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Review: Cell-free fetal DNA in the maternal circulation as an indication of placental health and disease

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Abstract

In human pregnancy, the constant turnover of villous trophoblast results in extrusion of apoptotic material into the maternal circulation. This material includes cell-free (cf) DNA, which is commonly referred to as “fetal”, but is actually derived from the placenta. As the release of cf DNA is closely tied to placental morphogenesis, conditions associated with abnormal placentation, such as preeclampsia, are associated with high DNA levels in the blood of pregnant women. Over the past five years, the development and commercial availability of techniques of massively parallel DNA sequencing have facilitated noninvasive prenatal testing (NIPT) for fetal trisomies 13, 18, and 21. Clinical experience accrued over the past two years has highlighted the importance of the fetal fraction (*ff*) in cf DNA analysis. The *ff* is the amount of cell-free fetal DNA in a given sample divided by the total amount of cell-free DNA. At any gestational age, *ff* has a bell-shaped distribution that peaks between 10 and 20% at 10–21 weeks. *ff* is affected by maternal body mass index, gestational age, fetal aneuploidy, and whether the gestation is a singleton or multiple. In approximately 0.1% of clinical cases, the NIPT result and a subsequent diagnostic karyotype are discordant; confined placental mosaicism has been increasingly reported as an underlying biologic explanation. Cell-free fetal DNA is a new biomarker that can provide information about the placenta and potentially be used to predict clinical problems. Knowledge gaps still exist with regard to what affects production, metabolism, and clearance of feto-placental DNA.

Keywords

Cell-free fetal DNA; Noninvasive prenatal testing (NIPT); Confined placental mosaicism; Aneuploidy; Placenta

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Conflict of interest statement

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1. Introduction

A decade ago, one of us (D.W.B.) spoke at a prior International Federation of Placenta Associations meeting in Mainz, Germany. The article that summarized that lecture was entitled “Circulating fetal DNA: its origin and potential—a review” [1]. The theme of the lecture and manuscript was on what basic science can teach us that could help to develop clinical applications for the use of cell-free fetal (cff) DNA in the maternal circulation. Ten years ago many of our scientific questions focused on the tissue source of the cell-free nucleic acids. Did they derive from the placenta or fetal membranes, fetal hematopoietic cells, or other organs undergoing apoptosis? Despite a lack of certainty of the origin of the fetal nucleic acids, real-time PCR amplification of DNA sequences that were uniquely fetal was already being used to determine fetal sex and Rhesus D genotype. In addition, it was already known that cff DNA levels were increased in pregnancies that were complicated by preeclampsia. In the interim years, a major advance in genetics and genomics has been the ability to efficiently and cost-effectively sequence the human genome. This has allowed techniques of massively parallel sequencing (MPS) to be applied to cff DNA. The clinical experience that has accumulated since the introduction of noninvasive prenatal testing (NIPT) using MPS of maternal plasma DNA for prenatal diagnosis has, in turn, provided new basic science insights regarding the placenta and fetus. Given the recent rapid advances in this field, we have created a timeline highlighting key scientific and clinical research developments in the use of cff DNA for analysis of placental health and disease (Fig. 1) [2–18].

2. Origin of cell-free fetal DNA in maternal blood

2.1. In vitro evidence that “fetal” DNA originates from the placenta

At the cellular level, the placenta is a dynamic organ. There is constant turnover of villous trophoblast. As elegantly described by Huppertz and Kingdom [19], there is a physiological cycle of apoptosis in the placenta that begins with proliferation and differentiation of cytotrophoblast. This is followed by fusion of cytotrophoblast with syncytiotrophoblast, further differentiation, and extrusion of apoptotic material. In normal pregnancies, grams of placental material are shed daily into the maternal circulation without causing inflammation. Bischoff and colleagues [20] flow sorted apoptotic bodies isolated from the plasma of pregnant women. Using electron microscopy, they showed that these bodies contained nucleosomes and chromatin. Subsequently, other mechanistic experiments connected oxidative stress, placental apoptosis, and the production of cell-free fetal DNA [21]. Villous explants from full-term placentas were cultured under normoxic (10% O₂) and hypoxic (0.5% O₂)/reoxygenation conditions. Significantly increased levels of cell-free β globin DNA were measured after 20 h of culture; this correlated with increased caspase 3 staining that localized to syncytiotrophoblast. Additional research has directly examined the placental origin of cff DNA *in vitro*. Gupta et al. conducted a study in which they derived syncytiotrophoblast microparticles from placental tissue [22]. They are released *in vivo* as part of the syncytiotrophoblast fusion process [19]. Fetal DNA was detected within the derived microparticles and in the culture supernatant of the villous explants. These results

correlate well with clinical findings, as cff DNA has been identified in maternal serum and plasma, both within microparticles and as freely circulating molecules [2,20].

