ANNUAL REVIEWS

Annual Review of Genomics and Human Genetics

The Next Frontier in Noninvasive Prenatal Diagnostics: Cell-Free Fetal DNA Analysis for Monogenic Disease Assessment

Lilian Pok Wa Zhong and Rossa W.K. Chiu

Department of Chemical Pathology, The Chinese University of Hong Kong, Shatin, Hong Kong, China; email: zlpw2304@link.cuhk.edu.hk, rossachiu@cuhk.edu.hk

Annu. Rev. Genom. Hum. Genet. 2022. 23:413–25

First published as a Review in Advance on March 22, 2022

The *Annual Review of Genomics and Human Genetics* is online at genom.annualreviews.org

https://doi.org/10.1146/annurev-genom-110821-113411

Keywords

cell-free fetal DNA, relative mutation dosage, relative haplotype dosage, next-generation sequencing, droplet digital PCR

Abstract

With the widespread clinical adoption of noninvasive screening for fetal chromosomal aneuploidies based on cell-free DNA analysis from maternal plasma, more researchers are turning their attention to noninvasive prenatal assessment for single-gene disorders. The development of a spectrum of approaches to analyze cell-free DNA in maternal circulation, including relative mutation dosage, relative haplotype dosage, and size-based methods, has expanded the scope of noninvasive prenatal testing to sex-linked and autosomal recessive disorders. Cell-free fetal DNA analysis for several of the more prevalent single-gene disorders has recently been introduced into clinical service. This article reviews the analytical approaches currently available and discusses the extent of the clinical implementation of noninvasive prenatal testing for single-gene disorders.

1. INTRODUCTION

To date, more than 2,300 single-gene disorders (SGDs) with a known molecular basis have been reported (5). The increasing adoption of expanded carrier screening means that more couples with inheritable disease alleles are identified (18), and the demand for prenatal testing from individuals with at-risk pregnancies may therefore increase. Conventionally, pregnancies with suspicious ultrasound findings, parental carrier status, or family history relevant for SGDs may warrant a definitive fetal genetic diagnosis, possibly necessitating amniocentesis or chorionic villus sampling, which poses a small but nonzero risk of miscarriage (1). The discovery of cell-free fetal DNA (cffDNA) in maternal plasma (27) offered opportunities for the development of DNA-based noninvasive prenatal testing (NIPT). Because NIPT can be done starting early in a pregnancy, it is widely used to determine fetal rhesus D blood group status (17) and screen for chromosomal aneuploidies (10). Lo et al. (26) found that the whole fetal genome and mutational profile can be constructed by cffDNA analysis, rendering noninvasive testing for SGDs theoretically possible. In contrast to the rapid adoption of noninvasive prenatal screening for aneuploidies, NIPT for SGDs has been making slow but steady progress (39). Heterogeneity in the mutational profiles of SGDs means that a range of testing approaches are needed (**Figure 1**), and one size may not fit all. Here, we focus on reviewing the more recent technological developments and the pace of clinical adoption of NIPT for SGDs.

2. ANALYTICAL APPROACHES

Cell-free DNA molecules are present extracellularly in circulation and are thought to be released after cell death. During pregnancy, DNA from placental cells is present in maternal plasma and is termed cffDNA. cffDNA typically amounts to approximately 10% of DNA in maternal plasma, with the remaining cell-free DNA molecules contributed by maternal cells. The motivation for analyzing maternal plasma DNA is mainly to access the cffDNA information in order to assess fetal chromosomal or genetic status. Because cell-free DNA molecules circulate in naturally fragmented forms, to gather genetic or genomic information, one generally needs to analyze many molecules while determining the sequence content of those molecules. Sequencing and PCR platforms have been used to assess the sequence content and/or quantify cell-free DNA molecules. The fetal (placental) or maternal origin of cell-free DNA molecules has been distinguished based on single-nucleotide polymorphisms (SNPs), sequence variants, and fragment size (because cffDNA is generally shorter than maternally derived cell-free DNA). The pool of cffDNA molecules could be further categorized as those showing sequence content inherited from the father, termed paternally inherited, and those showing sequence content inherited from the mother, termed maternally inherited.

2.1. Fetal Sex Determination

Fetal sex determination has a role in the assessment and management of pregnancies known to be at risk for sex-linked disorders or congenital adrenal hyperplasia. A female fetus would have lesser manifestations of a sex-linked disease than a male fetus and may obviate the need to perform invasive prenatal testing. On the other hand, a female fetus with congenital adrenal hyperplasia would be at risk of virilization and may be considered for steroid therapy. Fetal sex could be assessed by ultrasound and/or cffDNA analysis. cffDNA analysis has been performed to confirm ultrasound findings in order to determine fetal sex with a higher degree of confidence (24). In the case of congenital adrenal hyperplasia, genital virilization in an affected female fetus begins at approximately nine weeks of gestation. cffDNA analysis would allow earlier fetal sex assessment than ultrasonography and may therefore minimize unnecessary dexamethasone prescriptions

