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DOI:
10.1111/1471-0528.14050

The accuracy of cell-free fetal DNA based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis

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Running title: Cell-free fetal DNA based NIPT in singleton pregnancies

Word Count: 4295

Abstract

Background. Cell-free fetal DNA (cffDNA) non-invasive prenatal testing (NIPT) is rapidly expanding and being introduced at varying rates depending on country and condition.

Objectives. Determine accuracy of cffDNA-based NIPT for all conditions. Evaluate influence of other factors on test performance.

Search strategy. Medline, Embase, CINAHL, Cochrane Library, 1997-April 2015.

Selection criteria. Cohort studies reporting cffDNA-based NIPT performance in singleton pregnancies.

Data collection and analysis. Bivariate or univariate meta-analysis and sub-group analysis performed to explore influence of test type and population risk. .

Main results. 117 studies included which analysed 18 conditions. Bivariate meta-analysis demonstrated sensitivities and specificities respectively for: fetal sex 0.989(95%CI 0.980-0.994) and 0.996(95%CI 0.989-0.998) 11,179 tests; Rhesus D 0.993(0.982-0.997) and 0.984(0.964-0.993) 10,290 tests; trisomy 21 0.994(0.983-0.998) and 0.999(0.999-1.00) 148,344 tests; trisomy 18 0.977(0.952-0.989) and 0.999(0.998-1.00) 146,940 tests; monosomy X 0.929(0.741-0.984) and 0.999(0.995-0.999) 6,712 tests. Trisomy 13 was analysed by univariate meta-analysis with a summary sensitivity of 0.906(95%CI 0.823-0.958) and specificity of 1.00(95%CI 0.999-0.100) 134,691 tests. False and inconclusive results were poorly reported across all conditions. Test type did affect sensitivity and specificity, but there was no evidence that population risk did.

Conclusions. Performance of cffDNA-based NIPT is affected by condition under investigation. For fetal sex and Rhesus status NIPT can be considered diagnostic. For trisomy 21, 18 and 13, the lower sensitivity, specificity and disease prevalence combined with the biological influence of confined placental mosaicism designates it a screening test. These factors must be considered when counselling patients and assessing the cost of introduction into routine care.

Systematic review registration. PROSPERO CRD42014007174

Keywords. cell-free fetal DNA, non-invasive prenatal testing, diagnostic accuracy

Tweetable abstract. cffDNA NIPT accuracy high, can be diagnostic for fetal sex and Rhesus, but only screening test in aneuploidy

Introduction

Non-invasive prenatal testing (NIPT) utilises cell-free fetal DNA (cffDNA) present in maternal plasma and believed to originate from trophoblast. It was first detected by Lo et al. in 1997 (1) and used to note the presence of the Y chromosome to diagnose fetal sex. NIPT can now be used to test for aneuploidy, and single gene disorders such as cystic fibrosis, Huntington's disease or thanatophoric dysplasia (2-6). Its advantage is that it is non-invasive, avoiding the 0.5-1% risk of miscarriage associated with amniocentesis/chorionic villus sampling (7) and allows timely therapeutic intervention in conditions such as congenital adrenal hyperplasia (CAH) (8). cffDNA is cleared from plasma (in hours) following delivery ensuring individuality for each pregnancy (9). Non-invasive prenatal testing also has health economic implications eliminating the need to give all Rhesus negative women anti-D immunoglobulin prophylaxis.

NIPT is being introduced into routine antenatal care across the world at differing speeds, largely influenced by technological advances facilitated by the commercial sector. Current guidance in North America and from the International Society for Prenatal Diagnosis advises a positive NIPT for aneuploidy to be confirmed by invasive testing (10-12) due to the low risk of a false positive result secondary to confined placental mosaicism (CPM). Inconclusive results occur in up to 8.1% (10), with a repeat sample being successful in up to 80% participants (13).

Several systematic reviews and meta-analyses evaluating test accuracy have been published (14-18). However these have several limitations: i) they evaluate individual conditions (e.g. fetal sex, Rhesus status or aneuploidy) thus not allowing comparison; ii) have a high risk of bias as they include case-control studies; iii) utilise inferior statistical techniques for meta-analysis and iv) include studies with a significant risk of verification bias due to all participants not receiving a reference test (e.g. karyotype).

The aim of our paper is to produce the most comprehensive systematic review and meta-analysis of NIPT and address these issues: include only cohort studies to reduce bias (19); perform bivariate meta-analysis where possible and thirdly to encompass all

indications for antenatal use, so as to enable a more uniformed comparison for the use of NIPT in clinical practice. We also aim to assess aspects of test accuracy that might influence how cffDNA is implemented in the clinical pathway e.g. effect of technique on accuracy and evaluation of false positive, false negative and inconclusive results.

Methods

This review was performed according to recommended methods (20-23) and an *a priori* designed and registered protocol (PROSPERO CRD42014007174).

Identification of studies

Medline, Web of Science, Embase, CINAHL and the Cochrane Library databases were searched for relevant articles by FLM. Grey literature and reference lists were hand searched. The search terms used were 'noninvasive', 'non-invasive', 'non invasive', 'prenatal diagnosis', 'cell free fetal DNA' and 'cell-free fetal DNA'. The full search strategy is available as online supplementary material (Appendix S1). The date of publication was limited from 1997 to 13 April 2015. There was no limitation on language.

Study selection

Study selection was performed in duplicate (FLM, RKM) involving screening of titles and abstracts, then reviewing full manuscripts of selected articles. Disagreements in selection were resolved by MDK. Articles were included based on the following criteria:

Population: Women with a singleton pregnancy, any gestation. Populations could include women of varying risk with high-risk women defined as attending for testing due to pre-existing risk factors: a personal or family history of the condition being tested for, high-risk on routine biochemical screening, abnormal ultrasound scan, and/or raised maternal age. Women were considered low-risk if they had none of the above risk factors.