2.2. In vivo evidence that “fetal” DNA originates from the placenta

Cff DNA has been detected in cases in which only placental tissue is present, as in anembryonic gestations [23] and before the placental circulation is established [5]. Some of the strongest evidence for the placental origin of cff DNA comes from clinical studies on pregnancy pathologies. Case reports examining placenta increta found a relative increase in cff DNA with this invasive placental phenotype [24,25]. Under normal conditions, cff DNA is cleared relatively rapidly after delivery [26]. In cases of therapeutic abortion, in which expulsion of the placenta is incomplete, cff DNA is still detectable [4].

Further evidence has been drawn from pregnancies in which the placenta has a different genotype than the fetus. This is known as confined placental mosaicism (CPM). An initial case series described three women whose pregnancies all had chromosomal abnormalities confined to the placenta. These abnormalities directly correlated with the circulating fetal DNA [7]. Faas et al. reported a pregnancy in which the cytotrophoblast layer had a genotype of 45, X, although the mesenchymal core tissue was 46, XX [27]. The cff DNA in maternal plasma also exclusively had a genotype of 45, X, strongly promoting the idea of a trophoblast origin.

Prenatal diagnostic and treatment procedures also give clinical evidence of a placental origin, although results vary based on the specific technique used. Chorionic villous sampling (CVS) did not appear to alter levels of cff DNA when compared to control women who did not undergo an invasive procedure [28]. However, laser ablation for treatment of twin-to-twin transfusion has been associated with a clear increase in fetal DNA [29]. These two reports highlight that the release of cff DNA may be related to the extent of placental perturbation.

2.3. Quantitative cff DNA analysis: a useful tool for placental study

As the release of cff DNA is closely tied to placental morphogenesis, conditions that affect the placenta can directly impact its levels in maternal circulation. Preeclampsia has been one of the most well studied examples [30]. In preeclamptic placentas, oxidative stress leads to increased trophoblast apoptosis and shedding of syncytiotrophoblast microparticles, which results in increased release of cff DNA into the maternal circulation [30,31]. Importantly, higher cff DNA levels, compared to unaffected women, have been noted prior to the onset of clinical symptoms. These occur in a bimodal pattern, with levels increasing above controls between 17 and 28 weeks of gestation, followed by a second rise around three weeks prior to the development of clinical preeclampsia [8]. Given the kinetics of fetal DNA observed in preeclampsia, screening by measurement of levels of circulating cff DNA may be informative both prior to symptom onset and throughout disease progression. Indeed, a sustained increase in circulating cff DNA may also indicate other pathological sequelae. For example, high concentrations of cff DNA in maternal serum have also been associated with an increased risk of preterm labor [9].

Increased circulating cff DNA is also seen in pregnancies with intrauterine growth restriction (IUGR). Higher levels of cff DNA were particularly observed in patients with IUGR due to placental insufficiency [32]. Interestingly, this elevation was noted in patients both with and without preeclampsia, suggesting that fetal DNA release is closely tied to placental function independent of disease state.

2.4. Cff DNA primarily derives from apoptotic cells, not placental volume

In 2005, Wataganara and colleagues investigated whether placental volume influenced the quantity of cff DNA in maternal circulation [33]. This study correlated 3D placental volume measurements with cff DNA levels, as measured by Y chromosome sequences in male fetuses. Interestingly, placental volume did not correlate with the levels of fetal genetic material. These results suggest that fetal DNA is released via a controlled process that is independent of placental size.

Apoptosis appears to be the main mechanism controlling release of fetal DNA from the placenta. As stated above, cff DNA is likely released from the syncytiotrophoblast layer as a part of physiologic placental cell turnover throughout pregnancy [22]. Syncytiotrophoblast stress or placental oxidation can also enhance the release of cff DNA via apoptosis [21]. Other studies have suggested that shedding of fetal DNA occurs via a combined mechanism of apoptosis/necrosis. Termed “aponecrosis” by Hahn et al. [34], these authors proposed the initiation of an apoptotic pathway within the syncytiotrophoblast followed by necrosis. This process ultimately releases cff DNA into maternal circulation. Taken together, the combination of *in vitro* and *in vivo* evidence strongly suggests that cff DNA primarily derives from the placenta. Circulating fetal DNA can therefore provide valuable evidence for placental health and disease.

3. Lessons learned from clinical integration of noninvasive prenatal testing

The focus of most prenatal screening and diagnosis is on fetal whole chromosome abnormalities. Many professional societies, such as the American College of Obstetrics and Gynecology, recommend that all pregnant women be offered screening for fetal aneuploidy [35]. The standard approaches involve a combination of first trimester serum screening with an ultrasound measurement of the nuchal translucency, with or without a measurement of second trimester analytes. Over the past two years an alternative screen, noninvasive prenatal testing (NIPT) using massively parallel sequencing of cff DNA in maternal plasma, has become clinically and commercially available.

