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A unified approach to risk assessment for fetal aneuploidies

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KEYWORDS: Bayes' theorem; cell-free DNA; fetal fraction; first-trimester screening; nuchal translucency; serum free β-hCG; serum PAPP-A; trisomy 13; trisomy 18; trisomy 21

ABSTRACT

Objective *To examine the potential impact of combining measures from cell-free DNA (cfDNA) testing with maternal age and first-trimester biomarkers in screening for fetal trisomies.*

Methods *This was a theoretical study using Bayes' theorem to combine the* a priori *risk for fetal trisomy 21 derived from maternal age with likelihoods from nuchal translucency thickness, serum pregnancy-associated plasma protein-A, serum free β-human chorionic gonadotropin and plasma cfDNA. We adopted a binomial counting model for the cfDNA likelihoods and developed a model to account for errors in estimating fetal fraction.*

Results *When Bayes' theorem was used to combine the* a priori *risk for trisomy 21 derived from the first-trimester combined test with likelihoods from the cfDNA test, and when the true fetal fraction was known, the detection rate increased from 62% at a fetal fraction of 4% to 100% at a fetal fraction of ≥ 9%; the positive likelihood ratio (trisomic/euploid) increased from 620 to 1000 and the negative likelihood ratio (euploid/trisomic) increased from 3 to > 10 000. When the fetal fraction is < 4%, the cfDNA test has traditionally been considered to be a failure, but the cfDNA results can be used to improve the performance of screening by the combined test.*

Conclusions *In contingent policies that use the first-trimester combined test for first-line screening to select the subgroup for cfDNA testing, the data from the latter should be used to update the risk from the former. Individual patient results from cfDNA testing depend crucially on the fetal fraction and the precision of its measurement. Copyright © 2014 ISUOG. Published by John Wiley & Sons Ltd.*

INTRODUCTION

First-trimester combined-test screening for trisomies 21, 18 and 13 by a combination of maternal age, fetal nuchal translucency thickness (NT), serum free β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) can detect around 90% of affected pregnancies at a false-positive rate (FPR) of 5%, and this method is now well established[1,2]. In this test, estimates of individual patient risk are derived through the application of Bayes' theorem to modify the maternal age-specific prior risk of each trisomy with the likelihoods for NT, PAPP-A and free β-hCG levels.

Recent evidence suggests that analysis of cell-free DNA (cfDNA) in maternal blood can detect about 99% of cases of trisomy 21, 97% of trisomy 18 and 92% of trisomy 13, with respective FPRs of about 0.1%, 0.2% and 0.2%[3]. In most cases, the results from cfDNA testing are reported as positive/negative or as a risk > 99%/<1:10 000, and they do not take into account the prior risk from maternal age or other methods of screening. A notable exception is the Forte algorithm, which uses a risk-based approach incorporating fetal fraction and maternal age[4]. The basis for cfDNA testing using counting methods is that, in trisomic pregnancies, the number of molecules derived from the extra fetal chromosome, as a proportion of all sequenced molecules in maternal plasma, is higher than in euploid pregnancies. 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 the maternal plasma (fetal fraction)[4–7]. When the fetal fraction is < 4%, which occurs in 1–3% of pregnancies, the cfDNA test is usually presented as a failure and no result is reported[4,5].

The primary objective of this study was to examine the potential impact of combining results of the cfDNA test with maternal age and first-trimester biomarkers in screening for trisomies, in terms of providing accurate patient-specific risks and improving the overall performance of screening. A secondary objective was to demonstrate the effect of errors in estimated fetal fractions and the implications of this on the assessment of cfDNA *Z*-scores.

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METHODS

In cfDNA testing, the counts of specific cfDNA fragments arising from a chromosome under investigation are compared in euploid and trisomic pregnancies and the results are presented as *Z*-scores. The separation between the distributions of *Z*-scores from euploid and trisomic pregnancies increases with deeper levels of sequencing and an increasing fetal fraction[4–7]. However, there are limited data on the depth of sequencing and the precision of estimated fetal fractions. Moreover, the limited published evidence shows substantial differences in the distribution of *Z*-scores in trisomic fetuses from different providers[4,5].

