Noninvasive fetal genotyping of paternally inherited alleles

ISBN: 978-90-5335-545-9

Layout: Nikki Vermeulen, Ridderprint BV, Ridderkerk, The Netherlands
Print: Ridderprint BV, Ridderkerk, The Netherlands
Cover illustration: Lonneke Haer-Wigman and Peter Scheffer
Centrifuged sample of blood in a test tube. Photographed at Sanquin Research Amsterdam.

Noninvasive fetal genotyping of paternally inherited alleles

Niet-invasieve foetale genotypering van paternaal overgeërfde allelen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag
van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit
van het college voor promoties in het openbaar te verdedigen op
dinsdag 22 mei 2012 des ochtends te 10.30 uur

door

Peter Gerhard Scheffer
geboren op 15 februari 1981 te Utrecht

Promotores:	Prof. dr. C.E. van der Schoot
	Prof. dr. G.H.A. Visser
Co-promotores:	Dr. M. de Haas
	Dr. G.C.M.L. Page-Christiaens

Aan mijn oma†
Aan mijn ouders

Contents

Chapter 1

General introduction and scope of the thesis

Cell-free fetal nucleic acids in the blood of a pregnant woman are a source of fetal genetic material that can be obtained from the expectant mother by a simple venipuncture. In contrast to invasive procedures in which fetal genetic material is obtained from fetal cells directly from the uterus through either chorionic villous sampling between 11 and 14 weeks of gestation or amniocentesis after 15 weeks, a maternal blood draw does not possess the small but finite risk of a procedure-related fetal loss.

In the nearly fifteen years since the discovery of cell-free fetal DNA in the maternal circulation, noninvasive fetal genotyping has translated into clinical practice for several defined prenatal indications. The present emergence of powerful molecular biological techniques will lead to a further widespread clinical use of cell-free fetal nucleic acids as a tool for clinical diagnosis and screening for a variety of genetic conditions including chromosomal aneuploidies, in the near future.

Brief historical overview

Noninvasive prenatal genetic diagnosis has been a long-sought-after goal in prenatal medicine; avoiding the around 1%-risk of miscarriage associated with invasive procedures such as chorionic villous sampling and amniocentesis.[1,2] From the late 1960s, research has focused on the detection and analysis of fetal nucleated cells in the maternal circulation, but the scarcity of these cells (about 1-6 per millilitre of maternal blood), the low efficiency of enrichment methods and difficulties with chromosomal analysis because of dense compact nuclei in some cells have precluded their use as a clinical tool.[3-8] Moreover, as fetal progenitor cells can persist in the maternal circulation many years postpartum,[9] cells derived from previous pregnancies can cause false results. The real breakthrough for noninvasive prenatal genetic diagnosis was in 1997 when the presence of cell-free fetal DNA in maternal plasma and serum was discovered.[10]

The first evidence of circulating nucleic acids in human blood was provided by Mandel and Métais in 1948.[11] Their work was largely overlooked by the scientific community until subsequent researchers detected circulating DNA in the blood from patients suffering from systemic lupus erythematosus,[12] rheumatic arthritis[13] and cancer.[14,15] In particular, in the 1990s it was shown that a proportion of this extracellular (cell-free) DNA in some cancer patients contained tumour-derived genetic signatures.[16-18] Reasoning that the rapidly growing fetus and placenta possessed tumour-like qualities, Lo and colleagues[10] in 1997 demonstrated the presence of cell-free fetal DNA in maternal plasma and serum by detecting Y chromosomal DNA sequences in pregnant women bearing male fetuses, using conventional polymerase chain reaction (PCR) technology and agarose gel electrophoresis. This groundbreaking observation set the stage for extensive research in the field of cell-free fetal nucleic acids and the exploration of the use of these molecules for noninvasive prenatal diagnosis.

Characteristics of cell-free fetal DNA

Several lines of evidence point to the placenta as the predominant source of cell-free fetal DNA in the maternal circulation.[19,20] Firstly, cell-free fetal DNA can be detected in the maternal blood as early as 5 weeks of gestation[21,22] or 18 days after embryo transfer in pregnancies conceived by assisted reproductive techniques,[23] before fetoplacental circulation is established. Secondly, cell-free fetal DNA has been detected in maternal plasma in anembryonic pregnancies,[24] in which placental but no early fetal tissue is present. Thirdly, in cases of confined placental mosaicism, the cell-free DNA sequences detected in maternal blood reflect the placental genetic makeup.[25,26] Finally, the epigenetic signature of cell-free fetal DNA is similar to that of placental tissue.[27-29]

It is generally accepted that cell-free fetal DNA is released from trophoblastic cells undergoing apoptosis.[30] As a consequence of this apoptotic breakdown, cell-free fetal DNA in the maternal circulation typically consists of fragments less than 150 bp in size,[31-33] suggesting its presence as mononucleosomal DNA (the basic unit of DNA packaging in eukaryotes, consisting of a segment of DNA wound around a histone protein core).[34-36]