With NIPT, cell-free fetal and maternal DNA fragments around 150–200 base pairs (bp) in size are isolated from maternal plasma. The first 36 bp are sequenced, and then aligned to the human genome. Fragments that are mapped to the genome are known as tags. The tags are counted. The tags on the chromosome of clinical interest (e.g., 21) are then compared to a reference chromosome or chromosomes. Excess tags on the chromosome of interest suggest that there is an extra copy of that chromosome present.

3.1. Biological factors affecting cff DNA analysis: influence of the fetal fraction

To date, clinical experience with NIPT has highlighted the importance of the fetal fraction. Fetal fraction measurement differs from cff DNA levels discussed earlier in the paper. Cff DNA levels can be examined independently in relation to gestational pathologies, usually by quantitative polymerase chain reaction amplification. MPS is performed on both the maternal and fetal cell-free DNA in a given plasma sample. The fetal fraction (ff) is the amount of cff DNA divided by the amount of total cell-free DNA (Fig. 2). ff can be measured using molecular counting, amplification of differentially-methylated sequences, or detection of unique fetal polymorphisms [36,37]. As an example, the ff can be determined by examining genetic elements that differ between maternal and fetal DNA. Genes on the Y chromosome are the most commonly used distinguishing marker, because in most cases the mother does not have them [2]. Rare exceptions exist. While the fetal portion derives from the placenta, maternal cell free DNA mainly originates from apoptosis of hematopoietic cells [38] and/or adipose tissue [39].

At any given gestational age, ff has a bell-shaped distribution that peaks between 10 and 20% between 10 and 21 weeks of gestation. The ff is of clinical interest because it affects how well fetal genetic material can be detected in a sample. For example, a low ff can affect the detection limit for cff DNA analysis, causing false negative test results [40]. As a ratio, the ff is a dynamic value that can be influenced by several different factors. Technical artifacts resulting from sample extraction and processing may result in ff variations. Biological factors related to the mother and/or fetus can be another significant influence. Here we discuss four main biological influences that can affect the ff including maternal weight, gestational age, multiple gestations and fetal aneuploidy (Fig. 2).

Maternal weight and/or maternal body mass index (BMI) has the most significant effect on ff . Obesity during pregnancy is associated with lower ff s, as has been consistently demonstrated in several independent studies [12,13,40,41]. Lower ff s in gestational obesity may be due to a dilutional effect from increased maternal circulatory volume [40]. In addition, there may be an increase in maternal cell free DNA release from apoptosis/necrosis of stromal vascular cells and adipose tissue in obese pregnant women [39].

Gestational age is an additional factor that can affect the ff . Wang and colleagues noted that levels of fetal DNA increase throughout pregnancy, with an initial rise of 0.1% per week from 10 to 20 weeks of gestation, followed by a sharper increase of 1% per week after 21 weeks to term [13]. Different fetal aneuploidies have varied effects on the ff , depending on the affected chromosome. In comparison to euploid fetuses, fetuses with trisomy 21 have an increased fetal fraction. In contrast, trisomies 13 and 18 and monosomy X have decreased ff [42]. One possible explanation for the higher levels in trisomy 21 is the increase in trophoblast apoptosis that has been observed in affected cases compared to euploid placentas [43].

Currently, NIPT is recommended only for singleton gestations, although many companies are beginning to offer testing for twin gestations. Twin gestations have two placentas, and it would be reasonable to assume that fetal fraction would double. This is not what has been observed clinically.

Compared to singleton pregnancies, *ff* in twin gestations is increased by about a third [14]. This may be due to the fact there does not appear to be a relationship between placental volume and circulating DNA [33]. Using the ratio of X chromosome tags in twin gestations to reference female fetuses, Srinivasan et al. noted that the overall *ff* per fetus was reduced by as much as 50% [44]. This was independent of the type of chorionicity. The clinical significance is that in 10–15% of twin gestations, the *ff* per fetus may be too low for an accurate classification as euploid or aneuploid.

3.2. Discordant positive results: lessons from confined placental mosaicism

Worldwide, over 200,000 NIPTs have been performed to date. Testing has shown >99% sensitivity for detection of trisomy 21. When aneuploidy is detected, it is recommended that a follow-up invasive diagnostic procedure be performed to determine the fetal karyotype. In approximately 0.1% of cases the positive NPT result is discordant with the fetal metaphase karyotype. One important reason for this discrepancy is the presence of confined placental mosaicism (CPM).

