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Implications of non-invasive prenatal testing for identifying and managing high-risk pregnancies

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Implications of non-invasive prenatal testing for identifying and managing high-risk pregnancies

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Non-invasive prenatal testing is regularly used to screen for aneuploidies and Rhesus status of a fetus. Since 1997 when free fetal DNA (ffDNA) in the maternal circulation was first identified, it has been hypothesized that it may be possible to use non-invasive prenatal testing (NIPT) to identify high-risk pregnancies including pre-eclampsia, growth restriction and preterm birth. Since then there has been much interest in this area as a way to identify and understand disease processes. This review presents the current evidence for this approach. For pre-eclampsia the hypothesis is that ffDNA would increase but the evidence for this is heterogenous across studies and trimesters. There is however increasing agreement between studies that by the third trimester ffDNA is more likely to be raised in pre-eclamptic patients than controls. For preterm birth, again, the main hypothesis is that ffDNA should increase. The results are also heterogenous, with some studies finding increased ffDNA prior to preterm birth, and others finding no change. For fetal growth restriction, there are competing theories for reduced and increased ffDNA and some studies suggest that levels are raised and some reduced. There are complexities in interpreting all of this evidence as the studies' designs, patient populations, and especially in the context of growth restriction, the definitions are not clear. Furthermore, authors use different biochemical tests and different units to describe their results, making meta-analysis difficult. All of these issues and conflicting findings lead us to the conclusion that currently there is yet no definitive place in clinical practice for NIPT to support the diagnosis and management

of high-risk pregnancies. However, it is vital that this research continues as it could open the door to better understanding of the disease process and novel approaches to management.

Keywords: NIPT, ffDNA, pre-eclampsia, growth restriction, preterm birth

Implications of non-invasive prenatal testing for identifying and managing high-risk pregnancies

Non-invasive prenatal testing (NIPT) became a possibility when fetal DNA in maternal blood was identified by Lo et al in 1997.[1] This breakthrough led to the development and establishment of NIPT to screen for aneuploidies and define the fetal Rhesus status.[2][3]

Cell-free fetal DNA (ffDNA) is likely to come from the placenta as it is detectable from the 5th week of pregnancy and is also detectable in anembryonic pregnancies.[4] Its levels increase with advancing pregnancy,[5] but it is then rapidly cleared following delivery.[6]

These findings led to the theory that ffDNA could be used to identify high-risk pregnancies based on the hypotheses that poor clearance of the ffDNA in pregnancies with abnormal placentation[7], leads to ffDNA rises immediately prior to onset of preterm labour [8], and that a lower ffDNA fraction could be due to a smaller placental mass of a dysfunctional placenta.[9]

Why is there such interest in NIPT for high-risk pregnancies?

NIPT could enable early diagnosis of a condition (e.g. pre-eclampsia) and indicate impending disease.[7] It may also aid difficult diagnoses, for example invasive placenta.[10] Along with the relative ease of the simple blood test, is the benefit of predicting such conditions from the first or early second trimester. A pregnancy specific risk is provided due to the speed of

clearance of ffDNA. It may be particularly useful in an unselected population to define who needs further monitoring[11] or other interventions e.g. Aspirin.[12]

What factors can affect ffDNA levels?

Different factors can alter ffDNA. Several studies suggested that the fetal fraction is lower in women with raised BMI.[12–16] Similarly, smoking lowers ffDNA, [15,16] as does maternal age, IVF, chronic hypertension and diabetes.[9]

The fetal fraction is increased in women with low PAPP-A [15] as early as the first trimester.[16] Ethnic origin plays a role, with the ffDNA being higher in Afro-Caribbean,[12,15] East-Asian,[15] and Hispanic women[12] compared to Caucasian women.

What evidence will be discussed in this review?

We will discuss the current evidence around ffDNA and high-risk pregnancies including pre-eclampsia, intrauterine growth restriction, preterm birth and abnormal placentation.

Pre-Eclampsia

Pre-eclampsia is a multi-organ disease complicating 2-8% of pregnancies. Although the pathogenesis is not fully understood, it is thought to be linked to poor placental function in early pregnancy.[17] Early prediction is important to enable initiation of aspirin before 16 weeks gestation to reduce risk.[18]

Pre-eclampsia is a target for ffDNA measurement due to abnormal placentation. The impaired trophoblastic invasion is thought to lead to placental hypoxia and vascular endothelial cell dysfunction. In turn, due to the placental ischemia, there is thought to be increased necrotic and apoptotic fragments of ffDNA being released.[19]

One of the earliest studies found a 5 fold increase in ffDNA in pre-eclampsia patients compared to controls in the third trimester.[20] Subsequent studies have been heterogenous in terms of populations, gestation at testing, and findings. Studies investigating ffDNA in pre-eclampsia included in this review are presented in table 1.