Test: NIPT based on cffDNA in maternal blood, irrespective of condition being examined.

Reference standard: Studies must have compared all the cffDNA results with either: karyotype results or birth outcome (either blood sample or phenotype) as appropriate in all participants.

Study design: Cohort studies.

Exclusion criteria: pre-implantation testing, fetal cell testing, case-control studies, case series with <5 participants.

Data extraction

Data were extracted in duplicate on the relevant 2x2 tables comparing the non-invasive test with the reference test used for definitive diagnosis. Data were also extracted on factors which may affect test accuracy: participant characteristics (e.g. obstetric history); and test characteristics (e.g. cut offs used, test technique [e.g. PCR, MPS, mass spectrometry]). Information regarding false results and inconclusive results was obtained.

When a study used similar laboratory protocols on the same blood samples (e.g. different number of replicates performed) only the best results were included. When a study used different laboratory protocols on different blood samples, but the same type of test technique, these samples were grouped together for analysis. If a study sub-divided samples based on population characteristics (e.g. high-risk vs. low-risk for a condition, or 1st trimester vs. 2nd trimester vs. 3rd trimester) these were grouped together for the summary statistics, and analysed as a sub-group where appropriate.

Quality Assessment

The quality of the studies was assessed using the QUADAS-2 tool (24).

Data synthesis

For each study the 2x2 data were used to calculate sensitivity and specificity with 95% confidence intervals. Heterogeneity was explored by assessing the distribution of results in the Forest plots and summary receiver operating characteristic curves (SROC). Summary measures including sensitivities, specificities, diagnostic odds ratio, positive and negative likelihood ratios along with 95% confidence intervals were

147 calculated using bivariate logistic regression model with an unstructured correlation.
148 This model allows for the correlation between sensitivity and specificity from the same
149 study and for the sensitivities and specificities to have different random effects (25).
150 Meta-analysis was performed when there were more than 5 studies per condition using
151 STATA 13 (StataCorp. 2012, College Station, Texas) (see Appendix S2 for more
152 detail). Sub-group analysis and meta-regression was planned *a priori* to assess effects
153 of study level covariates on test accuracy, namely: population characteristics (level of
154 risk for condition where appropriate i.e. not performed in fetal sex or Rhesus D); test
155 technique (e.g. PCR, MPS) and quality aspects according to QUADAS-2. We used
156 sub-group analyses (as opposed to meta-regression) to assess the influence of all
157 categorical covariates due to model convergence difficulties (26).
158
159 **Results**
160 The search revealed 4433 studies for inclusion. After reviewing the full article, 117
161 studies (1, 27-143) were eligible reporting on 18 different conditions, and 472,935 tests
162 (Figure S1). The study characteristics are outlined in Table S1.
163 We were able to produce summary results using the fully unstructured bivariate model
164 for: fetal sex, Rhesus D, trisomy 21, trisomy 18 and monosomy X (Table S2). For
165 trisomy 13, despite a sufficient number of studies (n=15) there was no heterogeneity in
166 specificities across studies so the bivariate model, which takes into account the
167 correlation between the sensitivities and specificities, failed to converge and
168 consequently we fitted a univariate model. Because of this, these results are less
169 methodologically robust. The HSROC curves are presented in Figure S2 and the
170 results from our sub-group analyses in Table S2.
171 There were 5 studies (n=394,130 tests) in which there was differential verification of
172 results, in that some participants had their result confirmed by karyotype and others by
173 phenotype (35, 91, 93, 114, 133). These 5 studies all assessed fetal aneuploidy and
174 utilised NIPT as a screening test in a low-risk population. A sensitivity analysis

removing these 5 studies demonstrated no significant effect on the summary results, thus these studies are included in all analyses and Forest plots.

The following 12 conditions had insufficient studies for meta-analysis: Rhesus C, Rhesus E, 47XXX, 47XXY, 47XYY, trisomy 16, congenital adrenal hyperplasia, deletion-duplication syndromes, sickle cell anaemia, thalassaemia, human platelet antigen 1a, and KEL 1. The Forest plots of these 12 conditions are presented in Figure S3.

Methodological quality of included studies

This was assessed according to the Quality Assessment tool for Diagnostic Accuracy Studies (QUADAS-2) (24), the results are demonstrated in Figure S4 and further described in Appendix S3.

False results and inconclusive results

Reporting of causes and implications of false positive, false negative and inconclusive results was poor, and varied across all conditions (Table S3). The included studies reported an inconclusive result rate of 0.32-5.3%. This issue was further compounded by a myriad of varying quality control (QC) standards, some studies excluding samples that failed their QC and others implementing no QC steps and therefore reporting some results as false negatives which other studies would have excluded from analysis. Some studies investigated the reasons for their false and inconclusive results and reported these clearly, accounting for all samples. Other studies reported inconclusive results as false negatives or did not report them at all. We describe these results in more detail for each of the conditions investigated.

Results from bivariate meta-analysis

Fetal Sex

Sixty studies (11,179 tests) evaluated fetal sex and are represented in the Forest plot in Figure 1. Bivariate meta-analysis produced a summary sensitivity of 0.989 (95% CI 0.980 to 0.994) and specificity of 0.996 (95% CI 0.989 to 0.998), a positive likelihood ratio of 255 (95% CI 89 to 729) and negative likelihood ratio of 0.011 (95% CI 0.006 to 0.019). Other summary measures are in Table S2.

No significant effect on sensitivity was found with test technique. However there was a difference in specificity with real-time quantitative PCR 0.999 (95%CI 0.991 to 1.00) performing better than conventional PCR 0.939 (95%CI 0.872 to 0.972). For fetal sex, 11/60 studies reported inconclusive results, of these, 5 studies documented an explanation (in order of frequency): assay failure, no reason given, insufficient number of markers present from pre-specified cut-off and low fetal fraction. The commonest reasons given by the authors of the studies for the false results were: no reason given, low fetal fraction (although cffDNA not quantified), low fetal fraction confirmed by authors quantifying cffDNA, possible contamination/DNA degradation/vanishing twin/test failure although not confirmed, and previous male pregnancy, although the latter reason has since been disproven as cell-free fetal DNA is cleared from the maternal circulation hours post-delivery (9).

