

Performance of screening for aneuploidies by cell-free DNA analysis of maternal blood in twin pregnancies

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KEYWORDS: aneuploidies; cell-free DNA; screening test; trisomies; twin pregnancies

ABSTRACT

Objectives To report clinical implementation of cell-free DNA (cfDNA) analysis of maternal blood in screening for trisomies 21, 18 and 13 in twin pregnancies and examine variables that could influence the failure rate of the test.

Methods cfDNA testing was performed in 515 twin pregnancies at 10–28 weeks' gestation. The failure rate of the test to provide results was compared with that in 1847 singleton pregnancies, and logistic regression analysis was used to determine which factors among maternal and pregnancy characteristics were significant predictors of test failure.

Results Failure rate of the cfDNA test at first sampling was 1.7% in singletons and 5.6% in twins. Of those with a test result, the median fetal fraction in twins was 8.7% (range, 4.1–30.0%), which was lower than that in singletons (11.7% (range, 4.0–38.9%)). Multivariable regression analysis demonstrated that twin pregnancy, higher maternal weight and conception by in-vitro fertilization provided significant independent prediction of test failure. Follow-up was available in 351 (68.2%) of the twin pregnancies and comprised 334 with euploid fetuses, 12 discordant for trisomy 21 and five discordant for trisomy 18. In all 323 euploid cases with a result, the risk score for each trisomy was < 1:10 000. In 11 of the 12 cases with trisomy 21 and in the five with trisomy 18, the cfDNA test gave a high-risk result, but in one case of trisomy 21, the score was < 1:10 000.

Conclusion In twin pregnancies screening by cfDNA testing is feasible, but the failure rate is higher and detection rate may be lower than in singletons. Copyright © 2014 ISUOG. Published by John Wiley & Sons Ltd.

INTRODUCTION

In singleton pregnancies, cell-free DNA (cfDNA) analysis of maternal blood provides effective screening for trisomies 21, 18 and 13 with respective detection rates of about 99%, 97% and 92%, at a combined false-positive rate (FPR) of 0.4%¹. Such high performance of screening has been reported for both high-risk pregnancies and in the general population^{1–5}.

The incidence of multiple pregnancy has increased worldwide, mainly owing to the increased use of assisted reproductive techniques and the increasing maternal age of the population^{6–8}. Both the risk for aneuploidies and the risk of miscarriage from invasive testing are higher in twin pregnancies than in singletons^{9,10}. It would therefore be useful if cfDNA testing could be used for effective screening in twins. However, in twin pregnancies cfDNA testing is more complex than in singleton pregnancies because in dizygotic twins only one fetus is likely to have aneuploidy when detected, and the contribution of cfDNA of the two fetuses into the maternal circulation can vary by nearly two-fold^{11,12}. Consequently, if the fetal fraction of the affected fetus is below the threshold of 4% necessary for successful cfDNA analysis, but there is a high contribution from the normal cotwin, so that the total fetal fraction is satisfactory, the wrong conclusion can be reached – that the pregnancy is not trisomic. To avoid this potential mistake, it has been proposed that, in cfDNA testing in twin pregnancies, the lower fetal fraction of the two fetuses, rather than the total, should be used in the assessment of risk for aneuploidies¹³. However, an inevitable consequence of such a policy is that the no-result rate in twins is likely to be higher than in singleton pregnancies¹⁴.

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The objective of this study was to report the clinical implementation of cfDNA analysis of maternal blood in screening for trisomies 21, 18 and 13 in a large series of twin pregnancies and examine variables that could influence the failure rate of the test.

SUBJECTS AND METHODS

In this prospective multicenter study, 515 twin pregnancies underwent screening for trisomies 21, 18 and 13 by cfDNA testing at 10–28 weeks' gestation between May 2013 and September 2014. All patients received detailed pretest counseling and provided written informed consent for the test. Maternal blood was obtained by venepuncture (20 mL, in Streck cfDNA BCT™ tubes) and sent via courier to the USA for chromosome-selective cfDNA testing (Harmony™ Prenatal Test, Ariosa Diagnostics, Inc., San Jose, CA, USA)^{13–15}. The information given to the laboratory for each sample was patient-unique identifier, maternal age, method of conception and date of blood collection. Risk scores for each trisomy were provided as a percentage with ranges capped at > 99% and < 0.01%.

