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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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29 **Abstract**

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

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

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

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

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

40 **Main results.** 117 studies included which analysed 18 conditions. Bivariate meta-
41 analysis demonstrated sensitivities and specificities respectively for: fetal sex
42 0.989(95%CI 0.980-0.994) and 0.996(95%CI 0.989-0.998) 11,179 tests; Rhesus D
43 0.993(0.982-0.997) and 0.984(0.964-0.993) 10,290 tests; trisomy 21 0.994(0.983-
44 0.998) and 0.999(0.999-1.00) 148,344 tests; trisomy 18 0.977(0.952-0.989) and
45 0.999(0.998-1.00) 146,940 tests; monosomy X 0.929(0.741-0.984) and 0.999(0.995-
46 0.999) 6,712 tests. Trisomy 13 was analysed by univariate meta-analysis with a
47 summary sensitivity of 0.906(95%CI 0.823-0.958) and specificity of 1.00(95%CI 0.999-
48 0.100) 134,691 tests. False and inconclusive results were poorly reported across all
49 conditions. Test type did affect sensitivity and specificity, but there was no evidence
50 that population risk did.

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

57 **Systematic review registration.** PROSPERO CRD42014007174

58

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

60 **Tweetable abstract.** cffDNA NIPT accuracy high, can be diagnostic for fetal sex and

61 Rhesus, but only screening test in aneuploidy

62

63   **Introduction**

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

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

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

88   The aim of our paper is to produce the most comprehensive systematic review and
89   meta-analysis of NIPT and address these issues: include only cohort studies to reduce
90   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.

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

122 *Study design:* Cohort studies.

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

125 **Data extraction**

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

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

139 **Quality Assessment**

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

141 **Data synthesis**

142 For each study the 2x2 data were used to calculate sensitivity and specificity with 95%
143 confidence intervals. Heterogeneity was explored by assessing the distribution of
144 results in the Forest plots and summary receiver operating characteristic curves
145 (SROC). Summary measures including sensitivities, specificities, diagnostic odds ratio,
146 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

175 removing these 5 studies demonstrated no significant effect on the summary results,
176 thus these studies are included in all analyses and Forest plots.
177 The following 12 conditions had insufficient studies for meta-analysis: Rhesus C,
178 Rhesus E, 47XXX, 47XXY, 47XYY, trisomy 16, congenital adrenal hyperplasia,
179 deletion-duplication syndromes, sickle cell anaemia, thalassaemia, human platelet
180 antigen 1a, and KEL 1. The Forest plots of these 12 conditions are presented in Figure
181 S3.

182

183 ***Methodological quality of included studies***

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

187

188 ***False results and inconclusive results***

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

199

200 ***Results from bivariate meta-analysis***

201

202 ***Fetal Sex***

203 Sixty studies (11,179 tests) evaluated fetal sex and are represented in the Forest plot
204 in Figure 1. Bivariate meta-analysis produced a summary sensitivity of 0.989 (95% CI
205 0.980 to 0.994) and specificity of 0.996 (95% CI 0.989 to 0.998), a positive likelihood
206 ratio of 255 (95% CI 89 to 729) and negative likelihood ratio of 0.011 (95% CI 0.006 to
207 0.019). Other summary measures are in Table S2.

208 No significant effect on sensitivity was found with test technique. However there was a
209 difference in specificity with real-time quantitative PCR 0.999 (95%CI 0.991 to 1.00)
210 performing better than conventional PCR 0.939 (95%CI 0.872 to 0.972). For fetal sex,
211 11/60 studies reported inconclusive results, of these, 5 studies documented an
212 explanation (in order of frequency): assay failure, no reason given, insufficient number
213 of markers present from pre-specified cut-off and low fetal fraction. The commonest
214 reasons given by the authors of the studies for the false results were: no reason given,
215 low fetal fraction (although cffDNA not quantified), low fetal fraction confirmed by
216 authors quantifying cffDNA, possible contamination/DNA degradation/vanishing
217 twin/test failure although not confirmed, and previous male pregnancy, although the
218 latter reason has since been disproven as cell-free fetal DNA is cleared from the
219 maternal circulation hours post-delivery (9).

220

221 ***Rhesus D***

222 Thirty studies (10,290 tests) evaluated fetal Rhesus D status and are represented in
223 Figure 2. Bivariate meta-analysis produced a summary sensitivity of 0.993 (95% CI
224 0.982 to 0.997) and specificity of 0.984 (95% CI 0.964 to 0.993) a positive likelihood
225 ratio of 61 (95% CI 22 to 167) and negative likelihood ratio of 0.007 (95% CI 0.003 to
226 0.186). There was a significant difference between test techniques with real-time
227 quantitative PCR sensitivity: 0.997 (95% CI 0.987 to 0.999) demonstrating a higher
228 sensitivity than conventional PCR 0.924 (95%CI 0.832 to 0.968), although it was not
229 possible to assess if there was a difference in those which utilised mass spectrometry
230 (despite sufficient studies, due to convergence issues as detailed in the discussion),

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

239

240 ***Trisomy 21***

241 Thirty-one studies (148,344 tests) assessed trisomy 21 and are represented in Figure
242 3A. Bivariate meta-analysis produced a summary sensitivity of 0.994 (95% CI 0.983 to
243 0.998) and specificity of 0.999 (95% CI 0.999 to 1.00) a positive likelihood ratio of 1720
244 (95% CI 1111 to 2662) and negative likelihood ratio of 0.006 (95% CI 0.002 to 0.017).
245 Test technique and population risk had no significant effect. For trisomy 21, 14/31
246 studies reported inconclusive results, of these, 7 studies documented an explanation
247 (in order of frequency): assay failure, confirmed low fetal fraction, no reason given,
248 presumed low fetal fraction/inadequate sequencing depth. The commonest reasons
249 given for false results were: confirmed low fetal fraction, confirmed mosaicism, no
250 reason given, test failure, maternal CNV.

251

252 ***Trisomy 18***

253 Twenty-four studies (146,940 tests) assessed trisomy 18 and are represented in Figure
254 3B. Bivariate meta-analysis produced a summary sensitivity of 0.977 (95% CI 0.952 to
255 0.989) and specificity of 0.999 (95% CI 0.998 to 1.00) and a positive likelihood ratio of
256 1569 (95% CI 810 to 3149) and negative likelihood ratio of 0.023 (95% CI 0.011 to
257 0.048). Neither test technique or population risk had a significant effect. For trisomy 18,
258 12/24 studies reported inconclusive results, of these 7 studies documented an

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

263

264 ***Monosomy X***

265 Eight studies (6712 tests) assessed monosomy X and are represented in Figure 3C.
266 Bivariate meta-analysis produced a summary sensitivity of 0.929 (95% CI 0.741 to
267 0.984) and specificity of 0.999 (95% CI 0.995 to 0.999) and a positive likelihood ratio of
268 1337 (95% CI 213 to 8407) and negative likelihood ratio of 0.071 (95% CI 0.017 to
269 0.292). There was no significant difference with test technique. It was not possible to
270 assess the effect of population risk as there were insufficient low-risk studies. For
271 monosomy X, 5/8 studies reported inconclusive results, of these, 3 studies documented
272 an explanation (in order of frequency): low fetal fraction, presumed human error and no
273 reason given. The commonest reasons given for false results were: mosaicism and no
274 reason given.

