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Noninvasive prenatal diagnosis of single gene diseases - the next frontier

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Abstract**Background**

Cell-free fetal DNA (cffDNA) is present in the maternal blood from around 4 weeks gestation and makes up 5-20% of the total circulating cfDNA in maternal plasma. Presence of cffDNA has allowed development of non-invasive prenatal diagnosis (NIPD) for single gene disorders. This can be performed from 9 weeks gestation and offers a definitive diagnosis without the miscarriage risk associated with invasive procedures. One of the major challenges is distinguishing fetal mutations in the high background of maternal cfDNA, and research is currently focusing on the technological advances required in order to solve this problem.

Content

Here, the authors review the literature to describe the current status of NIPD for monogenic disorders and discuss how the evolving methodologies and technologies are expected to impact this field in both the commercial and public healthcare setting.

Summary

Non-invasive prenatal diagnosis for single gene diseases was first reported in 2000 and took 12 years to be approved for use in a public health service. Implementation has remained slow but is expected to increase as this testing becomes cheaper, faster, and more accurate. There are still many technical and analytical challenges ahead, and it is vital that discussions surrounding the ethical and social impact of NIPD take account of the considerations required to implement these services safely into the healthcare setting, whilst keeping up with the technological advances.

Background

Since the identification of cell-free fetal DNA (cffDNA) in maternal plasma in 1997 there have been rapid strides in exploiting its presence for prenatal diagnosis and screening (Table 1). The first proof of principle studies using cfDNA to detect fetal aneuploidy were published in 2008 (1, 2), following which there was rapid commercialisation and now non-invasive prenatal testing (NIPT) for aneuploidy is widely used across the globe as a screening test for the major fetal trisomies (3), either as a first line or contingent test following traditional combined testing. Unlike non-invasive prenatal testing, where a positive result requires confirmation following an invasive test, non-invasive prenatal diagnosis (NIPD)

offers the advantage of a definitive diagnosis without an invasive procedure and the associated miscarriage risk (4). This is because confined placental mosaicism, which can cause false positive results for aneuploidy NIPT, does not occur with NIPD for monogenic disorders as these are targeted tests, and false positive results from maternal DNA can be avoided by analysing the maternal germline DNA in parallel with the cell free DNA. NIPD can also be offered earlier in pregnancy compared to invasive testing, from 7 weeks for fetal sexing or 9 weeks for monogenic disorders. This can reduce parental anxiety and allow more time for decision making and planning (5). Progress with NIPD for monogenic disorders however has been much slower than NIPT. Although the first report of NIPD for a monogenic disorder, myotonic dystrophy (6), was in 2000 it wasn't until 2012 that it was used outside of a research setting when NIPD for achondroplasia and thanatophoric dysplasia were approved for use in the UK National Health Service (NHS) (7, 8). Since then implementation remains slow with most testing outside of the UK still being delivered in a research setting (9). This is unlike implementation of NIPT for aneuploidy which has been rapid and global, largely due to the significant commercial drive (3), unlike NIPD for monogenic disorders which has attracted less interest, no doubt because this represents a much smaller market opportunity and many cases have to be provided on a bespoke, patient or disease-specific basis. The methods and workflows are labour-intensive and not readily scalable (9).

There are significant challenges to overcome for NIPD. Circulating cffDNA, which is released from the placenta from about 4 weeks gestation, makes up only 5-20% of total circulating cfDNA in maternal plasma. This percentage increases with gestation and is influenced by factors such as maternal weight, smoking and pregnancy complications such as pre-eclampsia. Optimised techniques and highly sensitive detection approaches are therefore required in order to detect variants in the fetal DNA. Fetal fraction must be calculated in order to confirm that there are sufficient levels of cffDNA present and to avoid false negative results. For male fetuses this can be achieved using detection of SRY sequences, but for female fetuses use of human leukocyte antigen (HLA) or a specific fetal load assay, consisting of a panel of informative heterozygous single nucleotide polymorphisms (SNPs), is required. An alternative method that has been explored is using the methylation pattern of the promoter of *RASSF1A* in order to distinguish placental-derived hypermethylated sequences from maternal-derived hypomethylated sequences (10). Another issue is the short fragment length of cffDNA, which makes detection of triplet repeats and large deletions or duplications challenging. There is also a risk of false positive results due to a vanishing twin, as the placenta continues to shed DNA for some time after the demise of the fetus (11), or maternal somatic mosaicism, as testing analyses all cfDNA in maternal plasma. This necessitates the need for ultrasound scans for the detection of additional empty gestational sacs and parallel testing of maternal genomic DNA, respectively.

Current status – where are we now?

