Noninvasive prenatal detection of genetic defects.

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Contents

List of abbreviations

Cf(f)DNA	Cell-free (fetal) DNA
CPM	Confined placental mosaicism
CVS	Chorionic villus sampling
DMR	Differentially methylated region
EAR	Epigenetic allelic ratio
FCT	First trimester combined test
HTT	Huntingtin gene
LNA	Locked nucleic acid
LTC	Long-term culture
MP	Microparticle
MPS	Massive parallel sequencing
MSP	Methylation specific PCR
NGS	Next Generation Sequencing
NIPD	Noninvasive prenatal diagnostics
NIPT	Noninvasive prenatal testing
NCV	Normalized chromosome value
NT	Nuchal translucency
PE	Paired-end
QF-PCR	Quantitative fluorescent PCR
(m)*RASSF1A*	(methylated) Ras association (RalGDS/AF-6) domain family member 1, isoform or transcript variant A
RHD	Rhesus D gene
RMD	Relative mutation dosage
RSTD	Relative sequence tag density
SMS	Single molecule sequencing
SNP	Single nucleotide polymorphism
SRY	Sex determining region Y
STC	Short-term culture
T13	Trisomy 13, Patau syndrome
T18	Trisomy 18, Edwards syndrome
T21	Trisomy 21, Down syndrome
TFM	True fetal mosaicism
WGS	Whole genome sequencing

Chapter 1

General introduction

Chapter 1: General introduction

1.1. Current prenatal testing in the Netherlands

In the Netherlands, prenatal screening has been offered to pregnant women since the 1970s. The main focus has been on screening for fetal aneuploidies, such as Down syndrome (trisomy 21, T21). In the past few decades, screening approaches have changed. Originally risk assessments were only based on alpha-fetoprotein concentrations in maternal serum. Currently, prenatal screening is performed by use of a first trimester combined test (FCT), together with a 20-week anomaly scan to screen for neural tube defects and other structural abnormalities. FCT is a noninvasive screening method that uses an individualized risk-calculation to estimate the chance of carrying a fetus with a fetal trisomy (Gezondheidsraad, 2001; Verweij, 2014). In the first trimester, several parameters have been established for FCT calculations, such as biomarker measurement in maternal serum (e.g. intact or total human chorionic gonadotropin and pregnancy-associated plasma protein A) and a nuchal translucency (NT) scan. During this scan, the amount of fluid at the back of the neck of the fetus is determined. Increased amounts of this fluid will result in an increased NT and this may indicate that the fetus has a chromosomal abnormality. Both these parameters, combined with maternal age are subsequently used to calculate the *a posteriori* risk for women of carrying a fetus with fetal trisomy 13, 18 or 21 (Mol *et al.*, 2004). Subsequently, women can be offered amniocentesis or chorionic villi sampling (CVS) for further prenatal testing. Both methods involve an invasive procedure to obtain fetal DNA for subsequent DNA analysis in the laboratory and have a small procedure-related risk of miscarriage (Mujezinovic *et al.*, 2007; Tabor *et al.*, 2010; Akolekar *et al.*, 2014). Because of this risk, currently only women with an increased risk after FCT (i.e. an *a priori* risk of >1:200) or women at risk because of genetic indications (e.g. a familial monogenic disease or a previous child with a chromosomal anomaly) are offered invasive prenatal testing (www.rivm.nl).

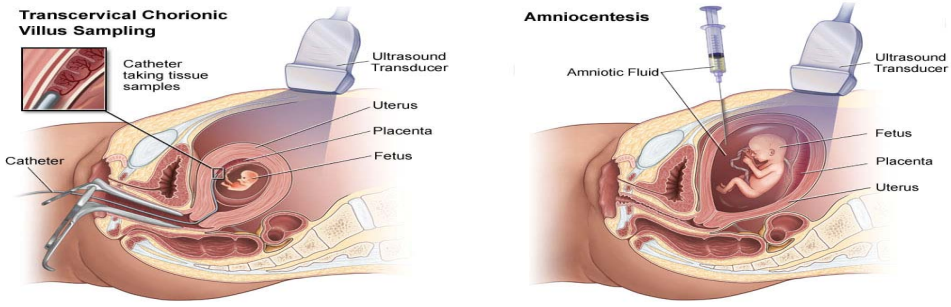

Figure 1: Transcervical chorionic villus sampling of fetal material from the chorion membrane of the placenta (left) and amniocentesis; sampling of amniotic fluid from the uterus (right). Adapted from www.hopkinsmedicine.org.

CVS is usually performed around 11-14 wks of gestation and can be executed both transcervically or transabdominally. Ultrasound is used to guide the catheter/ forceps to obtain placental tissue for DNA isolation (**Fig. 1**). However, when performing genetic testing on chorionic villi DNA, false positive and false negative results are possible due to confined placental mosaicism (CPM; **Appendix 1**). In case of suspected mosaicism amniocentesis is recommended to determine the type of mosaicism (i.e. CPM or true fetal mosaicism (TFM)). With am-

niocentesis or amniotic fluid sampling a small amount of amniotic fluid is sampled from the amniotic sac surrounding a developing fetus (**Fig. 1**). Amniocentesis can be performed as early as 15 weeks though it is usually performed between 16 and 22 weeks of gestation.

Fetal genetic material obtained through either one of these invasive procedures is subsequently used for prenatal diagnosis in the laboratory and this analysis can be performed with cytogenetic as well as molecular techniques. Cytogenetic techniques include G-banding analysis/ karyotyping or FISH (fluorescent in situ hybridization) analysis of chromosomes and are used to facilitate interpretation of possible translocations and/or mosaic findings (**Appendix 1**). Both amniocytes and chorionic villi can be cultured *ex vivo*. For CVS analysis in general a combined approach of both direct preparation/short-term culture (STC) and long-term culture (LTC) is preferred. This culturing process on average takes about two weeks. Direct DNA isolation or DNA isolation from cultured cells for molecular analysis can be performed in a shorter time frame. Results of subsequent molecular analysis can be obtained within a few hours. An example of a molecular technique for prenatal diagnosis is quantitative fluorescent polymerase chain reaction (QF-PCR). QF-PCR was introduced as a fast, precise and cost-effective alternative to analyze the most common autosomal and sex chromosomal aneuploidies and is based on multiplex PCR amplification of multiple genetic markers on chromosome 13, 18, 21, X and Y, together with some additional controls on different autosomes. Karyotyping, which is still considered to be the gold standard for prenatal genetic testing, should be performed additionally to QF-PCR to confirm results in case of an aneuploidy. Moreover, karyotyping is performed in case of a parental/familial chromosomal anomaly such as translocations (balanced/ unbalanced) or inversions, often complimented with additional FISH analysis or chromosomal microarrays. The diagnostic accuracy of performing prenatal diagnosis on fetal material obtained in these invasive procedures is virtually 100%. However, similar to every other medical intervention, the invasive procedures used for obtaining the fetal genetic material are associated with procedure-related risks such as miscarriage (Mujezinovic *et al.*, 2007; Tabor *et al.*, 2010; Akolekar *et al.*, 2014). Therefore, researchers have been exploring other minimally invasive or noninvasive ways to sample fetal material, to diminish such procedure associated risks of miscarriage and to have prenatal diagnosis available to more women.

In addition to invasive testing, with the start of the TRIDENT study (TRial by Dutch laboratories for Evaluation of Noninvasive prenatal Testing) in April 2014, in the Netherlands women with high risk pregnancies (i.e. *a priori* risk >1:200 in the FCT) are offered an additional option for fetal trisomy screening. During this national implementation study, women with high risk pregnancies may opt for noninvasive prenatal testing (NIPT) in which fetal trisomy screening is performed by use of Next Generation Sequencing (NGS). With the availability of this additional screening method, the number of invasive procedures and subsequent risk of miscarriage can be reduced.

1.2. Noninvasive prenatal diagnosis

In the past decades, a lot of effort has been put into developing tests for noninvasive prenatal diagnosis (NIPD) that would eliminate the small but significant risk (< 1%) of procedure-related fetal loss and would be equally reliable as the results of prenatal testing after invasive sampling (Mujezinovic *et al.*, 2007; Tabor *et al.*, 2010; Akolekar *et al.*, 2014). Already at the end of the 19th century, maternal blood was considered to be a useful source of fetal genetic material for noninvasive prenatal diagnosis when the first observation of fetal cells present in maternal circulation was published (Schmorl, 1893).

1.2.1. Fetal cells

Georg Schmorl is considered to be the first researcher to document on feto-maternal cellular trafficking after identifying trophoblast cells in lung capillaries of woman dying of eclampsia (Schmorl, 1893; Lapaire *et al.*, 2007). He also speculated that feto-maternal trafficking might occur in normal gestations. This theory has indeed been confirmed in the past century by many others scientists that have reported findings of a variety of fetal cells present in maternal circulation.

The presence of fetal leukocytes in maternal circulation was first described by the group of Walknowska *et al.* (Walknowska *et al.*, 1969). They identified metaphases of male origin in cultured lymphocytes isolated from blood of healthy pregnant woman, who subsequently gave birth to a male infant. Fetal leukocytes do not have a limited life span and are therefore likely to persist between pregnancies. In addition to the study of Walknowska *et al.*, Bianchi *et al.* described the prolonged persistence of male progenitor cells in a woman who had her last son 27 years prior to blood sampling (Bianchi *et al.*, 1996). As fetal leukocytes may persist through multiple pregnancies for long periods of time, they are not likely to be considered the best source for NIPD on fetal cells, since it is very difficult to distinguish between cells derived from the current or a previous pregnancy.

In contrast to fetal leukocytes, nucleated red blood cells (NRBCs) and their precursors could be a better source of fetal genetic material for NIPD. They are of interest because they are mononuclear and present in abundance in the fetus in the first trimester, while rare in maternal blood (Bianchi, 1995). In contrast to fetal leukocytes, NRBCs do have a limited life span with a relatively short half-life of about 25-35 days (Pearson, 1967).

Placental trophoblast cells are another potential source of fetal material to be sampled from maternal blood. Trophoblasts are the main cellular components of the placenta and originate from the trophectoderm of the blastocyst early in pregnancy (Straszewski-Chavez *et al.*, 2005). One of the potential drawbacks to the use of throphoblasts for noninvasive fetal cytogenetic diagnosis includes the previously mentioned phenomenon of CPM (**Appendix 1**). As a developing organ, the placenta undergoes constant tissue remodeling. The turnover time of a villous trophoblast cell is around 3-4 wks (Huppertz *et al.*, 2004). Deportation of the detached end stage syncytial cells to the maternal lung is a process that occurs in all human pregnancies. The number of cells increases with gestation and has been described to increase even further in patients with pathologic conditions such as (pre-)eclampsia (Tjoa *et al.*, 2006; Benirschke *et al.*, 2010).

Even though there are some cell types that can be a potential source of fetal genetic material to be used in NIPD, the number of fetal cells that can be isolated from maternal blood is limited. Isolation and enrichment of these cells remains therefore challenging because of their low frequency in maternal blood (Oosterwijk *et al.*, 1996).

1.2.2. Cell-free fetal DNA

Instead of looking for intact fetal cells, in 1997 the group of Lo *et al.* was the first to show the presence of fetal genetic material in the acellular component of blood. They demonstrated the detection of Y chromosomal sequences in plasma and serum of woman pregnant with male fetuses (Lo *et al.*, 1997). However, they were actually not the first scientists to observe elevated levels of genetic material in maternal plasma. Elevated levels of nucleic acids (DNA and RNA) in plasma of a pregnant woman had already been observed over 60 years ago by Mandel

and Métais, who were the first to measure nucleic acids in human plasma in 10 healthy subjects and 15 subjects with various conditions, including 1 pregnant female at 7 months of gestation (Mandel *et al.*, 1948). In two independent measurements they found distinct elevated levels of nucleic acids in maternal plasma and already expressed their interest in a follow-up of this interesting study object. A few decades later increased quantities of DNA were also found in serum of cancer patients in the study of Leon *et al.* (Leon *et al.*, 1977). Reasoning that the rapidly growing fetus and placenta possessed "pseudomalignant" tumor-like qualities, the group of Lo *et al.* was the first to show that there is significantly more placental DNA present in cell-free plasma (or serum) of pregnant women as compared to the number of intact fetal cells in the cellular fraction of maternal blood during pregnancy (Lo *et al.*, 1997). Moreover, placental DNA fragments could also be detected in maternal urine (Tsui *et al.*, 2012). These circulating placental DNA fragments in the maternal circulation are (perhaps erroneously) referred to as cell free fetal DNA (cffDNA), since the placenta may not always fully reflect the actual fetal karyotype.

Low levels of circulating cell free DNA (cfDNA) in plasma are a common phenomenon in every individual as a result of clearance of cells dying of apoptosis. However, during pregnancy, total levels of circulating cfDNA increase due to the additional placental contribution. These levels of placental cffDNA have been reported to be present from as early as 4 weeks of gestation (Illanes *et al.*, 2007). Nevertheless, the fetal fraction or percentage of placental cffDNA in plasma is relatively small in the first trimester. The majority of total cfDNA in maternal plasma is of maternal hematopoietic origin (Lui *et al.*, 2002). Fetal fraction is one of most crucial factors influencing NIPD or NIPT results and has been subject of study in many publications (Chu *et al.*, 2010; Jiang *et al.*, 2012b; Canick *et al.*, 2013; Hudecova *et al.*, 2014). Blood withdrawal for noninvasive testing is usually performed around 10-11 weeks of gestation. The percentage of cffDNA in the first trimester is on average ~10%, but differs quite extensively in range depending on gestational age and between individuals (Lo *et al.*, 1998; Lun *et al.*, 2008a; Go *et al.*, 2010; Sikora *et al.*, 2010; Chiu *et al.*, 2011a; Hahn *et al.*, 2011). Sampling later on in pregnancy (e.g. second or third trimester) will consequently result in a higher percentage of placental sequences and thus a higher fetal fraction. However, the great advantages of NIPD and NIPT is that it can be applied already very early on in gestation. Moreover, since maternal blood withdrawal has no risk for the fetus, it is preferred over invasive sampling procedures.

High maternal weight and inherent increased body-mass index (BMI) have been shown to negatively influence the amount of placental DNA recovered from maternal plasma and consequently influence the success rate of downstream testing (Palomaki *et al.*, 2011; Sparks *et al.*, 2012a; Ashoor *et al.*, 2013; Wang *et al.*, 2013; Hudecova *et al.*, 2014). There is an increased turnover of adipocytes in obese women. A high level of apoptosis in maternal blood may result in a lower fetal fraction, since the total amount of maternal cfDNA in plasma increases, at the expense of the percentage of placental DNA fragments. There has been some debate about whether BMI or maternal weight alone would be the best indicator for success rate. BMI corrects for length. However, tall women may also have a higher total blood volume as compared to smaller women with similar weight. This higher blood volume may also result in a dilution effect on the percentage of cffDNA (Wang *et al.*, 2013). For counting-based technologies such as shotgun whole genome sequencing or targeted sequencing a fetal fraction > 4% is required for analysis (Ehrich *et al.*, 2011; Palomaki *et al.*, 2011; Palomaki *et al.*, 2012). Several studies show that the failure rate of testing with insufficient fetal fraction increases extensively with a maternal weight of 100 kg or higher (Wang *et al.*, 2013; Canick *et al.*, 2013; Ashoor *et al.*, 2013). The ultimate covariates for the prediction of the success rate for NIPD or

NIPT have not been determined yet. Preferably, covariates such as weight, height, body type and BMI should be provided and documented with each request for NIPT to determine which of these covariates would be the best predictor of the success rate and ultimately results in the best fetal fraction determination in a large data set.

Total cfDNA can be isolated from maternal plasma. Plasma separation from blood is mostly performed by centrifugation. More recently, options for microscale methods for plasma isolation have become available with microfluidic chips. Plasma can be isolated from blood cells passively either through sedimentation of cells, microfiltration of cells through pores or cell deviation of cells flowing in microchannels. Another option is active separation by use of an external field (e.g. acoustic, electric or magnetic) (Kersaudy-Kerhoas *et al.*, 2013; Kersaudy-Kerhoas *et al.*, 2014). CffDNA in plasma from pregnant women appears to be stable up to 5 days after blood withdrawal (Muller *et al.*, 2011). Maternal blood cells on the other hand are not stable. Lysis of maternal blood cells will lead to a massive increase of the total amount of cfDNA, resulting in a dilution effect of the fetal fraction. Several studies show that performing plasma separation within 24 hrs after collection is essential to prevent maternal cell lysis (Muller *et al.*, 2011; Buysse *et al.*, 2013). The addition of formaldehyde may reduce or prevent cell lysis and has been described to enrich for cffDNA (Dhallan *et al.*, 2004). However, conflicting results have been obtained in other studies (Chung *et al.*, 2005; Chinnapapagari *et al.*, 2005).

Enrichment of placental sequences can be achieved through an epigenetic approach by using methylation specific/dependent techniques (e.g. bisulfite conversion in combination with methylation specific PCR, restriction enzyme digestion or MeDIP (methylated DNA immunoprecepitation)) (Old *et al.*, 2007; Tong *et al.*, 2007; Papageorgiou *et al.*, 2009). At sequence level, there are no differences between the maternal contribution to the fetal genome and the maternal genome itself. At epigenetic level however, several markers have been described that differ between mother and fetus. Epigenetic modifications are somatic alterations to the DNA that do not alter the actual genetic sequence but do affect gene expression. One of the most common and best-known forms of modifications is methylation. Cytosine methylation at the 5-carbon position is the only known stable base modification found in the mammalian genome (Patra *et al.*, 2008). It typically occurs at the cytosine-phosphate-guanine (CpG) sites where DNA methyltransferases turn a cytosine into a 5-methylcytosine (Bachmann *et al.*, 2012). CpG rich sites are mainly located in the promoter region of genes and DNA methylation results in differential expression of maternally and paternally inherited genes due to transcriptional silencing of either one of these genes (i.e. genomic imprinting) (Bachmann *et al.*, 2012).

A difference in size has been observed between maternal and placental cfDNA. This difference may permit the development of size-based strategy for selective enrichment of the fetal fraction from maternal plasma (Li *et al.*, 2004; Jorgez *et al.*, 2009; Yu *et al.*, 2014). In earlier studies, a difference in size had already been noticed between circulating DNA molecules in plasma of pregnant and non-pregnant women (Chan *et al.*, 2004). Analysis of a range of amplicon sizes targeting the *leptin* and *SRY* (sex determining region Y) genes, showed that the plasma of pregnant women contained a higher percentage of smaller size fragments (<201 bp), suggesting that the fetal/placental contribution to circulating cfDNA molecules is causative for this size difference. Post-transplantation chimerism studies in sex-mismatched bone marrow transplantation recipients showed that DNA in plasma and serum is predominantly of hematopoietic origin (Lui *et al.*, 2002). Moreover, data from paired-end (PE) massively parallel sequencing (MPS) of plasma DNA samples from sex-mismatched hematopoietic stem cell transplant recipients and one liver transplant recipient indicated that non-hematopoietically

derived DNA, resembling the fetal fraction, is shorter than hematopoietically derived DNA, which can be considered the bulk of maternal cfDNA (Lui *et al.*, 2002; Zheng *et al.*, 2012). Concordant to this, several studies indeed demonstrate that placental cffDNA fragments are shorter than maternal cfDNA (Chan *et al.*, 2004; Li *et al.*, 2004; Lo *et al.*, 2010; Fan *et al.*, 2010). Analysis of paired-end sequencing reads show that the entire fetal genome is represented in maternal plasma, displaying an average fetal fragment size of ~143 bp against an average maternal fragment size of ~166 bp (Lo *et al.*, 2010; Zheng *et al.*, 2012). These measured sizes of fragmented DNA seem to correspond to the number of bp that is packaged in a single nucleosome. A nucleosome is the fundamental repeating subunit of chromatin and consists of two of each of the histones H2A, H2B, H3 and H4 that come together to form a histon octamer (Annunziato, 2008). Chromosomal DNA is tightly wrapped around such a histon octamer forming a nucleosome. This way of packaging allows the DNA to be condensed into a smaller volume. An octamer binds on average 1.7 turns or 146 bp of chromosomal DNA. H1 histone with a binding capacity of minimal 20 bp stabilizes the two full turns of DNA around a single octamer, creating a nucleosome with a total length of at least 166 bp. Each chromosome consists of hundreds of thousands of nucleosomes, which are joined together by H1 bound to linker DNA (varying in size between ~20-80 bp in length) like beads on a string (Olins *et al.*, 2003; Luger, 2003). The difference in average size between placental and maternal fragmented DNA is likely due to presence of linker fragments (Lo *et al.*, 2010; Beaudet, 2011). This may also indicate that placental DNA is cleaved or degraded in a different non-hematological manner in the maternal circulation.

The kinetics of the cffDNA contribution within the maternal circulation suggests that the placenta is the predominant source of this DNA. Non-reproductive syncytial cells of the trophoblast are cleared effectively as they enter the pulmonary circulation (Benirschke, 1994). Appropriate removal of dying cells prior to the release of its intracellular components is critical for the prevention of fetal rejection (Abrahams *et al.*, 2004). The clearance of these apoptotic cells is driven by apoptosis or programmed cell death in which macrophages play a key role (Abrahams *et al.*, 2004; Straszewski-Chavez *et al.*, 2005; Tjoa *et al.*, 2006; Benirschke *et al.*, 2010). Degeneration of these non-reproductive syncytial cells results in the release of placental cffDNA and cffRNA into the maternal circulation within microparticles that protects them from degradation by plasma nucleases (Bianchi, 2004; Bischoff *et al.*, 2005; Tjoa *et al.*, 2006; Alberry *et al.*, 2007; Faas *et al.*, 2012; Hui *et al.*, 2013). Concentrated plasma pellets subjected to electron microscopic analysis demonstrated the presence of nucleosomes among structures containing chromatin that are likely to be ruptured apoptotic bodies (Bischoff *et al.*, 2005). In addition, the same group flow-sorted nucleic acid positive material from the acellular fraction of plasma samples taken from maternal plasma samples at 12-16 wks of gestation. Microscopic analysis revealed the presence of apoptotic bodies and nucleosomes. They further demonstrated that fetal Y chromosome sequences could be amplified from these apoptotic bodies, showing that at least a part of the circulating cfDNA is packed in apoptotic bodies or microparticles (MPs) (Bischoff *et al.*, 2005; Orozco *et al.*, 2008). Because MPs are heterogeneous in nature, further characterization is required before clinical use. If fetal-specific MPs would express unique surface markers from their original cells (i.e. trophoblasts), these markers could be used for enrichment strategies. By isolating fetal specific MPs, the fetal fraction could be optimized (Orozco *et al.*, 2008). However, no such unique markers have yet been described.

Placental cffDNA is cleared rapidly from maternal plasma following delivery with a short circulation half-life of ~16 min in a range between 4-30 min. Results from quantitative detection of the Y chromosomal marker *SRY* by PCR showed that cffDNA is virtually undetectable in the maternal circulation within 2 hrs postpartum (Lo *et al.*, 1999). A more recent study by Yu *et al.* describes the use of MPS for the detection of fetal sequences in maternal plasma and urine and describes a somewhat different clearance pattern. This study shows that clearance of cffDNA occurred in 2 phases; an initial rapid phase with a mean half-life of approximately 1 hr and a subsequent slow phase with a mean half-life of approximately 13 hrs, with a complete clearance at about 1-2 days postpartum (Yu *et al.*, 2013). This rapid clearance makes NIPD on cffDNA pregnancy specific and in addition, brings clear benefits of early testing, improved safety and ease of access.

1.3. The use of cffDNA in clinical practice

In the years since the discovery of cffDNA in maternal plasma, remarkable developments in noninvasive prenatal diagnosis have taken place. Early efforts focused on the detection of paternally inherited sequences absent in the maternal genome. Recent development in technologies have also enabled the detection of fetal trisomies and have allowed analysis of several monogenic disorders. Ever since, many of these applications have made the step from research to clinically applicable and available technologies.

1.3.1. Rhesus D genotyping and fetal sex determination

The first and currently leading application of NIPD in the Netherlands has been Rhesus D (*RhD*) genotyping in maternal plasma at around 27 weeks of gestation (www.rivm.nl). *RhD* blood group incompatibility between mother and fetus can occasionally result in maternal alloimmunization; an immune response to foreign antigens of the same species. Anti-D antibodies can subsequently cross the placenta and attack fetal red cells, causing fetal anaemia and ultimately fetal death. Knowledge of the fetal antigen status of the *RhD* locus is beneficial to facilitate pregnancy management in alloimmunized women with a heterozygote partner or for *RhD* negative women carrying a *RhD* positive foetus (Moise, Jr., 2008). Fetal *RhD* genotyping is currently performed as a standard screening in the Netherlands (de Haas *et al.*, 2014). Around 12 weeks of gestation, all women are screened for blood group and *RhD* status. Around 27 weeks of gestation additional fetal *RhD* typing is performed on cffDNA in maternal blood of *RhD* negative women (www.rivm.nl, www.sanquin.nl). Historically, fetal testing could only be performed after birth using cord blood (van der Schoot *et al.*, 2008). Performing *RhD* genotyping on cffDNA makes it possible to restrict immunoprofylaxis (administered antenatal in the 30th week of gestation and postnatal) only to non-immunized *RhD* negative women carrying a *RhD* positive foetus (Faas *et al.*, 1998; Scheffer *et al.*, 2011; de Haas *et al.*, 2014).

Since half of the fetal genotype is similar to the maternal genotype, most of the earlier NIPD applications were based on the detection of differences between mother and fetus, such as paternally inherited sequences. Amplification of a fetal marker that confirms the presence of cffDNA allows a negative result to be interpreted as a true negative result. Failure to detect these sequences could be due to lack of amplification of the targeted sequence or may be indicative of low concentrations or even complete absence of cffDNA in maternal plasma, and thus may lead to a false negative result.

The most studied fetal markers for male pregnancies are sequences of the Y chromosome, such as *DYS14*, a multicopy marker located within the *TSPY(1)* (testis specific protein, Y-linked (1)) gene or specific loci on the *SRY* gene (Arnemann *et al.*, 1987; Sato *et al.*, 2010). Both these markers have been studied extensively in fetal sex determination, which is also one of the first and well described applications for the use of cffDNA in diagnostics in addition to fetal *RhD* genotyping. Fetal sex determination is important in case of X-linked genetic conditions where pregnancies with male fetuses are primarily at risk. Furthermore, early determination of fetal sex is also clinically indicated for those at risk of conditions associated with ambiguous development of the external genitalia (e.g. congenital adrenal hyperplasia or CAH). Early maternal treatment with dexamethasone can reduce the degree of virilisation of female fetuses with CAH (Forest *et al.*, 1998; Rijnders *et al.*, 2002). Noninvasive determination of fetal sex can also be performed by ultrasound at as early as 11 weeks' gestation, though not always reliably (Odeh *et al.*, 2009). In contrast, Y chromosomal sequences in maternal plasma can be detected as early as 4 wks of gestation, although reliably from 7 wks onwards (Illanes *et al.*, 2007; Devaney *et al.*, 2011). Both *SRY* and *DYS14* have been used for the identification of male cffDNA in maternal plasma (Zimmermann *et al.*, 2005; Boon *et al.*, 2007; Lun *et al.*, 2008a; White *et al.*, 2012; Kolialexi *et al.*, 2012). This was mostly performed by quantitative Real-time PCR. Even though Y chromosomal sequences can be detected with high sensitivity and specificity early in gestation, a positive result can only be obtained in pregnancies with a male fetus, and alternative markers are required to confirm the presence of female cffDNA in maternal plasma in an universal and sex independent fashion.

1.3.2. Universal fetal markers

A sex independent approach to confirm the presence of fetal DNA is to analyze panels of SNPs or insertion/deletion polymorphisms for the detection of paternally inherited sequences (Alizadeh *et al.*, 2002; Page-Christiaens *et al.*; 2006; Tynan *et al.*, 2011). However, this method of detection can be quite laborious when not all markers are informative. In this case, a large panel of different markers needs to be tested for both biological parents along with the plasma sample.