275

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

279

280 ***Trisomy 13 – univariate meta-analysis***

281 Sixteen studies which equates to 134,691 tests examined trisomy 13, represented in
282 Figure 3D. There was a summary sensitivity of 0.906 (95% CI 0.823 to 0.958) and
283 specificity of 1.00 (95% CI 0.999 to 1.00). The positive likelihood ratio was 453 (95% CI
284 26 to 7864) and negative likelihood ratio was 0.188 (95% CI 0.080 to 0.44039) with a
285 diagnostic odds ratio of 2788 (95% CI 285 to 27252). For trisomy 13, 6/16 studies
286 reported inconclusive results, of these, 4 studies documented an explanation for

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

290

291 ***Results where meta-analysis not possible***

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

293

294 **Clinical application for NIPT for Down's syndrome screening**

295 Using published data from the National Down Syndrome Cytogenetic Register
296 (NDSCR) 2012 Annual report we have produced a table detailing the estimated
297 outcomes (livebirth rate, invasive test rate, euploid pregnancy loss rate, undiagnosed
298 aneuploidy livebirth rate) from the current standard Down's Syndrome Screening
299 (DSS) i.e. first trimester combined screening pathway (maternal age, nuchal
300 translucency, beta human chorionic gonadotrophin and pregnancy associated plasma
301 protein A) and from a pathway with NIPT as both contingent (i.e. NIPT offered to
302 women with a positive screen after first trimester combined screening) and first line
303 screening for a population of 100,000 women using crude rates (144) (Table S4). We
304 use the prevalence reported by NDSCR¹ (trisomy 21: 2.2 per 1000 women, trisomy 18:
305 0.64 per 1000, trisomy 13 0.26 per 1000). This assumes that standards for the first
306 trimester combined screening are "achievable" as described by Fetal Anomaly
307 Screening Programme (FASP) guidance i.e. for trisomy 21 a detection rate of 85% for
308 a screen positive rate of 2% (145). For NIPT the summary measures are those from
309 our meta-analysis. For the contingent screening model the cut-off for high risk is 1:1000
310 from first trimester combined screening with a detection rate of 96% and false positive
311 rate of 12% (146). This model assumes that all women accept screening when offered
312 as it is not possible to determine yet what the uptake of NIPT would be if offered as a
313 first-line test. It also assumes that all women are required to have an invasive test for
314 karyotyping after a screen positive result from combined or NIPT prior to considering

315 termination of pregnancy, thus the invasive test rates will be higher than in a real-life
316 population. It assumes a 0.5% pregnancy loss rate from invasive testing (146).
317
318 These data demonstrate the influence of disease prevalence on test performance. If we
319 compare combined screening with a 1:150 cut-off (i.e. current NHS practice) with NIPT
320 as a first-line test we can reduce the invasive test rate from 2000 to 319 per 100,000
321 women, the euploid pregnancy loss rate from 9 to 1 per 100,000 and the undiagnosed
322 trisomy 21 live births rate from 32 to 1 per 100,000. If NIPT was used as a contingent
323 screening test for a 1:1000 combined screening cut-off (i.e. as a 2nd test following a
324 positive combined screening result at a 1:1000 cut-off) then these figures are reduced
325 even further compared to combined screening with a 1:150 cut-off: 2000 to 222 per
326 100,000 women invasive test rate; 9 to 0 euploid pregnancy loss rate, although there is
327 less of a reduction in undiagnosed trisomy 21 live birth rate from 32 to 10. If NIPT was
328 used as a contingent screening test for a 1:150 combined screening cut-off then these
329 figures are: 2000 per 100,000 women invasive test rate; 0 euploid pregnancy loss and
330 34 undiagnosed trisomy 21 livebirth rate. A two stage contingent screening pathway
331 with a 1:1000 cut-off when compared to NIPT as a first line test affords a reduction in
332 false positive results (12 versus 100 per 100,000 women) that are found at the time of
333 NIPT as the prevalence of disease in the population now undergoing NIPT is much
334 higher. This is at the expense of a 10 fold increase in undiagnosed aneuploidy live
335 births (1 versus 10 per 100,000 women) due to the increased number of false
336 negatives at the first stage of screening that do not undergo NIPT. A cut-off of 1:150 at
337 the first stage for the combined test compared to a 1:150 cut-off for NIPT as a
338 contingent screening test has little effect on the number false negatives (33 versus 34),
339 however the invasive test rate is reduced (2000 versus 188 per 100,000 women).

340

341 **Discussion**

342 ***Main findings***

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

348

349 ***Strengths and limitations***

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

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

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

391

392 ***Interpretation***

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

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

404

405 ***Conclusion***

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

409

410 ***Implications for clinical practice***

411 The findings of this analysis support the use of NIPT as a diagnostic test for fetal sex
412 and Rhesus status due to the nature of these conditions and the populations being
413 tested. For assessment of aneuploidy the test must be considered a “screening test”
414 despite high accuracy due to the low prevalence of disease and influence of biological
415 factors such as CPM. We are aware that the National Screening Committee (NSC) is
416 currently reviewing all the evidence for aneuploidy, and is likely to recommend NIPT as
417 a contingency screening test in the UK (Dr Pranav Pandya, Personal Communication,
418 2015). While for Down’s syndrome screening (DSS) this will ensure access to an
419 accurate, non-invasive test and ensure equity for many more women (i.e. test threshold
420 has less of an impact on offering invasive testing and test can be offered throughout
421 gestation not just in a small first trimester window) this must be balanced with
422 consideration of the important ethical repercussions which need addressing (i.e. a test
423 that can assess for multiple conditions and those with a milder phenotype and also test
424 for conditions within the mother e.g. sex-chromosome anomaly or cancers) (160).
425 There are also counselling implications as access to a non-invasive, highly accurate
426 test still needs careful consideration by parents.

