

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

Mackie, Fiona; Hemming, Karla; Allen, Stephanie; Morris, R. Katie; Kilby, Mark; MacKie, Fiona

DOI:
[10.1111/1471-0528.14050](https://doi.org/10.1111/1471-0528.14050)

License:
Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version
Peer reviewed version

Citation for published version (Harvard):
Mackie, F, Hemming, K, Allen, S, Morris, RK, Kilby, M & MacKie, F 2016, 'The accuracy of cell-free fetal DNA based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis', *BJOG: An International Journal of Obstetrics & Gynaecology*. <https://doi.org/10.1111/1471-0528.14050>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

Checked for eligibility: 27/04/2016. This is the peer reviewed version of the following article: Mackie FL, Hemming K, Allen S, Morris RK, Kilby MD. The accuracy of cell-free fetal DNA-based non-invasive prenatal testing in singleton pregnancies: a systematic review and bivariate meta-analysis. *BJOG* 2016; DOI: 10.1111/1471-0528.14050. , which has been published in final form at <http://onlinelibrary.wiley.com/doi/10.1111/1471-0528.14050/full>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving."

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

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

3 Fiona L Mackie (Clinical Research Fellow)¹, Karla Hemming (Senior Lecturer Public
4 Health, Epidemiology and Biostatistics)², Stephanie Allen (Consultant Clinical Scientist
5 Genetics)³, R Katie Morris (Senior Lecturer/Honorary Consultant Maternal Fetal
6 Medicine)^{1,4}, Mark D Kilby (Professor Fetal Medicine)^{1,4}

7 1. Centre for Women's & Children Health and the School of Clinical and Experimental
8 Medicine, College of Medical and Dental Sciences, University of Birmingham,
9 Birmingham, B15 2TT, UK.

10 2. Public Health, Epidemiology and Biostatistics, School of Health and Population
11 Sciences, College of Medical and Dental Sciences, University of Birmingham,
12 Birmingham, B15 2TT, UK.

13 3. West Midlands Regional Genetics Laboratory, Birmingham Women's Hospital NHS
14 Foundation Trust, Mindelsohn Way, Edgbaston, Birmingham, B15 2TG, UK.

15 4. Fetal Medicine Centre, Birmingham Women's Hospital NHS Foundation Trust,
16 Birmingham, B15 2TG, UK.

17

18 **Corresponding Author:** Dr Fiona Mackie. 3rd Floor Academic Department,
19 Birmingham Women's Hospital NHS Foundation Trust, Mindelsohn Way, Edgbaston,
20 Birmingham, B15 2TG, UK. fionamackie@doctors.org.uk +44-121-626-4535

21

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

23

24 **Word Count: 4295**

25

26

27

28

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

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

95

96 **Methods**

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

99 ***Identification of studies***

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

107 ***Study selection***

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

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

117 *Test:* NIPT based on cffDNA in maternal blood, irrespective of condition being
118 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

454 **Acknowledgements:** The articles were translated by FLM and RKM, and Dr Carman
455 Lai. Some of these data have been presented at the British Maternal and Fetal
456 Medicine Society Annual Scientific Conference, 2015 (Mackie FL, Morris RK, Hemming
457 K, Allen S, Kilby MD. Cell-free fetal DNA based non-invasive prenatal testing: a
458 systematic review and meta-analysis of diagnostic accuracy. *Br J Obstet Gynecol*
459 2015;122:Supp 2)

460

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

463

464 **Contribution to authorship:** FLM extracted the data, contributed to the analysis and
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
471 the study.

472

473 **Details of ethical approval:** not required

474

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

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.

- 534 22. Khan K, Dinnes J, Kleijnen J. Systematic reviews to evaluate diagnostic tests. *Eur J*
535 *Obstet Gynecol Reprod Biol.* 2001;95:6-11.
- 536 23. Irwig L, Tosteson A, Gatsonis C, Lau J, Colditz G, Chalmers T. Guidelines for meta-
537 analyses evaluating diagnostic tests. *Ann Intern Med.* 1994;120:667-76.
- 538 24. Whiting P, Rutjes A, Westwood M, Mallett S, Deeks J, Reitsma J, et al. QUADAS-2: A
539 Revised Tool for the Quality Assessment of Diagnostic Accuracy Studies. *Ann Intern Med.*
540 2011;155(8):529-36.
- 541 25. Reitsma J, Glas A, Rutjes A, Scholten R, Bossuyt P, Zinderman A. Bivariate analysis of
542 sensitivity and specificity produces informative summary measures in diagnostic reviews. *J Clin*
543 *Epidemiol.* 2005;58(982-90).
- 544 26. Takwoingi Y, Guo B, Riley R, Deeks J. Performance of methods for meta-analysis of
545 diagnostic test accuracy with few studies or sparse data. *Stats Methods Med Res.* 2015.
- 546 27. Achargui S, Tijane M, Benchemsi N. Génotypage RHD fœtal par PCR dans le plasma de
547 femmes enceintes D négatif. *Transfusion Clinique et Biologique.* 2011;18(1):13-9.
- 548 28. Aghanoori MR, Vafaei H, Kavoshi H, Mohamadi S, Goodarzi HR. Sex determination
549 using free fetal DNA at early gestational ages: a comparison between a modified mini-STR
550 genotyping method and real-time PCR. *Am J Obstet Gynecol.* 2012;207(3):202.e1-e8.
- 551 29. Akolekar R, Farkas DH, VanAgtmael AL, Bombard AT, Nicolaides KH. Fetal sex
552 determination using circulating cell-free fetal DNA (ccffDNA) at 11 to 13 weeks of gestation.
553 *Prenat Diagn.* 2010;30(10):918-23.
- 554 30. Alberti A, Salomon L, Le Lorc'h M, Couloux A, Bussieres L, Goupil S, et al. Non-invasive
555 prenatal testing for trisomy 21 based on analysis of cell-free fetal DNA circulating in the
556 maternal plasma. *Prenat Diagn.* 2015;35:471-6.
- 557 31. Al-Yatama M, Mustafa A, Ali S, Abraham S, Khan Z, Khaja N. Detection of Y
558 chromosome-specific DNA in the plasma and urine of pregnant women using nested
559 polymerase chain reaction. *Prenat Diagn.* 2001;21(5):399-402.
- 560 32. Al-Yatama M, Mustafa A, Al-Kandari F, Khaja N, Zohra K, Monem R, et al. Polymerase-
561 Chain-Reaction-Based Detection of Fetal Rhesus D and Y-Chromosome-Specific DNA in the
562 Whole Blood of Pregnant Women during Different Trimesters of Pregnancy. *Med Princ Pract.*
563 2007;16(5):327-32.
- 564 33. Aykut A, Cotulu O, Onay H, Satol S, Ozkinay F. Determination of fetal rhd status by
565 maternal plasma DNA analysis. *Clin Genet.* 2010;78:S108.
- 566 34. Barrett A, McDonnell T, Chan K, Chitty L. Digital PCR Analysis of Maternal Plasma for
567 Noninvasive Detection of Sickle Cell Anemia. *Clin Chem.* 2012;58(6):1026-32.
- 568 35. Bianchi D, Parker R, Wentworth J, Mandankumar R, Saffer C, Das A. DNA sequencing
569 versus standard prenatal aneuploidy screening. *New Eng J Med.* 2014;370:799-808.
- 570 36. Bijok J, Gorzelnik K, Massalska D, Ilnicka A, Pawlowska B, Zimowski J, et al. Non-
571 invasive prenatal diagnosis of the most common aneuploidies with cell-free fetal DNA in
572 maternal serum - preliminary results. *Ginekol Pol.* 2014;85:208-13.
- 573 37. Bombard AT, Akolekar R, Farkas DH, VanAgtmael AL, Aquino F, Oeth P, et al. Fetal RHD
574 genotype detection from circulating cell-free fetal DNA in maternal plasma in non-sensitized
575 RhD negative women. *Prenat Diagn.* 2011;31(8):802-8.
- 576 38. Boon EMJ, Schleeht HB, Martin P, Daniels G, Vossen R, Den Dunnen JT, et al. Y
577 chromosome detection by Real Time PCR and pyrophosphorolysis-activated DNA isolated from
578 maternal polymerisation using free fetal plasma. *Prenatal Diagnosis.* 2007;27(10):932-7.
- 579 39. Bustamante-Aragones A, Rodriguez De Alba M, Gonzalez-Gonzalez C, Trujillo-Tiebas
580 MJ, Diego-Alvarez D, Vallespin E, et al. Foetal sex determination in maternal blood from the
581 seventh week of gestation and its role in diagnosing haemophilia in the foetuses of female
582 carriers. *Haemophilia.* 2008;14(3):593-8.
- 583 40. Chen H, Wang T, He G, Zhu L, Ma T. Gene analysis of free fetal DNA in maternal
584 plasma. *Journal of Tongji Medical University.* 2001;21(4):329-31.