CPM refers to the presence of a chromosome abnormality in the placenta with a normal fetal karyotype. When CVS is performed, CPM is found in 1–2% of first trimester placenta samples [45]. CPM is further subdivided as Types 1, 2 and 3, dependent upon whether the aberrant cell line is identified in the cytotrophoblast alone (direct, short-term culture), mesenchymal core (long-term) or both layers, respectively [46]. When CPM is present, higher rates of fetal growth restriction, pregnancy loss, and hypertension have all been reported [47–50]. There is controversy as to whether the type of CPM, the specific chromosome involved, or the extent of abnormal placental cell lines contributes disproportionately to the risk of adverse pregnancy outcomes [49,51]. In approximately one-third of cases of types 2 and 3 CPM, there is rescue disomy. The disomic fetus may possess two chromosomes inherited from the same parent. This is called uniparental disomy (UPD), which may also affect outcomes. This is of particular relevance in trisomies involving chromosomes 2, 6, 7, 11, 14, 15, 16, or 20, for which abnormal phenotypes are associated [52]. While follow-up of the children in pregnancies complicated by CPM has been limited, reassuring developmental outcomes and normal growth have been generally noted [49,53]. In one study, lower weight and height compared to controls was observed in later life when growth restriction was not present at birth [54].

More attention is being paid to CPM as discordant NIPT cases are identified. When the placenta and the fetus have different karyotypes, the placental information contained in cff DNA may not accurately reflect the fetal genome. Several instances of CPM detected at chorionic villus sampling (CVS) have been reported in association with discordant positive results from NIPT (Table 1). Mennuti et al. noted two cases of type 1 CPM, both in association with NIPT results that were positive for chromosome 13 [15]. In each of these cases, NIPT positive results for trisomy 13 were further evaluated by CVS. In the first case, trisomy 13 was detected only in the direct sample (CPM type 1). The long-term culture had a normal karyotype. This pregnancy was terminated; FISH studies showed disomy 13 and post-mortem culture of decidua and villi revealed 46, XY. In the second case, the CVS direct preparation revealed 46, XX, +13, der(13;13) (q10;q10), consistent with the NIPT results.

Long-term culture and amniocentesis results were 46, XX. The pregnancy was structurally normal by ultrasound and ongoing at the time of publication [15]. Additional examples of the role of CPM in discordant NIPT-fetal karyotype results are outlined in Table 1.

As more pregnant women undergo NIPT, we may be able to detect a higher percentage of CPM in the future. Increased diagnosis of CPM could unveil a previously unrecognized etiology for unexplained growth restriction, pregnancy loss and or gestational hypertension.

4. Conclusions

Integration of NIPT into clinical care has identified new aspects of perinatal biology. Cff DNA is a new biomarker that can provide information about the placenta and potentially be used to predict clinical problems. Knowledge gaps exist with regard to what affects the production, metabolism, and clearance of cff DNA. Does apoptosis directly correlate with higher fetal fractions? What accounts for the significant inter-individual variation in fetal fraction? Why doesn't fetal DNA increase arithmetically in multiple gestations? Does the pregnant woman's metabolism limit the amount of foreign circulating DNA? Lastly, as more women undergo NIPT, it is likely that more cases of CPM will be found. Whereas CPM was previously identified in pregnant women at high risk for aneuploidy undergoing CVS, NIPT may allow more widespread detection. Will this provide an underlying mechanistic explanation for children who fail to thrive and/or develop? By partnering clinicians with scientists studying the placenta, the answers to these questions will hopefully be forthcoming in the next decade.

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Fig. 1. Highlighted studies span initial scientific investigation of cff DNA through its eventual integration into clinical practice. MPS: Massively parallel sequencing; NIPT: Noninvasive prenatal testing; ff: fetal fraction, CPM: Confined placental mosaicism.

Fig. 2.

The fetal fraction is the ratio of the fetal to total circulating cell-free DNA. Throughout gestation, the fetal fraction is influenced by many exogenous factors.

Table 1

Influence of confined placental mosaicism on the discordance between NIPT results and fetal karyotype.

NIPT result	Fetal karyotype	Explanation	References
Autosomal aneuploidy			
Trisomy 13	46, XX (amniocentesis)	CPM: 46, XX, +13, der (13; 13) (q10; q10) [5] in direct preparation	Mennuti et al. [15]
Trisomy 13	46, XY (fetal tissue)	CPM: 47,XY, +13 in direct preparation	Mennuti et al. [15]
Trisomy 13	46, XY (amniocentesis and cord blood)	CPM:47, XY, +13 [10]/46, XY [12]	Hall et al. [16]
Trisomy 21	46, XX, upd (21) mat	CPM: Trisomy 21	Pan et al. [17]
Trisomy 22	Cord blood normal by sequencing	CPM: Trisomy 22	Choi et al. [18]
Sex chromosome aneuploidy			
47, XXY	46, XY (amniocentesis)	CPM: 49, XXX, +7, +21 [24]/46, XY [6]	Lau et al. [55]