Letter to the Editor

Non-invasive prenatal diagnosis of single-gene disorders from maternal blood

Ana Bustamante-Aragonés [a],[*], Marta Rodríguez de Alba [a], Sara Perlado [a], María José Trujillo-Tiebas [a], Javier Plaza Arranz [b], Joaquín Díaz-Recasens [b], Juan Troyano-Luque [c], Carmen Ramos [a]

[a] Genetics Service, Fundación Jiménez Diaz-IIS, Avda. Reyes Católicos, 2. 28040, Madrid, Spain
[b] Gynecology & Obstetrics Department, Fundación Jiménez Diaz-Capio, Avda. Reyes Católicos, 2. 28040, Madrid, Spain
[c] Obstetrics & Gynecology Department, University Hospital of Canary Islands, OFRA s/n 38002 Tenerife, Spain

ARTICLE INFO

ABSTRACT

Prenatal diagnosis (PD) is available for pregnancies at risk of monogenic disorders. However, PD requires the use of invasive obstetric techniques for fetal-sample collection and therefore, involves a risk of fetal loss. Circulating fetal DNA in the maternal bloodstream is being used to perform non-invasive prenatal diagnosis (NIPD). NIPD is a challenging discipline because of the biological features of the maternal blood sample. Maternal blood is an unequal mixture of small (and fragmented) amounts of fetal DNA within a wide background of maternal DNA. For this reason, initial NIPD studies have been based on the analysis of specific paternally inherited fetal tracts not present in the maternal genome so as to ensure their fetal origin. Following this strategy, different NIPD studies have been carried out, such as fetal-sex assessment for pregnancies at risk of X-linked disorders, RhD determination, and analysis of single-gene disorders with a paternal origin. The study of the paternal mutation can be used for fetal diagnosis of dominant disorders or to more accurately assess the risk of an affected child in case of recessive diseases. Huntington's disease, cystic fibrosis, or achondroplasia are some examples of diseases studied using NIPD. New technologies are opening NIPD to the analysis of maternally inherited fetal tracts. NIPD of trisomy 21 is the latest study derived from the use of next-generation sequencing (NGS).

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Circulating cell-free fetal DNA (ccffDNA) present in maternal blood during pregnancy can be used for non-invasive prenatal diagnosis (NIPD). While fetal sex assessment, fetal RhD determination and, recently, diagnosis of trisomy 21 in maternal blood have been incorporated into routine clinical practice (Bustamante-Aragones et al., 2008a; Finning and Chitty, 2008; Palomaki et al., 2011), other applications such as diagnosis of single-gene disorders are being validated for translation into clinical practice and fetal aneuploidies in the near future.

In median terms, only 10% of DNA present in maternal plasma is fetally derived; the remaining 90% is maternal DNA (Lun et al., 2008a). Thus far, NIPD has mainly focused on the analysis of *de novo* or paternally inherited traits to ensure their fetal origin. Different genetic diseases, with both dominant and recessive inheritance patterns, have been diagnosed in maternal blood, including Huntington's disease, achondroplasia, cystic fibrosis, and beta thalassemia, among others.

The diagnosis of dominant diseases that are paternally inherited or occur *de novo* is possible due to the absence of the mutant allele in the maternal genome. Presence/absence of the paternal mutant allele in the maternal plasma may be associated, respectively, the affected/non-affected status in the fetus.

- Achondroplasia, myotonic dystrophy, and Huntington's disease are examples of dominant diseases diagnosed in maternal plasma.
- Autosomal recessive disorders are more complex to diagnose since doing so requires maternal alleles to be distinguished from fetal ones. Therefore, diagnosis of recessive disorders has mainly been performed in those couples in which the maternal and the paternal mutations are different. In spite of this limitation, the study of the paternal defect in maternal plasma can significantly reduce the number of invasive diagnoses required, eliminating further invasive testing in cases where the fetus does not carry the paternal defect. If the mutation is present in the fetal genome, however, invasive testing would be strongly indicated since the risk of the disease increases from one in four to one in two. Cystic fibrosis, metabolic diseases, hemoglobinopathies, and retinopathies are some recessive diseases studied in maternal plasma.

Several technical approaches have been used for the detection of ccffDNA, from basic molecular methods to more sophisticated procedures. Depending on the molecular basis of the disease, different analytical methods need to be employed. For clinical application, the study of ccffDNA should be done by affordable technical methods. These should be accurate, rapid, easy handling, and inexpensive. The incorporation of NIPD into routine clinical practice is directly related to advances in analytical techniques. As techniques become more and more sensitive, they are increasingly able to detect ccffDNA in early stages of gestation, thus increasing the array of NIPD applications which can be used in treating patients.

