Cell-free fetal DNA: the new tool in fetal medicine

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Introduction

Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma in 1997 there has been rapid progress in harnessing this as a source of fetal genetic material for prenatal diagnosis. The majority of cell-free DNA (cfDNA) is maternal in origin, with the fetal proportion emanating from the placenta, detectable in the maternal circulation from around 5 weeks’ gestation and constituting around only 10% of cfDNA in early pregnancy. However, as cfDNA is cleared rapidly from the maternal circulation after delivery, it offers great potential as a source of fetal genetic material for prenatal diagnosis. Initially, in view of the high background of maternal cfDNA, technological restrictions only enabled the detection or exclusion of alleles that were not present in the mother but were present in the fetus because they were paternally inherited or arose de novo at conception. Thus, early indications were for the fetus because they were paternally inherited or arose de novo at conception. Early cffDNA testing and only offered in pregnancies in which the fetus is known to be female. However, close co-ordination with fetal-medicine services is required as testing is only reliable after 7 weeks’ gestation, with false-positive results possible in twin pregnancies or in those with early fetal demise of a cotwin.

X-linked disorders and disorders of genital ambiguity

The earliest clinical use of cffDNA was for the determination of fetal sex. This relies on the detection of sequences, SRY or DYS14, in the maternal plasma that derive from the Y-chromosome. The technique has already become incorporated into standard care in several European countries, including the UK, for management of pregnancies at risk of severe X-linked genetic disorders, such as Duchenne muscular dystrophy. It has the potential to reduce the incidence of invasive testing for such conditions by up to 50% by allowing targeted testing in male-bearing pregnancies. In pregnancies at risk of congenital adrenal hyperplasia, determining fetal sex can enable early cessation of steroid treatment in male-bearing pregnancies or, as occurs in several centers, steroid administration could be delayed until fetal sex is determined through early cffDNA testing and only offered in pregnancies in which the fetus is known to be female. However, close co-ordination with fetal-medicine services is required as testing is only reliable after 7 weeks’ gestation, with false-positive results possible in twin pregnancies or in those with early fetal demise of a cotwin.

Ambiguity of the genitalia is a rare finding on ultrasound, and even with the advent and improvement of three-dimensional (3D) imaging techniques, differentiation between clitoromegaly in the female fetus and hypospadias in the male remains difficult. In cases in which genital ambiguity is isolated and cffDNA testing indicates that the fetus is male, the most likely diagnosis is hypospadias, although some rare endocrine disorders cannot be excluded completely without sequencing of the androgen receptor gene. If, however, cffDNA testing indicates that the fetus is female genetically, referral to a team specialized in disorders of sexual development is advised as abnormalities in SRY can cause disorders of sexual differentiation and multiple markers should be assessed for determination of fetal sex in these cases. Another relatively common association with hypospadias is FGR; therefore, maternal uterine artery Doppler examinations should be performed and, if Down syndrome screening is performed, maternal serum biomarker results should be reviewed for low levels of pregnancy-associated plasma protein A (PAPP-A) and high levels of human chorionic gonadotropin (hCG) or ß-fetoprotein levels.

The use of cffDNA for sex determination can be an extremely useful aid to sonographic diagnoses of a number of genetic syndromes that present with multiple

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abnormalities, usually genital ambiguity in which determination of genetic sex can be diagnostic when combined with the presence of other relevant sonographic findings (Table 2). For example, in cases of campomelic dysplasia, which presents with varying degrees of lower limb shortening and bowing, talipes and micrognathia, at least 50% of affected male fetuses have genital ambiguity or complete sex reversal. Therefore, if a fetus has ambiguous or female genitalia with these sonographic features and cffDNA testing indicates a genetic female, the diagnosis can be made (Table 2 and Figure 2). Non-invasive fetal sex determination using cffDNA can also be very useful in the presence of some urogenital anomalies, such as bladder cloacal extrophy, as knowledge of the genetic sex can aid counseling with regard to long-term outcome.\(^1\)