Our analysis is based on the binomial counting model applied by Benn and Cuckle[7] to define the distribution of *Z*-scores, assuming that fetal fractions are known without error. We configured this model so that, for the distribution of fetal fractions in our data, an overall detection rate (DR) of 99% and a FPR of 0.1% were achieved among pregnancies with a successful cfDNA test and with a fetal fraction of at least 4%. The choice of a FPR of 0.1% and a DR of 99% was based on the results of a meta-analysis of clinical validation and implementation studies on cfDNA testing for fetal trisomies[3]. The distribution of fetal fraction was obtained from our series of 7749 pregnancies undergoing routine cfDNA testing at 10–13 weeks' gestation (Harmony™ Prenatal Test, Ariosa Diagnostics, Inc., San Jose, CA, USA). Assuming 1.67% for the proportion of fragments from the targeted chromosome in a euploid pregnancy[7], the depth of sequencing should provide counts from 1.87 million DNA fragments. For a targeted approach, in which the proportion is 50%, this is reduced to 30 000 fragments.

The performance of cfDNA testing in screening for trisomy 21 was determined using likelihoods derived from the distribution of *Z*-scores in trisomic and euploid pregnancies. We based our modeling on the distributions appropriate for trisomy 21, but the principal findings apply to other trisomies. Bayes' theorem was used to combine the *a priori* risk for fetal trisomies derived from maternal age with likelihoods from NT, PAPP-A and free β-hCG levels and cfDNA testing. Likelihoods for unaffected and trisomy-21 pregnancies were computed under the assumption of conditional independence, given outcome, between fetal NT, biochemical markers and cfDNA. Distributional characteristics of biomarker values in trisomy 21 and unaffected pregnancies were taken from the literature[8,9]. The binomial counting model described by Benn and Cuckle[7] was applied to obtain the likelihoods for cfDNA for a given *Z*-score and fetal fraction. We compared simulated data from the binomial counting model with published evidence from the literature[4,5] and extended the model to include errors in measured fetal fractions that provide a plausible mechanism for departures from the simple binomial counting model.

The performance of screening using risks depends on maternal age and gestational age as well as the value of the known or estimated fetal fraction. We assumed the maternal age distribution of pregnancies in England and Wales in 2011[10]. Computation of DRs and FPRs was as follows: for a given risk cut-off, age-specific DRs and FPRs were determined for each year of maternal age, from 12 to 50 years. The overall DR and FPR were computed by taking the weighted average of the age-specific rates according to the maternal age distribution of trisomic and euploid pregnancies in England and Wales in 2011 at 12.5 weeks' gestation[10,11]. Results are presented separately for cases in which fetal fraction is assumed to be known and where it is estimated.

The purpose of this paper was to provide a conceptual explanation of our work. Full technical details are available from the authors. The statistical software package R (R Foundation for Statistical Computing, Vienna, Austria) was used for data analysis[12].

RESULTS

Distribution of *Z*-scores in euploid and trisomic pregnancies

In euploid pregnancies, the distribution of *Z*-scores is Gaussian, with a mean and SD of 0 and 1, respectively. In trisomic pregnancies, the mean of the *Z*-score is greater than 0 and increases with increasing fetal fraction[5]. This paper presents theoretical results obtained from statistical modeling of the *Z*-score distributions in euploid and trisomic pregnancies. For a known fetal fraction, the distribution of the *Z*-score in trisomic pregnancies can be derived from the binomial distribution. For a fixed number of reads, this distribution has a mean proportion to the fetal fraction and an SD that can be determined from the proportions of counts expected in a euploid fetus, and the fetal fraction. In general, this SD is approximately 1.0.

Simulated *Z*-scores from the binomial model with a sample of 100 trisomic and 1000 euploid pregnancies are shown in Figure 1a. The distributions of *Z*-scores are consistent with the published results of Sparks *et al.*[4]. However, they are inconsistent with the larger series of 212 trisomic pregnancies in the publication of Palomaki *et al.*[5], which exhibits a curvilinear relationship with fetal fraction, a positively-skewed distribution and a greater degree of spread that increases with fetal fraction.