Within the maternal circulation, cell-free fetal DNA is present among an overwhelming background of maternal cell-free DNA.[37] The maternal cell-free DNA is predominantly of haematopoietic origin,[38] but transplant models have shown other tissues as a source of cell-free DNA as well.[39-43] The mean fractional concentration of cell-free fetal DNA in maternal plasma is approximately 9.7%, 9.0% and 20.4% during first, second and third trimester, respectively, as measured by digital PCR.[44] Earlier real-time PCR measurements of cell-free fetal DNA showed mean quantities of 25.4 genome equivalents per millilitre (GE/mL) of plasma (range 3.3–69.4) in early pregnancy and 292.2 GE/mL of plasma (range 76.9–769) in late pregnancy (representing 3.4% and 6.4% of the total fraction of cell-free DNA, respectively),[37] but quantities vary between and within pregnancies.[45-47] The fractional concentration of cell-free fetal DNA in maternal serum is lower than that in maternal plasma because of the lysis of maternal nucleated blood cells during the clotting process, leading to an increased proportion of maternal DNA in the blood sample.[37,48,49]

After birth, cell-free fetal DNA is cleared from the maternal circulation within several hours, with an observed half-life of 16 minutes.[50] This rapid breakdown implies that during pregnancy cell-free fetal DNA is continuously liberated in large quantities into the maternal circulation. Calculations have suggested a liberation rate of 2.24×10^4 copies per minute.[50] As opposed to fetal cells, which can persist in the maternal circulation for many years,[9] cell-free fetal DNA is not detected in the blood from non-pregnant mothers,[51-53] making it specific for the ongoing pregnancy. It is not yet known how exactly cell-free fetal DNA is cleared from the maternal circulation. Cell-free fetal DNA has been demonstrated in the urine of pregnant women,[54,55] suggesting renal involvement. On the other hand, prolonged postpartum persistence of fetal DNA has been observed in a woman affected by acute fatty liver of pregnancy, indicating

that hepatic disease impairs clearance.[56] These findings suggest that the clearance of cell-free fetal DNA from the maternal circulation is not organ-specific or restricted to the mononuclear phagocyte system. Indeed, animal studies have suggested a role for the liver, kidney and spleen, as well as plasma nucleases for the clearance of cell-free DNA.[57]
In addition to cell-free fetal DNA, cell-free fetal mRNA and placental microRNAs can also be detected in the maternal circulation during pregnancy.[58-61]

Clinical applications

The extraction and subsequent analysis of cell-free fetal DNA from a maternal blood sample offers opportunities for noninvasive prenatal genetic diagnosis.[62,63] Although the entire fetal genome is represented among the cell-free fetal DNA molecules in maternal plasma,[35] the preponderance of maternal DNA sequences currently limits the clinical applications of cell-free fetal DNA to diagnoses that rely on the detection of unique fetal sequences not shared by the maternal genome, i.e. those that the fetus has inherited from its father or mutations that have arisen *de novo*.
Real-time quantitative PCR is the most widely applied technique for the detection and identification of specific cell-free fetal DNA sequences, as it combines high sensitivity with a closed detection system, thereby minimising the risk of contamination.[62] Other methods, such as conventional and nested PCR,[54] pyrophosphorolysis-activated polymerisation[64] and mass spectrometry[65,66] have also been described. More recently, single molecule counting techniques, such as digital PCR and next generation sequencing, have been introduced for noninvasive prenatal genetic diagnosis,[67] allowing the detection and identification of fetal DNA sequences beyond those that are paternally inherited. Although these developments are very exciting, in their current form they are still too complex and costly and will need to be simplified considerably for their optimal translation to the clinic.[68]

Fetal sex determination

Given the relative ease with which Y chromosomal DNA sequences of a male fetus can be distinguished from maternal DNA, fetal sex determination has been one of the first clinical applications of cell-free fetal DNA.[10,69,70] In the first trimester, reliable determination of the fetal sex cannot be performed by means of ultrasonography, because development of the external genitalia is not complete.[71] In pregnancies at risk of X-linked chromosomal abnormalities (estimated cumulative incidence of 5/10,000 live births[72]), such as Duchenne/Becker muscular dystrophy or haemophilia, fetal sex determination using cell-free fetal DNA can spare most female fetuses from unnecessary invasive diagnostic testing and provide decisive answers to expecting parents earlier in pregnancy.[73] In addition, early knowledge of the fetal sex can be useful in pregnancies at risk of congenital adrenal hyperplasia (CAH), in which dexamethasone is administered to the mother as soon as pregnancy is established to reduce virilisation of

potentially affected female fetuses.[74] Finally, noninvasive fetal sex determination using cell-free fetal DNA can also be of help for the assessment of ambiguous genitalia identified on ultrasound examination.[75]

Several Y chromosome-specific sequences have been described to detect male fetuses in maternal plasma.[76,77] The multicopy DYS14 marker sequence of the *TSPY* gene and single copy *SRY* gene sequence are the most widely applied, the former having a 10-fold lower detection limit than the latter.[78] Because female fetuses are merely inferred by a negative result for Y chromosome-specific sequences and noninvasive fetal sex determination is usually performed early in pregnancy when cell-free fetal DNA concentrations are still relatively low, a control assay to confirm the actual presence of cell-free fetal DNA is indicated.

