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

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

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

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21 **Abbreviations:**

22 cffDNA; cell free fetal DNA

23 NIPT; non-invasive prenatal testing

24 NGS; next generation sequencing

25 CFM; confined placental mosaicism

- 26 ddPCR; droplet digital PCR
- 27 NIPD; non-invasive prenatal diagnosis
- 28 RVD; relative variant dosage
- 29 RMD; relative mutation dosage
- 30
- 31 **Human Genes:**
- 32 *RHD*; Rh blood group D antigen
- 33 *HBB*; hemoglobin subunit beta
- 34

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

49

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

60

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

67

68 In this issue of *Clinical Chemistry*, Constantinou et al. (9) push non-invasive prenatal
69 diagnosis (NIPD) for monogenic diseases one step forward by using ddPCR to
70 detect the IVSI-110G>A (HBB:c.93-21G>A) mutation in beta-thalassemia, common
71 in Cyprus, where this mutation represents 79% of the total carriers for the condition
72 (10). Other key mutations in the *HBB* gene in Cypriot carriers include IVSI-6 T>C
73 (HBB:c.92 + 6T>C), IVSI-1G>A (HBB:c.92 + 1G>A) and IVSII-745 C>G (HBB:c.316-
74 106C>G) (10).

75

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

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

88

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

96

97 Constantinou et al. (9) describe optimization of the ddPCR assay for the IVSI-
98 110G>A mutation, leading to a sensitive and specific assay that could be used in the
99 clinic. Optimization of ddPCR assays often relies on minimizing rain droplets that
100 occur between the clusters of negative and positive droplets, alongside maximizing
101 the number of positive droplets. Previous approaches to minimizing rain droplets
102 included changing chemical and physical parameters in the ddPCR (12).

103 Constantinou et al. (9) tried overnight plate incubation at 4°C, increasing the primer
104 concentration, decreasing the ramp rate for the PCR steps, increasing the
105 denaturation time and increasing the number of PCR cycles. In their setting, only
106 incubation of the ddPCR plate overnight before reading the fluorescence and
107 increasing the number of cycles from 40 to 45 were found beneficial. These
108 approaches showcase the importance of optimizing ddPCR assays to minimize rain
109 droplets before clinical testing. One future caveat would be to ensure assay

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

117

118 Constantinou et al. (9) used a reasonably small number of samples for validation
119 (forty) and so there would be a benefit to larger studies to confirm this proof of
120 principle approach, with clear details of paternal genotype, as well as maternal
121 genotype. There was no difference in the results from the tube used for blood
122 collection (EDTA or Streck). Previous work has suggested that EDTA tubes may
123 lead to lower amounts of cffDNA available for analysis making it harder to detect
124 (13). The advantage of the current study is the relatively low starting volume of
125 plasma (2mL) and the speed of collection of the plasma from the whole blood (< 2
126 hours). The current study had an accuracy of 97%, sensitivity of 100% and
127 specificity of 95%. Another study (14) using ddPCR has recently been published
128 looking at the IVSI-110 G>A mutation and the β^039 mutation, the most common
129 Mediterranean mutations. When both parents were carriers for IVSI-110 G>A, 5 out
130 of 5 fetuses were genotyped correctly by ddPCR, and when both parents were
131 carriers for β^039 , 1 out of 1 fetus was genotyped correctly (14). When only the
132 mother was a carrier for IVSI-110 G>A, 11 out of 11 fetuses were genotyped
133 correctly by ddPCR and when only the mother was a carrier for β^039 , 12 out of 13

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

136

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

144

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

152

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

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

161

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

168

169 Conflict of Interest disclosure: Nothing to declare.

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