The failure rate of the cfDNA test to provide results was compared with that in prospectively collected samples from 1847 singleton pregnancies undergoing cfDNA testing between January 2013 and September 2014, at the department of Obstetrics and Gynecology, University Hospital Brugmann, Brussels, Belgium, at which all data from the participating centers were collated in a unique database. The study was approved by the local ethics committee.

Statistical analysis

In cases with a cfDNA test result, the median of the lower fetal fraction of twin pregnancies was compared with the corresponding median in singleton pregnancies using the Mann–Whitney *U*-test. In the whole population of singleton and twin pregnancies, regression analysis was used to investigate the effect on test failure rate at first sampling of twin pregnancy (yes/no), maternal smoking status (smoker/non-smoker), method of conception (*in-vitro* fertilization (IVF)/non-IVF) and origin of oocyte (mother/donor), as categorical variables and maternal weight (kg) and gestational age at test (weeks), as continuous numerical variables. In twin pregnancies, regression analysis was used to investigate the effect on test failure rate of maternal smoking status, method of conception, origin of oocyte and chorionicity (monochorionic/dichorionic) as categorical variables and maternal weight and gestational age as continuous numerical variables.

Data were analyzed with the statistical software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), and Excel version 9.0 (Microsoft, Redmond, WA, USA). Two-sided $P < 0.05$ was considered to be statistically significant.

RESULTS

Study population

In total, 1847 singleton and 515 twin pregnancies underwent cfDNA testing either because prior screening by the first-trimester combined test or second-trimester triple/quadruple biochemistry test or ultrasound examination identified them as being at high risk for fetal trisomy or the women wanted to have the new test as a primary method of screening. The median maternal age was 36.8 (range, 19.0–50.3) years and the median maternal weight was 64.4 (range, 42.0–148.0) kg. The test was performed at a median gestational age of 13.6 (range, 10.0–34.7) weeks for singletons and 13.0 (range, 10.0–28.0) weeks for twins; the gestational age was < 14 weeks in 1027 (55.6%) of the singletons and in 353 (68.5%) of the twins. Conception was by IVF in 189 (10.2%) of the singletons and in 272 (52.8%) of the twin pregnancies.

Fetal fraction

The median of the lower fetal fraction in twin pregnancies was 8.7% (range, 4.1–30.0%), which was lower than the corresponding median in singleton pregnancies (11.7% (range, 4.0–38.9%)) ($P < 0.001$). In the cases tested before 14 weeks' gestation, the median of the lower fetal fraction in twins was 8.6% (range, 4.1–30.0%) and the median fetal fraction in singletons was 11.3% (range, 4.0–38.9%) ($P < 0.001$).

No result from cfDNA testing

The cfDNA test failed to provide results in the first sample obtained from patients in 32 (1.7%) singletons and in 29 (5.6%) twins (Figure 1). In the cases tested before 14 weeks' gestation, the cfDNA test did not provide results in 22 (2.1%) of the singletons and in 16 (4.5%) of the twins.

Univariable regression analysis demonstrated that significant predictors of failure of the test were twin pregnancy, higher maternal weight and conception by IVF, but not gestational age at test, cigarette smoking or origin of oocyte. Multivariable regression analysis demonstrated that significant independent predictors of test failure were twin pregnancy, higher maternal weight and conception by IVF (Table 1 and Figure 2). In twin pregnancies, univariable regression analysis demonstrated that significant predictors of failure of the test were higher maternal weight and conception by IVF, but not gestational age at test, cigarette smoking, origin of oocyte or chorionicity. Multivariable regression analysis demonstrated that significant independent predictors of test failure were higher maternal weight and conception by IVF (Table 2).