**Cell-free fetal DNA and management of complications arising from blood-group antigens**

The second clinical application of cffDNA testing was for the determination of fetal RhD status in pregnant RhD-negative mothers.\(^2\) As with fetal sex determination, this is possible because an RhD-negative mother does not produce any copies of the RhD gene (RHD), and thus the RHD identified in maternal blood originates from the fetus who has inherited the gene from the father. For the past decade, fetal RHD genotyping in RhD-negative women with significant titers of anti-RhD immunoglobulin has been possible using labor-intensive polymerase chain reaction (PCR)-based methods.\(^3\) This approach to management of these high-risk pregnancies has avoided the need for invasive testing that was required previously. In addition to avoiding the associated risks of miscarriage, NIPT circumvents the need for assessment of paternal phenotype, which may not be known or available. If the fetus is RhD positive, increased surveillance in a tertiary center to monitor for the development of fetal anemia or hydrops is required, whereas if the fetus is predicted to be RhD negative, there is no risk of hemolytic disease of the newborn (HDN) and standard antenatal care is appropriate.\(^4\)
Introduction of high-throughput technologies for mass fetal RHD genotyping\textsuperscript{22} has provided potential for routine fetal genotyping and targeted administration of anti-D immunoglobulin, a human blood product. Currently, many countries offer routine antenatal prophylaxis to all RhD-negative mothers. This has significantly decreased the incidence of Rhesus sensitization, and cases resulting in fetal anemia are now rare. However, this policy results in the unnecessary administration of anti-D immunoglobulin to around 38\% of RhD-negative women who are carrying a RhD-negative fetus\textsuperscript{23}. Routine fetal RHD genotyping and targeted anti-D prophylaxis have been introduced recently into routine obstetric care at 26–28 weeks’ gestation for RhD-negative women in The Netherlands and Denmark\textsuperscript{24,25}. However, a UK study has shown that high-throughput RHD genotyping is highly accurate from 11 weeks’ gestation\textsuperscript{26}. Introduction at this earlier stage in pregnancy would result in further avoidance of administration of anti-D immunoglobulin for sensitizing events that occur in early pregnancy. Subsequently, there have been calls for implementation of high-throughput RHD genotyping into routine antenatal care in the UK\textsuperscript{27}. Current routine immunoprophylaxis programs do not achieve complete uptake as some women decline anti-D immunoglobulin treatment. Routine fetal RHD genotyping is likely to be very acceptable to women\textsuperscript{28} and may improve immunoprophylaxis uptake, thereby targeting women at highest risk, causing a further decline in rates of alloimmunization and, subsequently, the need for in-utero transfusion.

Although anti-RhD is the most common cause of HDN, other antibodies, in particular anti-c and anti-K (and less commonly anti-C and anti-E), are responsible for an increasing proportion of cases. Unlike anti-D, there is no prophylaxis, so the use of cfDNA as routine screening is unlikely; however, it remains an important investigation in sensitized pregnant women. The accuracy of fetal genotyping in these cases approaches 100\%, thereby obviating the need for invasive testing in these pregnancies\textsuperscript{21,29,30}.

Fetal or neonatal alloimmune thrombocytopenia (FNAIT) is caused by production of maternal alloantibodies directed against paternally inherited antigens present on fetal platelets\textsuperscript{21}. Complications include intracranial hemorrhage, occurring in up to 20\% of cases, which may cause severe long-term consequences to the child. Until recently, in cases with a heterozygous father, invasive testing was required to determine whether the fetus was affected by FNAIT, which can occur only if they are positive for human platelet antigen-1a (HPA-1a). Analysis of cfDNA in maternal blood can detect the HPA-1a gene\textsuperscript{31,32}, which again avoids the need for invasive testing in women with a heterozygous partner.

### Non-invasive prenatal testing for monogenic disorders

The use of cfDNA in the detection of monogenic disorders is considerably more challenging technically than fetal RHD genotyping or sex determination and is currently in clinical use only for the detection of alleles that have arisen de novo at conception, for example, achondroplasia\textsuperscript{9}, or that are inherited from the father\textsuperscript{34,35} or by use of the differential methylation in fetal and maternal DNA\textsuperscript{36}; as yet, the reliability of these methods for use in routine clinical practice requires further development and evaluation. It is likely that this will become possible universally in the future, perhaps by exploiting the fact that fetal DNA is shorter than maternal cfDNA\textsuperscript{37}. In recessive conditions, whereby the parents carry different allele mutations, exclusion or detection of

| cfDNA result | Differential diagnosis | Other aids for management |
|--------------|------------------------|--------------------------|
| Male         | Isolated hypospadias   | Look for markers of FGR: |
|              |                        | review PAPP-A, hCG and MSAFP results if available |
|              | FGR                    | Review maternal Doppler   |
|              | Inadequate production of testosterone because of Leydig cell hypoplasia or rare abnormalities of steroid metabolic pathway | Consider referral to DSD team for sequencing of androgen receptor gene |
|              | Partial androgen insensitivity syndrome | |
|              | 5α-reductase deficiency  | |
|              | True hermaphrodite      | |
| Female       | Congenital adrenal hyperplasia | Refer to DSD team for further investigations |
|              | 21-hydroxylase deficiency | Amniotic steroid levels |
|              | 11-hydroxylase deficiency | Maternal serum androgen levels |
|              | 3β-hydroxysteroid dehydrogenase deficiency | Maternal urinary estrogen levels |
|              | True hermaphrodite      | Maternal ovarian scan for multicystic change |
|              | Maternally derived androgens (e.g. luteoma of pregnancy) |
|              | Placental aromatase deficiency |