Effect of errors in estimating fetal fraction on the distribution of *Z*-scores

A plausible explanation for the abovementioned departure from the theoretical model is that the fetal fractions in the published series are subject to estimation errors[5]. Noting that the individual *Z*-scores plotted against true fetal fraction in Figure 1a are the same as those plotted against estimated fetal fraction in Figure 1b, estimation errors in fetal fraction cause horizontal shifts to the left or right, depending on whether the error is negative or positive. Consequently, the relationship between *Z*-score and estimated fetal fraction is altered, and the scatter diagram changes from that shown in Figure 1a to that in Figure 1b. This 'errors-in-variables' model has the

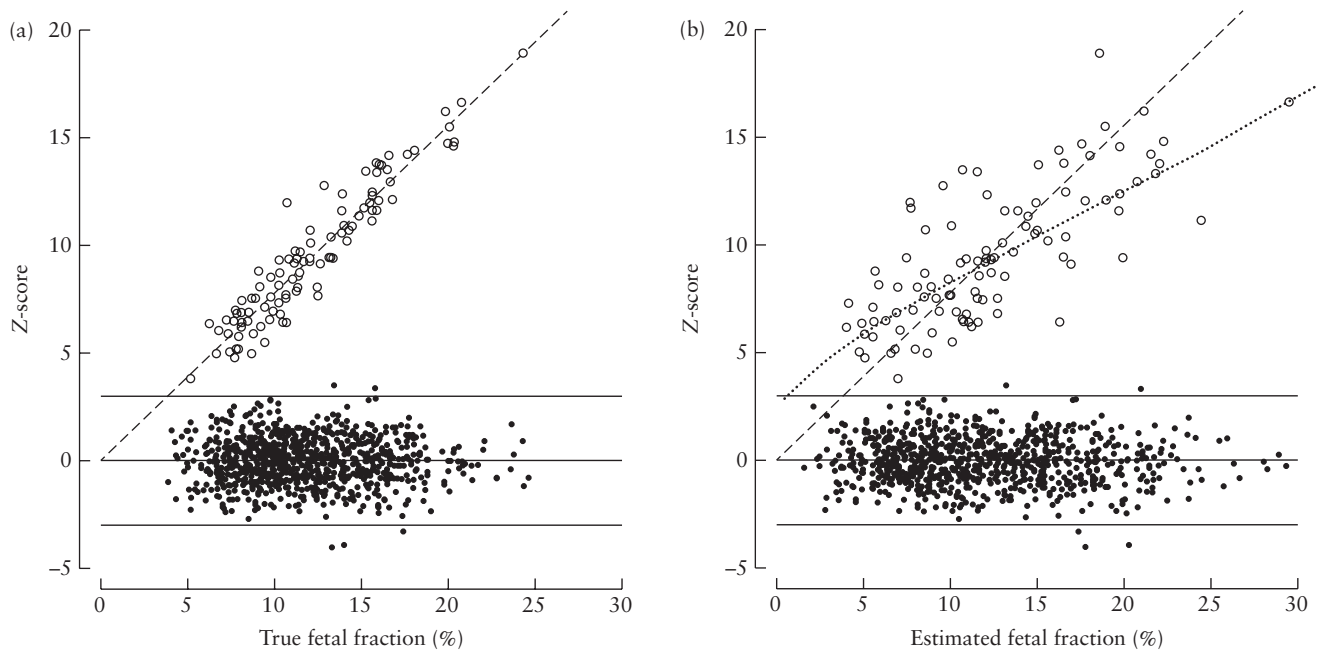

Figure 1 Simulated *Z*-scores from euploid (●) and trisomy-21 (○) pregnancies with the assumption that the true fetal fraction is known (a) or estimated (b). Horizontal lines represent mean ± 3SD of *Z*-scores in euploid pregnancies. Dashed regression lines demonstrate the relationship between *Z*-scores and true fetal fraction in trisomy-21 pregnancies. Curvilinear dotted line in (b) is regression line for *Z*-scores with estimated fetal fraction.

characteristics of a curvilinear relationship with fetal fraction, skewness and increasing spread with estimated fetal fraction exhibited in the data of Palomaki *et al.*[5]. With regard to Figure 1b, it is notable that, at estimated fetal fractions close to zero, there is more separation than there is for the true fetal fractions. This occurs because the lower estimated fetal fractions tend to arise from higher true fetal fractions with negative errors. At the higher estimated fetal fractions the opposite occurs and there is less separation because the estimated fetal fractions tend to arise from lower true fetal fractions with positive errors. This behavior is a form of regression dilution[13].