Application of the latest technologies (i.e., next-generation sequencing) featuring both high sensitivity and accuracy is broadening the scope of NIPD to diseases that are both paternally and maternally inherited. However, these methods are still far from becoming part of routine clinical practice.

2. Single-gene disorders

A single-gene disorder, also known as a Mendelian disorder, is one that is determined primarily by the alleles at a single locus. The online version of *Mendelian Inheritance in Man* (OMIM) (www.ncbi.nlm.nih.gov/omim) currently lists 3173 diseases with known molecular bases. Although individually rare, as a group they are responsible for a significant proportion of childhood diseases and mortality.

Single-gene disorders are inherited in specific inheritance patterns and involve different recurrence risks. They are characterized by their pattern of transmission in families which depends chiefly on two factors:

a) whether the phenotype is dominant (expressed when only one chromosome of a pair carries the mutant allele and the other chromosome has a wild-type allele at that locus) or recessive (expressed only when both chromosomes of a pair carry mutant alleles at that locus); and
b) the chromosomal location of the gene locus, which may be on an autosome (chromosomes 1 to 22) or on a sex chromosome (chromosomes X and Y).

An individual with an autosomal dominant disease has a 50% risk of having a child with the same disorder. Couples with a previous child affected by an autosomal recessive disease have a 25% probability of conceiving a fetus affected by the condition in their next pregnancy. In X-linked recessive disorders, a female carrier has a 25% risk of having an affected child. Furthermore, 50% of her daughters will be carriers of the disease, although a majority of female carriers of X-linked diseases remain unaffected. Males affected by an X-linked disease are not at risk of having affected sons, although their daughters will be carriers.

Because of the risk of recurrence, reproductive genetic counselling is made available to patients in order to help them have healthy children. Prenatal diagnosis is the most accessible reproductive option available for pregnancies at risk of single-gene disorders.

3. Conventional (invasive) prenatal diagnosis

Prenatal diagnosis (PD) of single-gene disorders through the use of invasive techniques is an accepted part of clinical practice and is performed when there is a family history of a particular disease or, less frequently, ultrasonographic findings. Conventional prenatal diagnosis requires invasive obstetric procedures (chorion biopsy or amniocentesis) that entail a risk of fetal loss which is estimated at 0.5–1% depending on the method used (Kozlowski et al., 2008; Mujezinovic and Alfirevic, 2007).

Although either amniocentesis or CVS can be used for diagnostic purposes, the latter is the preferred method for the following reasons:

1) more fetal DNA can be obtained easily. Accurate diagnosis of the molecular basis of the disease is then possible in the same way as in postnatal genetic testing
2) genetic study may be followed by termination of pregnancy, producing a psychological effect which might be worse in advanced stages of gestation.

After fetal-sample collection, the method/s to be applied for the genetic analysis are chosen depending on the molecular basis of the familiar disease. There is a wide range of molecular techniques available for this aim, but those most commonly used are restriction analysis, automated sequencing and minisequencing for the study of point mutations, QF-PCR for the analysis of dynamic mutations, and MLPA or Real-Time PCR for the analysis of deletions/duplications.

4. Non-invasive prenatal diagnosis

The existence of ccffDNA in maternal plasma allows for non-invasive prenatal diagnosis of several fetal conditions, including single-gene disorders. However, the detection of fetal point mutations from ccffDNA is extremely challenging due to the predominance of maternal DNA sequences.

4.1. Circulating cell-free fetal DNA (ccffDNA)

Although circulating fetal cells in the maternal bloodstream were the primary source used for NIPD (Herzenberg et al., 1979), the major boost for the NIPD field came with the discovery of ccffDNA. The scarcity of fetal cells together with the painstaking manipulation required for their collection and isolation caused ccffDNA to become the preferred technique for NIPD studies (Lo et al., 1997). The entire fetal genome is represented in the maternal circulation (Lo et al., 2010); thus, all types of genetic prenatal studies are potentially feasible in maternal blood. At present, it is widely accepted that apoptosis of the trophoblastic cells is the primary source of ccffDNA (Alberry et al., 2007), which explains the high turnover of circulating DNA and its rapid clearance after delivery (Lo et al., 1999). It has been reported that fetal DNA fragments are shorter than 313 base pairs (Chan et al., 2004) and are smaller than those having a maternal origin. ccffDNA can be detected in maternal plasma from the 5th week of gestation (Bustamante-Aragones et al., 2008a; Guibert et al., 2003), which makes early NIPD possible. In median terms, only 10% of DNA in maternal plasma is fetally derived; the other 90% is maternal DNA (Lun et al., 2008a). This is the main limitation of NIPD, as the presence of large amounts of maternal DNA in the plasma sample poses an obstacle for the identification of fetal sequences. Hence, NIPD has been focused on the study of those fetal tracts which have a paternal origin and are absent in the maternal genome (i.e., *SRY* gene) to ensure their fetal origin (Table 1).