Rhesus D

Thirty studies (10,290 tests) evaluated fetal Rhesus D status and are represented in Figure 2. Bivariate meta-analysis produced a summary sensitivity of 0.993 (95% CI 0.982 to 0.997) and specificity of 0.984 (95% CI 0.964 to 0.993) a positive likelihood ratio of 61 (95% CI 22 to 167) and negative likelihood ratio of 0.007 (95% CI 0.003 to 0.186). There was a significant difference between test techniques with real-time quantitative PCR sensitivity: 0.997 (95% CI 0.987 to 0.999) demonstrating a higher sensitivity than conventional PCR 0.924 (95%CI 0.832 to 0.968), although it was not possible to assess if there was a difference in those which utilised mass spectrometry (despite sufficient studies, due to convergence issues as detailed in the discussion),

and no difference in specificity was seen (Table S2). For Rhesus D, 13/30 studies reported inconclusive results, of these, 10 studies documented an explanation (in order of frequency): no reason given, RHD gene variant, insufficient number of markers present from pre-specified cut-off, test failure, low fetal fraction. The commonest reasons given for false results were: presumed low fetal fraction (although not quantified by authors), no reason given, presumed RHD gene variant (although not confirmed), confirmed RHD gene variant, test failure, possible contamination/DNA degradation/pipetting error/incorrect neonatal blood testing.

Trisomy 21

Thirty-one studies (148,344 tests) assessed trisomy 21 and are represented in Figure 3A. Bivariate meta-analysis produced a summary sensitivity of 0.994 (95% CI 0.983 to 0.998) and specificity of 0.999 (95% CI 0.999 to 1.00) a positive likelihood ratio of 1720 (95% CI 1111 to 2662) and negative likelihood ratio of 0.006 (95% CI 0.002 to 0.017). Test technique and population risk had no significant effect. For trisomy 21, 14/31 studies reported inconclusive results, of these, 7 studies documented an explanation (in order of frequency): assay failure, confirmed low fetal fraction, no reason given, presumed low fetal fraction/inadequate sequencing depth. The commonest reasons given for false results were: confirmed low fetal fraction, confirmed mosaicism, no reason given, test failure, maternal CNV.

Trisomy 18

Twenty-four studies (146,940 tests) assessed trisomy 18 and are represented in Figure 3B. Bivariate meta-analysis produced a summary sensitivity of 0.977 (95% CI 0.952 to 0.989) and specificity of 0.999 (95% CI 0.998 to 1.00) and a positive likelihood ratio of 1569 (95% CI 810 to 3149) and negative likelihood ratio of 0.023 (95% CI 0.011 to 0.048). Neither test technique or population risk had a significant effect. For trisomy 18, 12/24 studies reported inconclusive results, of these 7 studies documented an

explanation (in order of frequency): low fetal fraction, test failure, no reason given, mosaicism. The commonest reasons given for false results were: confirmed low fetal fraction, confirmed mosaicism, presumed low fetal fraction/human error, maternal CNV, no reason given.

Monosomy X

Eight studies (6712 tests) assessed monosomy X and are represented in Figure 3C. Bivariate meta-analysis produced a summary sensitivity of 0.929 (95% CI 0.741 to 0.984) and specificity of 0.999 (95% CI 0.995 to 0.999) and a positive likelihood ratio of 1337 (95% CI 213 to 8407) and negative likelihood ratio of 0.071 (95% CI 0.017 to 0.292). There was no significant difference with test technique. It was not possible to assess the effect of population risk as there were insufficient low-risk studies. For monosomy X, 5/8 studies reported inconclusive results, of these, 3 studies documented an explanation (in order of frequency): low fetal fraction, presumed human error and no reason given. The commonest reasons given for false results were: mosaicism and no reason given.

The 5 aneuploidy studies which evaluated an unselected obstetric population reported inconclusive results rates of 0.29-5.1% and provided the same reasons for their false and inconclusive results as with the high-risk aneuploidy populations.

Trisomy 13 – univariate meta-analysis

Sixteen studies which equates to 134,691 tests examined trisomy 13, represented in Figure 3D. There was a summary sensitivity of 0.906 (95% CI 0.823 to 0.958) and specificity of 1.00 (95% CI 0.999 to 1.00). The positive likelihood ratio was 453 (95% CI 26 to 7864) and negative likelihood ratio was 0.188 (95% CI 0.080 to 0.44039) with a diagnostic odds ratio of 2788 (95% CI 285 to 27252). For trisomy 13, 6/16 studies reported inconclusive results, of these, 4 studies documented an explanation for

inconclusive results: low fetal fraction, different fragmentation rate, contamination, assay failure and human error. The only reason given for false results was confirmed low fetal fraction.

Results where meta-analysis not possible

The results for these conditions are presented as Forest plots in S3.

Clinical application for NIPT for Down's syndrome screening

Using published data from the National Down Syndrome Cytogenetic Register (NDSCR) 2012 Annual report we have produced a table detailing the estimated outcomes (livebirth rate, invasive test rate, euploid pregnancy loss rate, undiagnosed aneuploidy livebirth rate) from the current standard Down's Syndrome Screening (DSS) i.e. first trimester combined screening pathway (maternal age, nuchal translucency, beta human chorionic gonadotrophin and pregnancy associated plasma protein A) and from a pathway with NIPT as both contingent (i.e. NIPT offered to women with a positive screen after first trimester combined screening) and first line screening for a population of 100,000 women using crude rates (144) (Table S4). We use the prevalence reported by NDSCR[1] (trisomy 21: 2.2 per 1000 women, trisomy 18: 0.64 per 1000, trisomy 13 0.26 per 1000). This assumes that standards for the first trimester combined screening are "achievable" as described by Fetal Anomaly Screening Programme (FASP) guidance i.e. for trisomy 21 a detection rate of 85% for a screen positive rate of 2% (145). For NIPT the summary measures are those from our meta-analysis. For the contingent screening model the cut-off for high risk is 1:1000 from first trimester combined screening with a detection rate of 96% and false positive rate of 12% (146). This model assumes that all women accept screening when offered as it is not possible to determine yet what the uptake of NIPT would be if offered as a first-line test. It also assumes that all women are required to have an invasive test for karyotyping after a screen positive result from combined or NIPT prior to considering

termination of pregnancy, thus the invasive test rates will be higher than in a real-life population. It assumes a 0.5% pregnancy loss rate from invasive testing (146).