We used a beta-distribution for the unknown true values of the fetal fraction and assumed that the estimates are composed of the true value of the fetal fraction plus a random error. The error distribution assumed that, for a given true value, estimates arise from beta-distribution centered on the true value. The choice of beta-distribution in this 'errors-in-variables' model ensures that values of fetal fraction lie between 0 and 1. The fitted distributions of the true and estimated values, together with our data, are shown in Figure 2. In our data, estimated fetal fractions below 4% were not provided and are excluded from the histogram. For fetal fractions of ≥ 4%, the fitted distribution of estimates (solid-line curve) is a good fit to the histogram. The underlying distribution of true values (dashed-line curve) exhibits less spread than does the distribution of estimates; 3.0% of the distribution of estimates fall below 4%, compared to 0.4% of true fetal fractions. This reflects the fact that the lower estimated fetal fractions tend to arise from larger true values with negative errors of estimation.

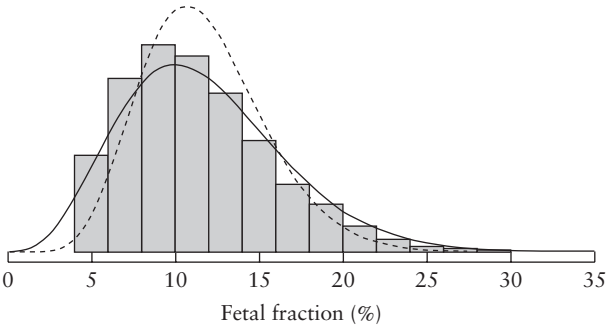

Figure 2 Histogram presenting distribution of fetal fraction in 7749 singleton pregnancies undergoing cell-free DNA testing at 10–13 weeks' gestation. Solid curve shows distribution of estimated fetal fractions and dashed curve shows distribution of true fetal fractions from our model.

Likelihood ratios

The Gaussian distributions of *Z*-scores for euploid and trisomic pregnancies and corresponding likelihood ratios for true fetal fractions of 2%, 4% and 8% are shown in Figure 3. With a fetal fraction of 2%, there is considerable overlap between the distributions, and the likelihood ratio is relatively flat. With a fetal fraction of 8%, there is virtually no overlap and the likelihood ratio is extremely steep, so that in the vast majority of pregnancies, cfDNA would completely dominate information from other markers and maternal age. With a fetal fraction of 4%, there is some degree of overlap, and in the majority of pregnancies risk assessment could be usefully informed by data from maternal age and other biomarkers. The distributions of *Z*-scores and likelihood ratios obtained from the 'errors-in-fetal-fraction' model are shown in Figure 4.