In 26 of the 29 twin pregnancies in which the first sample did not provide a result from cfDNA testing, a second sample was examined and a result was obtained in 13 (50.0%). In singleton pregnancies, the test was repeated in 28 of the 32 cases with a failed first sample and a result was obtained in 19 (67.9%).

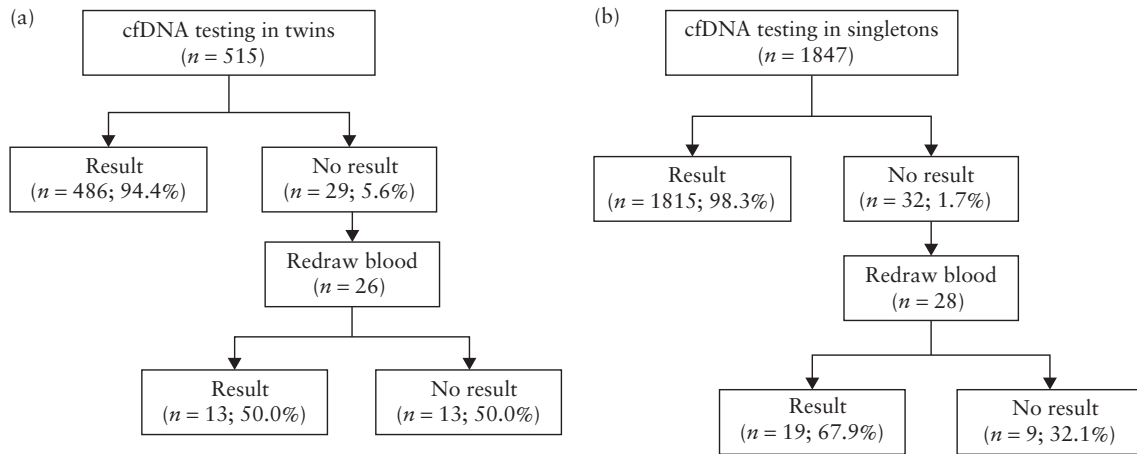


Figure 1 Flowcharts of prospective screening for trisomies 21, 18 and 13 by cell-free DNA (cfDNA) testing of maternal blood for aneuploidies in twin (a) and singleton (b) pregnancies.

Table 1 Regression analysis in prediction of effect of different variables on failure of cell-free DNA testing for aneuploidies in twin and singleton pregnancy (n = 2362)

Variable	Value	Univariable analysis		Multivariable analysis	
		Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Twin pregnancy					
Yes	515 (21.8)	3.384 (2.028–5.650)	< 0.001	2.331 (1.245–4.362)	0.008
No	1847 (78.2)	1		1	
Maternal smoker*					
Yes	91 (3.9)	1.362 (0.417–4.448)	0.609		
No	2130 (90.2)	1			
Maternal weight (kg)†	64.4 (42.0–148.0)	1.049 (1.034–1.065)	< 0.001	1.057 (1.040–1.075)	< 0.001
Gestational age at test (weeks)	13.6 (10.0–34.7)	0.954 (0.876–1.041)	0.284		
Method of conception					
IVF	461 (19.5)	4.496 (2.692–7.508)	< 0.001	3.781 (2.025–7.057)	< 0.001
Non-IVF	1901 (80.5)	1		1	
Origin of oocyte					
Mother	2303 (97.5)	1			
Donor	59 (2.5)	2.074 (0.631–6.819)	0.230		

Data given as n (%) or median (range). *Data available for 2221 (94.0%) patients. †Data available for 2296 (97.2%) patients. IVF, *in-vitro* fertilization.