Markers that are differentially methylated between mother and fetus could also be used to confirm the presence of fetal DNA in maternal plasma in a sex-independent approach. The use of genomic imprinting in NIPD was first shown by the group of Poon *et al.* displaying methylation differences between mother and fetus in a region of the human *IGF2-H19* locus (Poon *et al.*, 2002). Since it has been shown that cffDNA in maternal plasma originates from trophoblast cells of the placenta, the search for differentially methylated markers has focused on genes expressed in placental tissues (Bianchi, 2004; Bischoff *et al.*, 2005; Tjoa *et al.*, 2006; Alberry *et al.*, 2007; Benirschke *et al.*, 2010; Faas *et al.*, 2012; Hui *et al.*, 2013). The two main fetal specific markers that have been studied in NIPD are *SERPINB5* (serpin peptidase inhibitor, clade B (ovalbumin) member 5, also known as *MASPIN* or mammary serine protease inhibitor) and *RASSF1A* (Ras association (RalGDS/AF-6) domain family member 1, isoform or transcript variant A) (Chim *et al.*, 2005; Chan *et al.*, 2006; Chiu *et al.*, 2007; Tsui *et al.*, 2007; Bellido *et al.*, 2010; Della *et al.*, 2010; Zhao *et al.*, 2010; White *et al.*, 2012; Lee *et al.*, 2013).

Both *SERPINB5* and *RASSF1A* are tumor suppressor genes. *SERPINB5* is located on chromosome 18q21.3 and is differentially expressed during human placental development (Dokras *et al.*, 2002). In maternal blood *SERPINB5* is hypermethylated, while in the placenta this gene is hypomethylated (Chim *et al.*, 2005). In contrast to *SERPINB5*, the methylation pattern of *RASSF1A* in the developing placenta shows an opposite pattern, with fetal *RASSF1A*

being hypermethylated, while maternal blood cells are hypomethylated (Chan *et al.*, 2006; Chiu *et al.*, 2007; White *et al.*, 2012). The *RASSF1* locus at 3p21.3 contains eight exons. Alternative splicing and usage of two different promoters give rise to eight different transcripts; *RASSF1A*-*RASSF1H* (Donninger *et al.*, 2007; Richter *et al.*, 2009). The *RASSF1A* isoform is a 39 kDa protein and the gene is frequently inactivated by methylation rather than mutational events (Agathanggelou *et al.*, 2005). Inactivation through promoter region hypermethylation of *RASSF1A* has also been reported in a large variety of tumors in both adult and childhood cancers, including lung, breast, kidney, neuroblastoma and gliomas (Agathanggelou *et al.*, 2005). During fetal development, the promoter region of *RASSF1A* is described to be differentially methylated between mother and fetus, which makes it an interesting universal marker to quantify or confirm the presence of cffDNA in maternal plasma (Chan *et al.*, 2006; Chiu *et al.*, 2007; Zejskova *et al.*, 2010; White *et al.*, 2012). Additionally, several studies show that the concentrations of fetal hypermethylated *RASSF1A* sequences not only increase according to advancing gestation, but also before the onset of clinical manifestation of pregnancy complications secondary to placental dysfunction, such as preeclampsia (Hromadnikova *et al.*, 2010; Kim *et al.*, 2013; Papantoniou *et al.*, 2013).

1.3.3. Fetal aneuploidy screening

The majority of requests for prenatal diagnosis after invasive sampling are related to fetal aneuploidy testing due to aberrant results after FCT. Therefore, the need for novel reliable noninvasive sampling and/or screening methods for subsequent fetal aneuploidy detection had created a strong interest in the field of NIPT. The main focus has been on the detection of fetal T21 with a prevalence of 1 in 700 live born children, although many studies also address T18 detection, T13 detection and/or aneuploidy of the sex chromosomes (Megarbane *et al.*, 2009). Even though the percentage of placental cffDNA in maternal plasma is relatively small, the addition or absence of a particular chromosome in the fetus can be detected with high accuracy using various approaches, such as a targeted or whole genome sequencing approach.

Targeted approach

Besides the use as fetal specific epigenetic markers for the confirmation of the presence of cffDNA in maternal plasma, several markers located on chromosome 21 or 18 have also been described for use in fetal aneuploidy detection. These markers are located in regions with a difference in methylation pattern which are described as differentially methylated regions (DMRs). *SERPINB5* has been described as a differentially methylated marker for fetal T18 detection in NIPT. The group of Tong *et al.* showed that the aneuploidy status of the fetus could be determined using bisulfite modification followed by methylation specific PCR (MSP). The epigenetic allelic ratio (EAR) of a SNP present within differentially methylated *SERPINB5* promoter sequences in maternal plasma can be calculated to determine fetal aneuploidy status for T18 in a fetus as compared to a control group of euploid fetuses (**Appendix 2**) (Tong *et al.*, 2006).

For T21 detection 3 epigenetic markers (i.e. *HLCS*, *PDE9A* and *DSCR4*) on chromosome 21 have been described for NIPT. The putative promoter of *HLCS* is hypermethylated in the placenta while hypomethylated in maternal blood cells. The group of Tong *et al.* first developed a male specific test for detection of T21 by comparing chromosome dosage (**Appendix 2**) of the number of copies from the *HLCS* marker to the *ZFY* (zinc finger protein, Y-linked) on chromosome Y to determine the presence of an additional copy of chromosome 21 (Tong *et al.*, 2010b). Additionally, they developed a sex independent test where they used meth-

ylation-sensitive restriction endonuclease digestion followed by Real-Time or digital PCR to analyze chromosome dosage (**Appendix 2**). Instead of using sequences on the Y-chromosome, in the subsequent study they compared the results of the digestion-resistant *HLCS* gene to a paternally inherited SNP (Tong *et al.*, 2010a). Another marker that has been described for epigenetic based T21 detection is *PDE9A*, which is hypomethylated in placental tissues and hypermethylated in maternal blood (Chim *et al.*, 2008; Lim *et al.*, 2011b). Here, differences in levels of maternal methylated (*M-PDE9A*) and fetal unmethylated (*U-PDE9A*) levels were quantified for detection of T21 detection using quantitative MSP with two different primer sets specific for either *M-PDE9A* or *U-PDE9A* sequences after bisulfite conversion (Lim *et al.*, 2011b). Levels of *U-PDE9A* were significantly elevated in women carrying T21 fetuses as compared to women carrying normal fetuses. *DSCR4* is also considered to be a candidate fetal specific marker for fetal T21 detection and the promoter region shows a similar methylation pattern compared to *PDE9A* (Du *et al.*, 2011). Other groups have also described the search for more candidate DMR for noninvasive T21 detection (Chim *et al.*, 2008; Papageorgiou *et al.*, 2011; Lim *et al.*, 2014).

For fetal aneuploidy detection using digital PCR the focus is not on detecting specific fetal markers, mutations or sequences. For this approach it is no longer required to distinguish between maternal or fetal sequences. Digital PCR is a single molecule counting technique that allows the quantification of DNA by counting one molecule at the time. Single molecules are isolated by dilution and individually amplified by PCR. Each PCR product is then analyzed individually. This technique is very useful in quantifying the contribution of an additional chromosome, for example an additional copy of chromosome 21 in case of fetal T21, when compared to euploid pregnancies (Lo *et al.*, 2007a; Fan *et al.*, 2007; Zimmermann *et al.*, 2008; Fan *et al.*, 2009).

For identifying fetal trisomies, also SNP based approaches have been described. Total cfDNA isolated from maternal plasma is amplified in a single multiplex PCR reaction targeting 11,000 SNPs on chromosome 13, 18, 21, X and Y (Zimmermann *et al.*, 2012). Statistical methods that incorporate parental genotypes are used to determine copy number of these chromosomes. Even higher sensitivity and specificity of the detection of fetal aneuploidies could be obtained when expanding the number of polymorphic loci to 19,488 SNPs (Samango-Sprouse *et al.*, 2013). Ghanta *et al.* analyzed highly heterozygous tandem SNP sequences as short haplotypes by using capillary electrophoresis (Ghanta *et al.*, 2010). Heterozygous informative tandem SNPs from maternal buccal swaps were subsequently measured in maternal plasma by capillary electrophoresis and were used to determine fetal aneuploidy status through haplotype ratio analysis (**Appendix 2**) (Ghanta *et al.*, 2010). A similar approach described by Sparks *et al*., also enriched cfDNA for chromosomes of interest (Sparks *et al.*, 2012b). They developed the digital analysis of selected regions (DANSR™) method, which was developed to reduce the amount of sequencing required for NIPT. This method selectively evaluates specific clinical relevant genomic fragments or loci for each chromosomes of interest (i.e. 13, 18 and 21) to estimate the chromosome proportion and fetal fraction by calculating the chromosome to reference chromosome ratio for each of the chromosomes of interest (e.g. chr. 21 from sample vs reference chr. 21). The DANSR method can be combined with the additional FORTE™ (fetal fraction optimized risk of trisomy evalution) algorithm to calculate the likelihood of fetal trisomy. In addition to the fetal fraction, also age-related risks are taken into account in this algorithm to provide an individualized risk score for fetal trisomy (Sparks *et al.*, 2012a; Sparks *et al.*, 2012b; Juneau *et al.*, 2014).

Whole Genome approach

Instead of using specific markers or SNPs, shotgun massively parallel whole genome sequencing (WGS) permits locus independent simultaneous sequencing of extreme large quantities of fetal and maternal DNA molecules. In 2008, the first studies that described the application of WGS for fetal aneuploidy screening were published (Fan *et al.*, 2008; Chiu *et al.*, 2008). Since, many studies for the validation and implementation of fetal aneuploidy screening have been published (Chiu *et al.*, 2011a; Sehnert *et al.*, 2011; Ehrich *et al.*, 2011; Lau *et al.*, 2012b; Bianchi *et al.*, 2012). The majority of these studies have used Illumina sequence analyzers, although the SOLiD (Sequencing by Oligonucleotide Ligation and Detection) platform and Ion Torrent have been described as well for noninvasive aneuploidy detection (Faas *et al.*, 2012; Yuan *et al.*, 2013). Both SOLiD and Illumina use fluorescently labeled nucleotides for visualization. In contrast to SOLiD sequencing by ligation, the Illumina platform uses sequencing by synthesis technology, tracking the addition of fluorescently labeled nucleotides as the DNA chain is copied, in a massively parallel fashion. Also the Ion Torrent platform or ion semiconductor sequencing platform is a sequencing by synthesis method. However, this method is based on the detection of hydrogen ions that are released during dNTP incorporation. The semiconductor chip measures differences in pH with each incorporation.

After sequencing each fragment (i.e. read) can be assigned back to the chromosome of origin. If a fetal aneuploidy is present, there should be a relative excess or deficit for the chromosome in question. However, it is necessary to sequence many millions of fragments in WGS to ensure sufficient counts since, for instance, chromosome 21 represents only ~1.5% of the human genome. The count of fragments or reads mapped back to a particular chromosome can subsequently be compared with the expected counts for euploid fetuses to determine the presence of a fetal aneuploidy (**Appendix 3**). With the improvement of the techniques, the possibility of running multiple samples simultaneously (i.e. multiplexing) is available. The addition of a sample specific bar-code or tag sequence to each fragment allows the identification of the fragment to the sample of origin. Originally, the first multiplex studies described only duplexed samples (2-plex) since the total number of reads produced by the sequence analyzers was relatively low. Currently with improved technology, sufficient numbers of reads are produced to run 8-plexed, 12-plexed or even 24-plexed samples for noninvasive aneuploidy detection on the Illumina platform (Lau *et al.*, 2012b; Bianchi *et al.*, 2014, Bayindir *et al*., 2015).

In the majority of these studies for NIPT, the main focus is on T21 screening. When comparing DNA sequencing to standard prenatal aneuploidy screening (i.e. FCT), the false positive rates when using cfDNA from maternal plasma were significantly lower than those with standard first trimester screening (Bianchi *et al.*, 2014). Both the sensitivity and specificity of fetal T21 detection exceed 99% (Benn *et al.*, 2012; Mersy *et al.*, 2013; Gil *et al.*, 2014). In addition, performance for fetal trisomy 18 and 13 screening has also been reported, with detection rates of 96.8% and 92.1% respectively (Gil *et al.*, 2014).

Preferential amplification of sequences has been observed on PCR-based MPS platforms (Fan *et al.*, 2008; Dohm *et al.*, 2008). Many studies reported have suggested that this lower performance for T18 and T13 detection is due to the guanine and cytosine (GC) content (Chen *et al.*, 2011; Bianchi *et al.*, 2012; Lau *et al.*, 2012b; Palomaki *et al.*, 2012; Norton *et al.*, 2012; Sparks *et al.*, 2012b). Together with the dynamics and development in the sequencing technology, bioinformatics software and analysis tools are constantly changing and improved. New algorithms used for the analysis of WGS and targeted NGS data are continuously developed and upgraded. For example, the RAPID (Reliable Accurate Prenatal non-Invasive Diag-

nosis) analysis method is available as RAPIDR, an open source R package for the detection of monosomy X and fetal sex in addition to trisomy 13, 18 and 21. This pipeline implements a combination of several published and validated NIPT analysis methods such as NCV (Normalized Chromosomal Value; **Appendix 3**) calculations and correction to account for GC bias (Lo *et al.*, 2014). With the WISECONDOR (WIthin SamplE COpy Number aberration DetectOR) tool, copy number aberrations can be detected and, in contrast to the RAPIDR method, analysis is no longer restricted to chromosome 13, 18 and 21 only (Straver *et al.*, 2014).

1.3.4. Monogenic disorders

The large majority of prenatal requests in the laboratory are related to fetal aneuploidy detection. With NIPT for common aneuploidies already available, the next step is to focus on NIPD for monogenic disorders. This research area represents a smaller part of the total diagnostic field in noninvasive prenatal genetic testing. Nevertheless, there is also a request from patients and physicians to expand the NIPD repertoire.

NIPD for single gene disorder has been described for a variety of monogenic disorders, such as achondroplasia, cystic fibrosis and α- and β-thallassaemia (Gonzalez-Gonzalez *et al.*, 2002; Li *et al.*, 2007; Lim *et al.*, 2011a; Yan *et al.*, 2011; Phylipsen *et al.*, 2012). NIPD can be applied for both autosomal dominant and recessive cases, most efficiently when the mother does not carry the mutant allele or carries a different mutation as the biological father (Bustamante-Aragones *et al.*, 2012; Daley *et al.*, 2014). In addition, detection of *de novo* mutations can be performed as well. Several approaches to perform NIPD for the detection of paternally inherited mutations or *de novo* mutations have been described, ranging from more basic molecular methods such as quantitative PCR and QF-PCR to more complex methods such as MALDI-TOF mass spectrometry (Gonzalez-Gonzalez *et al.*, 2003a; Li *et al.*, 2007; Sirichotiyakul *et al.*, 2012; Chitty *et al.*, 2013). The detection of maternally inherited mutations or autosomal recessive monogenic diseases with parents sharing identical mutations is more challenging in NIPD. Since maternally inherited fetal alleles are genotypically identical to the maternal background, one cannot determine fetal status by simply detecting the presence of a maternal mutation in maternal plasma. A relative mutation dosage (RMD) approach using digital PCR is an example of an approach that can be used for NIPD of monogenic diseases for cases where the mother also carries a mutation (**Appendix 3**) (Lun *et al.*, 2008a; Zimmermann *et al.*, 2008; Chiu *et al.*, 2009). By measuring the relative amounts of the maternal mutant and wild type alleles in maternal plasma, the inherited dosage of the mutant fetal allele can be determined.

1.4. Scope of the thesis

There is a growing need in the field of prenatal diagnostics for alternative laboratory tests to complement and/or replace current invasive testing. With the discovery of the presence of cell-free fetal DNA (cffDNA) in maternal plasma, an alternative method for obtaining fetal genetic material is now available. Many studies describing applications for the use of cffDNA show promising results for noninvasive prenatal diagnosis (NIPD) or noninvasive prenatal testing (NIPT). The aim of this thesis is to develop and validate new applications for the use of cffDNA that may complement or replace current diagnostic tests.

In order to study the use of cffDNA, it is crucial that the presence of cffDNA in maternal plasma can be confirmed. Many studies have shown that confirmation can be accomplished in a sex-dependent manner through the detection of Y-chromosomal sequences. However, in

case of a female fetus, this sex-dependent method is not informative. In **chapter 2** we describe the validation of a novel approach for the detection of fetal methylated *RASSF1A* (*mRASSF1A*) in maternal plasma. We describe the use of bisulfite conversion in combination with pyrophosphorolysis-activated polymerization (PAP) to confirm the presence of fetal DNA in maternal plasma in a sex-independent manner.

An application for NIPT that previously has been described is fetal trisomy screening by means of shotgun massive parallel sequencing (MPS). With this technique millions of short sequencing reads are produced that can be mapped back to the chromosome of origin to determine fetal aneuploidy status through a relative overrepresentation for chromosome 21 in case of fetal T21. Studies have reported that high-throughput next generation sequencing (NGS) platforms previously tested use a PCR step during sample preparation, which results in amplification bias in GC-rich areas of the human genome. This GC bias may result in a lower sensitivity for fetal trisomy screening. In **chapter 3** we describe an alternative method for fetal trisomy 21 (T21) detection by means of single molecule sequencing (SMS) on the Helicos platform to eliminate this bias and we compare SMS to the previously described Illumina platform. In addition to this, we also describe the application of single molecule sequencing for trisomy 18 (T18) and trisomy 13 (T13) detection in **chapter 4**.

Instead of the detection of a relative overrepresentation of an entire chromosome, NIPD can also be used for the detection of paternally inherited pathogenic repeats or alleles. Detecting low levels of fetal sequences in the excess of maternal cell-free DNA is still challenging. Whole genome shotgun NGS as currently applied for NIPT may not always be the fastest and available method of choice for the detection of merely 1 or 2 variants at a short turn-around time as is required when performing prenatal diagnostics for monogenic disorders. Moreover, the detection of large repeat sequences with NGS is currently difficult, if not impossible. In **chapter 5** we describe the development of a sensitive, mutation specific and fast alternative for NGS-mediated NIPD. We report a novel PCR based application of high-resolution melting curve analysis in combination with a blocking locked nucleic acid (LNA) probe to detect paternally inherited mutations in both autosomal dominant and recessive disorders. In **chapter 6** we additionally show the application of NIPD for the detection of paternally inherited CAG repeats in maternal plasma. We describe the validation for use of NIPD aimed at the detection of polymorphic paternally inherited CAG repeats in the Huntingtin (*HTT*) gene for fetuses at risk of Huntingon disease (HD).

A general discussion of the data is presented in **chapter 7** and a summary of the major findings of this thesis is presented in **chapter 8**.

Chapter 2

mRASSF1A-PAP, a novel methylation-based assay for the detection of cell-free fetal DNA in maternal plasma

Chapter 2: mRASSF1A-PAP, a novel methylation-based assay for the detection of cell-free fetal DNA in maternal plasma

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Abstract

Objectives: *RASSF1A* has been described to be differentially methylated between fetal and maternal DNA and can therefore be used as a universal sex-independent marker to confirm the presence of fetal sequences in maternal plasma. However, this requires highly sensitive methods. We have previously shown that Pyrophosphorolysis-activated Polymerization (PAP) is a highly sensitive technique that can be used in noninvasive prenatal diagnosis. In this study, we have used PAP in combination with bisulfite conversion to develop a new universal methylation-based assay for the detection of fetal methylated *RASSF1A* sequences in maternal plasma.

Methods: Bisulfite sequencing was performed on maternal genomic (g)DNA and fetal gDNA from chorionic villi to determine differentially methylated regions in the *RASSF1A* gene using bisulfite specific PCR primers. Methylation specific primers for PAP were designed for the detection of fetal methylated *RASSF1A* sequences after bisulfite conversion and validated.

Results: Serial dilutions of fetal gDNA in a background of maternal gDNA show a relative percentage of ~3% can be detected using this assay. Furthermore, fetal methylated *RASSF1A* sequences were detected both retrospectively as well as prospectively in all maternal plasma samples tested (n=71). No methylated *RASSF1A* specific bands were observed in corresponding maternal gDNA. Specificity was further determined by testing anonymized plasma from non-pregnant females (n=24) and males (n=21). Also, no methylated *RASSF1A* sequences were detected here, showing this assay is very specific for methylated fetal DNA. Combining all samples and controls, we obtain an overall sensitivity and specificity of 100% (95% CI 98.4%-100%).

Conclusions: Our data demonstrate that using a combination of bisulfite conversion and PAP fetal methylated *RASSF1A* sequences can be detected with extreme sensitivity in a universal and sex-independent manner. Therefore, this assay could be of great value as an addition to current techniques used in noninvasive prenatal diagnostics.

Introduction

Over the past years, the use of cell-free fetal DNA (cffDNA) for noninvasive prenatal diagnosis (NIPD) has proven its clinical potential in a wide range of fields. Although the possibilities for using cffDNA in NIPD are numerous, they do require highly sensitive and specific techniques to detect the low levels of fetal sequences in the pool of maternal plasma DNA early in gestation.

For the detection and/or quantification of fetal DNA, many investigators have based their strategy on the detection of Y-chromosomal-specific sequences (*SRY* and *DYS14*), or on the use of paternally inherited SNPs or polymorphic loci that are either absent or different in the mother (Tang *et al.*, 1999; Alizadeh *et al.*, 2002; Page-Christiaens *et al.*, 2006; Hill *et al.*, 2010; Scheffer *et al.*, 2010). Even though Y-chromosomal sequences can be detected using several different techniques with high sensitivity and specificity early in gestation, a positive result can only be obtained in pregnancies with a male fetus. Additional detection of paternally inherited sequences could be used to discriminate between a true negative result in case of a female pregnancy, or a false negative result in case of low levels of circulating cffDNA. However, these methods are quite laborious since both biological parents need to be tested along with the plasma sample and not all SNPs and loci tested will be informative. Therefore, a large panel of different markers need to be tested for each individual case (Scheffer *et al.*, 2010).

Other fetal identifiers have been described which are based on epigenetic differences between fetus and mother. These differences are caused by so-called genomic imprinting and are characterized by differential expression of maternally and paternally inherited genes due to transcriptional silencing of either one of these genes through DNA methylation (Bachmann *et al.*, 2012). The use of genomic imprinting in NIPD was first shown by the group of Poon *et al.* displaying differences in methylation status between fetal and maternal sequences in a region of the human *IGF2-H19* locus (Poon *et al.*, 2002). Since it has been shown that cffDNA in maternal plasma originates from trophoblast cells of the placenta, the search for differentially methylated patterns has focused on genes expressed in placental tissues (Tjoa *et al.*, 2006; Alberry *et al.*, 2007; Bianchi, 2004; Faas *et al.*, 2012; Chim *et al.*, 2005; Chiu *et al.*, 2007; Chim *et al.*, 2008; Tong *et al.*, 2006; Tong *et al.*, 2007; Papageorgiou *et al.*, 2009; Bellido *et al.*, 2010). One of such genes that have been reported to be differentially methylated between mother (hypomethylated) and fetus (hypermethylated) is Ras-Association Domain Family Member 1, transcript variant A (*RASSF1A*) (Chan *et al.*, 2006; Chiu *et al.*, 2007; Lun *et al.*, 2007; Della *et al.*, 2010; Bellido *et al.*, 2010; Zhao *et al.*, 2010; Tsui *et al.*, 2007; White *et al.*, 2012). Previous studies used these differences in methylation in *RASSF1A* to confirm the presence of cffDNA in maternal plasma, independent of fetal sex and without the restriction of only detecting paternally inherited sequences (Chan *et al.*, 2006; Chiu *et al.*, 2007; Lun *et al.*, 2007; Della *et al.*, 2010; Bellido *et al.*, 2010; Zhao *et al.*, 2010; Tsui *et al.*, 2007; White *et al.*, 2012). Methylation-sensitive restriction enzyme digestion, (Real-Time) methylation specific PCR (MSP), mass spectrometry and bisulfite conversion in combination with direct sequencing were the main techniques used in these studies. Some of the aforementioned techniques require a relatively high DNA input. This may indicate that not all of these techniques are sensitive enough to detect the low levels of cffDNA in maternal plasma early in gestation. We have previously shown that Pyrophosphorolysis-activated polymerization (PAP) is a highly sensitive method for the detection of fetal sequences in a large pool of maternal plasma (Boon *et al.*, 2007; Phylipsen *et al.*, 2012). PAP was initially developed to detect rare known mutations with high selectivity in an excess of wild-type template (Liu *et al.*, 2000). It utilizes unidirectional

(PAP) or bidirectional (bi-PAP) blocked oligonucleotides on the 3'end. These blocks need to be removed by pyrophosphorolysis for DNA extension to occur. This is only possible when the oligonucleotides completely match the template strand. This makes PAP a highly specific and sensitive method to use in NIPD (Liu *et al.*, 2000; Boon *et al.*, 2007; Shi *et al.*, 2007; Liu *et al.*, 2004; Phylipsen *et al.*, 2012).

In this study we have used this method to develop a new universal sex-independent methylation-based assay to detect fetal methylated *RASSF1A* (*mRASSF1A*) sequences in maternal plasma for NIPD.

Materials and Methods

Samples

Written informed consent was obtained and this study was approved by the Medical Ethics Committee (CME) of the Leiden University Medical Center. Maternal peripheral blood samples (10-20 mL) were collected in EDTA coated tubes from pregnant women for noninvasive fetal sexing at the Laboratory for Diagnostic Genome Analysis of the Leiden University Medical Center (LUMC), Leiden, the Netherlands. Maternal blood samples (n=71) were drawn at a median gestational age of 10.6 weeks (range 8.0 – 18.1 wks.) and were processed within 24 hrs. after collection as described previously (van den Oever *et al.*, 2012). The retrospective samples used were previously tested for fetal sexing (n=60) using a combination of Real-Time PCR and Pyrophosphorolysis-activated polymerization (Y-PAP) for the detection of Y-chromosomal sequences as previously described (Boon *et al.*, 2007). All fetal gender was confirmed by karyotyping or after birth. In the prospective samples (n=11) fetal sexing was determined using a combination of tests mentioned above, supplemented with Real-Time PCR detection of a panel of 8 high frequency paternal deletion/insertion polymorphisms (Alizadeh *et al.*, 2002). As a control, anonymized plasma control samples from males (n=21) and non-pregnant females (age>48, n=24) were used.

DNA isolation

Cell-free DNA was isolated from plasma with the EZ1 Virus Mini Kit v2.0 on the EZ1 Advanced (QIAGEN, Venlo, The Netherlands; www.qiagen.com) according to the manufacturer's instructions with an input volume of 800 (2*400) µL plasma and an elution volume of 120 (2*60) µL.

Bisulfite conversion

Bisulfite conversion was performed using the EZ DNA Methylation-Gold™ kit (Zymo Research, USA) according to manufactures' instructions, with an input of 100 ng gDNA per reaction (maximum DNA reaction volume of 50 µL) and an elution volume of 10 µL. Bisulfite conversion of plasma DNA was performed as mentioned previously, with an input of 2*50 µL total cell-free DNA (cfDNA) from plasma per bisulfite reaction. (N.B. two corresponding plasma DNA samples were pooled after conversion and purified over 1 column). Elution volume used was 10 µL.

