Chapter 6

Noninvasive prenatal diagnosis of Huntington Disease; detection of the paternally inherited expanded CAG repeat in maternal plasma

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Jessica van den Oever

Emilia Bijlsma

Ilse Feenstra

Nienke Muntjewerff

Inge Mathijssen

Mariëtte Hoffer

Bert Bakker

Martine van Belzen

Elles Boon

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Abstract

Objective: With a shift towards noninvasive testing, we have explored and validated the use of noninvasive prenatal diagnosis (NIPD) for Huntington disease (HD).

Methods: Fifteen couples have been included, assessing a total of n=20 pregnancies. Fetal paternally inherited CAG repeat length was determined in total cell-free DNA from maternal plasma using a direct approach by PCR and subsequent fragment analysis.

Results: All fetal HD (n=7) and intermediate (n=3) CAG repeats could be detected in maternal plasma. Detection of repeats in the normal range (n=10) was successful in n=5 cases where the paternal repeat size could be distinguished from maternal repeat patterns after fragment analysis. In all other cases (n=5) the paternal peaks coincided with the maternal peak pattern. All NIPD results were concordant with results from routine diagnostics on fetal genomic DNA from chorionic villi.

Conclusion: In this validation study we demonstrated that all fetuses at risk for HD could be identified noninvasively in maternal plasma. Additionally, we have confirmed results from previously described case reports that NIPD for HD can be performed using a direct approach by PCR. For future diagnostics, parental CAG profiles can be used to predict the success rate for NIPD prior to testing.

Introduction

Huntington disease (HD, OMIM #143100) is an autosomal dominant progressive neurodegenerative disorder, characterized by irrepressible motor symptoms, cognitive impairment and psychiatric disturbances (Landles *et al.*, 2004). HD is caused by the expansion of a polymorphic trinucleotide (CAG)n repeat in exon 1 of the huntingtin (*HTT*) gene (previously known as *IT15*) which is located on chromosome 4p16.3 (The Huntington's Disease Collaborative Research Group, 1993). CAG repeats are classified in 3 major categories: Alleles < 27 CAG repeats are classified as normal, the range between 27 and 35 as intermediate and > 36 repeats as causing HD. Repeats in the intermediate range can be unstable and may expand into the affected range over generations, predominantly upon paternal germline transmission (Semaka *et al.*, 2010). As a consequence, the offspring is at risk for developing HD.

Prospective parents in families with HD may opt for prenatal testing which can be accomplished via in vitro fertilization (IVF) in combination with preimplantation genetic diagnosis (PGD) or prenatal molecular testing. The latter option can be performed either by means of a direct approach, testing the expanded CAG repeat and/or by linkage analysis of informative markers (de Die-Smulders *et al.*, 2013). In contrast to PGD, prenatal molecular testing is offered by many labs (de Die-Smulders *et al.*, 2013). Prenatal diagnosis for HD, as for many other genetic disorders, is performed on fetal DNA derived from invasive procedures such as chorionic villus sampling (CVS) or amniocentesis. These procedures are associated with a small but significant procedure-related risk of fetal loss of ~0.5-1% (Nicolaides *et al.*, 1994; Tabor *et al.*, 2010). After the discovery of the presence of circulating cell-free fetal DNA (cffDNA) in maternal plasma, a shift towards noninvasive prenatal diagnosis (NIPD) occurred as an alternative for prenatal testing (Lo *et al.*, 1997). Several NIPD studies have since been incorporated into daily clinical practice, including fetal sex determination, fetal Rhesus D (*RHD*) determination and the diagnosis of monogenetic disorders caused by single mutations or small duplications/deletions (Daley *et al.*, 2014; van den Oever *et al.*, 2013). However, only a few case studies have been reporting on disorders caused by the expansion of large polymorphic trinucleotide repeats. Four previous papers from one group describe NIPD for a total of 7 unique cases of fetuses at risk for HD (Gonzalez-Gonzalez *et al.*, 2003a; Gonzalez-Gonzalez *et al.*, 2003b; Gonzalez-Gonzalez *et al.*, 2008; Bustamante-Aragones *et al.*, 2012). In these studies a direct approach for NIPD was used by determining paternally inherited fetal CAG repeat length in maternal plasma using (semi-)quantitative fluorescent polymerase chain reaction. In 5 out of these 7 cases this direct approach was applied successfully.