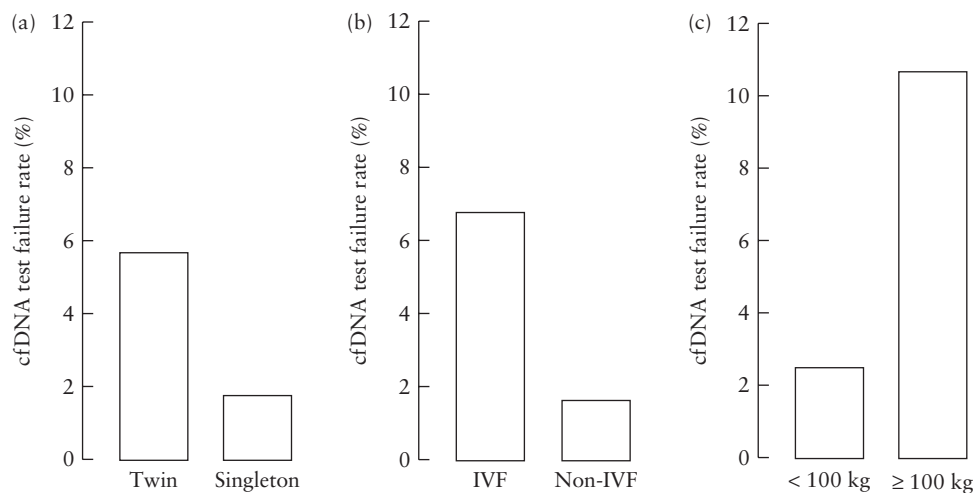


Figure 2 Relationship of maternal and pregnancy characteristics (pregnancy status (a), mode of conception (b) and maternal weight (c)) with failure of cell-free DNA (cfDNA) testing of maternal blood for aneuploidies. IVF, *in-vitro* fertilization.

Table 2 Regression analysis in prediction of effect of different variables on failure rate of cell-free DNA testing for aneuploidies in twin pregnancy ($n = 515$)

Variable	Value	Univariable analysis		Multivariable analysis	
		Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
Maternal smoker*					
Yes	19 (3.7)	1.871 (0.405–8.642)	0.422		
No	355 (68.9)	1			
Maternal weight (kg)†	64.0 (43.9–117.0)	1.058 (1.028–1.090)	< 0.001	1.064 (1.031–1.097)	< 0.001
Gestational age at test (weeks)	13.0 (10.0–28.0)	0.918 (0.886–1.143)	0.918		
Method of conception					
IVF	272 (52.8)	2.967 (1.244–7.074)	< 0.014	3.557 (1.372–9.217)	< 0.01
Non-IVF	243 (47.2)	1		1	
Origin of oocyte					
Mother	467 (90.7)	1			
Donor	48 (9.3)	0.709 (0.163–3.076)	0.646		
Chorionicity‡					
Monochorionic	67 (13.0)	1			
Dichorionic	301 (58.4)	2.313 (0.527–10.145)	0.266		

Data given as n (%) or median (range). *Data available for 374 (72.6%) patients. †Data available for 449 (87.2%) patients. ‡Data available for 368 (71.5%) patients. IVF, *in-vitro* fertilization.

Pregnancy outcome in twins and performance of the cfDNA test

Outcomes were divided into (1) normal if the karyotype of chorionic villi, amniotic fluid or neonatal blood was normal or the neonate was phenotypically normal ($n = 334$); (2) trisomies 21, 18 or 13 if the karyotype of chorionic villi, amniotic fluid or neonatal blood demonstrated the relevant trisomy (12 cases discordant for trisomy 21 and five discordant for trisomy 18); (3) unknown karyotype because the pregnancy resulted in miscarriage or stillbirth and no karyotyping of fetal tissue was carried out ($n = 7$); and (4) outcome unknown because the pregnancies were continuing ($n = 19$) or were lost to follow-up ($n = 138$).

cfDNA testing provided risk scores after first or second sampling in 340 (96.9%) of the 351 twin pregnancies with known outcome. In the 323 euploid cases in which a cfDNA test result was obtained, the risk score for each trisomy was < 1:10 000. In 11 of the 12 cases with trisomy 21 and in the five with trisomy 18, the cfDNA test gave a high-risk result, but in one case of trisomy 21 the score was < 1:10 000.