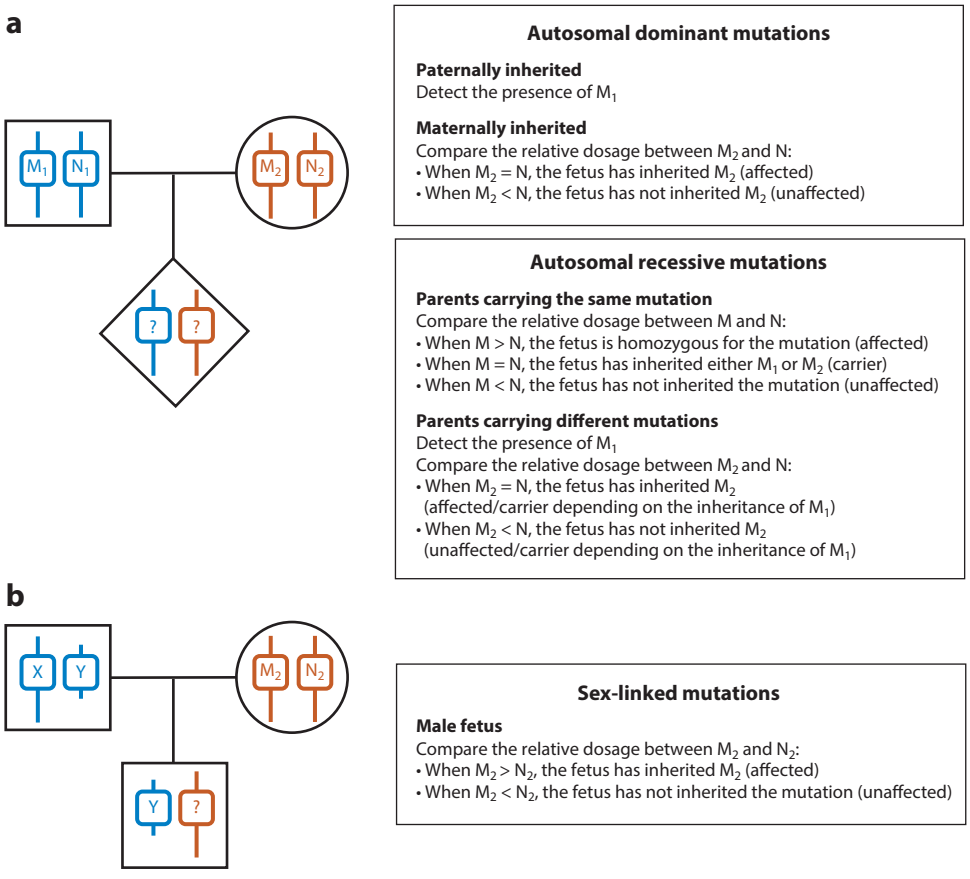

Figure 1

Schematic approaches for detecting (*a*) autosomal dominant and autosomal recessive mutations and (*b*) sex-linked mutations. M_1 and M_2 denote the paternal and maternal mutant alleles, respectively; N_1 and N_2 denote the paternal and maternal wild-type alleles, respectively; and X and Y denote the X chromosome and Y chromosome, respectively.

for pregnancies with male fetuses. However, the field has since moved on from relying only on fetal sex determination for SGD management. Approaches for the assessment of maternally inherited mutations on the X chromosome or autosomes have been developed to facilitate a more holistic fetal genotype assessment using cell-free DNA analysis. The analytical approach to adopt depends on whether the fetus is at risk for an autosomal or sex-linked condition or for an autosomal dominant or recessive disease and on the configuration of the maternal and paternal mutations. **Figure 1** summarizes the approach to take depending on the inheritance mode of the disease in question and the parental genotypes.

2.2. Autosomal Dominant Disorders Caused by De Novo or Paternally Inherited Mutations

cffDNA is present within a large background of cell-free maternal DNA in the maternal circulation. Interference by the background maternal DNA in maternal plasma is therefore an inevitable challenge in NIPT for SGDs. It is relatively more straightforward to detect mutations that are not

present in the mother—in other words, the paternally inherited mutations or de novo mutations that are not inherited from either parent. Soon after the discovery of cffDNA in maternal blood, a study showed that PCR and restriction enzyme digestion analysis can detect paternally inherited autosomal dominant disorders (38). Some of these tests have since been introduced into clinical practice; for example, cffDNA analysis was used to facilitate prenatal testing of *FGFR3*-related achondroplasia (6) and thanatophoric dysplasia (7) based on suspicious ultrasound findings specified in the testing criteria (31). A positive signal in cffDNA analysis would increase the likelihood that a paternally inherited or de novo *FGFR* mutation is present in the fetus.

For some time, testing has been based mainly on analyzing a small panel of mutations with PCR. Thanks to technological advancements, next-generation sequencing has offered new options for NIPT for SGDs. Detection of achondroplasia and thanatophoric dysplasia by a next-generation sequencing platform resulted in improved sensitivity and specificity compared with PCR-based methods, and a much expanded range of mutations can be identified in a single run (8). Dan et al. (13) used targeted sequencing to diagnose fetal lethal skeletal dysplasia by identifying de novo mutations in 16 relevant genes. Furthermore, Zhang et al. (51) utilized targeted sequencing, together with unique molecular identifiers (UMIs), to detect the prevalent autosomal dominant disorders. UMIs are used in a molecular barcoding technique that tags each original template DNA fragment with a unique short sequence before amplification or sequencing. All derivative products of the same template DNA molecule should have the same sequence and would have the same UMI. UMIs could therefore be used to distinguish genomic sequence variants from sequence errors introduced during laboratory analysis (51). Detection of paternally inherited traits has even been demonstrated for the detection of Huntington disease, which is caused by expanded trinucleotide repeat alleles (42).