Fetal red cell blood group genotyping

Red cell blood group antigen incompatibility between a pregnant women and her fetus can result in maternal alloimmunisation and consequently haemolytic disease of the fetus and newborn (HDFN), a condition characterised by fetal anaemia, hydrops and intrauterine death, and neonatal hyperbilirubinaemia and kernicterus.[79] Reported prevalences of severe HDFN, defined as perinatal death or the need for intrauterine transfusions and/or exchange or top-up transfusions in the first week of life, vary from 3/100,000 to 80/100,000.[80] Pregnancies complicated by red cell alloimmunisation – usually identified by standard antibody screening at first booking before twelve weeks of gestation – require careful monitoring for fetal anaemia with serial assessment of maternal antibody titres and activity, fetal ultrasound measurements of the peak systolic velocity in the middle cerebral artery and, if indicated, intrauterine fetal blood sampling.[81] The D antigen of the rhesus (Rh) blood group system is the most frequently involved antigen in HDFN, and despite the widespread use of prophylactic antenatal and postnatal anti-D immunoglobulin, Rh D alloimmunisation is still a significant cause of fetal and neonatal morbidity and mortality. In addition, alloimmunisation to the Rh c antigen and the K antigen of the Kell blood group system can also cause severe HDFN.[82-84]

In red cell alloimmunised pregnant women, knowledge of the fetal antigen status is beneficial to tailor the pregnancy management. If the fetus is negative (i.e. does not carry the implicated antigen), it is not at risk of HDFN and extensive monitoring for fetal anaemia can be omitted. In addition, in nonimmunised D-negative pregnant women, knowledge of the fetal D antigen status can restrict the use of antenatal anti-D immunoprophylaxis, administered between 28 and 34 weeks of gestation, and after invasive procedures, to the around 60% of pregnant women carrying a D-positive child,[85] saving on a valuable human blood product produced from volunteer donors.

In the Caucasian population, the D-negative phenotype is generally caused by a homozygous deletion of the *RHD* gene.[86] Given this fact, after the initial demonstration that Y chromosomal DNA sequences could be detected in the plasma from women carrying male fetuses, the

detection of fetal *RHD* sequences in the plasma from D-negative pregnant women to determine the fetal D phenotype was rapidly seized upon.[87,88] However, it should be emphasised that, partly due to the structure of the *RH* locus, in which the two highly homologues *RH* genes, *RHCE* and *RHD*, are present in opposite orientation,[89] many variant *RH* genes have evolved through rearrangements of these genes as well as through mutations. In particular, in the African population, many variant *RH* genes exist and in the majority of D-negative African Blacks the *RHD* gene is not deleted but rather inactivated.[90-92] So, although detection of a single region of *RHD* in the plasma from D-negative pregnant women can provide a correct fetal phenotype in the majority of cases, it is prudent to design noninvasive fetal *RHD* genotyping assays in such a way that false results do not arise from the presence of inactivated maternal *RHD* genes or *RHD* variant genes. Therefore, in most assays, two or three exons of the *RHD* gene are targeted.[93,94]

At present, noninvasive fetal *RHD* genotyping in D-alloimmunised pregnant women has translated into routine clinical practice in several European countries.[95,96] What is more, following large-scale fetal *RHD* genotyping studies,[97-99] nation-wide fetal *RHD* screening programmes to ascertain the requirement of anti-D immunoprophylaxis in nonimmunised D-negative pregnant women have recently been introduced in Denmark and The Netherlands.[100] Noninvasive fetal blood group genotyping assays for other clinically relevant red cell antigens have also been developed.[101-103] However, since the differences between the maternal and fetal alleles of most blood group antigens are much more subtle as compared with *RHD* (e.g. only differing by one single nucleotide rather than by the complete absence/presence of a whole gene locus), some assays are hampered by nonspecific amplification of maternal DNA and therefore only a few laboratories offer these tests diagnostically.[96]

Fetal platelet blood group genotyping

Similar to HDFN, platelet blood group antigen incompatibility between a pregnant women and her fetus can result in fetal or neonatal alloimmune thrombocytopenia (FNAIT), a potentially devastating condition, which may lead to intracranial haemorrhage in the fetus or neonate, often with death or major neurological damage as consequence.[104,105] FNAIT complicates about 0.1% of all pregnancies, most cases caused by alloimmunisation to the human platelet antigen (HPA)-1a.[106,107] Antenatal treatment may consist of weekly administration of intravenous immunoglobulins, to the mother, with or without corticosteroids, or fetal blood sampling with platelet infusions.[108,109] In the absence of screening programmes, treatment is currently only provided in pregnancies subsequent to the birth of a previously affected child or pregnancies known to be at risk because of a family history.[110,111] Currently, no reliable noninvasive fetal genotyping assays to predict the fetal human platelet antigen phenotype are clinically available and in some countries invasive testing to assess fetal risk in platelet alloimmunised pregnant women is still performed.[112]

