higher in 18 (26.5%) of the 68 pregnancies (fig. 1). This is the cut-off considered by the UK National Screening Committee for classifying pregnancies as high risk.

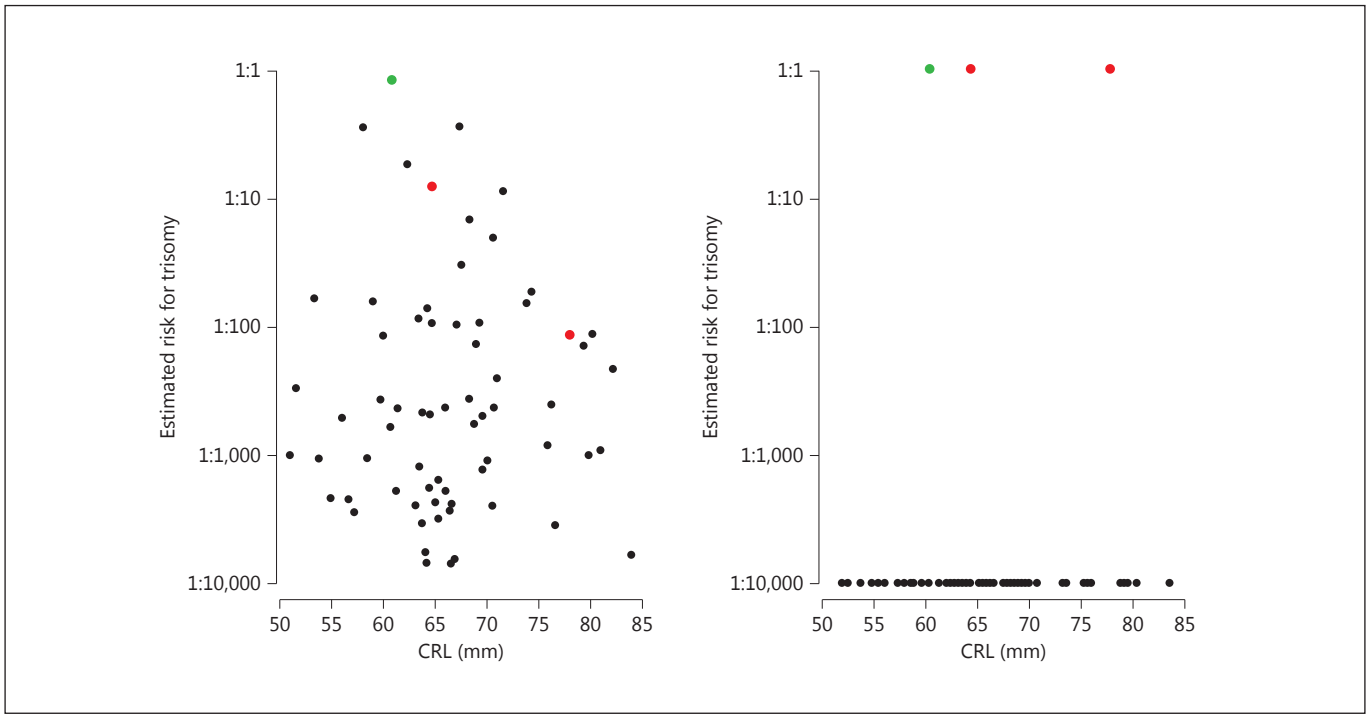

Fig. 1. Estimated risk for trisomies 21, 18 or 13 in the pregnancies with trisomy 21 (red circle), trisomy 18 (green circle) and assumed euploid (black circle) fetuses by the combined test (left) and maternal blood cfDNA test (right).

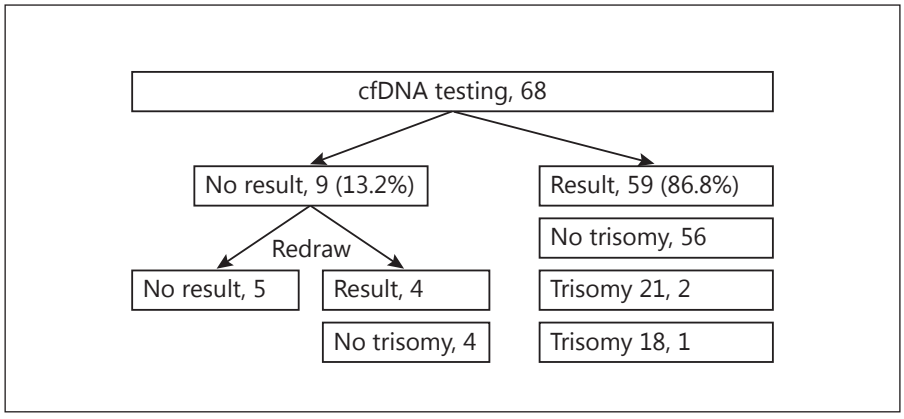

Fig. 2. Flow chart of prospective screening for trisomies 21, 18 or 13 in twin pregnancies by cfDNA testing in maternal blood.

The median time interval between blood sampling and arrival of the samples to the laboratory for cfDNA testing was 2 (range 1–5) days and the interval between blood sampling and receiving results was 10 (range 7–16) days with 97.1% (66 of 68) being available within 14 days from sampling. The median lower fetal fraction was 7.4% (IQR 5.9–10.0%).

Risk scores from cfDNA testing were provided for 59 (86.8%) of the 68 cases (fig. 2) on the first blood draw. In 9 cases, including 8 dichorionic and 1 monochorionic, no result was provided because of low fetal fraction. In these 9 cases a further blood sample was obtained and a risk score was provided in 4. Therefore, cfDNA results were provided for a total of 63 (92.6%) cases. In 60 of the 63 cases with a result, the risk score for each of the 3 triso-

mies 21, 18 and 13 was less than 1:10,000, in 2 the score for trisomy 21 was >99% and the score for trisomies 18 and 13 was less than 1:10,000 and in 1 case the risk score for trisomy 18 was 59% and the score for trisomies 21 and 13 was less than 1:10,000 (fig. 1).

The 60 pregnancies with risk scores for each trisomy of less than 1:10,000 are continuing except one pregnancy that has resulted in a live birth of a phenotypically normal neonate. In the 3 high-risk pregnancies, invasive testing was carried out and karyotyping confirmed the suspected trisomy in 1 of the twins for all cases. In the case of trisomy 18 and 1 of the 2 with trisomy 21, chorionic villous sampling was performed at 13 and 14 weeks, respectively, followed by selective fetocide a few days later using ultrasound-guided injection of potassium chloride into the heart of the affected fetus; these pregnancies are now continuing normally at 27 and 22 weeks, respectively. In the second pregnancy with trisomy 21, cfDNA testing was carried out at 13.4 weeks, the results were available at 14.7 weeks, amniocentesis was performed at 15 weeks and selective fetocide at 15.7 weeks. At 20 weeks there was preterm prelabor amniorrhexis and a few days later miscarriage of the whole pregnancy.

