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First Trimester Noninvasive Prenatal Diagnosis of Maternally Inherited Beta-Thalassemia Mutations

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First trimester non-invasive prenatal diagnosis of maternally-inherited beta-thalassemia mutations

Running header: Non-invasive prenatal testing of beta-thalassemia

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Abbreviations:

cffDNA; cell free fetal DNA

NIPT; non-invasive prenatal testing

NGS; next generation sequencing

CFM; confined placental mosaicism

ddPCR; droplet digital PCR

NIPD; non-invasive prenatal diagnosis

RVD; relative variant dosage

RMD; relative mutation dosage

Human Genes:

RHD; Rh blood group D antigen

HBB; hemoglobin subunit beta

The discovery of circulating free fetal DNA (cffDNA) in maternal plasma (1) has allowed non-invasive prenatal testing (NIPT), reducing the number of women undergoing invasive procedures (amniocentesis and chorionic villous sampling), which carry a small risk of miscarriage (2). NIPT using cffDNA was initiated with the assessment of paternally-inherited genetic markers (for example, Y chromosome and *RHD* blood group gene) using real time PCR (3); subsequently NIPT moved to next generation sequencing (NGS) approaches for fetal aneuploidies (3) but has taken longer to be implemented for monogenic diseases. This is primarily due to the required sensitivity for detecting maternally-inherited variants in cffDNA. Whereas NIPT for aneuploidy detection remains a screening test due to the risk of discordant results from confined placental mosaicism (CFM), maternal neoplasms, maternal chromosomal rearrangements or a vanishing twin, NIPT for monogenic diseases generates a diagnosis with no risk from CFM and can account for the maternal genome in the analysis (4).

Monogenic diseases are conditions caused by mutations in a single gene and include the thalassemias, sickle cell anemia, cystic fibrosis, Huntington's disease, and Duchenne muscular dystrophy (5). Thalassemias result from the reduction or absence of one or more globin chain types. Beta-thalassemia is most common in people from the Mediterranean, Africa, India, Southeast Asia and Indonesia, with a worldwide incidence of approximately 1 in 100,000 individuals in the general population for symptomatic cases (6). Approximately 1.5% of the global population (currently an estimated 120 million people) are carriers of beta-thalassemia (6). More than 400 beta-thalassemia alleles have been identified (7), which can make prenatal diagnosis complex.

While cffDNA can be analyzed with technologies such as NGS, the high cost and the required bioinformatic expertise mean cheaper and more straightforward techniques are needed for routine clinical diagnostics. Droplet digital PCR (ddPCR) is one option that allows PCR amplification to occur on a nanoliter sized scale with each reaction partitioned into thousands of droplets (8), meaning valuable cffDNA samples can be preserved.

In this issue of *Clinical Chemistry*, Constantinou et al. (9) push non-invasive prenatal diagnosis (NIPD) for monogenic diseases one step forward by using ddPCR to detect the IVSI-110G>A (HBB:c.93-21G>A) mutation in beta-thalassemia, common in Cyprus, where this mutation represents 79% of the total carriers for the condition (10). Other key mutations in the *HBB* gene in Cypriot carriers include IVSI-6 T>C (HBB:c.92 + 6T>C), IVSI-1G>A (HBB:c.92 + 1G>A) and IVSII-745 C>G (HBB:c.316-106C>G) (10).

Constantinou et al (9) utilize ddPCR with a relative variant dosage (RVD) approach to identify fetal genotypes as an extension of the relative mutation dosage (RMD) approach successfully used previously (4). RMD has the advantage of only requiring a maternal blood sample, as opposed to needing samples from both parents as well as affected proband(s) or unaffected siblings. RMD using digital PCR (11) is needed when an expectant mother is heterozygous for the investigated mutation. If the fetus is also heterozygous, the maternal and fetal genotypes are identical, are in allelic balance and the allelic ratio would be 1. If the fetus is homozygous normal, the mutant allele is underrepresented with respect to the wild type allele, there is allelic

imbalance and the allelic ratio would be <1. If the fetus is homozygous for the mutant allele, the mutant allele is overrepresented with respect to the wild type allele, there is allelic imbalance and the allelic ratio would be >1 (11).