First trimester

Some studies have found no differences in first trimester ffDNA between women who developed pre-eclampsia and controls. Levine et al conducted a nested cohort study within the Calcium for Preeclampsia Prevention trial cohort of healthy nulliparous women. They found no differences between the 120 cases and controls.[7] Poon et al looked at a cohort of 1949 pregnancies where 64 went on to develop pre-eclampsia, and found that ffDNA concentrations were not increased in the first trimester for those women.[15] Thurik et al conducted a nested case control study in the 'Risk Estimation for Pregnancy complications to provide tailored care' cohort. There were 37 cases of preeclampsia and 96 controls and there were no differences in ffDNA concentrations.[16] Silver et al used a nested case control study within a trial of vitamin C and E. They studied 175 women with pre-eclampsia and 175 controls. Although they measured both total and cell free fetal DNA, they were

72 unable to detect a meaningful level of ffDNA in either group. However total cell free DNA
73 was not different between pre-eclampsia and controls.[12]

74 Several other studies found that the ffDNA concentration is increased during the first
75 trimester in women who later develop pre-eclampsia. Sifakis et al's case-control study
76 included 44 cases of pre-eclampsia (11 delivered before 34 weeks) and 176 controls. FfDNA
77 was higher in women delivering before 34 weeks compared to controls, but not in those
78 developing pre-eclampsia later.[21] Similarly Illanes et al investigated ffDNA in women who
79 went on to have pre-eclampsia and growth restriction. They included 24 controls and 24
80 cases (9 delivered before 35 weeks). They showed no differences between controls and
81 those delivering after 35 weeks, but ffDNA was higher in those delivering before 35 weeks
82 compared to controls.[22] Kim et al investigated women attending for routine screening
83 they recruited 161 controls and 22 pre-eclampsia patients. Of these there were 36 controls
84 and 4 cases of pre-eclampsia with first trimester results. FfDNA concentrations were higher
85 In the 4 patients than those of controls.[23] Papantoniou et al examined the concentrations
86 of the RASSF1A gene (non sex-dependent) to establish the ffDNA. They found that median
87 cffDNA concentration was increased in pre-eclamptic patients compared to controls.[24]

88 More recent studies have examined the fetal fraction, which is the proportion of total cell
89 free DNA that contains ffDNA. Norton et al used a database of 18,955 ffDNA tests to
90 examine the fetal fraction. The mean percentage of ffDNA in the pre-eclampsia patients was
91 higher, however, it was not different between those who developed severe pre-eclampsia
92 compared to any pre-eclampsia.[25] Another study screened women for risk of pre-
93 eclampsia and fetal growth restriction alongside taking a routine ffDNA test for
94 chromosomal abnormalities. Across a group of 4317 patients it was found that the lower the

fetal fraction.[9] In contrast to the study of Norton and colleagues, they found a relationship between more severe pre-eclampsia and lower fetal fraction.[9] Papantoniou et al found a higher proportion of fetal DNA in pre-eclampsia patients compared to controls.[24]

Second trimester:

Second trimester findings, whilst not entirely consistent, lend more weight to their being increased levels of ffDNA in women who develop pre-eclampsia. Leung et al reported higher concentrations in 18 cases than in 33 controls.[26] Zhong et al reported similar findings in 10 cases and 40 controls. Concentrations in cases were at least three times higher than in controls.[27]

Several other studies agreed with these findings. Cotter et al identified 88 cases of pre-eclampsia and 176 controls. They found that when using whole blood samples there was an odds ratio for developing pre-eclampsia of 8.3 (95% CI 2.9-23.4).[28] Farina et al examined low risk women; there were 6 cases matched with 5 controls. Pregnancies developing pre-eclampsia had 2.39 times higher levels of ffDNA compared to controls.[11] In the study by Levine et al, second and third trimester samples were taken in addition to the first trimester ones. Whilst in the first trimester the concentrations of ffDNA were comparable, from 17 weeks the concentration of ffDNA seemed to increase in cases compared to controls. However this relationship was only significant in the pre-eclamptic patients who delivered before 37 weeks.[7]

More recently, Kim et al reported second trimester results for 78 controls and 5 pre-eclampsia patients. FfDNA concentrations were 3.3 times higher in the pre-eclampsia group.[23]