Bisulfite sequencing and mRASSF1A-PAP primer design

Two sets of Bisulfite Sequencing Primers (BSP) containing an M13 tag for Sanger sequencing were designed for two subsequent fragments (BisA 191 bp and BisB 297 bp, Fig. 1, Table 1) in the promoter region of the *RASSF1A* gene (NM_007182.4) outside predicted CpG islands or other potentially methylated cytosines using MethPrimer v1.1 beta (Li *et al.*, 2002; Chiu *et al.*, 2007; Maat *et al.*, 2007). After bisulfite conversion, we assessed methylation patterns of these two regions by conventional Sanger sequencing using these 2 sets of BSP-M13 primers and SeqScape Software (Applied Biosystems). Three sets of fetal gDNA derived from chorionic villus samples (CVS) and corresponding maternal gDNA sequences from maternal blood cells were compared to determine differentially methylated regions of the *RASSF1A* gene at nucleotide level. Methylation specific PAP primers for the detection of *mRASSF1A* were subsequently designed and a so-called bi-PAP reaction was performed.

mRASSF1A-PAP

The *mRASSF1A*-PAP reaction mixture contained 1x PAP-PCR buffer (250 mM Tris-HCl pH 7.5 (Gibco, Life Technologies Corporation), 80 mM $(NH_4)_2SO_4$ (J.T. Baker), 17.5 mM $MgCl_2$ (J.T. Baker), 125 µM of each of the four dNTP's (Thermo Scientific), 450 µM Na_4PPi pH 8.0 (Sigma-Aldrich)), 2.5 IU Klentaq S (ScienTech Corp), 4 µM of each PAP-primer (Biolegio, Nijmegen, the Netherlands, Table 1) and 10 µL of bisulfite converted cfDNA from maternal plasma. Cycling conditions were 15 s 94°C, 40 s 60°C, 40 s 64°C, 40 s 68°C and 40 s 72°C for a total of 45 cycles. PAP reaction product was visualized on a 3.5% 1x TBE agarose gel.

As an internal negative control, maternal gDNA from the buffy coat (input 100 ng) was always converted and analyzed together with the cfDNA isolated from the corresponding maternal plasma sample. A fully methylated human cell line (CpGenome, S7821, Merck Millipore) and/or a gDNA sample from CVS (both 100 ng input per reaction) were used as positive controls to check the bisulfite conversion and the PAP reaction. For the latter, this control had been converted in an independent separate reaction, aliquoted and stored at -20°C until further use.

Serial dilutions (range 1000-7 pg) of fetal gDNA from CVS in H_2O were performed to determine the analytical sensitivity of the assay. In addition, comparable serial dilutions of fetal gDNA in a background of 1000 pg maternal gDNA were performed. Input mentioned is the total amount of fetal gDNA per bisulfite conversion reaction.

Results

Determination of differentially methylated regions in RASSF1A

To determine regions in the *RASSF1A* gene which are differentially methylated between mother and fetus, bisulfite sequencing was performed on maternal gDNA and fetal gDNA from CVS (n=3 sets). Two different regions (BisA and BisB) were analyzed by conventional Sanger sequencing using two sets of BSP-M13 primers (Fig. 1, Table 1). Differentially methylated sequences were found in both regions (Fig. 2). *mRASSF1A*-PAP primers PAP primers were designed in the region covered by the BisB BSP primers and are specific for fetal methylated sequences after bisulfite conversion (Figure 3, Table 1). This region was also previously described by the group of Chiu and colleagues (Chiu *et al.*, 2007). We considered this region the

most suitable for PAP primer design since it contains many methylated cytosines in the fetal (hypermethylated) sequences, while in the mother, these cytosines are unmethylated and will convert into uracil after bisulfite conversion. This resulted in 5 mismatches between each PAP primer and maternal DNA template and will increase the specificity of this assay (Fig. 3). To increase specificity of the PAP primers even more, the length of the oligonucleotides was at least 28 nt. In addition, this assay was designed as a bi-PAP, containing a 3'ddC block on both the forward as well as the reverse primer.

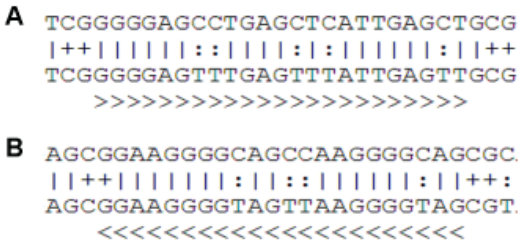

Figure 1: Sequences after Methprimer prediction.
Predicted sequences of the *RASSF1A* for Bisulfite Specific Primers (BSP) design using Methprimer (Li *et al.*, 2002). BSP primers are located outside differentially methylated regions. Methylated nucleotides are indicated with +, unmethylated nucleotides with : and other nucleotides with |. A: The predicted sequence of the BisB forward primer (indicated as >>>). B: The predicted sequence of the BisB reverse primer (indicated as <<<).

Analytical sensitivity and specificity of the mRASSF1A-PAP assay

The analytical sensitivity of the *mRASSF1A*-PAP assay was first determined by testing serial dilutions of gDNA derived from CVS in water. Our results show that this assay is sensitive enough to detect fetal sequences in amounts as low as 16 pg in a 50 µL sample reaction volume (data not shown). To simulate the situation in maternal plasma, gDNA from CVS was serially diluted in a background of maternal gDNA. Our data show that in a background of 1000 pg maternal gDNA, as low as 30 pg of fetal gDNA can be detected, representing a relative percentage of around 3% (Fig. 4). These serial dilutions thus showed that this assay is highly sensitive.

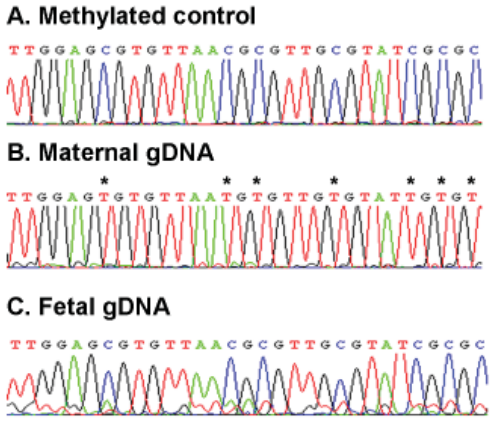

Figure 2: Differentially methylated regions after bisulfite sequencing.
Sanger sequencing results for *RASSF1A* of a fully methylated control cell line (A), maternal gDNA (B) and fetal gDNA derived from CVS (C) after bisulfite sequencing. A representative part of the complete sequence is shown. All unmethylated cytosines are converted to uracil after bisulfite sequencing. Differences between maternal and fetal (methylated) sequences are indicated with an *.

Table 1: Bisulfite sequencing primers and PAP primers.

Target	*Name*	*Sequence (5'- 3')*	*Product size (bp)*	*Primer type*
RASSF1A	*RASSF1A*_BISaF-**M13**	**TGTAAAACGACGGCCAGT**ACTTTTTCTATTTACCTTTTTATTG	227*	BSP
RASSF1A	*RASSF1A*-BISaR-**M13**	**CAGGAAACAGCTATGACC**AACTCAATAAACTCAAACTCCCC		BSP
RASSF1A	*RASSF1A*_BISbF-**M13**	**TGTAAAACGACGGCCAGT**GGGGAGTTTGAGTTTATTGAGTT	333*	BSP
RASSF1A	*RASSF1A*_BISbR-**M13**	**CAGGAAACAGCTATGACC**CTACCCCTTAACTACCCCTTCC		BSP
RASSF1A	*M-RASSF1A*_PAPF2	GTTGGAGCGTGTTAACGCGTTGCGTAT-ddC	110	PAP
RASSF1A	*M-RASSF1A*_PAPR2	ACGTAACGAACCCCGCGAACTAAAAACGATAA-ddC		PAP

Table 1: Primer sequences. M13 tag used for Sanger sequencing is depicted in bold. BSP: Bisulfite Specific Primer, PAP: Pyrophosphorolysis-activated Polymerization. *Product sizes for BSP primers are including the M13 tags.

Table 2: Summary of sample characteristics from the retrospective study.

Samples	*Gestational Age (range in weeks)*	*SRY Real-Time PCR*	*Y-PAP*	*mRASSF1A*	*Fetal Gender*	*Confirmation*	*Concordance*
n = 53	8.0 - 18.1	Undet.	Undet.	Pos.	Female	Yes	Yes
n = 7	9.0 - 15.4	Y	Y	Pos.	Male	Yes	Yes

Table 2: Summary of sample characteristics used in the retrospective study. Undet.: Undetermined (e.g. no Y chromosomal sequences were detected); Pos: positive; Y: Y chromosomal sequences were detected; Y-PAP: Y-chromosomal specific PAP-assay; Karyo: Full karyotyping performed on these samples; birth; fetal gender confirmed at birth.

To demonstrate that this assay is also highly specific for methylated fetal DNA sequences, several controls were tested. As an internal negative control, corresponding maternal gDNA samples were always converted and analyzed in parallel to the maternal plasma samples. No *mRASSF1A* specific bands were observed in these samples. In addition, anonymized plasma samples were tested from non-pregnant females (age >48, n=24) and males (n=21). Also, no *mRASSF1A*-specific products were observed here. Therefore, the assay is also very specific since no false positives were present in all control samples tested (n=116), resulting in an analytical sensitivity and specificity of 100% (95% CI 97.4%-100%).

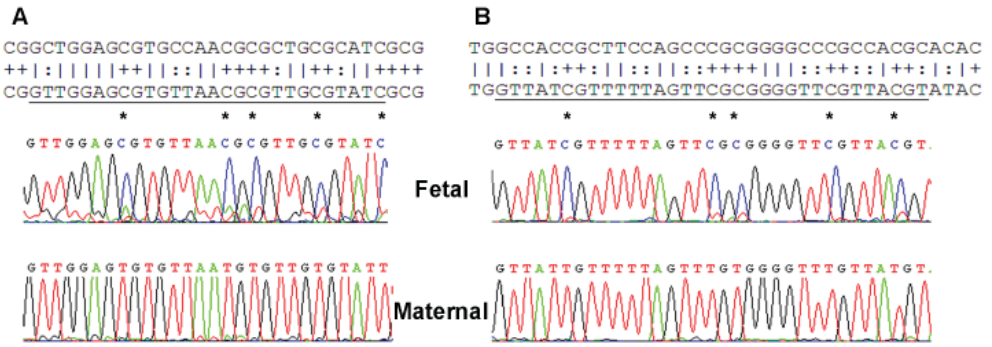

Figure 3: Predicted and confirmed sequences for PAP-primer design.
Sequences of the *RASSF1A* gene were analyzed after bisulfite sequencing of maternal gDNA and fetal gDNA derived from CVS. Differentially methylated regions of the BisB region predicted by MethPrimer (Li et al., 2002) could be confirmed using bisulfite sequencing. Both forward (A, upper panel, underlined) and reverse PAP-primer (B, upper panel, underlined) are specific for fetal sequences (middle panels) after bisulfite conversion and both primers have several mismatches to the maternal sequences (lower panels). Mismatches between fetal specific PAP-primers and maternal sequences are indicated with an * for each primer.

Testing of retrospective and prospective clinical samples

In a retrospective study, fifty three samples previously tested for fetal gender using a combination of Real Time PCR and Y-PAP (Boon *et al.*, 2007) and indicated as undetermined (no Y chromosomal sequences detected), were tested for the presence of cffDNA using the *mRASSF1A*-PAP assay (Table 2). Fetal *mRASSF1A* sequences were detected in all maternal plasma samples tested (n= 53). As a control, the presence of *mRASSF1A* was also confirmed in 7 samples already tested positive for Y-chromosomal sequences. In these samples the presence of fetal DNA could be confirmed both in a sex-dependent and sex-independent assay, showing that the results from the *mRASSF1A* detection are concordant with the detection of Y chromosomal sequences. The data also confirmed that since this assay is sex-independent, it can be applied to all pregnancies. Altogether, the presence of cffDNA in maternal plasma was shown retrospectively with a sensitivity and specificity of 100% (95% CI 95.0%-100%).

Moreover, in a prospective study, several clinically relevant samples (n=11) were tested with the *mRASSF1A*-PAP assay in parallel to our current diagnostic protocol for fetal sexing using the detection of Y-chromosomal sequences and, in case of a negative (e.g. undetermined or no Y chromosomal sequences detected) result, additional testing for 8 paternal deletion/insertion polymorphisms (Table 3) (Alizadeh *et al.*, 2002; Boon *et al.*, 2007). Although a panel of high frequency polymorphisms was used, informative polymorphisms were either not present (n=4), not inherited (n=3) or results for the detection of these polymorphisms did not

meet our quality criteria (e.g. at least 2/3 Ct values < 40) used for diagnostics (n=1) (Table 3). In parallel, these samples were tested using the *mRASSF1A*-PAP. In all 11 cases tested, the presence of fetal sequences could be confirmed using this new assay. In combination with both Real-Time PCR and Y-PAP results fetal gender could be determined as female (Table 3) and in all cases, our results were concordant with fetal gender determined after additional testing later on in gestation or after birth (Table 3).

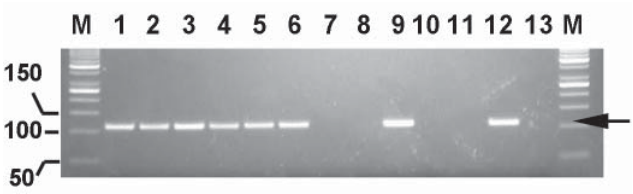

Figure 4: mRASSF1A-PAP serial dilution range of gDNA from CVS.
Serial dilutions were performed with gDNA from CVS in a background of 1000 pg maternal gDNA. Input (pg) mentioned is the total amount of fetal gDNA. M= 50 bp marker, 1= 1000 pg, 2= 500 pg, 3= 250 pg, 4= 125 pg, 5= 60 pg, 6= 30 pg, 7= 15 pg, 8= 7 pg, 9= positive control for bisulfite conversion, 10= NTC for bisulfite conversion, 11= negative control for bisulfite conversion (non-bisulfite converted fetal gDNA), 12= positive control for *mRASSF1A*-PAP, 13= NTC for *mRASSF1A*-PAP. A 110 bp product (arrow) is obtained in cases where *mRASSF1A* sequences could be detected using *mRASSF1A*-PAP.

Discussion

We have developed a novel sex- and polymorphism independent, methylation-based diagnostic test for the detection and confirmation of fetal DNA sequences in maternal plasma. In contrast to methods most widely used in noninvasive diagnostics (e.g. detection of Y chromosomal sequences or paternal polymorphisms), this assay can be applied to all pregnancies. Our test, based on the epigenetic differences between maternal (hypomethylated) and fetal (hypermethylated) *RASSF1A* sequences, was found to be 100% reliable.

Differentially methylated regions between mother and fetus have previously been identified in several genes. However, this was mainly done by techniques such as cloning, mass spectrometry and array. These techniques only produced methylation patterns without high resolution since CpG islands were analyzed as a whole (Chim *et al*., 2005; Chan *et al*., 2006; Bellido *et al*., 2010; Della *et al*., 2010; Papageorgiou *et al*., 2009; Poon *et al*., 2002). As we were interested in designing sequence-specific primers, we needed to study these methylation patterns at nucleotide level and therefore decided to perform bisulfite sequencing. The sequences of the BSP primers are located around predicted CpGs and other possible methylated cytosines (Li *et al*., 2002). Since these sequences are not influenced by bisulfite conversion, it is possible to study differentially methylated regions, both before and after conversion and subsequently to design methylation specific primers for Pyrophosphorolysis-activated Polymerization (PAP) (Liu *et al*., 2000; Liu *et al*., 2004; Shi *et al*., 2007). PAP requires an allele specific oligo with a dideoxyoligonucleotide block at the 3'end. If and only when the sequence of the oligo completely matches the template strand, the dideoxyoligonucleotide can be removed in the presence of pyrophosphate before the oligo can be extended subsequently. We have designed the *mRASSF1A*-PAP primers specific to the fetal (hypermethylated) sequences after bi-

Table 3: Sample characteristics from the prospective study.

Sample	*Gestational Age (weeks)*	*SRY Real-Time PCR*	*Y-PAP*	*# IF Pols*	*# IF Pols detected*	*mRASSF1A*	*Fetal Gender*	*Confirmation (karyo, QF-PCR, US, Birth)*	*Concord-ance*
1	10.7	Undet.	Undet.	3 IF	0 IF[A]	Pos.	Female	QF-PCR	Yes
2	8.6	Undet.	Undet.	1 IF	0 IF[A]	Pos.	Female	QF-PCR	Yes
3	13.6	Undet.	Undet.	0 IF[B]	-	Pos.	Female	US	Yes
4	9.0	Undet.	Undet.	0 IF[B]	-	Pos.	Female	US	Yes
5	10.4	Undet.	Undet.	1 IF	1 IF	Pos.	Female	Karyo	Yes
6	8.1	Undet.	Undet.	1 IF	1 IF	Pos.	Female	QF-PCR	Yes
7	9.0	Undet.	Undet.	1 IF	0 IF[C]	Pos.	Female	US	Yes
8	14.0	Undet.	Undet.	0 IF[B]	-	Pos.	Female	Birth	Yes
9	8.3	Undet.	Undet.	2 IF	0 IF[A]	Pos.	Female	US	Yes
10	9.0	Undet.	Undet.	1 IF	1 IF	Pos.	Female	QF-PCR	Yes
11	8.7	Undet.	Undet.	0 IF[B]	-	Pos.	Female	US	Yes

[A] No informative polymorphisms detected/ inherited, [B] no informative polymorphism present, [C] results did not meet our quality criteria (only 1/3 Ct values ≤ 40).
Table 3: **Sample characteristics of clinical samples (prospective study)**. Undet: Undetermined (e.g. no Y chromosomal sequences were detected); IF: Informative; Pols: Polymorphisms, Pos.: Positive, QF-PCR: Quantitative Fluorescent PCR; US: Ultrasound.

sulfite conversion. Compared to these fetal sequences, maternal (hypomethylated) sequences will differ quite extensively after bisulfite conversion, resulting in several mismatches between each primer and the maternal DNA template. This will prevent the PAP reaction from occuring since the 3' block cannot be removed prior to extension, which makes this assay very specific. Although many other methods for minority allele enrichment have been described, PAP has been described to provide the highest selectivity (Milbury *et al*., 2009). This selectivity could even be enhanced using a bidirectional modification of two opposing allele-specific 3' dideoxyoligonucleotides (Milbury *et al*., 2009; Liu *et al*., 2000; Liu *et al*., 2004; Shi *et al*., 2007). The *mRASSF1A*-PAP is based on this bi-directional principle. We previously demonstrated the use of PAP for noninvasive fetal sex determination using a combination of Real-Time PCR and PAP for the detection of Y-chromosomal sequences (Boon *et al*., 2007). This was successfully validated in our facility by testing a large amount of samples for noninvasive fetal sexing (n=213), resulting in a diagnostic sensitivity and specificity of both 100% (95% CI 98.6%-100%) (unpublished data). Samples from the latter validation study were used for this *mRASSF1A*-PAP validation study as well. In daily clinical practice, we have also tested the *mRASSF1A*-PAP by using this assay in parallel with routine diagnostics for noninvasive fetal sexing. For the cases with undetermined results (e.g. no Y chromosomal sequences detected (Boon *et al*., 2007) we started out testing the *mRASSF1A*-PAP in parallel with Real-Time PCR detection of a panel of 8 high frequency paternal polymorphisms (Alizadeh *et al*., 2002). In some cases, no informative polymorphisms were present that could be used for a diagnostic conclusion. Thus despite using a panel of polymorphisms, the presence of cffDNA in maternal plasma could not be confirmed in 67% of the cases. *mRASSF1A*-PAP was also performed on these samples. In these cases *mRASSF1A*-PAP results were positive and fetal gender was determined and indeed confirmed as female showing that this assay could serve as a valuable supplemental test in diagnostics.

However, there are exceptions where it is preferable to use paternally inherited polymorphisms to confirm the presence of fetal DNA instead of detecting methylated *RASSF1A*. Several recent studies have reported that aberrant methylation in the promoter region of *RASSF1A* can also be used as potential marker for (early) diagnosis of several types of cancer (Zhang *et al*., 2013; Majchrzak-Celinska *et al*., 2013; Ponomaryova *et al*., 2013). This could mean that there is a potential risk for false positive results in the *mRASSF1A*-PAP assay. Although this risk is considered to be small, given the prevalence of cancer in the reproductive age group, it should be taken into account when including women for NIPD. When there is a history of cancer, this should be reported to the lab which is testing the samples. In these cases, testing of paternally inherited polymorphisms to confirm the presence of fetal DNA is preferable over testing methylated *RASSF1A*.

Although the percentage cffDNA early in gestation differs between individuals, most reports agree that the fetal contribution is around 10% in the first trimester (Lo *et al*., 1998; Lun *et al*., 2008a; van den Oever *et al*., 2012). On average, we isolate 2-3 ng of total cfDNA from maternal plasma, thus expecting around 200-300 pg cffDNA as input for the the *mRASSF1A*-PAP assay. Our data show that using PAP, we can reproducibly detect amounts much lower than these average expected amounts of fetal DNA, even in the range of a few genome equivalents (30 pg). This demonstrates the extreme sensitivity of PAP. Using serial dilutions, we could even detect amounts in the range of only 1-2 genome equivalents (6-15 pg). However, since only a few copies of the gene of interest are present, these results were less reproducible. We have used this *mRASSF1A*-PAP assay as a control test in fetal sexing. However, it can also be useful in other applications such as noninvasive prenatal testing (NIPT) for fetal trisomies

using Next Generation Sequencing. Since the assay is universal and sex-independent, it can be applied to all samples and reliably confirms the presence of fetal DNA within a sample.

In conclusion, this study confirmed that methylated *RASSF1A* sequences can be used as informative universal markers for detecting the presence of cffDNA in maternal plasma, irrespective of fetal sex. Moreover, the PAP technique used provides an extremely sensitive method for the detection of fetal sequences in a large pool of maternal plasma DNA early in gestation. Therefore, this assay could be of great value as an addition to current techniques used in noninvasive prenatal diagnostics.

Acknowledgements

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Chapter 3

Single Molecule Sequencing of Free DNA from Maternal Plasma for Noninvasive Trisomy 21 Detection

Chapter 3: Single Molecule Sequencing of Free DNA from Maternal Plasma for Noninvasive Trisomy 21 Detection

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Abstract

Background: Noninvasive fetal aneuploidy detection using free DNA from maternal plasma has recently been shown to be achievable by whole genome shotgun sequencing. The high-throughput Next Generation Sequencing platforms previously tested use a PCR step during sample preparation, which results in amplification bias in GC rich areas of the human genome. To eliminate this bias, and thereby experimental noise, we have used single molecule sequencing as an alternative method.

Methods: For noninvasive trisomy 21 detection, single molecule sequencing was performed on the Helicos platform using free DNA isolated from maternal plasma from 9 weeks of gestation onwards. Relative sequence tag density ratios were calculated and results were directly compared to the previously described Illumina GAII platform.

Results: Sequence data generated without an amplification step show no GC-bias. Therefore, using single molecule sequencing all trisomy 21 fetuses could be distinguished more clearly from euploid fetuses.

Conclusion: This study shows for the first time that single molecule sequencing is an attractive and easy to use alternative for reliable noninvasive fetal aneuploidy detection in diagnostics. Using this approach, previously described experimental noise associated with PCR amplification, such as GC bias, can be overcome.

Introduction

Trisomy 21 (T21) is the most common chromosomal abnormality in live-born children. The diagnosis can be made early in pregnancy using invasive testing (e.g. chorionic villus sampling (CVS) or amniocentesis). These invasive procedures however, are associated with a risk of miscarriage. Therefore, these tests are commonly only offered to women at increased risk for fetal trisomy. Risk assessment used to be based on maternal age. More recently, this was refined by adding serum markers for trisomy and ultrasound measurement of the fetal nuchal translucency (Driscoll *et al.*, 2009). Current screening programs have detection rates for T21 of around 80% with a false positive rate of 5%, meaning that one in every 20 women screened is offered invasive testing with its inherent risks, while carrying a healthy fetus (Wapner *et al.*, 2003; Mujezinovic *et al.*, 2007).

The discovery of cell-free fetal (cff) DNA and RNA in maternal plasma opened possibilities for noninvasive prenatal diagnosis (NIPD) (Lo *et al.*, 1997). Although cffRNA has been used for noninvasive T21 detection (Lo *et al.*, 2007b; Picchiassi *et al.*, 2010; Tsui *et al.*, 2010; Deng *et al.*, 2011), the majority of approaches use cffDNA for NIPD of T21. In the first trimester, the percentage of cffDNA in maternal plasma is on average 1-10% and differs quite extensively in range depending on gestational age and between individuals (Go *et al.*, 2010; Chiu *et al.*, 2011b; Lo *et al.*, 1998; Lun *et al.*, 2008a; Sikora *et al.*, 2010; Hahn *et al.*, 2011). Therefore, it remains challenging to detect fetal sequences in a large pool of maternal DNA. Previously, several papers have shown that noninvasive T21 detection is possible by using single nucleotide polymorphisms (SNPs) (Dhallan *et al.*, 2007; Ghanta *et al.*, 2010) and epigenetics (Old *et al.*, 2007; Chim *et al.*, 2008; Tong *et al.*, 2010b; Papageorgiou *et al.*, 2011) although these methods have a number of limitations.

In 2008, noninvasive T21 detection by Next Generation Sequencing (NGS) was introduced (Fan *et al.*, 2008; Chiu *et al.*, 2008), opening a whole new way of analysis. No longer only fetal specific sequences were analyzed, but all free DNA in plasma, from both fetal and maternal origin, is sequenced with this technique. Two recent papers confirmed the potential value of NGS for noninvasive fetal T21 detection in multiplexed plasma DNA samples in a clinical setting (Ehrich *et al.*, 2011; Chiu *et al.*, 2011a). Both the Illumina Genome Analyzer (GA) II (Fan *et al.*, 2008; Chiu *et al.*, 2008; Fan *et al.*, 2010; Ehrich *et al.*, 2011; Chiu *et al.*, 2011a) and the SOLiD platform (Chiu *et al.*, 2010) have been used for noninvasive T21 detection by NGS. These platforms use amplification steps by polymerase chain reaction (PCR) which are known to introduce preferential amplification of sequences depending on different GC content (Fan *et al.*, 2008; Chiang *et al.*, 2009).

In the present study, we have tested single molecule sequencing (tSMS, Helicos Heliscope™ Single Molecule Sequencer) for noninvasive T21 detection. The Helicos platform utilizes visual imaging across the flow cell for direct DNA measurement by recording the incorporation of fluorescently labeled nucleotides (Gupta, 2008; Milos, 2009). The use of single molecule sequencing has been described previously (Harris *et al.*, 2008) and this technique should largely overcome the limitations associated with PCR amplification and bias as mentioned above. Although the sequencing time on the Helicos platform is longer compared to the Illumina platform (4 days respectively 2 days), Helicos sample preparation is simple, 3 times faster (1 day compared to 3 days) and therefore relatively cheap. Furthermore, this method requires low amounts of DNA, which could be of special interest early in gestation.

Here, we present a comparison of the application of single molecule sequencing for noninvasive T21 detection using cffDNA from maternal plasma to the previously described PCR-based Illumina NGS platform.

Materials and Methods

Subjects

Pregnant women undergoing prenatal diagnosis were recruited at the Department of Obstetrics of the Leiden University Medical Center (LUMC), Leiden, The Netherlands. Informed consent was obtained and this study was approved by the Institution's Medical Ethics Committee.

Sample Processing and Isolation

Maternal peripheral blood samples (10-20 mL) were collected in EDTA coated tubes at the LUMC and were processed within 24 hrs after collection. All blood samples were drawn at a median gestational age of 12 +2 weeks (range 9 +3 to 16 +6 wks). Preferably blood samples were drawn before an invasive procedure, if this was not possible samples were drawn at least 5 days after the invasive procedure to minimize any disturbance with fetal material due to this procedure.

Blood was centrifuged at 1200g (without brake) for 10 min at room temperature. Plasma was transferred to 15 mL micro centrifuge tubes and centrifuged at 2400g for 20 min (with brake) at room temperature to remove residual cells. Cell-free plasma was divided into 800 µL aliquots and stored at -80^{o}C until further processing.

Because both sequencing platforms require different amounts of input DNA, cell-free DNA was isolated from plasma with the EZ1 Virus Mini Kit v2.0 on the EZ1 Advanced (QIAGEN, Venlo, The Netherlands; www.qiagen.com) for Helicos sample preparation or manually with the QiaAmp MinElute Virus Spin Kit (QIAGEN) for Illumina sample preparation according to the manufacturer's instructions.