The RVD method in the current study (9) used the allelic ratios and Z scores with an optimization set of samples to define the thresholds for the different fetal genotypes (homozygous wild type, heterozygous and homozygous mutant), but also to define a crucial inconclusive range of values. If samples were to give such inconclusive values in a clinical setting, there would be retesting of samples. The RVD approach is key for the detection of **both** maternally- and paternally-inherited variants in the fetus, opening up the ddPCR method to all pregnancies at risk of beta-thalassemia.

Constantinou et al. (9) describe optimization of the ddPCR assay for the IVSI-110G>A mutation, leading to a sensitive and specific assay that could be used in the clinic. Optimization of ddPCR assays often relies on minimizing rain droplets that occur between the clusters of negative and positive droplets, alongside maximizing the number of positive droplets. Previous approaches to minimizing rain droplets included changing chemical and physical parameters in the ddPCR (12). Constantinou et al. (9) tried overnight plate incubation at 4°C, increasing the primer concentration, decreasing the ramp rate for the PCR steps, increasing the denaturation time and increasing the number of PCR cycles. In their setting, only incubation of the ddPCR plate overnight before reading the fluorescence and increasing the number of cycles from 40 to 45 were found beneficial. These approaches showcase the importance of optimizing ddPCR assays to minimize rain droplets before clinical testing. One future caveat would be to ensure assay

reproducibility between independent laboratories, without the need for further ddPCR optimization. With the addition of an overnight cold temperature step, there will be an increased turnaround time for the results that may play a crucial part in the move towards adoption of this assay in the clinic. Changing the manual thresholds did not alter the genotyping results but this may be a limitation in the clinical setting, where minimal human intervention is required to allow for automated workflows and reduction of errors.

Constantinou et al. (9) used a reasonably small number of samples for validation (forty) and so there would be a benefit to larger studies to confirm this proof of principle approach, with clear details of paternal genotype, as well as maternal genotype. There was no difference in the results from the tube used for blood collection (EDTA or Streck). Previous work has suggested that EDTA tubes may lead to lower amounts of cffDNA available for analysis making it harder to detect (13). The advantage of the current study is the relatively low starting volume of plasma (2mL) and the speed of collection of the plasma from the whole blood (< 2 hours). The current study had an accuracy of 97%, sensitivity of 100% and specificity of 95%. Another study (14) using ddPCR has recently been published looking at the IVSI-110 G>A mutation and the $\beta^0$39 mutation, the most common Mediterranean mutations. When both parents were carriers for IVSI-110 G>A, 5 out of 5 fetuses were genotyped correctly by ddPCR, and when both parents were carriers for $\beta^0$39, 1 out of 1 fetus was genotyped correctly (14). When only the mother was a carrier for IVSI-110 G>A, 11 out of 11 fetuses were genotyped correctly by ddPCR and when only the mother was a carrier for $\beta^0$39, 12 out of 13

fetuses were genotyped correctly (14). Addition of an assessment for fetal fraction would benefit the current assay and be key for its clinical development.

There has always been an impetus to perform NIPT as early in pregnancy as possible, informing clinical management of the pregnancy. As cffDNA levels increase during pregnancy amounting to approximately 10-20% of all DNA in maternal plasma (4), it is easier to detect conditions in the fetus from the end of the first trimester onwards. Constantinou et al. (9) have used samples from 8-13 weeks of gestation, whereas D'Aversa et al. (14) detected the IVSI-110 G>A mutation as early as 7 weeks of gestation in maternal carriers.

It is possible that low fetal fraction led to the misclassification of one of the samples (9) and situations like these could be avoided in future by performing technical replicates. All cffDNA samples were analyzed in six replicates, which could be increased in a clinical setting to maximize the chance of correct scoring, with minimal impact on turnaround time. The danger with misclassified false positive results in the clinical setting is the impact that such results may have on clinical management of the pregnancy and the potential risk for the termination of a healthy fetus.