427

428 ***Implications for future research***

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

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

452

453

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463
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465 data interpretation, and drafted the manuscript. RKM assisted extracting the data,
466 contributed to the analysis and data interpretation and amended the manuscript. KH
467 conducted the bivariate meta-analysis and data interpretation and amended the
468 manuscript. SA assisted with data extraction, interpretation of the results and amended
469 the manuscript. MDK conceived, designed and oversaw the work, made final decisions
470 where there were discrepancies and amended the manuscript. MDK is guarantor for
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477 478 **References**

- 479 1. Lo Y, Corbetta N, Chamberlain P, Rai V, et al. Presence of fetal DNA in maternal plasma
480 and serum. *Lancet*. 1997;350(9076):485-7.
481 2. Bréchet P, Mouawia H, Saker A. Diagnostic prénatal non invasif de la mucoviscidose.
482 *Arch Pédiatr*. 2011;18(1):111-8.

- 483 3. Chitty L, S, Barrett A, McKay F, Lench N, Daley R, Jenkins L. Non-invasive prenatal
484 diagnosis of achondroplasia and thanatophoric dysplasia: next-generation sequencing allows
485 for a safer, more accurate, and comprehensive approach. *Prenat Diagn.* 2015;35:656-62
- 486
- 487 4. Bustamante-Aragones A, Trujillo-Tiebas M, Gallego-Merlo J, Rodriguez de Alba M,
488 Gonzalez-Gonzalez C, Cantalapiedra D, et al. Prenatal diagnosis of Huntington disease in
489 maternal plasma: direct and indirect study. *European Journal of Neurology.* 2008;15(12):1338-
490 44.
- 491 5. González-González M, Garcia-Hoyos M, Trujillo-Tiebas M, Bustamante Aragonés A,
492 Rodriguez de Alba M, Alvarez D, et al. Improvement in strategies for the non-invasive prenatal
493 diagnosis of Huntington disease. *J Assist Reprod Genet.* 2008;25(9-10):477-81.
- 494 6. Lench N, Barrett A, Fielding S, McKay F, Hill M, Jenkins L, et al. The clinical
495 implementation of non-invasive prenatal diagnosis for single-gene disorders: challenges and
496 progress made. *Prenat Diagn.* 2013;33(6):555-62.
- 497 7. Tabor A, Alfirevic Z. Update on procedure-related risks for prenatal diagnosis
498 techniques. *Prenat Diagn.* 2010;27(1):1-7.
- 499 8. Lo Y, Tein M, Lau T, Haines C, Leung T, al. e. Quantitative analysis of fetal DNA in
500 maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet.*
501 1998.
- 502 9. Lo Y, Zhang J, Leung T, Lau T, Chang A, Hjelm N. Rapid clearance of fetal DNA from
503 maternal plasma. *Am J Hum Genet.* 1999;64:218-24.
- 504 10. SMFM. Society for Maternal-Fetal Medicine (SMFM) Consult Series #36: Prenatal
505 aneuploidy screening using cell free DNA. *Am J Obstet Gynecol.* 2015;epub ahead of print.
- 506 11. Langlois S, Brock J. SOGC Committee Opinion: Current status in non-invasive prenatal
507 detection of Down syndrome, trisomy 18, and trisomy 13 using cell-free fDNA in maternal
508 plasma. *J Obstet Gynecol Can.* 2013;35:177-81.
- 509 12. ISPD. Position statement from the chromosome abnormality screening committee on
510 behalf of the board of the International Society for Prenatal Diagnosis Charlottesville, VA:
511 International Society for Prenatal Diagnosis; 2015 [cited 06 May 2015]. Available from:
512 <http://www.ispdhome.org/public/news/2015/PositionStatementFinal04082015.pdf>.
- 513 13. Sonek J, Cuckle H. What will be the role of first-trimester ultrasound if cell-free DNA
514 screening for aneuploidy becomes routine. *Ultrasound Obstet Gynecol.* 2014;44:621-30.
- 515 14. Gil M, Quezada M, Revello R, Akolekar R, Nicolaides K. Analysis of cell-free DNA in
516 maternal blood in screening for fetal aneuploidies: updated meta-analysis. *Ultrasound Obstet*
517 *Gynecol.* 2015;45:249-66.
- 518 15. Devaney S, Palomaki G, Scott J, Bianchi D. Noninvasive fetal sex determination using
519 cell-free fetal DNA. *JAMA.* 2011;306:627-36.
- 520 16. Wright C, Wei Y, Higgins J, Sagoo G. Non-invasive prenatal diagnostic test accuracy for
521 fetal sex using cell-free DNA a review and meta-analysis. *BMC Res Notes.* 2012;5:1-11.
- 522 17. Geifman-Holtzman O, Grotegut C, Gaughan J. Diagnostic accuracy of noninvasive fetal
523 Rh genotyping from maternal blood - a meta-analysis. *Am J Obstet Gynecol.* 2006;195:1163-
524 75.
- 525 18. Zhu Y, Zheng Y, Li L, Zhou H, Liao X, Guo J, et al. Diagnostic accuracy of non-invasive
526 fetal RhD genotyping using cell-free fetal DNA: a meta-analysis. *J Maten Fetal Neonatal Med.*
527 2014;27(18):1839-44.
- 528 19. Rutjes A, Reitsma J, Di Nusio M, Smidt N, van Rijn J, Bossuyt P. Evidence of bias and
529 variation in diagnostic accuracy studies. *CMAJ.* 2006;174:469-76.
- 530 20. Cochrane. Cochrane methods working group on systematic reivews of screening and
531 diagnostic tests: recommended methods. Cochrane, editor2011.
- 532 21. Deeks J. Systematic reviews in health care: systematic reviews of diagnostic and
533 screening tests. *BMJ.* 2001;323:157-62.