DISCUSSION

Main findings of the study

This prospective 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. In twins, the rate of reporting results from cfDNA testing was 94.4%, which improved to 96.9% after repeat sampling, and in those with results the median of the lower fetal fraction was 8.7%. These rates were lower than the respective values of 98.3% and 99.3% observed in singleton pregnancies, in which the median fetal fraction was 11.7%. The reason for the

lower reporting rate in twins was low fetal fraction, which is the inevitable consequence of selecting the lower fetal fraction of the two fetuses, rather than the total fetal fraction, in estimating the risk for aneuploidies¹. The rationale for this choice is to avoid a false-negative result in a dizygotic twin pregnancy discordant for aneuploidy, for which the total fetal fraction is satisfactory but the contribution of the affected fetus could be less than 4%.

In twins, the cfDNA test did not provide any false-positive results and it detected 16 of the 17 pregnancies discordant for trisomy 21 or 18. However, many of the pregnancies were continuing or were lost to follow-up and therefore the study cannot provide accurate assessment of detection rate.

Significant predictors of failure of the cfDNA test to provide results are high maternal weight and IVF conception, which was by far more common in the twin than in the singleton pregnancies (53% *vs* 10%). Although the failure rate was twice as high in dichorionic than in monozygotic twins, this difference was not significant. The likely source of fetal cfDNA in maternal plasma is dying cells in the placenta, and the inverse association between fetal fraction and maternal weight could be attributed to a dilutional effect^{16–18}. Another possible explanation for such an association is that, in obese women, there is an accelerated turnover of adipocytes, which releases an increased amount of cfDNA of maternal origin into the circulation, hence relatively less fetal cfDNA^{19,20}.

The finding of lower fetal fraction in IVF conceptions may be the consequence of a smaller placental mass and lower number of apoptotic cells than in natural conceptions. We have previously reported significant associations between fetal fraction and serum pregnancy-associated plasma protein-A (PAPP-A) and free β -human chorionic gonadotropin, whose concentration provides an indirect measure of placental mass^{17,18,20}. Evidence for impaired placentation in IVF conceptions is provided by reports

that in such pregnancies the serum concentration of PAPP-A is decreased by 10–25%^{21–23} and the incidence of pre-eclampsia is increased²⁴.

Limitations of the study

The main limitations of the study relate to the small number of trisomic pregnancies and the lack of data on pregnancy outcome in about one third of the cases, which prevents definitive conclusions from being drawn as to the performance of screening by cfDNA testing through chromosome-selective sequencing in twins.

Comparison with previous studies

There are four previous studies on screening for trisomies by cfDNA testing in twin pregnancies. In three studies, massively parallel shotgun sequencing was used to examine stored plasma or prospectively collected blood from a combined total of 226 twin pregnancies^{25–27}. In these studies, no attempt was made to determine the fetal fraction for each twin and it was assumed that the contribution from each fetus to the maternal plasma cfDNA was adequate for accurate results. There were 206 cases with euploid fetuses, 15 discordant and two concordant for trisomy 21, two discordant for trisomy 18 and one discordant for trisomy 13. The cfDNA test provided results for all cases and correctly classified all cases except one of the cases of trisomy 18, which was reported as normal. Therefore, the detection rate for all trisomies was 95.0% (19 of 20), with an FPR of 0%.

One previous study used chromosome-selective sequencing and an algorithm that relied on the lower fetal fraction of the twins, as in the present study, to assess the risk for trisomies in stored plasma samples obtained at 11–13 weeks' gestation from 207 twin pregnancies¹⁴. Risk scores for trisomies 21, 18 and 13 by cfDNA testing were provided for 192 (92.8%) cases, including 181 with euploid fetuses, 10 discordant for trisomy 21 and one discordant for trisomy 13. The test correctly identified nine of the 10 cases of trisomy 21 and the one case of trisomy 13 with no false-positive results¹⁴.

In the cumulative data from the literature and the present study on cfDNA testing, in a total of 758 twin pregnancies with known outcome, comprising 710 with euploid fetuses, 39 with trisomy 21, seven with trisomy 18 and two with trisomy 13, the respective detection rates for the trisomies were 95%, 86% and 100%, at an FPR of 0%.