With the success of using cell-free DNA analysis to detect paternally inherited autosomal dominant disorders, scientists began investigating the feasibility of noninvasively assessing autosomal recessive diseases (15). Information on paternally inherited alleles can also be useful in excluding fetal autosomal recessive disorders (11): If a paternally inherited wild-type allele appears in maternal plasma, the fetus is predicted to be unaffected. This approach has been applied to exclude fetal cystic fibrosis and beta thalassemia using either massively parallel sequencing (22) or PCR (12).

2.3. Sex-Linked and Autosomal Recessive Disorders

Unlike alleles transmitted by the father and de novo mutations that are absent from the maternal genome, maternally transmitted alleles are invariably present in the maternal plasma sample among the cell-free DNA molecules contributed by maternal cells. The mere detection of a maternal mutation in a maternal plasma sample may not yield conclusive information regarding the fetal inheritance status. Hence, more sophisticated strategies are needed to analyze maternal plasma DNA for the purpose of determining the fetal inheritance of mutations transmitted by the mother that are relevant to the prenatal assessment of sex-linked and autosomal recessive disorders.

2.3.1. Relative mutation dosage. Using cell-free DNA analysis to determine which maternal allele the fetus has inherited was previously thought to be impossible due to the interference of the maternal DNA background. Relative mutation dosage (RMD) is an approach that measures the quantitative ratio between a wild-type allele and a maternal mutant allele among the fragmented cell-free DNA molecules circulating in maternal plasma to determine the fetal genotype at the tested locus (29). If the ratio between the alleles in maternal plasma is in balance, then the fetus is deemed to be heterozygous. If there is an allelic imbalance—meaning that one of the two alleles is overrepresented—then the fetus is deemed to have inherited the overrepresented allele from the

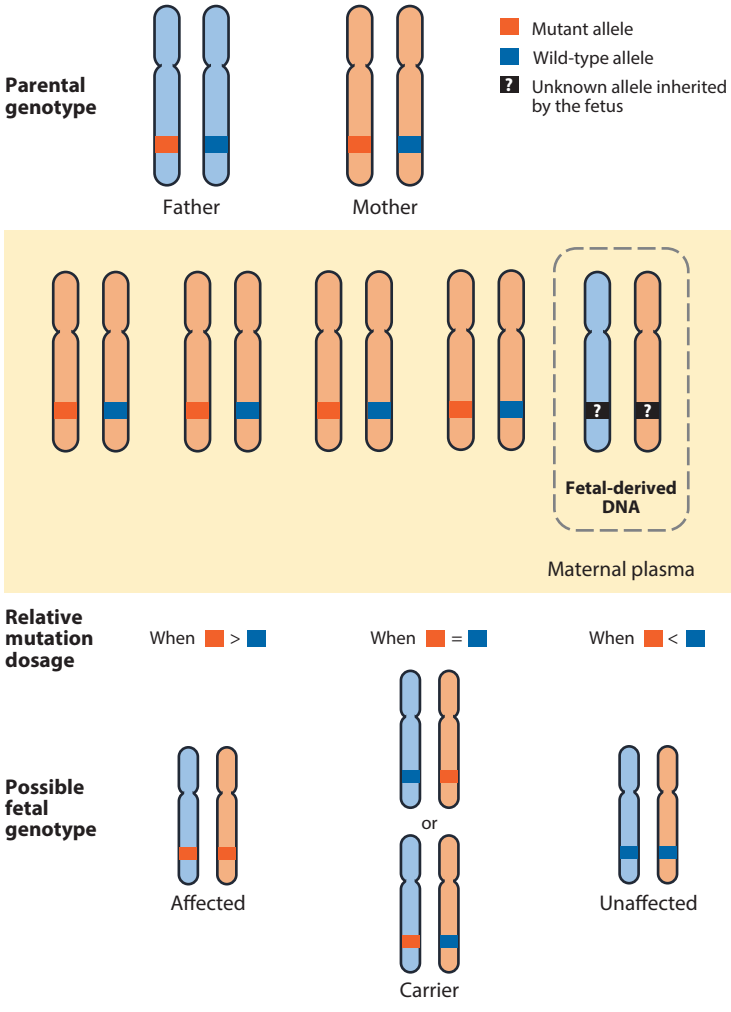

Figure 2

Example of the use of relative mutation dosage analysis to detect autosomal recessive disorders when the parents carry the same mutant allele (*orange*) and a wild-type allele (*blue*). The shaded area in the middle schematically presents the distributions of the mutant allele and wild-type allele in a maternal plasma sample containing a 20% fetal fraction. The fetal genotype is interpreted by measuring the ratio between the mutant and wild-type alleles. If the mutant allele is over- or underrepresented in the maternal plasma, then the fetus is homozygous for the mutant allele or wild-type allele, respectively. If the allelic ratio between the mutant and wild-type allele is balanced, then the fetus is heterozygous for the mutation.

mother (**Figure 2**). For example, if the mutant allele is overrepresented, then the fetus has likely inherited the mutant allele from the mother.

Droplet digital PCR, a highly precise quantification method based on the premise that the sample has a sufficient fetal fraction (the proportion of DNA of fetal origin in the tested maternal plasma sample), has been successfully deployed to differentiate slight differences in allelic representation. In combination with the concept of RMD, it has been applied to detect both paternally inherited alleles and maternally inherited alleles, with a reported accuracy of up to 96% (35).

Other studies have applied targeted sequencing for RMD analysis to detect common mutations associated with beta thalassemia (47) and sickle cell anemia (41).