22. Khan K, Dinnes J, Kleijnen J. Systematic reviews to evaluate diagnostic tests. *Eur J Obstet Gynecol Reprod Biol.* 2001;95:6-11.
23. Irwig L, Tosteson A, Gatsonis C, Lau J, Colditz G, Chalmers T. Guidelines for meta-analyses evaluating diagnostic tests. *Ann Intern Med.* 1994;120:667-76.
24. Whiting P, Rutjes A, Westwood M, Mallett S, Deeks J, Reitsma J, et al. QUADAS-2: A Revised Tool for the Quality Assessment of Diagnostic Accuracy Studies. *Ann Intern Med.* 2011;155(8):529-36.
25. Reitsma J, Glas A, Rutjes A, Scholten R, Bossuyt P, Zwinderman A. Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews. *J Clin Epidemiol.* 2005;58(9):82-90.
26. Takwoingi Y, Guo B, Riley R, Deeks J. Performance of methods for meta-analysis of diagnostic test accuracy with few studies or sparse data. *Stats Methods Med Res.* 2015.
27. Achargui S, Tijane M, Benchemsi N. Génotypage RHD fœtal par PCR dans le plasma de femmes enceintes D négatif. *Transfusion Clinique et Biologique.* 2011;18(1):13-9.
28. Aghanoori MR, Vafaei H, Kavoshi H, Mohamadi S, Goodarzi HR. Sex determination using free fetal DNA at early gestational ages: a comparison between a modified mini-STR genotyping method and real-time PCR. *Am J Obstet Gynecol.* 2012;207(3):202.e1-e8.
29. Akolekar R, Farkas DH, VanAgtmael AL, Bombard AT, Nicolaides KH. Fetal sex determination using circulating cell-free fetal DNA (ccffDNA) at 11 to 13 weeks of gestation. *Prenat Diagn.* 2010;30(10):918-23.
30. Alberti A, Salomon L, Le Lorc'h M, Couloux A, Bussières L, Goupil S, et al. Non-invasive prenatal testing for trisomy 21 based on analysis of cell-free fetal DNA circulating in the maternal plasma. *Prenat Diagn.* 2015;35:471-6.
31. Al-Yatama M, Mustafa A, Ali S, Abraham S, Khan Z, Khaja N. Detection of Y chromosome-specific DNA in the plasma and urine of pregnant women using nested polymerase chain reaction. *Prenat Diagn.* 2001;21(5):399-402.
32. Al-Yatama M, Mustafa A, Al-Kandari F, Khaja N, Zohra K, Monem R, et al. Polymerase-Chain-Reaction-Based Detection of Fetal Rhesus D and Y-Chromosome-Specific DNA in the Whole Blood of Pregnant Women during Different Trimesters of Pregnancy. *Med Princ Pract.* 2007;16(5):327-32.
33. Aykut A, Cotulu O, Onay H, Satol S, Ozkinay F. Determination of fetal rhd status by maternal plasma DNA analysis. *Clin Genet.* 2010;78:S108.
34. Barrett A, McDonnell T, Chan K, Chitty L. Digital PCR Analysis of Maternal Plasma for Noninvasive Detection of Sickle Cell Anemia. *Clin Chem.* 2012;58(6):1026-32.
35. Bianchi D, Parker R, Wentworth J, Mandankumar R, Saffer C, Das A. DNA sequencing versus standard prenatal aneuploidy screening. *New Eng J Med.* 2014;370:799-808.
36. Bijok J, Gorzelnik K, Massalska D, Ilnicka A, Pawlowska B, Zimowski J, et al. Non-invasive prenatal diagnosis of the most common aneuploidies with cell-free fetal DNA in maternal serum - preliminary results. *Ginek Pol.* 2014;85:208-13.
37. Bombard AT, Akolekar R, Farkas DH, VanAgtmael AL, Aquino F, Oeth P, et al. Fetal RHD genotype detection from circulating cell-free fetal DNA in maternal plasma in non-sensitized RhD negative women. *Prenat Diagn.* 2011;31(8):802-8.
38. Boon EMJ, Schleeht HB, Martin P, Daniels G, Vossen R, Den Dunnen JT, et al. Y chromosome detection by Real Time PCR and pyrophosphorolysis-activated DNA isolated from maternal polymerisation using free fetal plasma. *Prenatal Diagnosis.* 2007;27(10):932-7.
39. Bustamante-Aragones A, Rodriguez De Alba M, Gonzalez-Gonzalez C, Trujillo-Tiebas MJ, Diego-Alvarez D, Vallespin E, et al. Foetal sex determination in maternal blood from the seventh week of gestation and its role in diagnosing haemophilia in the foetuses of female carriers. *Haemophilia.* 2008;14(3):593-8.
40. Chen H, Wang T, He G, Zhu L, Ma T. Gene analysis of free fetal DNA in maternal plasma. *Journal of Tongji Medical University.* 2001;21(4):329-31.

41. Chen SP, Lau TK, Zhang CL, Xu CM, Xu ZF, Hu P, et al. A method for noninvasive detection of fetal large deletions/duplications by low coverage massively parallel sequencing. *Prenat Diagn.* 2013;33(6):584-90.
42. Chi C, Hyett J, Finning K, Lee C, Kadir R. Non-invasive first trimester determination of fetal gender: a new approach for prenatal diagnosis of haemophilia. *BJOG.* 2006;113(2):239-42.
43. Chitty L, Finning K, Wade A, Soothill P, Martin B, Oxenford K, et al. Diagnostic accuracy of routine antenatal determination of fetal RHD status across gestation: population based cohort study. *BMJ.* 2014;349(g5243).
44. Chiu R, Lau T, Leung T, Chow K, et al. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *The Lancet.* 2002;360(9338):998-1000.
45. Clausen FB, Christiansen M, Steffensen R, Jorgensen S, Nielsen C, Jakobsen MA, et al. Report of the first nationally implemented clinical routine screening for fetal RHD in D-pregnant women to ascertain the requirement for antenatal RhD prophylaxis. *Transfusion.* 2012;52(4):752-8.
46. Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y. First-trimester fetal sex determination in maternal serum using real-time PCR. *Prenat Diagn.* 2001;21(12):1070-4.
47. Costa JM, Giovangrandi Y, Ernault P, Lohmann L, Nataf V, El Halali N, et al. Fetal RHD genotyping in maternal serum during the first trimester of pregnancy. *Br J Haematol.* 2002;119(1):255-60.
48. Cremonesi L, Galbiati S, Foglieni B, Smid M, Gambini D, Ferrari A, et al. Feasibility Study for a Microchip-Based Approach for Noninvasive Prenatal Diagnosis of Genetic Diseases. *Ann N Y Acad Sci.* 2004;1022(1):105-12.
49. Davalieva K, Dimcev P, Efremov GD, Plaseska-Karanfilska D. Non-invasive fetal sex determination using real-time PCR. *J Matern Fetal Neonatal Med.* 2006;19(6):337-42.
50. Deng ZH, Wu GG, Li Q, Zhang X, Liang YL, Li DC, et al. Noninvasive genotyping of 9 Y-chromosome specific STR loci using circulatory fetal DNA in maternal plasma by multiplex PCR. *Prenat Diagn.* 2006;26(4):362-8.
51. Fan C, Blumenfeld Y, Chitkara U, Hudgins L, Quake S. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *PNAS.* 2008;105(42):16266-71.
52. Fernandez-Martinez FJ, Galindo A, Garcia-Burguillo A, Vargas-Gallego C, Nogues N, Moreno-Garcia M, et al. Noninvasive fetal sex determination in maternal plasma: a prospective feasibility study. *Genet Med.* 2012;14(1):101-6.
53. Ferres M, Lichten L, Sachs A, Lau K, Bianchi D. Early experience with noninvasive DNA testing for aneuploidy in prenatal care. *Prenat Diagn.* 2013;33(Suppl 1):S72.
54. Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion.* 2007;47:2126-33.
55. Gautier E, Benachi A, Giovangrandi Y, Ernault P, Olivi M, Gaillon T, et al. Fetal RhD genotyping by maternal serum analysis: A two-year experience. *Am J Obstet Gynecol.* 2005;192(3):666-9.
56. Ge Q, Bai Y, Liu Z, Liu Q, Yan L, Lu Z. Detection of fetal DNA in maternal plasma by microarray coupled with emulsions PCR. *Clin Chim Acta.* 2006;369:82-8.
57. Ghanta S, Mitchell M, Ames M, Hidestrand M, Simpson P, Goetsch M, et al. Non-invasive prenatal detection of trisomy 21 using tandem single nucleotide polymorphisms. *PloS one.* 2010;5(10):1.
58. Gorduza E, Popescu R, Caba L, Ivanov I, Martiniuc V, Nedelea F, et al. Prenatal diagnosis of 21 trisomy by quantification of methylated fetal DNA in maternal blood: study on 10 pregnancies. *Rev Romana Med Lab.* 2013;21:275-84.
59. Grill S, Banzola I, Li Y, Rekhviashvili T, Legler TJ, Muller SP, et al. High throughput non-invasive determination of foetal Rhesus D status using automated extraction of cell-free foetal DNA in maternal plasma and mass spectrometry. *Arch Gynecol Obstet.* 2009;279(4):533-7.