Conclusions

There are sufficient data to suggest that with cfDNA testing for trisomies in twin pregnancies, firstly, the FPR is very low, as in singletons, secondly, the detection rate is high, albeit the total number of affected cases is too small for accurate conclusions to be drawn, and thirdly, the method of estimating the fetal fraction from each twin and ensuring that the lower of the two is at least 4%, aiming

to minimize the risk of providing false-negative results, is associated with a higher failure rate than methods that ignore assessment of the contribution of each fetus to the maternal cfDNA concentration.

In twin pregnancies, the rate of failure to obtain results from cfDNA testing increases with maternal weight, as in singleton pregnancies, and is higher in IVF than in natural conceptions.

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REFERENCES

1. Gil MM, Akolekar R, Quezada MS, Bregant B, Nicolaides KH. Analysis of cell-free DNA in maternal blood in screening for aneuploidies: meta-analysis. *Fetal Diagn Ther* 2014; 35: 156–173.
2. Nicolaides KH, Syngelaki A, Ashoor G, Birdir C, Touzet G. Noninvasive prenatal testing for fetal trisomies in a routinely screened first-trimester population. *Am J Obstet Gynecol* 2012; 207: 374.e1–6.
3. Song Y, Liu C, Qi H, Zhang Y, Bian X, Liu J. Noninvasive prenatal testing of fetal aneuploidies by massively parallel sequencing in a prospective Chinese population. *Prenat Diagn* 2013; 33: 700–706.
4. Bianchi DW, Parker RL, Wentworth J, Madankumar R, Saffer C, Das AF, Craig JA, Chudova DI, Devers PL, Jones KW, Oliver K, Rava RP, Sehnert AJ; CARE Study Group. DNA sequencing versus standard prenatal aneuploidy screening. *N Engl J Med* 2014; 370: 799–808.
5. Quezada MS, Gil MM, Francisco C, Oròsz G, Nicolaides KH. 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. *Ultrasound Obstet Gynecol* 2015; 45: 36–41.
6. Reynolds MA, Schieve LA, Martin JA, Jeng G, Macaluso M. Trends in multiple births conceived using assisted reproductive technology, United States, 1997–2000. *Pediatrics* 2003; 111: 1159–1162.
7. Luke B, Martin JA. The rise in multiple births in the United States: who, what, when, where, and why. *Clin Obstet Gynecol* 2004; 47: 118–133.
8. Khoshnood B, Blondel B. Regional variations in trends for multiple births: a population-based evaluation in France, 1972–2003. *Twin Res Hum Genet* 2007; 10: 406–415.
9. Hansen M, Kurinczuk JJ, Milne E, de Klerk N, Bower C. Assisted reproductive technology and birth defects: a systematic review and meta-analysis. *Hum Reprod Update* 2013; 19: 330–353.
10. Royal College of Obstetricians and Gynaecologists (RCOG). *Amniocentesis and Chorionic Villus Sampling*. Green-Top Guideline No. 8. RCOG Press: London, 2010.
11. Qu JZ, Leung TY, Jiang P, Liao GJ, Cheng YK, Sun H, Chiu RW, Chan KC, Lo YM. Noninvasive prenatal determination of twin zygosity by maternal plasma DNA analysis. *Clin Chem* 2013; 59: 427–435.
12. Leung TY, Qu JZ, Liao GJ, Jiang P, Cheng YK, Chan KC, Chiu RW, Lo YM. Noninvasive twin zygosity assessment and aneuploidy detection by maternal plasma DNA sequencing. *Prenat Diagn* 2013; 33: 675–681.
13. Struble CA, Syngelaki A, Oliphant A, Song K, Nicolaides KH. Fetal fraction estimate in twin pregnancies using directed cell-free DNA analysis. *Fetal Diagn Ther* 2014; 35: 199–203.