In the 3 cases with proven fetal aneuploidies, the risk for trisomies 21, 18 or 13 by the combined test was 1:2, 1:8 and 1:121, respectively. In 2 of the 4 cases with no result from cfDNA testing, the risk from the combined test was >1:100 and in these cases invasive testing demonstrated normal fetal karyotype.

In our study population the expected number of pregnancies with trisomy 21, 18 or 13, in at least 1 of the fetuses, on the basis of the maternal age distribution and the age-related risk for these trisomies at the 11- to 13-week scan was 2.12 [17, 18]. The expected number on the basis of the combined test was 2.7 [11–14], which is similar to the observed number of 3. On the assumption that all continuing pregnancies are normal, in the 63 cases with a cfDNA test result there were 3 trisomic and 60 unaffected pregnancies. The false positive rates were 0% for cfDNA testing and 23.3% (14 of 60) for the combined test.

Discussion

Main Study Findings

This study demonstrates the feasibility of chromosome-selective sequencing of cfDNA in maternal blood for the assessment of risk for fetal trisomies 21, 18 and 13 in twin pregnancies at 10–13 weeks' gestation. In the study of stored plasma samples, a risk score was provided for 93% of cases and the method correctly classified 11 of the 12 trisomic pregnancies with no false positive results. In the prospective study, risk scores for trisomies were provided for 92.6% of cases and the method correctly identified 3 trisomic pregnancies with no false positive results. The detection rate is uncertain until all pregnancies with a low-risk score have a pregnancy outcome.

Result Rate and Low Fetal Fraction

In the prospective study the rate of reporting results from chromosome-selective sequencing of cfDNA in maternal blood was 86.8% and this was improved to 92.6% after repeat sampling. These rates are considerably lower than the respective values of 95 and 98% observed in singleton pregnancies at 10 weeks' gestation using the same method of sequencing [15].

The reason for the lower reporting rate in twins was low fetal fraction and this is the inevitable consequence of selecting the lower fetal fraction of the 2 fetuses, rather than the total, in estimating the risk for aneuploidies [9]. The median of the lower fetal fraction in the prospective study of twins was 7.4% (IQR 5.9–10.0%), which was lower than in our previous study in singletons (median 10.0%, IQR 7.8–13.0%) [19]. The rationale for this choice is to avoid a false negative result in a dizygotic twin pregnancy discordant for aneuploidy where the total fetal fraction is satisfactory but the contribution of the affected fetus could be less than 4%.

In our study of stored plasma samples there was 1 case of trisomy 21 with discordant cfDNA and invasive testing results. This case had the lowest fetal fraction of all the trisomy 21 pregnancies at 5.3% and it is the only case with a cfDNA screen-negative result where the risk score was 1:714; in all other euploid cases in the retrospective study and the presumed euploid cases in the prospective study the risk score was less than 1:10,000. This case emphasizes the importance of knowing the fetal fraction in interpreting results from any cfDNA testing method because accurate counting of the incremental cfDNA fragments from the trisomic fetus, in both singletons and twins, at low fetal fractions is reduced and thus differentiation between euploid and aneuploid is less clear. One practical option that is likely to reduce the potential for a false negative result from cfDNA testing is to rely on the results of the combined test in deciding in favour or against invasive testing when the fetal fraction is low and the risk score from cfDNA testing is higher than 1:10,000. Such cases are very uncommon and it is therefore unlikely that this policy would result in a significant increase in the rate of invasive testing.

Comparison with the Combined Test

In our prospective study, most pregnancies are continuing and it is therefore not possible at present to assess the sensitivity of the screening tests in identifying the trisomic fetuses. However, the observed number of affected pregnancies is similar to that estimated from the maternal age distribution of the study population and the results of the combined test. On the assumption that there are no fetuses with trisomy 21, 18 or 13 among the continuing pregnancies, the combined test, at the recommended risk cut-off of 1:100, identified 2 of the 3 affected pregnancies and cfDNA testing detected all 3. Other major advantages of cfDNA testing, compared to the combined test, highlighted by our study are firstly, reporting of results as a very high or very low risk which makes it easier for parents to decide in favour or against invasive testing (fig. 1) and secondly, a substantial reduction in the false positive rate.

The patients attending the Fetal Medicine Centre were self-referred and the high false positive rate of 23% from the combined test is likely to be the consequence of the high maternal age of the study population, with 78% being 35 years or older, and at least in some a high-risk result from prior screening by the combined test in their own hospital. Two thirds of the pregnancies were conceived by in vitro fertilization and these women were reluctant to undergo invasive testing.

Results of Previous Studies

Three previous studies of cfDNA testing in twins used massively parallel shotgun sequencing to assess the risk for trisomies 21, 18 and 13 [5, 20, 21]. The first study examined 5 twin pregnancies, 4 in a training set and 1 in a test set, and correctly categorized the 2 with trisomy 21 fetuses (both in the training set) and the 3 with euploid fetuses [5]. The second study examined stored plasma samples from 25 twin pregnancies, including 17 with euploid fetuses, 5 discordant and 2 concordant for trisomy 21 and 1 discordant for trisomy 13, and correctly classified all cases [20]. In the third study, cfDNA testing was carried out prospectively in 12 twin pregnancies, including 11 with euploid fetuses and 1 discordant for trisomy 21, and the correct classification was made in all cases [21].

Only one of the studies provided data on fetal fraction [20]. The total fetal fraction was measured; it was assumed that the contribution of each fetus was equal and it was estimated that each twin contributes about one third less cfDNA in maternal blood than in a singleton pregnancy. It was therefore suggested that reliable results from cfDNA testing in a twin pregnancy discordant for trisomy necessitates that the minimum total fetal fraction should be 6%, rather than the 4% used in singleton pregnancies [20].

Clinical Implications

In the last 20 years the rate of twinning has increased, mainly due to the widespread use of assisted reproduction techniques and the increasing maternal age of the population. For example, in the USA and Canada the rates of twin live births increased from about 20 per 1,000 live births in 1991 to more than 30 in 2009 [22]. Most of the increase relates to dizygotic twins with major implications in terms of screening and diagnosis of aneuploidies and subsequent management of pregnancies discordant for such aneuploidies.

In dizygotic pregnancies, the maternal age-related risk for major trisomies for each twin may be the same as in singleton pregnancies and therefore the chance that at least 1 fetus is affected by a trisomy is twice as high as in singleton pregnancies. Furthermore, since the rate of dizygotic twinning increases with maternal age the proportion of twin pregnancies with trisomies is higher than in singleton pregnancies. Consequently, the proportion of twin pregnancies that are screen-positive by the traditional methods of screening is considerably higher than in singleton pregnancies.

The risk of miscarriage from invasive testing in twins is likely to be higher than in singletons [23]. If the pregnancies are discordant for an aneuploidy and the parents choose to have selective fetocide, the subsequent risk of miscarriage or early preterm delivery increases with gestational age at fetocide [24]. The risks of miscarriage from chorionic villus sampling and amniocentesis in twins are similar [25]. It is therefore preferable that in twin pregnancies screening for aneuploidies, invasive testing and when necessary selective fetocide are performed in the first rather than during the second trimester.