60. Gunel T, Kalelioglu I, Ermis H, Aydinli K. Detection of fetal RhD gene from maternal blood. *J Turk Ger Gynecol Assoc.* 2010;11(2):82-5.
61. Gutensohn K, Müller S, Thomann K, Stein W, Suren A, Körtge-Jung S, et al. Diagnostic accuracy of noninvasive polymerase chain reaction testing for the determination of fetal rhesus C, c and E status in early pregnancy. *BJOG.* 2010;117(6):722-9.
62. Han S, Ryu J, Bae S, Kim Y, Yang Y, Lee K. Noninvasive fetal RhD genotyping using circulating cell-free fetal DNA from maternal plasma in RhD-negative pregnant women. *J Mol Diagn.* 2012;14(6):648.
63. Hill M, Finning K, Martin P, Hogg J, Meaney C, Norbury G, et al. Non-invasive prenatal determination of fetal sex: translating research into clinical practice. *Clin Genet.* 2011;80(1):68-75.
64. Ho SS, Damayanti Z, Chua WY, Ng BL, Peh CM, Biswas A, et al. Non-invasive prenatal diagnosis of fetal gender using real-time polymerase chain reaction amplification of SRY in maternal plasma. *Ann Acad Med Singapore.* 2004;33(5):S61-2.
65. Hofmann W, Entezami M, Haug K, Blank C, Wustemann M, Schulze B. Diagnostic accuracy for the noninvasive prenatal detection of common autosomal aneuploidies. *Prenat Diagn.* 2013;33(Suppl 1):75.
66. Hromadnikova I, Vechetova L, Vesela K, Benesova B, Doucha J, Vlk R. Non-invasive fetal RHD and RHCE genotyping using real-time PCR testing of maternal plasma in RhD-negative pregnancies. *J Histochem Cytochem.* 2005;53(3):301-5.
67. Hromadnikova I, Vesela K, Doucha J, Nekovarova K, Duskova D, Schrollova R, et al. Non-invasive determination of fetal c and E allele of RHCE gene via real-time PCR testing of extracellular DNA extracted from maternal plasma samples using QIAamp DSP virus kit. *Journal of the Turkish German Gynecology Association.* 2007;8(2):140-5.
68. Hwa H, Ko T, Yen M, Chiang Y. Fetal gender determination using real-time quantitative polymerase chain reaction of maternal plasma. *J Formos Med Assoc.* 2004;103(5):364-68.
69. Hyett J, Gardener G, Stojilkovic-Mikic T, Finning K, Martin P, Rodeck C, et al. Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. *Prenatal Diagnosis.* 2005;25(12):1111-6.
70. Hyland C, Gardener G, Davies H, Ahvenainen M, Flower R, Irwin D, et al. Evaluation of non-invasive prenatal RHD genotyping of the fetus. *Med J Aust.* 2009;191(1):21-5.
71. Kim SY, Lim JH, Park SY, Kim MY, Choi JS, Ryu HM. Non-invasive prenatal determination of fetal gender using QF-PCR analysis of cell-free fetal DNA in maternal plasma. *Clin Chim Acta.* 2012;413(5-6):600-4.
72. Kolialexi A, Tounta G, Apostolou P, Vrettou C, Papantoniou N, Kanavakis W, et al. Early non-invasive detection of fetal Y chromosome sequences in maternal plasma using multiplex PCR. *Eur J Obstet Gynecol Reprod Biol.* 2012;161(1):34-7.
73. Lau TK, Chen F, Pan X, Pooh RK, Jiang F, Li Y. Noninvasive prenatal diagnosis of common fetal chromosomal aneuploidies by maternal plasma DNA sequencing. *J Matern Fetal Neonatal Med.* 2012;25:1370-74.
74. Li Y, Edoardo Di N, Vitucci A, Zimmermann B, et al. Detection of Paternally Inherited Fetal Point Mutations for [beta]-Thalassemia Using Size-Fractionated Cell-Free DNA in Maternal Plasma. *JAMA.* 2005;293(7):843-9.
75. Li Y, Finning K, Daniels G, Hahn S, Zhong X, Holzgreve W. Noninvasive genotyping fetal Kell blood group (KEL1) using cell-free fetal DNA in maternal plasma by MALDI-TOF mass spectrometry. *Prenat Diagn.* 2008;28(3):203-8.
76. Li P-Q, XZhang J, Fan J-H, Zhang Y-Z, Hou H-Y. Development of noninvasive prenatal diagnosis of trisomy 21 by RT-MLPA with a new set of SNP markers. *Arch Gynecol Obstet.* 2014;289:67-73.
77. Liao C, Fu Y-G, Huang S-Y, Fu F, Xie G-E. Rapid noninvasive prenatal diagnosis of Down syndrome with Ion Proton. *Prenat Diagn.* 2013;33(Suppl 1):76.