14. Gil MM, Quezada MS, Bregant B, Syngelaki A, Nicolaides KH. Cell-free DNA analysis for trisomy risk assessment in first-trimester twin pregnancies. *Fetal Diagn Ther* 2014; **35**: 204–211.
15. Sparks AB, Struble CA, Wang ET, Song K, Oliphant A. Noninvasive prenatal detection and selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21 and trisomy 18. *Am J Obstet Gynecol* 2012; **206**: 319.e1–9.
16. Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, van den Boom D, Bombard AT, Deciu C, Grody WW, Nelson SF, Canick JA. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. *Genet Med* 2011; **13**: 913–920.
17. Ashoor G, Poon L, Syngelaki A, Mosimann B, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: effect of maternal and fetal factors. *Fetal Diagn Ther* 2012; **31**: 237–243.
18. Ashoor G, Syngelaki A, Poon LC, Rezende JC, Nicolaides KH. Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound Obstet Gynecol* 2013; **41**: 26–32.
19. Haghiac M, Vora NL, Basu S, Johnson KL, Presley L, Bianchi DW, Hauguel-de Mouzon S. Increased death of adipose cells, a path to release cell-free DNA into systemic circulation of obese women. *Obesity (Silver Spring)* 2012; **20**: 2213–2219.
20. Poon LC, Musci T, Song K, Syngelaki A, Nicolaides KH. Maternal plasma cell-free fetal and maternal DNA at 11–13 weeks' gestation: relation to fetal and maternal characteristics and pregnancy outcomes. *Fetal Diagn Ther* 2013; **33**: 215–223.
21. Kagan KO, Wright D, Spencer K, Molina FS, Nicolaides KH. First-trimester screening for trisomy 21 by free beta-human chorionic gonadotropin and pregnancy-associated plasma protein-A: impact of maternal and pregnancy characteristics. *Ultrasound Obstet Gynecol* 2008; **31**: 493–502.
22. Amor DJ, Xu JX, Halliday JL, Francis I, Healy DL, Breheny S, Baker HW, Jaques AM. Pregnancies conceived using assisted reproductive technologies (ART) have low levels of pregnancy-associated plasma protein-A (PAPP-A) leading to a high rate of false-positive results in first trimester screening for Down syndrome. *Hum Reprod* 2009; **24**: 1330–1338.
23. Engels MA, Pajkrt E, Groot DT, Schats R, Twisk JW, van Vugt JM. Validation of correction factors for serum markers for first-trimester Down syndrome screening in singleton pregnancies conceived with assisted reproduction. *Fetal Diagn Ther* 2013; **34**: 217–224.
24. Chaveeva P, Carbone IF, Syngelaki A, Akolekar R, Nicolaides KH. Contribution of method of conception on pregnancy outcome after the 11–13 weeks scan. *Fetal Diagn Ther* 2011; **30**: 9–22.
25. Canick JA, Kloza EM, Lambert-Messerlian GM, Haddow JE, Ehrich M, van den Boom D, Bombard AT, Deciu C, Palomaki GE. DNA sequencing of maternal plasma to identify Down syndrome and other trisomies in multiple gestations. *Prenat Diagn* 2012; **32**: 730–734.
26. Lau TK, Jiang F, Chan MK, Zhang H, Lo PS, Wang W. Non-invasive prenatal screening of fetal Down syndrome by maternal plasma DNA sequencing in twin pregnancies. *J Matern Fetal Neonatal Med* 2013; **26**: 434–437.
27. Huang X, Zheng J, Chen M, Zhao Y, Zhang C, Liu L, Xie W, Shi S, Wei Y, Lei D, Xu C, Wu Q, Guo X, Shi X, Zhou Y, Liu Q, Gao Y, Jiang F, Zhang H, Su F, Ge H, Li X, Pan X, Chen S, Chen F, Fang Q, Jiang H, Lau TK, Wang W. Noninvasive prenatal testing of trisomies 21 and 18 by massively parallel sequencing of maternal plasma DNA in twin pregnancies. *Prenat Diagn* 2014; **34**: 335–340.