Single gene disorders

Noninvasive diagnosis of dominant diseases that are paternally inherited or occur *de novo* as a result of spontaneous mutation is possible due to the absence of the disease causing allele in the maternal genome. Several case reports on the diagnosis of autosomal dominant diseases in maternal plasma have been published,[63,113] including the diagnosis of Huntington's disease,[114,115] achondroplasia[116-119] and myotonic dystrophy.[120] For autosomal recessive diseases in which both parents carry a different mutation, the absence of the paternal mutated allele and/or presence of the normal paternal allele in maternal plasma can rule out the possibility of the fetus being compound heterozygous.[121,122] This approach has been described for, among others, cystic fibrosis[123,124] and β-thallasaemia.[65,125-127]

The detection of monogenetic diseases other than those caused by unique paternally inherited mutations is much more challenging as the maternally inherited alleles are genotypically identical to the maternal background DNA. Recently, however, Lun et al.,[128] using digital PCR, reported the successful determination of the fetal inheritance of haemoglobin E and β-thallasaemia mutations in mothers carrying either mutation. Similarly, on the same platform, Tsui et al. [129] were able to detect whether a male fetus had inherited a causative mutation for haemophilia from its mother.

Fetal aneuploidy

Considered the 'holy grail' of prenatal diagnosis, noninvasive fetal aneuploidy detection in maternal plasma has been subject of intensive research by many groups. Various approaches have been developed to detect the small increased proportion of DNA from the extra fetal chromosome in maternal plasma.[130-137] The most promising approach appears to be based on massively parallel sequencing.[138-141] Awaiting large prospective studies in low-risk populations, fetal aneuploidy detection in maternal plasma is likely to become a clinical reality in the foreseeable future.

Complications of pregnancy

Various pregnancy-related disorders, including preeclampsia, HELLP syndrome, intrauterine growth restriction and invasive placentation have been linked to increased concentrations of cell-free fetal DNA.[142-146] It has therefore been suggested that quantitative analysis of cell-free fetal DNA may be of value in prediction models for pregnancy-related complications.[147,148] However, since absolute levels of cell-free fetal DNA in the maternal circulation fluctuate over short periods of time[46] and vary with both ethnicity[149] and maternal weight,[150] cell-free fetal DNA levels are likely not discriminative enough to be used as a stand-alone marker.[151] Notably, increased levels of maternal cell-free DNA have also been observed in pregnancies complicated by preeclampsia and HELLP syndrome.[152,153]

Scope and outline of the thesis

The research presented in this thesis focuses on the performance of noninvasive fetal genotyping of paternally inherited alleles in clinical practice.

One of the first clinical applications of cell-free fetal DNA has been fetal sex determination. Yet, variable results have been reported by different laboratories, most groups only describing their achievements in a research setting. In the majority of studies, a positive control for the presence of fetal DNA was not included. This has hampered a fruitful introduction of noninvasive fetal sex determination in clinical practice. We therefore investigate the diagnostic accuracy and clinical applicability of fetal sex determination using cell-free fetal DNA isolated from maternal plasma by the analysis of a 6-year clinical service of noninvasive fetal sex determination in our laboratory (**chapter 2**).

We next evaluate a 7-year clinical experience of noninvasive fetal blood group genotyping of Rh D, c, E and of K in red cell alloimmunised pregnant women. The results of these assays were used to guide the clinical and laboratory management of the alloimmunised pregnancies. Knowledge of the fetal blood group antigen status in red cell alloimmunised pregnant women is beneficial to determine whether a fetus is at risk of haemolytic disease of the fetus and newborn in which case extensive monitoring for fetal anaemia during pregnancy is required (**chapter 3**).

In pregnancies complicated by the presence of maternal anti-human-platelet-antigen (HPA)-1a-alloantibodies, knowledge of the fetal HPA-1a status is beneficial to determine whether a fetus is at risk of fetal or neonatal alloimmune thrombocytopenia, in which case antenatal treatment is required. However, so far no reliable noninvasive fetal HPA-1a genotyping assay has been available, since all assays were hampered by nonspecific amplification of the maternal HPA-1b allele. We therefore aimed to develop a specific and sensitive noninvasive fetal genotyping assay of human platelet antigen-1a. Results of these studies are described in **chapter 4**.