The first NIPD testing to be approved for use in clinical practice in the UK National Health Service (NHS) was in 2011 for fetal sex determination and in Europe Rhesus D status in *RHD* negative mothers (9, 12, 13). Fetal sex determination using NIPD is now routinely used for families with X-linked disorders, such as Duchenne muscular dystrophy and

haemophilia, where invasive testing can be limited to male fetuses and therefore reduced by 50% (14), or suspected congenital adrenal hyperplasia (avoiding the unnecessary treatment of male fetuses with dexamethasone) (15).

Following the success of cell-free fetal sex determination came the development and implementation in the UK of NIPD for autosomal dominant disorders caused by *de novo* or paternally inherited mutations, where variants in the fetal DNA can easily be distinguished in the high background of maternal cfDNA (9). The original approach for these NIPD tests was PCR followed by restriction enzyme digest (PCR-RED), and this was approved for use in the UK NHS for achondroplasia (7) and thanatophoric dysplasia (8) in 2012, but only allowed testing of individual mutations and due to the subjective nature of the analysis there was a high inconclusive rate of ~8%. With sequencing developments these approaches have been superseded to allow development of NGS panels, which allow multiple variants to be assessed in a single and more accurate test, and this approach was approved for use in clinical practice in the UK NHS in 2014 (16). Since then panel-based NGS has been used to offer testing for autosomal dominant disorders such as *FGFR3*-related skeletal dysplasia and *FGFR2*-related craniosynostosis syndromes (9). The same principle is also applied to paternal mutation exclusion for autosomal recessive disorders such as cystic fibrosis and β -thalassaemia. In this situation a paternal exclusion test can be offered if parents are heterozygous for different mutations. Invasive testing will then only be required if the paternal mutant allele is detected (17, 18). Bespoke tests can also be designed and carried out for a range of monogenic disorders for families at risk of a recurrence of a rare disease where the mutation is known, however this is expensive and labour-intensive as it is developed on an individual family basis (19).

NIPD for X-linked and autosomal recessive disorders where both parents are carriers of the same mutation has posed a greater challenge. This is due to the need to assess differences in low levels of fetal mutant alleles in a high background of maternal mutant allele already present in the circulating cfDNA. This means that the relative proportions of mutant:wild-type allele need to be determined rather than the presence or absence of an allele not present in the mother. Simply put, the presence of a fetus with a different genotype to the carrier mother (in other words either homozygous mutant or homozygous wild-type) will slightly alter the mutant to wild-type ratio in the cfDNA. Relative mutation dosage (RMD) analysis can be used to determine the relative proportions of wild-type and mutant allele present in the cfDNA (Figure 1) (20). Droplet-digital PCR or NGS can be used to target a single mutation for this analysis, and this requires specific probes to be designed for the familial mutation.

A further extension to this is relative haplotype dosage analysis (RHDO) (Figure 2), which identifies the maternal and paternal haplotypes associated with the disease and then in turn determines the relative proportions of the wild-type and mutant haplotype present in the cfDNA (21, 22). A major advantage of RHDO is that it can be used for genes that are not amenable to direct sequencing, for example due to the presence of a pseudogene, or for more complex mutations and rearrangements such as the *F8* inversion in severe haemophilia A. The RHDO approach inherently measures the fetal fraction of the cfDNA, and more robustly than RMD as multiple informative single nucleotide polymorphisms (SNPs) are used to identify paternal haplotypes. In addition, multiplexing allows a number of genes and mutations to be assessed simultaneously. This approach can therefore theoretically be used for any disease, resulting in reduced work-up and validation efforts, but it does require access to relatives or DNA from affected offspring for haplotyping.

Furthermore, the recombination rate of the target genes must be considered. Despite these limitations this technique has been shown to be successful for β -thalassaemia (23) and is now in clinical practice in the UK NHS for cystic fibrosis (24), spinal muscular atrophy (25) and Duchenne muscular dystrophy (26). Encouragingly, these authors have reported detection of recombination events which is a concern when applying a linkage approach to prenatal diagnosis.

Future applications – where are we going?

Direct haplotyping methods for RHDO

RHDO has the potential to enable NIPD for many different single gene disorders that result from a wide range of mutations. However, the need for a sample from a proband or unaffected sibling in order to obtain parental haplotype information limits this analysis to families with children or where DNA has been stored. The goal is therefore to develop proband-free RHDO, which requires a method to haplotype the parents directly. This has so far been shown to be possible by two different approaches: targeted locus amplification (TLA) (27) and microfluidics-based linked-read sequencing (28, 29).