Prenatal studies with ccffDNA are pursuing the same goals as conventional PD, meaning that researchers are attempting to perform them earlier in gestation, as happens in genetic studies in CVS.

4.2. Clinical application of NIPD: Prenatal diagnosis of single-gene disorders

Prenatal diagnosis is currently an alternative for couples at risk of having children with single-gene disorders. However, there is also a risk of fetal loss associated with sample collection by CVS or amniocentesis (0.5–1%). NIPD aims to replace these invasive methods and perform a genetic study of the fetus by studying ccffDNA in the maternal plasma. Therefore, NIPD studies are mainly performed in the first trimester of pregnancy as a pre-test leading up to conventional PD. In pregnancies resulting from preimplantation genetic diagnosis (PGD) in which prenatal diagnosis is recommended, NIPD also has great potential. These couples are unwilling to undergo PD due to

Table 1
ccffDNA features and their impact in NIPD.

ccffDNA features	NIPD
Entire fetal genome is represented	Any fetal genomic region can be analyzed
Trophoblastic origin	High turnover
Rapid clearance after delivery	No presence of ccffDNA from previous pregnancies
Early detection	Early NIPD studies
Mixed with maternal DNA	Difficult detection of maternally-inherited fetal genomic sequences
Low percentage	High sensitive analytical techniques needed
Fragmented (<313 bp)	Small PCR amplicons are preferred

Table 2
Data of the validation study for fetal sex determination. Results at the different gestational ages tested are shown. Sensitivity: number of male-bearing samples correctly identified/total number of male-bearing samples tested. Specificity: number of female-bearing samples correctly identified/total number of female-bearing samples tested. Positive predictive value (PPV): number of male-bearing samples correctly identified/total number of positive results through real-time PCR. Negative predictive value (NPV): number of female-bearing samples correctly identified/total number of negative results through Real-time PCR. Accuracy: total number of male- and female-bearing samples correctly identified/total number of samples tested. (Bustamante-Aragones et al, 2008a, Haemophilia).

Gestational Ages (weeks)	5	6	7	8	9	10	11	12
Samples analyzed (n=316)	11	22	27	54	58	55	58	31
Female fetuses (n=155)	7	4	17	28	29	23	28	19
Male fetuses (n=161)	2	14	10	26	29	32	30	12
Falses negatives	2	4	0	0	0	0	0	0
Sensitivity (%)	50	77	100	100	100	100	100	100
Specificity (%)	100	100	100	100	100	100	100	100
PPV (%)	100	100	100	100	100	100	100	100
NPV (%)	78	50	100	100	100	100	100	100
Accuracy (%)	81	81	100	100	100	100	100	100

their difficulties in conceiving. They do not accept the risk of fetal loss associated with invasive prenatal testing, so NIPD represents a safe alternative for them.

Although NIPD of single-gene disorders is technically challenging due to the predominance of maternal DNA sequences, several genetic diseases have been currently diagnosed in maternal blood.

4.2.1. X-linked disorders

With the exception of congenital adrenal hyperplasia (CAH), sex-linked diseases are caused by mutations in the X-chromosome. They mostly have a recessive inheritance pattern and mostly affect males. These affected males have no risk of recurrence since their daughters will be carriers and their sons will be healthy. However, female carriers have a 25% risk of giving birth to an affected male.

As described in Section 4.1, most NIPD studies are based on the analysis of paternal defects not present in the maternal genome. Then, in female carriers of X-linked disorders, NIPD of the causative mutation in the fetus is challenging. The first alternative that could be offered to these patients based on the analysis of ccffDNA is the fetal sex determination in maternal blood. Since X-linked disorders mainly affect males, early fetal sex determination reduces the need for subsequent invasive testing only in pregnancies with male fetuses. This was the first clinical application derived from the discovery of ccffDNA in maternal blood. It began when scientists identified Y-chromosome-derived sequences in maternal blood from pregnant women carrying a male fetus (Lo et al., 1997). The presence of Y-chromosome sequences (i.e., SRY or DYS14 genes) in the maternal circulation was associated with male fetuses. However, pregnancies exhibiting absence of Y-chromosome sequences are presumed to bear a female fetus which is not at risk of major manifestations of the sex-linked diseases, and hence patients could be spared invasive prenatal diagnosis. The clinical impact derived from the introduction of fetal sex determination in maternal blood as a pre-test leading up to conventional PD has been a 50% reduction in invasive tests in pregnancies at risk of sex-linked disorders (Finning and Chitty, 2008).