- 585 41. Chen SP, Lau TK, Zhang CL, Xu CM, Xu ZF, Hu P, et al. A method for noninvasive
586 detection of fetal large deletions/duplications by low coverage massively parallel sequencing.
587 *Prenat Diagn.* 2013;33(6):584-90.
- 588 42. Chi C, Hyett J, Finning K, Lee C, Kadir R. Non-invasive first trimester determination of
589 fetal gender: a new approach for prenatal diagnosis of haemophilia. *BJOG.* 2006;113(2):239-
590 42.
- 591 43. Chitty L, Finning K, Wade A, Soothill P, Martin B, Oxenford K, et al. Diagnostic accuracy
592 of routine antenatal determination of fetal RHD status across gestation: population based
593 cohort study. *BMJ.* 2014;349(g5243).
- 594 44. Chiu R, Lau T, Leung T, Chow K, et al. Prenatal exclusion of beta thalassaemia major by
595 examination of maternal plasma. *The Lancet.* 2002;360(9338):998-1000.
- 596 45. Clausen FB, Christiansen M, Steffensen R, Jorgensen S, Nielsen C, Jakobsen MA, et al.
597 Report of the first nationally implemented clinical routine screening for fetal RHD in D-
598 pregnant women to ascertain the requirement for antenatal RhD prophylaxis. *Transfusion.*
599 2012;52(4):752-8.
- 600 46. Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y. First-trimester fetal
601 sex determination in maternal serum using real-time PCR. *Prenat Diagn.* 2001;21(12):1070-4.
- 602 47. Costa JM, Giovangrandi Y, Ernault P, Lohmann L, Nataf V, El Halali N, et al. Fetal RHD
603 genotyping in maternal serum during the first trimester of pregnancy. *Br J Haematol.*
604 2002;119(1):255-60.
- 605 48. Cremonesi L, Galbiati S, Foglieni B, Smid M, Gambini D, Ferrari A, et al. Feasibility Study
606 for a Microchip-Based Approach for Noninvasive Prenatal Diagnosis of Genetic Diseases. *Ann N*
607 *Y Acad Sci.* 2004;1022(1):105-12.
- 608 49. Davalieva K, Dimcev P, Efremov GD, Plaseska-Karanfilska D. Non-invasive fetal sex
609 determination using real-time PCR. *J Matern Fetal Neonatal Med.* 2006;19(6):337-42.
- 610 50. Deng ZH, Wu GG, Li Q, Zhang X, Liang YL, Li DC, et al. Noninvasive genotyping of 9 Y-
611 chromosome specific STR loci using circulatory fetal DNA in maternal plasma by multiplex PCR.
612 *Prenat Diagn.* 2006;26(4):362-8.
- 613 51. Fan C, Blumenfeld Y, Chitkara U, Hudgins L, Quake S. Noninvasive diagnosis of fetal
614 aneuploidy by shotgun sequencing DNA from maternal blood. *PNAS.* 2008;105(42):16266-71.
- 615 52. Fernandez-Martinez FJ, Galindo A, Garcia-Burguillo A, Vargas-Gallego C, Nogues N,
616 Moreno-Garcia M, et al. Noninvasive fetal sex determination in maternal plasma: a prospective
617 feasibility study. *Genet Med.* 2012;14(1):101-6.
- 618 53. Ferres M, Lichten L, Sachs A, Lau K, Bianchi D. Early experience with noninvasive DNA
619 testing for aneuploidy in prenatal care. *Prenat Diagn.* 2013;33(Supp 1):S72.
- 620 54. Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c
621 and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion.* 2007;47:2126-33.
- 622 55. Gautier E, Benachi A, Giovangrandi Y, Ernault P, Olivi M, Gaillon T, et al. Fetal RhD
623 genotyping by maternal serum analysis: A two-year experience. *Am J Obstet Gynecol.*
624 2005;192(3):666-9.
- 625 56. Ge Q, Bai Y, Liu Z, Liu Q, Yan L, Lu Z. Detection of fetal DNA in maternal plasma by
626 microarray coupled with emulsions PCR. *Clin Chim Acta.* 2006;369:82-8.
- 627 57. Ghanta S, Mitchell M, Ames M, Hidestrand M, Simpson P, Goetsch M, et al. Non-
628 invasive prenatal detection of trisomy 21 using tandem single nucleotide polymorphisms. *PLoS*
629 *one.* 2010;5(10):1.
- 630 58. Gorduza E, Popescu R, Caba L, Ivanov I, Martiniuc V, Nedelea F, et al. Prenatal
631 diagnosis of 21 trisomy by quantification of methylated fetal DNA in maternal blood: study on
632 10 pregnancies. *Rev Romana Med Lab.* 2013;21:275-84.
- 633 59. Grill S, Banzola I, Li Y, Rekhviashvili T, Legler TJ, Muller SP, et al. High throughput non-
634 invasive determination of foetal Rhesus D status using automated extraction of cell-free foetal
635 DNA in maternal plasma and mass spectrometry. *Arch Gynecol Obstet.* 2009;279(4):533-7.