TLA involves the cross-linking, digestion, and *in situ* ligation of sequences surrounding the gene of interest. The proximity ligation primarily yields intra-chromosomal ligation products, therefore after selectively amplifying and then sequencing ligation products with SNPs of interest, any variants detected within the same ligation product are assigned to the same haplotype. One of the major advantages of this approach is that no specialist equipment is required and therefore it is possible to introduce into any clinical diagnostic laboratory. Linked-read sequencing on the other hand requires a specific 10x™ Genomics instrument, making this technique more expensive and less amenable to diagnostic labs. This approach utilises molecular barcodes to tag reads that originate from the same long DNA fragment. High molecular weight genomic DNA is partitioned into barcoded gel beads. Following amplification and barcoding, the DNA is sheared into short reads and then sequenced. Reads that contain the same barcode can then be re-assembled to form pseudo long reads.

A third possible direct haplotyping method for parental samples, yet to be explored in this context, is long-read sequencing using either the Pacific Biosciences (PacBio) or Oxford Nanopore Technologies (ONT) platform. These technologies are able to produce sequence reads which are on average >10 kb in length (30).

Despite the differences in the detailed methods, all three of these approaches enable direct haplotyping of parental samples, and therefore have the potential to make RHDO possible for families where no proband sample is available.

Non-invasive prenatal screening for monogenic diseases and the commercial offering

Currently, the majority of NIPD strategies reported, and certainly those available in the UK NHS, are available for cases where the familial mutation is known or there is a strong indication of the condition based on sonographic findings. As discussed above, compared to NIPT testing for aneuploidy, the commercial investment in developing NIPD for rare diseases has been less, largely due to the individual nature of the test required increasing costs. However, recently there has been a growing market of commercially-available NIPD tests offering screening to low risk pregnancies for a range of single gene diseases.

One of the greatest concerns within the healthcare community surrounding commercial screening tests is the lack of pre- and post-test counselling that is offered to provide patients with the appropriate knowledge of what a positive or negative result will mean for them and their family. For rare diseases, sensitivity and specificity figures can be misleading, and may rely on small datasets. One of the commercial single gene screening tests on offer quotes a combined analytical sensitivity and specificity of >99%, although the detection rate for two of the 30 genes on their panel is less than 50% (31). The quoted figures provided also only relate to the specific genes or mutations under investigation, which in some cases will not cover the full spectrum of causative variants. The problem is confounded by a lack of follow-up of patients who have undergone testing, plus uptake by low-risk women, which brings a greater risk of false positive results. A lack of counselling also means patients are often unaware of factors such as incomplete penetrance, variable expressivity of disease, or incomplete coverage of the genes in question. Many genes tested by these companies do in fact contribute to diseases with variable expressivity, making their claims of zero false negative results potentially unreliable, particularly as one report had follow-up on less than 50% of their cases (31). On the technical side, issues such as incomplete sequence coverage of some genes or exons, allele drop-out, and the difficulty in detecting copy number variants and structural rearrangements, all contribute to an uncertainty surrounding whether or not all genes and mutations are covered. Again, the lack of genetic counselling means many patients will be unaware of these potential issues. Until these issues have been resolved, the commercial offering of prenatal screening using cfDNA will continue to lack any recommendation or endorsement by a professional society.

NIPD for monogenic disorders is still an emerging technique and there remains a lot to learn. For example we do not fully understand the production mechanism of cfDNA and how this may affect the detection of variants in the fetal DNA (32). In fact we are so far aware of two unexplained false negative cases. The first of these is from a commercial screen, which reported no pathogenic or likely pathogenic variants to a patient who subsequently gave birth to an affected child. In this case the report quoted a fetal fraction of 13% but did not include any information regarding the coverage of the genes tested, just that they were included in the test. The second case is from a bespoke assay carried out in an accredited NHS diagnostic genetics laboratory, and the cause of the false negative result remains unexplained. This laboratory has subsequently reviewed all of its cases and the false negative rate is 1 out of 135 amplicons (0.74%); this limitation is included on any report where a mutation has not been detected.

That being said, if correctly validated and implemented cautiously with close monitoring, non-invasive prenatal screening for monogenic disorders in this manner could hold a great many advantages over direct testing for known familial variants. It has the potential to deliver a definitive diagnosis early in pregnancy and therefore provide more information to parents regarding prognosis and recurrence risk. In this way it can also aid plans for delivery or early postnatal treatment, or even help to direct targeted in utero treatment or fetal surgery, as is currently being demonstrated in the research setting (33). Needless to say, it is crucial that patients and healthcare professionals fully understand the limitations of this screening, confirm positive findings with invasive testing and be fully aware of the possibilities for false negatives.

Non-invasive prenatal whole exome/genome sequencing

The natural extension of technological advances leads to non-invasive whole exome sequencing (WES) or whole genome sequencing (WGS) for abnormal ultrasound scan findings suggestive of a monogenic disorder. By obtaining a diagnosis at this stage, it may help safely limit the postnatal diagnostic odyssey that these families would otherwise inevitably be facing. In addition to serving the patients directly, by gathering a wealth of information this will in time help to build a better understanding of fetal phenotypes in order to aid the interpretation of ultrasound scans and genotype information in the future.