Since the accuracy of fetal sex assessment has been reported by several groups to be higher than 95% (Bustamante-Aragones et al., 2008a; Scheffer et al., 2010), it has been the first NIPD application translated into clinical practice. A recent systematic review and meta-analysis concluded that this test is highly accurate and useful in clinical practice (Devaney et al., 2011). Our service first introduced fetal sex assessment in 2008. Clinical application was preceded by a large-scale validation study in 316 plasma samples collected from the fifth to the twelfth week of gestation (Bustamante-Aragones et al., 2008a) (Table 2). Although ccffDNA was detectable from the fifth week of gestation, the results showed that the accuracy was 100% starting from the seventh week (Fig. 1).

Therefore, the clinical protocol we are currently using calls for the collection of two independent maternal plasma samples collected at the 7–8th week and the 9-10th week, respectively. Since 2008, we have studied a total of 171 cases including 23 different pathologies. Hemophilia and Duchenne muscular dystrophy are the two most prevalent disorders.

Since sex determination is based on the presence/absence of the Y-chromosome sequences, the risk of false negative results (FN) (a male fetus considered female) could have important clinical consequences. Therefore, a parallel independent test could be performed to confirm the existence of ccffDNA in those female fetuses. However, the high accuracy shown by the method and the low percentage of FN results led some authors to consider this parallel analysis as superfluous. The study of two independent samples reduces these risks.

The next step in NIPD of sex-linked disorders is the possibility of studying the maternal defect in maternal plasma and then to diagnose the disease status in the male fetuses. A recent study reported the analysis of maternal mutations in 7 pregnancies at risk of hemophilia (Tsui et al., 2011). The method is based on a relative mutation dosage (RMD) approach. A dosage imbalance between mutant and wild-type alleles in maternal plasma is used to infer the fetal genotype. Quantification of both alleles requires highly sensitive techniques like digital PCR. Moreover, data interpretation is made by statistical analysis, meaning that for a sufficient statistical power, multiple replicates of each sample must be studied. These conditions make this approach too expensive to be applied in the clinical routine.

4.2.2. Autosomal single-gene disorders

Detection of both autosomal dominant and recessive disorders have been reported in maternal plasma. Most of these studies are based on the analysis of de novo or paternally inherited DNA sequences so as to ensure fetal origin. In spite of being limited to the study of paternal defects, NIPD should not be underestimated since it can eliminate the need for conventional PD in several situations:

– Pregnancies at risk of a dominant disease with paternal origin: In at-risk pregnancies due to an affected father, the study of the paternal mutation or alleles in maternal plasma could determine the fetal condition with regard to the pathology. Therefore, NIPD will spare invasive prenatal testing in 100% of these pregnancies. Huntington's disease (Bustamante-Aragones et al., 2008e; Gonzalez-Gonzalez et al., 2003a, 2003b, 2008), achondroplasia (Chitty et al., 2011; Li et al., 2004, 2007; Lim et al., 2010; Saito et al., 2000), myotonic dystrophy (Amicucci et al., 2000), and early onset primary dystonia (Meaney and Norbury, 2009) are the pathologies with a dominant inheritance pattern studied to date in maternal plasma (Table 3).

– **NIPD of Huntington's disease**: As a reference laboratory for Huntington's disease studies, our experience in the NIPD of this pathology includes the study of 13 cases (only 6 of which have been published)(Bustamante-Aragones et al., 2008e; Gonzalez-Gonzalez et al., 2003a, 2003b). Results have shown a high
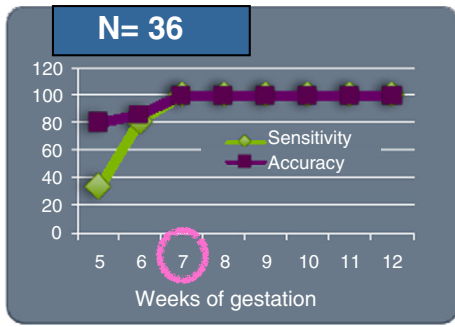
Fig. 1. Sensitivity and accuracy of the validation study for fetal sex determination. X-axis: weeks of gestation. Y-axis: % value. Green line: sensitivity. Pink line: accuracy. Accuracy from the seventh week of gestation is 100%. (Bustamante-Aragones et al, 2008a, Haemophilia).
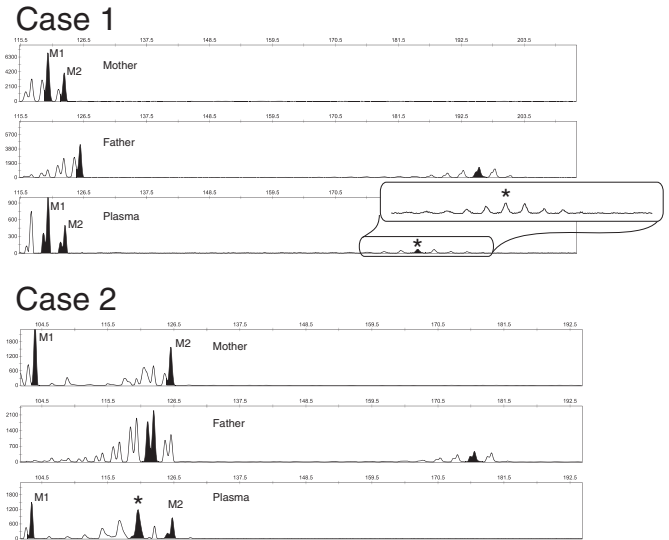