- 636 60. Gunel T, Kalelioglu I, Ermis H, Aydinli K. Detection of fetal RhD gene from maternal
637 blood. *J Turk Ger Gynecol Assoc.* 2010;11(2):82-5.
- 638 61. Gutensohn K, Müller S, Thomann K, Stein W, Suren A, Körtge-Jung S, et al. Diagnostic
639 accuracy of noninvasive polymerase chain reaction testing for the determination of fetal
640 rhesus C, c and E status in early pregnancy. *BJOG.* 2010;117(6):722-9.
- 641 62. Han S, Ryu J, Bae S, Kim Y, Yang Y, Lee K. Noninvasive fetal RhD genotyping using
642 circulating cell-free fetal DNA from maternal plasma in RhD-negative pregnant women. *J Mol*
643 *Diagn.* 2012;14(6):648.
- 644 63. Hill M, Finning K, Martin P, Hogg J, Meaney C, Norbury G, et al. Non-invasive prenatal
645 determination of fetal sex: translating research into clinical practice. *Clin Genet.* 2011;80(1):68-
646 75.
- 647 64. Ho SS, Damayanti Z, Chua WY, Ng BL, Peh CM, Biswas A, et al. Non-invasive prenatal
648 diagnosis of fetal gender using real-time polymerase chain reaction amplification of SRY in
649 maternal plasma. *Ann Acad Med Singapore.* 2004;33(5):S61-2.
- 650 65. Hofmann W, Entezami M, Haug K, Blank C, Wustemann M, Schulze B. Diagnostic
651 accuracy for the noninvasive prenatal detection of common autosomal aneuploidies. *Prenat*
652 *Diagn.* 2013;33(Supp 1):75.
- 653 66. Hromadnikova I, Vechetova L, Vesela K, Benesova B, Doucha J, Vlk R. Non-invasive fetal
654 RHD and RHCE genotyping using real-time PCR testing of maternal plasma in RhD-negative
655 pregnancies. *J Histochem Cytochem.* 2005;53(3):301-5.
- 656 67. Hromadnikova I, Vesela K, Doucha J, Nekovarova K, Duskova D, Schrollova R, et al.
657 Non-invasive determination of fetal c and E allele of RHCE gene via real-time PCR testing of
658 extracellular DNA extracted from maternal plasma samples using QIAamp DSP virus kit. *Journal*
659 *of the Turkish German Gynecology Association.* 2007;8(2):140-5.
- 660 68. Hwa H, Ko T, Yen M, Chiang Y. Fetal gender determination using real-time quantitative
661 polymerase chain reaction of maternal plasma. *J Formos Med Assoc.* 2004;103(5):364-68.
- 662 69. Hyett J, Gardener G, Stojilkovic-Mikic T, Finning K, Martin P, Rodeck C, et al. Reduction
663 in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early
664 pregnancy. *Prenatal Diagnosis.* 2005;25(12):1111-6.
- 665 70. Hyland C, Gardener G, Davies H, Ahvenainen M, Flower R, Irwin D, et al. Evaluation of
666 non-invasive prenatal RHD genotyping of the fetus. *Med J Aust.* 2009;191(1):21-5.
- 667 71. Kim SY, Lim JH, Park SY, Kim MY, Choi JS, Ryu HM. Non-invasive prenatal determination
668 of fetal gender using QF-PCR analysis of cell-free fetal DNA in maternal plasma. *Clin Chim Acta.*
669 2012;413(5-6):600-4.
- 670 72. Kolialexi A, Tounta G, Apostolou P, Vrettou C, Papantoniou N, Kanavakis W, et al. Early
671 non-invasive detection of fetal Y chromosome sequences in maternal plasma using multiplex
672 PCR. *Eur J Obstet Gynecol Reprod Biol.* 2012;161(1):34-7.
- 673 73. Lau TK, Chen F, Pan X, Pooh RK, Jiang F, Li Y. Noninvasive prenatal diagnosis of
674 common fetal chromosomal aneuploidies by maternal plasma DNA sequencing. *J Matern Fetal*
675 *Neonatal Med.* 2012;25:1370-74.
- 676 74. Li Y, Edoardo Di N, Vitucci A, Zimmermann B, et al. Detection of Paternally Inherited
677 Fetal Point Mutations for [beta]-Thalassemia Using Size-Fractionated Cell-Free DNA in
678 Maternal Plasma. *JAMA.* 2005;293(7):843-9.
- 679 75. Li Y, Finning K, Daniels G, Hahn S, Zhong X, Holzgreve W. Noninvasive genotyping fetal
680 Kell blood group (KEL1) using cell-free fetal DNA in maternal plasma by MALDI-TOF mass
681 spectrometry. *Prenat Diagn.* 2008;28(3):203-8.
- 682 76. Li P-Q, XZhang J, Fan J-H, Zhang Y-Z, Hou H-Y. Development of noninvasive prenatal
683 diagnosis of trisomy 21 by RT-MLPA with a new set of SNP markers. *Arch Gynecol Obstet.*
684 2014;289:67-73.
- 685 77. Liao C, Fu Y-G, Huang S-Y, Fu F, Xie G-E. Rapid noninvasive prenatal diagnosis of Down
686 syndrome with Ion Proton. *Prenat Diagn.* 2013;33(Supp 1):76.