These data demonstrate the influence of disease prevalence on test performance. If we compare combined screening with a 1:150 cut-off (i.e. current NHS practice) with NIPT as a first–line test we can reduce the invasive test rate from 2000 to 319 per 100,000 women, the euploid pregnancy loss rate from 9 to 1 per 100,000 and the undiagnosed trisomy 21 live births rate from 32 to 1 per 100,000. If NIPT was used as a contingent screening test for a 1:1000 combined screening cut-off (i.e. as a 2nd test following a positive combined screening result at a 1:1000 cut-off) then these figures are reduced even further compared to combined screening with a 1:150 cut-off: 2000 to 222 per 100,000 women invasive test rate; 9 to 0 euploid pregnancy loss rate, although there is less of a reduction in undiagnosed trisomy 21 live birth rate from 32 to 10. If NIPT was used as a contingent screening test for a 1:150 combined screening cut-off then these figures are: 2000 per 100,000 women invasive test rate; 0 euploid pregnancy loss and 34 undiagnosed trisomy 21 livebirth rate. A two stage contingent screening pathway with a 1:1000 cut-off when compared to NIPT as a first line test affords a reduction in false positive results (12 versus 100 per 100,000 women) that are found at the time of NIPT as the prevalence of disease in the population now undergoing NIPT is much higher. This is at the expense of a 10 fold increase in undiagnosed aneuploidy live births (1 versus 10 per 100,000 women) due to the increased number of false negatives at the first stage of screening that do not undergo NIPT. A cut-off of 1:150 at the first stage for the combined test compared to a 1:150 cut-off for NIPT as a contingent screening test has little effect on the number false negatives (33 versus 34), however the invasive test rate is reduced (2000 versus 188 per 100,000 women).

Discussion

Main findings

Our results demonstrate that for fetal sex and Rhesus D status, cffDNA-based NIPT has a high sensitivity and specificity. For aneuploidies: trisomy 21, and in particular trisomy 18 and 13 we have demonstrated improved accuracy from other recent systematic reviews likely due to technological developments. Importantly we found that false results and inconclusive results were poorly reported across all conditions.

Strengths and limitations

This review was performed according to rigorous methodology with efforts made to reduce bias in participant selection and clinical applicability by excluding case-control studies, performing bivariate meta-analysis and meta-regression analysis and assessing the impact of differential verification (i.e. different reference standards). Bivariate meta-analysis is the recommended approach for the meta- analysis of diagnostic test accuracy studies. This is because a conventional univariate analysis makes assumptions that are known not to be tenable (that the sensitivity and specificity from the same study are independent). However, the bivariate meta-analysis model is a technically difficult model to fit and it is well known that these models might not converge when there are a small number of studies, or when there are zero cells (i.e. sensitivity or specificity close to 100) (26). We observed no indication that other model fits were unstable and so have no reason to be concerned about the statistical validity of the other results. Our review also evaluates more conditions than previously. In addition, our paper has been able to assess the impact of test technique and population risk. We were unable to evaluate the number of samples which failed QC measures as this was reported in varying degrees. When considering the implementation of a new test, information regarding failed tests (147, 148), and inconclusive results is vital. We investigated the reasons for false positive and false negative results within and across studies and attempted to summarise these. This was again hampered by poor reporting with a common reason being low fetal fraction which is difficult to measure accurately and thus has led to variations in approach between

studies. It is especially important to consider this further as low fetal fraction has been shown to be associated with trisomy 18 and triploidies.

A limitation of this work is that it was not possible to account for the many subtle differences in laboratory techniques such as comparing the different combinations of genetic markers used for each condition; or the myriad of adjustments made to bioinformatics algorithms as these were so varied. This is where the results from the large studies in screening populations are especially important as there is QC across laboratories and standardisation of techniques (35, 91, 93, 114, 133). In the process of publishing this review, the search was re-run from April 2015 - September 2015 in view of the rapid progression in this area. This yielded 78 new citations, of which 11 additional papers would be eligible for inclusion (3, 149-158), which comprise 10,191 women in total. These studies examine fetal sex (n=436 women), Rhesus D status (n=2965), trisomy 21 (n=6661), trisomy 18 (n=6701), trisomy 13 (n=6495), and monosomy X (n=40), which equate to a small proportion of additional tests, compared to the studies we have already analysed. There is also now one study which investigates thanatophoric dysplasia (n=108), although this cannot be included in a meta-analysis as it is the only study to look at this condition thus far. As the search was under a year old when the publication was accepted we have not included these 11 studies in our results. We are confident that if these studies were included they would not impact on our results and conclusions.

Interpretation

It is recognised that there are fewer studies in our meta-analyses for trisomy 13 and monosomy X compared to a previous large meta-analysis (14) but this is due to excluding case-control studies and limiting to singletons. This has led to us reporting higher summary sensitivities and specificities than existing analyses, demonstrating how NIPT is advancing, and supporting the belief that NIPT will be used as the first-line screening test in the future. Our clinical application model has highlighted the

importance of low prevalence of disease on the positive predictive value and false positive rate in the case of aneuploidies. Although positive and negative predictive values are useful indicators of test accuracy as they take into account disease prevalence (159), we have not presented these values within this paper due to variation in disease prevalence among included study populations.