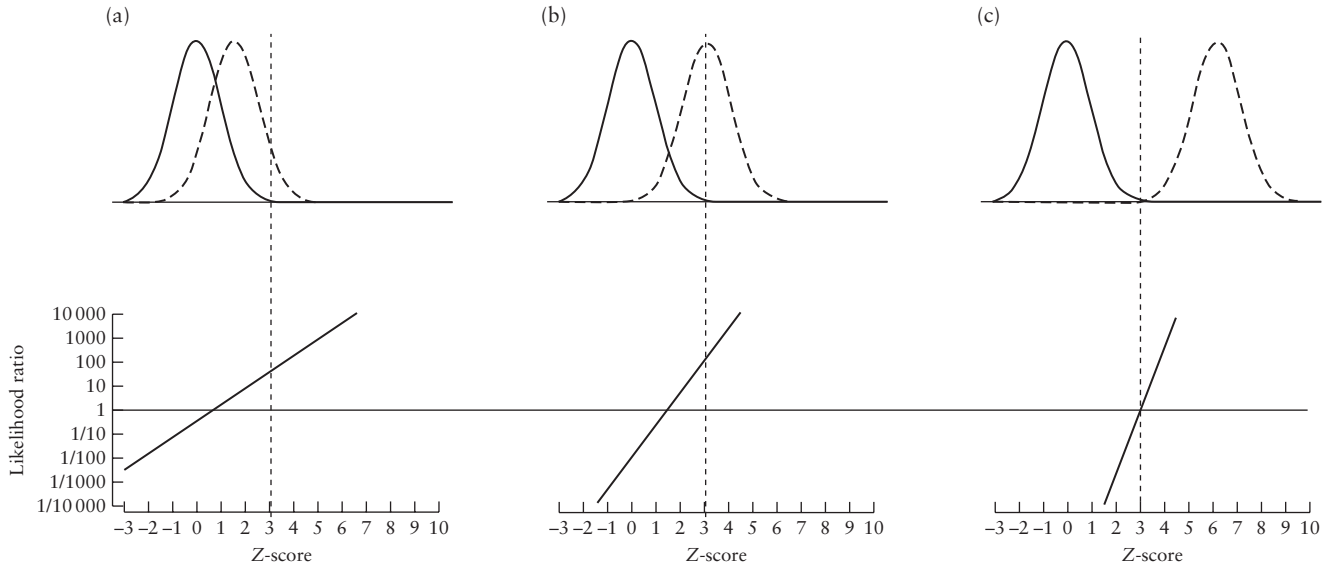

Figure 3 Gaussian distributions of *Z*-scores for euploid (——) and trisomic (– – –) pregnancies and corresponding likelihood ratios for true fetal fractions of 2% (a), 4% (b) and 8% (c). Dashed vertical line represents a *Z*-score of 3.

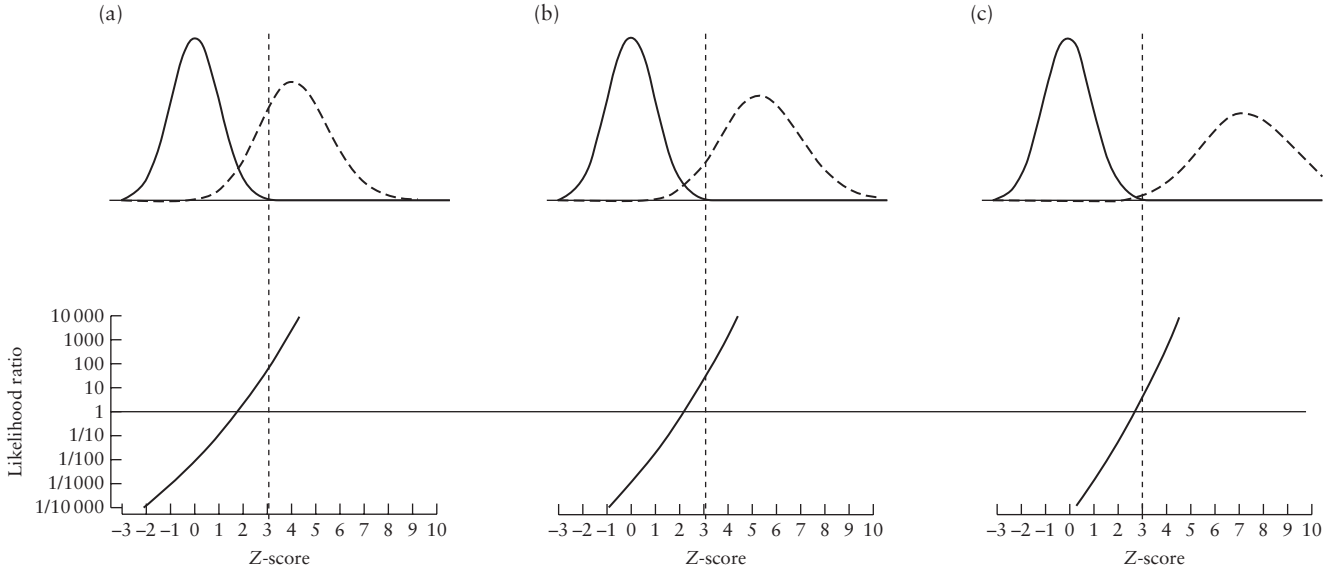

Figure 4 Gaussian distributions of *Z*-scores for euploid (——) and trisomic (– – –) pregnancies and corresponding likelihood ratios for estimated fetal fractions of 2% (a), 4% (b) and 8% (c). Dashed vertical line represents a *Z*-score of 3.

