Noninvasive prenatal detection of genetic defects

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Chapter 1

General introduction

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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).

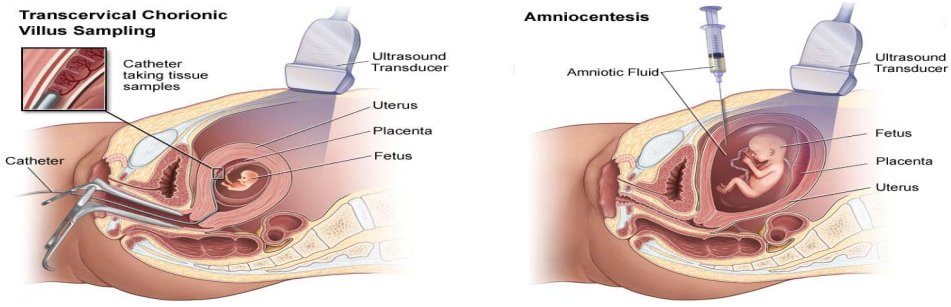

Figure 1: Transcervical chorionic villus sampling of fetal material from the chorion membrane of the placenta (left) and amniocentesis; sampling of amniotic fluid from the uterus (right). Adapted from www.hopkinsmedicine.org.

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**.