- 687 78. Liao C, Yin A-H, Peng C-F, Fu F, Yang J-X, Li R, et al. Noninvasive prenatal diagnosis of
688 common aneuploidies by semiconductor sequencing. *PNAS*. 2014;111:7415-20.
- 689 79. Lim J, Park S, Kim S, Kim D, Choi J, Kim M, et al. Effective detection of fetal sex using
690 circulating fetal DNA in first-trimester maternal plasma. *FASEB J*. 2012;26(1):250-8.
- 691 80. Liu F-M, Wang X-Y, Feng X, Wang W, Ye Y-X, Chen H. Feasibility study of using fetal
692 DNA in maternal plasma for non-invasive prenatal diagnosis. *Acta Obstet Gynecol Scand*.
693 2007;86(5):535-41.
- 694 81. Lo Y, Hjelm N, Fidler C, Sargent I, Murphy M, Chamberlain P, et al. Prenatal diagnosis of
695 fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med*. 1998;339(24):1734-8.
- 696 82. Machado I, Castilho L, Pellegrino Jr J, Barini R. Fetal rhd genotyping from maternal
697 plasma in a population with a highly diverse ethnic background. *Rev Assoc Med Bras*
698 2006;52(4):232-5.
- 699 83. Manzanares S, Entrala C, Sanchez-Gila M, Fernandez-Rosado F, Cobo D, Martinez E, et
700 al. Noninvasive fetal RhD status determination in early pregnancy. *Fetal Diagn Ther*. 2013;35:7-
701 12.
- 702 84. Martinhago C, de Oliveira R, Tomitão Canas M, Vagnini L, Alcantara Oliveira J, Petersen
703 C, et al. Accuracy of fetal gender determination in maternal plasma at 5 and 6 weeks of
704 pregnancy. *Prenat Diagn*. 2006;26(13):1219-23.
- 705 85. Minon JM, Gerard C, Senterre JM, Schaaps JP, Foidart JM. Routine fetal RHD
706 genotyping with maternal plasma: a four-year experience in Belgium. *Transfusion*.
707 2008;48(2):373-81.
- 708 86. Mohammed N, Kakal F, Somani M, Zafar W. Non-invasive prenatal determination of
709 fetal RhD genotyping from maternal plasma: a preliminary study in Pakistan. *J Coll Physicians*
710 *Surg Pak*. 2010;20(4):246-9.
- 711 87. Moise K, Boring N, O'Shaughnessy R, Simpson L, Wolfe H, Baxter J, et al. Circulating
712 cell-free fetal DNA for the detection of RHD status and sex using reflex fetal identifiers. *Prenat*
713 *Diagn*. 2013;33:95-101.
- 714 88. Mortarino M, Garagiola I, Lotta L, Siboni S, Semprini A, Peyvandi F. Non-invasive tool
715 for foetal sex determination in early gestational age. *Haemophilia*. 2011;17(6):952-6.
- 716 89. New M, Tong Y, Yuen T, Jiang P, Pina C, Chan K, et al. Noninvasive prenatal diagnosis of
717 congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. *J Clin Endocrinol*
718 *Metab*. 2014;99:1022-30.
- 719 90. Nicolaidis KH, Syngelaki A, Gil M, Atanasova V, Markova D. Validation of targeted
720 sequencing of single-nucleotide polymorphisms for non-invasive prenatal detection of
721 aneuploidy of chromosomes 13, 18, 21, X, and Y. *Prenat Diagn*. 2013;33(6):575-9.
- 722 91. Nicolaidis K, Syngelaki A, Ashoor G, Birdir C, Touzet G. Noninvasive prenatal testing for
723 fetal trisomies in a routinely screened first-trimester population. *Am J Obstet Gynecol*.
724 2012;207(5):374.e1-e6.
- 725 92. Norton M, Brar H, Weiss J, Karimi A, Laurent L, Caughey A, et al. Non-Invasive
726 Chromosomal Evaluation (NICE) Study: results of a multicenter prospective cohort study for
727 detection of fetal trisomy 21 and trisomy 18. *Am J Obstet Gynecol*. 2012;207(2):137.e1-e8.
- 728 93. Norton M, Jacobsson B, Swamy G, Laurent L, Ranzini A, Brar H, et al. Cell-free DNA
729 Analysis for Noninvasive Examination of Trisomy. *N Engl J Med*. 2015;0:null.
- 730 94. Pergament E, Cuckle H, Zimmermann B, Banjevic M, Sigurjonsson S, Ryan A, et al.
731 Single-nucleotide polymorphism-based noninvasive prenatal screening in a high-risk and low-
732 risk cohort. *Obstet Gynecol*. 2014;124:210-18.
- 733 95. Perlado-Marina A, Bustamente-Aragones A, Horcajada L, Trujillo-Tiebas M, Lorda-
734 Sanchez I, Ramos M, et al. Overview of five-years of experience performing non-invasive fetal
735 sex assessment in maternal blood. *Diagnostics*. 2013;3:283-90.
- 736 96. Picchiassi E, Coata G, Fanetti A, Centra M, Pennacchi L, Di Renzo G. The best approach
737 for early prediction of fetal gender by using free fetal DNA from maternal plasma. *Prenat*
738 *Diagn*. 2008;28(6):525-30.

739 97. Polin H, Reiter A, Brisner M, Danzer M, Weinberger J, Gabriel C. Clinical application of
740 non-invasive fetal blood group genotyping in Upper Austria. *Transfus Med Hemother*.
741 2013;40(Supp 1):36-7.

742 98. Porreco R, Garite T, Maurel K, Marusiak B, Ehrich M, van den Boom D. Noninvasive
743 prenatal screening for fetal trisomies 21, 18, 13 and common sex chromosome aneuploidies
744 from maternal blood using massively parallel genomic sequencing of DNA. *Am J Obstet*
745 *Gynecol*. 2014;211:e1-12.

746 99. Quezada M, Gil M, Francisco C, Orosz G, Nicolaides K. Screening for trisomies 21, 18
747 and 13 by cell-free DNA analysis of maternal blood at 11-13 weeks' gestation and the
748 combined test at 11-13 weeks. *Ultrasound Obstet Gynecol*. 2015;45:36-41.

749 100. Rijnders R, Christiaens G, Bossers B, van de Smagt J, van der Schoot, E, de Haas M.
750 Clinical applications of cell-free fetal DNA from maternal plasma. *Obstet Gynecol*.
751 2004;130:157-64.

752 101. Rijnders R, van der School CE, Bossers B, de Vroede M, Christiaens G. Fetal sex
753 determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia.
754 *Obstet Gynecol*. 2001;98:374-78.

755 102. Rong Y, Gao JJ, Jiang XQ, Zheng F. Multiplex PCR for 17 Y-Chromosome Specific Short
756 Tandem Repeats (STR) to Enhance the Reliability of Fetal Sex Determination in Maternal
757 Plasma. *Int J Mol Sci*. 2012;13(5):5972-81.

758 103. Rouillac-Le S, Sérazin V, Brossard Y, Oudin O, Le Van Kim C, Colin Y, et al. Noninvasive
759 fetal RHD genotyping from maternal plasma: Use of a new developed Free DNA Fetal Kit RhD®.
760 *Transfus Clin Biol*. 2007;14(6):572-7.

761 104. Sbarsi I, Isernia P, Montanari L, Badulli C, Martinetti M, Salvaneschi L. Implementing
762 non-invasive RHD genotyping on cell-free foetal DNA from maternal plasma: the Pavia
763 experience. *Blood transfus*. 2012;10(1).

764 105. Scheffer PG, van der School CE, Page-Christiaens G, Bossers B, van Erp F, de Haas M.
765 Reliability of Fetal Sex Determination Using Maternal Plasma. *Obstet Gynecol*.
766 2010;115(1):117-26.

767 106. Scheffer P, Ait Soussan A, Verhagen O, Page-Christiaens G, Oepkes D, de Haas M, et al.
768 Noninvasive fetal genotyping of human platelet antigen-1a. *BJOG*. 2011;118(11):1392-5.

769 107. Sehnert AJ, Rhees B, Comstock D, de Feo E, Heilek G, Burke J, et al. Optimal Detection
770 of Fetal Chromosomal Abnormalities by Massively Parallel DNA Sequencing of Cell-Free Fetal
771 DNA from Maternal Blood. *Clin Chem*. 2011;57(7):1042-9.

772 108. Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo M, Saito H, et al. Accuracy of fetal
773 gender determination by analysis of DNA in maternal plasma. *Clin Chem*. 2001;47:1856-58.

774 109. Sesarini C, Gimenez M, Redal M, Izbizky G, Aiello H, Argibay P, et al. Non invasive
775 prenatal genetic diagnosis of fetal RhD and sex through the analysis of free fetal DNA in
776 maternal plasma. *Arch Argent Pediatr*. 2009;107(5):405-9.