RMD is highly favorable when it is used to detect single and common mutations in the population, but it requires specific primers or probes for each mutation and may be more challenging to use for the detection of disease loci associated with structural chromosomal changes or pseudogenes (9). The utility of each RMD assay is dependent on the prevalence of the targeted mutation for the disease in question. Moreover, the robustness of the test is dependent on the accurate measurement of the fetal fraction in maternal plasma. The statistical strength of the determination of fetal inheritance is affected if the tested mutation is not in the panel or the fetal fraction is below the testing threshold, leading to inconclusive or discordant results (2). These limitations must be overcome before the RMD approach can be translated into clinical use.

2.3.2. Relative haplotype dosage. Expanding from RMD, relative haplotype dosage (RHDO) counts the SNP alleles on haplotypes linked with the wild-type and mutant alleles (26). The presence or absence of a statistically significant imbalance between the counts of alleles belonging to the respective haplotypes reveals the haplotype the fetus has inherited, thereby predicting whether the fetus has inherited the mutant allele (**Figure 3**). To identify the paternal inheritance of the fetus, heterozygous SNPs in the father and homozygous SNPs in the mother are classified as informative; to identify the maternal inheritance of the fetus, homozygous SNPs in the father and heterozygous SNPs in the mother are classified as informative. The presence of the paternal mutant allele or mutant-linked paternal haplotype in maternal plasma suggests that the fetus has inherited the paternal mutation. Overrepresentation of the mutant-linked maternal haplotype as compared with the wild-type-linked maternal haplotype indicates that the fetus has likely inherited the mutant allele from the mother. This approach has been shown to be feasible in detecting maternally inherited congenital adrenal hyperplasia (32), Duchenne and Becker muscular dystrophies (34), spinal muscular atrophy (33), beta thalassemia (26), and cystic fibrosis (4) in either a whole-genome or targeted manner. When applying RHDO, careful selection of the set of polymorphisms is warranted to minimize the chance of a recombination event confounding the test result interpretation (23, 32, 34).

Implementing RHDO requires haplotype information from the maternal genome. The parental haplotypes may be deduced if DNA from a family member known to be affected by the disease (the proband) is available (32). Proband genomic DNA is extracted to facilitate the phasing (i.e., to determine the order of SNP alleles along a chromosome) of mutant-linked and wild-type-linked haplotypes in the parents, but this approach would prohibit the application of RHDO in pregnancies without a known family history. Moreover, haplotype information on the affected proband or family members is not always available for phasing.

Direct haplotyping of parents is potentially a solution for the unavailability of proband DNA and maximizes the applicability of the haplotype-based approach to most families. Zeevi et al. (50) constructed the universal founder haplotype to map the common mutation of Gaucher disease in the Ashkenazi Jewish population. In 2017, two research groups (23, 44) published promising approaches for performing direct haplotyping on parental DNA, thereby facilitating RHDO analysis of different kinds of SGDs. This greatly widened the testing spectrum for SGDs. Hui et al. (23) performed microfluidics-based linked-read sequencing to reconstruct parental haplotypes. Linked-read sequencing applies the same label to short sequence tags derived from each high-molecular-weight DNA molecule, while short sequence tags from other high-molecular-weight DNA molecules are labeled differently. This approach allows the short sequence reads originally derived from any one long DNA molecule to be reidentified as belonging to the same