Other studies however disagreed with these findings. Stein et al used a subset of a large cohort study testing RhD status. Of 611 included women, 44 went on to develop pre-eclampsia. No association was shown between the concentration of ffDNA and pre-eclampsia.[29] Crowley et al carried out a nested case control study from a cohort of 1993 women. Pre-eclampsia was diagnosed in 16 women, and there were 72 controls. Median ffDNA levels were similar in cases and controls.[30]

Third trimester:

In the third trimester there was more agreement, with most studies finding higher levels of ffDNA in pre-eclampsia patients. Lo et al studied 20 pre-eclamptic women and 20 controls. The median ffDNA increased fivefold in the pre-eclamptic patients.[20] Swinkels et al reported a 6.1 fold increase in ffDNA in 7 pre-eclamptic women compared to 10 normotensive controls.[31] Tsui et al studied ten pre-eclamptic women recruited at diagnosis together with 20 controls. There was a 4.3 fold higher concentration in pre-eclamptic pregnancies.[32] Alberry et al reported higher ffDNA concentrations in 48 women with pre-eclampsia compared to 77 controls. Furthermore the ffDNA concentrations were higher in the severe compared to mild pre-eclampsia.[13]

Zhong et al recruited 53 controls and 44 women with pre-eclampsia, of which 32 were mild-moderate and 12 were severe. The ffDNA was significantly higher in those with severe vs the less severe forms of pre-eclampsia, with the lowest values in the control group.[33] Lau et al studied 10 control and 7 pre-eclamptic patients (5 severe and 2 mild). Samples were taken before delivery and then at regular intervals until 6 hours post-delivery. There was a 2.3 fold increase in ffDNA between pre-eclampsia and controls.[34] More recently, in a study by Kim et al with 115 controls and 20 women with pre-eclampsia, the ffDNA concentrations were approximately three times higher in the pre-eclampsia group.[23]

Agreements about these findings however has not been universal. In a study by Levine et al, third trimester concentrations of ffDNA seemed to be raised in PET compared to controls. However, this only reached significance for severe pre-eclampsia and pre-eclampsia associated with small for gestations age infants.[7]

Some studies have taken a different approach to quantifying fetal cells in maternal blood, by looking for mRNA rather than DNA. In the two studies discussed here, circulating corticotrophin releasing hormone (CRH) mRNA was quantified. This is important as it is an approach that can be used for male as well as female fetuses. Ng et al obtained samples from 12 women with pre-eclampsia and 10 controls before and 2 hours after delivery. The concentrations were 10.5 times higher in pre-eclampsia.[35] Farina et al recruited 17 controls and 17 cases at a median of 37 weeks and found higher concentrations in women with pre-eclampsia.[36]

Preterm Birth

Preterm birth (PTB) is a leading cause of perinatal morbidity and mortality.[37] Having a test to predict it could lead to insights into pathogenesis[38] and allow appropriate management to be initiated.

The potential of ffDNA to contribute to the prediction of PTB was considered by Lo et al in their seminal paper, as levels would be increased due to the placental barrier being broken down in preparation for delivery.[1] A subsequent study showing an increase in ffDNA levels with preterm birth sparked interest in ffDNA as a potential maker for PTB. [8]

The key PTB studies are summarised in table 2. As with pre-eclampsia, the studies for PTB are heterogenous in population, study design, method of measurement and results.

Studies suggesting ffDNA is increased in PTB

Jakobsen et al undertook a cohort study of 876 women who had participated in a routine Rhesus screening program, with samples taken between 23 and 28 weeks. They found that ffDNA over 95th centile was associated with a 6.3 times higher odds of birth before 37 weeks and a 16.6 fold increase in the odds of birth before 34 weeks.[39] Dugoff et al performed a retrospective analysis of a cohort of 1349 women at increased risk of aneuploidy. Among those tested at 14-20 weeks, ffDNA at or above the 95th percentile was associated with an increased risk of birth before 37 weeks (OR 4.59) and before 34 weeks (OR 22.0). There was no difference for tests performed at 10-14 weeks.[40]

190

191 Leung et al found increased median levels of ffDNA in preterm birth in a case-control study
192 of 13 preterm births and 17 controls at the time of presentation with threatened pre-term
193 labour.[8] Farina et al recruited 71 women admitted for threatened preterm labour. They
194 were then divided into 3 groups, women delivering over 36 weeks, women delivering below
195 36 weeks and women who delivered below 36 weeks with preterm pre-labour rupture of
196 membranes. The samples were collected at a median of 31.6 weeks. They established an
197 arbitrary cut off range for ffDNA concentration and reported that the risk of PTB <30 weeks
198 below that cutoff was 14% whereas it was 45% above it. When considering all PTB <37
199 weeks this changed to 66% below the cutoff and 73% above. They reported a hazard ratio
200 for women with a ffDNA above the cut off of 1.57.[41]