777 110. Shaw S, Chen C-Y, Hsiao C-H, Ren Y, Tian F, Tsai C. Non-invasive prenatal testing for
778 whole fetal chromosome aneuploidies: a multi-center prospective cohort trial in Taiwan.
779 *Prenat Diagn*. 2013;33(supp1):81.

780 111. Sirichotiyakul S, Charoenkwan P, Sanguanserm Sri T. Prenatal diagnosis of homozygous
781 alpha-thalassaemia-1 by cell-free fetal DNA in maternal plasma. *Prenat Diagn*. 2011;32:45-9.

782 112. Siva S, Johnson S, McCracken S, Morris J. Evaluation of the clinical usefulness of
783 isolation of fetal DNA from the maternal circulation. *Aust N Z J Obstet Gynaecol*.
784 2003;43(1):10-5.

785 113. Song K, Ashoor G, Syngelaki A, Wagner M, Birdir C, Struble C, et al. Clinical evaluation
786 of a directed cfDNA analysis method for non-invasive prenatal fetal trisomy detection. *Prenat*
787 *Diagn*. 2012;32:1-35.

788 114. Song Y, Liu C, Qi H, Zhang Y, Bian X, Liu J. Noninvasive prenatal testing of fetal
789 aneuploidies by massively parallel sequencing in a prospective Chinese population. *Prenat*
790 *Diagn*. 2013;33(7):700-6.

- 791 115. Sparks A, Struble C, Wang E, Song K, Oliphant A. Noninvasive prenatal detection and
792 selective analysis of cell-free DNA obtained from maternal blood: evaluation for trisomy 21
793 and trisomy 18. *Am J Obstet Gynecol.* 2012;206(4):319.e1-.e9.
- 794 116. Stumm M, Entezami M, Trunk N, Beck M, Löcherbach J, Wegner R-D, et al. Noninvasive
795 prenatal detection of chromosomal aneuploidies using different next generation sequencing
796 strategies and algorithms. *Prenat Diagn.* 2012;32(6):569-77.
- 797 117. Stumm M, Entezami M, Haug K, Blank C, Wustemann C, Schulze B, et al. Diagnostic
798 accuracy of random massively parallel sequencing for non-invasive prenatal detection of
799 common autosomal aneuploidies: a collaborative study in Europe. *Prenat Diagn.* 2014;34:185-
800 91.
- 801 118. Tong Y, Jin S, Chiu R, Ding C, Chan K, Leung T, et al. Noninvasive Prenatal Detection of
802 Trisomy 21 by an Epigenetic-Genetic Chromosome-Dosage Approach. *Clinical Chemistry.*
803 2010;56(1):90-8.
- 804 119. Tsang JCH, Charoenkwan P, Chow KCK, Jin Y, Wanapirak C, Sanguanserm Sri T, et al.
805 Mass spectrometry-based detection of hemoglobin E mutation by allele-specific base
806 extension reaction. *Clin Chem.* 2007;53(12):2205-9.
- 807 120. Tungwiwat W, Fucharoen G, Fucharoen S, Ratanasiri T, Sanchaisuriya K, Sae-Ung N.
808 Application of maternal plasma DNA analysis for noninvasive prenatal diagnosis of Hb E- β -
809 thalassemia. *Transl Res.* 2007;150(5):319-25.
- 810 121. Tungwiwat W, Fucharoen S, Fucharoen G, Ratanasiri T, Sanchaisuriya K. Accuracy of
811 fetal gender detection using a conventional nested PCR assay of maternal plasma in daily
812 practice. *Aust N Z J Obstet Gynaecol.* 2008;48(5):501-4.
- 813 122. Turner M, Martin C, O'Leary J. Detection of fetal Rhesus D gene in whole blood of
814 women booking for routine antenatal care. *Eur J Obstet Gynecol Reprod Biol.* 2003;108(1):29-
815 32.
- 816 123. Tynan J, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed
817 analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD
818 testing. *Am J Obstet Gynecol.* 2011;204(3):251.e1-.e6.
- 819 124. Van den Oever JME, Balkassmi S, Johansson LF, van Scheltema PNA, Suijkerbuijk RF,
820 Hoffer MJV, et al. Successful Noninvasive Trisomy 18 Detection Using Single Molecule
821 Sequencing. *Clin Chem.* 2013;59(4):705-9.
- 822 125. Van den Oever JME, Balkassmi S, Verweij EJ, van Iterson M, van Scheltema PNA,
823 Oepkes D, et al. Single Molecule Sequencing of Free DNA from Maternal Plasma for
824 Noninvasive Trisomy 21 Detection. *Clin Chem.* 2012;58(4):699-706.
- 825 126. Vecchione G, Tomaiuolo M, Sarno M, Colaizzo D, Petraroli R, Matteo M, et al. Fetal Sex
826 Identification in Maternal Plasma by Means of Short Tandem Repeats on Chromosome X. *Ann*
827 *N Y Acad Sci.* 2008;1137(1):148-56.
- 828 127. Verweij E, deBoer M, van Scheltema P, van den oever J, Boon E, Oepkes D. Non-
829 invasive prenatal diagnosis of trisomy 21: replacing invasive testing or replacing screening?
830 *Am J Obstet Gynecol.* 2012;S313.
- 831 128. Vora N, Johnson K, Peter I, Tighiouart H, Ralston S, Craigo S, et al. Circulating cell-free
832 DNA levels increase variably following chorionic villus sampling. *Prenat Diagn.* 2010;30(4):325-
833 8.
- 834 129. Wagner J, Džijan S, Pavan-Jukić D, Wagner J, Lauc G. Analysis of multiple loci can
835 increase reliability of detection of fetal Y-chromosome DNA in maternal plasma. *Prenat Diagn.*
836 2008;28(5):412-6.
- 837 130. Wang X, Wang B, Ye S, Liao Y, Wang L, He Z. Non-invasive foetal RHD genotyping via
838 real-time PCR of foetal DNA from Chinese RhD-negative maternal plasma. *Eur J Clin Invest.*
839 2009;39(7):607-17.
- 840 131. Wei C, Saller D, Sutherland J. Detection and Quantification by Homogeneous PCR of
841 Cell-free Fetal DNA in Maternal Plasma. *Clin Chem.* 2001;47(2):336-8.