There is extensive evidence that in singleton pregnancies cfDNA analysis in maternal blood can lead to the detection of about 99% of fetuses with trisomy 21 at a false positive rate less than 0.1% and a no reporting rate of 2% from testing at 10–13 weeks [1, 15]. The number of twin pregnancies undergoing cfDNA analysis in this and previous studies is too small to provide accurate assessment of the performance of the test. Nevertheless, it is likely that the sensitivity will be high and the false positive rate low. However, the high no reporting rate at first sampling is of major concern; this would shift the option of prenatal diagnosis and selective fetocide from the first to the second trimester with consequent increase in the rate

of miscarriage. This problem is exemplified by one of our pregnancies discordant for trisomy 21 where selective fetocide at 15 weeks' gestation was followed by miscarriage at 20 weeks.

In twin pregnancies, the 11- to 13-week scan is essential for firstly, accurate determination of gestational age from the CRL of the largest fetus, secondly, diagnosis of major abnormalities that would allow for earlier and safer selective fetocide and thirdly, assessment of chorionicity which defines the risk for most pregnancy complications and therefore the subsequent management [26]. In twin pregnancies, as in singletons, there are essentially two options in the introduction of cfDNA testing in such a way as to retain the advantages of the 11- to 13-week scan [15, 27]. One option is to carry cfDNA testing in all women at 10 weeks' gestation followed by a scan at 12 weeks; in patients with a high-risk score from cfDNA testing, invasive diagnostic testing and selective fetocide can be carried out in the first trimester. In cases with no result from cfDNA testing, pregnancy management could be based on the results of the combined test.

The alternative to universal screening by the cfDNA test is a strategy of cfDNA testing contingent on the results of first-line screening by ultrasound and biochemical testing. This approach retains the major advantages of cfDNA testing in increasing the detection rate of trisomies and decreasing the false positive rate, but at considerably lower cost than offering the test to the whole population [27]. The disadvantage of this approach, arising from delay or failure to obtain a result, is the resultant shift in diagnosis from the first to the second trimester. This could be partly ameliorated by offering invasive testing when the estimated risk from the combined test is very high and reserving cfDNA testing for the intermediate-risk group.

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Study 3

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Fetal fraction of cell-free DNA in maternal plasma in the prediction of spontaneous preterm delivery

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KEYWORDS: cell-free DNA; fetal fraction; first-trimester screening; preterm birth

ABSTRACT

Objectives *To investigate whether, in pregnancies complicated by spontaneous preterm delivery, fetal fraction of cell-free DNA (cfDNA) in maternal plasma at 11–13 weeks' gestation is altered and if this measurement could be useful in the prediction of preterm delivery.*

Methods *Fetal fraction of cfDNA was measured at 10 + 0 to 13 + 6 weeks' gestation in 3169 pregnancies, 3066 (96.7%) that delivered at ≥ 37 weeks and 103 (3.3%) with spontaneous delivery at < 37 weeks, including 21 that delivered at < 34 weeks and 82 that delivered at 34–37 weeks. The measured fetal fraction was converted to multiples of the median (MoM), corrected for maternal characteristics and gestational age, and the Mann–Whitney* U*-test was used to determine the significance of differences in the median values in the spontaneous preterm delivery groups from that in the term delivery group.*

Results *In the spontaneous preterm delivery groups (< 34 weeks' gestation, 34–37 weeks, < 37 weeks), compared to the term delivery group, there was no significant difference in the median fetal fraction MoM (1.004, 0.922 and 0.946, respectively,* vs *1.015).*

Conclusion *Measurement of fetal fraction in maternal plasma at 11–13 weeks' gestation is not predictive of spontaneous preterm delivery.*

INTRODUCTION

Preterm delivery is the leading cause of perinatal death and disability in children, and the vast majority of mortality and morbidity relates to early delivery before 34 weeks' gestation[1,2]. Spontaneous delivery before 34 weeks occurs in about 1% of singleton pregnancies. Studies have shown that the patient-specific risk for spontaneous early delivery can be determined at 11–13 weeks' gestation by combining maternal demographic characteristics and obstetric history, with an estimated detection rate of 28%, at a false-positive rate of 10%[3]. This performance of screening is not improved by biophysical or biochemical markers of placental perfusion or function, but the detection rates increased to 36% and 55% by the addition of maternal serum α-fetoprotein and cervical length measurement, respectively, at 11–13 weeks[3–5]. There is also some evidence that plasma cell-free DNA (cfDNA) is increased in pregnancies complicated by spontaneous preterm birth, with a suggested mechanism of early initiation of breakdown of the placental barrier in anticipation of labor[6–8]. However, there is contradictory evidence as to whether, in cases of spontaneous preterm birth, the increase in cfDNA precedes the clinical event[9–12].

The aims of this study were to explore whether, in pregnancies that are complicated by spontaneous preterm birth, fetal fraction of cfDNA in maternal plasma at 11–13 weeks' gestation is altered and if this measurement is useful in the prediction of this pregnancy complication.

METHODS

The data for this study were derived from clinical implementation of cfDNA testing in screening for trisomies 21, 18 and 13 at 10 + 0 to 13 + 6 weeks' gestation in women with singleton pregnancies, attending the Fetal Medicine Centre in London, UK, between October 2012 and November 2013.

We recorded maternal characteristics and medical history, provided pretest counseling and obtained written consent for cfDNA testing. Patients were asked to complete a questionnaire on maternal age, racial origin (Caucasian, Afro-Caribbean, South Asian, East Asian or mixed), method of conception (spontaneous or assisted conception requiring the use of ovulation drugs or

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in-vitro fertilization), cigarette smoking during pregnancy (yes or no) and parity (parous or nulliparous if no previous pregnancy at or after 24 weeks' gestation). The questionnaire was then reviewed by a doctor together with the patient. Maternal weight was measured and recorded. An ultrasound examination was carried out to determine if the pregnancy was singleton with a live fetus and to estimate gestational age from measurement of the fetal crown–rump length (CRL)[13]. 20 mL of maternal blood was obtained by venepuncture, using Streck cfDNA BCT™ tubes, and cfDNA testing was subsequently performed using the Harmony™ Prenatal Test (Ariosa Diagnostics, Inc., San Jose, CA, USA).

Data on pregnancy outcome were collected from obstetricians, general medical practitioners or the women themselves. The outcome measures were spontaneous delivery at < 34 weeks' gestation (early preterm), at 34 + 0 to 36 + 6 weeks (late preterm) and at < 37 weeks (total preterm). Spontaneous preterm birth included those with spontaneous onset of labor and those with preterm prelabor rupture of membranes (PPROM).