All diagnostic testing for HD in the Netherlands is performed in our facility. Due to a general shift towards less invasive sampling techniques in the Netherlands, there is also an appeal for NIPD for HD. Here we describe a validation study for the detection of the paternally inherited CAG repeat in maternal plasma for fetuses at risk for HD.

Patients and Methods

Patients

From 2010 onwards, pregnant couples of which the male was at risk for developing HD and opting for prenatal diagnosis, were asked to participate in this study and to provide additional blood samples for NIPD. Inclusion criteria for this study were (1) only the prospective

father is a carrier for a CAG repeat in the intermediate or HD range, (2) a singleton pregnancy with a gestational age from 8 weeks onwards and (3) signed informed consent. Exclusion criteria for participation were (1) invasive procedure performed prior to blood sampling, (2) fetal demise at the time of blood sampling, (3) inability to understand the study information and (4) age at time of sampling < 18 yrs. Sixteen couples directly met all inclusion criteria mentioned above. Two cases were excluded afterwards: one pregnancy resulted in early fetal demise after blood sampling and subsequent karyotyping revealed triploidy. The blood sample from the other pregnancy did not contain fetal DNA. One couple was included later in pregnancy. In this case, a period of > 4 wks between the invasive procedure and blood sampling was considered sufficient to exclude procedure related effects on cffDNA levels in maternal plasma. In total 15 couples were included in this study assessing 20 plasma samples from singleton pregnancies (Table 1). For 14 couples, full CAG repeat profiles from genomic DNA (gDNA) analysis were available for both parents. For 1 couple only the maternal profile was known. The father was at 50% risk for developing HD and at the time of prenatal diagnosis he refrained from molecular genetic testing. Written informed consent was obtained for all cases and all procedures were approved by the ethical standards of the Medical Ethics Committee (METC) of the Leiden University Medical Center.

Sample preparation

Maternal blood withdrawal was performed from 8 weeks of gestation onwards (range 7+6 – 16+1 wks+days, see Table 1). Maternal plasma was processed within 24 hrs after withdrawal and total cell-free DNA from plasma (input 800 µL) was isolated as previously described. (van den Oever *et al.*, 2012) Isolated cell-free DNA was concentrated to 20 µL using the Zymo Clean & Concentrator™-5 kit (Zymo Research, USA). Paternal plasma (n=4) was obtained and processed similar to maternal plasma and was used as a control during optimization. Parental gDNA was isolated from peripheral blood cells using automated isolation (QIAGEN, the Netherlands). Fetal gDNA from CVS was isolated on the QIAcube according to manufacturer's instructions (QIAGEN, the Netherlands).

PCR amplification and fragment analysis:

A combination of PCR and subsequent automated fragment analysis was used to determine CAG repeat size. PCR for NIPD was performed in a final reaction volume of 25 µL containing 5 µL of concentrated plasma DNA, 5 pmol of each primer (modified from Warner *et al.* (Warner *et al.*, 1993); Fw (HD1*): 5' 6-FAM*-ATG AAG GCC TTC GAG TCC CTC AAG TCC TTC-3' and Rev (HD3): 5'-GGC GGT GGC GGC TGT TGC TGC TGC-3' (Biolegio, the Netherlands)) and 12.5 µL OneTaq Hot Start 2x Master Mix with GC buffer (New Engeland Biolabs, USA). Cycling conditions were 94°C for 5 min, 40 cycles of 94°C 30 sec, 63°C 1 min and 68°C 2 min, followed by a final extension at 68°C for 5 min. Each maternal plasma sample was tested in duplicate. In case of inconclusive results (i.e. no paternally inherited allele was detected), the test was repeated. Subsequent automated fragment analysis was performed on the 3130XL Genetic Analyzer (Applied Biosystems, USA) using Gene Scan™ 500 LIZ Size Standard (Applied Biosystems) and data was analyzed with GeneMarker Software version 2.4.0 (Softgenetics, USA) using an empirical determined and validated panel to convert fragment length (bp) into the number of CAG repeats. Fetal gDNA from CVS and parental gDNA samples (input 1 ng gDNA per reaction) were also tested with this NIPD protocol as additional controls. Findings from NIPD on maternal plasma were compared to results from routine prenatal diagnosis using fetal gDNA from CVS. All diagnostic testing for HD in our facility is performed under the

guidelines of the European Molecular Genetics Quality Network and the Clinical Molecular Genetics Society (EMQN/CMGS).