78. Liao C, Yin A-H, Peng C-F, Fu F, Yang J-X, Li R, et al. Noninvasive prenatal diagnosis of common aneuploidies by semiconductor sequencing. *PNAS*. 2014;111:7415-20.
79. Lim J, Park S, Kim S, Kim D, Choi J, Kim M, et al. Effective detection of fetal sex using circulating fetal DNA in first-trimester maternal plasma. *FASEB J*. 2012;26(1):250-8.
80. Liu F-M, Wang X-Y, Feng X, Wang W, Ye Y-X, Chen H. Feasibility study of using fetal DNA in maternal plasma for non-invasive prenatal diagnosis. *Acta Obstet Gynecol Scand*. 2007;86(5):535-41.
81. Lo Y, Hjelm N, Fidler C, Sargent I, Murphy M, Chamberlain P, et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med*. 1998;339(24):1734-8.
82. Machado I, Castilho L, Pellegrino Jr J, Barini R. Fetal rhd genotyping from maternal plasma in a population with a highly diverse ethnic background. *Rev Assoc Med Bras*. 2006;52(4):232-5.
83. Manzanares S, Entrala C, Sanchez-Gila M, Fernandez-Rosado F, Cobo D, Martinez E, et al. Noninvasive fetal RhD status determination in early pregnancy. *Fetal Diagn Ther*. 2013;35:7-12.
84. Martinhago C, de Oliveira R, Tomitão Canas M, Vagnini L, Alcantara Oliveira J, Petersen C, et al. Accuracy of fetal gender determination in maternal plasma at 5 and 6 weeks of pregnancy. *Prenat Diagn*. 2006;26(13):1219-23.
85. Minon JM, Gerard C, Senterre JM, Schaaps JP, Foidart JM. Routine fetal RHD genotyping with maternal plasma: a four-year experience in Belgium. *Transfusion*. 2008;48(2):373-81.
86. Mohammed N, Kakal F, Somani M, Zafar W. Non-invasive prenatal determination of fetal RhD genotyping from maternal plasma: a preliminary study in Pakistan. *J Coll Physicians Surg Pak*. 2010;20(4):246-9.
87. Moise K, Boring N, O'Shaughnessy R, Simpson L, Wolfe H, Baxter J, et al. Circulating cell-free fetal DNA for the detection of RHD status and sex using reflex fetal identifiers. *Prenat Diagn*. 2013;33:95-101.
88. Mortarino M, Garagiola I, Lotta L, Siboni S, Semprini A, Peyvandi F. Non-invasive tool for foetal sex determination in early gestational age. *Haemophilia*. 2011;17(6):952-6.
89. New M, Tong Y, Yuen T, Jiang P, Pina C, Chan K, et al. Noninvasive prenatal diagnosis of congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. *J Clin Endocrinol Metab*. 2014;99:1022-30.
90. Nicolaides KH, Syngelaki A, Gil M, Atanasova V, Markova D. Validation of targeted sequencing of single-nucleotide polymorphisms for non-invasive prenatal detection of aneuploidy of chromosomes 13, 18, 21, X, and Y. *Prenat Diagn*. 2013;33(6):575-9.
91. Nicolaides K, 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(5):374.e1-e6.
92. Norton M, Brar H, Weiss J, Karimi A, Laurent L, Caughey A, et al. Non-Invasive Chromosomal Evaluation (NICE) Study: results of a multicenter prospective cohort study for detection of fetal trisomy 21 and trisomy 18. *Am J Obstet Gynecol*. 2012;207(2):137.e1-e8.
93. Norton M, Jacobsson B, Swamy G, Laurent L, Ranzini A, Brar H, et al. Cell-free DNA Analysis for Noninvasive Examination of Trisomy. *N Engl J Med*. 2015;0:null.
94. Pergament E, Cuckle H, Zimmermann B, Banjevic M, Sigurjonsson S, Ryan A, et al. Single-nucleotide polymorphism-based noninvasive prenatal screening in a high-risk and low-risk cohort. *Obstet Gynecol*. 2014;124:210-18.
95. Perlado-Marina A, Bustamente-Aragones A, Horcajada L, Trujillo-Tiebas M, Lorda-Sanchez I, Ramos M, et al. Overview of five-years of experience performing non-invasive fetal sex assessment in maternal blood. *Diagnostics*. 2013;3:283-90.
96. Picchiassi E, Coata G, Fanetti A, Centra M, Pennacchi L, Di Renzo G. The best approach for early prediction of fetal gender by using free fetal DNA from maternal plasma. *Prenat Diagn*. 2008;28(6):525-30.

97. Polin H, Reiter A, Brisner M, Danzer M, Weinberger J, Gabriel C. Clinical application of non-invasive fetal blood group genotyping in Upper Austria. *Transfus Med Hemother*. 2013;40(Supp 1):36-7.
98. Porreco R, Garite T, Maurel K, Marusiak B, Ehrich M, van den Boom D. Noninvasive prenatal screening for fetal trisomies 21, 18, 13 and common sex chromosome aneuploidies from maternal blood using massively parallel genomic sequencing of DNA. *Am J Obstet Gynecol*. 2014;211:e1-12.
99. Quezada M, Gil M, Francisco C, Orosz G, Nicolaides K. Screening for trisomies 21, 18 and 13 by cell-free DNA analysis of maternal blood at 11-13 weeks' gestation and the combined test at 11-13 weeks. *Ultrasound Obstet Gynecol*. 2015;45:36-41.
100. Rijnders R, Christiaens G, Bossers B, van de Smagt J, van der Schoot, E, de Haas M. Clinical applications of cell-free fetal DNA from maternal plasma. *Obstet Gynecol*. 2004;130:157-64.
101. Rijnders R, van der School CE, Bossers B, de Vroede M, Christiaens G. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. *Obstet Gynecol*. 2001;98:374-78.
102. Rong Y, Gao JJ, Jiang XQ, Zheng F. Multiplex PCR for 17 Y-Chromosome Specific Short Tandem Repeats (STR) to Enhance the Reliability of Fetal Sex Determination in Maternal Plasma. *Int J Mol Sci*. 2012;13(5):5972-81.
103. Rouillac-Le S, Sérazin V, Brossard Y, Oudin O, Le Van Kim C, Colin Y, et al. Noninvasive fetal RHD genotyping from maternal plasma: Use of a new developed Free DNA Fetal Kit RhD®. *Transfus Clin Biol*. 2007;14(6):572-7.
104. Sbarsi I, Isernia P, Montanari L, Badulli C, Martinetti M, Salvaneschi L. Implementing non-invasive RHD genotyping on cell-free foetal DNA from maternal plasma: the Pavia experience. *Blood transfus*. 2012;10(1).
105. Scheffer PG, van der School CE, Page-Christiaens G, Bossers B, van Erp F, de Haas M. Reliability of Fetal Sex Determination Using Maternal Plasma. *Obstet Gynecol*. 2010;115(1):117-26.
106. Scheffer P, Ait Soussan A, Verhagen O, Page-Christiaens G, Oepkes D, de Haas M, et al. Noninvasive fetal genotyping of human platelet antigen-1a. *BJOG*. 2011;118(11):1392-5.
107. Sehnert AJ, Rhees B, Comstock D, de Feo E, Heilek G, Burke J, et al. Optimal Detection of Fetal Chromosomal Abnormalities by Massively Parallel DNA Sequencing of Cell-Free Fetal DNA from Maternal Blood. *Clin Chem*. 2011;57(7):1042-9.
108. Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo M, Saito H, et al. Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem*. 2001;47:1856-58.
109. Sesarini C, Gimenez M, Redal M, Izbizky G, Aiello H, Argibay P, et al. Non invasive prenatal genetic diagnosis of fetal RhD and sex through the analysis of free fetal DNA in maternal plasma. *Arch Argent Pediatr*. 2009;107(5):405-9.
110. Shaw S, Chen C-Y, Hsiao C-H, Ren Y, Tian F, Tsai C. Non-invasive prenatal testing for whole fetal chromosome aneuploidies: a multi-center prospective cohort trial in Taiwan. *Prenat Diagn*. 2013;33(supp1):81.
111. Sirichotiyakul S, Charoenkwan P, Sanguanserm Sri T. Prenatal diagnosis of homozygous alpha-thalassaemia-1 by cell-free fetal DNA in maternal plasma. *Prenat Diagn*. 2011;32:45-9.
112. Siva S, Johnson S, McCracken S, Morris J. Evaluation of the clinical usefulness of isolation of fetal DNA from the maternal circulation. *Aust N Z J Obstet Gynaecol*. 2003;43(1):10-5.
113. Song K, Ashoor G, Syngelaki A, Wagner M, Birdir C, Struble C, et al. Clinical evaluation of a directed cfDNA analysis method for non-invasive prenatal fetal trisomy detection. *Prenat Diagn*. 2012;32:1-35.
114. 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(7):700-6.