Conclusion

This work demonstrates that there is a sufficient body of evidence for the accuracy and reproducibility of cffDNA-based NIPT to allow its introduction into routine clinical practice within the UK, however its role is yet to be decided.

Implications for clinical practice

The findings of this analysis support the use of NIPT as a diagnostic test for fetal sex and Rhesus status due to the nature of these conditions and the populations being tested. For assessment of aneuploidy the test must be considered a “screening test” despite high accuracy due to the low prevalence of disease and influence of biological factors such as CPM. We are aware that the National Screening Committee (NSC) is currently reviewing all the evidence for aneuploidy, and is likely to recommend NIPT as a contingency screening test in the UK (Dr Pranav Pandya, Personal Communication, 2015). While for Down’s syndrome screening (DSS) this will ensure access to an accurate, non-invasive test and ensure equity for many more women (i.e. test threshold has less of an impact on offering invasive testing and test can be offered throughout gestation not just in a small first trimester window) this must be balanced with consideration of the important ethical repercussions which need addressing (i.e. a test that can assess for multiple conditions and those with a milder phenotype and also test for conditions within the mother e.g. sex-chromosome anomaly or cancers) (160). There are also counselling implications as access to a non-invasive, highly accurate test still needs careful consideration by parents.

Implications for future research

The authors would recommend that the same rigorous assessment of the evidence and accuracy as we have performed be applied in multiple pregnancies once the evidence base is sufficient.

The NIHR funded RAPID study which has used NIPT in an NHS setting for women in whom combined testing gave a risk of ≥ 1:1000 will soon be published. This study aims to assess the uptake of NIPT and whether the addition of NIPT to the DSS pathway affects the uptake of DSS and invasive testing; a detailed health economic evaluation using a tool developed in conjunction with the UK NSC; optimal ways to deliver education to women and healthcare professionals; and sensitivity and specificity of NIPT for aneuploidy when performed in an NHS regional genetics laboratory. The results from our review indicate the latter (accuracy results from an NHS regional genetics laboratory) will be an important outcome as it will remove the influence of results from the commercial sector and poor reporting. This will allow for improved QC, enable continued assessment on a national basis, and ensure that the cost of NIPT will improve further. Similarly, the conditions for which NIPT will be used are likely to increase; 11 studies which examined single gene mutations and microdeletions could not be included in our meta-analysis due to having fewer than 5 participants; even whilst writing this review larger studies are being reported on these conditions (161). However, an economic evaluation of this first-line screening with NIPT would also need to include maintaining access to a high quality first trimester ultrasound scan including nuchal translucency (NT) assessment, to allow dating, viability, multiple pregnancy, structural anomaly and adnexal assessment, and importantly the assessment of the risk of cardiac anomalies and increased pregnancy loss associated with raised NT.

Acknowledgements: The articles were translated by FLM and RKM, and Dr Carman Lai. Some of these data have been presented at the British Maternal and Fetal Medicine Society Annual Scientific Conference, 2015 (Mackie FL, Morris RK, Hemming K, Allen S, Kilby MD. Cell-free fetal DNA based non-invasive prenatal testing: a systematic review and meta-analysis of diagnostic accuracy. *Br J Obstet Gynecol* 2015;122:Supp 2)

Disclosure of interest: We have no disclosures of interests to declare. The ICMJE disclosure forms are available as online supporting information.

Contribution to authorship: FLM extracted the data, contributed to the analysis and data interpretation, and drafted the manuscript. RKM assisted extracting the data, contributed to the analysis and data interpretation and amended the manuscript. KH conducted the bivariate meta-analysis and data interpretation and amended the manuscript. SA assisted with data extraction, interpretation of the results and amended the manuscript. MDK conceived, designed and oversaw the work, made final decisions where there were discrepancies and amended the manuscript. MDK is guarantor for the study.

Details of ethical approval: not required

Funding: FLM is funded by the Richard and Jack Wiseman Trust (Registered charity number: 1036690).

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Supplementary material legends

Figure S1 Study selection from initial search

Figure S2 HSROC curves for bivariate analyses

Figure S3 Forest plots of studies bivariate not possible

Figure S4 Bar chart demonstrating quality assessment of included studies from QUADAS-2 risk of bias assessment

Table S1 Characteristics of included studies

Table S2 Bivariate results

Table S3 Reasons for false positives and false negatives and inconclusive results

Table S4 Clinical application for Trisomy 21

934 **Appendix S1** Search strategy

935 **Appendix S2** Additional statistical methods

936 **Appendix S3** Quality assessment results

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Figure 1: Forest plot of studies testing fetal sex using cell-free fetal DNA