To verify fetal gender and to measure the total quantity of cell-free DNA, we respectively performed a pyrophosphorylation-activated polymerization assay on the Y chromosome (Y-PAP) and a Real-Time Taqman PCR assay on *CCR5* for quality control purposes as described previously (Boon *et al.*, 2007). In addition, for male fetuses we estimated the percentage of cffDNA based on sequencing data of chromosome X (Fan *et al.*, 2008) and by Real-Time Taqman PCR assay on *SRY*, for which we used a standard curve from male genomic DNA to determine the range of cffDNA percentages in maternal plasma. Percentages were estimated by dividing the amount of *SRY* (pg/µL) by the maternal fraction of *CCR5* from 1 allele (*SRY*/(0.5**CCR5*$_{total}$ – *SRY*)), taken into account that the PCR efficiency of both genes is similar.

Library preparation and sequencing

A total of 24 plasma samples was included in this retrospective study, containing 20 samples from singleton pregnancies, of which 11 cases (5 female and 6 male fetuses) of T21, 9 cases of disomy (D21) pregnancies (1 female and 8 male fetuses) and 4 plasma control samples from anonymous adult male blood donors. All samples were de-identified to the investigators before sample preparation and data analysis. These results were not revealed to the investigators until after data analysis. Material from the invasive procedure was sent to the

Cytogenetics Lab for full karyotyping. Fetal gender was confirmed by karyotype or after birth.

All cell-free plasma DNA samples were sequenced on both the Helicos (Helicos BioSciences Corporation, Cambridge, MA, USA, www.helicosbio.com) and the Illumina (Illumina Inc., San Diego, CA, USA, www.illumina.com) GA II platform. Owing to the relatively short length (Fan *et al.*, 2008) and fragmented nature of free DNA in plasma, no additional shearing step was performed during library preparation.

Helicos sample preparation was performed according to the manufacturer's ChIP-Seq Direct Tailing Procedure with an input of 400 µL plasma for DNA isolation with the EZ1 (QIAGEN) and the maximum amount of input for tailing. As a quality control, size of the fragments and template size distribution were determined by running a High Sensitivity DNA chip on the Agilent Technologies 2100 Bioanalyzer. A standard 120-cycle run was performed on the Heliscope™ Single Molecule Sequencer, which resulted in an average read length of 35 nucleotides.

Illumina sample preparation was performed according to the manufacturer's ChIP-Seq protocol with an input of 1600 µL plasma per sample per column for manual DNA isolation and a maximum amount of input for this protocol. Sixteen out of 24 samples were sequenced in a duplex assay (T21 n=7, D21 n=7 and male plasma controls n=2). For this, unique synthetic 6 nucleotide barcodes (indexes) were used. The barcode was ligated to the plasma DNA molecule prior to the PCR amplification step. Indexed samples were additionally purified on a 3% TAE Agarose gel prior to the quality control run on the Bioanalyzer as mentioned above. A 36-cycle run was performed on the Illumina GA II.

Data analysis

Helicos sequencing data were analyzed with the Helicos Helisphere resequencing pipeline using default settings. Data were aligned against hg19 and gaps and repeats were filtered out. Filtered data were sorted and binned per 50 kb.

Illumina raw data from duplexed samples were pre-analyzed by splitting the data per indexed barcode with in-house Linux command lines. Sequencing data were analyzed with NextGENe software (SoftGenetics, State College, PA, USA, www.softgenetics.com). Data were mapped to the annotated Human Genome GFCh37-dbSNP 131(4/14/2010) (hg19) for Illumina data compatible with NextGENe software. Expression reports per 50 kb were created. Only unique reads with at most 1 mismatch, which could be aligned to the reference genome, were used for calculations.

For all samples (both T21 and D21) used for noninvasive fetal trisomy 21 detection in maternal plasma, ratios of relative sequence tag density (RSTD) were calculated. First, for each sample the total number of reads was calculated per chromosome, by summing the read counts of all 50 kb bins belonging to a particular chromosome. Second, for each sample, the total summed number of reads was normalized by the median value of the autosomes. Finally, ratios of RSTD were calculated by dividing these normalized values by the averaged normalized value of the disomy samples (Fan *et al.*, 2008) or, in addition, by the normalized average of male plasma control samples. As the data were obtained by two separate runs for both sequencing technologies, ratios were determined for each run separately.

Statistical analysis

Statistical analysis was conducted with PASW Statistics version 17.0 (SPSS Inc., Chicago IL, USA, www.spss.com), Prism 5 (version 5.00, GraphPad Software, Inc. La Jolla CA, USA, www.graphpad.com) and R version 2.13 (R Development Core Team (2011)). R: A language environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN

Table 1: Overview of included maternal plasma samples.

Sample number	*Karyotype*	*Maternal Age*	*Indication*	*AD Blood*	*CVS/ Amnio*	*AD Procedure*
1	46,XY	35	Family history of mental retardation/ ICSI pregnancy	12 +2	Amnio	16 +1
2	46,XY	39	Advanced maternal age	9 +6	Amnio	17 +0
3	47,XX,+21	41	Ultrasound abnormality: Hygroma colli and generalized oedema	15 +3	CVS	14 +2
4	47,XY,+21	43	Advanced maternal age	10 +2	Amnio	16 +0
5	46,XX	40	Advanced maternal age	10 +1	Amnio	16 +0
6	46,XY	38	Advanced maternal age	11 +2	Amnio	17 +1
7	Male	39	Increased NT, advanced maternal age	9 +3	-	-
8	Male	37	Increased NT, advanced maternal age	13 +2	-	-
9	47,XY,+21	34	Ultrasound abnormality	13 +2	CVS	12 +0
10	Male	38	Increased NT, advanced maternal age	11 +0	-	-
11	47,XX+21	41	Advanced maternal age	16 +6	Amnio	16 +1
12	47,XY,+21	41	Advanced maternal age	14 +4	CVS	12 +3
13	47,XX,+21	41	Ultrasound abnormality: Hydrothorax, cystic hygroma	11 +2	CVS	11 +3
14	46,XY	40	Advanced maternal age	11 +5	CVS	11 +5
15	46,XY	39	Advanced maternal age	16 +6	Amnio	16 +6
16	47,XY,+21	38	Ultrasound abnormality	12 +2	CVS	12 +2
17	47,XY,+21	39	Ultrasound abnormality: Hygroma colli	12 +1	CVS	12 +1
18	47,XX,+21	36	Ultrasound abnormality: Hygroma colli	14 +2	CVS	14 +2
19	47,XY,+21	35	Increased NT	12 +4	CVS	12 +4
20	47,XY,+21	34	Ultrasound abnormality: Hygroma colli	13 +1	CVS	13 +1

Table 1: *AD Blood*: Gestational age at the time of blood collection depicted as weeks +days, *CVS*: Chorionic Villus Sampling, *Amnio*: Amniocentesis, *AD Procedure*: Gestational age at the time of the invasive procedure depicted as weeks +days, *ICSI:* Intracytoplasmic Sperm Injection, *Increased NT*: Increased nuchal translucency thickness.

3-900051-07-0, www.R-project.org). Differences between the numbers of uniquely mapped reads between groups were determined by independent samples T-test. Correlation between the number of reads and RSTD were determined by non-parametric Spearman correlation. P values of less than 0.05 are considered statistically significant.

Results

Included samples

A total of 20 maternal plasma samples were included in this study and were taken at a median gestational age of 12 +2 weeks (range 9 +3 to 16 +6 weeks). In 4 out of 20 cases blood samples were drawn after the invasive procedure (on average > 1 week afterwards). No correlation between the time of sampling (before or after the invasive procedure) and the ratios was observed. All details on the included samples are depicted in Table 1. For the noninvasive detection of fetal T21, DNA isolated from 20 maternal plasma samples and 4 anonymous male plasma controls were sequenced on both the Helicos and the Illumina GA II platform. One D21 sample, that did pass the quality controls prior to sequencing, failed the quality controls after sequencing for both platforms. For this sample hardly any reads were obtained for the Helicos platform and sequencing results from the Illumina platform showed preferential amplification of only a few regions. This sample was therefore excluded for further analysis.

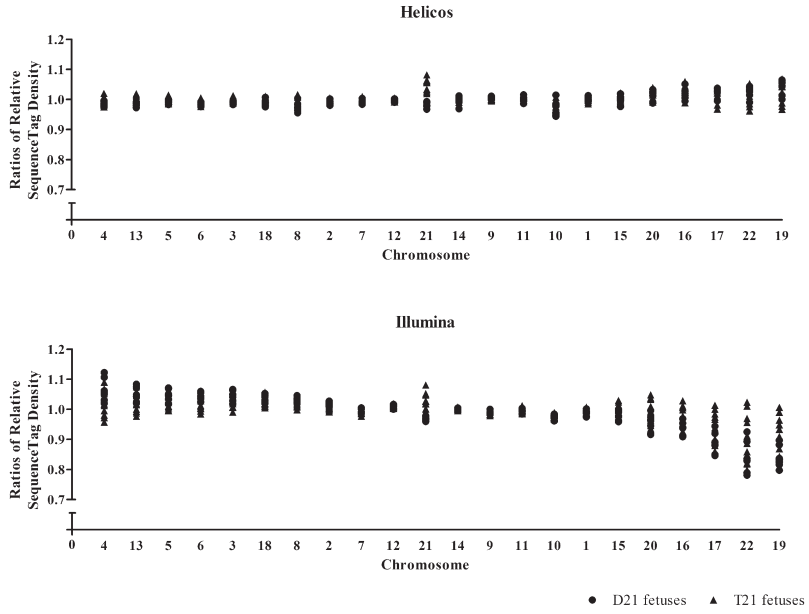


Figure 1: Ratios of normalized relative sequence tag density (RSTD) from all autosomes.
Ratios are calculated against averaged normalized read counts from male plasma controls. Data are shown for each Next Generation Sequencing platform (T21 n=11 and D21 n=8). Chromosomes are ordered by increasing GC content. Upper panel: Helicos, Lower panel: Illumina GA II.

Sample statistics

For each NGS platform, the mean number of raw reads, the percentage of filtered reads and the mean and median number of uniquely mapped reads are depicted in Table 2. For Helicos, our data show one D21 sample with the overall lowest amount of reads, to have the lowest RSTD ratio, but overall we observed no correlation between RSTD ratio and the amount of uniquely mapped reads for both platforms (Helicos, Spearman r = -0.088, 95%CI [-0.532-0.394], P = 0.7210 and Illumina, Spearman r = -0.232, 95% CI [-0.629, 0.263], P = 0.3401). Furthermore, the number of uniquely mapped reads between T21 and D21 was similar (Helicos P = 0.128 and Illumina P = 0.810). When looking at the duplexed Illumina samples (n=16), no bias in read counts was observed towards any specific barcode after splitting (P= 0.9551).

The percentage of cffDNA in maternal plasma was calculated using 2 different methods. When using the method based on Illumina sequence data from chromosome X by the group of Fan *et al*. (Fan *et al.*, 2008), we estimated the percentage of fetal DNA for male pregnancies (n=6) to be on average ~7% (range 1-18%). Concordant results were obtained by Real-Time PCR on the *SRY* gene (average ~9%, range 3-18%).

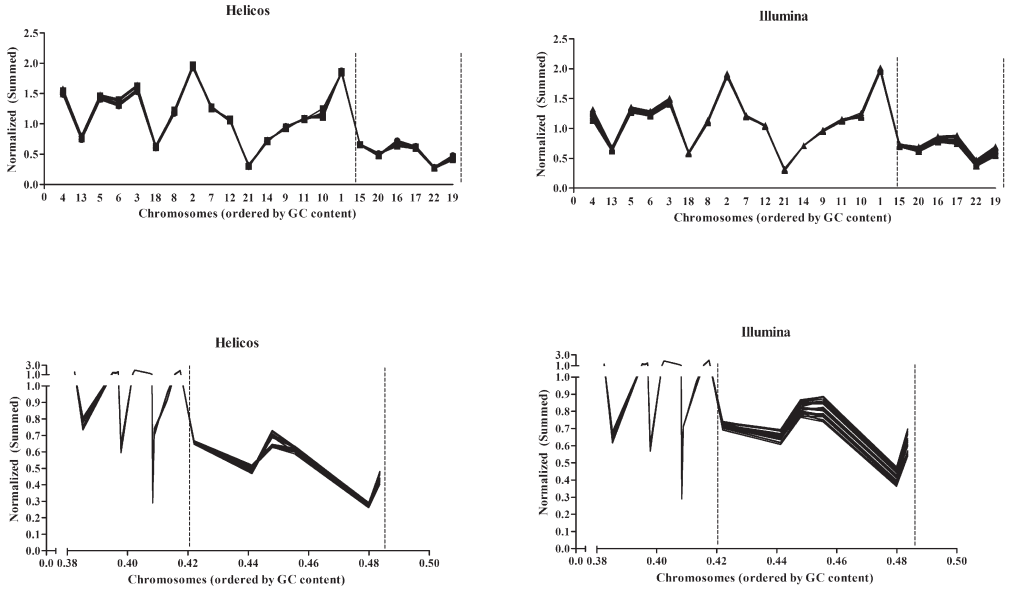


Figure 2: Normalized total number of reads per chromosome against GC content.
Normalized reads are shown in order of GC content per chromosome (upper panel) and by GC percentage (lower panel) for both the Helicos (left) and Illumina (right) platform. Chromosomes subject to possible GC bias because of high GC content are depicted within dashed lines (upper and lower panel).

Noninvasive T21 detection

For the detection of noninvasive fetal T21, RSTD ratios for all 19 maternal plasma samples are shown per chromosome for each NGS platform (Fig. 1). The autosomes were ordered by increasing GC content (Fan *et al.*, 2008). The overall distribution of reads across the genome

is similar between both platforms and seems independent of GC content (data not shown). However, our data show a clear difference in read coverage between platforms. For Helicos, the RSTD ratios for all chromosomes (Fig. 1), the normalized total number of reads per chromosome (Fig. 2) and the average amount of reads per bin (Fig. 3) were quite uniform between samples and virtually independent of GC content of the chromosome, while as reported before (Fan *et al.*, 2008), Illumina results showed increased read density in GC rich areas of the genome (Fig. 1-3).

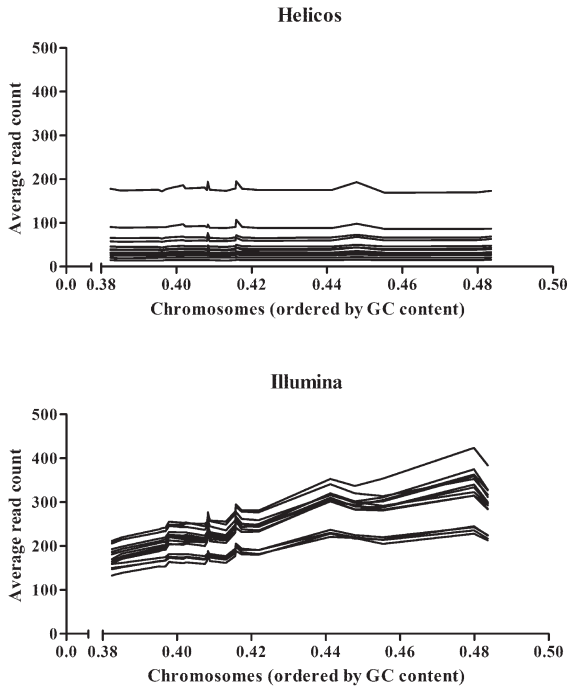


Figure 3. Average read count per 50 kb bin against GC content.
For both platforms, the average number of reads per bin was determined by dividing the summed total number of reads by the number of bins. Chromosomes are ordered by GC content.

Our data show RSTD ratios for T21 samples in a range of 1.04-1.11 for Helicos and a range of 1.03-1.12 for Illumina. For D21 samples we obtained RSTD ratios from 0.98-1.01 and 0.99-1.01 respectively (Fig. 4). Our data show a clear distinction between plasma samples from women carrying a T21 fetus and woman carrying a D21 fetus for both platforms when looking at the overrepresentation of the affected chromosome (Fig. 4). All maternal plasma samples of women carrying a fetus with Down syndrome were correctly classified as T21 (n=11). In addition, all euploid samples (n=8) were correctly identified as D21, resulting in a sensitivity and specificity of both 100% (95% CI [87.0-100]). When constructing a 99% confidence interval of the distribution of RSTD from all D21 samples, all T21 samples lie outside the upper boundary of 1.01 and all D21 samples on or below this boundary. Overall, we show that noninvasive detection of T21 can be performed on both NGS platforms, although Helicos results show a better distinction between T21 and D21 samples (Fig. 4).

Table 2: Overview of mean and median number of uniquely mapped reads[A].

Platform	*Mean # of raw reads*	*% of filtered reads*	*Mean # of alligned reads*	*Median # of alligned reads*
Helicos	1.06×10^7 (0.46×10^7)	35.09 (1.52)	4.65×10^6 (3.6×10^6)	2.65×10^6
Illumina	2.47×10^7 (0.38×10^7)[B]	76.07 (5.14)[B]	1.26×10^7 (0.40×10^7)	1.26×10^7

Table 2: [A] Results are indicated by Next Generation Sequencing Platform. Data for each platform are represented as mean (SD) for n=23 samples. [B]For the Illumina platform, the mean number of raw reads and % filtered reads is depicted for the duplexed samples n=15 (T21 n=7, D21 n=6, male plasma control n=2).

Calculation methods

We have based our calculations on the method of Fan *et al.* (Fan *et al.*, 2008), which uses read counts to calculate ratios of RSTD. Samples can be normalized against averaged normalized RSTD from both adult male plasma controls (Fig. 1) or disomy samples (Fig. 4). Our results look similar when applying either one of these methods to data from both NGS platforms. Recently, a new calculation method for the detection of fetal chromosomal abnormalities was published by the group of Sehnert *et al.* (Sehnert *et al.*, 2011). With this method, samples can be classified as affected (i.e. aneuploid for that chromosome) or unaffected by calculating a normalized chromosome value (NCV) using data from a previously analyzed training set consisting of unaffected samples (i.e. maternal plasma samples from women carrying a euploid fetus). When applying this new calculation method to our Illumina data, all Illumina samples were again correctly classified as either T21 or D21, within the criteria as described (See Supplemental Figure S1) (Sehnert *et al.*, 2011). Since these criteria are only determined for Illumina data, they are not applicable on our Helicos results and thus first need to be established.

a

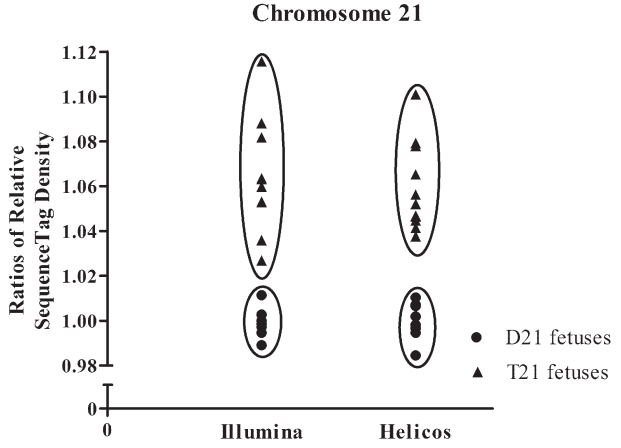


Figure 4. Ratios of normalized relative sequence tag density (RSTD) from chromosome 21.
Ratios are calculated against averaged normalized read counts from disomy samples. Data are shown for each Next Generation Sequencing platform (T21 n = 11 and D21 n = 8).

Discussion

Noninvasive fetal aneuploidy detection using free DNA from maternal plasma has evolved dramatically the past few years with the introduction of NGS. The majority of studies use the Illumina GA II platform for whole genome shotgun detection of T21. Data obtained in these studies have shown that limitations due to low percentages of cffDNA in maternal plasma, no longer seem to be a major problem. However, the Illumina platform is PCR-based and the amplification step could initiate several negative side effects, such as read density bias in GC rich areas of the genome.

In this study, we show successful fetal T21 detection using free DNA from maternal plasma by single molecule sequencing on the Helicos platform and compared it to the Illumina GA II platform (Fan *et al.*, 2008; Chiu *et al.*, 2008; Ehrich *et al.*, 2011; Chiu *et al.*, 2011a). For Illumina, we could confirm previously described findings (Fan *et al.*, 2008). Moreover, we demonstrate a more distinct separation between T21 and D21 samples in Helicos data versus Illumina. We show that as early as 9 +3 wks of gestational age, cffDNA samples from maternal plasma can be classified correctly with high sensitivity and specificity. Because for single molecule sequencing only small amounts of free DNA are required as input for sample preparation and direct sequencing is performed, we hypothesize that this method might therefore be more suitable for early noninvasive aneuploidy detection.

Also, our study confirms that data obtained on the Helicos platform is not biased in GC rich areas, leading to an increased accuracy of analysis. Previously, a strong correlation between GC rich areas and read coverage was observed on the Illumina platform, with increased number of reads in areas containing elevated GC content (Dohm *et al.*, 2008; Hillier *et al.*, 2008; Fan *et al.*, 2008). There has been discussion whether this is a biological effect relating to chromatin structure or originates from PCR artifacts introduced during sample preparation, cluster formation or the sequencing process itself. Since GC bias is not observed in single molecule sequencing it is less likely that this is a true biological effect or be due to the sequencing process. We therefore hypothesize that it is introduced in the pre-amplification step for DNA enrichment or during local amplification for cluster formation on the flow cell. The exact reason, however, remains to be elucidated.

Before implementing noninvasive trisomy detection into routine diagnostics several quality controls criteria need to be determined and validated. The QUADAS criteria can be applied, which take into account the experimental bias and variation (Whiting *et al.*, 2003). Equally important are the quality controls before and during sample preparation. Since the percentage of cell free DNA in maternal plasma differs between samples and at different times of gestation (Lo *et al.*, 1998; Lun *et al.*, 2008a), it is difficult to determine the most appropriate time of gestation for testing. However, for diagnostics inclusion criteria including time of gestation need to be determined. Measurement of the amount of cffDNA and its correlation to reliable diagnosis and time of gestation needs to be studied more thoroughly in large validation studies. Before sequencing isolated free DNA, combined Real-Time PCR results on *CCR5* and *SRY* could help estimate the ratio of maternal and fetal DNA in maternal plasma, the percentage of fetal DNA and the quality of DNA as shown in our data. After sequencing, percentages of cffDNA can then be verified by using data from chromosome X as described previously (Fan *et al.*, 2008). Both methods however are limited to male pregnancies only. When encountering samples containing low percentages of fetal sequences or large amounts of contaminating maternal sequences, restrictions for the detection limit should be taken into account. For female pregnancies a sex-and polymorphism-independent method based on epi-

genetic differences could be used for quantification (Nygren *et al.*, 2010), although it needs to be established whether differences in methylation are stable and comparable between individuals to be used reliably in diagnostics.

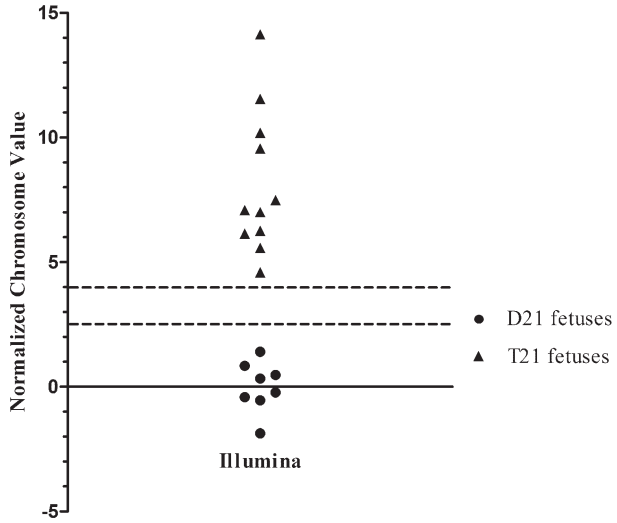
Another issue that should be taken into account are maternal copy number variations (CNVs). These can be of particular interest for the interpretation of trisomy detection using NGS data in diagnostics. Pre-determination of CNVs in the maternal genome could be an useful control in diagnostics, because these findings may influence the interpretation of data when looking at the overrepresentation of a specific chromosome, regardless the NGS platform used.

In summary, this study shows for the first time that single molecule sequencing can be a reliable and easy-to-use alternative for noninvasive T21 detection in diagnostics. By using single molecule sequencing, previously described experimental noise associated with PCR amplification, such as GC bias, can be overcome. This method is therefore not only promising for noninvasive T21 detection, but is potentially also useful for the detection of other aneuploidies.

Acknowledgements

We would like to thank Jennie Verdoes for including pregnant women, Michiel van Galen for bioinformatics, Yavuz Ariyurek and Henk Buermans for technical support and BIOKÉ (The Netherlands) for NextGENe software assistance.

Supplemental data



Supplemental figure S1. Normalized Chromosome Value for Chromosome 21.
Normalized Chromosome Value (NCV) for chromosome 21 was calculated according to the method described in Sehnert *et al*. (Sehnert *et al.,* 2011). An NCV > 4.0 was used to classify the sample as aneuploid for chromosome 21 and an NCV < 2.5 to classify a chromosome as unaffected. Samples with an NCV between 2.5 and 4.0 were classified as "no call" (dashed lines).

Chapter 4

Successful Noninvasive Trisomy 18 Detection Using Single Molecule Sequencing

Chapter 4: Successful Noninvasive Trisomy 18 Detection Using Single Molecule Sequencing

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Abstract

Background: Noninvasive trisomy 21 detection using massively parallel sequencing is achievable with high diagnostic sensitivity and low false positive rates. Detection of fetal trisomy 18 and 13 has been reported as well, but seems to be less accurate using this approach. Reduced accuracy can be explained by PCR introduced guanine-cytosine (GC) bias influencing sequencing data. Previously, we demonstrated that sequence data generated by single molecule sequencing show virtually no GC bias and result in a more pronounced noninvasive detection of fetal trisomy 21. In this study, single molecule sequencing was used for noninvasive detection of trisomy 18 and 13.

Methods: Single molecule sequencing was performed on the Helicos platform using free DNA isolated from maternal plasma from 11 weeks of gestation onwards (n=17). Relative sequence tag density ratios were calculated against male control plasma samples and results were compared to those of previous karyotyping.

Results: All trisomy 18 fetuses were identified correctly with a diagnostic sensitivity and specificity of 100%. However, low diagnostic sensitivity and specificity was observed for fetal trisomy 13 detection.

Conclusions: We successfully applied single molecule sequencing in combination with relative sequence tag density calculations for noninvasive trisomy 18 detection using free DNA from maternal plasma. However, noninvasive trisomy 13 detection was not accurate and seemed to be influenced by more than just GC content.

Recent large studies have confirmed that noninvasive prenatal diagnosis (NIPD) for fetal aneuploidies is achievable (Fan *et al.*, 2008; Chiu *et al.*, 2008; Chiu *et al.*, 2011a; Ehrich *et al.*, 2011; Palomaki *et al.*, 2011; Lau *et al.*, 2012a; Bianchi *et al.*, 2012). Using massively parallel sequencing (MPS) and subsequent quantification of chromosome specific sequences, overrepresentation of a specific chromosome can be determined with high diagnostic accuracy. Successful detection of fetal trisomy 21 (T21) in maternal plasma was shown in several clinical validation studies (Chiu *et al.*, 2011a; Palomaki *et al.*, 2011; Ehrich *et al.*, 2011; Lau *et al.*, 2012a; Bianchi *et al.*, 2012). For noninvasive detection of trisomy 18 (Edwards Syndrome, T18) and trisomy 13 (Patau Syndrome, T13), however, it seems to be more difficult to achieve similar results (Chen *et al.*, 2011; Lau *et al.*, 2012a; Palomaki *et al.*, 2012; Bianchi *et al.*, 2012). Although theoretically molecules from different regions of a genome should be sequenced uniformly by MPS, preferential amplification of sequences, depending on different guanine-cytosine (GC) content, has been observed (Dohm *et al.*, 2008; Fan *et al.*, 2008; van den Oever *et al.*, 2012). In contrast to an average GC content of chromosome 21, chromosomes 13 and 18 have a relatively low GC content (Fan *et al.*, 2008; van den Oever *et al.*, 2012). Therefore, non-uniform amplification of these chromosomes could occur on PCR based MPS platforms. As a result, several studies have used specific algorithms or internal references to correct for GC content to optimize noninvasive detection rates for T18 and T13 (Chen *et al.*, 2011; Palomaki *et al.*, 2012; Sparks *et al.*, 2012a; Lau *et al.*, 2012b).