Fig. 2. Examples of NIPD of Huntington's disease. Case 1: An affected fetus. The * shows the paternally inherited fetal mutation. Case 2: Healthy fetus. The * shows the healthy paternal allele inherited by the fetus. Image taken from Bustamante-Aragones, A., et al., 2008e. Eur. J. Neurol. 15 (12), 1338–1344.

accuracy in the first trimester of pregnancy. Only in two cases was it not possible to ascertain the fetal condition because no healthy or mutated paternal alleles were observed. The explanation in one case was the anomalous length of the expanded fetal allele (more than 400 bp). Because of the fragmentation of the ccffDNA, amplification of such as long amplicons is very difficult to attain. Our approximation to the analysis of HD in maternal plasma is based on the routine methods used in the laboratory for the study of this disorder, i.e., study of the expanded allele and STRs analysis by QF-PCR (Quantitative Fluorescent-PCR) (Fig. 2).

– **Pregnancies at risk of a dominant disease because of a *de novo* defect**: Pregnancies suspected of having a dominant disorder due to ultrasonography findings or previous affected children are candidates for NIPD. Achondroplasia is one example of this application because about 90% of the patients are born to unaffected parents. Achondroplasia is the most frequent form of chondrodysplasia and has a prevalence of one child in every 15,000. This type of dwarfism is characterized by short limbs, hyperlordosis, short hands, and macrocephaly with a high forehead and saddle nose. Some of these features can be predicted prenatally by ultrasonography as of the third trimester of pregnancy.

- **Pregnancies at risk of a compound heterozygote fetus for a recessive disease**: In couples carrying different mutations for a recessive pathology, the absence of the paternal mutation and/or detection of normal paternal alleles in maternal plasma rules out the possibility of the fetus manifesting the disease. Therefore, invasive testing could be avoided. On the other hand, detection of the paternal mutation or alleles associated with the defect would increase the risk of the fetus from 1 in 4 to 1 in 2. Then, conventional PD would be recommended to assess the status of the fetus with regard to the maternal mutation and, therefore, the

disease. This application of NIPD is more suitable in pathologies with a high mutational spectrum. Then, the chance that both parents carry different mutations is higher. This is very characteristic of the metabolic diseases. However, in other pathologies in which there is a prevalent causative mutation, i.e., Delta508 mutation in cystic fibrosis, NIPD is more restricted. (Bustamante-Aragones et al., 2008b, 2008c, 2008d; Chan et al., 2010; Chiu et al., 2002a, 2002b; Fucharoen et al., 2003; Gonzalez-Gonzalez et al., 2002; Herzenberg et al., 1979; Li et al., 2009; Lo and Chiu, 2010; Lun et al., 2008b; Nasis et al., 2004; Papasavva et al., 2006, 2008; Tungwiwat et al., 2006, 2007; Yi et al., 2010a, 2010b)

The list of publications reporting NIPD of Mendelian disorders in maternal plasma appears in Table 3. In addition to these published studies, other personal data not published yet include the NIPD of: Leber congenital amaurosis (1 case), McKusick type metaphyseal chondrodysplasia (1 case), Hurler syndrome (1 case), Huntington's disease (9 cases), epidermolysis bullosa (1 case), achondroplasia (5 cases), oculodentodigital dysplasia (1 case), Osler–Rendu–Weber disease (1 case) and Ellis-van Creveld syndrome (1 case).

Initial attempts to achieve non-invasive prenatal diagnosis of monogenic diseases focused on the detection of paternal mutations. Hence, NIPD was not feasible for pregnancies at risk of a maternal dominant disease or pregnancies from parents carrying the same mutation.

Table 3
List of pathologies and publications about NIPD of single-gene disorders in maternal blood.