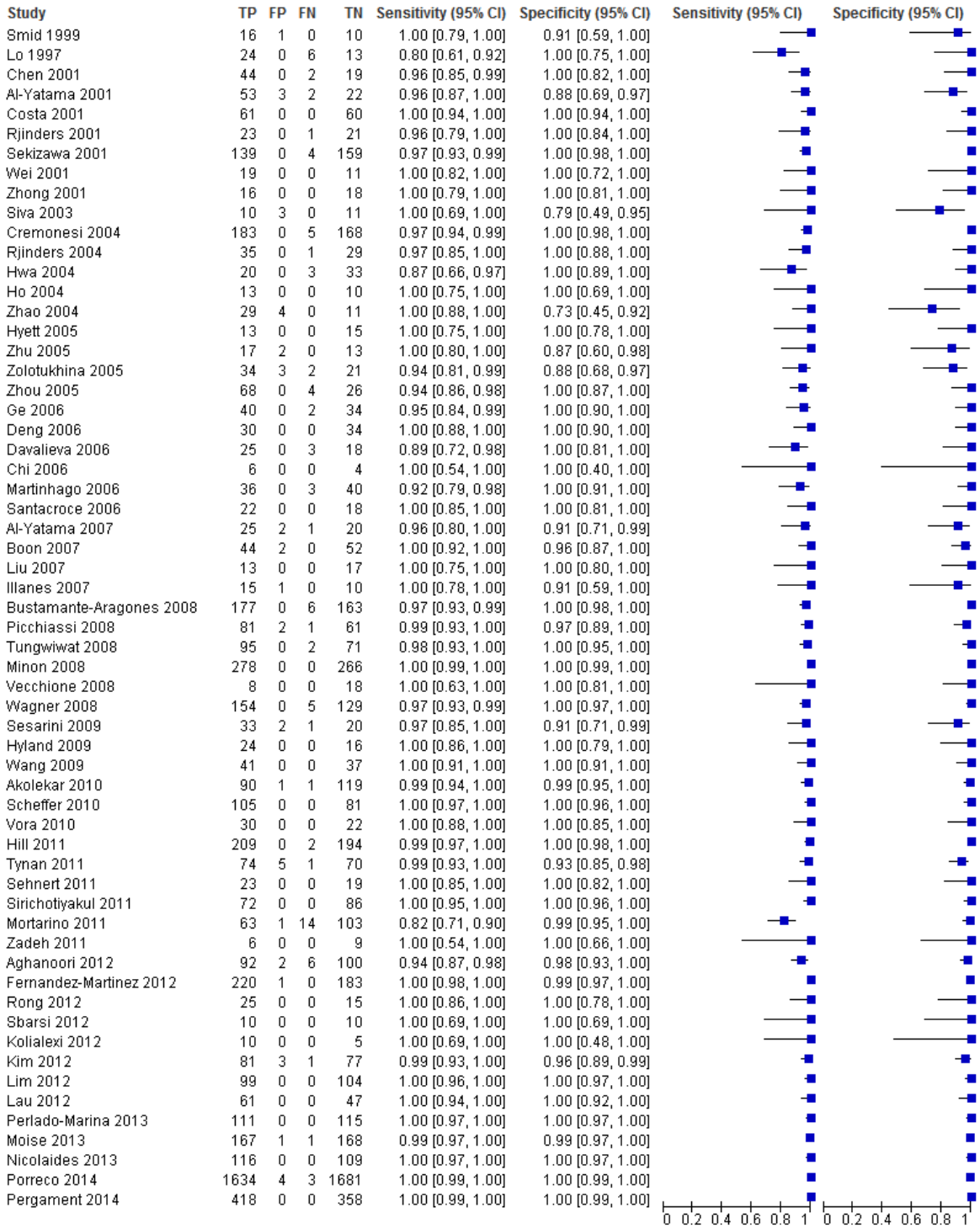

Figure 2: Forest plot of studies testing Rhesus D status using cell-free fetal DNA