We previously demonstrated that sequence data generated by single molecule sequencing show virtually no GC bias (van den Oever *et al.*, 2012). This specific method of sequencing requires no PCR amplification step during sample preparation or during flow cell processing and results in a more pronounced noninvasive detection of T21. Therefore, this approach could also be applicable for the detection of other common fetal aneuploidies such as T18 and T13.

To test this hypothesis, a retrospective study was performed on first and second trimester pregnant women with an increased risk for fetal aneuploidy based on previous serum screening and/or ultrasound results. Maternal peripheral blood samples were collected in EDTA coated tubes and processed within 24 hrs after collection. All blood samples were drawn at a median gestational age of 12 weeks + 6 days (range 11w +4d to 22w +1d, see Table 1) prior to an invasive procedure, except for one sample, which was obtained 6 days after amniocentesis. Plasma was obtained by double centrifugation of the blood samples and stored at -80^{o}C until further processing. Material from all invasive procedures was sent to our cytogenetics laboratory for karyotyping as the gold standard.

A total of 21 plasma samples were used in this study. Four plasma control samples from anonymous male blood donors and 17 samples of singleton pregnancies (Table 1), consisting of 9 cases of T18 (2 female and 7 male fetuses), 4 cases of T13 (2 female and 2 male fetuses), and 4 euploid pregnancies (all male fetuses) were included. All maternal blood samples were processed within 24 hrs after collection. Cell-free DNA was isolated from plasma using the EZ1 Virus Mini Kit v2.0. For quality control purposes, fetal sex and the total amount of free DNA in maternal plasma were determined by Real-Time Taqman PCR assays as described previously (Boon *et al.*, 2007; van den Oever *et al.*, 2012). In addition, using this data, the percentage of cell-free fetal DNA (cffDNA) for male pregnancies was estimated (van den Oever *et al.*, 2012). All samples were de-identified to the investigators before sample preparation and data analysis. Libraries were prepared according to manufacturer's ChipSeq protocol and a standard 120-cycle sequencing run was performed on the Helicos platform (Helicos BioSciences, www.helicosbio.com).

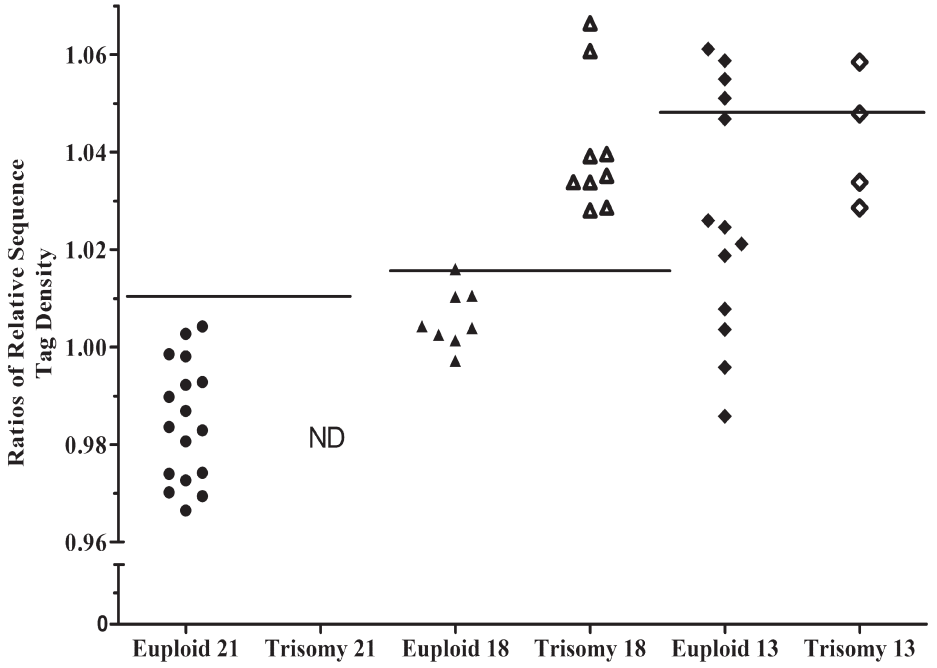

Figure 1. Ratios of normalized Relative Sequence Tag Density for noninvasive fetal aneuploidy detection.
Ratios were calculated against anonymous male plasma controls (n=4). Samples are divided in either disomic (closed symbols) or trisomic (open symbols) for that specific chromosome. Chromosome 21 is displayed as circles, chromosome 18 as triangles and chromosome 13 as diamonds. 99% Confidence intervals for disomic samples were calculated for each chromosome and upper boundaries are depicted in the graph as a line. Euploid fetuses (n=4), T18 fetuses (n=9), T13 fetuses (n=4). ND: Not determined.

Raw data analysis was performed with the HeliSphere software package. Ratio calculations and statistics were executed as described previously (van den Oever *et al.*, 2012). In short, for fetal trisomy detection, ratios of relative sequence tag density (RSTD) were calculated by dividing the normalized total summed number of reads for each sample by the normalized mean of male plasma controls for each chromosome of interest. After alignment against hg19 reference genome and filtering of gaps and repeats a mean of $1.21x10^6 + 0.69x10^6$ (SD) reads, with a median of $1.12x10^6$, were obtained per sample. In 11 maternal plasma samples from women carrying a male fetus, the percentage of cffDNA was estimated, resulting in a mean percentage of 11 % (Table 1).

For noninvasive T18 detection we showed that using RSTD calculations for chromosome 18, all T18 samples (n=9) were correctly identified as being aneuploid and all euploid controls and T13 samples as being disomic for chromosome 18. When constructing a 99% confidence interval from all samples disomic for chromosome 18 (n=8), all T18 samples were outside the upper boundary of the 99% CI [0.991, 1.016], while all euploid controls and T13 samples were on or below this upper boundary (Fig. 1). For noninvasive T13 detection, only 1 out of 4 T13 samples was correctly identified. False positive results (4/13) were observed in both euploid (n=2) and T18 (n=2) samples when using RSTD ratio and 99% CI calculations for chromosome

13, resulting in a diagnostic sensitivity and specificity of 25% and 69% respectively (Fig. 1). As a control we calculated RSTD ratios for chromosome 21 for all samples tested in this study (n=17) using the 99% CI previously published (VAN DEN OEVER *et al.*, 2012). All samples tested in this study were indeed identified as disomic for chromosome 21 (Fig 1). When calculating a 99% CI using RSTD results from this study a similar upper boundary was obtained, thus confirming this result.

As a follow up on noninvasive T21 detection using single molecule sequencing, in the present study we demonstrated successful noninvasive detection of T18 (100% diagnostic sensitivity and specificity) using free DNA from maternal plasma from 11w + 4d of gestation onwards. The mean percentage of cffDNA in maternal plasma in the first trimester was 4.03% and we observed an increase in fetal fraction during the second trimester, with a mean percentage of 21.1%. This observation is concordant with previous reports (LUN *et al.*, 2008a; LO *et al.*, 1998). Even though the percentage increased, we still observed quite a large range between individuals with an approximate 4-fold change for the second trimester pregnancies, up to a 13-fold difference between first trimester samples.

Compared to noninvasive detection of T18, our data showed low diagnostic sensitivity and specificity for detection of T13 using single molecule sequencing. Previous publications from other groups also reported reduced diagnostic sensitivity and/or specificity for noninvasive T13 detection (CHEN *et al.*, 2011; LAU *et al.*, 2012b; PALOMAKI *et al.*, 2012; BIANCHI *et al.*, 2012). However, the values were not as low as observed in this study. Furthermore, in these cases it was thought to be related to the GC content of chromosome 13 given that PCR based Next Generation Sequencing (NGS) platforms were used. As shown in our previous study, data for chromosome 13 are biased on such platforms (VAN DEN OEVER *et al.*, 2012). Chromosome 13, compared to 18 and 21, has the lowest GC content of all three (38.5%) (DUNHAM *et al.*, 2004). This low GC content could be reason for a misrepresentation of the amount of sequencing reads coming from these PCR based NGS platforms. However, in the current study, single molecule sequencing results were not influenced by a chromosome's GC content, implying that other factors might be involved in lowering the diagnostic sensitivity and specificity for noninvasive trisomy 13 detection.

The fetal contribution of free DNA in maternal plasma is derived from syncytiotrophoblasts undergoing apoptosis (ALBERRY *et al.*, 2007). Placental apoptosis is a naturally occurring process during gestation in both normal and abnormal pregnancies, resulting in fragmented fetal DNA circulating in the maternal circulation (HEAZELL *et al.*, 2008; ALBERRY *et al.*, 2007). Some studies have demonstrated the difference in size between fetal and maternal free DNA fragments and have even shown that the entire fetal genome is present (LO *et al.*, 2010). However, virtually no studies have considered that fetal DNA from chromosomes of different sizes and/or those of differing GC contents may fragment at different rates. Considering that chromosome 13 is the largest acrocentric chromosome with the lowest gene density among all human chromosomes (DUNHAM *et al.*, 2004), its stability may differ from chromosome 18 and 21. A less stable chromosome is hypothesized to degrade faster, which could lead to a skewed number of DNA fragments from this particular chromosome in maternal plasma. Also, several segmental duplications with at least 90% homology and regions with a high SNP density due to the presence of paralogous sequence variants have been shown for chromosome 13 (DUNHAM *et al.*, 2004). This may influence data analysis, resulting in improper assignment of reads to a certain chromosome during alignment. Which factors exactly play a role is not clear at this point and needs to be studied in more detail; however, our study suggests that sequencing

Table 1. Overview of included maternal plasma samples.

Sample number	*Karyotype*	*Maternal age, years*	*Indication*	*GA Blood*	*CVS/ Amnio*	*SRY (pg/ 10 µL)*
1	46,XY	28	Ultrasound abnormality	12 w + 1d	CVS	2
2	46,XY	33	Ultrasound abnormality	12 w + 1d	CVS	4
3	46,XY	30	Increased NT/ serum screening	12 w + 6d	CVS	11
4	46,XY	30	Ultrasound abnormality	13 w + 5d	CVS	10
5	47,XY,+18	44	Ultrasound abnormality	11 w + 4d	CVS	5
6	47,XY,+18	44	Ultrasound abnormality	11 w + 6d	CVS	5
7*	47,XY,+18	33	Ultrasound abnormality	22 w + 1d	Amnio	5
8	47,XY,+18	39	Ultrasound abnormality	13 w + 1d	CVS	9
9	47,XY,+18	42	Ultrasound abnormality	11 w + 6d	CVS	ND
10	47,XY,+18	39	Ultrasound abnormality	21 w + 3d	Amnio	6
11	47,XY,+18	38	Increased NT/ serum screening	12 w + 6d	CVS	14
12	47,XX,+18	36	Ultrasound abnormality	13 w + 3d	CVS	ND
13	47,XX,+18	41	Ultrasound abnormality	13 w + 4d	CVS	ND
14	47,XX,+13	37	Advanced maternal age	11 w + 4d	CVS	ND
15	47,XX,+13	35	Ultrasound abnormality	15 w +2d	Amnio	ND
16	47,XY,+13	37	Ultrasound abnormality	12 w + 3d	CVS	4
17	47,XY,+13	29	Ultrasound abnormality	12 w + 3d	CVS	3

Table 1: GA blood, gestational age at the time of blood collection depicted as weeks + days; amnio, amniocentesis; CVS, chorionic villus sampling; NT, nuchal translucency measurement; SRY, fetal DNA concentrations of the SRY gene were determined by quantitative real-time Taqman PCR. *This blood sample was obtained 6 days after amniocentesis. Other blood samples were obtained prior to the invasive procedure.

data are influenced by more than just GC content alone. Data analysis for noninvasive fetal trisomy 13 detection may therefore require a different approach.

In summary, we demonstrate successful noninvasive T18 detection using a combination of single molecule sequencing and relative sequence tag density ratio calculations, while non-invasive T13 detection is not accurate using this approach.

Acknowledgements:

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Chapter 5

A novel targeted approach for noninvasive detection of paternally inherited mutations in maternal plasma

Chapter 5: A novel targeted approach for noninvasive detection of paternally inherited mutations in maternal plasma

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Abstract

The challenge in noninvasive prenatal diagnosis (NIPD) for monogenic disorders lies in the detection of low levels of fetal variants in the excess of maternal cell-free plasma DNA. Next Generation Sequencing (NGS), which is the main method used for noninvasive prenatal testing and diagnosis, can overcome this challenge. However this method may not be accessible to all genetic laboratories. Moreover, shotgun NGS as for instance currently applied for noninvasive fetal trisomy screening may not be suitable for the detection of inherited mutations. We have developed a sensitive, mutation specific and fast alternative for NGS-mediated NIPD using PCR methodology. For this proof of principle study, noninvasive fetal paternally inherited mutation detection was performed using cell-free DNA from maternal plasma. Preferential amplification of the paternally inherited allele was accomplished through a personalized approach using a blocking probe against maternal sequences in a high resolution melting curve analysis (HR-MCA) based assay. Enhanced detection of the fetal paternally inherited mutation was obtained for both an autosomal dominant and a recessive monogenic disorder by blocking the amplification of maternal sequences in maternal plasma.

Introduction

Since the successful introduction of noninvasive prenatal testing for fetal trisomy screening, there has also been a growing request to expand the repertoire for noninvasive prenatal diagnostics (NIPD). NIPD can be performed on small fragments of cell-free fetal DNA (cffDNA) that are present in maternal plasma (Lo *et al.*, 1997). On average, from about 7-9 weeks in gestation the amount of cffDNA is sufficient to be detected noninvasively in maternal plasma (Hill *et al.*, 2010). Current clinical application of NIPD include fetal sex determination, fetal Rhesus D (*RhD*) determination and the diagnosis of several monogenic disorders. For the latter, NIPD can be applied in both autosomal dominant and recessive cases, most efficiently when the mother does not carry the mutant allele and/or carries a different mutation compared to the father respectively (Daley *et al.*, 2014; Bustamante-Aragones *et al.*, 2012).

One of the biggest challenges of noninvasive detection of paternally inherited sequences in the fetus, is the excess of maternal cell-free DNA (cfDNA) in plasma. Here a parallel can be drawn towards cancer genetics, which faces similar challenges in the need to detect mosaic or low level somatic mutations in the presence of excess wild-type sequences (Oh *et al.*, 2010). Deep sequencing approaches using various Next Generation Sequencing (NGS) platforms can be used to overcome these challenges for both NIPD and cancer genetics (e.g. targeted NGS approaches for both cancer detection and therapy) (Chang *et al.*, 2013; Hagemann *et al.*, 2013; Lo *et al.*, 2010). Even though the application of NGS for both these purposes is expanding, currently implementation and proper validation of novel applications for NGS in diagnostics is still quite expensive, especially when this method is applied for the detection of merely 1 or 2 variants. Moreover, NGS may be less suitable for the detection of variants in certain regions of the genome, such as GC rich regions and repeat areas and may therefore not be the most eligible method of choice for mutation detection. Therefore this study is aimed to develop an alternative noninvasive paternal mutation detection method that does not require NGS. Such an alternative needs to be accessible for genetic diagnostic laboratories and needs to be sensitive enough to detect the low levels of fetal sequences in maternal plasma.

High-resolution melting curve analysis (HR-MCA) is a relatively simple, fast and low-cost technique for genotyping and mutation scanning and is frequently used in routine molecular and cancer diagnostics (Montgomery *et al.*, 2007; Oh *et al.*, 2010; Almomani *et al.*, 2009; van der Stoep *et al.*, 2009). It combines (asymmetric) PCR with a short post-PCR melting step to detect sequence variations using a saturating double-stranded DNA binding dye (Montgomery *et al.*, 2007). Although HR-MCA is a relatively sensitive technique, the detection of mosaic or low level mutations may still be challenging and variant dependent (Out *et al.*, 2015). Therefore, variations in traditional HR-MCA methods have been developed to overcome this challenge (Chou *et al.*, 2005; Oh *et al.*, 2010; Laughlin *et al.*, 2010; Warshawsky *et al.*, 2011; Macher *et al.*, 2012). The majority of these studies describe the use of either peptide nucleic acid (PNA) or locked nucleic acid (LNA) probes. Addition of such probes to the PCR reaction results in clamping or blocking specific undesired PCR products by inhibiting amplification (Chou *et al.*, 2005; Oh *et al.*, 2010; Laughlin *et al.*, 2010; Warshawsky *et al.*, 2011). LNA is a bicyclic high affinity nucleic acid analogue that contains a ribonucleoside link between the 2'-oxygen and the 4'-carbon atoms with a methylene unit (2'-O,4'-C-methylene bridge) (Mouritzen *et al.*, 2003; Warshawsky *et al.*, 2011). The thermal stability, binding capacity and affinity of LNA to complementary DNA increases substantially with each LNA base incorporated, resulting in suppressed amplification of these complementary sequences (Mouritzen *et al.*, 2003; Warshawsky *et al.*, 2011). More importantly, in case of a mismatch, the LNA probe does not bind

to the template with high affinity, enabling primer extension and preferential amplification of the allele of interest. This principle of allele specific blocking could be of use in NIPD to obtain preferential amplification of the paternally inherited allele through targeted blocking of the maternal allele. By first determining both parental genotypes, target specific LNA probes against maternal sequences could be designed, enabling preferential amplification and specific detection of the paternally inherited mutation in maternal plasma.

In this proof of principle study we describe a fast and sensitive alternative for NGS-mediated NIPD using a PCR-based methodology. We have explored the use of HR-MCA in combination with target specific blocking LNA probes to obtain allele specific blocking of maternal sequences for the enhanced detection of the fetal paternally inherited allele in maternal plasma. We show that this novel approach for NIPD can be applied in both an autosomal dominant and recessive monogenic disorder.

Methods

Patients

Two couples who opted for prenatal diagnosis visited the department of Clinical Genetics. Both mothers underwent an invasive procedure (chorionic villus sampling (CVS)) for prenatal diagnosis to determine fetal genotype for a familial mutation. In case 1, the father was a carrier of a pathogenic *BRCA2* mutation (c.5682C>G, p.Tyr1894*). In case 2, both parents were carriers of a different heterozygous mutation in the *HBB* gene. The mother was heterozygous for the HbC mutation (c.19G>A, p.Glu7Lys) and the father was heterozygous for the HbS mutation (c.20A>T, p.Glu7Val). A previous child was also shown to be a carrier of the HbS mutation. Maternal blood withdrawal was performed at 10+6 and 11+1 weeks of gestation for case 1 and 2 respectively after informed consent was obtained.

Sample processing

Maternal (n=2) and paternal (n=1) plasma (input 800 µL) was isolated, processed and measured as previously described (van den Oever *et al.*, 2012). Isolated plasma DNA was concentrated to 20 µL using the Zymo Clean & Concentrator™ -5 kit (Zymo Research, Irvine, USA). As a control, the total amount of cell-free DNA (fetal + maternal) was determined by Real Time PCR detection of *CCR5* as previously described (Boon *et al.*, 2007). A total concentration of 112 pg/µL and 350 pg/µL was obtained for the *BRCA2* and *HBB* case respectively. Genomic DNA (gDNA) from all parents was isolated from peripheral blood cells using automated isolation (QIAGEN, Venlo, the Netherlands). Fetal gDNA was isolated from CVS on the QIAcube according to manufacturer's instructions (QIAGEN, Venlo, the Netherlands).

Control samples

Several positive and negative control samples (gDNA and freshly isolated anonymized wild type (WT) plasma DNA) were used to optimize the assay. All control samples were isolated similarly to the parental DNA samples. For *BRCA2* a total of n= 20 control samples were analyzed: anonymized WT plasma DNA (n=12), WT gDNA (n=6) and gDNA from individuals heterozygous for the *BRCA2* mutation (n=4). For *HBB* a total of n=23 control samples were analyzed: anonymized WT plasma DNA (n=12), WT gDNA (n=4), gDNA heterozygous for HbC

(n=2), gDNA heterozygous for HbS (n=2), gDNA homozygous for HbC (n=1), gDNA homozygous for HbS (n=1) and gDNA from an individual compound heterozygous for HbC/HbS (n=1).

Assay design

PCR for HR-MCA was performed using target specific primers and a mutation specific unlabeled detection probe (from now on referred to as "mutation detection probe") with a 3' C3-spacer (Biolegio, Nijmegen, the Netherlands) and was executed both with and without the addition of a target specific blocking LNA probe (from now on referred to as "target blocking probe") (Exiqon, Vedbaek, Denmark) that binds to the WT or mutant maternal allele. Primer/ probe design was based on parental Sanger sequencing results of the region of interest (Table 1). Amplicons of 117 bp and 115 bp were designed for the detection of the familial *BRCA2* and *HBB* mutations respectively using LightScanner Primer Design (Idaho Tech/ BioFire Diagnostics, Salt Lake City, USA). In both cases, mutation detection probes were designed against the forward strand. Target blocking probes were designed against maternal templates in the same region as the mutation detection probes and were directed to the reverse strand.

PCR and HR-MCA

PCR and HR-MCA without target blocking probe were performed as previously described (van der Stoep *et al.*, 2009; Almomani *et al.*, 2009). In short, asymmetric PCR (to preferentially amplify the forward strand) was performed in 96-well non-transparent plates (Framestar, 4titude, Surray, United Kingdom) in a total reaction volume of 10 µL containing 1x LightScanner Master mix (Idaho Tech/ BioFire Defense), 5 pmol forward primer, 1 pmol reverse primer, 5 pmol mutation detection probe and 2 ng gDNA template. Primer specific optimal annealing temperature (Ta) for both primer sets was determined using a PCR gradient (58-64°C). Asymmetric PCR was performed with a reverse primer, forward primer and mutation detection probe ratio at 1:5:5 respectively. All samples were tested in duplicate. A range from 50 to 98°C was used for HR-MCA melting. Melt temperature (Tm) of normalized melting peaks was determined using the unlabeled probe genotyping analysis tool of the LightScanner software (Idaho Tech/ BioFire Diagnostics, Salt Lake City, USA). Target blocking probe was titrated into each reaction in a mutation detection probe to target blocking probe ratio from 1:1 to 1:10 (i.e. 5-50 pmol/reaction) and optimized for each set. Cycling protocol for testing the target blocking probe was 95°C for 5 min, 50 cycles of 10 s at 95°C, 20 s at 72°C and 30 s at the primer specific Ta of 58°C or 63°C for *BRCA2* and *HBB* respectively to obtain target blocking probe binding prior to amplification (modified from Oh *et al.*) (Oh *et al.*, 2010).

Determining the detection limit of the assay

As a control, the detection limit of the assay was determined using a mix of paternal gDNA (mutation carrier (MUT)) heterozygous for the familial mutation and maternal gDNA for each case, mimicking an artificial pregnancy (with the paternal gDNA representing the fetus). A relative serial dilution range from 33% to 1% paternal gDNA mixed into maternal gDNA was created using a total amount of ~425 pg mixed gDNA (maternal and paternal) per reaction. Both parental samples were also tested separately (100% paternal or 100% maternal gDNA). All samples were tested in duplicate using the optimal ratio of mutation detection probe to target blocking probe of 1:5 and 1:2 for *BRCA2* and *HBB* respectively in each PCR reaction.

Conditions for testing maternal plasma samples

Maternal plasma samples were tested together with corresponding parental gDNA, CVS gDNA and several positive and negative controls (see Control samples) using the cycling protocol for target blocking probes. When testing plasma samples total reaction volume was increased 1.5x enabling an input of 7.5 µL of concentrated plasma DNA template per reaction. Plasma samples were tested at least in duplicate. Total gDNA input per reaction for control samples was 2 ng. Results were confirmed in at least 2 independent tests.

Results

Optimization of HR-MCA

To optimize parameters for HR-MCA mutation scanning using a mutation detection probe, DNA samples from all parents and several controls with known genotypes (anonymized plasma DNA and gDNA) were utilized. With the optimal Ta for the primers determined (i.e. 58°C or 63°C for *BRCA2* and *HBB* respectively), the target blocking probes specific for the maternal allele(s) were tested subsequently, together with the mutation detection probe. The selected PCR conditions used for testing the target (WT) specific blocking LNA probes enabled binding of the target blocking probe to unwanted target sequences prior to primer extension (Fig. 1). To determine optimal concentrations, target blocking probe was titrated into each PCR reaction, resulting in optimal ratios of mutation detection probe to WT target blocking probe of 1:5 and 1:2 for *BRCA2* and *HBB* respectively.

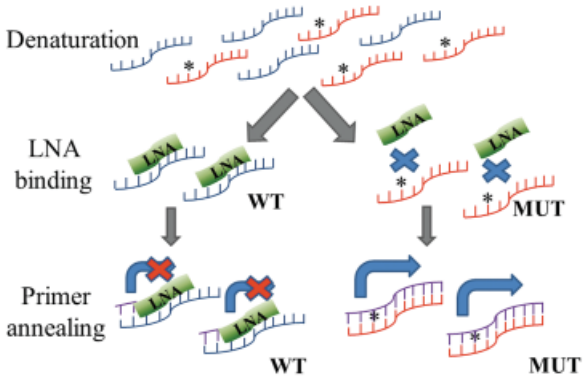

Figure 1: Principle of target blocking LNA probe binding in HR-MCA.
Target blocking probes designed against maternal wild type (WT) sequences are able to bind denatured single stranded WT sequences. No primer extension and amplification can occur (blue arrow, red cross). In case of a mutation (*) target blocking probes will not bind to paternal mutant (MUT) sequences (blue cross), enabling primer extension and amplification.

Next, we determined the detection limit of this assay. For each case, paternal (MUT) gDNA was mixed into maternal gDNA mimicking an artificial pregnancy using amounts of gDNA resembling the quantities of cfDNA found in maternal plasma early in gestation. Without the addition of a target blocking probe, a dilution effect is observed in the detection signal

of the MUT allele, while the detection signal of the WT specific melting peak was increased because of the high background of WT sequences (Fig. 2A). Without blocking, the mutant allele could no longer be detected in a relative paternal gDNA percentage of ~16% and lower (i.e. ~10 genome equivalents (GE), based on a conversion factor of 6.6 pg of DNA per cell). However, addition of a target blocking probe directed against maternal sequences resulted in preferential amplification and enhanced detection of the paternal mutation at ~1% - 2% paternal gDNA (i.e. ~0.5-2 GE) in a background of maternal sequences.

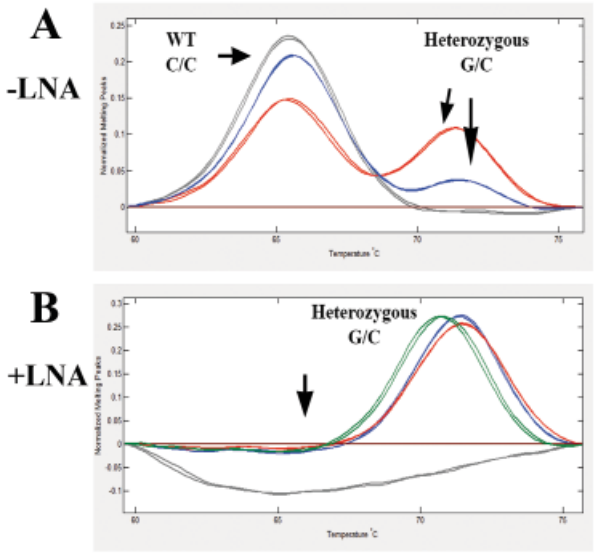

Figure 2: HR-MCA results using the *BRCA2* mutation detection probe.
Panel A: Without target blocking LNA probe: Gray: Wild type (WT) plasma DNA; Red: heterozygous paternal gDNA; Blue: control paternal gDNA (25%) diluted in WT maternal gDNA. Panel B: With target blocking LNA probe: WT signal is blocked (arrow). Gray: maternal WT gDNA; Red: heterozygous CVS gDNA; Blue: heterozygous paternal gDNA; Green: maternal plasma.