For trisomic pregnancies, there is increased spread and skewness similar to the published data of Palomaki *et al.*[5]. It is notable that there is more separation and a steeper likelihood ratio for the estimated fetal fraction of 2% than for the true fetal fraction of 2%. This occurs because the lower estimated fetal fractions generally arise from higher true fetal fractions. At higher values there is more separation and a steeper likelihood ratio for the true fetal fractions than for the estimated fetal fractions. A noteworthy feature of both Figures 3 and 4 is that there are values of the *Z*-score of < 3 that have likelihood ratios greater than 1 that would result in an increase in any prior-risk estimate. This can be contrasted with the approach of applying a *Z*-score cut-off of 3 and giving a negative result. This occurs at true fetal fractions of 2% and 4% and at estimated fetal fractions of 2%, 4% and 8%.

Screening with known fetal fractions using a *Z*-score cut-off

The theoretical screening performance based on cfDNA testing for the model with known fetal fractions, using a *Z*-score cut-off of 3.09 to achieve a FPR of 0.1%, is given in Table 1. The DR increased from 62.1% at a fetal fraction of 4% to 100% at a fetal fraction of $\geq 9\%$. The positive likelihood ratio (trisomic/euploid) increased from 620 to 1000 and the negative likelihood ratio (euploid/trisomic) increased from 3 to $> 10\,000$. Consequently, if the results of the cfDNA test are presented as positive or negative depending on the *Z*-score, the clinical interpretation depends crucially on the fetal fraction. When the cfDNA test suggests that the fetus is unaffected, the *a priori* risk from maternal age or

Table 1 Theoretical screening performance for trisomy 21 by cell-free DNA (cfDNA) testing with positive and negative likelihood ratios according to known fetal fraction (FF)

True FF (%)	Detection rate (%)	Likelihood ratio	
		Positive	Negative
4	62.1	620	3
5	87.4	870	8
6	97.6	980	42
7	99.8	990	410
8	100.0	1000	7350
≥ 9	100.0	1000	> 10 000
All	99.0	991	99.9

Z-score cut-off was determined to achieve a false-positive rate of 0.1%. Results apply to maternal-age distribution of pregnancies in England and Wales in 2011[10] and are conditional on a successful cfDNA test with a true FF of ≥ 4%.

the first-trimester combined test would be reduced by a factor of 3 if the fetal fraction is 4% and by > 10 000 if the fetal fraction is ≥ 9%. When the cfDNA test suggests trisomy, the *a priori* risk is increased by a factor of 620 if the fetal fraction is 4% and by 1000 if the fetal fraction is ≥ 9%.

Screening with known fetal fractions using a risk cut-off

The theoretical performance of screening by the cfDNA test together with maternal age or the first-trimester combined test, using a risk cut-off at the time of screening of 1 in 100, is given in Table 2. In a population with the maternal-age distribution of pregnancies in England and Wales in 2011[10], the DR and FPR in screening by maternal age alone are 37.2% and 6.0%, respectively, and in screening by the first-trimester combined test alone the DR is 85.9% and the FPR is 2.6%. The results suggest that with fetal fractions of 0.0–3.9, when the cfDNA test is traditionally considered to be a failure, the data from the test can actually be used to improve the performance of screening by maternal age alone or the combined test alone. Screening by the first-trimester combined test and the cfDNA test gave an overall DR and FPR of 99.9 and 0.02%, respectively.

Screening with estimated fetal fractions

Tables 3 and 4 present results according to estimated fetal fraction in a situation in which the true fetal fraction is unknown. Performance at lower estimated fetal fractions is better than the corresponding performance at the true fetal fractions (Tables 1 and 2). As mentioned previously, this is a reflection of the fact that lower estimated fetal fractions tend to arise from higher true fetal fractions. At higher fetal fractions, the situation is reversed and screening performance based on estimated fetal fractions is poorer than it is with the corresponding true fetal fractions. Screening by the first-trimester combined test and the cfDNA test gave an overall DR and FPR for the whole population of 99.8% and 0.05%, respectively, while for the subgroup with a fetal fraction of ≥ 4%, the overall DR and FPR were 99.8% and 0.04%, respectively.

DISCUSSION

Principal findings of the study

The findings of this study demonstrate that in screening for fetal trisomies by cfDNA analysis of maternal blood, the DR at a given FPR and the positive and negative likelihood ratios depend on the fetal fraction.