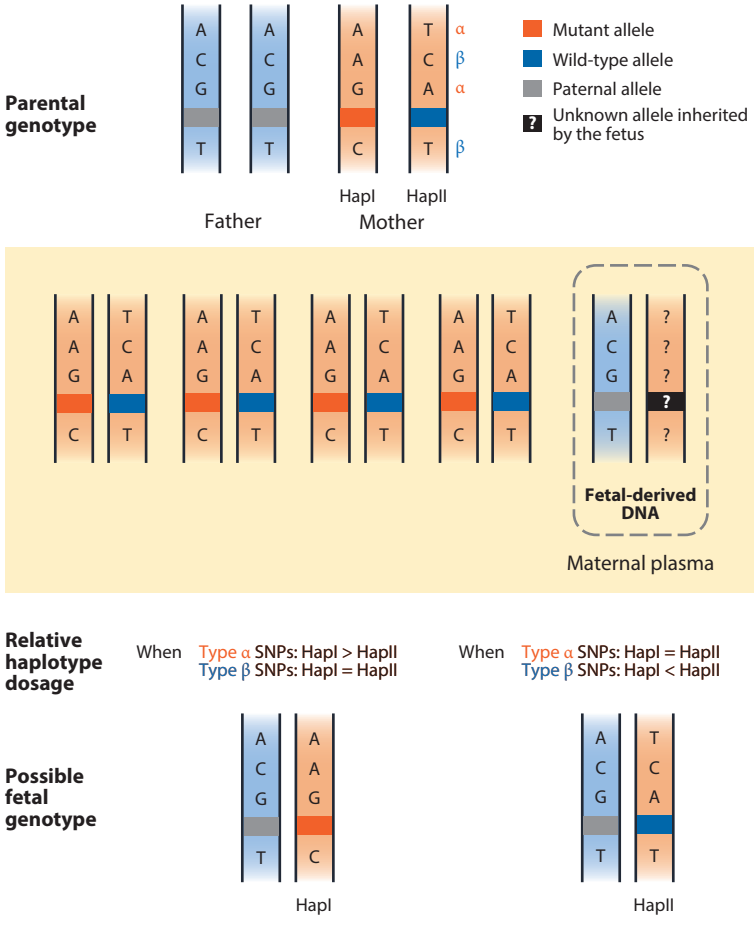

Figure 3

Example of the use of relative haplotype dosage analysis to detect fetal inheritance of a maternal mutation. The shaded area in the middle presents hypothetical distributions of a mutant allele (*orange*) and wild-type allele (*blue*) in maternal plasma containing a 20% fetal fraction. Informative single-nucleotide polymorphisms (SNPs) from the mother are selected to construct the maternal mutant-linked haplotype and wild-type-linked haplotype. Paternal alleles that are the same as those in maternal haplotype I (HapI) are defined as type α SNPs, while paternal alleles that are the same as those in maternal haplotype II (HapII) are defined as type β SNPs. The fetal genotype is interpreted by measuring the relative ratio of the mutant-linked and wild-type-linked haplotypes.

haplotype. After targeted sequencing, Hui et al. (23) linked and phased sequence reads that contained the same DNA label as the mutant allele into the mutant-linked haplotype, while reads that contained the same DNA label as the wild-type allele were linked and phased into the wild-type-linked haplotype. Vermeulen et al. (44) proposed the use of targeted locus amplification, which is a cross-linking-based direct haplotyping approach. In brief, neighboring sequences that are in close spatial proximity are first cross-linked by formaldehyde and enzymatically digested. The cross-linked fragments are then religated and de-cross-linked to form a large DNA circle for inverse PCR amplification and sequencing. In theory, SNPs that can be found in the same ligation product are from the same chromosome and therefore will be phased to a haplotype.

2.3.3. Differentiating fetal and maternal DNA by molecular size. cffDNA is known to be shorter than cell-free maternal DNA (3, 49). Circulating single-molecule amplification and resequencing technology (cSMART) is a PCR-based method that can potentially reduce amplification bias and hence facilitates the detection of fetal mutations in maternal plasma (30). Each cell-free DNA molecule is uniquely barcoded, amplified, and then denatured to form a single-stranded circular product. A pair of inverse primers are designed near the mutation locus to target the fragments of interest and form a linear DNA product for sequencing. Uniquely barcoded sequences of different sizes are counted to determine the fetal allelic ratio. This assay has been applied to the noninvasive prenatal detection of Wilson disease (30) and phenylketonuria (16) using a set of primers covering the known pathogenic mutations and polymorphisms in the region of interest. Computationally, Rabinowitz et al. (36) developed a bioinformatic algorithm that uses DNA size to distinguish the origins of DNA fragments. They applied a Bayesian statistic in the algorithm to estimate the probability that fragments are derived from the fetus based on the size information to determine the fetal alleles and then interpret the genotype of the fetus. These approaches are still in a proof-of-concept stage and require more comprehensive data for validation.

3. CLINICAL IMPLEMENTATION

cffDNA analysis for the prenatal assessment of paternally inherited autosomal dominant disorders (including achondroplasia and thanatophoric dysplasia) (15) and paternal mutation exclusion of cystic fibrosis (22) has become clinically available. The use of RHDO for the noninvasive prenatal assessment of autosomal recessive and X-linked disorders, such as spinal muscular atrophy (48), Duchenne and Becker muscular dystrophies (48), and cystic fibrosis (4, 48), launched recently. Patients with a known family history of the disorder were recruited to provide both parental and proband DNA samples for haplotype phasing. For autosomal recessive disorders, genomic DNA was required from trios, including the mother, the father, and a proband, who could be either an affected child or an unaffected noncarrier child. If the parents were carriers for different mutations, a carrier child could serve as the proband. For sex-linked disorders, only women shown to be pregnant with a male fetus were accepted, and genomic DNA from the mother and a male proband (regardless of his genotype status) was required. Maternal plasma DNA was extracted, target captured, and sequenced. When the paternal-specific allele was determined to be wild-type linked with a demonstration of statistical significance, a report could be generated to conclude that the fetus was likely to be unaffected. Otherwise, the analysis would proceed to RHDO to determine the fetal inheritance of the maternal alleles.

Based on the experiences from laboratories offering RHDO-based testing, the reliability of RHDO testing depends on several factors:

1. The fetal fraction must be adequate (4, 23, 32, 44, 48, 51). Fetal DNA typically constitutes approximately 10% of the maternal plasma DNA on average (28), and an adequate fetal fraction is a key factor because increasing the sequencing depth does not improve the results of samples with a low fetal fraction (48). A minimum fraction of 4% has been recommended in order to produce a confident result (4, 41). The fetal fraction in maternal plasma can be affected by preanalytical factors, including specimen handling and transportation conditions. Selecting an appropriate blood collection tube is important to preserve cell-free DNA and avoid excessive cell lysis. For example, certain collection tubes contain preservatives that can stabilize the nucleated blood cells for up to 5 days at room temperature (45). Blood samples can be stored at room temperature in ethylenediaminetetraacetic acid (EDTA) tubes for up to 6 h, and tubes with cell-stabilizing preservatives can be stored for up to 14 days (40). An insufficient volume or hemolyzed blood is not optimal for NIPT for SGDs. Moreover, a

double centrifugation protocol should be adopted to minimize contamination by maternal cellular DNA.