DSD, disorders of sexual development; FGR, fetal growth restriction; hCG, human chorionic gonadotropin; MSAFP, maternal serum alpha-fetoprotein; PAPP-A, pregnancy-associated plasma protein A.
Examples of genetic syndromes for which sex determination using cell-free fetal DNA (cffDNA) or targeted non-invasive prenatal testing for single-gene disorders may aid ultrasound diagnosis:

| Syndrome | Genitalia | Other sonographic findings | Differential diagnosis |cffDNA result | Other aids for diagnosis |
|----------|-----------|---------------------------|------------------------|--------------|-------------------------|
| Bardet–Biedl | Ambiguous | Large echogenic kidneys, polydactyly, cleft lip, renal anomalies | Trisomy 13 | Male | Family history, consanguinity, maternal urinary steroids |
| Smith-Lemli–Opitz | Ambiguous | Polysyndactyly, cardiac and CNS anomalies, other genetic syndromes | Trisomy 13 | Male | Family history, consanguinity, maternal urinary steroids, maternal urinary steroids |
| Malpeuch syndrome | Ambiguous | Cleft lip, FGR, renal anomalies | Trisomy 18 | Male | Family history, consanguinity |
| Campomelic dysplasia | Ambiguous | Osteogenesis imperfecta | Type III/IV | Female | Micrognathia, micrognathia, macrocephaly, trident hands, polyhydramnios (small chest) |
| Achondroplasia | Normal | Rhizomelic shortening of long bones at >24 weeks, bowed femora, frontal bossing, relative macrocephaly, macrocephaly, trident hands, polyhydramnios, small chest, short fingers | Normal limb length at <24 weeks' gestation | Normal | FGFR3 mutn |
| Thanatophoric dysplasia | Normal | Early-onset shortened long bones, bowed femora, frontal bossing, relative macrocephaly, cloverleaf skull | Normal | Normal | FGFR3 mutn |
| Apert syndrome | Normal | Abnormal skull shape, mitten hands and feet | Male | Male | FGFR2 |

Table 2: Examples of genetic syndromes for which sex determination using cell-free fetal DNA (cffDNA) or targeted non-invasive prenatal testing for single-gene disorders may aid ultrasound diagnosis.

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9 weeks’ gestation, earlier than invasive testing, which cannot be performed safely until 11 weeks, and much earlier than an ultrasound scan for women not wanting to put the pregnancy at risk by an invasive test.

The use of cfDNA for the diagnosis of monogenic disorders has great potential. In the UK it has been approved for use in clinical National Health Service (NHS) practice to screen for mutations in the FGFR3 (achondroplasia and thanatophoric dysplasia) and FGFR2 (Apert syndrome) genes and for paternal exclusion of common cystic fibrosis mutations. With the introduction of these safer tests we are seeing a dramatic decrease in the use of invasive testing for monogenic disorders (Table 3), with a further decline in the likely need for invasive testing as more non-invasive tests are developed and validated.