Pathology (D: dominant; R: recessive; X: X-linked)	Reference
Myotonic dystrophy (D)	(Amicucci et al., 2000)
Huntington's disease (D)	(Gonzalez-Gonzalez et al., 2003a; 2003b; 2008; Bustamante-Aragones et al., 2008e)
Achondroplasia (D)	(Chitty et al., 2011; Li et al., 2004, 2007; Lim et al., 2010; Saito et al., 2000)
Early onset primary dystonia I (D)	(Meaney and Norbury, 2009)
XLRP (X)	(Bustamante-Aragones et al., 2006)
Hemophilia (X)	(Tsui et al., 2011)
Cystic fibrosis (R)	(Bustamante-Aragones et al., 2008b; Gonzalez-Gonzalez et al., 2002; Nasis et al., 2004)
Alpha-thalassemia (R)	(Tungwiwat et al., 2006)
Beta-thalassemia (R)	(Chan et al., 2010; Chiu et al., 2002a; Fucharoen et al., 2003; Li et al., 2009; Lo and Chiu, 2010; Lo et al., 2010; Lun et al., 2008b; Papasavva et al., 2006, 2008; Tungwiwat et al., 2007; Yi et al., 2010a, 2010b)
Propionic acidemia (R)	(Bustamante-Aragones et al., 2008c)
Congenital adrenal hyperplasia (R)	(Chiu et al., 2002b)
Leber congenital amaurosis (R)	(Bustamante-Aragones et al., 2008d)

However, recent advances in the use of the new technologies have studied both paternal and maternal mutations (Tsui et al., 2011) and a genome-wide genetic map of the fetus has been constructed from information about paternal and maternal haplotype (Lo et al., 2010). These preliminary results are promising, but the methods and data analysis they require are expensive and time-consuming.

5. Prospects

Some months ago, most of the efforts in the NIPD field were mainly focused on the development and clinical application of a test to diagnose the most common aneuploidies. The incorporation of NGS to the study of ccffDNA in maternal blood has opened the possibilities of the field for the detection of both aneuploidy and monogenic diseases. Hence, since March 2012, the first NIPD test for detection of trisomy 21, 13, and 18 is available for the at-risk population. However, other alternatives are being explored for the development of a similar test using different methodologies. A recently developed technology based on the study of fetal-maternal differentially methylated regions (DMRs) has demonstrated accuracy for the detection of trisomy 21 (Papageorgiou et al., 2011). A great advantage of this alternative method is that the laboratory equipment required is more affordable for standard diagnostic laboratories.

All the efforts applied to the diagnosis of the most common aneuploidies in a non-invasive way will also imply a great progress in the diagnosis of monogenic disorders. The recent demonstration by the group of Prof. Lo that all fetal genome is represented in maternal blood implies that this diagnosis will be available for any Mendelian disorder independently of the parental origin.

6. Summary and conclusions

Fourteen years after the discovery of the presence of circulating fetal DNA in materna maternal blood, NIPD is a reality in clinical practice. It is currently available as a preliminary test to conventional PD leading up to determine fetal sex, fetal RhD, and paternally inherited fetal mutations. NIPD also has great potential in pregnancies resulting from a Pre-implantation Genetic Diagnosis, since the future parents have had difficulty conceiving and usually do not wish to run the risk of fetal loss.

In pregnancies at risk of sex-linked disorders, the incorporation of the fetal sex assessment into pregnancy management has brought about a reduction of around 50% in invasive prenatal methods. Fetal RhD determination in maternal blood is also available for these RhD (−) pregnant women, thus reducing anti-D administration to only those pregnancies with D (+) fetuses. Additionally, the analysis of paternally inherited mutations is being used for the first time prior to PD to determine the real need of subsequent invasive testing. However, analysis of fetal aneuploidies and maternally inherited fetal defects, is still being investigated. NIPD methods for many of the most common aneuploidies have been described, clearly proving that genetic study of the fetus from maternal blood is possible. However, researchers are still searching for the best technical approach and are validating methods that may be manageable within clinical practice.

References

Alberry, M., et al., 2007. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. Prenat. Diagn. 27 (5), 415–418 May.

Amicucci, P., Gennarelli, M., Novelli, G., Dallapiccola, B., 2000. Prenatal diagnosis of myotonic dystrophy using fetal DNA obtained from maternal plasma. Clin. Chem. 46 (2), 301–302.

Bustamante-Aragones, A., et al., 2006. Detection of a paternally inherited fetal mutation in maternal plasma by the use of automated sequencing. Ann. N. Y. Acad. Sci. 1075, 108–117.

Bustamante-Aragones, A., et al., 2008a. Foetal sex determination in maternal blood from the seventh week of gestation and its role in diagnosing haemophilia in the foetuses of female carriers. Haemophilia 14, 593–598.

Bustamante-Aragones, A., et al., 2008b. New strategy for the prenatal detection/exclusion of paternal cystic fibrosis mutations in maternal plasma. J. Cyst. Fibros. 7 (6), 505–510.