The use of multiplex ddPCR for aneuploidy detection in NIPT opened up the possibility of detecting more than one trisomy in the same assay (such as trisomy 18 and 21 (15)). It could be argued that as there are so many mutations that can cause beta-thalassemia, using multiplex ddPCR with either changes in primer/probe concentrations or different fluorescent dyes could allow the simultaneous detection of several mutations, while retaining sensitivity. However, the dominance of certain

mutations in geographical areas may make this redundant. It must be remembered that ddPCR can only detect known mutations, as opposed to *de novo* mutations.

NIPT and NIPD need to be fast, cost-effective and not require high levels of human intervention. Constantinou et al. (9) illustrate how quickly ddPCR is evolving to assess maternally- and paternally-inherited monogenic diseases in pregnancy, indicative that this is the principal way forward for NIPD in clinical practice. With early detection of the relevant beta-thalassemia mutations, it will allow for improved clinical management during pregnancy and informed choice for the parents.

Conflict of Interest disclosure: Nothing to declare.

References

1. Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, Wainscoat JS. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485-7.

2. Salomon LJ, Sotiriadis A, Wulff CB, Odibo A, Akolekar R. Risk of miscarriage following amniocentesis or chorionic villus sampling: systematic review of literature and updated meta-analysis. Ultrasound Obstet Gynecol 2019;54:442-51.

3. Chiu RW, Lo YM. Clinical applications of maternal plasma fetal DNA analysis: translating the fruits of 15 years of research. Clin Chem Lab Med 2013;51:197-204.

4. Scotchman E, Shaw J, Paternoster B, Chandler N, Chitty LS. Non-invasive prenatal diagnosis and screening for monogenic disorders. Eur J Obstet Gynecol Reprod Biol 2020;253:320-7.

5. National Human Genome Research Institute. Genetic Disorders. 2022 https://www.genome.gov/For-Patients-and-Families/Genetic-Disorders (Accessed May 2022)

6. Galanello R, Origa R. Beta-thalassemia. Orphanet J Rare Dis 2010;5:11.

7. Kountouris P, Lederer CW, Fanis P, Feleki X, Old J, Kleanthous M. IthaGenes: an interactive database for haemoglobin variations and epidemiology. PLoS One 2014;9:e103020.

8. Bio-Rad Laboratories, Inc. Digital PCR. 2022 https://www.bio-rad.com/en-in/category/digital-pcr?ID=M9HE2R15 (Accessed May 2022)

9. Constantinou CG, Karitzi E, Byrou S, Stephanou C, Michailidou K, Makariou C et al. Optimized droplet digital PCR assay on cell-free DNA samples for non-invasive prenatal diagnosis: application to beta-thalassemia. Clin Chem 2022 [Note to copy-ed: Please ensure that the correct volume and page info is supplied for this article to appear in the same issue as the current editorial].

10. Kountouris P, Kousiappa I, Papasavva T, Christopoulos G, Pavlou E, Petrou M, et al. The molecular spectrum and distribution of haemoglobinopathies in Cyprus: a 20-year retrospective study. Sci Rep 2016;6:26371.

11. 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.

12. Witte AK, Mester P, Fister S, Witte M, Schoder D, Rossmanith P. A systematic investigation of parameters influencing droplet rain in the Listeria monocytogenes prfA assay - reduction of ambiguous results in ddPCR. PLoS One 2016;11:e0168179.

13. Sillence KA, Roberts LA, Hollands HJ, Thompson HP, Kiernan M, Madgett TE, et al. Fetal sex and RHD genotyping with digital PCR demonstrates greater sensitivity than real-time PCR. Clin Chem 2015;61:1399-407.

14. D'Aversa E, Breveglieri G, Boutou E, Balassopoulou A, Voskaridou E, Pellegatti P, et al. Droplet digital PCR for non-invasive prenatal detection of fetal single-gene point mutations in maternal plasma. Int J Mol Sci 2022;23:2819.

15. Tan C, Chen X, Wang F, Wang D, Cao Z, Zhu X, et al. A multiplex droplet digital PCR assay for non-invasive prenatal testing of fetal aneuploidies. Analyst 2019;144:2239-47.