Different laboratories use quite different methods for the estimation of fetal fraction; some use data generated from the sequencing, some use a separate assay based on epigenetic differences between maternal and fetal DNA, and some rely on single-nucleotide polymorphic differences. Consequently, the 'error in estimating fetal fraction' could actually be attributable to differences in

Table 2 Theoretical screening performance of cell-free DNA testing together with maternal age and first-trimester combined test, for a risk cut-off of 1 in 100 at time of screening, according to when fetal fraction (FF) is known

True FF (%)	Frequency (%)	False-positive rate (%)		Detection rate (%)	
		Maternal age	Combined test	Maternal age	Combined test
0.0–0.9	0.00	6.0	2.5	42.4	86.8
1.0–1.9	0.00	6.2	2.2	59.2	89.6
2.0–2.9	0.05	4.8	1.6	75.4	93.0
3.0–3.9	0.32	3.1	1.0	86.9	95.9
4.0–4.9	1.08	1.5	0.58	93.8	98.0
5.0–5.9	2.55	0.7	0.26	97.4	99.1
6.0–6.9	4.62	0.26	0.10	99.1	99.7
7.0–7.9	6.94	0.07	0.03	99.7	99.9
8.0–8.9	9.02	0.02	0.01	99.9	100.0
9.0–9.9	10.44	0.01	0.00	100.0	100.0
≥ 10.0	64.98	0.00	0.00	100.0	100.0
All	100.0	0.07	0.02	99.7	99.9
FF ≥ 4%	99.63	0.07	0.02	99.7	99.9

Results apply to maternal age distribution of pregnancies in England and Wales in 2011[10].

Table 3 Theoretical screening performance for trisomy 21 by cell-free DNA (cfDNA) testing with positive and negative likelihood ratios according to estimated fetal fraction (FF)

Estimated FF (%)	*Detection rate (%)*	*Likelihood ratio*	
		Positive	*Negative*
4	95.7	957	23
5	97.6	976	42
6	98.7	987	74
7	99.2	992	132
8	99.6	996	234
9	99.8	998	415
10	99.9	999	738
11	99.9	999	1313
12	100.0	1000	2342
13	100.0	1000	4186
14	100.0	1000	7500
15	100.0	1000	13 480
16	100.0	1000	> 10 000
All	99.0	990	100

Z-score cut-off was determined to achieve a false-positive rate of 0.1%. Results apply to maternal-age distribution of pregnancies in England and Wales in 2011[10] and are conditional on a successful cfDNA test with an estimated FF of ≥ 4%.

depth of sequencing, extent of DNA enrichment, or other assay variables.

When Bayes' theorem is used to combine the *a priori* risk for fetal trisomy derived from maternal age with likelihoods from the cfDNA test, and the true fetal fraction is ≥ 9%, almost all cases of trisomy 21 can be detected at a FPR of less than 0.1%. When this fetal fraction is 4–8%, the performance of screening by cfDNA testing is improved by the addition of the maternal-age-related risk and more so by the inclusion of results from the first-trimester combined test. When the fetal fraction is < 4%, the results from the cfDNA test can potentially be used to improve the performance of screening by the combined test.

Limitations of the study

In this study we adopted a binomial model for the distribution of *Z*-scores according to the true fetal fraction. We configured the model so that, for the distribution of fetal fractions in our large database of pregnancies undergoing cfDNA testing at 10–13 weeks' gestation, the overall DR was 99% and the FPR was 0.1%[3]. We extended the model to take into account errors of estimation in fetal fraction. Although these were theoretical models, the distribution of *Z*-scores in trisomy-21 pregnancies was compatible with those reported from clinical studies[4,5].

Implications for clinical practice

The reported performance of screening for trisomy 21 by the cfDNA test, with a DR of 99% and a FPR of < 0.1%[3], is superior to that of all other methods of screening. Consequently, the test is gaining widespread acceptability. However, the high cost of the cfDNA test limits its application to high- and intermediate-risk patients, identified as such by another traditional first-line method of screening.