Cell-free DNA testing for aneuploidy

The use of cfDNA testing for aneuploidy is bringing the most radical change to the practice of fetal medicine. Early attempts at providing NIPT for Down syndrome relied on quantifying the amount of placenta-specific 4 (PLAC4) in the maternal plasma. This gene originates from chromosome 21, is expressed only in the placenta and is therefore fetal in origin. By detection of two separate alleles in a 1:1 ratio, the fetus can be assumed to be euploid. If there is duplication of an allele (because of an extra copy of chromosome 21), the ratio will be 2:1 and the fetus can be assumed to be trisomic for chromosome 21. Although groundbreaking, this initial approach was flawed as it required the identification of genetic differences in the parents and was therefore only applicable in around 40% of pregnancies. The advent of NGS brought a new approach that could be applied universally. Rather than detection of a gene product derived from chromosome 21, all DNA circulating in maternal plasma is sequenced. This, by definition, includes both maternal and fetal DNA, and sequencing will analyze the entire fetal and maternal genome. There are three NGS-based approaches to NIPT for aneuploidy: whole-genome NGS; targeted NGS; and single nucleotide polymorphisms (SNPs). The whole-genome approach requires sequencing of cfDNA from maternal plasma to generate millions of short sequence reads from the whole genome. These are then mapped to a reference human genome sequence to determine from which chromosome the fragment is derived; the number of fragments mapped uniquely to the chromosome of interest are then counted and compared with the number of counts obtained from other chromosomes. A variety of bioinformatics algorithms have been developed to determine whether there is an increase or decrease in the expected number of counts around a set threshold which is suggestive of aneuploidy; for example, if the fetus has trisomy 21, more fragments from chromosome 21 will be present than expected in maternal plasma. Alternative NGS approaches involve the selective amplification of specific genomic loci on the chromosome of interest followed by sequencing. This approach may be more economical as the amount of sequencing required is reduced but has the limitation that only the preselected regions of interest can be studied and the development of these tests is potentially more labor intensive, although it does allow estimation of fetal fraction. Early studies validated this approach for the detection of trisomies 21 and 18 in high-risk pregnancies but, more recently, the results of a large general-population study have shown similar high performance in pregnancies at low prior risk. The third approach, a variation of the
Figure 3 Detection of a mutation in the fibroblast growth factor receptor 3 (FGFR3) gene causing thanatophoric dysplasia, showing the increasing ease of interpretation between polymerase chain reaction (PCR)-based method (a), digital PCR (b) and digital readout obtained from sequencing (c). PCR-based method (a) relies on subjective interpretation; very faint bands for mutant alleles in affected cell-free (cf) DNA can be seen (bottom arrows). The wild-type (normal) allele is strongly present in all samples (upper arrow). This compares with digital PCR (b) for detection of the mutant allele c.742 C>T (blue dot) and wild-type alleles (red dot). Each row represents one sample. Wild-type signals are present in all samples but the mutant allele is only present in the positive control (panel 1) and test sample (panel 2). Panel 3 is the result obtained from a normal pregnancy and shows only wild-type alleles present. The digital readout obtained from sequencing (c) reveals a very high wild-type allele count (blue), as this represents both maternal and fetal alleles, and a lower mutant allele (pink) count, but is still very high compared with the counts for other disease-causing mutations, indicating that the fetus has thanatophoric dysplasia as a result of the c.742 C>T mutation.

Table 3 Shift from invasive to non-invasive prenatal testing (NIPT) for achondroplasia and thanatophoric dysplasia in the UK from 2009 to 2013, as tests became validated and approved for use in the North East Thames Regional National Health Service genetics laboratory

| Year      | Achondroplasia Invasive | NIPT | Thanatophoric dysplasia Invasive | NIPT |
|-----------|--------------------------|------|---------------------------------|------|
| 2009–2010 | 28                       | 0    | 16                              | 0    |
| 2010–2011 | 27                       | 13   | 21                              | 0    |
| 2011–2012 | 28                       | 14   | 25                              | 2    |
| 2012–2013 | 20                       | 22   | 17                              | 11   |
| 2013–     | 10                       | 14   | 7                               | 18   |

Data are given as n. Other conditions for which NIPT has been performed in high-risk families include Apert syndrome (n = 7), Crouzon syndrome (n = 2), Fraser’s syndrome (n = 4), autosomal polycystic kidney disease, osteogenesis imperfecta (n = 2) and cystic fibrosis.

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have largely been developed using artificially produced samples, and reasonable validation data describing sensitivity, specificity and the positive predictive value in maternal plasma samples are yet to be published. Whilst this approach might increase the detection of pathogenic mutations, this targeted approach will only detect around 25% of pathogenic rearrangements as these occur across all chromosomes. There is also concern that using extended NIPT may increase the false-positive rate, potentially reversing the downward trend seen in invasive testing subsequent to the introduction of NIPT for aneuploidy. If this approach is to be used, it would seem sensible to confine its use to cases in which there is an increased incidence of pathogenic rearrangements, for example in euploid fetuses with multiple ultrasound anomalies76–78.

**Conclusions**

The use of cfDNA for the diagnosis of fetal genetic and chromosomal conditions is having a profound effect on the practice of fetal medicine worldwide. The advent of NIPT for aneuploidy is reducing the need for invasive testing, the rate of which has declined dramatically in some countries79, a fall that is met with approval from both women and health professionals alike as we move toward safer and earlier prenatal diagnosis80,81. The use of cfDNA to direct invasive testing or treatment in sex-linked diseases is also decreasing the need for invasive testing, whilst making prenatal diagnosis safer and more acceptable to high-risk families82; however, this may increase the economic burden on health services as
an increasing number of families elect to undergo NIPT for information. These changes will inevitably impact on care pathways and fetal medicine in general, in respect to both training and service provision, as the indications for invasive tests decrease. The pace of change has been rapid and we must urgently address how we structure our services so that we can provide safe services for those who continue to need invasive testing and treatment.

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