Aside from these advantages, prenatal diagnosis using WES or WGS poses a great number of technical and analytical challenges. Prenatal diagnosis using next generation sequencing analysis of invasive samples is still very much in its infancy, and it is becoming clear that the fetal phenotypes may not always reflect the recognised postnatal phenotype (34). This therefore makes interpreting the identified sequence variants (of which there will be a great number from whole exome or genome sequencing) incredibly challenging. In addition, the prenatal setting adds the further complication of the counselling issues that arise for variants of uncertain significance (VUS), incidental and secondary findings (35). One solution is to limit the analysis to large gene panels or a targeted exome in order to reduce these findings, however this brings us back to the difficulty in designing these panels due to the paucity of information on fetal genotype-phenotype correlations. Lastly, we must also consider the time and cost implications associated with this testing. Fetal structural anomalies are most often detected at the 20 week ultrasound scan and this therefore puts a very tight time-limit on the analysis of data from the subsequent prenatal testing. The costs associated with the deep sequencing that would be required for NIPD, analysis, data storage and potential re-interpretation, and counselling must also be evaluated.

Most of the issues discussed above apply to both invasive and non-invasive prenatal WES/WGS, however one challenge unique to NIPD is that of identifying fetal variants in a background of maternal cfDNA (21). Not every fragment of cfDNA will contain an informative marker in order to identify it as fetal or maternal in origin. Obtaining adequate sequence coverage and accurate variant calling are therefore some of the greatest challenges faced by this field, and currently make any diagnostic approach based on these approaches unfit for routine clinical practice.

Prenatal diagnosis based on fetal cells in the maternal circulation

Another emerging technique for NIPD is single cell sequencing of circulating fetal cells isolated from the maternal blood (36, 37) or trophoblastic cells extracted from the external parts of the cervix (38). The use of fetal cells circumvents the issue of trying to detect fetal variants in a high maternal background, and also offers non-fragmented DNA which is more amenable to sequencing analysis. However, fetal cells are extremely low in number in the maternal circulation and are therefore difficult to isolate (37, 39). Another major issue when considering carrying out WES or WGS using DNA isolated from these cells is the allele drop-out rate following whole genome amplification. There is also concern surrounding sampling bias associated with single cell analysis. This fetal cell approach has promise, however there is a lot of work required in order to validate the use of these cells in the clinical diagnostic setting.

What do we need to consider?

When discussing the advances in non-invasive prenatal diagnosis it is important to consider the social and economic impact on patients and healthcare providers. Firstly, there is concern among some that, due to the inherent non-invasive nature of this testing, parents will not think as carefully as they would prior to deciding whether or not to have an invasive test (40). There is a worry that the taking of blood for NIPD may become lost among the many other blood tests that are carried out during pregnancy, and therefore not given the consideration it requires. It is vital that patients opting for NIPD for a monogenic disease are counselled as fully as they would be for any other genetic test, and are aware of the implications not only for themselves and their pregnancy but also for their extended family. One other concern that has been raised is what the limits of the safer NIPD should be, or where is the line (5, 40)? Currently, at least in the UK non-invasive prenatal diagnosis follows the same guidance as current invasive testing when it comes to what conditions should be included and who should be offered testing.

The development of NIPD services within the public healthcare setting must inevitably be guided by cost-benefit analysis. For autosomal dominant disorders or paternal exclusion tests, NIPD for *de novo* and paternal mutations is cheaper than invasive testing (41), whereas RMD, RHDO and bespoke assays are currently much more expensive. Centralising testing, as is being done in the UK NHS Genomic Medicine service, to increase throughput and allow multiplexing, and advances in technology will help decrease costs. However, developing bespoke testing on an individual family basis will remain costly until we develop a WES approach to NIPD. There are some that argue that for those families with a previous *de novo* dominant condition who are at very low gonadal mosaic risks, this should not be offered (42), but they do not take account of the benefits realised in terms of early reassurance or the fact that some parents would opt for invasive testing if NIPD were not available.

Conclusion

The data summarized in this review is largely focused on the UK, as this is the leading centre for the translation to clinical practice for NIPD. Community and healthcare engagement in the development and implementation of NIPD will be context specific; lessons learned from the UK experience may help inform responsible implementation in other countries, but it is unlikely to be replicated in identical models as health service delivery is so variable across the globe.

NIPD for monogenic diseases is a rapidly developing field and is bringing safer and more accurate testing that is welcomed by both patients and the healthcare community. There are however still significant technical and analytical challenges ahead, as well as the need for clear ethical guidelines in advance of implementing these techniques into mainstream antenatal care. This includes the need for high quality genetic counselling both before and after testing to ensure that expectant parents fully understand the reported result and the implications for not only themselves but also their family. It is also crucial that commercial laboratories offering NIPD for single gene diseases are held to the same high laboratory and counselling standards as public healthcare services.