Testing maternal plasma samples

Paternal mutation detection was performed on total cfDNA from maternal plasma using a mutation detection probe and target blocking probe(s) for selective blocking of maternal template amplification during PCR amplification.

For case 1, results from WT plasma DNA show that with the use of only the *BRCA2* mutation detection probe, one WT specific normalized melting peak is present in HR-MCA, as expected in a WT individual, with a Tm calling at around 66°C (Fig. 2A, gray line). Paternal gDNA shows two melting peaks, with a Tm calling at 66°C for the WT and 72° for the *BRCA2* MUT specific peak respectively, as expected for an individual heterozygous for this mutation (Fig. 2A, red line). Similar results were obtained for paternal plasma (data not shown). As a control we mixed heterozygous paternal gDNA with maternal WT gDNA (25% paternal gDNA in 100% maternal gDNA). As expected, without the use of a target blocking probe a dilution effect of the MUT specific melting peak was observed (Fig. 2A, blue line). The detection signals were skewed towards detection of the WT specific melting peak as also previously observed in the aforementioned serial dilution range of mixed parental gDNA. To improve paternal mutation detection, we used a WT target blocking probe together with the *BRCA2* mutation de-

tection probe, resulting in inhibition of amplification of the WT *BRCA2* allele during PCR (Fig. 2B, arrow). As a result, the *BRCA2* mutation detection probe can no longer detect a WT PCR product in HR-MCA as shown for WT maternal gDNA (Fig. 2B, gray line). In CVS (red line) and paternal (blue line) gDNA samples heterozygous for the *BRCA2* mutation, only the *BRCA2* MUT specific normalized melting peak could be detected (Fig. 2B). In the maternal plasma sample, the paternally inherited mutation in the fetus could only be detected when the maternal WT template was blocked, showing that for this mutation, the addition of a single target specific blocking LNA probe is sufficient to enhance the detection of the paternally inherited *BRCA2* mutation (Fig.2B, green line).

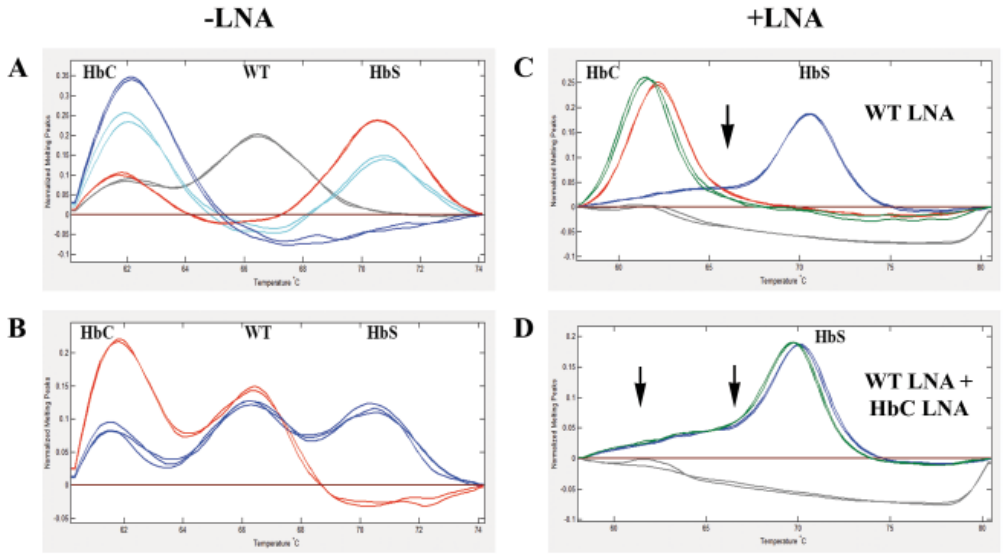

Figure 3: Representation of the HR-MCA melting peak patterns for *HBB* from controls, parents and fetus using the HbS mutation detection probe.

Panel A: Selection of the positive and negative controls scanned for optimization of settings for HR-MCA tested without a target blocking LNA probe. Gray: Wild type (WT); Dark blue: control homozygous for HbS; Red: control homozygous for HbC; Light blue: control compound heterozygous for HbS/HbC. Panel B: Melting peak patterns of maternal (red; heterozygous for HbC), paternal and CVS gDNA (blue; both heterozygous for HbS), without addition of a target blocking LNA probe to the PCR reaction. Panel C: Samples tested with WT target blocking LNA probe directed to block only the maternal WT sequences (arrow). Gray: WT gDNA; Blue: paternal gDNA heterozygous for HbS; Red: maternal gDNA heterozygous for HbC; Green: maternal plasma. Panel D: Additional blocking with an HbC target blocking LNA probe directed to the maternal HbC allele together with a WT blocking LNA probe (arrows). Gray: maternal gDNA heterozygous for HbC; Blue: paternal gDNA heterozygous for HbS; Green: maternal plasma.

For case 2, the situation is more challenging. In this case both parents carry a different mutation in the *HBB* gene. These mutations even affect the same codon and the position of the mutations is only 1 bp apart. As a result, the template region covered by the paternal HbS mutation detection probe, also covers the adjacent maternal HbC mutation. When using only this HbS mutation detection probe (Fig. 3A and 3B, without LNA), results from parental, CVS and control gDNA samples show a specific normalized melting peak pattern in the HR-MCA assay for all 3 different alleles (HbC, WT and HbS with Tm calling at 62, 66 and 70°C respectively

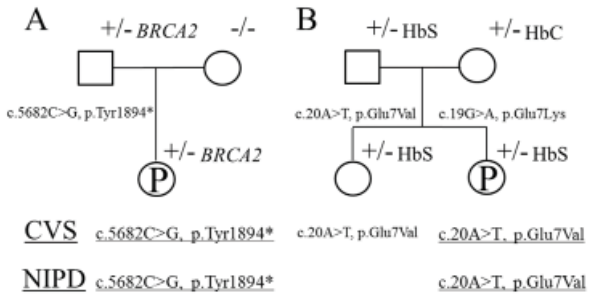

Figure 4: Family pedigrees from participating couples.
Panel A: Case 1: Both father and fetus are heterozygous for BRCA2 mutation c.5682C>G, p. Tyr1894*. Panel B: Case 2: Both parents are heterozygous for a different mutation in the HBB gene. Mother is heterozygous for c.19G>A, p.Glu-7Lys (HbC), while father, daughter and the fetus are heterozygous for c.20A>T, p.Glu7Val (HbS).

(Fig. 3A and 3B). As expected, maternal gDNA shows a melting peak for the HbC and WT allele, since mother is heterozygous for HbC mutation (Fig. 3B, red line). Results from paternal gDNA (heterozygous for the HbS mutation) show two peaks for both the WT and HbS allele respectively (Fig. 3B, blue line). CVS gDNA displays a pattern similar to father (Fig. 3B, blue line).

Table 1: Primer and probe sequences.

Description	*Sequences 5'- 3*
*BRCA2*_NIPD_MCA_F	5'-CAA CGA GAA TAA ATC AAA AAT TTG-3'
*BRCA2*_NIPD_MCA_R	5'-TGC GTG CTA CAT TCA TCA TTA-3'
*BRCA2*_NIPD_MCA_P_Me*	5'-CCG TCC AAC AAT CCT CCG TAA CCT-3'
*BRCA2*_LNA (WT)	5'-T+T+G+T+TA+C+G+A+G+GC-3'
*HBB*_NIPD_MCA_F	5'-GAC ACA ACT GTG TTC ACT AGC A-3'
*HBB*_NIPD_MCA_R	5'-CCA CCA ACT TCA TCC ACG TTC A-3'
*HBB*_NIPD_MCA_P_Me*	5'-GCA GAC TTC TCC ACA GGA GTC AG-3'
*HBB*_LNA1 (WT)	5'-+T+G+A+C+TC+C+T+G+A+G-3'
*HBB*_LNA2 (HbC)	5'-C+T+C+C+T+A+A+G+G+A+G-3'

Table 1: Primer and probe sequences used for PCR and HR-MCA.
Forward (F) and reverse (R) primers are depicted for both cases. Mutation detection probes (P) contain a 3' C3-spacer (Me*). LNA (locked nucleic acid) modified bases in the target blocking probes are depicted with + prior to the base. Target blocking probes were designed to perfectly match maternal sequences. Position of the altered nucleotide is underlined.

The addition of a WT target (*HBB*) blocking probe to the PCR reaction, completely blocked amplification of the WT *HBB* allele. As expected, no PCR product can be detected by the HbS mutation detection probe in WT control plasma DNA (data not shown) and WT gDNA (Fig. 3C, gray line and arrow), while in heterozygous maternal and paternal gDNA only the HbC and HbS MUT peaks are visible (Fig. 3C, red line (HbC) and blue line (HbS) respectively). More importantly, results from maternal plasma show that blockage of only the maternal WT *HBB* allele is not sufficient to detect the fetal paternally inherited HbS mutation (Fig. 3C, green line). In maternal plasma only the maternal HbC specific melting peak is visible, since the excess of HbC allele is not blocked by the WT *HBB* target blocking probe (Fig. 1). Hence, an HbC target

blocking probe was designed and additionally titrated into the PCR reactions together with both the HbS mutation detection probe and the WT (HBB) target blocking probe. The optimal ratio between HbS mutation detection probe, WT target blocking probe and HbC target blocking probe per reaction was shown to be 1:2:2 respectively. As expected, no signal is detected in maternal gDNA (Fig. 3D, gray line) when simultaneously blocking WT and HbC templates (Fig. 3D, arrows). In paternal gDNA only the HbS peak is visible (Fig. 3D, blue line). Subsequently, in maternal plasma the paternally inherited HbS mutation in the fetal cfDNA can now be detected after simultaneously blocking amplification of both maternal WT *HBB* and HbC allele (Fig. 3D, green line).

For both case 1 and case 2, successful detection of the fetal paternally inherited mutation in maternal plasma was achieved using this LNA-mediated targeted blocking approach in HR-MCA for NIPD. In case 1 this meant that the fetus would be affected and in case 2 the fetus would either be a carrier or affected with the disease. All results were concordant to Sanger sequencing results from CVS derived gDNA obtained after invasive procedures. (Fig. 4).

Discussion

The use of cffDNA isolated from maternal plasma for prenatal molecular testing or diagnostics has increased rapidly. Noninvasive prenatal testing (NIPT) for fetal trisomy screening has been introduced successfully in the past few years. Maternal plasma is easily obtainable and very early in pregnancy sufficient amounts of cffDNA are present. All this, together with the low risk for the fetus and continuous improvements of detection methods, have provided many advances for the use of NIPD in favor of invasive testing procedures early in gestation (Daley *et al.*, 2014).

Advantage and application of the HR-MCA approach in NIPT

In this proof of principle study, we demonstrate the use of LNA target specific blocking probes in HR-MCA. These target blocking LNA probes are directed against maternal background sequences in order to enhance the detection of fetal paternally inherited mutations in maternal plasma DNA. We choose to explore this approach since this methodology is sensitive, mutation specific and has a short turnaround time. Moreover, HR-MCA is easy to implement in diagnostics and also equipment that is required to perform HR-MCA is relatively inexpensive. This makes this method more manageable for genetic laboratories rather than for example an NGS mediated approach.

High throughput whole genome shotgun sequencing as currently performed for NIPT is not efficient for the detection of a single paternally inherited mutation since this method will require a much higher vertical coverage of the data than currently is obtained. Targeted sequencing may be a good alternative NGS method to use for mutation detection since good vertical coverage can be obtained. Pooling of multiple samples is required to obtain cost reduction. However, in case of prenatal testing, a short turnaround time is demanded. Therefore batching of samples might not always be feasible because of insufficient sample number. The advantage of HR-MCA is that it can always be performed within a short turnaround time regardless of the sample number.

When performing paternally inherited mutation detection using this novel HR-MCA based approach in NIPD, for autosomal dominant disorders it is restricted to cases where the mother does not carry the mutation, while for autosomal recessive disorders mother and

father should carry different mutations (Daley *et al.*, 2014). In this proof of principle study, we have pursued a personalized approach and we have used these differences in parental genotype to design target blocking LNA probes for use in HR-MCA which are specifically directed against the maternal sequences. This way, amplification of maternal cfDNA in plasma, including the maternally inherited fetal allele, will be blocked, providing enhanced sensitivity and specific detection of paternally inherited mutations by mutation specific detection probes. Such an approach could be a first step towards expanding the current repertoire for NIPD towards a more general application by detecting recurrent pathogenic mutations or genotypes linked to a pathogenic haplotype.

Detection of paternally inherited mutations in maternal plasma DNA using HR-MCA.

In this study, we describe the application of this approach for 2 different cases; one autosomal dominant (*BRCA2*) and one autosomal recessive monogenic disorder (*HBB*). While for case 1 (*BRCA2*) maternal sequences could be blocked with the use of only a single blocking LNA probe, for case 2 (*HBB*) the situation was more challenging. Both parents were heterozygous for a different mutation in the *HBB* gene and these mutations involved the same codon/amino acid by affecting a bp substitute 1 bp apart. Therefore, the template region covered by the HbS specific detection probe and the WT specific blocking LNA probe, also covered the adjacent maternal HbC mutation. Consequently, this implicated that the WT specific blocking LNA probe would have a mismatch on the other maternal (HbC) allele and amplification of this HbC allele could therefore still occur. Blocking only the maternal WT allele in this case appeared insufficient for selective detection of the paternally inherited mutation because of the excess of amplified HbC specific template in maternal plasma after PCR. Both the maternal WT and HbC alleles needed to be blocked simultaneously to provide enough background reduction of maternal cfDNA to detect the paternally inherited mutation in the fetus. Considering the recessive inheritance of the disease, additional confirmation of the actual fetal genotype through an invasive procedure was still required for this case, to determine whether the fetus would be affected or a carrier of the disease. Nevertheless, in cases where the paternally inherited mutation is excluded an invasive procedure could be avoided using this approach (in ~ 50% of the cases).

Applying HR-MCA method in NIPD.

As shown in this study, this method can be used successfully for NIPD. Do note that additional controls to confirm the presence of cffDNA in plasma are essential in NIPD to exclude false negative results, especially when no paternally inherited mutation was detected (Boon *et al.*, 2007; van den Oever *et al.*, 2013). Due to the fragmented nature of circulating cfDNA, there is a restriction for designing primers and probes. Fetal cfDNA is on average around 143-146 bp in size, which limits amplicon size for PCR (Lo *et al.*, 2010).

HR-MCA has previously been proposed as a useful method for NIPD (Yenilmez *et al.*, 2013; Macher *et al.*, 2012; Phylipsen *et al.*, 2012). In these studies no blocking LNA probe was used. The use of a blocking LNA probe could however be essential for the detection of fetal mutations in case of low fetal fraction or for the detection of more challenging mutations. In the study of Yenilmez and colleagues HR-MCA without a blocking LNA probe was performed and was not successful in case of early gestation (Yenilmez *et al.*, 2013). Levels of cffDNA may differ extensively between individuals and have been described to increase as gestation progresses (Lo *et al.*, 1998; Lun *et al.*, 2008a). Early in gestation, fetal paternally inherited variants may not be distinguished from the maternal background, since the levels of cffDNA

are too low to detect. We have previously shown that the lowest detectable fraction of a variant or mosaic by a conventional HR MCA approach (without a blocking probe) is very variant dependent and can be limited to only 25% (Out *et al.*, 2015) Therefore, it will be particularly challenging for some variants to be detected at low levels of template DNA, not only in gDNA but especially in plasma DNA. For future NIPD, the use of target blocking probes to block the amplification of undesired PCR products may therefore be extremely useful for mutation detection early in gestation, if not essential.

In summary, in this proof of principle study we have successfully demonstrated a PCR-based target specific detection HR-MCA approach that is suitable for the detection of paternally inherited mutations in cffDNA from maternal plasma by making use of a target specific LNA blocking probe. We have used a personalized approach by designing primers, paternal allele specific mutation detection probes and maternal allele specific target blocking probes based on parental sequences. The application of this method was shown for NIPD in both an autosomal dominant and recessive monogenic disorders and can be used as a sensitive and fast alternative for NGS-based approaches.

Acknowledgements

The authors would like to thank Dave van Heusden for technical assistance.

Chapter 6

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

Chapter 6: Noninvasive prenatal diagnosis of Huntington Disease; detection of the paternally inherited expanded CAG repeat in maternal plasma

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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).

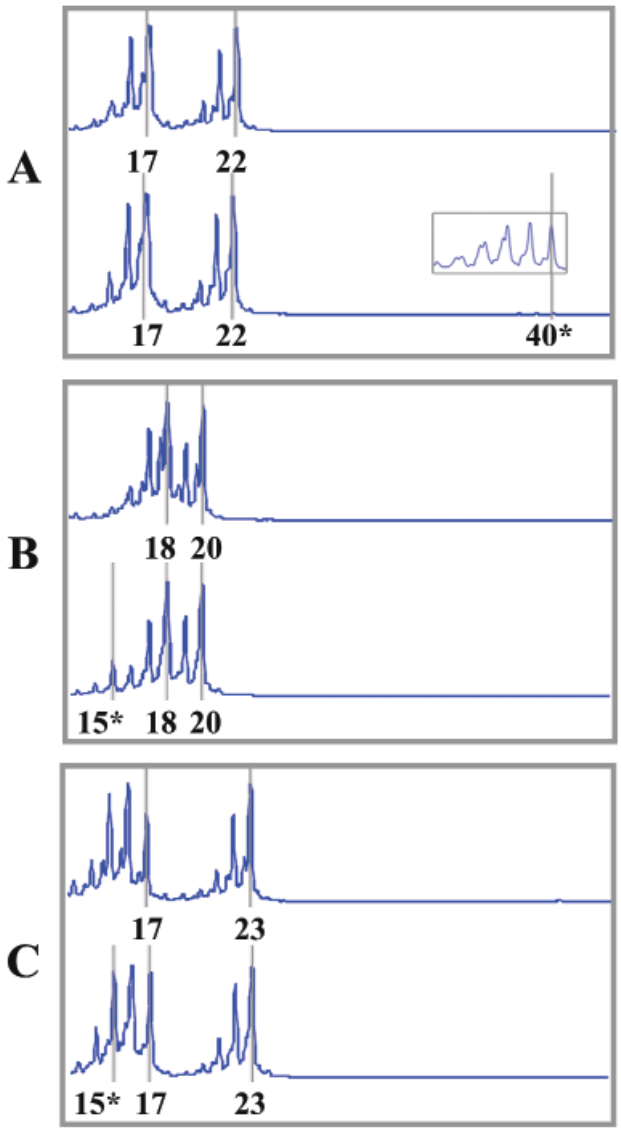

Figure 1: Representative results for direct CAG repeat analysis on maternal plasma for 3 different pregnancies. In each panel, the top part represents the result from maternal gDNA. The bottom part represents results from maternal plasma with the fetal paternally inherited repeat size indicated with *. 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.

Acknowledgements

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Chapter 7

Discussion

Discussion

Fragmented cell-free fetal DNA (cffDNA) present in maternal plasma is a potential source for prenatal genetic analysis of the fetus early in gestation. Although the amount of fetal fragments in maternal plasma is relatively low as compared to the excess of maternal DNA, results from the study of Lo *et al.* showed that the entire fetal genome is present in maternal plasma (Lo *et al.*, 2010). In theory, this could mean that every genetic defect in the fetus can be detected in maternal plasma by noninvasive prenatal diagnosis (NIPD). Since the whole fetal genome is present, a genome wide approach for NIPD could be used. Shotgun Whole genome sequencing (WGS) has already been applied for NIPT. However, for NIPD, shotgun WGS currently has its limitations. Many millions of sequencing reads can be obtained genome wide. Yet, shotgun sequencing occurs randomly and is not mutation specific. Hence, the coverage of a mutation or region of interest may be too low for reliable NIPD. A universal approach, such as WGS, for NIPD may therefore not be achievable or the preferred method of choice yet. Novel applications as well as other approaches could be considered and explored. There is a growing demand for less invasive alternatives in prenatal diagnostics. Therefore, it is to be expected that in the near future the number of available tests will increase dramatically. In this thesis, we have described several applications that make use of cfDNA from maternal plasma in NIPD and NIPT, such as fetal trisomy screening and targeted detection of paternally inherited mutations.

Fetal markers

In both NIPD and NIPT determination of the fetal fraction in maternal plasma can be important. Male specific markers (e.g. *DYS14* and *SRY*) are used most often for this purpose. Despite the high sensitivity and specificity obtained in diagnostic tests with these markers, a positive result can only be obtained in case of a male fetus. Preferably a universal and sex independent marker such as fetal methylated *RASSF1A* (*mRASSF1A*) should be used to confirm the presence of cffDNA in maternal plasma. This universal marker is differentially expressed between mother and fetus. In **chapter 2** we have described a novel approach for the detection of *mRASSF1A* in maternal plasma. We have used a combination of bisulfite conversion and pyrophosphorolysis-activated polymerization (PAP) for the detection of *mRASSF1A*. Bisulfite conversion is necessary to reveal the differences in methylation of cytosines between maternal and fetal sequences. PAP is an extremely sensitive and specific technique which can be used to detect specific sequences within a high background, in this case fetal specific sequences within the maternal background. Previously, the use of PAP in NIPD has proven to be of value in fetal sexing (Boon *et al.*, 2007). PAP has also been described for the detection of residual tumour cells in blood, serum or plasma (Maat *et al.*, 2008). *RASSF1A* is a tumour suppressor gene and *RASSF1A* promoter hypermethylation is associated with loss of expression in tumour cells (Honorio *et al.*, 2003). The fact that *RASSF1A* expression is also tumour related is a very important consideration. Unknown underlying malignancies or circulating tumour cells in the maternal circulation may result in false positive results, although the prevalence of cancer in the reproductive age group is considered to be small. The laboratory should therefore always be informed when women opting for NIPD have a history of cancer. Other alternative tests could then be performed to confirm the presence of cffDNA in plasma, such as the determination of paternally inherited SNPs (Alizadeh *et al.*, 2002; Page-Christiaens *et al.,* 2006). This assay is a good, although labour intensive, alternative for the PAP assay. Also, one should keep

in mind that SNPs may not always be informative. Parents could have all tested SNPs in common, or the fetus did not inherit an informative paternal SNP.

The *mRASSF1A*-PAP we currently perform for fetal sexing is merely a qualitative test, rather than quantitative. Quantitative detection of a sex independent fetal marker would be extremely beneficial in both NIPD and NIPT to determine the fetal fraction in a sample. A digitalized quantitative PAP assay has previously been described, though these assays were performed without prior bisulfite conversion of the DNA (Madic *et al.*, 2012; Song *et al.*, 2014; Bidard *et al.*, 2014). It has to be elucidated whether this combination of bisulfite conversion and subsequent digitalized PAP for the detection of *mRASSF1A* in maternal plasma would work. It would be very advantageous to combine such a digitalized PAP assay with NIPD of monogenic disorders. Not only the fetal paternally inherited mutation could then be detected, also the amount of cffDNA in the sample could be determined to define fetal fraction using the same sample. This in contrast to NIPT, where the amount of fetal DNA is determined either prior or after sample prep. At present, it is not yet clear which one of these determinations is the best predictor for the actual fetal fraction of a sample, since it is also not yet clear how these results will best represent the fetal fraction in the actual NGS data.

In the last few years, the search for other differentially methylated regions (DMRs) has continued (Papageorgiou *et al.*, 2009; Ioannides *et al.*, 2014; Xiang *et al.*, 2014). Most of this research is performed with methylated DNA immunoprecipitation sequencing (MeDIP-Seq) together with comparative arrays to provide global methylation landscapes. The differences in methylation between mother and fetus are based on relative differences in expression or methylation level. Some candidate DMRs show a high level of heterogeneity between individual fetuses. Yet, good candidate DMRs for use in NIPT or NIPD require a high level of homogeneity between individual fetuses and should be able to exclude maternal DNA easily (Xiang *et al.*, 2014). Even though MeDIP-Seq will give a good indication of which regions are differentially methylated, for use in a PAP assay, it has to be confirmed at nucleotide level whether these regions are indeed good candidate DMRs. The focus for the discovery of novel DMRs has been on chromosome 21 and 18. These DMRs have mostly been used for fetal trisomy screening (Tong *et al.*, 2006; Chim *et al.*, 2008; Tong *et al.*, 2010a; Tong *et al.*, 2010b). Furthermore, recent studies have shown the determination of the fetal methylome and transcriptome (Lun *et al.*, 2013; Tsui *et al.*, 2014). In the near future, perhaps more candidate DMRs will be discovered that can be useful for NIPD or NIPT.

NIPT

For NIPT, we have proposed Helicos Single Molecule Sequencing (SMS) as an alternative non-PCR-based sequencing platform in **chapter 3 and 4**. In contrast to PCR-based platforms, Helicos SMS data show no GC bias. In this study, SMS was successful for both T21 and T18 detection. For T13 however, it appeared less optimal. In several NIPT studies performed on PCR-based platforms, the detection rate for NIPT of fetal trisomy 13 was reported to be lower as compared to T21 and T18 detection (Gil *et al.*, 2014). In several other studies, the lower detection rate has been explained by the difference in GC content between these chromosomes, mainly because chromosome 13 has the lowest average GC content of these three chromosomes (Chen *et al.*, 2011; Bianchi *et al.*, 2012; Lau *et al.*, 2012b; Palomaki *et al.*, 2012; Norton *et al.*, 2012; Sparks *et al.*, 2012a). In chapter 3 we have shown that the mean number of reads per 50 kb bin increases when GC content increases on a PCR based sequencing platform. For Helicos the mean read count was not influenced by GC content, thus Helicos data display no GC bias. Therefore, the difference in GC content could not explain the lower detection rate of

T13 with SMS. Kalousek *et al.* showed that all placentas from T13 and T18 fetuses examined in their study were mosaic (Kalousek *et al.*, 1996). In case of such confined placental mosaicism (CPM), the presence of large quantities of cells with different karyotypes may influence results of fetal aneuploidy detection. Even though CPM occurs frequently in T13 and T18 placenta's, in our preliminary study using SMS possible CPM could also not explain lower performance observed for T13, since full karyotyping of the samples showed CPM was not present. It should however be noted, that the number of samples tested for these preliminary studies using SMS has been low. Extending this data set would be very useful to elucidate the reason for the lower detection rate of T13 screening and to investigate the role of GC content and/or bias for this particular chromosome. Unfortunately, since 2012 Helicos services were no longer available because of bankruptcy of the company and these tests could therefore not be performed for this thesis. A revival of the company might open possibilities to run additional samples to extend this data for fetal trisomy screening by use of SMS.

We have compared Helicos SMS with the Illumina GAII platform, which was mostly used for noninvasive fetal trisomy screening at that time. Since then, many improvements in the Illumina sequencing technology have been extremely beneficial for NIPT purposes and at present, Illumina sequencers (e.g. HiSeq) are most frequently used for NIPT. Newer updates with features such as a rapid run modus and decreased turn-around time are extremely beneficial for NIPT. With the introduction of novel sequencers such as HiSeq X Ten, which combines Nano technology to obtain sufficient data for a few genomes per single run, the costs for running NIPT samples may even further be reduced (Buermans *et al.*, 2014; Hayden, 2014).

Calculation methods and downstream analysis pipelines for NIPT have been improved in the last few years. Studies that use ratio calculations for fetal trisomy detection as described in chapter 3 and 4 have not been reported since. Currently the Z-score, NCV (**Appendix 3**) and student's t-test-based methods are mostly used, either as stand-alone methods or combined (Chiu *et al.*, 2008; Sehnert *et al.*, 2011; Jiang *et al.*, 2012a; Lo *et al.*, 2014; Straver *et al.*, 2014; Bayindir *et al.*, 2015). Thousands of NIPT samples have been processed in the past few years by many different groups and all these groups have been putting a lot of effort into optimizing NIPT for use in their laboratory setting, most frequently using the Illumina platform for sequencing.