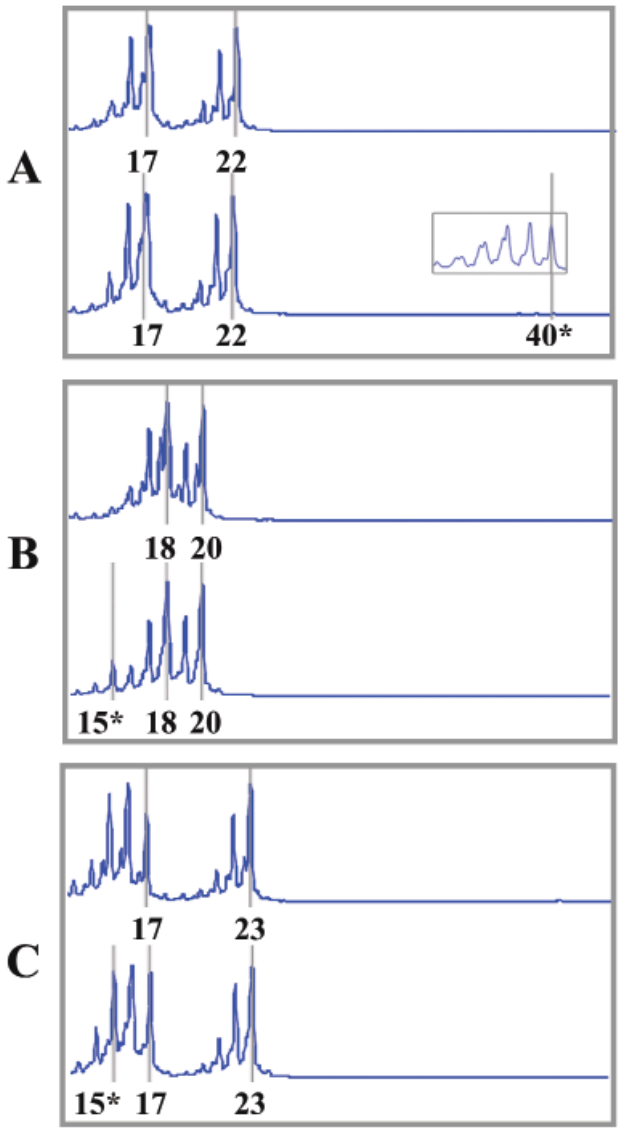

Figure 1: Representative results for direct CAG repeat analysis on maternal plasma for 3 different pregnancies. In each panel, the top part represents the result from maternal gDNA. The bottom part represents results from maternal plasma with the fetal paternally inherited repeat size indicated with *. Panel A: Family 78457; Fragment analysis on maternal plasma shows maternal CAG repeat of 17 and 22 together with the fetal paternally inherited repeat of 40 CAG repeats (insert). Panel B: Family 56092; The maternal profile of 18 and 20 CAG repeats is shown with a third distinct peak at 15 CAG repeats corresponding to the paternally inherited allele of the fetus. Panel C: Family 8062; Results show a maternal CAG repeat of 17 and 23. The paternally inherited fetal repeat of 15 CAG repeats coincides with the maternal stutter peak pattern and could not be confirmed.

Table 1: Overview of ncluded samples.