Laboratory analysis

Maternal venous blood samples were sent via courier to the USA for analysis using a chromosome-selective assay (Harmony Prenatal Test). The method relies on the assay of non-polymorphic and polymorphic loci, in which fetal alleles differ from maternal alleles, enabling simultaneous determination of chromosome proportion and fetal fraction[14]. Risk scores for trisomies 21, 18 and 13 and fetal fraction were provided in the test report[14–16].

Statistical analysis

Descriptive data are presented as median and interquartile range for continuous variables and as *n* (%) for categorical variables. Comparisons between outcome groups were performed using the Mann–Whitney *U*-test for continuous variables and the χ^2 test or Fisher's exact test for categorical variables.

The measured fetal fraction was $\log_{10}$ transformed to make the distribution Gaussian. Normality of distribution was assessed using probability plot. Regression analysis was used to examine the significance of association between $\log_{10}$ fetal fraction and gestational age at delivery. In the term delivery group, backward stepwise multiple regression was used to determine which of the factors among fetal CRL, maternal age, weight, racial origin, smoking status, parity and method of conception were significant predictors of $\log_{10}$ fetal fraction. The distributions of fetal fraction were converted to multiples of the median (MoM) in all cases, corrected for maternal characteristics and gestational age as determined. The Mann–Whitney *U*-test was used to determine the significance of differences in the median values between the outcome groups.

The statistical software package SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

RESULTS

During the study period, cfDNA testing was performed in 3483 singleton pregnancies with a live fetus at 10 + 0 to 13 + 6 weeks' gestation. We excluded 314 (9.0%) cases owing to loss to follow-up or incomplete data on pregnancy outcome ($n = 128$), termination of pregnancy because of aneuploidies or other fetal abnormalities ($n = 57$), miscarriage ($n = 49$), cfDNA assay failure ($n = 12$) or iatrogenic delivery at < 37 weeks ($n = 68$). Thus the study population comprised 3169 pregnancies. In 3123 of these cases, the reported result from cfDNA analysis was low risk for the three trisomies and the fetal fraction was ≥ 4%. In 46 (1.5%) cases, the fetal fraction was < 4% and no risk was given for the three trisomies; these pregnancies resulted in the birth of phenotypically normal neonates and for the purpose of the analysis the fetal fraction was assumed to be 3%.

The study population comprised 3066 (96.7%) pregnancies that delivered at ≥ 37 weeks' gestation and 103 (3.3%) with spontaneous delivery at < 37 weeks. The maternal and pregnancy characteristics of each of the outcome groups are summarized in Table 1. In both early and late spontaneous preterm delivery groups (< 34 weeks and 34 + 0 to 36 + 6 weeks), compared to the term delivery group, the median gestational age at delivery and neonatal birth weight were significantly reduced.

The frequency distribution of $\log_{10}$ fetal fraction is shown in Figure 1. In the total study group, there was no significant correlation between $\log_{10}$ fetal fraction and gestational age at delivery ($r = 0.030$, $P = 0.095$; Figure 2). In the term delivery group, multiple regression analysis demonstrated that for the prediction of $\log_{10}$ fetal fraction, significant independent contributions were provided by fetal CRL, maternal weight, South Asian racial origin, parity and method of conception (Table 2). In the spontaneous preterm delivery groups (< 34 weeks, 34 + 0 to 37 weeks, < 37 weeks), compared to the term delivery group, there was no significant difference in the median fetal fraction (Table 3, Figure 3).

DISCUSSION

Main findings of the study

The findings of this study demonstrate that in pregnancies resulting in early and late spontaneous preterm delivery, the median fetal fraction of cfDNA in the maternal plasma at 11–13 weeks' gestation is not significantly different from that of those delivering at term.

In pregnancies that result in term delivery, the fetal fraction of cfDNA in maternal plasma increases with fetal CRL, decreases with maternal weight, is higher in parous than nulliparous women, lower in women of South Asian racial origin than in Caucasians and lower in women who had assisted, rather than spontaneous, conception. Consequently, the estimated fetal fraction was adjusted for these variables before comparing results between outcome groups.

Table 1 Characteristics of the study population of 3169 women with a singleton pregnancy, according to term, early-preterm or late-preterm delivery

	GA at delivery (weeks)		
Characteristic	*≥ 37 (term)* (n = *3066*)	*< 34 (preterm)* (n = *21*)	*34 + 0 to 36 + 6 (late preterm)* (n = *82*)
Maternal age (years)	36.8 (33.8–39.6)	38.8 (35.6–41.1)	35.9 (33.1–39.3)
Maternal weight (kg)	63.0 (57.5–71.0)	62.7 (54.2–67.0)	60.5 (54.9–69.9)
Crown–rump length (mm)	64.7 (60.4–69.4)	65.0 (58.9–68.4)	65.2 (60.1–71.1)
Racial origin			
Caucasian	2673 (87.2)	18 (85.7)	70 (85.4)
Afro-Caribbean	63 (2.1)	—	1 (1.2)
South Asian	174 (5.7)	3 (14.3)	7 (8.5)
East Asian	103 (3.4)	—	3 (3.7)
Mixed	53 (1.7)	—	1 (1.2)
Parity			
Nulliparous	1384 (45.1)	11 (52.4)	43 (52.4)
Parous	1682 (54.9)	10 (47.6)	39 (47.6)
Cigarette smoker	23 (0.8)	—	1 (1.2)
Conception			
Spontaneous	2620 (85.5)	18 (85.7)	72 (87.8)
Assisted reproduction	446 (14.5)	3 (14.3)	10 (12.2)
GA at delivery (weeks)	39.6 (38.9–40.3)	31.6 (27.9–33.2)*	35.7 (35.3–36.3)*
Birth weight (g)	3405 (3133–3710)	1839 (1016–2003)*	2670 (2300–2935)*
Birth-weight percentile	53.3 (28.8–78.3)	44.3 (36.3–66.0)	53.5 (20.6–76.8)
Birth-weight percentile < 5th	97 (3.2)	—	7 (8.5)

Data are given as median (interquartile range) or *n* (%). *Statistically significant difference on comparison between each preterm-delivery group with term-delivery group (Mann–Whitney *U*-test with *post hoc* Bonferroni correction and χ^2-test or Fisher's exact test for categorical variables, $P < 0.025$). GA, gestational age.

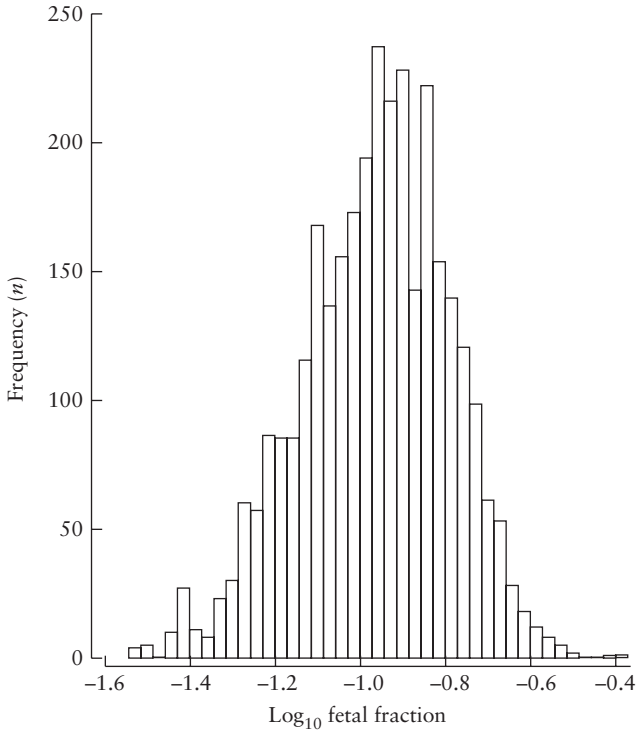

Figure 1 Frequency distribution of $\log_{10}$-transformed fetal fraction in maternal plasma cell-free DNA in the total study group of 3169 singleton pregnancies.