Other single molecule sequencing platforms beside Helicos SMS have been developed in the past years, although not every platform may be as suitable for NIPT purposes. The PacBio sequencer by Pacific Biosciences is a single molecule real-time (SMRT) DNA sequencing system that records light pulses emitted as a by-product of nucleotide incorporation. One of the most important features of SMRT sequencing is fast run time (within hrs) and the extremely long read length that can be obtained. Compared to the 8-9 day running time of the Helicos flow cells, the PacBio sequencer is faster and scalable in runtime, which is favourable for NIPT turn-around time. Depending upon PacBio starting library, over half of the data are in reads > 14,000 base pairs long, starting from around 3000 bp with the longest reads over 40,000 bp. Such long read lengths can indeed be beneficial for several applications, nevertheless cfDNA in maternal plasma is on average a hundredfold shorter than the reads that can be obtained with this platform. Therefore, PacBio is not a suitable platform for NIPT. Also Oxford Nanopores, a platform that uses nanopores as biosensors, has similar advantages (e.g. fast and scalable run time) as compared to the PacBio. Sequencing with the Oxford Nanopores platform can also result in very long read lengths, although in contrast to PacBio where the fragment of interest is analysed multiple times, the read length on the Nanopore platform equals fragment size. Although Nanopore sequencers seem to have good features for NIPT

purposes (e.g. single molecule, base detection without labels, low GC bias, multiplexing and scalable in data output), the use of this platform for NIPT has not been described yet (Buermans *et al.*, 2014; Zhang *et al.*, 2014).

Ion Torrent Technology on the other hand has both the advantage of producing short reads in a fast and cost-efficient manner, although the total amount of data per run is relatively low compared to the previously mentioned sequencing platforms. NIPT with semiconductor sequencing has indeed been described successfully (Yuan *et al.*, 2013; Liao *et al.*, 2014). With the Ion Proton and/or improved chips for the Ion Torrent that will yield a larger number of reads, it is possible to run multiple samples at once and to handle a true clinical throughput of multiple samples per week (Wang *et al.*, 2014; Jeon *et al.*, 2014). Hence, the improved Ion Torrent and especially the Ion Proton could be good alternative sequencing platforms for NIPT purposes.

Many different factors may influence the success rate of NIPT, such as the presence of (unknown) maternal chromosomal abnormalities, CPM, true fetal mosaicism (TFM) or a vanishing twin (Bianchi *et al.*, 2012; Hall *et al.*, 2013; Wang *et al.*, 2013; Futch *et al.*, 2013; Lau *et al.*, 2014). As mentioned previously fetal fraction is one of the key factors for successful NIPT and NIPD. Fetal fraction is determined with Real-Time PCR or is calculated from NGS data. With Real-Time PCR it is difficult to determine fetal fraction when the percentage is very low. If this is the case, the data are not robust since measurement is performed in only a few copies of the fetal genome. When calculating fetal fraction from the actual sequencing data by using Y chromosomal reads, one should take into account that there is also a small percentage of reads from maternal origin that can be incorrectly assigned to the Y chromosome (Chiu *et al.*, 2011a; Hudecova *et al.*, 2014). Fetus specific Y chromosomal reads need to be deduced from the maternal background. The downside of these two methods is that they are sex dependent. In a genome wide or a targeted NGS approach, millions of reads are produced from the cfDNA present in the sample. It is generally accepted that for NIPT ~10 million unique mappable reads are required for reliable analysis. Some studies additionally stress out that for such counting-based technologies a fetal fraction of at least 4% is required for analysis (Ehrich *et al.*, 2011; Palomaki *et al.*, 2011; Palomaki *et al.*, 2012). Low fetal fraction may result in sample rejection or incorrect outcome. When the percentage of fetal sequences in a sample is too low as compared to the maternal background sequences, the relative contribution of a third chromosome in case of fetal trisomy is too low for this sample to be distinguished from the euploid foetus.

As previously described for fetal markers, both the presence and in this case quantification of cffDNA should preferably be performed with a sex independent method. The DANSR assay is an example of a NGS based sex-independent approach to determine fetal fraction. Here a set of 192 SNPs-containing loci on chromosome 1-12 was assessed for fetal trisomy detection (Sparks *et al.*, 2012a; Ashoor *et al.*, 2013; Brar *et al.*, 2013). This SNP based approach is a targeted approach, since analysis involves a selection of autosomes. However, for WGS such a SNP based targeted approach is not cost efficient at this moment, since it requires a much higher horizontal and especially vertical coverage of SNPs to be analysed. The study of Lo *et al.*, in which they show by using WGS that the complete fetal genome is present in maternal plasma, already indicated that extremely large amounts of reads are required in WGS to have sufficient coverage of a given SNP (Lo *et al.*, 2010). At present, sufficient sequencing depth cannot be accomplished without extremely high costs and is therefore not feasible yet. Another method to determine fetal fraction in a sex independent way that could be of interest for NIPT was described by Yu *et al.* They describe the analysis of library fragment size by

determining a ratio between short fetal and longer maternal fragments (Yu *et al.*, 2014). The advantage of this method is that it does not require additional experiments since library fragment size determination is widely used in standard protocols and is therefore easily applied for this purpose as well.

Monogenic disorders

For the detection of monogenic disorders, at present a genome wide approach by use of NGS is not cost-efficient. To ensure that a given mutation in the fetus will be covered sufficiently to be detected in maternal background, this would require high vertical coverage of the region of interest (Lo *et al.*, 2010). For NIPT only ~10 million unique mappable reads are required for analysis. In the paper by Lo *et al.* where a genome wide approach was used for the detection of a monogenic disorder, almost 4 billion reads were produced, equivalent to an average 65-fold coverage of the human genome (Lo *et al.*, 2010). Even then, the fetal-specific read sequencing depth of a SNP ranged from only 1-8 reads per SNP. The detection of monogenic disorders by use of a genome wide approach would ultimately lead to much higher sequencing costs since the amount of data necessary for analysis is many times higher as compared to NIPT. A whole exome sequencing (WES) approach would therefore be a better alternative for NIPD of monogenic disorders. Instead of focussing on the entire genome, only the protein-coding genes are targeted, representing ~1% of the human genome. However, WES also has its limitations for NIPD. Even though mutations in exons are more likely to have severe consequences, intronic mutations can occur and these intronic mutations will not be targeted by use of exome sequencing. Targeted gene or disease specific approaches may be custom designed for some genetic conditions, targeting genes with causative mutations. However, certain regions of the genome or exome may be difficult to target, such as GC rich regions and repeats.

Instead of using NGS, in **chapter 5 and 6** we have described alternative methods for the detection of monogenic disorders. Because of the wide variety in genetic defects, (e.g. point mutation or repeat expansion) each defect may require a different approach. In **chapter 5** we have pursued the option of blocking the maternal background in maternal plasma to enhance the detection of the fetal paternally inherited mutation. We showed that the detection of a mutation in the fetus can be enhanced by blocking the maternal background by use of complementary locked nucleic acid (LNA) probes in high resolution melting curve analysis (HR-MCA). These short LNA probes were designed to perfectly match maternal wild-type (WT) sequences. Binding of these LNA probes subsequently resulted in prevention of PCR amplification of these maternal sequences. Moreover preferential amplification and enhanced detection of the paternally inherited allele is enabled. This method can only be applied in case of PCR based targeted detection of single mutations and possibly small insertions or duplications. LNA probes are short, only around 12 nt in size. The target mutation should therefore not exceed this size. Beside HR-MCA, the use of LNA probes in Real-Time PCR could also be an attractive application in NIPD, to block maternal sequences and monitor the mutant allele in real-time. LNA probes will only bind with high affinity to complementary sequences. Because of the LNA modifications, the probe will not interfere in the PCR itself by acting as a potential primer or target. When LNA probes are used for blocking purposes, it remains essential that the parental genotypes are known, because there are highly polymorphic regions present in the human genome. In these regions the chance is high that there is a SNP present in the maternal genome at a position covered by the LNA probe. Hence, the LNA probe will not bind and consequently no inhibition of the amplification of unwanted sequences will occur. Design of

multiple LNA probes each containing one of the SNP variants could solve this problem.

As described previously for NIPT, all PCR based techniques are influenced by the GC content of the template. Therefore, GC rich areas of the human genome (e.g. promoter areas) are more difficult to amplify or target. The same holds for AT rich areas. First attempts to design HR-MCA detection and LNA probes for a familial mutation in a GC rich area in exon 4 of the *NOTCH3* gene resulted in non-functional primers and a detection probe with a very high melting temperature (Tm) (unpublished data). A difference between Tm of the LNA probe and Tm of the primers is required to facilitate LNA binding and thus blocking prior to primer binding and extension of the template sequences. The restriction of the amplicon size in combination with high GC content of the template was the main reason for failure in setting up an HR-MCA assay for this particular mutation. Increasing amplicon size could facilitate in the design of primers with a lower Tm when this genomic region is less GC rich. A strategy of blocking maternal background sequences is not necessary when the focus is on detecting polymorphic paternally inherited (extended) CAG repeats such as in Huntington disease (HD). Generally the maternal repeats are in the normal range. Size selection or selective blocking of shorter repeats in PCR cannot be accomplished with a blocking approach as was described for the detection of paternally inherited mutations. Because LNA probes target a specific sequences of ~12 nt, they are not able to distinguish between shorter or longer repeats, and in theory would block every repeat.

The size of fragmented cffDNA is also a restriction in PCR-based assays in general, for example in amplicon design. The large majority of cffDNA fragments have been shown to be on average <150 bp in length (Fan *et al.*, 2010; Lo *et al.*, 2010). This implies that these size constraints should be taken into account when designing NIPD assays to detect a given fetal target. In case a disease is caused by a repeat expansion, such as the CAG expansions in HD, the detection becomes more complex. These repeats vary greatly in length and are known to be unstable upon transmission. Therefore, repeat size is not predictable in advance and repeats may expand into a repeat size that exceeds the fragment size of cffDNA. When analysing a point mutation or small insertion or deletion, the constraint can be accommodated quite easily by designing small amplicons of ~100 bp to ensure efficient amplification of the target sequence. Fetal sequences >150 bp are also present in maternal plasma, although in minority. As shown in the original paper on the discovery of cffDNA in maternal plasma, Y chromosome specific primers designed to amplify a sequence (*DYS14*) with an amplicon size of 198 bp were used (Lo *et al.*, 1990; Lo *et al.*, 1997). This is larger than the average amplicon size for cffDNA of ~143 bp that was reported a few years later by the same group (Lo *et al.*, 2010). These results show that there is quite a range in cffDNA size. For PCR-based approaches for mutation detection, preferably amplicon sizes should be used that do not exceed the average reported size of ~143 bp.

In **chapter 6** we have described the application of NIPD for HD by directly measuring the fetal paternally inherited CAG repeat from the Huntingtin (*HTT*) gene in maternal plasma. This has been accomplished through a PCR based approach and subsequent fragment analysis of total cfDNA. For this approach to be successful for NIPD, it requires a PCR protocol that is optimized for low DNA input. Also, the Taq polymerase should prevent preferential amplification of smaller fragment as much as possible. Even though preferential amplification could not be completely prevented, the *Taq* polymerase used in PCR for NIPD of HD shows that longer fragments are being amplified in sufficient amounts to be detected in fragment analysis. This is absolutely essential for detecting the fetal expanded paternally inherited repeats in maternal plasma. When directly targeting fetal paternally inherited CAG repeats in Huntington disease,

we showed that we could detect a trinucleotide repeat up to 53 and even 70 CAG repeats, representing cffDNA fragments with a size of >170 bp and larger. As mentioned previously, fragments of this size have indeed been reported, though with a very low occurrence, which makes the detection of such rare long fragments even more challenging (Chan *et al.*, 2004). Besides restrictions in the maximum size of repeats in the expanded HD range, our results show that there are restrictions in detecting the paternally inherited repeats in the normal range. No paternally inherited repeat could be identified in approximately 50% of the cases presented, because the paternally inherited repeat coincided with the maternal repeat pattern. When NIPD results are inconclusive due to absent or low levels of cffDNA, prospective parents are given the option to provide a new blood sample for NIPD later on in gestation to retest for the paternally inherited fetal repeat. Nevertheless, in all cases where no paternal contribution in maternal plasma could be detected or in cases with inconclusive results, an invasive procedure to confirm the fetal genotype on fetal gDNA is indicated.

Fragment size analysis is at present the best strategy for NIPD for HD. Detection of large repeats for NIPD using other technologies such as NGS is currently difficult, if not impossible. CAG repeats that encode for long glutamine (Q) stretches are present all through the human genome. To date, a total of nine polyQ diseases have been reported (Fan *et al.*, 2014). A NGS mediated approach for repeat determination and/ or detection for NIPD of HD would require paired-end sequencing of a specified and targeted repeat, in this case the *HTT* gene. Although the repetitive sequence of a repeat is simple, errors in NGS data may interfere with correct data analysis. Moreover, since CAG repeat are common throughout the genome, bioinformatics will have to focus on HTT specific CAG repeats to determine repeat size confidently.

Concluding remarks

Noninvasive prenatal diagnostics and testing have strongly improved in time and more and more applications have become available. Since the entire fetal genome is present in maternal plasma, it is to be expected that in the future the large majority if not all of prenatal diagnostics is preferably be performed using cffDNA. Whether or not this will always be performed in a universal and genome wide approach using WGS has to be elucidated. Currently, there are still limitations in using WGS for some prenatal requests. With WGS large amounts of data can be generated, which can be useful in detecting known but also unknown variants or de novo mutations in fetuses without a prior history or predisposition of a familial mutation. However, shotgun sequencing is not mutation specific and is currently not always cost-efficient for use in the detection of these specific mutations.

The majority of prenatal requests are for fetal aneuploidy detection. In the Netherlands, during a two year national implementation study, NIPT is currently available for high risk pregnant women. It is to be expected that NIPT will soon become available for low risk pregnant women as well. In addition to this, in the near future it is desirable for genetic laboratories to have several NIPD alternatives available for prospective prenatal requests. As mentioned previously and as shown in this thesis, no universal approach, particularly for NIPD, is available yet. For each novel application, one should clearly consider the best approach in detecting the genetic defect using NIPD. Moreover, some applications (e.g. detection of point mutations) do not have to be restricted only to couples where the father is carrier of the mutant allele.

In conclusion, we show several novel applications for the use of cffDNA for NIPD and NIPT. We also show that each application at present may require a different approach. In the near future, it is to be expected that more noninvasive alternatives for a wider variety of genetic anomalies will become available for prenatal diagnostics early in gestation.

Chapter 8

Summary

Samenvatting

Summary

Current prenatal diagnostics is mainly based on obtaining fetal DNA through invasive procedures which are associated with a small, but significant risk of fetal loss. The discovery of the presence of cell-free fetal DNA (cffDNA) in maternal plasma opened possibilities for less or noninvasive alternative procedures.

CffDNA is comprised of small fragments of fetal extracellular DNA derived from placental cells that go into apoptosis. It circulates in maternal plasma and can be detected already very early in gestation. However, the majority of total cell-free DNA (cfDNA) in maternal plasma is of maternal origin and the fetal contribution is relatively small in the first trimester. Despite this high maternal background, the use of cffDNA in noninvasive prenatal diagnostics (NIPD) and noninvasive prenatal testing (NIPT) has been described successfully. In this thesis, we describe novel applications and approaches for the use of cffDNA in both NIPD and NIPT.

For all these novel applications, either NIPT or NIPD, it is important to know the fetal fraction in the maternal background. Independent confirmation of the presence of fetal DNA in the sample is therefore required. Most often male specific markers (e.g. *SRY* and *DYS14*) are used to confirm the presence of cffDNA or to determine the fetal fraction in a sample. However, using these markers a positive result can only be obtained in case of a male fetus. Therefore we have developed a novel approach to detect and confirm the presence of cffDNA in maternal plasma in a sex-independent way. **Chapter 2** describes a novel assay for the detection of fetal specific methylated *RASSF1A* (*mRASSF1A*) using a combination of bisulfite conversion and pyrophosphorolysis-activated polymerization. *RASSF1A* is differentially methylated between mother and fetus. These differences in methylation were used to specifically detect and thus confirm the presence of fetal *mRASSF1A* sequences. We have shown that this qualitative approach to determine cffDNA in maternal plasma can be used successfully with high sensitivity and specificity and can be applied early in gestation. In the future, digitalization and thus quantification of this assay could be beneficial in the determination of the fetal fraction.

NIPT for fetal aneuploidy screening is perhaps one of the best studied applications for the use of cffDNA and is frequently performed on PCR based sequencing platforms. These platforms are known for GC content related bias in sequencing data. **Chapter 3** describes the use of Helicos Single Molecule Sequencing (SMS) as an alternative non-PCR based sequencing platform for NIPT of fetal trisomy 21 (T21). We have directly compared SMS to the PCR based Illumina platform and show that when using SMS, the mean number of sequencing reads is not influenced by GC content, thereby showing that SMS sequencing data is not GC biased. While, using the Illumina platform, the mean number of sequencing reads generated increases when GC content of the chromosome increases. Moreover, we show that when using SMS, all fetuses with T21 can be correctly identified and distinguished more clearly from euploid fetuses. In **chapter 4**, we additionally describe the use of SMS for other fetal trisomies. We show that SMS is also successful for trisomy 18 (T18) screening. However, for trisomy 13 (T13) screening SMS turns out to be less successful. To elucidate the cause of this poor performance further testing is needed.

In contrast to NIPT, NIPD for monogenic disorders currently represents only a small part of the field of noninvasive prenatal genetic testing. NIPD can be used for the detection of paternally inherited sequences. However, not all NIPD can currently be addressed by a single or universal approach, such as for instance NGS. **Chapter 5** describes the use of High-Resolution Melting Curve Analysis (HR-MCA) as a non-NGS PCR mediated alternative for the detection of paternally inherited point mutations in maternal plasma. We show that by blocking the

maternal background with locked nucleic acid (LNA) blocking probes, the detection of the paternally inherited allele of the fetus can be enhanced. We show this proof of principle for both an autosomal dominant and an autosomal recessive disorder.

Chapter 6 additionally shows the use of another PCR based approach for the detection of fetal paternally inherited repeats in Huntington disease (HD). HD is caused by the expansion of a trinucleotide (CAG) repeat in the Huntingtin (*HTT*) gene. The fetal paternally inherited repeat can detected by use of a PCR based approach with primers flanking the repeat. We describe a validation study for NIPD for HD and showed that we could detect a fetal repeat of up to 70 CAG repeats in maternal plasma. In this validation study, all paternally inherited repeats for all fetuses at risk for HD could be detected. We additionally show that in cases where the fetus has inherited the normal paternal allele, the detection strongly depends on the parental genotypes.

In the future, the number of applications for NIPT and particularly NIPD is expected to increase. Even though the use of fragmented cffDNA from maternal plasma still has its challenges (e.g. relative low amounts, short in size), we show that by careful consideration of the most efficient approach for each application, these challenges may be overcome. This makes cffDNA a very potential source for use in NIPT and NIPD. Based on the ongoing improvements of these tests, both NIPT and NIPD will soon become available to more women, although a universal approach for all applications is not feasible yet. Nevertheless, it is to be expected that in the near future, the majority of prenatal diagnostics will be replaced by either NIPD or NIPT.

Samenvatting

De huidige prenatale diagnostiek is voornamelijk gebaseerd op analyse van foetaal DNA verkregen met behulp van invasieve procedures. Deze procedures zijn geassocieerd met een klein, maar significant risico op een miskraam. De ontdekking dat er kleine stukjes celvrij foetaal DNA (cffDNA) in het (bloed)plasma van moeder aanwezig zijn, opende mogelijkheden voor minder of niet invasieve alternatieven voor het verkrijgen van foetaal DNA voor prenatale diagnostiek.

CffDNA bestaat uit kleine fragmenten foetaal extracellulair DNA dat afkomstig is uit cellen van de placenta die in apoptose zijn gegaan (ofwel geprogrammeerde cel dood hebben ondergaan). Dit DNA circuleert in het plasma van de zwangere vrouw en kan al vroeg in de zwangerschap gedetecteerd worden. De meerderheid van het aanwezige cel-vrije DNA in maternaal plasma is echter van maternale origine en de bijdrage van de foetus in het eerste trimester van de zwangerschap is relatief klein. Ondanks deze hoge maternale achtergrond is het gebruik van cffDNA voor niet-invasieve prenatale diagnostiek (NIPD) en niet-invasieve prenatale testen (NIPT) al eerder succesvol beschreven. In dit proefschrift worden een aantal nieuwe applicaties en toepassingen besproken voor het gebruik van cffDNA voor zowel NIPD als NIPT.

Voor zowel NIPD of NIPT, is het van belang om de foetale fractie in de maternale achtergrond te weten. Daarom is het noodzakelijk om de aanwezigheid van cffDNA in een sample te bepalen. Voor het bevestigen van de aanwezigheid van foetaal DNA of voor het bepalen van de foetale fractie worden meestal markers gebruikt die op het Y chromosoom liggen en die dus specifiek zijn voor mannelijke sequenties, zoals *SRY* en *DYS14*. Helaas kunnen deze markers alleen een resultaat geven indien de foetus mannelijk is. Daarom is een nieuwe methode om de aanwezigheid van cffDNA te kunnen bevestigen ontwikkeld, die in elke zwangerschap gebruikt kan worden, dus ongeacht het geslacht van de foetus. In **hoofdstuk 2** wordt een nieuwe test beschreven voor de detectie van foetus specifieke sequenties van gemethyleerd *RASSF1A* (*mRASSF1A*), waarbij gebruik gemaakt wordt van een combinatie van bisulfiet omzetting en pyrophosphorolyse-geactiveerde polymerisatie (PAP). *RASSF1A* is differentieel gemethyleerd tussen moeder en foetus. Deze verschillen in methylatie patroon worden gebruikt om specifiek de foetale sequenties te kunnen detecteren en daarmee dus de aanwezigheid van deze sequenties te kunnen bevestigen. De resultaten laten zien dat deze kwalitatieve toepassing al vroeg in de zwangerschap met hoge sensitiviteit en specificiteit succesvol gebruikt kan worden. In de toekomst zou digitalisatie en kwantificatie van deze test tevens uitkomst kunnen bieden in het bepalen van de foetale fractie in een sample.

NIPT, voor het screenen naar foetale aneuploïdieën, is op dit moment een van de best bestudeerde toepassingen van het gebruik van cffDNA. Voor NIPT wordt meestal gebruik gemaakt van Next Generation Sequencing (NGS) platformen die PCR gebaseerd zijn. Deze platformen staan bekend om de invloed die het GC percentage van een chromosoom heeft op de hoeveelheid data die geproduceerd wordt. In **hoofdstuk 3** beschrijven we het gebruik van Helicos Single Molecule Sequencing (SMS) bij NIPT voor trisomy 21 (T21 ofwel Down Syndroom) detectie als een alternatief platform dat niet gebaseerd is op een PCR techniek. We hebben SMS een-op-een vergeleken met het PCR gebaseerde Illumina platform en laten zien dat het gemiddelde aantal sequenties bij SMS niet beïnvloed wordt door het GC percentage. Bij het Illumina platform daarentegen zien we dat het gemiddeld aantal sequenties toeneemt bij toenemend GC percentage. Daarbij laten we ook zien dat bij SMS alle T21 foetussen correct geïdentificeerd worden en zelfs nog beter te onderscheiden zijn van euploïde foetusen als

vergeleken met het Illumina platform. In **hoofdstuk 4** laten we bovendien de toepassing van SMS zien voor het screenen naar andere trisomieën. Het gebruik van SMS is ook succesvol voor trisomie 18 (T18 ofwel Edward's syndroom) screening. Voor trisomie 13 (T13 ofwel syndroom van Patau) is deze methode minder succesvol. Om de exacte reden van deze mindere prestatie beter te kunnen duiden, zou verder onderzoek uitgevoerd moeten worden.

In tegenstelling tot NIPT, beslaat NIPD voor monogene erfelijke aandoeningen slechts een klein deel van het veld van niet-invasief prenataal testen. NIPD kan gebruikt worden voor de detectie van paternaal overgeërfde sequenties. Momenteel is het nog niet mogelijk om alle NIPD uit te voeren met één enkele of universele aanpak, zoals bijvoorbeeld het gebruik van NGS. In **hoofdstuk 5** wordt het gebruik van PCR en hoge resolutie smelt curve analyse (HR-MCA) als een alternatief voor NGS beschreven. Door het blokken van de maternale achtergrond tijdens de PCR met behulp van een LNA probe, wordt de detectie van het paternaal overgeërfde allel in de foetus sterk verbeterd. Als "proof of principle" tonen we dit voor zowel een autosomaal dominant als een autosomaal recessief overervende aandoening aan.

Daarnaast wordt in **hoofdstuk 6** nog een andere PCR gebaseerde toepassing getoond voor de ziekte van Huntington (HD) en de detectie van het paternale allel in de foetus. HD wordt veroorzaakt door een expansie van een trinucleotide (CAG) herhaling/repeat in het huntingtine gen (*HTT*). De paternale overgeërfde repeat in de foetus kan worden gedetecteerd met behulp van een PCR waarbij gebruik gemaakt wordt van primers die rondom de repeat liggen. In deze validatie studie voor NIPD van HD konden we een foetale repeat van tot wel 70 CAG herhalingen aantonen in maternaal plasma. Bovendien laten we zien dat we bij alle foetussen die risico lopen op het krijgen van HD, de paternaal overgeërfde repeat werd aangetoond. Bij de foetussen die het normale allel van vader hebben gekregen, blijkt de detectie ervan sterk afhankelijk te zijn van het genotype van beide ouders.

Het is zeer waarschijnlijk dat het aantal toepassingen voor NIPT en zeker voor NIPD de komende tijd sterk zal toenemen. Ondanks het feit dat het gebruik van gefragmenteerd cffDNA uit maternaal plasma nog steeds uitdagingen kent (bijvoorbeeld de relatief lage hoe- veelheid en de korte lengte), laat dit proefschrift zien dat deze uitdagingen overwonnen kunnen worden door voor iedere toepassing de meest efficiënte aanpak te gebruiken. Kortom, cffDNA is een uitermate geschikte bron van foetaal DNA voor NIPT en NIPD. Met het verbeteren van de huidige technieken, zullen zowel NIPT als NIPD in de toekomst voor meer vrouwen beschikbaar komen, alhoewel het op dit moment nog niet mogelijk lijkt om hiervoor een universele aanpak voor te gebruiken. Desalniettemin is de verwachting dat in de toekomst het merendeel van invasieve procedures voor prenatale diagnostiek vervangen gaat worden door NIPD of NIPT.

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Curriculum vitae

Curriculum vitae

Jessica Maria Elisabeth van den Oever werd geboren op 11 mei 1980 te Roosendaal, gemeente Roosendaal en Nispen. In 1997 behaalde zij haar HAVO diploma aan het Gertrudiscollege te Roosendaal, waarna zij in datzelfde jaar startte met de opleiding Biologie en Medisch Laboratorium Onderzoek aan de Hogeschool Brabant, Faculteit Techniek en Natuur te Etten-Leur (tegenwoordig onderdeel van Avans Hogeschool gevestigd te Breda).

Tijdens haar afstudeerstage op de afdeling Klinische Genetica van de Erasmus Universiteit Rotterdam onder supervisie van Dr. Rob Willemsen, deed zij onderzoek naar het fragiele X syndroom. Hiervoor bestudeerde zij het transport van FMRP in een PC12 neuronale cellijn.

Na het behalen van haar diploma, startte zij in 2001 met haar studie Biologie aan de Universiteit Leiden via een HBO-instroom programma. Ter afronding van deze studie werd stage gelopen bij de sectie Moleculaire Biologie van het instituut voor Moleculaire Plantkunde bij de Faculteit Biologie van Universiteit Leiden. Onder begeleiding van Prof. Herman Spaink en Prof. Michael Richardson werd gestart met een pilot studie met vertebraten waarbij er onderzoek gedaan werd naar het expressie patroon van Selenium Binding Protein in zebravissen.