The interpretation of negative results in noninvasive fetal genotyping assays is challenging. As well as such a result being indicative of a negative phenotype (true negative), it could also arise from the absence of fetal DNA in the detection system (false negative). Noninvasive fetal blood group genotyping assays have been found to be generally robust and there has been discussion about the absolute need for positive controls to confirm the presence of fetal DNA in these assays. We therefore thoroughly review all available literature on the various types of controls to prevent false-negative results in **chapter 5** and discuss their value in noninvasive fetal blood group genotyping assays for different indications.

A nation-wide fetal *RHD* screening programme for targeted anti-D immunoglobulin prophylaxis has recently been introduced in The Netherlands. The challenge of the Dutch programme is to correctly predict the fetal D phenotype in a multiethnic population. As part of this thesis, the performance of the fetal *RHD* typing algorithm is evaluated (**chapter 6**).

Finally, in **chapter 7**, the findings in this thesis are summarised and some concluding remarks are made.

References

1 Alfirevic Z, Sundberg K, Brigham S. Amniocentesis and chorionic villus sampling for prenatal diagnosis. *Cochrane Database Syst Rev* 2003;CD003252.

2 Mujezinovic F, Alfirevic Z. Procedure-related complications of amniocentesis and chorionic villous sampling: a systematic review [published erratum appears in Obstet Gynecol 2008;111:779]. *Obstet Gynecol* 2007;110:687–94.

3 Lapaire O, Holzgreve W, Oosterwijk JC, Brinkhaus R, Bianchi DW. Georg Schmorl on trophoblasts in the maternal circulation. *Placenta* 2007;28:1–5.

4 Walknowska J, Conte FA, Grumbach MM. Practical and theoretical implications of fetal-maternal lymphocyte transfer. *Lancet* 1969;1:1119–22.

5 Oosterwijk JC. Prenatal diagnosis on fetal cells from maternal blood: approaches and perspectives. *Eur J Obstet Gynecol Reprod Biol* 1999;82:169–70.

6 van Wijk IJ, de Hoon AC, Jurhawan R, Tjoa ML, Griffioen S, Mulders MA et al. Detection of apoptotic fetal cells in plasma of pregnant women. *Clin Chem* 2000;46:729–31.

7 Bianchi DW, Simpson JL, Jackson LG, Elias S, Holzgreve W, Evans MI et al. Fetal gender and aneuploidy detection using fetal cells in maternal blood: analysis of NIFTY I data. National Institute of Child Health and Development Fetal Cell Isolation Study. *Prenat Diagn* 2002;22:609–15.

8 Babochkina T, Mergenthaler S, De Napoli G, Hristoskova S, Tercanli S, Holzgreve W et al. Numerous erythroblasts in maternal blood are impervious to fluorescent in situ hybridization analysis, a feature related to a dense compact nucleus with apoptotic character. *Haematologica* 2005;90:740–5.

9 Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 1996;93:705–8.

10 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

11 Mandel P, Métais P. Les acides nucléiques du plasma sanguin chez l'homme. *C R Acad Sci Paris* 1948;142:241–3.

12 Tan EM, Schur PH, Carr RI, Kunkel HG. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J Clin Invest* 1966;45:1732–40.

13 Lindstedt G, Lundberg PA, Iwarsson S, Lindberg J. Circulating heat-labile DNA binder(s) in chronic active hepatitis and rheumatoid arthritis. *Clin Chim Acta* 1975;62:183–5.

14 Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977;37:646–50.

15 Stroun M, Anker P, Lyautey J, Lederrey C, Maurice PA. Isolation and characterization of DNA from the plasma of cancer patients. *Eur J Cancer Clin Oncol* 1987;23:707–12.

16 Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat Med* 1996;2:1033–5.

17 Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996;2:1035–7.

18 Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Chen XQ et al. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. *Gastroenterology* 1997;112:1114–20.

19 Bianchi DW. Circulating fetal DNA: its origin and diagnostic potential - a review. *Placenta* 2004;25 Suppl A:S93–S101.

20 Bischoff FZ, Lewis DE, Simpson JL. Cell-free fetal DNA in maternal blood: kinetics, source and structure. *Hum Reprod Update* 2005;11:59–67.

21 Rijnders RJP, van der Luijt RB, Peters EDJ, Goeree JK, van der Schoot CE, Ploos van Amstel JK et al. Earliest gestational age for fetal sexing in cell-free maternal plasma. *Prenat Diagn* 2003;23:1042–4.

22 Illanes S, Denbow M, Kailasam C, Finning K, Soothill PW. Early detection of cell-free fetal DNA in maternal plasma. *Early Hum Dev* 2007;83:563–6.

23 Guibert J, Benachi A, Grebille AG, Ernault P, Zorn JR, Costa JM. Kinetics of SRY gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique. *Hum Reprod* 2003;18:1733–6.

24 Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N et al. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenat Diagn* 2007;27:415–8.

25 Flori E, Doray B, Gautier E, Kohler M, Ernault P, Flori J et al. Circulating cell-free fetal DNA in maternal serum appears to originate from cyto- and syncytio-trophoblastic cells. Case report. *Hum Reprod* 2004;19:723–4.