Family number	*Gestational age (weeks + days)*	*Paternal CAG repeat sizes*	*Maternal CAG repeat sizes*	*Fetal CAG repeat sizes*[A]	*Repeat range of the paternally inherited repeat*	*NIPD: paternal allele detected?*
68395	7 +6	Unknown	16-18	18-**23**	Normal	Yes
8181A	8 +0	17-30	15-17	15-**30**	Intermediate	Yes
8181B	8 +4			15-**29**[$]	Intermediate[I]	Yes
91361	10 +3	15-46	17-22	**15**-17	Normal	No
8064	16 +1	27-45	17-17	17-**27**	Intermediate	Yes
78032	11 +1	15-43	22-25	**15**-22	Normal	Yes
8131	11 +0	20-49	17-18	18-**20**	Normal	Yes
54593A	10 +3	25-42	17-31	31-**42**	HD	Yes
54593B	11 +0			17-**42**	HD	Yes
78457	10 +1	18-40	17-22	17-**40**	HD	Yes
79050	11 +4	17-41	15-18	**17**-18	Normal	No
79422A	11 +6	17-48	15-17	17-**47**[$]	HD	Yes
79422B	13 +5			15-**70**[#]	HD	Yes
8062	9 +6	15-46	17-23	**15**-17	Normal	No
60289	11 +1	17-39	17-22	**17**-17	Normal	No
65732A	8 +5	17-42	17-17	17-**42**	HD	Yes
65732B	10 +6			**17**-17	Normal	No
8033A	8 +3	18-48	23-25	23-**53**[#]	HD	Yes
8033B	11 +4			**18**-23	Normal	Yes
56092	11 +5	15-42	18-20	**15**-20	Normal	Yes

Table 1: Overview of samples included in this study and results obtained with NIPD for Huntington disease (HD). Fetuses from subsequent pregnancies are indicated with A and B. w+d, weeks + days (A) Fetal CAG repeat size results from chorionic villus gDNA, with the paternally inherited repeat depicted in bold. [#]Repeat expanded upon transmission. [$]Repeat contracted upon transmission.

Results

All fetal paternally inherited HD (n=7) and intermediate (n=3) CAG repeats could be detected in one or more replicates in maternal plasma (Table 1, Figure 1A). In our study, the CAG repeat had contracted upon transmission in two cases and expanded in two cases. The longest fetal repeat present in this cohort was 70 CAG repeats (Table 1). Transmission of repeats in the normal range could be detected in 50% of the cases (n=5). These repeat sizes were either at least 2 repeats larger or 3 repeats smaller than the nearest maternal CAG repeat (Figure 1B). In all other cases (n=5) results were inconclusive because either both parents shared a particular repeat size or the paternally inherited peak coincided with the maternal stutter peak profile in fragment analysis and could therefore not be distinguished (Figure 1C). All NIPD results were concordant to results obtained in routine prenatal diagnosis using fetal gDNA from CVS. The accuracy of this NIPD test is 100%, provided this test is performed in duplo or triplo.

Discussion and Conclusions

In the past few years, the use of NIPD in a clinical setting has already been established for applications such as fetal sexing and *RhD* detection. Nevertheless, little is known yet about NIPD for disorders caused by polymorphic repeat expansions, such as HD. In this validation study for NIPD of HD, we show that we can indeed detect paternally inherited CAG repeats in maternal plasma. We have hereby not only confirmed the results from previously described case studies reported by González-González *et al*. and Bustamante-Aragones *et al.*, we have also extended the number of cases tested (Gonzalez-Gonzalez *et al.*, 2003a; Gonzalez-Gonzalez *et al.*, 2003b; Gonzalez-Gonzalez *et al.*, 2008; Bustamante-Aragones *et al.*, 2012). Moreover, we show that NIPD for HD can also be used for successful detection of intermediate repeats in addition to normal and HD repeats. We did experience the same technical limitations for the detection of extremely large HD repeats and repeats in the normal range as previously described.

The success of detecting the paternal repeat in maternal plasma is influenced by several factors. Detection depends on the difference in size between the paternal and maternal repeats. Irrespective of the size range of the transmitted repeat (e.g. normal, intermediate or HD), our study shows that the paternal repeat can be detected in maternal plasma when there was a sufficient difference in size between paternal and maternal repeats. With respect to partially informative couples (i.e. parents share an allele size), this would mean that in NIPD only the extended paternal allele can be discriminated from the maternal profile. In case of informative couples (i.e. parents have 4 different CAG repeats) on the other hand, the terminology “informative” may be misleading in some cases. Even though in theory 4 different parental repeat sizes imply a high detection rate, results from NIPD may not always be informative when the paternal peak coincides with the stutter peak pattern of the maternal profile. Stutter peaks are a known phenomenon in repeat amplification (Walsh *et al.*, 1996). Each peak in the stutter lacks one core repeat unit relative to the main peak. When the paternal CAG repeat size is directly adjacent to the maternal CAG repeat size, it may be very difficult to distinguish the signals. Therefore, the use of both parental gDNA profiles as a reference is very helpful in fragment analysis since patterns observed in gDNA are quite similar to patterns observed in plasma DNA. In our study, one family (#68395) was included in which the paternal genotype was unknown at the time of maternal blood sampling. Results from NIPD showed