- 842 132. Zadeh NM, Mesbah-Namin A, Ala F. Noninvasive prenatal diagnosis of fetal sex by a
843 new highly sensitive Real-time PCR. *Clin Biochem.* 2011;44(13):S100-01.
- 844 133. Zhang H, Gao Y, Jiang F, Fu M, Yuan Y, Guo Y, et al. Non-invasive prenatal testing for
845 trisomies 21, 18 and 13: clinical experience from 146 958 pregnancies. *Ultrasound Obstet*
846 *Gynecol.* 2015;45(5):530-8.
- 847 134. Zhong X, Holzgreve W, Hahn S. Risk free simultaneous prenatal identification of fetal
848 Rhesus D status and sex by multiplex real-time PCR using cell free fetal DNA in maternal
849 plasma. *Swiss Med Wkly.* 2001;131:70-4.
- 850 135. Zhu B, Sun Q-W, Lu Y-C, Sun M-M, Wang L-J, Huang X-H. Prenatal fetal sex diagnosis by
851 detecting amelogenin gene in maternal plasma. *Prenat Diagn.* 2005;25(7):577-81.
- 852 136. Zhou L, Thorson JA, Nugent C, Davenport RD, Butch SH, Judd WJ. Noninvasive prenatal
853 RHD genotyping by real-time polymerase chain reaction using plasma from D-negative
854 pregnant women. *Am J Obstet Gynecol.* 2005;193(6):1966-71.
- 855 137. Zimmermann B, Hill M, Gemelos G, Demko Z, Banjevic M, Baner J, et al. Noninvasive
856 prenatal aneuploidy testing of chromosomes 13, 18, 21, X, and Y, using targeted sequencing of
857 polymorphic loci. *Prenat Diagn.* 2012;32(13):1233-41.
- 858 138. Zolotukhina TV, Shilova NV, Voskoboeva EY. Analysis of cell-free fetal DNA in plasma
859 and serum of pregnant women. *J Histochem Cytochem.* 2005;53(3):297-9.
- 860 139. Illanes S, Denbow M, Kailasam C, Finning K, Soothill PW. Early detection of cell-free
861 fetal DNA in maternal plasma. *Early Hum Dev.* 2007;83(9):563-6.
- 862 140. Santacroce R, Vecchione G, Tomaiuolo M, Sessa F, Sarno M, Colaizzo D, et al.
863 Identification of fetal gender in maternal blood is a helpful tool in the prenatal diagnosis of
864 haemophilia. *Haemophilia.* 2006;12(4):417-22.
- 865 141. Smid M, Lagona F, de Benassuti L, Ferrari A, Ferrari M, Cremonesi L. Evaluation of
866 Different Approaches for Fetal DNA Analysis from Maternal Plasma and Nucleated Blood Cells.
867 *Clin Chem.* 1999;45(9):1570-2.
- 868 142. Song Y, Huang S, Zhou X, Jiang Y, Qi Q, Bian X, et al. Non-invasive prenatal testing for
869 fetal aneuploidies in the first trimester of pregnancy. *Ultrasound Obstet Gynecol.*
870 2015;45(1):55-60.
- 871 143. Zhao X, Suzumori N, Ozaki Y, Sato T, Suzumori K. Examination of fetal cells and cell-free
872 fetal DNA in maternal blood for fetal gender determination. *Gynecol Obstet Invest.* 2004;58:57-
873 60.
- 874 144. Morris J, Springett A. The National Down Syndrome Cytogenetic Register for England
875 and Wales 2012 Annual Report: Queen Mary University of London, Barts and The London
876 School of Medicine and Dentistry 2014.
- 877 145. Programmes NS. Fetal Anomaly Screening Programme (FASP) Standards. London:
878 Public Health England; 2015.
- 879 146. Morris S, Karlsen S, Chung N, Hill M, Chitty L. Model-based analysis of costs and
880 outcomes of non-invasive prenatal testing for Down's Syndrome using cell free fetal DNA in
881 the UK National Health Service. *Plos One.* 2014;9(4):e935559.
- 882 147. Palomaki GE, Kloza EM, Lambert-Messerlian GM, van den Boom D, Ehrich M, Deciu C,
883 et al. Circulating cell free DNA testing: are some test failures informative? *Prenatal Diagnosis.*
884 2015;35(3):289-93.
- 885 148. Mennuti M, Cherry A, Morrissette J, Dugoff L. Is it time to sound an alarm about false-
886 positive cell free DNA testing for fetal aneuploidy? *Am J Obstet Gynecol.* 2013;209:415-9.
- 887 149. Ma J, Pan H, Fu J, Yu L, Yang H. Perspective study of non-invasive prenatal testing using
888 cell-free fetal DNA in high-risk population. *Zhonghua Yi Xue Za Zhi.* 2015;95(11):849-52.
- 889 150. Wax J, Cartin A, Chard D, Lucas F, Pinette M. Noninvasive prenatal testing: impact of
890 genetic counselling, invasive prenatal diagnosis, and trisomy 21 detection. *J Clin Ultrasound.*
891 2015;43(1):1-6.

- 892 151. Ke WL, Zhao WH, Wany XY. Detection of fetal cell-free DNA in maternal plasma for
893 Down Syndrome, Edward Syndrome and Patau syndrome of high risk fetus. *Int J Clin Exp Med*.
894 2015;8(6):9525-30.
- 895 152. Ahmadi M, Amirizadeh N, Azarkeyvan A, Valikhani A, Sayyadipoor F, Navirouyan M.
896 Fetal RHD genotyping in plasma of RH negative pregnant women by real time PCR. *Vox Sang*.
897 2015;109:302.
- 898 153. Finning K, Tovey S, Desay K, Latham T, Daniels G. UK NHS blood and transplant fetal
899 RHD screening - Giving anti-D only to those who need it! *Vox Sang*. 2015;109:282.
- 900 154. Gonenc G, Isci H, Yititer A, Hancer V, Buyukdotan M, Guducu N, et al. Non-invasive
901 prenatal diagnosis of fetal RhD by using free fetal DNA. *Clin Exp Obstet Gynecol*.
902 2015;42(3):344-46.
- 903 155. Hernandez-Gomez M, Ramirez-Arroyo E, Melendez-Hernandez R, Garduno-Zarazua L,
904 Mayen-Molina D. Non-invasive prenatal test (NIPT) in maternal blood by parallel massive
905 sequencing, initial experience in Mexican women and literature review. *Ginecol Obstet Mex*.
906 2015;83(5):277-88.
- 907 156. Sago H, Sekizawa A. Nationwide demonstration project of next-generation sequencing
908 of cell-free DNA in maternal plasma in Japan: 1-year experience. *Prenat Diagn*. 2015;35(4):331-
909 6.
- 910 157. Picchiassi E, Di Renzo G, Tarquini F, Bini V, Centra M, Pennacchi L, et al. Non-invasive
911 prenatal RHD genotyping using cell-free fetal DNA from maternal plasma: An Italian
912 experience. *Transfus Med Hemother*. 2015;42(1):22-8.
- 913 158. Tarquini F, Picchiassi E, Centra M, Pennacchi L, Galeone F, Bini V, et al. Maternal
914 smoking does not affect the amount of cell-free fetal DNA in maternal plasma during the 1st
915 trimester of pregnancy. *J Obstet Gynecol*. 2015;35(1):42-5.
- 916 159. Grace M, Hardisty E, Green N, Davidson E, Stuebe A, Vora N. Cell free DNA testing–
917 interpretation of results using an online calculator. *Am J Obstet Gynecol*. 2015;213(1):30.e1-
918 .e4.
- 919 160. Bianchi D. Pregnancy: prepare for unexpected prenatal test results. *Nature*.
920 2015;522:29-30.
- 921 161. Chitty L, Kroese M. Realising the promise of non-invasive prenatal testing. *BMJ*.
922 2015;350.