Figure Legends

Figure 1. Illustration of relative mutation dosage. Red represents the mutated allele and grey represents the wild-type allele. The fetal genotype can be deduced from the relative amounts of mutated and wild-type allele detected in the maternal plasma. Adapted from Lun *et al.* (20).

Figure 2. Illustration of relative haplotype dosage. Red represents the mutated allele and grey represents the wild-type allele. The maternal and paternal haplotypes associated with the disease are determined from informative heterozygous SNPs linked to the mutation site. The fetal genotype is deduced from the relative amounts of affected and unaffected haplotype detected in the maternal plasma.

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Declaration of Interest

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References

1. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci U S A* 2008;105:16266-71.
2. Lo YM, Chiu RW. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidies by maternal plasma nucleic acid analysis. *Clin Chem* 2008;54:461-6.
3. Minear MA, Lewis C, Pradhan S, Chandrasekharan S. Global perspectives on clinical adoption of nipt. *Prenat Diagn* 2015;35:959-67.
4. Alfirovic Z, Navaratnam K, Mujezinovic F. Amniocentesis and chorionic villus sampling for prenatal diagnosis. *Cochrane Database Syst Rev* 2017;9:CD003252.
5. Hill M, Suri R, Nash EF, Morris S, Chitty LS. 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* 2014;3:176-90.
6. Amicucci P, Gennarelli M, Novelli G, Dallapiccola B. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. *Clin Chem* 2000;46:301-2.

7. Chitty LS, Griffin DR, Meaney C, Barrett A, Khalil A, Pajkrt E, Cole TJ. 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* 2011;37:283-9.
8. Chitty LS, Khalil A, Barrett AN, Pajkrt E, Griffin DR, Cole TJ. Safe, accurate, prenatal diagnosis of thanatophoric dysplasia using ultrasound and free fetal DNA. *Prenat Diagn* 2013;33:416-23.
9. Jenkins LA, Deans ZC, Lewis C, Allen S. Delivering an accredited non-invasive prenatal diagnosis service for monogenic disorders and recommendations for best practice. *Prenat Diagn* 2018;38:44-51.
10. White HE, Dent CL, Hall VJ, Crolla JA, Chitty LS. Evaluation of a novel assay for detection of the fetal marker rassf1a: Facilitating improved diagnostic reliability of noninvasive prenatal diagnosis. *PLoS One* 2012;7:e45073.
11. Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, Soothill PW. Free fetal DNA in maternal plasma in anembryonic pregnancies: Confirmation that the origin is the trophoblast. *Prenat Diagn* 2007;27:415-8.
12. Hill M, Finning K, Martin P, Hogg J, Meaney C, Norbury G, et al. Non-invasive prenatal determination of fetal sex: Translating research into clinical practice. *Clin Genet* 2011;80:68-75.
13. Clausen FB. Integration of noninvasive prenatal prediction of fetal blood group into clinical prenatal care. *Prenat Diagn* 2014;34:409-15.
14. Hyett JA, Gardener G, Stojilkovic-Mikic T, Finning KM, Martin PG, Rodeck CH, Chitty LS. Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. *Prenat Diagn* 2005;25:1111-6.
15. Tardy-Guidollet V, Menassa R, Costa JM, David M, Bouvattier-Morel C, Baumann C, et al. New management strategy of pregnancies at risk of congenital adrenal hyperplasia using fetal sex determination in maternal serum: French cohort of 258 cases (2002-2011). *J Clin Endocrinol Metab* 2014;99:1180-8.
16. Chitty LS, Mason S, Barrett AN, McKay F, Lench N, Daley R, Jenkins LA. Non-invasive prenatal diagnosis of achondroplasia and thanatophoric dysplasia: Next-generation sequencing allows for a safer, more accurate, and comprehensive approach. *Prenat Diagn* 2015;35:656-62.
17. Hill M, Twiss P, Verhoef TI, Drury S, McKay F, Mason S, et al. Non-invasive prenatal diagnosis for cystic fibrosis: Detection of paternal mutations, exploration of patient preferences and cost analysis. *Prenat Diagn* 2015;35:950-8.
18. Xiong L, Barrett AN, Hua R, Tan TZ, Ho SS, Chan JK, et al. Non-invasive prenatal diagnostic testing for beta-thalassaemia using cell-free fetal DNA and next generation sequencing. *Prenat Diagn* 2015;35:258-65.
19. Hayward J, Chitty LS. Beyond screening for chromosomal abnormalities: Advances in non-invasive diagnosis of single gene disorders and fetal exome sequencing. *Semin Fetal Neonatal Med* 2018;23:94-101.
20. Lun FM, Tsui NB, Chan KC, Leung TY, Lau TK, Charoenkwan P, et al. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. *Proc Natl Acad Sci U S A* 2008;105:19920-5.