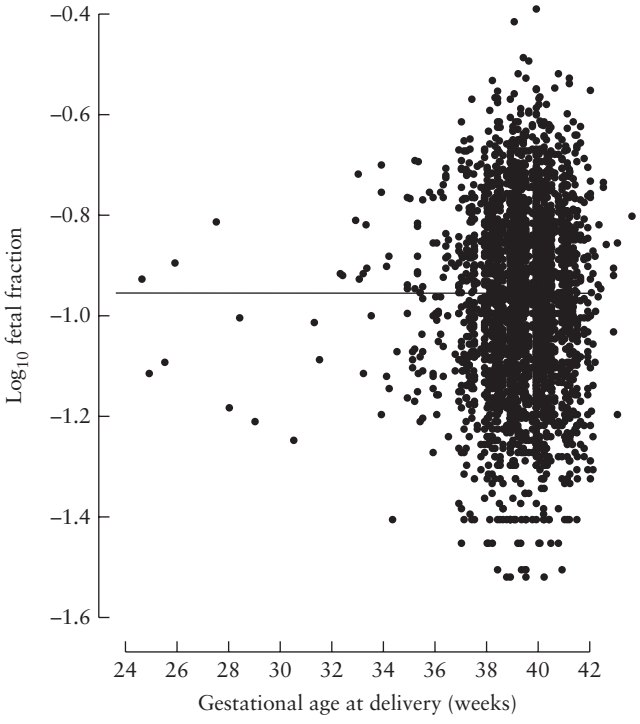

Figure 2 Association between $\log_{10}$-transformed fetal fraction of cell-free DNA in maternal plasma and gestational age at delivery in 3169 singleton pregnancies. Pearson correlation coefficient, $r = 0.030$; $P = 0.095$.

Limitations of the study

This was a cross-sectional study at 10 + 0 to 13 + 6 weeks' gestation and the conclusions, in relation to the inability of fetal fraction to distinguish between pregnancies that subsequently deliver preterm and those delivering at term, are confined to this early gestational age. Longitudinal studies are needed to examine whether a change in fetal fraction precedes the onset of labor and the interval between the two events.

Table 2 Fitted regression model for $\log_{10}$ fetal fraction at 10–13 weeks' gestation in 3169 women with a singleton pregnancy

Independent variable	*Regression coefficient (95% CI)*	*Standard error*	P
Intercept	−0.68531 (−0.74661 to −0.62491)	0.031263	< 0.0001
Fetal crown–rump length (mm)	0.0010460 (0.00022669 to 0.0018653)	0.00041786	0.012
Maternal weight (kg)	−0.0055255 (−0.0060105 to −0.0050405)	0.00024735	< 0.0001
South Asian racial origin	−0.033763 (−0.058070 to −0.0094556)	0.012397	0.007
Parous	0.045157 (0.033727 to 0.056587)	0.058293	< 0.0001
Assisted conception	−0.061662 (−0.077795 to −0.045529)	0.0082280	< 0.0001

Table 3 Fetal fraction of cell-free DNA in maternal plasma according to different pregnancy outcome groups in 3169 women with a singleton pregnancy

	Fetal fraction	
Outcome group	*Percent*	*MoM*
Term: delivery ≥ 37 weeks ($n = 3066$)	11.1 (8.3–14.3)	1.015 (0.787–1.284)
Early preterm: spontaneous delivery < 34 weeks ($n = 21$)	11.7 (7.8–12.5)	1.004 (0.705–1.184)
Late preterm: spontaneous delivery 34 + 0 to 36 + 6 weeks ($n = 82$)	10.8 (7.7–13.8)	0.922 (0.768–1.218)
*Preterm: spontaneous delivery < 37 weeks ($n = 103$)	10.8 (7.7–13.6)	0.946 (0.753–1.207)

Data are given as median (interquartile range). *Comprises both early-preterm and late-preterm deliveries. Early-preterm and late-preterm groups were each compared to term group (*P*-value cut-off 0.025) and preterm group was compared to term group (*P*-value cut-off 0.05) using Mann–Whitney *U*-test and with *post hoc* Bonferroni correction, with no significant difference found in any comparison. MoM, multiples of the median.

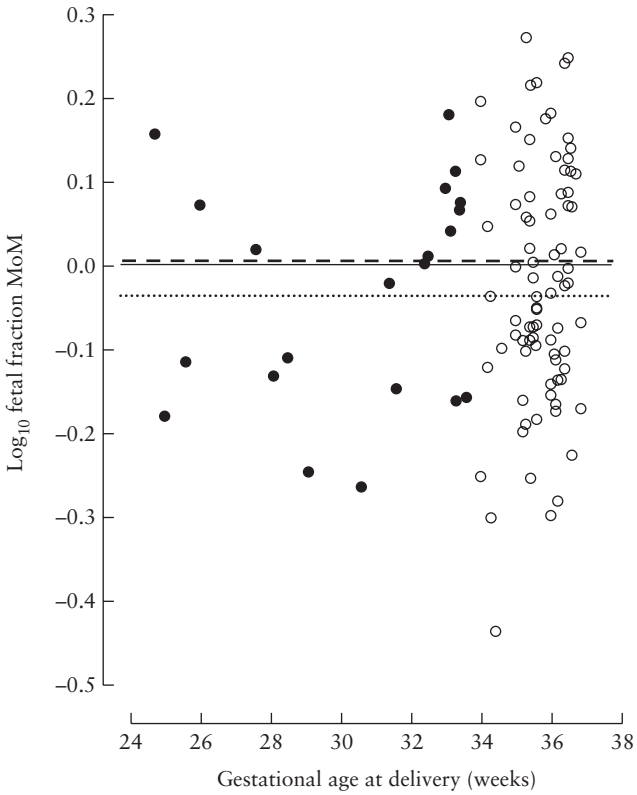

Figure 3 Association between $\log_{10}$-transformed fetal fraction multiples of the median (MoM) and gestational age at delivery in cases of spontaneous delivery at < 34 weeks' gestation (●; ——, median) and spontaneous delivery at 34–37 weeks (○; ....., median). – –, Median $\log_{10}$MoM of term delivery.