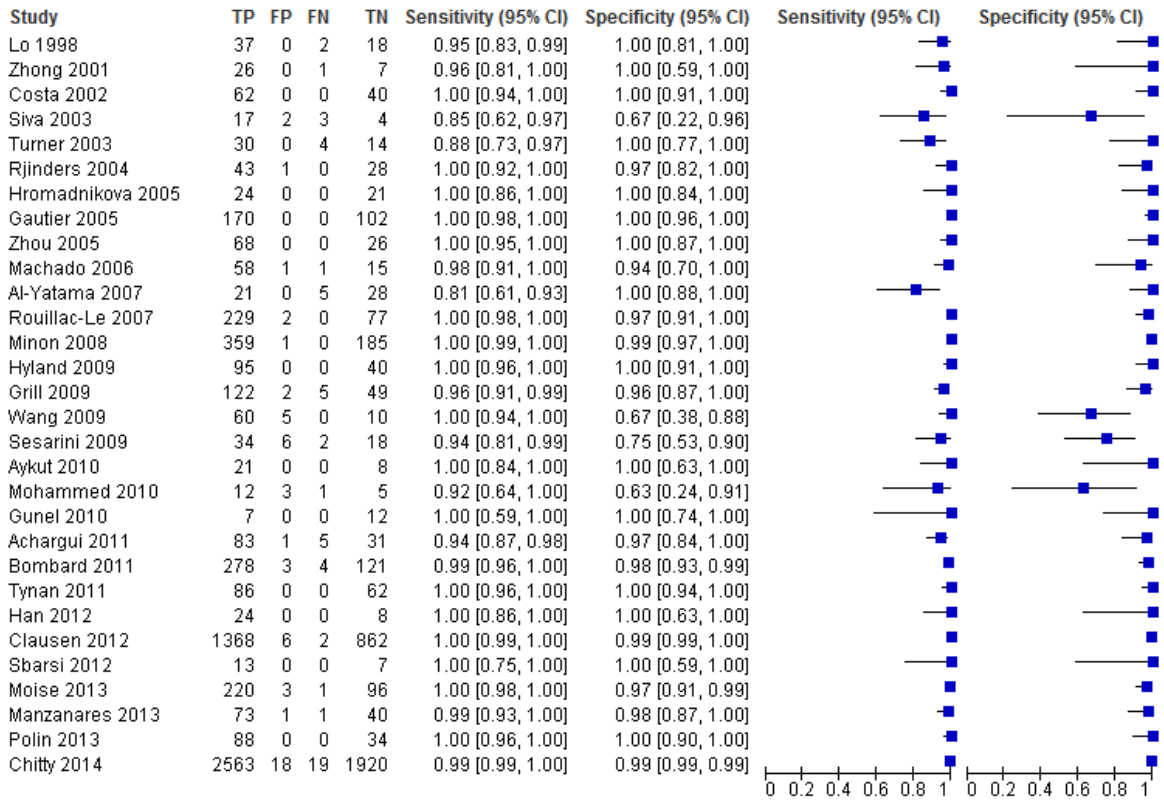

Figure 3A: Forest plot of studies testing Trisomy 21 using cell-free fetal DNA

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Fan 2008	9	0	0	9	1.00 [0.66, 1.00]	1.00 [0.66, 1.00]
Ghanta 2010	7	0	0	20	1.00 [0.59, 1.00]	1.00 [0.83, 1.00]
Tong 2010	5	1	0	23	1.00 [0.48, 1.00]	0.96 [0.79, 1.00]
Sehnert 2011	13	0	0	34	1.00 [0.75, 1.00]	1.00 [0.90, 1.00]
Nicolaides 2012	8	0	0	1941	1.00 [0.63, 1.00]	1.00 [1.00, 1.00]
Lau 2012	11	0	0	97	1.00 [0.72, 1.00]	1.00 [0.96, 1.00]
Norton 2012	81	1	0	2887	1.00 [0.96, 1.00]	1.00 [1.00, 1.00]
Van den 2012	11	0	0	8	1.00 [0.72, 1.00]	1.00 [0.63, 1.00]
Verweij 2012	17	0	1	486	0.94 [0.73, 1.00]	1.00 [0.99, 1.00]
Sparks 2012	36	0	0	131	1.00 [0.90, 1.00]	1.00 [0.97, 1.00]
Stumm 2012	8	0	0	34	1.00 [0.63, 1.00]	1.00 [0.90, 1.00]
Zimmermann 2012	11	0	0	134	1.00 [0.72, 1.00]	1.00 [0.97, 1.00]
Ferres 2013	4	0	0	195	1.00 [0.40, 1.00]	1.00 [0.98, 1.00]
Liao 2013	5	0	0	145	1.00 [0.48, 1.00]	1.00 [0.97, 1.00]
Gorduza 2013	5	0	1	5	0.83 [0.36, 1.00]	1.00 [0.48, 1.00]
Hofmann 2013	40	0	1	427	0.98 [0.87, 1.00]	1.00 [0.99, 1.00]
Shaw 2013	11	0	0	190	1.00 [0.72, 1.00]	1.00 [0.98, 1.00]
Nicolaides 2013	25	0	0	204	1.00 [0.86, 1.00]	1.00 [0.98, 1.00]
Song 2013	89	1	0	474	1.00 [0.96, 1.00]	1.00 [0.99, 1.00]
Song 2013a	8	0	0	1733	1.00 [0.63, 1.00]	1.00 [1.00, 1.00]
Bianchi 2014	5	6	0	1898	1.00 [0.48, 1.00]	1.00 [0.99, 1.00]
Li 2014	41	0	2	203	0.95 [0.84, 0.99]	1.00 [0.98, 1.00]
Liao 2014	55	2	0	458	1.00 [0.94, 1.00]	1.00 [0.98, 1.00]
Porreco 2014	137	3	0	3182	1.00 [0.97, 1.00]	1.00 [1.00, 1.00]
Pergament 2014	58	0	0	906	1.00 [0.94, 1.00]	1.00 [1.00, 1.00]
Stumm 2014	40	0	2	430	0.95 [0.84, 0.99]	1.00 [0.99, 1.00]
Alberti 2015	47	0	0	136	1.00 [0.92, 1.00]	1.00 [0.97, 1.00]
Quezada 2015	32	1	0	2803	1.00 [0.89, 1.00]	1.00 [1.00, 1.00]
Norton 2015	38	9	0	15794	1.00 [0.91, 1.00]	1.00 [1.00, 1.00]
Song Y 2015	2	0	0	202	1.00 [0.16, 1.00]	1.00 [0.98, 1.00]
Zhang 2015	715	59	6	111485	0.99 [0.98, 1.00]	1.00 [1.00, 1.00]