21. Lo YM, Chan KC, Sun H, Chen EZ, Jiang P, Lun FM, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med* 2010;2:61ra91.
22. New MI, Tong YK, Yuen T, Jiang P, Pina C, Chan KC, et al. Noninvasive prenatal diagnosis of congenital adrenal hyperplasia using cell-free fetal DNA in maternal plasma. *J Clin Endocrinol Metab* 2014;99:E1022-30.
23. Xiong L, Barrett AN, Hua R, Ho S, Jun L, Chan K, et al. Non-invasive prenatal testing for fetal inheritance of maternal beta-thalassaemia mutations using targeted sequencing and relative mutation dosage: A feasibility study. *BJOG* 2018;125:461-8.
24. Chandler N, Ahlfors H, Drury S, Mellis R, Hill M, McKay F, et al. Non-invasive prenatal diagnosis for cystic fibrosis: Implementation, uptake, outcome and implications. *Clin Chem* 2019;In press.
25. Parks M, Court S, Bowns B, Cleary S, Clokie S, Hewitt J, et al. Non-invasive prenatal diagnosis of spinal muscular atrophy by relative haplotype dosage. *Eur J Hum Genet* 2017;25:416-22.
26. Parks M, Court S, Cleary S, Clokie S, Hewitt J, Williams D, et al. Non-invasive prenatal diagnosis of duchenne and becker muscular dystrophies by relative haplotype dosage. *Prenat Diagn* 2016;36:312-20.
27. Vermeulen C, Geeven G, de Wit E, Verstegen M, Jansen RPM, van Kranenburg M, et al. Sensitive monogenic noninvasive prenatal diagnosis by targeted haplotyping. *Am J Hum Genet* 2017;101:326-39.
28. Hui WW, Jiang P, Tong YK, Lee WS, Cheng YK, New MI, et al. Universal haplotype-based noninvasive prenatal testing for single gene diseases. *Clin Chem* 2017;63:513-24.
29. Jang SS, Lim BC, Yoo SK, Shin JY, Kim KJ, Seo JS, et al. Targeted linked-read sequencing for direct haplotype phasing of maternal dmd alleles: A practical and reliable method for noninvasive prenatal diagnosis. *Sci Rep* 2018;8:8678.
30. van Dijk EL, Jaszczyszyn Y, Naquin D, Thermes C. The third revolution in sequencing technology. *Trends Genet* 2018;34:666-81.
31. Zhang J, Li J, Saucier JB, Feng Y, Jiang Y, Sinson J, et al. Non-invasive prenatal sequencing for multiple mendelian monogenic disorders using circulating cell-free fetal DNA. *Nat Med* 2019;25:439-47.
32. Sun K, Jiang P, Wong AIC, Cheng YKY, Cheng SH, Zhang H, et al. Size-tagged preferred ends in maternal plasma DNA shed light on the production mechanism and show utility in noninvasive prenatal testing. *Proc Natl Acad Sci U S A* 2018;115:E5106-E14.
33. Westgren M, Gotherstrom C. Stem cell transplantation before birth - a realistic option for treatment of osteogenesis imperfecta? *Prenat Diagn* 2015;35:827-32.
34. Jelin AC, Wilkins-Haug L, Vora N. Changing fetal phenotypes: One of the challenges for fetal sequencing. *Prenat Diagn* 2019;39:In press.
35. Horn R, Parker M. Opening pandora's box?: Ethical issues in prenatal whole genome and exome sequencing. *Prenat Diagn* 2018;38:20-5.
36. Breman AM, Chow JC, U'Ren L, Normand EA, Qdaisat S, Zhao L, et al. Evidence for feasibility of fetal trophoblastic cell-based noninvasive prenatal testing. *Prenat Diagn* 2016;36:1009-19.
37. Chen F, Liu P, Gu Y, Zhu Z, Nanisetti A, Lan Z, et al. Isolation and whole genome sequencing of fetal cells from maternal blood towards the ultimate non-invasive prenatal testing. *Prenat Diagn* 2017;37:1311-21.