Comparison with findings from previous studies

Two previous studies examined women presenting with contractions and/or PPROM, and reported that, in those with spontaneous preterm delivery, maternal plasma fetal cfDNA was increased[6,7]. Leung *et al.*[6], using real-time quantitative polymerase chain reaction (PCR) for the detection of the *SRY* gene, demonstrated that the median fetal cfDNA was higher in 13 pregnancies with male fetuses, presenting with threatened preterm labor and subsequent delivery at 26–34 weeks, when compared to 17 controls that delivered at term. Farina *et al.*[7] assessed the *DYS1* locus on the Y chromosome by real-time PCR to determine fetal cfDNA in 29 women with preterm labor delivering at < 36 weeks, 21 with PPROM delivering at < 36 weeks and 21 with preterm labor delivering at ≥ 36 weeks. They reported that cumulative rates of delivery at < 30 weeks and delivery at < 36 weeks were significantly higher for women with fetal cfDNA ≥ 1.82 MoM than for those with fetal cfDNA concentrations below this cut-off (early preterm delivery: 45% *vs* 14%; preterm delivery: 73% *vs* 66%). The observed increase in fetal cfDNA levels appears to be part of the process that initiates the onset of labor and subsequent delivery.

Some contradictory evidence suggests that, in cases of spontaneous preterm delivery, the increase in cfDNA precedes the clinical event. A cohort study of 876 women undergoing routine fetal rhesus D (RhD) genotyping, at 23–28 weeks' gestation, reported that if the fetal cfDNA level was above the 95th percentile there was a 6- and 16-fold increase in risk for spontaneous delivery at < 37 weeks ($n = 19$) and at < 34 weeks ($n = 8$), respectively[9]. In contrast, Stein *et al.*[12] reported that, in a cohort study of 611 women undergoing routine fetal RhD genotyping at 24–25 weeks' gestation, the levels of fetal cfDNA were not altered in pregnancies complicated by preterm delivery. A study examining 34 women with a short cervix and 22 women with normal cervical length at 22–24 weeks' gestation assessed the *DYS14* locus on the Y chromosome by real-time PCR to determine fetal cfDNA levels, and reported no significant difference in the level of fetal cfDNA between those that delivered before 37 weeks and those delivering at term[11]. More

recently, a study used chromosome-selective sequencing of non-polymorphic and polymorphic loci, in which fetal alleles differ from maternal alleles, to determine the cfDNA counts of fetal and maternal origin in maternal plasma at 11–13 weeks' gestation[17]. Both fetal and maternal cfDNA counts were affected by maternal characteristics, but the corrected values in 20 cases of spontaneous preterm delivery were not significantly different from those of 1805 unaffected pregnancies.

Implications for clinical practice

First-trimester screening for spontaneous preterm delivery can be achieved using a combination of factors, including maternal characteristics and obstetric history, cervical length and serum α-fetoprotein levels, at 11–13 weeks' gestation[3–5]. The benefit of such early identification of pregnancies at high risk for spontaneous preterm birth is the potential to reduce the prevalence of this complication through early surgical and pharmacological therapies[18]. The reported high performance of cfDNA analysis of maternal blood in screening for fetal trisomies will inevitably lead to widespread uptake of this technique, and an integral part of such aneuploidy screening is measurement of the fetal fraction[19]. A beneficial consequence of such measurement of the fetal fraction could have been the improved performance of early screening for spontaneous preterm delivery. However, as demonstrated by our study, the use of cfDNA testing is unlikely to be useful for the prediction of this pregnancy complication.

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CHAPTER 3 SUMMARY

3.1 English

Study 1:

The objective of this study was to report the results of clinical implementation of cfDNA testing for trisomies 21, 18 and 13 in the general population at 10-11 weeks' gestation and compare its performance to that of the first trimester combined test.

In 2,905 singleton pregnancies prospective screening for trisomies was performed by chromosome-selective sequencing of cfDNA in maternal blood at 10-11 weeks and by the combined test at 11-13 weeks.

The median maternal age was 36.9 (range 20.4-51.9) years. Results from cfDNA analysis were provided for 2,851 (98.1%) cases and these were available within 14 days from sampling in 2,848 (98.0%). The trisomic status of the pregnancies was determined by prenatal or postnatal karyotyping or clinical examination of the neonates. In the 2,785 pregnancies with cfDNA result and known trisomic status, cfDNA testing correctly identified all 32 cases with trisomy 21, nine of 10 with trisomy 18 and two of five with trisomy 13 with respective false positive rates of 0.04%, 0.19% and 0.07%. In cases with discordant results between cfDNA testing and fetal karyotype the median fetal fraction was lower than in those with concordant results (6% vs 11%). In the combined test, the estimated risk for trisomy 21 was $\geq$1:100 in all trisomic cases and in 4.4% of the non-trisomic pregnancies.

It was concluded that the performance of first-trimester cfDNA testing for trisomies 21 and 18 in the general population is similar to that in high-risk pregnancies. Most false positive and negative results from cfDNA testing could be avoided if the *a priori* risk from the combined test is taken into account in the interpretation of individual risk.

Study 2

The objective of this study was to examine the clinical implementation of chromosome-selective sequencing of cfDNA in maternal blood in the assessment of risk for trisomies in twin pregnancies.

Risk for trisomies 21, 18 and 13 by cfDNA testing were estimated in stored plasma samples obtained at 11-13 weeks' gestation from 207 pregnancies with known outcome and prospectively in 68 twin pregnancies undergoing screening at 10-13 weeks. Cell-free DNA was extracted from the maternal plasma or blood samples and chromosome- selective sequencing was carried out as in singleton pregnancies. However, in the assessment of risk for trisomies the smallest fetal fraction contribution of the two fetuses was considered.

Risk scores for trisomies were provided for 192 (92.8%) of stored plasma and for 63 (92.6%) of the prospective cases. In the retrospective study 10 of 11 trisomic pregnancies were correctly identified with no false positive results. In the prospective study three trisomic pregnancies were correctly identified with no false positive results. The median of the lower fetal fraction in the prospective study of twins was 7.4% (inter-quartile range 5.9-10.0%), which was lower than in our previous study in singletons (median 10.0%, IQR 7.8-13.0%).

It was concluded that cfDNA testing in twins is feasible but the reporting rate of results is lower than in singletons due to a lower fetal fraction. The number of twin pregnancies undergoing cfDNA analysis in this and previous studies is too small to provide accurate assessment of the performance of the test. Nevertheless, it is likely that the sensitivity will be high and the false positive rate low. However, the high no reporting rate at first sampling is of major concern; this would shift the option of prenatal diagnosis and selective fetocide from the first to the second trimester with consequent increase in the rate of miscarriage.

Study 3

The objective of this study was explore whether in pregnancies that are complicated by spontaneous preterm delivery fetal fraction of cfDNA in maternal plasma at 11-13 weeks' gestation is altered and if this measurement is useful in the prediction of preterm birth.