In 2005 begon zij als research analist op het project “Immunomodulatory properties of Mesenchymal Stem Cells” op de afdeling Immunohematologie en bloedtransfusie (IHB) van het Leids Universitair Medisch Centrum (LUMC) onder begeleiding van Prof. Wim Fibbe, Dr. Alma Nauta en Dr. Kirsten Canté-Barrett. Vanaf 2007 was zij tevens werkzaam als research analist op het project “Mechanismen van cytokine-geïnduceerde hematopoietische stam- en progenitorcel mobilisatie” onder begeleiding van Prof. Wim Fibbe en Dr. Melissa van Pel. Daarnaast heeft zij in die periode gewerkt als interim proefdiercoördinator voor de afdeling IHB.

Eind 2008 begon zij als research analist op het project “Gene expression profiling in a chronic restraint stress rat model” bij de afdeling Medische Farmacologie van het Leiden/ Amsterdam Center for Drug Research (LACDR)/ LUMC onder begeleiding van Dr. Nicole Datson en Prof. Ron de Kloet.

In oktober 2010 werd gestart met het promotie onderzoek “Noninvasive prenatal detection of genetic defects” bij de afdeling Klinische Genetica op het Laboratorium voor Diagnostische Genoomanalyse (LDGA) van het LUMC te Leiden. Onder leiding van Dr. Elles Boon en Prof. Bert Bakker werden de experimenten verricht die staan beschreven in dit proefschrift. Van juli t/m september 2015 heeft zij onder supervisie van Prof. Joris Vermeesch gewerkt als interim project manager voor targeted NIPT bij het Centrum Menselijke Erfelijkheid van de Katholieke Universiteit Leuven in België.

Publications and Presentations

Publications

- Brison, N., Van Den Bogaert, K., **van den Oever, J.M.**, Dehaspe, L., Janssens, K., Blaumeiser, B., Peeters, H., Van Esch, H., de Ravel, T., Legius, E., Devriendt, K., Vermeesch, J.R., *Maternal incidental findings during non-invasive prenatal testing for fetal aneuploidies*, submitted.
- **Van den Oever, J.M**., van Minderhout, I.J.H.M, Harteveld, C.L., den Hollander, N.S., Bakker, E., van der Stoep, N., Boon, E.M.J., *A novel targeted approach for noninvasive detection of paternally inherited mutations in maternal plasma*, J Mol Diagn. 2015.
- **Van den Oever, J.M**., Bijlsma, E.K., Feenstra, I., Muntjewerff, N., Mathijssen, I.B., Bakker, E., van Belzen, M.J., Boon, E.M.J., *Noninvasive prenatal diagnosis of Huntington disease: detection of the paternally inherited expanded CAG repeat in maternal plasma*, Prenat. Diagn. 2015.
- **Van den Oever, J.M**., Balkassmi, S., Segboer, T., Verweij, E.J., van der Velden, P.A., Oepkes, D., Bakker, E., Boon, E.M., *Mrassf1a-pap, a novel methylation-based assay for the detection of cell-free fetal DNA in maternal plasma*, PloS One, 2013.
- Datson, N.A., **van den Oever, J.M**., Korobko, O.B., Margarinos, A.M., de Kloet, E.R., McEwen, B.S., *Previous history of chronic stress changes the transcriptional response to glucocorticoid challenge in the dentate gyrus region of the male rat hippocampus*, Endocrinology 2013.
- **Van den Oever, J.M**., Balkassmi, S., Johansson, L.F., Adama van Scheltema, P.N., Suijkerbuijk, R.F., Hoffer, M.J., Sinke, R.J., Bakker, E., Sikkema-Raddatz, B., Boon, E.M., *Succesful noninvasive trisomy 18 detection using single molecule sequencing*, Clin Chem 2013.
- Polman, J.A., Hunter, R.G., Speksnijder, N., **van den Oever, J.M**., Korobko, O.B., McEwen, B.S., de Kloet, E.R., Datson, N.A., *Glucocorticoids modulate the mTOR pathway in the hippocampus: differential effects depending on stress history*, Endocrinology 2012.
- **Van den Oever, J.M**., Balkassmi, S., Verweij, E.J., van Iterson, M., Adama van Scheltema, P.N., Oepkes, D., van Lith, J.M., Hoffer, M.J. den Dunnen J.T., Bakker, E., Boon, E.M., *Single molecule sequencing of free DNA from maternal plasma for noninvasive trisomy 21 detection*, Clin Chem 2012.
- Verweij, E.J., **van den Oever, J.M**., de Boer, M.A., Boon, E.M., Oepkes, D., *Diagnostic accuracy of noninvasive detection of fetal trisomy 21 in maternal blood: a systematic review*, Fetal Diagn Ther 2012.
- De Jager, S.C., Canté-Barrett, K., Bot, I., Husberg, C., van Puijvelde, G.H., van Santbrink, P.J., Yndestad, A., **van den Oever, J.M**., Kuiper, J., van Berkel, T.J., Lipp, M., Zwaginga, J.J., Fibber, W.E., Aukrust, P., Biessen, E.A., *Impaired effector memory T-cell regulation facilitates graft versus host disease in CCR7-deficient bone marrow transplant chimeras*, Transplantation, 2009.

Presentations

- Invited speaker: Prenatal Molecular Diagnostics; Trends, advances & prospects, Lisbon, April 2015.
- *Noninvasive prenatal diagnosis of Huntington disease in the Netherlands*: DHDRN, Amsterdam, mei 2014.
- Invited speaker: Werkgroep prenatale diagnostiek en therapie/ werkgroep foetale

echoscopie: *Gemetyleerd RASSF1A: een universele biomarker ter bevestiging van de aanwezigheid van foetaal DNA in maternaal bloed*; najaarssymposium, Utrecht, oktober 2013.

- *Single Molecule Sequencing of Free DNA from maternal plasma for noninvasive trisomy testing*: Medical Genetics Centre PhD student workshop, Luxemburg, mei 2013
- Invited speaker: *Noninvasive fetal aneuploidy detection using Next Generation Sequencing: towards application in diagnostics*; Integrated Data Analysis meeting organised by the dept. of Epidemiology in collaboration with SASC, LUMC, Leiden, november 2012.
- Invited speaker: *Noninvasive fetal aneuploidy detection using Helicos: Third generation vs Next Generation Sequencing*: NBIC *Next Generation Sequencing meeting,* Leuven, juni 2012.
- *Noninvasive fetal sexing and maternal discrimination tests: a validation study for application and implementation in diagnostics*; NVHG voorjaarssymposium, Veldhoven, maart 2011.

Grants/ Awards:

- VIVA400 Award 2015 nominee in the category “Knappe koppen”.
- Travel grant Stichting Simonsfonds (2014).

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Appendices

Appendix 1: Confined placental mosaicism

Placental villi obtained with CVS can be analyzed by two distinct culturing methods. With a semi-direct method (short-term culture or STC) cells from the invading cytotrophoblast are analyzed. After culture (long-term culture or LTC) cells of the mesenchymal lineage are evaluated. Distinction between these two culturing methods and knowledge of the origin of the cells is very important for interpretation of the outcome of prenatal genetic testing on CVS material, since cells of different embryogenic progenitors are analyzed for chromosomal analysis of the fetus with these two distinct culturing methods (Bianchi *et al.*, 1993). When culturing is successful and sufficient sample is provided, preferably a combination of both STC and LTC should be used to interpret prenatal findings.

In the majority of pregnancies the karyotype of the placental cells is similar to the karyotype of the fetus. However, in ~2% of the pregnancies studied by CVS a cytogenetic abnormality is found, most often a trisomy (Kalousek *et al.*, 1996). The existence of a discrepancy between the karyotype from chorionic tissue and embryonic/fetal tissue is caused by complex developmental events during early embryogenesis. When a trisomy is formed soon after fertilization before the trophoblast and the inner cell mass are differentiated, the discrepancy (or mosaic) can be generalized to both placenta and fetal tissues. When it is formed after the separation of the fetal and placental compartments, the abnormal tissue may be confined to either the placenta (**confined placental mosaicism** or **CPM**) or the fetus, but not necessarily to both tissues (Simoni *et al.*, 1992). Therefore, in case of mosaicism it is very important to distinguish between a true fetal mosaicism (TFM) and CPM and confirmatory karyotyping on amniocytes is required to asses which type of mosaicism is present. Mosaicism can be classified according to the distribution of the abnormal cell line (Table 1) (Grati, 2014).

Type	*Nature*	*Trophoblast (direct)*	*Mesenchyme (culture)*	*Amniocytes*
I	CPM	Abnormal	Normal	Normal
II	CPM	Normal	Abnormal	Normal
III	CPM	Abnormal	Abnormal	Normal
IV	TFM	Abnormal	Normal	Abnormal
V	TFM	Normal	Abnormal	Abnormal
VI	TFM	Abnormal	Abnormal	Abnormal
Evaluated in:		**CVS(STC)/ NIPT**	**CVS (LTC)**	**Amniocentesis**

Table 1: Different types of mosaic outcome: (CPM; confined placental mosaisicm, TFM; true fetal mosaicism) found after chorionic villous and amniocytes karyotyping. Adapted from (GRATI, 2014).

Since cffDNA is derived from trophoblast cells, the presence of a possible CPM can also influence results of noninvasive prenatal testing (NIPT) for common fetal aneuploidies. Due to feto-placental mosaicism potential false positive (CPM type I or III) and false negative (TFM type V) results may occur for mosaics in which the trophoblast is cytogenetically discrepant from the fetus. Even though a discrepancy between karyotypes occurs only in around 2% of the cases, in the majority of cases, the mosaicism is confined to the placenta (i.e. CPM type I, ~35%). CPM type III and TFM type V are found in around 10% and 6% of the cases respectively (Grati, 2014). For NIPT, additional genetic analysis of either amniocytes or a combination of STC and LTC is therefore required to determine fetal karyotype in case of an aberrant result.

Appendix 2: Epigenetic allelic ratio, haplotype ratio analysis and relative mutation dosage.

Analysis of the epigenetic allelic ratio (EAR) is a method to assess copy number of a particular chromosome of interest for fetal aneuploidy detection by determining the ratio of an informative single nucleotide polymorphism (SNP) on fetal alleles. This SNP is present within a fetal specific amplified DNA molecule in a differentially methylated region (DMSR) on the chromosome of interest. Bisulfite converted DNA samples are amplified with methylation specific PCR (MSP). Subsequently, methylation differences are assessed with allele-specific primer extension. This primer extension utilizes internal primers which anneal to a PCR-generated template and terminate immediately 5' adjacent to the informative single base variation (Gonzalgo *et al.*, 1997). Extension of these allele specific primers can distinguish between an allele with a nucleotide that is not affected by the conversion (allele A) and an allele with a polymorphism that is affected by the bisulfite conversion (allele B with an unmethylated cytosine (C) which is converted into a thymidine). The extension reactions are designed to generate products of distinct masses (and thus distinct peaks) when analyzing the alleles with Mass Spectrometry.

In euploid fetuses there are equal amounts of allele A and B. Therefore, the theoretical relative peak frequency of allele A and B is both 50% (or 0.5). The EAR can subsequently be calculated by dividing the relative peak frequency of A by the relative peak frequency of B (1). Hence, for euploid fetuses, the EAR is 1.

In case of a fetus with a trisomy, instead of two alleles, three alleles are present. Moreover, there is an overrepresentation of one of these alleles (e.g. either allele A (AAB) or allele B (ABB)). For AAB, there is twice the amount of A alleles contributing to the total amount of three alleles (e.g. twice as much A compared to B). Therefore, the contribution of A relative to the total amounts of alleles is 2 out of 3 (i.e. 67% or 0.67), while relative the B allele is present in only 1 of the 3 alleles (i.e. a relative peak frequency of 33% or 0.33 for B). Therefore, the EAR for AAB is 2 (i.e. 0.67/0.33). In case a fetal trisomy with only one A allele and two B alleles (i.e. ABB), the relative contribution for A is 0.33 and 0.67 for B, resulting in an EAR for ABB of 0.5 (i.e. 0.33/0.67). An EAR that has deviated from 1 (i.e. euploid fetus) is indicative for a fetal trisomy. Adapted from (Tong *et al.*, 2006).

(1) $$EAR = \frac{Relative\ peak\ frequency\ A}{Relative\ peak\ frequency\ B}$$

Another method for fetal aneuploidy detection is the determination of the **haplotype ratio** (HR). In HR analysis only highly heterozygous tandem SNPs on the chromosomes of interest exhibiting three different alleles (haplotypes) are considered informative (i.e. two different maternal alleles and a third distinctive paternally inherited fetal allele) (**Fig. 1**). In contrast to EAR, for HR it is not required to have SNPs only present in DMR, since this method is not based on differences in methylation.

For HR, first Multiplexed Linear Amplification (MLA) is performed on DNA from maternal buccal swaps. This linear amplification product is used as a template in a "sequence specific" PCR and Cycling Temperature Capillary Electrophoresis (CTCE). When results of the maternal buccal swaps indicate that the maternal tandem SNP status is homozygous, the SNPs are not informative. When the maternal SNPs are heterozygous, maternal plasma can be processed similar to the maternal buccal DNA and analyzed subsequently. Fetal chromosome dosage can

be determined by calculating HR using the area under the curve of the three distinct peaks (p1, p2 and p3) in the electropherogram after CTCE (8). As a control, gDNA from maternal buccal swabs or maternal lymphocytes is analyzed and compared to the mixed profile of both mother and fetus in maternal plasma (**Fig. 1**). The maternal contribution to the fetal haplotype can be (quantitatively) compared to the paternally inherited haplotype (yellow peaks) to determine fetal aneuploidy status. In **Fig. 1**, each peak is schematically represented as a triangle and represents one haplotype; either only from the mother (e.g. no fetal contribution; white), only the fetus (e.g. the paternally inherited haplotype; only yellow) or a shared haplotype between mother and fetus (white and yellow). In a maternal plasma sample from a mother carrying a euploid fetus, the presence of three different alleles are informative (Figure 1 A and B). There is a unique non-shared maternal haplotype (white), a haplotype that is shared between mother and fetus (white and yellow) and a distinct unique paternally inherited haplotype (yellow). In a euploid fetus the paternally inherited peak p3 is equal to the relative difference between p1 and p2, resulting in HR = 1 (2). Both maternal and paternal contribution to the fetal genotype is equal.

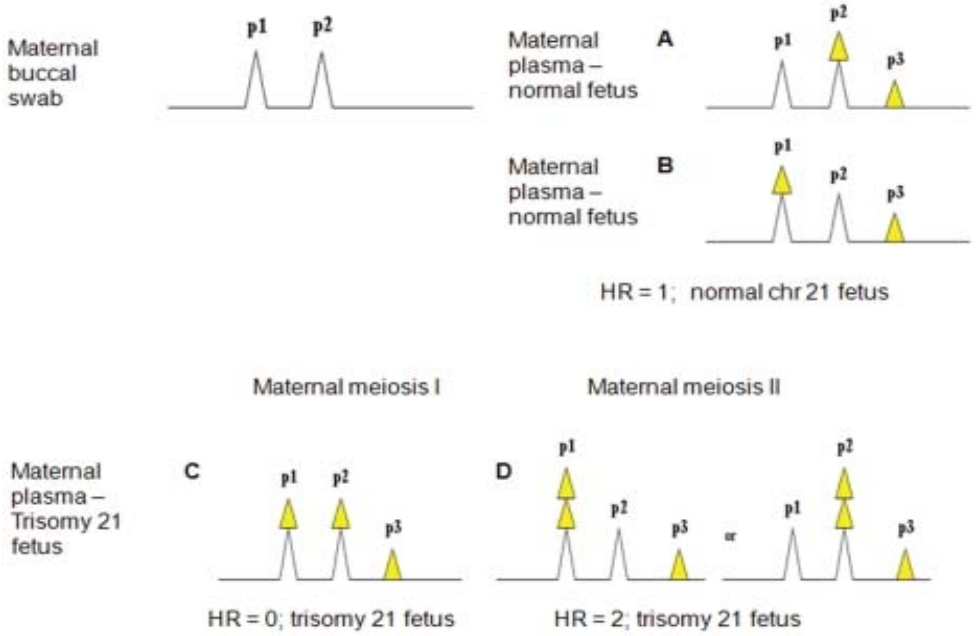

Figure 1: Theoretical CTCE electropherogram output from maternal buccal swab and maternal plasma using tandem SNP analysis. Adapted from (GHANTA et al., 2010).

In case of a plasma sample from a mother carrying a fetus with a trisomy, the CTCE electropherogram shows an uneven contribution between paternal (p3) and maternal haplotypes (p1 and/or p2) of the fetus. Fetal trisomy can be caused either by a familial form (e.g. Robersonian translocation) or, in the majority of cases, by a meiotic nondisjunction event. A normale gamete (ovum or sperm) has one copy of each chromosome, containing 23 chromosomes in total (n). With nondisjunction, chromosomes fail to separate normally, resulting in a gain or loss of a chromosome in a gamete.

(2) $$HR = \frac{(|p1 - p2|)}{p3}$$

Nondisjunction can occur both in mitosis and meiosis. Failure of sister chromatids to separate during mitosis may lead to mosaicism. Failure of a pair of homologue chromosomes to separate in meiosis I (i.e. primary nondisjunction) will result in both members of this homologues pair to be present into the same daughter cell (**Fig. 2 left**) resulting in a fertilized egg with an abnormal number of chromosomes (i.e. aneuploidy). Failure of the sister chromatids to separate during meiosis II (i.e. secondary nondisjunction) will result in both daughter chromosomes going into the same gamete, also resulting in an abnormal number of chromosome in the fertilized cell (**Fig. 2 right**).

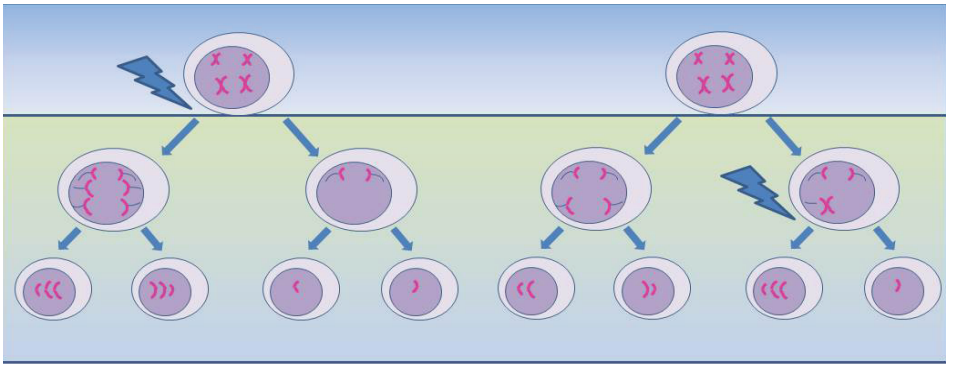

Figure 2: Nondisjunction occurring at meiosis I (left) and meiosis II (right). Nondisjunction in meiosis I will lead to two gametes with an extra chromosome (n+1) and two gametes with a missing chromosome (n-1). After fertilization this will result in a trisomy or monosomy respectively. Nondisjunction in meiosis II will lead to two normal gametes (n) and two abnormal gametes with either an extra chromosome (n+1) or missing one chromosome (n-1).

In case of fetal trisomy, the CTCE plots can also provide information about whether primary or secondary nondisjunction has occurred. When both maternal haplotypes (p1 and p2) are shared in the fetus, nondisjunction occurred during maternal meiosis I. The HR value for maternal meiosis I equals 0 (p1 = p2; therefore the relative difference between p1 and p2 = 0) (Figure 1 C). With secondary maternal nondisjunction, three alleles with different areas are reported whereas p1 or p2 is equal to twice the area of p3, resulting in HR = 2 (Figure 1 D). Adapted from (Ghanta *et al.*, 2010)

In addition to previous described methods used for fetal aneuploidy detection, also digital PCR can be used for fetal aneuploidy detection to determine the relative overrepresentation of a chromosome by calculating relative chromosome dosage (RCD). With RCD the total copy number of a chromosome is assessed in a sample to determine whether this chromosome is overrepresented when compared to a reference chromosome. However, the use of digital PCR is not only restricted to fetal aneuploidy detection in NIPT. Similar to RCD, the principle of digital relative mutation dosage (RMD) can also be applied to NIPD of monogenic diseases. With digital RMD it is no longer required to test only for paternally inherited mutations or fetal sequences that are different (e.g. methylated *RASSF1A*) or absent (e.g. *SRY*) in the mother. With RMD it is possible to compare and measure relative amounts of both the maternal mutant (M) and wild type alleles (N) in maternal plasma to determine the inherited dosage of the mutant allele by the fetus (**Fig. 3**). Therefore, it is no longer necessary to distin-

guish between fetal and maternal sequences. Digital RMD, performed by digital PCR, determines whether the M or N alleles are in balance in maternal plasma (**Fig. 3**). When a pregnant woman and her fetus are both heterozygous for a certain mutation, the amounts of the M allele and N allele are in balance (M=N). When the fetus is homozygous for the mutation, there will be an over-representation of the mutant allele (M>N). When the fetus is wild type, there will be an under-representation of the mutant allele (M<N) in the RMD. Adapted from (Chiu *et al.*, 2009; Lun *et al.*, 2008b).

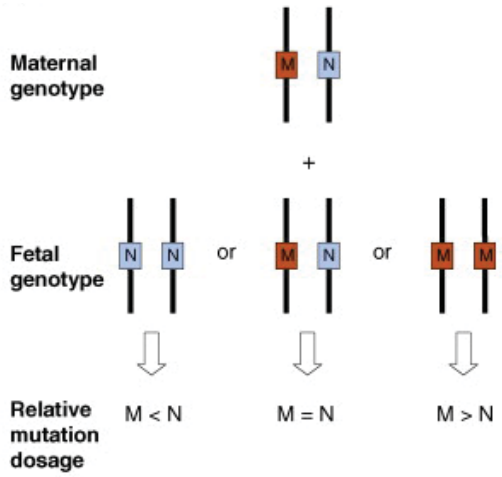

Figure 3: Relative mutation dosage of mutant (M) and normal (N) wild type alleles. Adapted from (Chiu et al., 2009)

Appendix 3: Calculations for trisomy detection

Relative sequence tag density (RSTD):

Shotgun sequencing of numerous of short cfDNA sequences produced after massive parallel sequencing (MPS) are mapped to the chromosome of origin. For each chromosome, these short sequence fragments or reads are counted and summed. In addition, the median of these summed reads from all the autosomes was calculated. To correct for input, for each sample the sum of the reads per chromosome is normalized by dividing this value by the median of all autosomes; the sequence tag density (STD). Male plasma samples or maternal plasma samples from women carrying male euploid fetuses can be used as a reference. STD was also calculated for the controls or reference samples, by first calculating the average of summed tags per chromosome from all reference samples. Subsequently, the median is determined over the values of the autosomes. The average of summed tags per chromosome is normalized by dividing this number by the median value of the autosomes (3). Relative sequence tag density (RSTD) can be determined for each sample by calculating ratios between normalized value per chromosome from each maternal plasma sample and normalized value per chromosome for the controls (4) (Adapted from (Fan *et al.*, 2008).

By determining these ratios, the over- or underrepresentation of any chromosome in maternal plasma contributed by an aneuploidy fetus can be detected. This method does not

require the differentiation between maternal and fetal sequence tags. When a woman carries a healthy fetus, both mother and fetus have 2 copies of each autosome. The RSTD between the normalized value for each autosome as compared to the normalized value of the controls is therefore ~1.

(3) $$Normalized\ chr\ N = \frac{\sum tags\ chr\ N}{median\ autosomes}$$

(4) $$RSTD = \frac{Normalized\ chr\ N_{sample}}{Normalized\ chr\ N_{controls}}$$

A fetus with a trisomy (e.g. trisomy 21) has an additional copy of chromosome 21 as compared to mother. Theoretically, the RSTD of chromosome 21 between mother and this affected fetus would be 1.5 (e.g. the mother has 2 copies of chromosome 21 while the fetus has 3 copies). However, in maternal plasma, the fetal contribution to cfDNA in maternal plasma is only ~10% in the first trimester. Therefore, the RSTD for a plasma sample from a fetus with trisomy 21 is expected to be between 1 and 1.5 due to the relatively small contribution of the additional fetal chromosome 21 as compared to the maternal background (**Table 2**).

Sample	*Fetal DNA content*	*↑ chr 21 (21)*	*↑ chr 21 (fold)*
CVS	100 %	50 %	1.5
Maternal plasma	~10 %	5 %	1.05

Table 2: Theoretical example of fold increase of chromosome 21 in fetal trisomy 21 in gDNA from chorionic villus sampling (CVS) and maternal plasma.

Z-scores:

In fetal trisomy detection, the Z-score refers to the number of standard deviations that the percentage of reads from a particular chromosome in a test sample differs from the mean % of that particular chromosome in a reference data set. Such reference set contains plasma samples from pregnancies of women carrying euploid fetuses. The advantage of a reference set and reference values is that they have to be established only once for a certain run setting. It is therefore no longer required to run control samples together with unknown samples.

For Z-score calculations, first the % representation of unique sequences mapped to a chromosome is calculated by dividing the number of unique count for chromosome N (chr N) by the total counts from that sample (5). Subsequently, the difference between % chr N (x) and the mean % of chr N (μ) in the reference set is determined and divided by the standard deviation (SD; σ) of the % chr N in the reference set to determine the Z score for chromosome N (6). With this Z-score, disease status of the fetus is determined by looking at the overrepresentation of a certain chromosome. For example, a maternal plasma sample with a % chr 21 that is > 3 SD from the mean of the % chr 21 of the euploid reference set is considered to be a fetal trisomy 21 (Adapted from (Chiu *et al.*, 2008) and ISPD preconference NGS Course, 2012).

(5) $$\%\ chr\ N = \frac{Unique\ count\ for\ chr\ N}{Total\ unique\ count}$$

(6) $$chr\ N\ Z-score\ for\ test\ sample = \frac{\%\ chr\ N_{sample} - mean\ \%\ chr\ N_{reference}}{SD\ \%\ chr\ N_{reference}}$$

Or $$Z = \frac{x-\mu}{\sigma}$$

NCV:

Normalized chromosome value (NCV) calculations are based on correction for the intrarun and interrun sequencing variation in the chromosomal distribution of sequence reads. These variations may obscure the effects of fetal aneuploidy on the distribution of mapped sequence sites. For NCV calculations, a chromosome ratio is calculated (7), in which the count of mapped sites for the chromosome of interest is normalized to counts of another predetermined chromosome (or set of chromosomes) of the same sample (8); Modified from (Sehnert *et al.*, 2011).

Sehnert *et al.* used a training set, consisting of pregnancies with unaffected fetuses. For each chromosomes of interest (e.g. chr 13, 18, and 21) they determined the denominator chromosome that minimized the variation of the chromosome ratios within and between the runs (**Table 3**). They also used the training set to determine parameters and boundaries for sample classification (i.e.. mean, SD and NCV classification). An NCV > 4.0 classifies the chromosome as affected (i.e. aneuploidy for that chromosome). An NCV < 2.5 classifies the chromosome as unaffected. Samples with an NCV between 2.5 and 4.0 were classified as "no call". Similar to Z-score calculations, this method does not require additional control sample to be sequenced together with maternal plasma samples when all parameters and boundaries have been established. Adapted and modified from (Sehnert *et al.*, 2011).

(7) $$ratio\ chr\ N = \frac{\#\ reads_{numerator}}{\#\ reads_{denominator}}$$

(8) $$NCV\ chr\ N = \frac{ratio\ chr\ N - mean\ ratio_{trainingset}}{SD\ ratio_{trainingset}}$$

Chromosome of interest	Numerator (chromosome mapped sites)	Denominator (chromosome mapped sites)
21	21	9
18	18	8
13	13	Sum (2-6)

Table 3: Numerator and denominator combinations for noninvasive trisomy detection.

Note: Determination of appropriate control groups and calculation methods that have been addressed in Appendix 3 were used for fetal aneuploidy detection as described in chapters 3 and 4.