201

202

203 *Studies suggesting no difference in ffDNA in PTB*

204

205 Illanes et al recruited 56 women who had cervical length assessment between 22-24 weeks.
206 Exactly when ffDNA samples were collected is unclear. They divided participants into three
207 groups: short cervix (<15mm) delivered at term, short cervix delivered before 37 weeks, and
208 normal length cervix delivering at term. They showed no differences in ffDNA between the
209 three groups.[42]

210

211 Other studies assessed unselected populations. Dugoff et al found no difference in PTB
212 when measuring ffDNA at 10-14 weeks.[40] Poon et al examined a cohort of 1949 women in
213 the first trimester. There were 20 spontaneous PTB before 34 weeks' and there was no

correlation between PTB and ffDNA.[15] Quezada et al measured ffDNA in the first trimester in 3169 women attending for ffDNA trisomy screening. Of those, 103 delivered spontaneously before 37 weeks and 21 at less than 34. They compared three groups <34, 34-37 and <37 weeks to the term delivery group and found no differences in ffDNA.[43] Thurik et al measured first trimester ffDNA levels and found no differences between the 49 women with PTB and those who delivered at term.[16]

Two studies used second trimester ffDNA levels to investigate PTB. Stein et al used a sub-set of a cohort study with 611 pregnancies. Of these, 76 delivered ≤ 37 weeks, 18 ≤ 34 weeks and 10 ≤ 32 weeks. The ffDNA concentrations were not different between these groups.[29] Bauer et al studied 84 women, including 7 women with spontaneous PTB, and revealed no differences between groups.[44]

Fetal Growth Restriction

The investigation into fetal growth restriction (FGR) and ffDNA is based on a variety of hypotheses. Some suggest that there is a lower placental mass due to placental dysfunction, leading to a reduced fetal fraction.[9] Others predict higher ffDNA due to increased trafficking of fetal cells into the maternal circulation[30,45] and also the possibility of increased degenerative changes in the syncytiotrophoblast.[22] Again, the results are mixed. The studies are summarized in table 3.

A key issue to consider is how FGR was defined in the studies. Many studies defined it as birthweight <10th centile, which is small for gestational age (SGA), but does not necessarily

represent a pathological process as some babies will be normally small. Whereas in FGR, placental insufficiency is the likely cause. Only one study implied that it took into account the process of placental insufficiency.[13] Several studies did not clarify how they identified FGR babies,[30,44,45] and many defined it as SGA.[15,22,23,29,46]

Studies showing increased levels of ffDNA in FGR

Caramelli et al performed a case control study of 8 women with abnormal uterine artery Doppler and 14 controls. Four cases developed FGR, although the definition of FGR is not clear. Women were enrolled at a median of 29 weeks. Cases had ffDNA concentrations of 1.81 MoM compared to controls. When considering those who developed FGR alone it was 2.16 MoM.[45] Alberdy et al's study recruited patients in the third trimester. They defined FGR as SGA, but also acknowledged that a proportion of these would be normally small and highlighted the need to identify fetuses with FGR secondary to placental insufficiency. There were 77 control and 12 with growth restriction; compared to the control group, ffDNA was higher in the FGR group.[13]

Kim et al had 43 women with SGA babies and 161 controls across all trimesters. They investigated the RASSF1A gene as a non-Y linked ffDNA measure. In the second and third trimesters they reported a significant ffDNA increase in cases.[23]

The study by Illanes et al was the only one to find increased ffDNA levels in the first trimester. They defined FGR as SGA in the context of preeclampsia and birth prior to or after 35 weeks. They found that the ffDNA taken at 11-14 weeks was significantly higher if

women delivered before 35 weeks. They also found that the likelihood of having pre-eclampsia or an SGA baby delivered before 35 weeks increases by 1.67 times for every 1GE/ml rise in ffDNA concentration.[22]

Studies showing no difference in ffDNA in FGR

Crowley et al undertook a case-control study in which samples were collected before 20 weeks from 72 controls and 36 cases. They defined FGR as not reaching growth potential, and detected ffDNA at similar concentrations in both groups.[30]