923

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

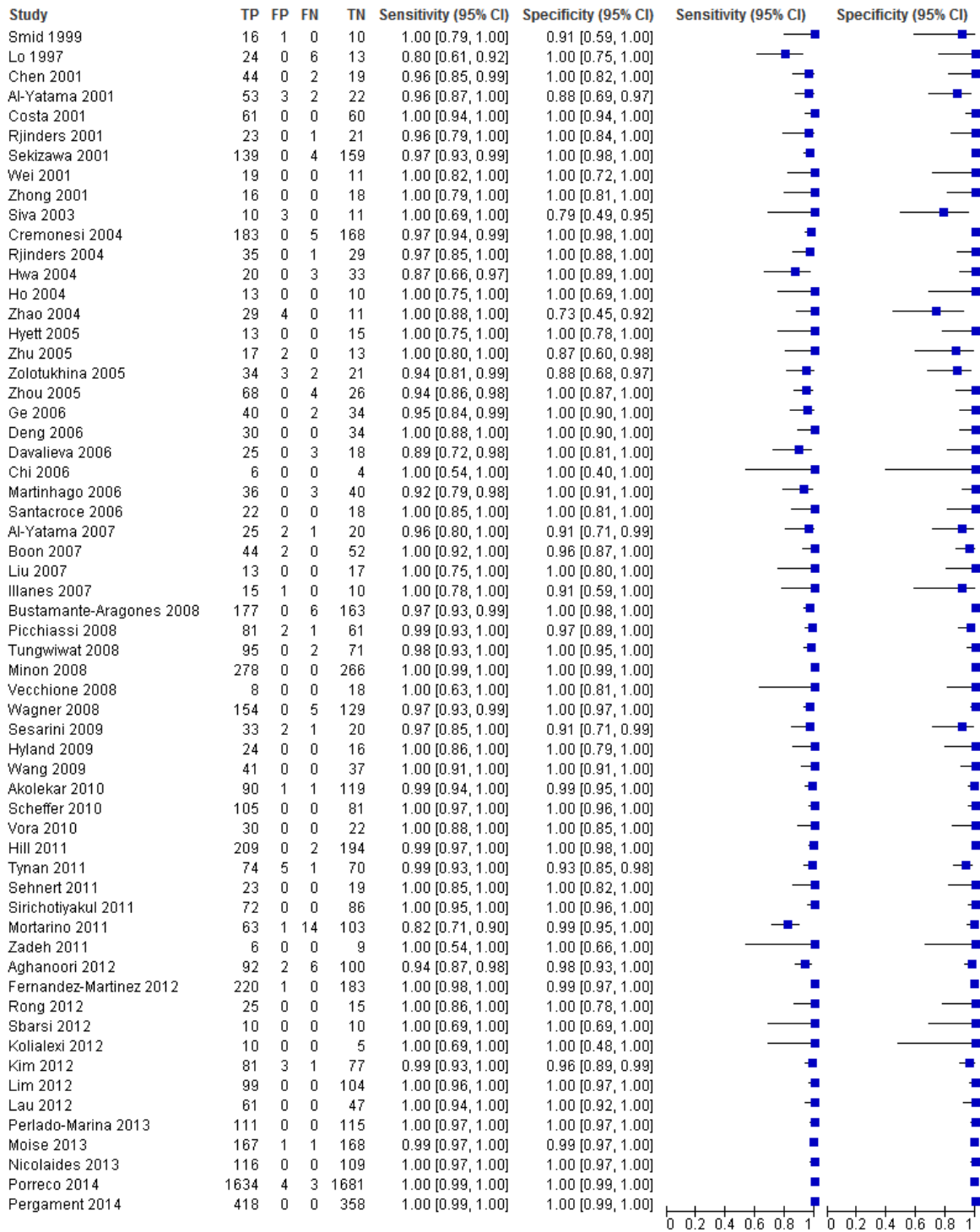


Figure 2: Forest plot of studies testing Rhesus D status using cell-free fetal DNA