2. A recombination event occurring between the tested RHDO SNPs and the disease locus would lead to test results that are discordant with the fetal genotype. Although a bidirectional RHDO analysis could be conducted to locate the site of recombination with higher accuracy, whether the site is proximal to the mutant allele would remain inconclusive (23, 48).
3. The number of informative SNPs also influences the robustness of RHDO analysis. This limiting factor is more frequently encountered in consanguineous pregnancies (25, 26); because of the genetic similarity of the parents, fewer informative SNPs can be identified.
4. cffDNA analysis becomes more challenging in certain situations, such as multiple pregnancies, prior organ transplantation in the pregnant woman, and the presence of maternal somatic mosaicism, confined placental mosaicism, or uniparental disomy (9). It is important to consider these issues, as they may lead to false-positive or false-negative results. Off-target copy number variants and variants of unknown significance, which could normally be detected by a conventional chromosomal array, may also not be apparent with cffDNA testing. Moreover, since the test is based on the haplotype inheritance from the parents, fetal de novo variants are not guaranteed to be detected. Therefore, parents should be counseled before proceeding with haplotype-based NIPT.

NIPT for SGDs has received generally positive comments from patients and health professionals during its clinical implementation (19, 20). NIPT provides a safe and convenient diagnostic option for parents and widens the number of choices available for prenatal management. Approximately 90% of potential service users would opt for NIPT for an SGD if it was clinically available (22). Furthermore, the fact that NIPT for SGDs is available from the eighth week of gestation, with a turnaround time of approximately 11 days (4, 48), can give parents more time to prepare themselves psychologically and further consider how to manage the pregnancy. Although both patients and health professionals have expressed similar expectations for prenatal testing strategies, patients had a greater emphasis on safety, while health professionals had more awareness of the timing and accuracy of the test (21). Cost analysis revealed that technical difficulties, the number of samples, and availability for batch testing were key factors influencing costs (43). Consequently, NIPT for autosomal dominant disorders was relatively cheaper than invasive diagnosis, but NIPT for autosomal recessive or sex-linked disorders generally cost more than invasive testing, depending on the analytical approach and care pathway.

Ethical considerations associated with NIPT for SGDs have been raised when the testing is solely informational (14) and regarding whether resources should be directed toward testing that would not change any aspect of pregnancy management, although it may provide psychological benefits and allow early preparation for the birth of an affected child. Another issue is that the relatively simple and risk-free sampling nature of NIPT could influence women's autonomous decision-making about whether to opt for testing (14). On the one hand, NIPT may facilitate this decision-making by providing pregnant women with an additional testing option, but on the other hand, it may prompt a feeling of pressure to accept the test and make a decision after receiving the results. Wider discussion surrounding these ethical issues should therefore be encouraged, with the goal of developing best practice guidelines.

4. PROSPECTS

Haplotype-based analysis is becoming a trend for NIPT for SGDs, and the successful demonstration of direct haplotyping inspires the thought of applying this concept more cost-effectively

and less laboriously. The use of third-generation long-read sequencing technologies, such as Single Molecule, Real-Time (SMRT) sequencing from Pacific Biosciences and Oxford Nanopore sequencing, may simplify the haplotype phasing procedures. Long read length combined with single-molecule resolution may allow the phasing of more informative SNPs into haplotype blocks and increase accuracy (37).

In addition to the continuous efforts to develop NIPT approaches for an increased range of SGDs, research efforts have been directed at optimizing the quality control steps and care pathways. Currently, determining the fetal fraction is a common quality control to avoid false-negative results. Haplotype accuracy—particularly for maternally inherited haplotypes—and crossover resolution are highly affected by the fetal fraction and number of informative SNPs (36). Typically, when an inconclusive result is reported, providing comprehensive genetic counseling services for a high-risk pregnancy is of the utmost importance, and options for invasive prenatal diagnosis could be offered to the patient for consideration. As the risk of miscarriage associated with chorionic villus sampling and amniocentesis is lower than previously described (46), the NIPT test failure rate needs to be extremely low to outperform other testing methods. More clinical information regarding the accuracy of the test and the standard care pathway is needed for successful NIPT implementation for SGDs.

5. CONCLUSION

With the successful implementation of NIPT for aneuploidies, research on NIPT for SGDs has gathered momentum. Initial efforts focused on the detection of paternally inherited mutations because it was relatively more straightforward to detect mutations that were not present in the mother. The development of RMD and RHDO analysis overcame the limitations caused by the maternal DNA background in plasma, enabling the detection of maternally inherited mutations by counting allelic ratio differences. A proband-free haplotype-based protocol reduced some of the restrictions previously imposed on cffDNA analysis for SGD assessment. The service provision of NIPT for SGDs has primarily received positive feedback from service users and professionals. We envisage that research into NIPT for SGDs will continue and will gradually be adopted in prenatal care. More research is ongoing to examine the feasibility of expanding the spectrum of tested SGDs and their clinical adoption.

DISCLOSURE STATEMENT

R.W.K.C. has filed patent applications and been granted patents on aspects of cell-free nucleic acid–based prenatal diagnostics, the latter of which have been licensed to Illumina, Sequenom, Xcelom, DRA, and Take2; is on the board of DRA and Take2; and has equities in Illumina, DRA, and Take2.

ACKNOWLEDGMENTS

This work was supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region government under the Theme-Based Research Scheme (T12-403/15-N).