115. Sparks A, Struble C, Wang E, 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(4):319.e1-.e9.

116. Stumm M, Entezami M, Trunk N, Beck M, Löcherbach J, Wegner R-D, et al. Noninvasive prenatal detection of chromosomal aneuploidies using different next generation sequencing strategies and algorithms. *Prenat Diagn.* 2012;32(6):569-77.

117. Stumm M, Entezami M, Haug K, Blank C, Wustemann C, Schulze B, et al. Diagnostic accuracy of random massively parallel sequencing for non-invasive prenatal detection of common autosomal aneuploidies: a collaborative study in Europe. *Prenat Diagn.* 2014;34:185-91.

118. Tong Y, Jin S, Chiu R, Ding C, Chan K, Leung T, et al. Noninvasive Prenatal Detection of Trisomy 21 by an Epigenetic-Genetic Chromosome-Dosage Approach. *Clinical Chemistry.* 2010;56(1):90-8.

119. Tsang JCH, Charoenkwan P, Chow KCK, Jin Y, Wanapirak C, Sanguansermstri T, et al. Mass spectrometry-based detection of hemoglobin E mutation by allele-specific base extension reaction. *Clin Chem.* 2007;53(12):2205-9.

120. Tungwiwat W, Fucharoen G, Fucharoen S, Ratanasiri T, Sanchaisuriya K, Sae-Ung N. Application of maternal plasma DNA analysis for noninvasive prenatal diagnosis of Hb E- β -thalassemia. *Transl Res.* 2007;150(5):319-25.

121. Tungwiwat W, Fucharoen S, Fucharoen G, Ratanasiri T, Sanchaisuriya K. Accuracy of fetal gender detection using a conventional nested PCR assay of maternal plasma in daily practice. *Aust N Z J Obstet Gynaecol.* 2008;48(5):501-4.

122. Turner M, Martin C, O'Leary J. Detection of fetal Rhesus D gene in whole blood of women booking for routine antenatal care. *Eur J Obstet Gynecol Reprod Biol.* 2003;108(1):29-32.

123. Tynan J, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. *Am J Obstet Gynecol.* 2011;204(3):251.e1-.e6.

124. Van den Oever JME, Balkassmi S, Johansson LF, van Scheltema PNA, Suijkerbuijk RF, Hoffer MJV, et al. Successful Noninvasive Trisomy 18 Detection Using Single Molecule Sequencing. *Clin Chem.* 2013;59(4):705-9.

125. Van den Oever JME, Balkassmi S, Verweij EJ, van Iterson M, van Scheltema PNA, Oepkes D, et al. Single Molecule Sequencing of Free DNA from Maternal Plasma for Noninvasive Trisomy 21 Detection. *Clin Chem.* 2012;58(4):699-706.

126. Vecchione G, Tomaiuolo M, Sarno M, Colaizzo D, Petraroli R, Matteo M, et al. Fetal Sex Identification in Maternal Plasma by Means of Short Tandem Repeats on Chromosome X. *Ann N Y Acad Sci.* 2008;1137(1):148-56.

127. Verweij E, deBoer M, van Scheltema P, van den oever J, Boon E, Oepkes D. Non-invasive prenatal diagnosis of trisomy 21: replacing invasive testing or replacing screening? *Am J Obstet Gynecol.* 2012:S313.

128. Vora N, Johnson K, Peter I, Tighiouart H, Ralston S, Craigo S, et al. Circulating cell-free DNA levels increase variably following chorionic villus sampling. *Prenat Diagn.* 2010;30(4):325-8.

129. Wagner J, Džijan S, Pavan-Jukić D, Wagner J, Lauc G. Analysis of multiple loci can increase reliability of detection of fetal Y-chromosome DNA in maternal plasma. *Prenat Diagn.* 2008;28(5):412-6.

130. Wang X, Wang B, Ye S, Liao Y, Wang L, He Z. Non-invasive foetal RHD genotyping via real-time PCR of foetal DNA from Chinese RhD-negative maternal plasma. *Eur J Clin Invest.* 2009;39(7):607-17.

131. Wei C, Saller D, Sutherland J. Detection and Quantification by Homogeneous PCR of Cell-free Fetal DNA in Maternal Plasma. *Clin Chem.* 2001;47(2):336-8.