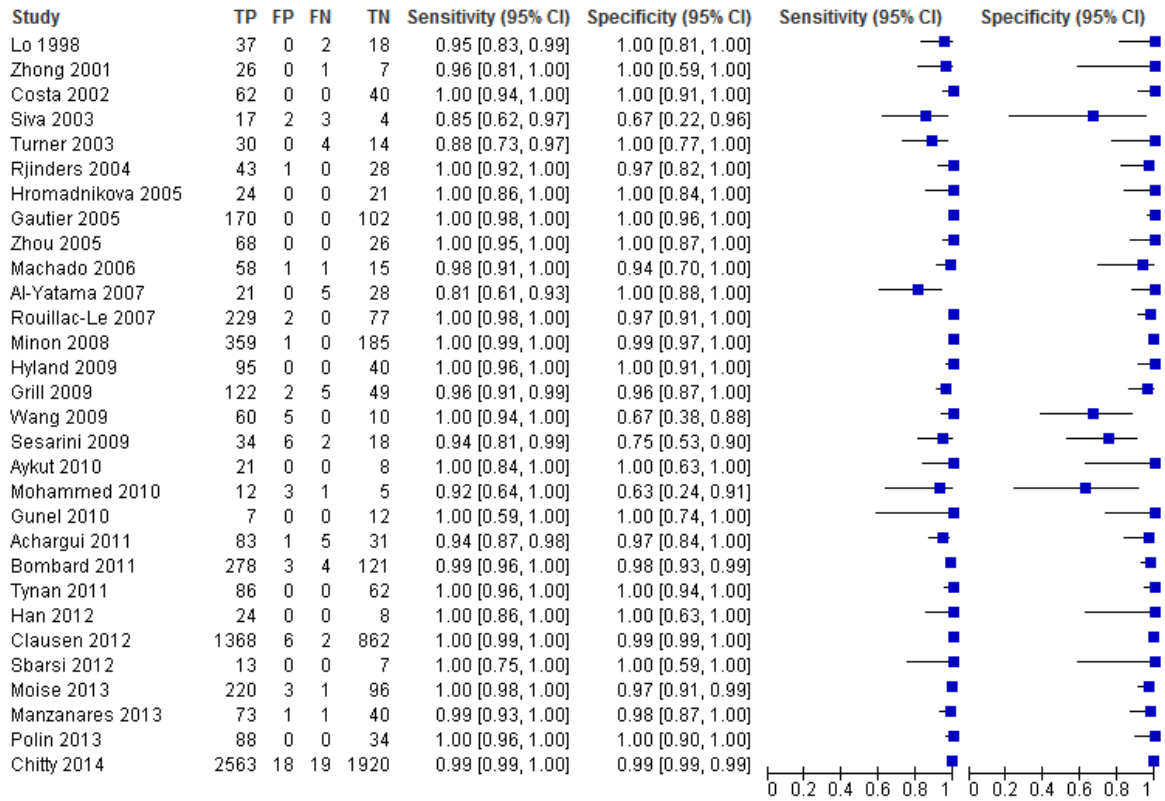


Figure 3A: Forest plot of studies testing Trisomy 21 using cell-free fetal DNA

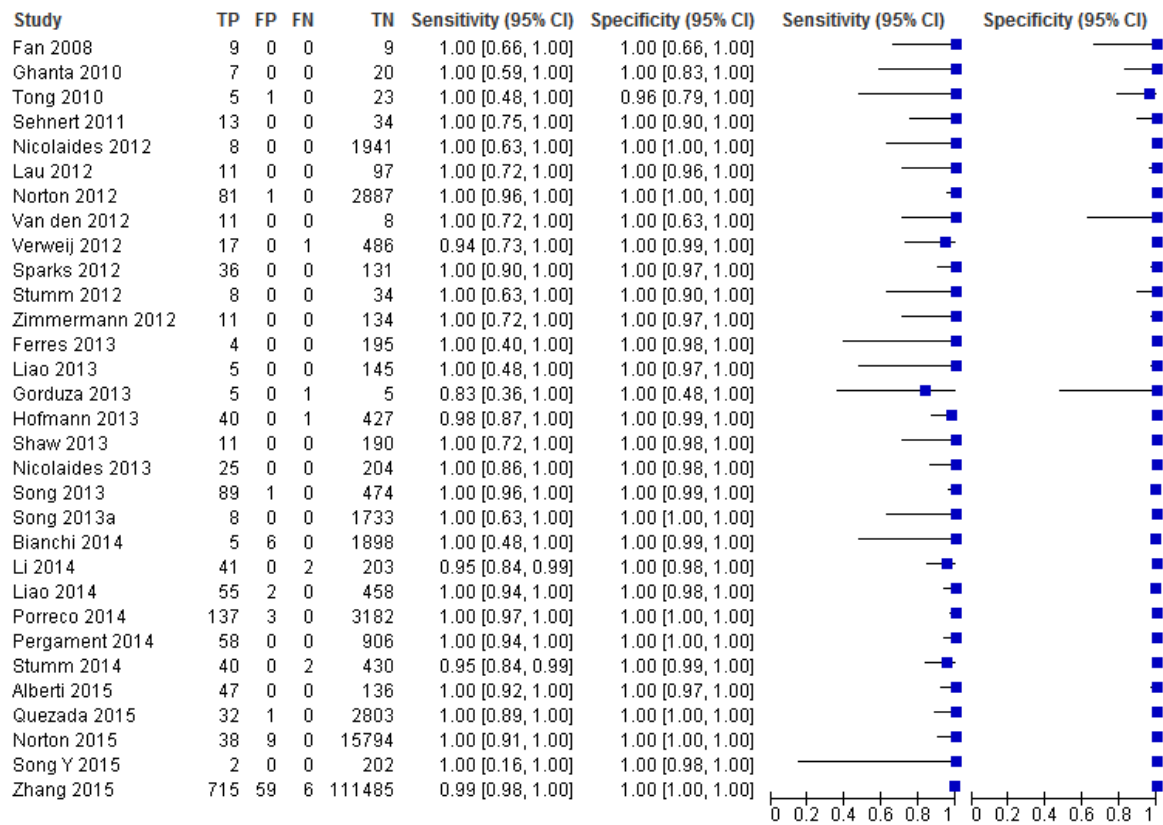


Figure 3B: Forest plot of studies testing Trisomy 18 using cell-free fetal DNA

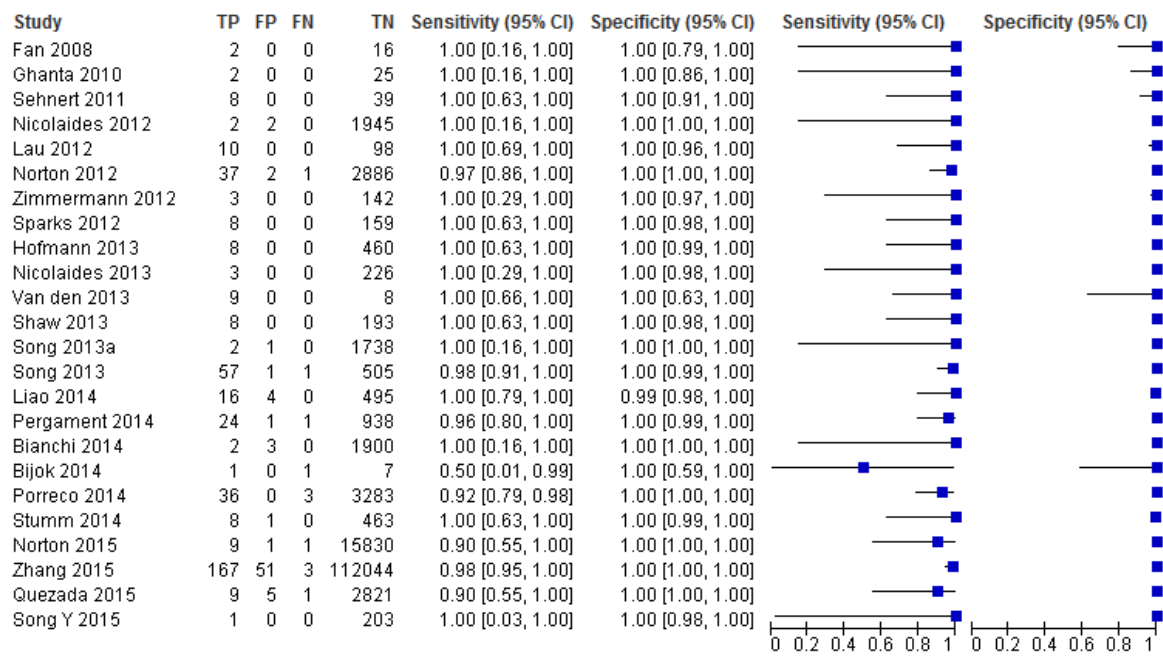


Figure 3C: Forest plot of studies testing Monosomy X using cell-free fetal DNA

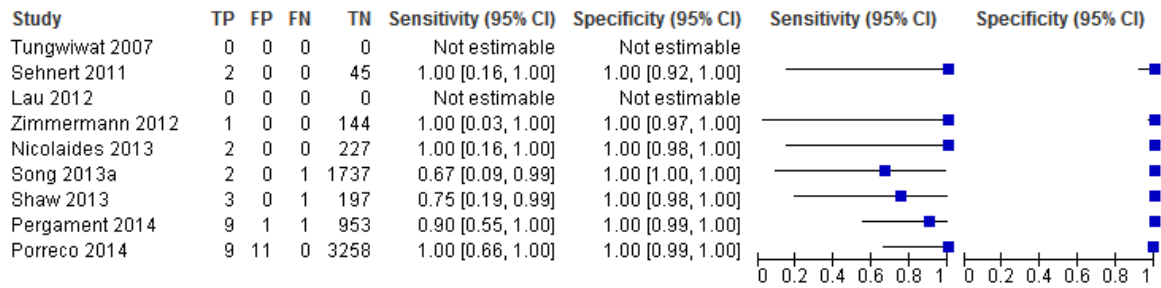


Figure 3D: Forest plot of studies testing Trisomy 13 using cell-free fetal DNA