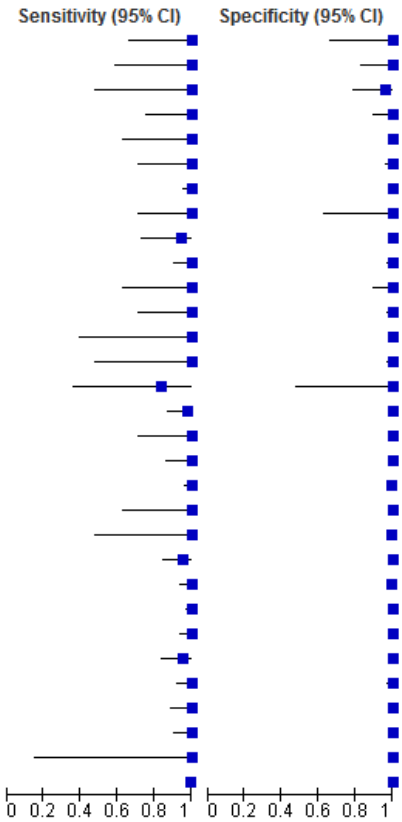

Figure 3B: Forest plot of studies testing Trisomy 18 using cell-free fetal DNA

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Fan 2008	2	0	0	16	1.00 [0.16, 1.00]	1.00 [0.79, 1.00]
Ghanta 2010	2	0	0	25	1.00 [0.16, 1.00]	1.00 [0.86, 1.00]
Sehnert 2011	8	0	0	39	1.00 [0.63, 1.00]	1.00 [0.91, 1.00]
Nicolaides 2012	2	2	0	1945	1.00 [0.16, 1.00]	1.00 [1.00, 1.00]
Lau 2012	10	0	0	98	1.00 [0.69, 1.00]	1.00 [0.96, 1.00]
Norton 2012	37	2	1	2886	0.97 [0.86, 1.00]	1.00 [1.00, 1.00]
Zimmermann 2012	3	0	0	142	1.00 [0.29, 1.00]	1.00 [0.97, 1.00]
Sparks 2012	8	0	0	159	1.00 [0.63, 1.00]	1.00 [0.98, 1.00]
Hofmann 2013	8	0	0	460	1.00 [0.63, 1.00]	1.00 [0.99, 1.00]
Nicolaides 2013	3	0	0	226	1.00 [0.29, 1.00]	1.00 [0.98, 1.00]
Van den 2013	9	0	0	8	1.00 [0.66, 1.00]	1.00 [0.63, 1.00]
Shaw 2013	8	0	0	193	1.00 [0.63, 1.00]	1.00 [0.98, 1.00]
Song 2013a	2	1	0	1738	1.00 [0.16, 1.00]	1.00 [1.00, 1.00]
Song 2013	57	1	1	505	0.98 [0.91, 1.00]	1.00 [0.99, 1.00]
Liao 2014	16	4	0	495	1.00 [0.79, 1.00]	0.99 [0.98, 1.00]
Pergament 2014	24	1	1	938	0.96 [0.80, 1.00]	1.00 [0.99, 1.00]
Bianchi 2014	2	3	0	1900	1.00 [0.16, 1.00]	1.00 [1.00, 1.00]
Bijok 2014	1	0	1	7	0.50 [0.01, 0.99]	1.00 [0.59, 1.00]
Porreco 2014	36	0	3	3283	0.92 [0.79, 0.98]	1.00 [1.00, 1.00]
Stumm 2014	8	1	0	463	1.00 [0.63, 1.00]	1.00 [0.99, 1.00]
Norton 2015	9	1	1	15830	0.90 [0.55, 1.00]	1.00 [1.00, 1.00]
Zhang 2015	167	51	3	112044	0.98 [0.95, 1.00]	1.00 [1.00, 1.00]
Quezada 2015	9	5	1	2821	0.90 [0.55, 1.00]	1.00 [1.00, 1.00]
Song Y 2015	1	0	0	203	1.00 [0.03, 1.00]	1.00 [0.98, 1.00]

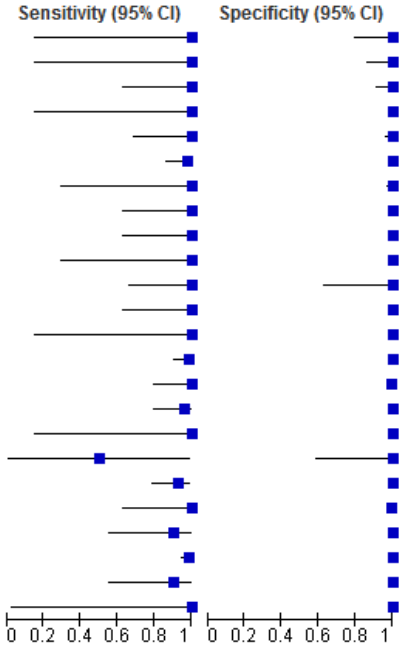

Figure 3C: Forest plot of studies testing Monosomy X using cell-free fetal DNA

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Tungwiwat 2007	0	0	0	0	Not estimable	Not estimable
Sehnert 2011	2	0	0	45	1.00 [0.16, 1.00]	1.00 [0.92, 1.00]
Lau 2012	0	0	0	0	Not estimable	Not estimable
Zimmermann 2012	1	0	0	144	1.00 [0.03, 1.00]	1.00 [0.97, 1.00]
Nicolaides 2013	2	0	0	227	1.00 [0.16, 1.00]	1.00 [0.98, 1.00]
Song 2013a	2	0	1	1737	0.67 [0.09, 0.99]	1.00 [1.00, 1.00]
Shaw 2013	3	0	1	197	0.75 [0.19, 0.99]	1.00 [0.98, 1.00]
Pergament 2014	9	1	1	953	0.90 [0.55, 1.00]	1.00 [0.99, 1.00]
Porreco 2014	9	11	0	3258	1.00 [0.66, 1.00]	1.00 [0.99, 1.00]

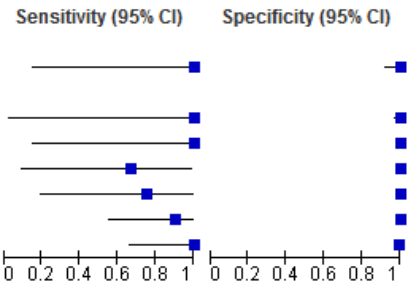

Figure 3D: Forest plot of studies testing Trisomy 13 using cell-free fetal DNA

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Fan 2008	1	0	0	17	1.00 [0.03, 1.00]	1.00 [0.80, 1.00]
Zimmermann 2012	2	0	0	143	1.00 [0.16, 1.00]	1.00 [0.97, 1.00]
Lau 2012	2	0	0	106	1.00 [0.16, 1.00]	1.00 [0.97, 1.00]
Van den 2013	1	4	3	9	0.25 [0.01, 0.81]	0.69 [0.39, 0.91]
Nicolaides 2013	1	0	0	228	1.00 [0.03, 1.00]	1.00 [0.98, 1.00]
Shaw 2013	3	0	0	198	1.00 [0.29, 1.00]	1.00 [0.98, 1.00]
Song 2013a	1	0	0	1740	1.00 [0.03, 1.00]	1.00 [1.00, 1.00]
Hofmann 2013	5	0	0	463	1.00 [0.48, 1.00]	1.00 [0.99, 1.00]
Porreco 2014	14	0	2	3306	0.88 [0.62, 0.98]	1.00 [1.00, 1.00]
Pergament 2014	12	0	0	953	1.00 [0.74, 1.00]	1.00 [1.00, 1.00]
Stumm 2014	5	0	0	467	1.00 [0.48, 1.00]	1.00 [0.99, 1.00]
Liao 2014	3	0	0	512	1.00 [0.29, 1.00]	1.00 [0.99, 1.00]
Zhang 2015	22	45	0	112198	1.00 [0.85, 1.00]	1.00 [1.00, 1.00]
Song Y 2015	1	0	0	203	1.00 [0.03, 1.00]	1.00 [0.98, 1.00]
Norton 2015	2	2	0	11181	1.00 [0.16, 1.00]	1.00 [1.00, 1.00]
Quezada 2015	2	2	3	2829	0.40 [0.05, 0.85]	1.00 [1.00, 1.00]