26 Masuzaki H, Miura K, Yoshiura K, Yoshimura S, Niikawa N, Ishimaru T. Detection of cell free placental DNA in maternal plasma: direct evidence from three cases of confined placental mosaicism. *J Med Genet* 2004;41:289–92.

27 Poon LLM, Leung TN, Lau TK, Chow KCK, Lo YMD. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin Chem* 2002;48:35–41.

28 Chim SSC, Tong YK, Chiu RWK, Lau TK, Leung TN, Chan LYS et al. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc Natl Acad Sci U S A* 2005;102:14753–8.

29 Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN et al. Hypermethylated RASSF1A in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.

30 Tjoa ML, Cindrova-Davies T, Spasic-Boskovic O, Bianchi DW, Burton GJ. Trophoblastic oxidative stress and the release of cell-free feto-placental DNA. *Am J Pathol* 2006;169:400–4.

31 Chan KCA, Zhang J, Hui ABY, Wong N, Lau TK, Leung TN et al. Size distributions of maternal and fetal DNA in maternal plasma. *Clin Chem* 2004;50:88–92.

32 Li Y, Zimmermann B, Rusterholz C, Kang A, Holzgreve W, Hahn S. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. *Clin Chem* 2004;50:1002–11.

33 Sikora A, Zimmermann BG, Rusterholz C, Birri D, Kolla V, Lapaire O et al. Detection of increased amounts of cell-free fetal DNA with short PCR amplicons. *Clin Chem* 2010;56:136–8.

34 Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Analysis of the size distributions of fetal and maternal cell-free DNA by paired-end sequencing. *Clin Chem* 2010;56:1279–86.

35 Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, Lun FMF et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med* 2010;2:61ra91.

36 Kimura M, Hara M, Itakura A, Sato C, Ikebuchi K, Ishihara O. Fragment size analysis of free fetal DNA in maternal plasma using Y-STR loci and SRY gene amplification. *Nagoya J Med Sci* 2011;73:129–35.

37 Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768–75.

38 Lui YYN, Chik KW, Chiu RWK, Ho CY, Lam CWK, Lo YMD. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. *Clin Chem* 2002;48:421–7.

39 Lo YM, Tein MS, Pang CC, Yeung CK, Tong KL, Hjelm NM. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. *Lancet* 1998;351:1329–30.

40 Minon JM, Senterre JM, Schaaps JP, Foidart JM. An unusual false-positive fetal RHD typing result using DNA derived from maternal plasma from a solid organ transplant recipient. *Transfusion* 2006;46:1454–5.

41 Partsalis T, Chan LYS, Hurworth M, Willers C, Pavlos N, Kumta N et al. Evidence of circulating donor genetic material in bone allotransplantation. *Int J Mol Med* 2006;17:1151–5.

42 Snyder TM, Khush KK, Valantine HA, Quake SR. Universal noninvasive detection of solid organ transplant rejection. *Proc Natl Acad Sci U S A* 2011;108:6229–34.

43 Zheng Y, Chan K, Sun H, Jiang P, Su X, Chen E et al. Nonhematopoietically derived DNA is shorter than hematopoietically derived DNA in plasma: a transplantation model. *Clin Chem* 2011;58:549–58.

44 Lun FMF, Chiu RWK, Allen Chan KC, Yeung Leung T, Kin Lau T, Dennis Lo YM. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. *Clin Chem* 2008;54:1664–72.

45 Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo M, Saito H et al. Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem* 2001;47:1856–8.

46 Hahn S, Zhong XY, Burk MR, Troeger C, Kang A, Holzgreve W. Both maternal and fetal cell-free DNA in plasma fluctuate. *Ann N Y Acad Sci* 2001;945:141–4.

47 Birch L, English CA, O'Donoghue K, Barigye O, Fisk NM, Keer JT. Accurate and robust quantification of circulating fetal and total DNA in maternal plasma from 5 to 41 weeks of gestation. *Clin Chem* 2005;51:312–20.

48 Lee TH, Montalvo L, Chrebtow V, Busch MP. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. *Transfusion* 2001;41:276–82.

49 Fernando MR, Chen K, Norton S, Krzyzanowski G, Bourne D, Hunsley B et al. A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. *Prenat Diagn* 2010;30:418–24.

50 Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999;64:218–24.

51 Benachi A, Steffann J, Gautier E, Ernault P, Olivi M, Dumez Y et al. Fetal DNA in maternal serum: does it persist after pregnancy? *Hum Genet* 2003;113:76–9.

52 Smid M, Galbiati S, Vassallo A, Gambini D, Ferrari A, Viora E et al. No evidence of fetal DNA persistence in maternal plasma after pregnancy. *Hum Genet* 2003;112:617–8.

53 Rijnders RJP, Christiaens GCML, Soussan AA, van der Schoot CE. Cell-free fetal DNA is not present in plasma of nonpregnant mothers. *Clin Chem* 2004;50:679–81.