LITERATURE CITED

1. Alfirevic Z, Navaratnam K, Mujezinovic F. 2017. Amniocentesis and chorionic villus sampling for prenatal diagnosis. *Cochrane Database Syst. Rev.* 9:CD003252
2. Barrett AN, McDonnell TC, Chan KA, Chitty LS. 2012. Digital PCR analysis of maternal plasma for noninvasive detection of sickle cell anemia. *Clin. Chem.* 58:1026–32

3. Chan KCA, Zhang J, Hui ABY, Wong N, Lau TK, et al. 2004. Size distributions of maternal and fetal DNA in maternal plasma. *Clin. Chem.* 50:88–92
4. Chandler NJ, Ahlfors H, Drury S, Mellis R, Hill M, et al. 2020. Noninvasive prenatal diagnosis for cystic fibrosis: implementation, uptake, outcome, and implications. *Clin. Chem.* 66:207–16
5. Chial H. 2008. Rare genetic disorders: learning about genetic disease through gene mapping, SNPs, and microarray data. *Nat. Educ.* 1:192
6. Chitty LS, Griffin DR, Meaney C, Barrett A, Khalil A, et al. 2011. New aids for the non-invasive prenatal diagnosis of achondroplasia: dysmorphic features, charts of fetal size and molecular confirmation using cell-free fetal DNA in maternal plasma. *Ultrasound Obstet. Gynecol.* 37:283–89
7. Chitty LS, Khalil A, Barrett AN, Pajkrt E, Griffin DR, Cole TJ. 2013. Safe, accurate, prenatal diagnosis of thanatophoric dysplasia using ultrasound and free fetal DNA. *Prenat. Diagn.* 33:416–23
8. Chitty LS, Mason S, Barrett AN, McKay F, Lench N, et al. 2015. Non-invasive prenatal diagnosis of achondroplasia and thanatophoric dysplasia: next-generation sequencing allows for a safer, more accurate, and comprehensive approach. *Prenat. Diagn.* 35:656–62
9. Chiu EKL, Hui WWI, Chiu RWK. 2018. cfDNA screening and diagnosis of monogenic disorders—where are we heading? *Prenat. Diagn.* 38:52–58
10. Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, et al. 2008. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *PNAS* 105:20458–63
11. Chiu RWK, Lau TK, Cheung PT, Gong ZQ, Leung TN, Lo YMD. 2002. Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. *Clin. Chem.* 48:778–80
12. Chiu RWK, Lau TK, Leung TN, Chow KC, Chui DH, Lo YMD. 2002. Prenatal exclusion of β thalassaemia major by examination of maternal plasma. *Lancet* 360:998–1000
13. Dan S, Yuan Y, Wang Y, Chen C, Gao C, et al. 2016. Non-invasive prenatal diagnosis of lethal skeletal dysplasia by targeted capture sequencing of maternal plasma. *PLOS ONE* 11:e0159355
14. Deans Z, Hill M, Chitty LS, Lewis C. 2013. Non-invasive prenatal testing for single gene disorders: exploring the ethics. *Eur. J. Hum. Genet.* 21:713–18
15. Drury S, Mason S, McKay F, Lo K, Boustred C, et al. 2016. Implementing non-invasive prenatal diagnosis (NIPD) in a national health service laboratory; from dominant to recessive disorders. In *Circulating Nucleic Acids in Serum and Plasma – CNAPS IX*, ed. PB Gahan, M Fleischhacker, B Schmidt, pp. 71–75. Cham, Switz.: Springer
16. Duan H, Liu N, Zhao Z, Liu Y, Wang Y, et al. 2019. Non-invasive prenatal testing of pregnancies at risk for phenylketonuria. *Arch. Dis. Child. Fetal Neonatal Ed.* 104:F24–29
17. Finning KM, Martin PG, Soothill PW, Avent ND. 2002. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 42:1079–85
18. Gregg AR, Edwards JG. 2018. Prenatal genetic carrier screening in the genomic age. *Semin. Perinatol.* 42:303–6
19. Hill M, Compton C, Karunaratna M, Lewis C, Chitty L. 2014. Client views and attitudes to non-invasive prenatal diagnosis for sickle cell disease, thalassaemia and cystic fibrosis. *J. Genet. Counsel.* 23:1012–21
20. Hill M, Karunaratna M, Lewis C, Forya F, Chitty L. 2013. Views and preferences for the implementation of non-invasive prenatal diagnosis for single gene disorders from health professionals in the United Kingdom. *Am. J. Med. Genet. A* 161A:1612–18
21. Hill M, Suri R, Nash EF, Morris S, Chitty LS. 2014. Preferences for prenatal tests for cystic fibrosis: a discrete choice experiment to compare the views of adult patients, carriers of cystic fibrosis and health professionals. *J. Clin. Med.* 3:176–90
22. Hill M, Twiss P, Verhoef TI, Drury S, McKay F, et al. 2015. Non-invasive prenatal diagnosis for cystic fibrosis: detection of paternal mutations, exploration of patient preferences and cost analysis. *Prenat. Diagn.* 35:950–58
23. Hui WWI, Jiang P, Tong YK, Lee WS, Cheng YKY, et al. 2017. Universal haplotype-based noninvasive prenatal testing for single gene diseases. *Clin. Chem.* 63:513–24
24. Hyett JA, Gardener G, Stojilkovic-Mikic T, Finning KM, Martin PG, et al. 2005. Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. *Prenat. Diagn.* 25:1111–16