This study has shown that the general practice of companies offering the cfDNA test to report results as positive/negative or as risk > 99%/<1:10 000 does not reflect the true estimate of individual patient-specific risk for a given trisomy, especially when the fetal fraction is < 10%. A more appropriate approach for the estimation of patient-specific risks is to use Bayes' theorem to combine all the available data from the cfDNA test with those of any prior method of screening. The advantages of such a practice are first, provision of more accurate patient-specific risks; second, improved performance of screening by the cfDNA test; and third, improved performance of the first-line method of screening when the fetal fraction is < 4% and the cfDNA test is reported to have failed. However, to achieve these objectives fully

Table 4 Theoretical screening performance of cell-free DNA testing together with maternal age and first-trimester combined test, for a risk cut-off of 1 in 100 at time of screening, according to when fetal fraction (FF) is unknown

Estimated FF (%)	*Frequency (%)*	*False-positive rate (%)*		*Detection rate (%)*	
		Maternal age	*Combined test*	*Maternal age*	*Combined test*
0.0–0.9	0.03	6.0	2.4	86.5	95.7
1.0–1.9	0.26	2.3	0.9	92.9	97.8
2.0–2.9	0.85	1.3	0.5	95.4	98.5
3.0–3.9	1.85	0.8	0.3	96.9	99.0
4.0–4.9	3.16	0.5	0.2	98.0	99.3
5.0–5.9	4.60	0.4	0.1	98.6	99.5
6.0–6.9	6.00	0.2	0.1	99.1	99.7
7.0–7.9	7.14	0.2	0.1	99.3	99.8
8.0–8.9	7.94	0.1	0.0	99.5	99.8
9.0–9.9	8.36	0.1	0.0	99.6	99.9
≥ 10.0	59.80	0.0	0.0	99.8	99.9
All	100.0	0.13	0.05	99.4	99.8
FF ≥ 4%	97.01	0.10	0.04	99.6	99.8

Results apply to maternal age distribution of pregnancies in England and Wales in 2011[10].

it would be necessary for the suppliers of the cfDNA test to report information on depth of sequencing, *Z*-scores and both the fetal fraction and its precision.

In the absence of the necessary data from the suppliers of the cfDNA test, it would be preferable for clinicians managing individual patients to use the risk estimate from the first-line method of screening as the prior risk and modify this by the appropriate positive or negative likelihood ratio for a given fetal fraction from the cfDNA test, rather than to counsel all patients that their result is positive or negative.

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REFERENCES

1. Kagan KO, Wright D, Baker A, Sahota D, Nicolaides KH. Screening for trisomy 21 by maternal age, fetal nuchal translucency thickness, free beta-human chorionic gonadotropin and pregnancy-associated plasma protein-A. *Ultrasound Obstet Gynecol* 2008; **31**: 618–624.
2. Kagan KO, Wright D, Valencia C, Maiz N, Nicolaides KH. Screening for trisomies 21, 18 and 13 by maternal age, fetal nuchal translucency, fetal heart rate, free beta-hCG and pregnancy-associated plasma protein-A. *Hum Reprod* 2008; **23**: 1968–1975.
3. 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.
4. 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.
5. 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.
6. Canick JA, Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE. The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. *Prenat Diagn* 2013; **33**: 667–674.
7. Benn P, Cuckle H. Theoretical performance of non-invasive prenatal testing for chromosome imbalances using counting of cell-free DNA fragments in maternal plasma. *Prenat Diagn* 2014; **34**: 778–783.
8. Wright D, Kagan KO, Molina FS, Gazzoni A, Nicolaides KH. A mixture model of nuchal translucency thickness in screening for chromosomal defects. *Ultrasound Obstet Gynecol* 2008; **31**: 376–383.
9. Wright D, Spencer K, Kagan K, Tørring N, Petersen OB, Christou A, Kallikas J, Nicolaides KH. First-trimester combined screening for trisomy 21 at 7–14 weeks' gestation. *Ultrasound Obstet Gynecol* 2010; **36**: 404–411.
10. Office for National Statistics. *Live Births in England and Wales by Characteristics of Mother 1, 2011*. http://www.ons.gov.uk/ons/publications/re-reference-tables.html?edition=tcm%3A77-241261.
11. Wright DE, Bray I. Estimating birth prevalence of Down's syndrome. *J Epidemiol Biostat* 2000; **5**: 89–97.
12. R Development Core Team. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing: Vienna, Austria, 2011. ISBN 3-900051-07-0, http://www.R-project.org/.
13. Hutcheon JA, Chiolero A, Hanley JA. Random measurement error and regression dilution bias. *BMJ* 2010; **340**: c2289.