Rafaeli-Yehudai et al collected third trimester samples from 28 patients with SGA fetuses and 39 controls and reported similar concentrations of ffDNA.[46] Stein et al sampled women in the second-third trimester and looked at SGA <10th centile (13 participants) and <5th centile (9 participants). The concentration of ffDNA in both groups were similar to controls.[29]

Two studies collected first trimester data. Poon and colleagues study had 68 SGA neonates. They found no difference in the ffDNA compared to controls.[15] When considering Kim et al's first trimester results, there was no difference between groups.[23]

Studies showing a lower fetal fraction in FGR:

Rolnik et al looked at the relationship between ffDNA and the biophysical markers of FGR, uterine artery pulsatility index and PAPP-A. Fetal fraction was inversely associated with

uterine artery Pulsatility index (which when raised predicts risk) and positively associated with PAPP-A (which when low predicts risk). This supports the idea that with FGR there may be reduced ffDNA.[9] Previously, when Poon et al looked at these possible predictors of FGR they found that as PAPP-A increased, so did the ffDNA and as the uterine artery pulsatility index increased, so did the ffDNA.[15] Similarly Thurik et al's study found a significant correlation between ffDNA level and PAPP-A.[16]

Other adverse outcomes

Two studies contribute on placenta previa/invasive placenta. The premise is that the trophoblast is destroyed by the maternal immune system.[10] Sekizawa reported on 7 women with placenta praevia, including two with invasive placentae, and 13 controls. Samples were collected in the third trimester. They reported higher ffDNA levels for placenta praevia.[10] Kim et al studied 14 patients with placenta praevia. There were no differences in ffDNA in the first trimester while in the second trimester ffDNA was increased in cases. In the third trimester, there was a trend towards a higher result, but it was not significant.[23]

Thurik et al examined pregnancy induced hypertension and gestational diabetes. Their samples were collected in the first trimester. In the 84 women who went on to develop pregnancy induced hypertension, they found lower concentrations of ffDNA, and a similar trend with gestational diabetes.[16] Bauer et al however reported similar ffDNA concentrations in patients with and without gestational diabetes.[44]

Potential role of ffDNA in clinical practice

310

311 This is a highly controversial area. The ongoing interest in the use of ffDNA as a predictive
312 marker or diagnostic test for adverse pregnancy outcomes is because of the need to better
313 understand disease processes and to enable improved treatments to be developed and
314 appropriately targeted.

315

316 When considering usefulness in clinical practice, several authors have suggested that ffDNA
317 does not enhance currently available pre-eclampsia screening.[9,21] Some have come to
318 this conclusion for PTB too.[43] However Levine et al suggested there are two clinical uses
319 for pre-eclampsia. To screen and then predict disease.[7]

320

321 In a meta-analysis, the detection rate for pre-eclampsia in the first trimester (based on one
322 study) was 18%, with a false positive rate of 10%.[19] The detection rate improved at 17-28
323 weeks to approximately 68.8%, with a false positive rate of 10%. Farina et al in testing ffDNA
324 at around 20 weeks, found a 33% detection rate for a false positive rate of 5%, and with a
325 false positive rate of 10% this was 50%.[11] A further study by Farina et al, also testing
326 women at approximately 20 weeks, found a detection rate of 46% for a false positive rate of
327 5%.[47] These findings suggest that there may be a role for ffDNA in future.

328

329 *Limitations of current studies*

330

331 For no conditions are all of the results suggesting changes in the same direction. There is
332 also no standardisation of ffDNA measurement. Initially ffDNA was quantified in male
333 fetuses using the male SRY gene.[1] Later more advanced PCR techniques allowed

measurement in both sexes,[32] and most recently there has been interest in measuring fetal RNA.[35] Furthermore, within each of these methods different laboratory techniques are used. Added to this there are a variety of units for quantifying concentrations, for example ge/ml and multiples of the median. This makes it difficult to compare anything but general trends between studies, and makes meta-analyses challenging.

Further difficulties arise as there is no agreed time period for ffDNA measurement. Some studies use routinely collected samples with others collected at the time of diagnosis. This heterogenous population makes comparisons of these relatively small studies difficult.

Conclusions

The evidence for ffDNA predicting adverse pregnancy outcomes is controversial. However, this research is vital for developing better understanding of disease processes. FfDNA testing is currently a long way off being useful in clinical practice.