the father had transmitted a CAG repeat in the normal range, that could clearly be distinguished from the maternal profile. Shortly after fetal results from routine prenatal diagnostics were reported, he had his genotype determined. Outcome showed he actually had two CAG repeats in the normal range and both these repeats differed sufficiently from both the maternal repeats. This also illustrates that future cases where the father does not want to have his profile determined can indeed be included for NIPD. However, it may be more challenging to distinguish between a true or false negative result and couples should be informed about the limitations of performing NIPD without the accessibility of a paternal reference profile. Preferably, profiles of both parents should be available prior to the start of NIPD to determine whether couples are eligible for this test and to estimate the success rate of NIPD based on CAG repeat size differences.

Besides the difference in repeat sizes between both parents, also repeat size itself can be of influence for the success of direct analysis for NIPD. With an average size of ~143 bp, the fragmented nature of cffDNA indeed makes the detection of expanded repeats in the fetus more challenging (Chan *et al.*, 2004; Lo *et al.*, 2010). Expanded repeats can be unstable. When the inherited paternal allele is expanded upon transmission, there is a possibility that it becomes too large to be detected in fragmented cffDNA. Flanking primers used for detection of the CAG repeat may not be able to bind both sides of the fragment and thus amplification will be hampered. The longest repeat size described in the study by the group of Bustamante-Aragones *et al.* was 114 repeats and could not be detected (Bustamante-Aragones *et al.*, 2008), while in our study the largest fetal CAG repeat was 70 (representing a PCR product of ~245 bp) which could be detected with NIPD. In this study we could detect all HD repeats, however such long repeats were not detected in every replicate. We therefore strongly advice to perform the test in duplo or triplo to obtain a more accurate and robust test result. Preferential amplification of small repeats (i.e. repeats in the normal range) is often observed after fragment analysis. As a consequence, there can be a large difference in the intensity of signals between different HD repeat ranges. The signal intensity of long CAG repeats is often much lower compared to the signal of smaller repeats and this phenomenon is observed in both gDNA as well as plasma DNA. In maternal plasma, the fetal contribution to the total amount of cell-free DNA is on average only ~10% in the first trimester, however this percentage may differ quite extensively between individuals (Lo *et al.*, 1998; Lun *et al.*, 2008a). The group of Chan *et al.* report in their study only 20% of the total amount of fetal sequences in maternal plasma have a size >193 bp and this percentage decreases when fragments are even larger (Chan *et al.*, 2004). The low signal intensity of long CAG repeats together with the low amount of fetal sequences in these size ranges may explain why such long fetal repeats are not detected in every plasma DNA replicate.

Another factor to consider, especially when sampling very early in gestation, is a low amount of cffDNA in maternal plasma itself. Very low levels of cffDNA may lead to inconclusive results. In case of inconclusive results, a second blood sample could be requested to retest later in pregnancy. Nevertheless, for all inconclusive results prenatal testing through an invasive procedure is recommended.

In summary we show that in this study all fetuses at risk for HD could be identified noninvasively in maternal plasma. Moreover, we have hereby confirmed the results from previously published cases for NIPD of HD in a larger cohort. Our data also illustrates that when a paternally inherited allele in the normal range is transmitted to the fetus, the detection rate strongly depends on the size difference between paternal and maternal CAG repeats. With this validation study we show that NIPD for HD can indeed be performed through direct test-

ing of paternally transmitted repeats in maternal plasma, although not every couple will be good candidates for this test. However, prior to testing, parental CAG profiles can be used to determine whether a couple is actually eligible for NIPD. In conclusion, we consider the approach of detecting the paternally inherited repeat in maternal plasma by means of PCR and subsequent fragment analysis very promising application for NIPD of HD.

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