Fetal fraction of cfDNA was measured at 10^{+0}-13^{+6} weeks' gestation in 3,066 (94.7%) pregnancies that delivered at $\geq$37 weeks' gestation and 103 (3.2%) with spontaneous delivery at <37 weeks, including 21 that delived at <34 weeks and 82 that delivered at 34-36 weeks. Fetal fraction measured was converted to multiples of median (MoM), corrected for maternal characteristics and gestational age. Mann-Whitney U test was used to determine the significance of differences in the median values in the spontaneous preterm delivery groups to that in the term delivery group.

In the spontaneous preterm delivery groups (<34 weeks, 34-37 weeks, <37 weeks), compared to the term delivery group, there was no significant difference in the median fetal fraction MoM (1.004, 0.922 and 0.946, respectively, vs. 1.015).

It was concluded that measurement of fetal fraction in maternal plasma at 11-13 weeks is not predictive of spontaneous preterm birth. This was a cross-sectional study and the conclusions in relation to the inability of fetal fraction to distinguish between pregnancies that subsequently deliver preterm and those delivering at term are confined to this early gestational age. Longitudinal studies are needed to examine whether a change in fetal fraction precedes the onset of labor and the interval between the two events.

3.2 Spanish

Estudio 1

El objetivo de este estudio era analizar los resultados de la implementación clínica del ADN fetal libre en sangre materna para las trisomías 21, 18 y 13 en la población general a las 10-11 semanas gestacionales, y compararlo con los resultados del cribado del primer trimestre. Se realizó un cribado prospectivo sequenciando el ADN fetal en sangre materna a las 10-11 semanas gestacionales y se realizó el test combinado en 2905 embarazos únicos.

La media de la edad materna fue de 36.9 años (rango 20,4-51,9). En 2851 (98.1%) casos se obtuvieron resultados del análisis del ADN fetal y estos estuvieron disponibles entre los primeros 14 días tras la extracción sanguínea en 2848 (98%). El estado trisómico de la gestación fue determinado por el cariotipo prenatal o postnatal, o por la examinación clínica del neonato. En 2785 embarazos con resultado de ADN fetal y con estado trisómico conocido, el análisis del ADN fetal identificó correctamente todos los 32 casos de trisomía 21, 9 de los 10 casos de trisomía 18, y dos de los 5 casos de trisomía 13, con una tasa de falsos positivos de 0,04%, 0,19% y 0,07%, respectivamente. En los casos discordantes entre los resultados de ADN fetal y de cariotipo fetal, la media de la fracción fetal fue más baja que en aquellos con un resultado concordante (6% vs 11%). En el test combinado, el riesgo para trisomía 21 fue >1:100 en todos los casos de trisomía y en 4.4% de los embarazos no trisómicos.

Se concluyó que la realización en el primer trimestre del ADN libre en sangre materna para las trisomías 21 y 18 en al población general es similar a la población de alto riesgo. La mayoría de los casos de falsos positivos y negativos obtenidos con el análisis del ADN libre en sangre materna se podrían haber evitado si se hubiese tenido en cuenta el riesgo a priori, obtenido con el test combinado, en la interpretación del riesgo individual.

Estudio 2

El objetivo del estudio fue examinar los resultados de la implementación clínica de la secuenciación selectiva del ADN libre en sangre materna en el asesoramiento del riesgo de trisomías en las gestaciones gemelares.

El riesgo de trisomía 21,18 y 13 calculado con el test de ADN libre, se obtuvo analizando plasma almacenado de muestras obtenidas a las 11-13 semanas de gestación de 207 embarazos con resultado conocido, y de 68 embarazos gemelares que realizaron el cribado a las 10-13 semanas. La secuenciación selectiva del ADN libre extraído del plasma materno o de las muestras sanguíneas, fue llevado a cabo como en las gestaciones únicas. Sin embargo, se consideró la contribución de la menor fracción fetal en el asesoramiento del riesgo de trisomías.

Se obtuvo resultado en 192 (92.8%) del plasma almacenado y en 63 (92.6%) de los casos prospectivos. En el estudio retrospectivo, 10 de 11 embarazos trisómicos fueron correctamente identificados sin falsos positivos. En el estudio prospectivo 3 embarazos trisómicos fueron correctamente identificados sin resultados de falsos positivos. En el estudio prospectivo de los gemelos la media de la fracción fetal más baja fue de 7.4% (rango interquartile 5.9-10.0%), que fue más baja que en nuestro estudio de gestaciones únicas. (media 10.0%, IQR 7.8-13%).

Se concluyó que es posible realizar el test ADN libre en sangre materna en gestaciones gemelares, pero la tasa de resultados es menor que en las gestaciones únicas, debido a una baja fracción fetal. El número de gestaciones gemelares que realizaron el test de ADN libre en sangre materna, en este y en estudios previos, es demasiado pequeño para proporcionar un asesoramiento preciso en la aplicación del test. Es probable que la sensibilidad sea mayor y que la tasa de falsos positivos menor. Sin embargo, resulta preocupante la alta tasa de resultados no obtenidos con el test, esto podría trasladar la opción de diagnóstico prenatal y de fetocidio selectivo

del primer al segundo trimestre, con las consecuencia de incremento de la tasa de abortos.

Estudio 3

El objetivo de este estudio era analizar si la fracción fetal del ADN libre en sangre materna a las 11-13 semanas de gestación se encuentra alterado en las gestaciones complicadas por un parto pretérmino, y si esta medida resulta útil en la predicción de parto pretérmino.

La fracción fetal de ADN libre en sangre materna fue medida a las 10^{+0}-13^{+6} semanas de gestación en 3,066 (94,7%) embarazadas que parieron a término ($\geq$ 37 semanas de gestación), y en 103 (3.2%) con partos espontáneos por debajo de la semana de gestación 37, incluyendo 21 que parieron antes de la semana de gestación 34, y 82 que parieron entre las semanas 34 y 36. La fracción fetal medida fue transformada a múltiplos de media (MoM), corregida por las características maternas y la edad gestacional. Se utilizó el test de Mann-Withney U para determinar la significancia de las diferencias en los valores medios entre el grupo del parto pretérmino y el grupo de parto a término.

Al comparar el grupo de parto pretérmino espontaneo (<34 semanas, 34-37 semanas, <37 semanas) con el grupo de parto a término, no se encontraron diferencias significativas de la media de la fracción fetal MoM (1.004, 0.922 y 0.946, respectivamente, vs 1.015).

Se concluyó que la medida de la fracción fetal en sangre materna a las 11-13 semanas gestacionales no es predictiva del parto pretérmino. Este ha sido un estudio transversal y las conclusiones en relación a la incapacidad del la fracción fetal de distinguir entre los embarazos que llegarían a término con los que no, se verán limitadas por la edad gestacional temprana. Son necesarios estudios longitudinales para analizar si el cambio en la fracción fetal precede al inicio del parto, y el intervalo entre estos dos eventos.

CHAPTER 4 DISCUSSION

The studies in this thesis have to a large extent fulfilled the aims as outlined in chapter 1.5.