25. Jenkins LA, Deans ZC, Lewis C, Allen S. 2018. Delivering an accredited non-invasive prenatal diagnosis service for monogenic disorders and recommendations for best practice. *Prenat. Diagn.* 38:44–51
26. Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, et al. 2010. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci. Transl. Med.* 2:61ra91
27. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, et al. 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet* 350:485–87
28. Lun FMF, Chiu RWK, Chan KCA, Leung TY, Lau TK, Lo YMD. 2008. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin. Chem.* 54:1664–72
29. Lun FMF, Tsui NBY, Chan KCA, Leung TY, Lau TK, et al. 2008. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. *PNAS* 105:19920–25
30. Lv W, Wei X, Guo R, Liu Q, Zheng Y, et al. 2015. Noninvasive prenatal testing for Wilson disease by use of circulating single-molecule amplification and resequencing technology (cSMART). *Clin. Chem.* 61:172–81
31. Mellis R, Chandler N, Jenkins L, Chitty LS. 2020. The role of sonographic phenotyping in delivering an efficient noninvasive prenatal diagnosis service for *FGFR3*-related skeletal dysplasias. *Prenat. Diagn.* 40:785–91
32. New MI, Tong YK, Yuen T, Jiang P, Pina C, et al. 2014. Noninvasive prenatal diagnosis of congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. *J. Clin. Endocrinol. Metab.* 99:E1022–30
33. Parks M, Court S, Bowns B, Cleary S, Clokie S, et al. 2017. Non-invasive prenatal diagnosis of spinal muscular atrophy by relative haplotype dosage. *Eur. J. Hum. Genet.* 25:416–22
34. Parks M, Court S, Cleary S, Clokie S, Hewitt J, et al. 2016. Non-invasive prenatal diagnosis of Duchenne and Becker muscular dystrophies by relative haplotype dosage. *Prenat. Diagn.* 36:312–20
35. Perlado S, Bustamante-Aragones A, Donas M, Lorda-Sanchez I, Plaza J, Rodríguez de Alba M. 2016. Fetal genotyping in maternal blood by digital PCR: towards NIPD of monogenic disorders independently of parental origin. *PLOS ONE* 11:e0153258
36. Rabinowitz T, Polsky A, Golan D, Danilevsky A, Shapira G, et al. 2019. Bayesian-based noninvasive prenatal diagnosis of single-gene disorders. *Genome Res.* 29:428–38
37. Rhoads A, Au KF. 2015. PacBio sequencing and its applications. *Genom. Proteom. Bioinform.* 13:278–89
38. Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. 2000. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet* 356:1170
39. Scotchman E, Chandler NJ, Mellis R, Chitty LS. 2020. Noninvasive prenatal diagnosis of single-gene diseases: the next frontier. *Clin. Chem.* 66:53–60
40. Ungerer V, Bronkhorst AJ, Holdenrieder S. 2020. Preanalytical variables that affect the outcome of cell-free DNA measurements. *Crit. Rev. Clin. Lab. Sci.* 57:484–507
41. van Campen J, Silcock L, Yau S, Daniel Y, Ahn JW, et al. 2020. A novel non-invasive prenatal sickle cell disease test for all at-risk pregnancies. *Br. J. Haematol.* 190:119–24
42. van den Oever JME, Bijlsma EK, Feenstra I, Muntjewerff N, Mathijssen IB, et al. 2015. Noninvasive prenatal diagnosis of Huntington disease: detection of the paternally inherited expanded CAG repeat in maternal plasma. *Prenat. Diagn.* 35:945–49
43. Verhoef TI, Hill M, Drury S, Mason S, Jenkins L, et al. 2016. Non-invasive prenatal diagnosis (NIPD) for single gene disorders: cost analysis of NIPD and invasive testing pathways. *Prenat. Diagn.* 36:636–42
44. Vermeulen C, Geeven G, de Wit E, Verstegen MJA, Jansen RP, et al. 2017. Sensitive monogenic noninvasive prenatal diagnosis by targeted haplotyping. *Am. J. Hum. Genet.* 101:326–39
45. Wienzek-Lischka S, Bachmann S, Froehner V, Bein G. 2020. Potential of next-generation sequencing in noninvasive fetal molecular blood group genotyping. *Transfus. Med. Hemother.* 47:14–22
46. Wulff CB, Gerds TA, Rode L, Ekelund CK, Petersen OB, et al. 2016. Risk of fetal loss associated with invasive testing following combined first-trimester screening for down syndrome: a national cohort of 147 987 singleton pregnancies. *Ultrasound Obstet. Gynecol.* 47:38–44
47. Xiong L, Barrett AN, Hua R, Ho S, Jun L, et al. 2018. Non-invasive prenatal testing for fetal inheritance of maternal β-thalassaemia mutations using targeted sequencing and relative mutation dosage: a feasibility study. *BJOG* 125:461–68

48. Young E, Bowns B, Gerrish A, Parks M, Court S, et al. 2020. Clinical service delivery of noninvasive prenatal diagnosis by relative haplotype dosage for single-gene disorders. *J. Mol. Diagn.* 22:1151–61
49. Yu SCY, Chan KCA, Zheng YWL, Jiang P, Liao GJW, et al. 2014. Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *PNAS* 111:8583–88
50. Zeevi DA, Altarescu G, Weinberg-Shukron A, Zahdeh F, Dinur T, et al. 2015. Proof-of-principle rapid noninvasive prenatal diagnosis of autosomal recessive founder mutations. *J. Clin. Investig.* 125:3757–65
51. Zhang J, Li J, Saucier JB, Feng Y, Jiang Y, et al. 2019. Non-invasive prenatal sequencing for multiple Mendelian monogenic disorders using circulating cell-free fetal DNA. *Nat. Med.* 25:439–47