54 Al-Yatama MK, Mustafa AS, Ali S, Abraham S, Khan Z, Khaja N. Detection of Y chromosome-specific DNA in the plasma and urine of pregnant women using nested polymerase chain reaction. *Prenat Diagn* 2001;21:399–402.

55 Shekhtman EM, Anne K, Melkonyan HS, Robbins DJ, Warsof SL, Umansky SR. Optimization of transrenal DNA analysis: detection of fetal DNA in maternal urine. *Clin Chem* 2009;55:723–9.

56 Nelson M, Eagle C, Langshaw M, Popp H, Kronenberg H. Genotyping fetal DNA by non-invasive means: extraction from maternal plasma. *Vox Sang* 2001;80:112–6.

57 Emlen W, Mannik M. Effect of DNA size and strandedness on the in vivo clearance and organ localization of DNA. *Clin Exp Immunol* 1984;56:185–92.

58 Poon LL, Leung TN, Lau TK, Lo YM. Presence of fetal RNA in maternal plasma. *Clin Chem* 2000;46:1832–4.

59 Ng EKO, Tsui NBY, Lau TK, Leung TN, Chiu RWK, Panesar NS et al. mRNA of placental origin is readily detectable in maternal plasma. *Proc Natl Acad Sci U S A* 2003;100:4748–53.

60 Chim SSC, Shing TKF, Hung ECW, Leung TY, Lau TK, Chiu RWK et al. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem* 2008;54:482–90.

61 Go ATJI, van Vugt JMG, Oudejans CBM. Non-invasive aneuploidy detection using free fetal DNA and RNA in maternal plasma: recent progress and future possibilities. *Hum Reprod Update* 2011;17:372–82.

62 Wright CF, Burton H. The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. *Hum Reprod Update* 2009;15:139–51.

63 Bustamante-Aragones A, Gonzalez-Gonzalez C, de Alba MR, Ainse E, Ramos C. Noninvasive prenatal diagnosis using ccffDNA in maternal blood: state of the art. *Expert Rev Mol Diagn* 2010;10:197–205.

64 Boon EMJ, Schlecht HB, Martin P, Daniels G, Vossen RHAM, den Dunnen JT et al. Y chromosome detection by Real Time PCR and pyrophosphorolysis-activated polymerisation using free fetal DNA isolated from maternal plasma. *Prenat Diagn* 2007;27:932–7.

65 Ding C, Chiu RWK, Lau TK, Leung TN, Chan LC, Chan AYY et al. MS analysis of single-nucleotide differences in circulating nucleic acids: Application to noninvasive prenatal diagnosis. *Proc Natl Acad Sci U S A* 2004;101:10762–7.

66 Zhong X, Holzgreve W. MALDI-TOF MS in Prenatal Genomics. *Transfus Med Hemother* 2009;36:263–72.

67 Chiu RWK, Cantor CR, Lo YMD. Non-invasive prenatal diagnosis by single molecule counting technologies. *Trends Genet* 2009;25:324–31.

68 Hahn S, Lapaire O, Tercanli S, Kolla V, Hosli I. Determination of fetal chromosome aberrations from fetal DNA in maternal blood: has the challenge finally been met? *Expert Rev Mol Med* 2011;13:e16.

69 Zhong XY, Holzgreve W, Hahn S. Detection of fetal Rhesus D and sex using fetal DNA from maternal plasma by multiplex polymerase chain reaction. *BJOG* 2000;107:766–9.

70 Honda H, Miharu N, Ohashi Y, Ohama K. Successful diagnosis of fetal gender using conventional PCR analysis of maternal serum. *Clin Chem* 2001;47:41–6.

71 Odeh M, Granin V, Kais M, Ophir E, Bornstein J. Sonographic fetal sex determination. *Obstet Gynecol Surv* 2009;64:50–7.

72 Baird PA, Anderson TW, Newcombe HB, Lowry RB. Genetic disorders in children and young adults: a population study. *Am J Hum Genet* 1988;42:677–93.

73 Costa JM, Benachi A, Gautier E. New strategy for prenatal diagnosis of X-linked disorders. *N Engl J Med* 2002;346:1502.

74 Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christiaens GCML. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. *Obstet Gynecol* 2001;98:374–8.

75 Pajkrt E, Petersen OB, Chitty LS. Fetal genital anomalies: an aid to diagnosis. *Prenat Diagn* 2008;28:389–98.

76 Finning KM, Chitty LS. Non-invasive fetal sex determination: impact on clinical practice. *Semin Fetal Neonatal Med* 2008;13:69–75.

77 Devaney SA, Palomaki GE, Scott JA, Bianchi DW. Noninvasive fetal sex determination using cell-free fetal DNA: a systematic review and meta-analysis. *JAMA* 2011;306:627–36.

78 Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. *Clin Chem* 2005;51:1598–604.