132. Zadeh NM, Mesbah-Namin A, Ala F. Noninvasive prenatal diagnosis of fetal sex by a new highly sensitive Real-time PCR. *Clin Biochem.* 2011;44(13):S100-01.
133. Zhang H, Gao Y, Jiang F, Fu M, Yuan Y, Guo Y, et al. Non-invasive prenatal testing for trisomies 21, 18 and 13: clinical experience from 146 958 pregnancies. *Ultrasound Obstet Gynecol.* 2015;45(5):530-8.
134. Zhong X, Holzgreve W, Hahn S. Risk free simultaneous prenatal identification of fetal Rhesus D status and sex by multiplex real-time PCR using cell free fetal DNA in maternal plasma. *Swiss Med Wkly.* 2001;131:70-4.
135. Zhu B, Sun Q-W, Lu Y-C, Sun M-M, Wang L-J, Huang X-H. Prenatal fetal sex diagnosis by detecting amelogenin gene in maternal plasma. *Prenat Diagn.* 2005;25(7):577-81.
136. Zhou L, Thorson JA, Nugent C, Davenport RD, Butch SH, Judd WJ. Noninvasive prenatal RHD genotyping by real-time polymerase chain reaction using plasma from D-negative pregnant women. *Am J Obstet Gynecol.* 2005;193(6):1966-71.
137. Zimmermann B, Hill M, Gemelos G, Demko Z, Banjevic M, Baner J, et al. Noninvasive prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of polymorphic loci. *Prenat Diagn.* 2012;32(13):1233-41.
138. Zolotukhina TV, Shilova NV, Voskoboeva EY. Analysis of cell-free fetal DNA in plasma and serum of pregnant women. *J Histochem Cytochem.* 2005;53(3):297-9.
139. Illanes S, Denbow M, Kailasam C, Finning K, Soothill PW. Early detection of cell-free fetal DNA in maternal plasma. *Early Hum Dev.* 2007;83(9):563-6.
140. Santacroce R, Vecchione G, Tomaiuolo M, Sessa F, Sarno M, Colaizzo D, et al. Identification of fetal gender in maternal blood is a helpful tool in the prenatal diagnosis of haemophilia. *Haemophilia.* 2006;12(4):417-22.
141. Smid M, Lagona F, de Benassuti L, Ferrari A, Ferrari M, Cremonesi L. Evaluation of Different Approaches for Fetal DNA Analysis from Maternal Plasma and Nucleated Blood Cells. *Clin Chem.* 1999;45(9):1570-2.
142. Song Y, Huang S, Zhou X, Jiang Y, Qi Q, Bian X, et al. Non-invasive prenatal testing for fetal aneuploidies in the first trimester of pregnancy. *Ultrasound Obstet Gynecol.* 2015;45(1):55-60.
143. Zhao X, Suzumori N, Ozaki Y, Sato T, Suzumori K. Examination of fetal cells and cell-free fetal DNA in maternal blood for fetal gender determination. *Gynecol Obstet Invest.* 2004;58:57-60.
144. Morris J, Springett A. The National Down Syndrome Cytogenetic Register for England and Wales 2012 Annual Report: Queen Mary University of London, Barts and The London School of Medicine and Dentistry 2014.
145. Programmes NS. Fetal Anomaly Screening Programme (FASP) Standards. London: Public Health England; 2015.
146. Morris S, Karlsen S, Chung N, Hill M, Chitty L. Model-based analysis of costs and outcomes of non-invasive prenatal testing for Down's Syndrome using cell free fetal DNA in the UK National Health Service. *Plos One.* 2014;9(4):e935559.
147. Palomaki GE, Kloza EM, Lambert-Messerlian GM, van den Boom D, Ehrich M, Deciu C, et al. Circulating cell free DNA testing: are some test failures informative? *Prenatal Diagnosis.* 2015;35(3):289-93.
148. Mennuti M, Cherry A, Morrisette J, Dugoff L. Is it time to sound an alarm about false-positive cell free DNA testing for fetal aneuploidy? *Am J Obstet Gynecol.* 2013;209:415-9.
149. Ma J, Pan H, Fu J, Yu L, Yang H. Perspective study of non-invasive prenatal testing using cell-free fetal DNA in high-risk population. *Zhonghua Yi Xue Za Zhi.* 2015;95(11):849-52.
150. Wax J, Cartin A, Chard D, Lucas F, Pinette M. Noninvasive prenatal testing: impact of genetic counselling, invasive prenatal diagnosis, and trisomy 21 detection. *J Clin Ultrasound.* 2015;43(1):1-6.

151. Ke WL, Zhao WH, Wany XY. Detection of fetal cell-free DNA in maternal plasma for Down Syndrome, Edward Syndrome and Patau syndrome of high risk fetus. *Int J Clin Exp Med*. 2015;8(6):9525-30.
152. Ahmadi M, Amirizadeh N, Azarkeyvan A, Valikhani A, Sayyadipoor F, Navirouyan M. Fetal RHD genotyping in plasma of RH negative pregnant women by real time PCR. *Vox Sang*. 2015;109:302.
153. Finning K, Tovey S, Desay K, Latham T, Daniels G. UK NHS blood and transplant fetal RHD screening - Giving anti-D only to those who need it! *Vox Sang*. 2015;109:282.
154. Gonenc G, Isci H, Yititer A, Hancer V, Buyukdotan M, Guducu N, et al. Non-invasive prenatal diagnosis of fetal RhD by using free fetal DNA. *Clin Exp Obstet Gynecol*. 2015;42(3):344-46.
155. Hernandez-Gomez M, Ramirez-Arroyo E, Melendez-Hernandez R, Garduno-Zarazua L, Mayen-Molina D. Non-invasive prenatal test (NIPT) in maternal blood by parallel massive sequencing, initial experience in Mexican women and literature review. *Ginecol Obstet Mex*. 2015;83(5):277-88.
156. Sago H, Sekizawa A. Nationwide demonstration project of next-generation sequencing of cell-free DNA in maternal plasma in Japan: 1-year experience. *Prenat Diagn*. 2015;35(4):331-6.
157. Picchiassi E, Di Renzo G, Tarquini F, Bini V, Centra M, Pennacchi L, et al. Non-invasive prenatal RHD genotyping using cell-free fetal DNA from maternal plasma: An Italian experience. *Transfus Med Hemother*. 2015;42(1):22-8.
158. Tarquini F, Picchiassi E, Centra M, Pennacchi L, Galeone F, Bini V, et al. Maternal smoking does not affect the amount of cell-free fetal DNA in maternal plasma during the 1st trimester of pregnancy. *J Obstet Gynecol*. 2015;35(1):42-5.
159. Grace M, Hardisty E, Green N, Davidson E, Stuebe A, Vora N. Cell free DNA testing—interpretation of results using an online calculator. *Am J Obstet Gynecol*. 2015;213(1):30.e1-e4.
160. Bianchi D. Pregnancy: prepare for unexpected prenatal test results. *Nature*. 2015;522:29-30.
161. Chitty L, Kroese M. Realising the promise of non-invasive prenatal testing. *BMJ*. 2015;350.

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924 **Supplementary material legends**

925 **Figure S1** Study selection from initial search

926 **Figure S2** HSROC curves for bivariate analyses

927 **Figure S3** Forest plots of studies bivariate not possible

928 **Figure S4** Bar chart demonstrating quality assessment of included studies from

929 QUADAS-2 risk of bias assessment

930 **Table S1** Characteristics of included studies

931 **Table S2** Bivariate results

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

933 **Table S4** Clinical application for Trisomy 21

934	Appendix S1 Search strategy
935	Appendix S2 Additional statistical methods
936	Appendix S3 Quality assessment results
937	

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

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

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

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