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524 Table 1: Summary of pre-eclampsia studies

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Publication	Participant numbers	Study design	Gestational age at sampling	Methods of Measurement of cell free DNA and units of quantification	Main findings
Studies sampling in the first trimester					
Illanes 2009[22]	Control= 24 Delivered < 35 weeks= 9 Delivered >35 weeks= 15	Case control	11-14 weeks	PCR for DSY14	FfDNA higher in patients with PE that delivered before 35 weeks (4.34ge/mL–1.61ge/mL). For every unit (1ge/mL) in which ffDNA increases, the likelihood of having PE before 35 weeks increases by 1.67 times.
Norton 2015[25]	15715 in cohort. PE = 504	Cohort	10-14 weeks	Harmony prenatal test	There was a reduction in the fetal fraction of ffDNA in PE (10.06%) compared to those not developing PE (11.04%). No difference between PE developing less than 34 weeks and whole PE Cohort.
Poon 2013[15]	46 PE Controls 1805	Cohort	11+0 - 13+6 weeks.	Harmony prenatal test	ffDNA not altered significantly in PE. Increases with increasing PAPP-A and BHCG, inversely related uterine artery pulsatility index.
Rolnik et al 2018[9]	4317	Retrospective cohort study	11+0 to 13+6	Commercially available testing	Mean arterial pressure and uterine artery pulsatility index negatively associated with fetal fraction, PAPP-A and PIGF associated positively with fetal fraction.
Sifakis 2009[21]	44 PE (11 needing <34/40 delivery), 176 controls	Case control study	11+0 to 13+6 weeks.	PCR for DSY14	Median ffDNA higher in pre-eclampsia with delivery pre-34 weeks (median 95.5 GE/ml) compared to late PE (median 50.8 GE/ml) or controls(51.5GE/ml).
Silver et al 2017[12]	175 with PE 175 controls	Case control	<13 weeks	PCR for RASSF1A	No difference in cell-free total DNA in first trimester women who go on to develop PE.
Thurik 2016[16]	226 cases, 301 controls	Nested case control	8 to <14 weeks	PCR for DSY14	No difference between cases and controls for PE
Studies collecting samples in second trimester					
Stein 2013[29]	611 in the cohort. 44 PE	nested cohort study	Up to 32 weeks mean 25.1	PCR for DSY	FfDNA not found to be a marker for PE/ in this otherwise low risk group.
Bauer 2006[44]	84 pregnancies. 26 with complicated outcomes.	Cohort	14-29 weeks	PCR of SRY and QF-PCR	No associations between adverse pregnancy outcomes and FFDNA levels.
Crowley 2007[30]	PE= 16	Case control	Before 20 weeks	PCR of SRY	SRY detected in all groups and the median concentrations were

	Controls=7 2				similar. median concentration 30.5GE/ml in PE vs 27.5 GE/ml in controls
Farina 2004[11]	PE =6 Control =30	Case control	Around 20 weeks	DSY14 PCR	2.39 higher levels of maternal plasma free DNA if go onto develop PE.
Farina 2004[47]	PE=8 Control = 40	Case control	Around 20 weeks	PCR for β - globin gene amplification	β -globin levels higher in patients going onto develop PE. PE compared to controls was 1.99 Mom.
Leung 2001[26]	PE=18 Control = 33	Case control	11-22 weeks	PCR SRY	Median fetal DNA concentrations higher in preeclamptic (41.9 GE/ml) than control (22.0 GE/ml) pregnancies.
Zhong 2002[27]	PE=10 Control=40	Case control	19-25 weeks	PCR SRY	ffDNA higher in PE than controls (4229 vs 128.5 copies/ml).
Studies collecting samples in the third trimester					
Rafaelli- Yehudai et al 2018[46]	Control 39 PE 21	Cross- sectional	3 rd trimester	cell free DNA rapid direct fluorescent assay	higher median maternal serum concentration in PE (802 ng/ml, 400–2272 ng/ml) compared to controls (499 ng/ml, 0–1892 ng/ml,)
Alberry 2009[13]	Control 77 PE 49	Cohort study	Recruited at time of diagnosis.	PCR for DSY14	ffDNA concentrations higher in PE pregnancies compared to normal pregnancies. Z scores for all PE showed a mean of +1.22, and +3.60 for severe PE.
Cotter 2004[28]	PE=88 Control=17 6	Case Control	Sampled at first AN visit Mean 15.7 weeks	PCR of SRY	Free fetal DNA associated with 8x increase in developing PE.
Lau 2002[34]	PE=7 Control = 10	Case control	Immediately pre and then up to 6 hours post delivery	PCR for SRY	Pre-delivery median ffDNA higher in preeclamptic women than controls (521 GE/ml vs 227 GE/ml). Post- delivery (208 GE/ml vs 0 GE/ml)
Lo 1999[20]	PE=20 Control =20	Case control	PE= mean 32 weeks Control = mean 33 weeks	PCR SRY	The median circulating free fetal DNA was increased fivefold in PE compared to controls.
Ng 2003[35]	PE= 12 Control =10	Case control	Before and 2 hours after delivery	RNA PCR for CRH	CRH mRNA concentrations 10.5 times higher in the PE than the controls.
Farina 2004[36]	PE= 17 Control = 17	Case control	27-40 weeks median 37	RNA PCR for CRH	There was correlation between CRH and pre-eclampsia, With the median in controls 100 copies/ml and in cases 962 copies/ml.
Swinkels 2002[31]	Controls =10 PE = 7 PE with HELLP=10	Case control	27-34+3	PCR for SRY	FfDNA 6.1-fold increase in women with preeclampsia compared with normotensive pregnancies. FfDNA increased with the severity of hypertensive disorder.
Tsui 2007[32]	PE (8 severe and	Case control	Just prior to delivery	PCR for RASSF1A	Concentrations 4.3 times higher in pre-eclamptic compared to controls.