Bustamante-Aragones, A., Pérez-Cerdá, C., Pérez, B., de Alba, M.R., Ugarte, M., Ramos, C., 2008c. Prenatal diagnosis in maternal plasma of a fetal mutation causing propionic acidemia. Mol. Genet. Metab. 95 (1–2), 101–103.

Bustamante-Aragones, A., et al., 2008d. Early noninvasive prenatal detection of a fetal CRB1 mutation causing Leber congenital amaurosis. Mol. Vis. 14, 1388–1394.

Bustamante-Aragones, A., et al., 2008e. Prenatal diagnosis of Huntington disease in maternal plasma: direct and indirect study. Eur. J. Neurol. 15 (12), 1338–1344.

Chan, K.C., et al., 2004. Size distributions of maternal and fetal DNA in maternal plasma. Clin. Chem. 50, 88–92.

Chan, K., Yam, I., Ky, Leung, Tang, M., Tk, Chan, Chan, V., 2010. Detection of paternal alleles in maternal plasma for non-invasive prenatal diagnosis of beta-thalassemia: a feasibility study in southern Chinese. Eur. J. Obstet. Gynecol. Reprod. Biol. 150 (1), 28–33.

Chitty, L.S., 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 (3), 283–289.

Chiu, R.W., Lau, T.K., Leung, T.N., Chow, K.C., Chui, D.H., Lo, Y.M., 2002a. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. Lancet 360 (9338), 998–1000.

Chiu, R.W., Lau, T.K., Cheung, P.T., Gong, Z.Q., Leung, T.N., Lo, Y.M., 2002b. Non invasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study. Clin. Chem. 48 (5), 778–780.

Devaney, S.A., Palomaki, G.E., Scott, J.A., Bianchi, D.W., 2011. Non invasive fetal sex determination using cell-free fetal DNA. A systematic review and Meta-analysis. JAMA 306 (6), 627–636 Aug 10.

Finning, K.M., Chitty, L.S., 2008. Non-invasive fetal sex determination: impact on clinical practice. Semin. Fetal Neonatal Med. 13 (2), 69–75 Apr.

Fucharoen, G., Tungwiwat, W., Ratanasiri, T., Sanchaisuriya, K., Fucharoen, S., 2003. Prenatal detection of fetal hemoglobin E gene from maternal plasma. Prenat. Diagn. 23 (5), 393–396.

Gonzalez-Gonzalez, M.C., et al., 2002. Prenatal detection of a cystic fibrosis mutation in fetal DNA from maternal plasma. Prenat. Diagn. 22 (10), 946–948.

Gonzalez-Gonzalez, M.C., Trujillo, M.J., Rodriguez de Alba, M., Ramos, C., 2003a. Early Huntington disease prenatal diagnosis by maternal semi quantitative fluorescent-PCR. Neurology 60 (7), 1214–1215.

Gonzalez-Gonzalez, M.C., et al., 2003b. Huntington disease-unaffected fetus diagnosed from maternal plasma using QF-PCR. Prenat. Diagn. 23 (3), 232–234.

Gonzalez-Gonzalez, M.C., et al., 2008. Improvement in strategies for the non-invasive prenatal diagnosis of Huntington disease. J. Assist. Reprod. Genet. 25 (9–10), 477–481.

Guibert, J., Benachi, A., Grebille, A.G., Ernault, P., Zorn, J.R., Costa, J.M., 2003. Kinetics of SRY gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique. Hum. Reprod. 18 (8), 1733–1736 Aug.

Herzenberg, L.A., Bianchi, D.W., Schröder, J., Cann, H.M., Iverson, G.M., 1979. Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell sorting. Proc. Natl. Acad. Sci. U.S.A. 76 (3), 1453–1455 Mar.

Kozlowski, P., Knippel, A., Stressig, R., 2008. Individual risk of fetal loss following routine second trimester amniocentesis: a controlled study of 20,460 cases. Ultraschall Med. 29 (2), 165–172 Apr.

Li, Y., Holzgreve, W., Page-Christiaens, G.C., Gille, J.J., Hahn, S., 2004. Improved prenatal detection of a fetal point mutation for achondroplasia by the use of size-fractionated circulatory DNA in maternal plasma—case report. Prenat. Diagn. 24 (11), 896–898.

Li, Y., Page-Christiaens, G.C., Gille, J.J., Holzgreve, W., Hahn, S., 2007. Non-invasive prenatal detection of achondroplasia in size-fractionated cell-free DNA by MALDI-TOF MS assay. Prenat. Diagn. 27 (1), 11–17.