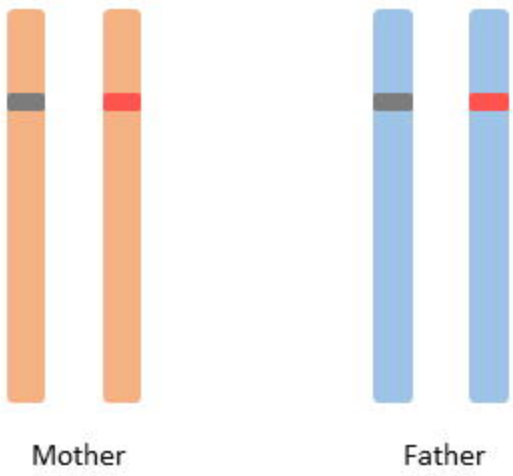
38. Pfeifer I, Benachi A, Saker A, Bonnefont JP, Mouawia H, Broncy L, et al. Cervical trophoblasts for non-invasive single-cell genotyping and prenatal diagnosis. *Placenta* 2016;37:56-60.
39. Huang ZW, Fong CY, Gauthaman K, Sukumar P, Mahyuddin AP, Barrett AN, et al. Biology of human primitive erythroblasts for application in noninvasive prenatal diagnosis. *Prenat Diagn* 2018;38:673-84.
40. Lewis C, Hill M, Chitty LS. Non-invasive prenatal diagnosis for single gene disorders: Experience of patients. *Clin Genet* 2014;85:336-42.
41. Verhoef TI, Hill M, Drury S, Mason S, Jenkins L, Morris S, Chitty LS. Non-invasive prenatal diagnosis (nipd) for single gene disorders: Cost analysis of nipd and invasive testing pathways. *Prenat Diagn* 2016;36:636-42.
42. Wilkie AOM, Goriely A. Gonadal mosaicism and non-invasive prenatal diagnosis for 'reassurance' in sporadic paternal age effect (pae) disorders. *Prenat Diagn* 2017;37:946-8.

Table 1. Development of cell-free fetal testing.

Milestone – NIPT		Milestone – NIPD	Technology	Clinical Availability
	1997	Discovery of cell-free fetal DNA (cffDNA) in the maternal plasma		
		Fetal sex determination using cffDNA	PCR	Research
	2000	NIPD test for single gene disorders	PCR	Research
	2001	Fetal sex determination using cffDNA available in clinical practice	PCR	Research
	2002	Prenatal exclusion for autosomal recessive conditions (cystic fibrosis, β -thalassaemia and CAH)	PCR	Research
Molecular counting for trisomy 21	2007		Digital PCR	Research
Detection of trisomies 21, 18 and 13	2008		NGS	Research
		NIPD for a monogenic disorder where parents carry the same mutation (male fetuses only)	Digital PCR and RMD	Research
	2010	Whole fetal genome mapping by parental haplotype analysis	NGS	Research
NIPT for trisomies 21, 18 and 13 offered by commercial companies in Hong Kong/China and the USA	2011		NGS	Commercial sector
		NIPD for fetal sex determination approved for use in the UK NHS	PCR	Public Health Service
		Routine 3 rd trimester RHD genotyping in all RhD- women offered to direct anti-RhD immunoglobulin administration	PCR	Public Health Service
	2012	NIPD for a monogenic disorder where parents carry the same mutation reported (male and female fetuses)	NGS	Research
		NIPD for selected single gene disorders approved for use in the UK NHS	PCR-RED	Public Health Service
	2013	NIPD for using gene panels approved for use in the UK NHS	NGS	Public Health Service
Widespread implementation of NIPT for aneuploidy			NGS	Private sector, some insurance reimbursement
Molecular karyotyping of subchromosomal abnormalities			NGS	Research
NIPT for subchromosomal abnormalities launched as a commercial service	2014		NGS	Commercial sector
	2016	NIPD for recessive conditions using RHDO	NGS	Public Health Service
NIPT implemented as a primary aneuploidy screen	2017		NGS	Public Health Service
	2018	cfDNA screening for monogenic disorders	NGS	Commercial sector