	2 mild) = 10 Controls = 20				
Zhong 2001[33]	PE=44 Control =53	Case control	Taken after PE diagnosed.	PCR SRY	FfDNA increased in pregnancies complicated by PE (3194.6 vs 332.8 copies/ml)
Papantonio 2013[24]	PE=24 Controls = 48	Case control	11-13 weeks	RASSF1A	FfDNA increased with women with 934.5GE/ml compared to controls 62 GE/ml
Studies collecting samples across multiple gestations					
Contro 2017[19]	severe PE: 82 cases; 1315 controls Any PE: 376 cases; 1270 controls	meta- analysis	11-13 weeks and 17-28 weeks.	9 studies included all using PCR	Detection rate for severe PE in first trimester 18%, 68.8% in the second. With a false positive rate of 10%. For any PE it was not possible to establish a detection rate in the first trimester, in the second it was 37%.
Kim 2013[23]	Controls = 161 PE= 22	Case control study	Samples 7- 41 gestational weeks	RASSF1A PCR	ffDNA concentrations higher in PE than controls across all trimesters. 119.6 vs 60.2 copies/ml in 1 st trimester, 188.8 vs 60.1copies/ml in the second trimester and 782.0 vs 256.8 copies/ml
Levine 2004[7]	PE = 120 Control = 120	Nested cohort study	8-20 weeks, 26-29 weeks, 36 weeks and at symptom onset	PCR for DYS1	Before PE develops at 17-28 weeks ffDNA higher in cases than controls (36 vs 16 GE/ml), 29 to 41 weeks (within 3 weeks of developing PE), cases had higher cffDNA than controls (176 vs 75 GE/ml) cffDNA higher after developing PE than before (219 vs 112 GE/ml)

GE/ml = genome equivalent/ml, PE=Pre-eclampsia, ffDNA – free fetal DNA

Table 2: Summary of pre-term labour studies

Publication	N	Study design	Methods	Methods of Measurement of cell free DNA	Main findings
Bauer 2006[44]	84 pregnancies. 26 with complicated outcomes.	Cohort	14-29 weeks	PCR of SRY and QF-PCR	No associations between adverse pregnancy outcomes and ffDNA levels.
Dugoff 2016[40]	Cohort = 1349 Birth <37/40 = 119 Birth <34/40=49	Retrospective cohort study	Samples between 10- 20 weeks.	methylation	No association with ffDNA between 10-14.1 weeks. At 14-20 weeks, when the ffDNA was at or above the 95 th percentile, there was an increased risk of birth below