Li, Y., et al., 2009. Size fractionation of cell-free DNA in maternal plasma improves the detection of a paternally inherited β-thalassemia point mutation by MALDI-TOF mass spectrometry. Fetal Diagn. Ther. 25, 246–249.

Lim, J.H., et al., 2010. Non-invasive prenatal detection of achondroplasia using circulating fetal DNA in maternal plasma. J. Assist. Reprod. Genet. 28 (2), 167–172.

Lo, Y.M., Chiu, R.W., 2010. Noninvasive approaches to prenatal diagnosis of hemoglobinopathies using fetal DNA in maternal plasma. Hematol. Oncol. Clin. North Am. 24 (6), 1179–1186 Dec.

Lo, Y.M., et al., 1997. Presence of fetal DNA in maternal plasma and serum. Lancet 16;350 (9076), 485–487 ANTES ERA EL 12.

Lo, Y.M., Zhang, J., Leung, T.N., Lau, T.K., Chang, A.M., Hjelm, N.M., 1999. Rapid clearance of fetal DNA from maternal plasma. Am. J. Hum. Genet. 64 (1), 218–224.

Lo, D.Y.M., et al., 2010. Maternal plasma DNA sequencing reveals the genome-wide genetics and mutational profile of the fetus. Sci. Transl. Med. 8;2 (61), 61ra91.

Lun, F.M., Chiu, R.W., Allen Chan, K.C., Yeung Leung, T., Kin Lau, T., Dennis Lo, Y.M., 2008a. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin. Chem. 54 (10), 1664–1672.

Lun, F.M., et al., 2008b. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. Proc. Natl. Acad. Sci. 105 (50), 19920–19925.

Meaney, C., Norbury, G., 2009. Noninvasive prenatal diagnosis of early onset primary dystonia I in maternal plasma. Prenat. Diagn. 29 (13), 1218–1221.

Mujezinovic, F., Alfirevic, Z., 2007. Procedure-related complications of amniocentesis and chorionic villous sampling: a systematic review. Obstet. Gynecol. 110 (3), 687–694 Sep.

Nasis, O., et al., 2004. Improvement in sensitivity of allele-specific PCR facilitates reliable noninvasive prenatal detection of cystic fibrosis. Clin. Chem. 50 (4), 694–701.

Palomaki, G.E., et al., 2011. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet. Med. 13 (11), 913–920 Nov.

Papageorgiou, E.A., Karagrigoriou, A., Tsaliki, E., Velissariou, V., Carter, N.P., Patsalis, P.C., 2011. Fetal-specific DNA methylation ratio permits noninvasive prenatal diagnosis of trisomy 21. Nat. Med. 17 (4), 510–513.

Papasavva, T., et al., 2006. Noninvasive prenatal diagnostic assay for the detection of beta-thalassemia. Ann. N. Y. Acad. Sci. 10751, 48–53.

Papasavva, T., Kalikas, I., Kyrri, A., Kleanthous, M., 2008. Arrayed primer extension for the noninvasive prenatal diagnosis of beta-thalassemia based on detection of single nucleotide polymorphisms. Ann. N. Y. Acad. Sci. 1137, 302–308.

Saito, H., Sekizawa, A., Morimoto, T., Suzuki, M., Yanaihara, T., 2000. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. Lancet 356 (9236), 1170.

Scheffer, P.G., van der Schoot, C.E., Page-Christiaens, G.C., Bossers, B., van Erp, F., de Haas, M., 2010. Reliability of fetal sex determination using maternal plasma. Obstet. Gynecol. 115, 117e26.

Tsui, N.B., et al., 2011. Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA. Blood 117 (13), 3684–3691 Mar 31.

Tungwiwat, W., Fucharoen, S., Fucharoen, G., Ratanasiri, T., Sanchaisuriya, K., 2006. Development and application of a real-time quantitative PCR for prenatal detection of fetal alpha(0)-thalassemia from maternal plasma. Ann. N. Y. Acad. Sci. 1075, 103–107.

Tungwiwat, W., Fucharoen, G., Fucharoen, S., Ratanasiri, T., Sanchaisuriya, K., Sae-Ung, N., 2007. Application of maternal plasma DNA analysis for noninvasive prenatal diagnosis of Hb E-beta-thalassemia. Transl. Res. 150 (5), 319–325.

Yi, P., et al., 2010a. Development of a pcr/ldr/capillary electrophoresis assay with potential for the detection of a beta-thalassemia fetal mutation in maternal plasma. J. Matern. Fetal Neonatal Med. 23 (8), 920–927.

Yi, P., et al., 2010b. Prenatal detection of beta-thalssemia CD71 (A->T) mutation by polymerase chain reaction/ligase detection reaction/capillary electrophoresis for fetal DNA in maternal plasma—a case report. Fetal Diagn. Ther. 27 (1), 25–31.