Confidential

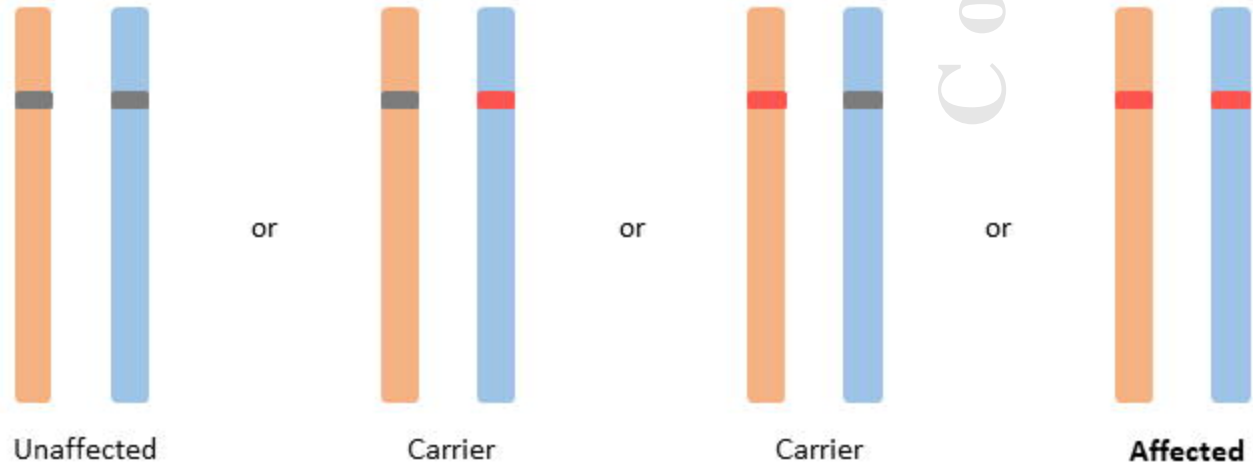
Parental DNA



Legend:

- Grey square: Wild-type allele
- Red square: Mutated allele

Fetal DNA

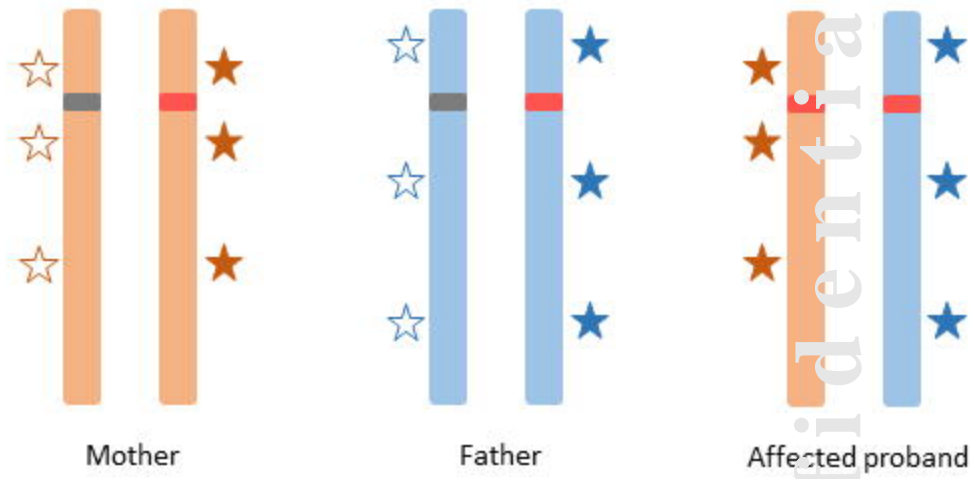


Relative mutation dosage in maternal plasma



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Family DNA



■ Wild-type allele

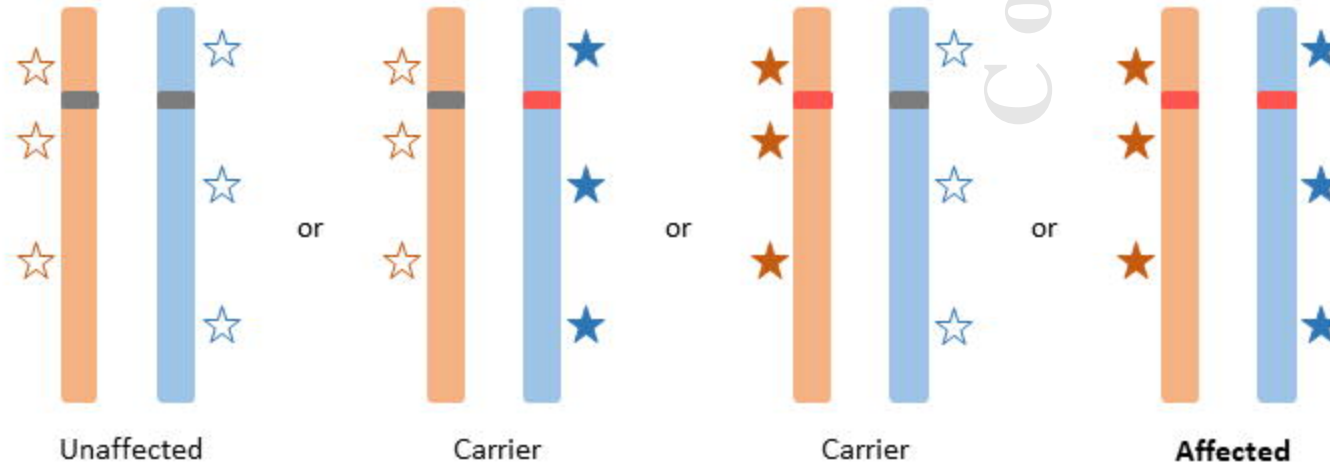
■ Mutated allele

Informative SNPs:

★ Affected haplotype

☆ Unaffected haplotype

Fetal DNA



Relative haplotype dosage
in maternal plasma

☆ > ★

☆ = ★

☆ = ★

☆ < ★