					37 weeks (OR 4.59 95%) and at 34 weeks (OR 22.0 95%).
Farina 2005[41]	PTL delivered >36/40 = 21, PTL delivered <36/40 =29 PPRM =21	Cross-sectional study	19.6 – 42.0 weeks	PCR DYS1	High concentrations of ffdDNA associated with increased risk of spontaneous PTB. They established an arbitrary cut off range for ffdDNA concentration (1.82 MoM). At <30 weeks below this cut off 14% were delivered compared to 45% above this. When considering all preterm birth <37 weeks this changed to 66% below the cutoff and 73% above.
Illanes 2011[42]	Cases 14, Controls 22 At risk (shot cervix) controls 20	Case control	22-24 weeks	PCR for DSY14	No difference between ffdDNA and gestational age at delivery or cervical length.
Jakobsen 2012[39]	Cohort 876 (883 with ffdDNA <95 th centile, 43 >95 th centile)	Cohort study	Samples at 25 weeks.	PCR for RHD	Association between ffdDNA levels above 95 th for study population and risk of PTB. High ffdDNA 6.3 times higher odds of birth before 37 weeks and a 16.6 fold increase in the odds of birth before 34 weeks
Leung 1998[8]	13 PTB, 17 controls	Case-control	Sampling on presentation with PTL	PCR SRY	The median ffdDNA concentrations were higher in preterm deliveries 124·8 copies/ml compared to controls 65·8 copies/ml.
Poon 2013[15]	20 PTB pre 34 weeks Controls 1805	Cohort	11+0 to 13+6 weeks.	Harmony prenatal test	ffdDNA not altered significantly in PTB
Quezada 2015[43]	3066 >37 weeks 103 < 37 weeks 82 34-37 weeks 21 <34 weeks	Cohort	11+0 to 13+6 weeks.	Harmony prenatal test	Fetal DNA fraction is no different in PTB compared to birth > 37 weeks. The median ffdDNA was 1.004 in the <34-week group, 0.922 in the 34-37 week group and 0.946 in the <37 week group, compared to 1.015 in the term group.
Stein 2013[29]	611 in the cohort. 76 PTB (<37) 18 PTB <34	Nested larger cohort study	Up to 32 weeks mean 24.8	PCR DSY	cffdDNA not found to be a marker for PTB in this otherwise low risk group.
Thurik 2016[16]	226 cases, 301 controls	Nested case control	8 – 13+6	PCR for DSY14	No difference between cases and controls for PTB

ffdDNA – Free Fetal DNA, PTB – Preterm Birth

Table 3: Summary of fetal growth restriction studies

Publication	N	Study design	Sample collected	Methods of Measurement of cell free DNA	Main findings
Alberry 2009[13]	Control 77 FGR 12	Cohort study	Recruited at time of diagnosis.	PCR for DSY14	FfDNA concentrations higher in PE and FGR pregnancies compared to normal pregnancies. Mean z-scores for growth restriction +1.67.
Bauer 2006[44]	84 pregnancies. 26 = complicated outcomes.	Cohort	14-29 weeks	PCR of SRY and QF-PCR	No associations between adverse pregnancy outcomes and ffDNA levels.
Caramelli 2003[45]	Cases = 8 Controls =14	Case control study	20-35 weeks (median 29)	PCR of SRY	Cases going onto develop FGR had a ffDNA concentration of 2.16MoM
Crowley 2007[30]	IUGR=36 Controls= 72	Case control	Before 20 weeks	PCR of SRY	Median concentrations were similar in cases (28ge/ml) to controls (27.5ge/ml).
Illanes 2009[22]	Control= 24 < 35 weeks= 9 >35 weeks= 15	Case control	11-14 weeks	PCR for DSY14	FfDNA higher in FGR that delivered <35 weeks (4.34ge/mL–1.61ge/mL). For every unit (1ge/mL) in which ffDNA increases, the likelihood of having a fetus growing under the tenth centile delivered before 35 weeks increases by 1.67 times.
Kim 2013[23]	Controls = 161 IUGR= 43	Case control study	7-41 weeks	RASSF1A PCR	Second trimester ffDNA concentration was raised with 101.2copies/ml compared to 60.1 copies/ml in controls. Third trimester 648.4 copies/ml vs 256.8 copies/ml in the controls. 1 st trimester no difference in ffDNA.
Poon 2013[15]	68 SGA, Controls 1805	Cohort	11+0 to 13+6 weeks.	Harmony prenatal test	ffDNA not altered significantly in FGR. Increases with increasing PAPP-A and BHCG, inversely related uterine artery pulsatility index.
Stein 2013[29]	611 in the cohort. 9 < 5 th Centile 13<10centile	nested cohort study	< 32 weeks mean 24.3	PCR DSY	FfDNA not found to be a marker for FGR in this otherwise low risk group.
Rolnik et al 2018[9]	4317	Retrospective cohort study	11+0 to 13+6	Commercially available testing	Mean arterial pressure and uterine artery pulsatility index negative association with fetal fraction, PAPP-A and PIGF associated positively with fetal fraction.

Rafaeli-Yehudai et al 2018[46]	Control 39 FGR 28	Cross-sectional	3 rd trimester	cell free DNA rapid direct fluorescent assay	No difference in ffDNA concentrations between controls and FGR
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FGR- fetal growth restriction, PE – pre-eclampsia, ffDNA – free fetal DNA

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