Implementation of cfDNA testing for trisomies in singleton pregnancies

This prospective study in 2,905 singleton pregnancies having routine first trimester screening for the major trisomies by cfDNA analysis of maternal blood and by the combined test, provided outcome data for nearly 98% of cases, which makes it possible to assess accurately the performance of screening. The combined test, at a risk cut-off of 1:100, identified all cases of trisomies 21, 18 and 13 with FPR of 4.4%. Screening by cfDNA analysis of maternal blood provided results in 98% of pregnancies and these were available within two weeks of sampling in 98% of cases. In the pregnancies with a cfDNA result all cases of fetal trisomy 21 were detected at FPR of 0.04%. The test also detected nine of the 10 cases with fetal trisomy 18 at FPR of 0.19%; in the one false negative case, QF PCR of chorionic villi reported disomy 18. The performance of screening for trisomy 13 was poorer with only two of five affected cases being detected.

The study has also highlighted that in cases of discordant results between cfDNA testing and fetal karyotype the fetal fraction was lower than in those with concordant normal or abnormal results.

A potential limitation of the study is that the patients were self-selected and inevitably a high proportion of women was of advanced age and conceived by assisted reproduction techniques. Nevertheless, the women did not have prior screening for trisomies by other methods and their results are applicable to a general population. Another limitation of the study relates to the high performance of the combined test. The results of cfDNA analysis were commonly available at the time of the ultrasound

scan for measurement of fetal NT and could have potentially biased the measurements.

Our findings on the performance of maternal blood cfDNA analysis in screening for trisomies 21 and 18 in a general population are compatible with the results of previous studies in high-risk pregnancies, but also those which examined general populations. The detection rate of trisomy 13 was lower, but the number of cases we have examined is too small for valid conclusions to be drawn. In the three previous prospective screening studies in the general population the median gestational age at cfDNA testing was 14-17 weeks (Song *et al.*, 2013; Bianchi *et al.*, 2014; Comas *et al.*, 2014). Our study has focused on the application of cfDNA testing at 10-11 weeks because first-trimester screening and diagnosis of aneuploidies lead to early reassurance of the majority of parents that their fetus is unlikely to be trisomic, and for the few with an affected fetus the parents have the option of an earlier and safer termination of pregnancy.

An important clinical implication of the data is that in the interpretation of cfDNA results, particularly in cases with low fetal fraction, the *a priori* risk should be considered. The ability to detect the small increase in the amount of a given chromosome in maternal plasma in a trisomic compared to a disomic pregnancy is directly related to the relative proportion of the fetal to maternal origin of the cfDNA in maternal plasma. In our cases with false positive and false negative results the median fetal fraction was lower than in those with concordant results between the cfDNA test and fetal karyotype.

Clinical implementation of cfDNA testing for trisomies in twin pregnancies

This study examined two datasets of twin pregnancies, first, stored plasma samples obtained at 11-13 weeks' gestation from 207 pregnancies with known outcome and second, 68 prospectively examined twin pregnancies undergoing screening at 10-13 weeks.

In both groups, the failure rate of cfDNA testing to provide a result was 7%, which is considerably higher than the 2% observed in singleton pregnancies using the same method of sequencing. The reason for the higher failure rate in twins was low fetal fraction and this is the inevitable consequence of selecting the lower fetal fraction of the two fetuses, rather than the total, in estimating the risk for aneuploidies (Struble et al., 2014). The rationale for this choice is to avoid a false negative result in a dizygotic twin pregnancy discordant for aneuploidy where the total fetal fraction is satisfactory but the contribution of the affected fetus could be less than 4%.

In terms of results, there were 12 cases of trisomy 21 and a correct diagnosis by cfDNA testing was made in 11; in one case there was a false negative result. There were two cases of trisomy 18 and a correct diagnosis by cfDNA testing was made in one; in one case there was no result. There were three cases of trisomy 13 and a correct diagnosis by cfDNA testing was made in one; in two cases there was no result. The case of trisomy 21 with a negative cfDNA result had a fetal fraction of only 5.3% which illustrates the fact that a low fetal fraction the accuracy of cfDNA testing, particularly in twins may be limited.

The number of twin pregnancies undergoing cfDNA analysis in this and previous studies is too small to provide accurate assessment of the performance of the test. Nevertheless, it is likely that the sensitivity will be high and the false positive rate low. However, the high no reporting rate at first sampling is of major concern; this would shift the option of prenatal diagnosis and selective fetocide from the first to the second trimester with consequent increase in the rate of miscarriage.

In twin pregnancies, as in singletons, there are essentially two options in the introduction of cfDNA testing in such a way as to retain the advantages of the 11-13 weeks scan (Gil et al, 2013; Nicolaides et al, 2014). One option is to carry cfDNA testing in all women at 10 weeks' gestation followed by a scan at 12 weeks; in patients with a high risk score from cfDNA testing, invasive diagnostic testing and selective fetocide can be carried out in the first trimester. In cases with no result from cfDNA testing, pregnancy management could be based on the results of the combined test.

The alternative to universal screening by the cfDNA test is a strategy of cfDNA testing contingent on the results of first-line screening by ultrasound and biochemical testing. This approach retains the major advantages of cfDNA testing in increasing the detection rate of trisomies and decreasing the false positive rate, but at considerably lower cost than offering the test to the whole population. The disadvantage of this approach, arising from delay or failure to obtain a result is the resultant shift in diagnosis from the first to the second trimester. This could be partly ameliorated by offering invasive testing when the estimated risk from the combined test is very high and reserving cfDNA testing for the intermediate risk group.

Cell-free DNA in the prediction of spontaneous preterm delivery

Preterm delivery is the leading cause of perinatal death and handicap in children. Consequently, a method of early prediction and prevention of such pregnancy complication could have a major impact on perinatal death and handicap. There is some contradictory evidence that in cases of spontaneous preterm birth fetal DNA in maternal blood may be increased before the clinical event.

Our study of 3,169 singleton pregnancies, including 103 (3.2%) with spontaneous preterm birth, demonstrated that in pregnancies resulting in early and late spontaneous preterm delivery, compared to those delivering at term, the median fetal fraction of cfDNA in the maternal plasma at 11-13 weeks' gestation is not significantly different.

A potential limitation of our study arises from its cross-sectional nature confined to 11-13 weeks. The conclusions in relation to the inability of fetal fraction to distinguish between pregnancies that subsequently deliver preterm and those delivering at term are confined to this early gestational age. Longitudinal studies are needed to examine whether a change in fetal fraction precedes the onset of labor and the interval between the two events.

The reported high performance of cfDNA analysis of maternal blood in screening for fetal trisomies will inevitably lead to widespread uptake of this technique and an integral part of such aneuploidy screening is measurement of the fetal fraction. A beneficial consequence of such measurement of the fetal fraction would have been improved performance of early screening for spontaneous preterm delivery. However, as demonstrated by our study the use of cfDNA testing is unlikely to be useful for the prediction of this pregnancy complication.

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