79 Urbaniak SJ, Greiss MA. RhD haemolytic disease of the fetus and the newborn. *Blood Rev* 2000;14:44–61.

80 Koelewijn, JM. Detection and prevention of pregnancy immunisation – The OPZI study. Thesis. Groningen: University of Amsterdam 2009.

81 Moise KJJ. Management of rhesus alloimmunization in pregnancy. *Obstet Gynecol* 2008;112:164–76.

82 van Wamelen DJ, Klumper FJ, de Haas M, Meerman RH, van Kamp IL, Oepkes D. Obstetric history and antibody titer in estimating severity of Kell alloimmunization in pregnancy. *Obstet Gynecol* 2007;109:1093–8.

83 Koelewijn JM, Vrijkotte TGM, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. *Transfusion* 2008;48:941–52.

84 Moise KJ. Fetal anemia due to non-Rhesus-D red-cell alloimmunization. *Semin Fetal Neonatal Med* 2008;13:207–14.

85 Race RR, Mourant AE. The Rh chromosomes in England. *Blood* 1948;3:689–95.

86 Colin Y, Cherif-Zahar B, Le Van Kim C, Raynal V, Van Huffel V, Cartron JP. Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by Southern analysis. *Blood* 1991;78:2747–52.

87 Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* 1998;352:1196.

88 Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734–8.

89 Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. *Blood* 2000;95:3662–8.

90 Faas BH, Beckers EA, Wildoer P, Ligthart PC, Overbeeke MA, Zondervan HA et al. Molecular background of VS and weak C expression in blacks. *Transfusion* 1997;37:38–44.

91 Daniels GL, Faas BH, Green CA, Smart E, Maaskant-van Wijk PA, Avent ND et al. The VS and V blood group polymorphisms in Africans: a serologic and molecular analysis. *Transfusion* 1998;38:951–8.

92 Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D-negative blood group phenotype. *Blood* 2000;95:12–8.

93 Grootkerk-Tax MGHM, Soussan AA, de Haas M, Maaskant-van Wijk PA, van der Schoot CE. Evaluation of prenatal RHD typing strategies on cell-free fetal DNA from maternal plasma. *Transfusion* 2006;46:2142–8.

94 Avent ND. RHD genotyping from maternal plasma: guidelines and technical challenges. *Methods Mol Biol* 2008;444:185–201.

95 Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002;42:1079–85.

96 Daniels G, van der Schoot CE, Olsson ML. Report of the fourth International Workshop on molecular blood group genotyping. *Vox Sang* 2011;101:327–32.

97 van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Page-Christiaens GCML, de Haas M. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006;13:53–7.

98 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* 2008;336:816–8.

99 Muller SP, Bartels I, Stein W, Emons G, Gutensohn K, Kohler M et al. The determination of the fetal D status from maternal plasma for decision making on Rh prophylaxis is feasible. *Transfusion* 2008;48:2292–301.

100 Clausen F, Christiansen M, Steffensen R, Jorgensen S, Nielsen C, Jakobsen M et al. Report of the first nationally implemented clinical routine screening for fetal RHD in D– pregnant women to ascertain the requirement for antenatal RhD prophylaxis. *Transfusion* 2011.

101 Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c, and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion* 2007;47:2126–33.

102 Geifman-Holtzman O, Grotegut CA, Gaughan JP, Holtzman EJ, Floro C, Hernandez E. Noninvasive fetal RhCE genotyping from maternal blood. *BJOG* 2009;116:144–51.

103 Gutensohn K, Muller SP, Thomann K, Stein W, Suren A, Kortge-Jung S et al. Diagnostic accuracy of noninvasive polymerase chain reaction testing for the determination of fetal rhesus C, c and E status in early pregnancy. *BJOG* 2010;117:722–9.

104 Williamson LM, Hackett G, Rennie J, Palmer CR, Maciver C, Hadfield R et al. The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PlA1, Zwa) as determined by antenatal screening. *Blood* 1998;92:2280–7.

105 Kamphuis MM, Paridaans N, Porcelijn L, de Haas M, van der Schoot CE, Brand A et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG* 2010;117:1335–43.

106 Mueller-Eckhardt C, Kiefel V, Grubert A, Kroll H, Weisheit M, Schmidt S et al. 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet* 1989;1:363–6.

107 Knight M, Pierce M, Allen D, Kurinczuk JJ, Spark P, Roberts DJ et al. The incidence and outcomes of fetomaternal alloimmune thrombocytopenia: a UK national study using three data sources. *Br J Haematol* 2011;152:460–8.

108 van den Akker ESA, Oepkes D, Lopriore E, Brand A, Kanhai HHH. Noninvasive antenatal management of fetal and neonatal alloimmune thrombocytopenia: safe and effective. *BJOG* 2007;114:469–73.

109 Berkowitz RL, Lesser ML, McFarland JG, Wissert M, Primiani A, Hung C et al. Antepartum treatment without early cordocentesis for standard-risk alloimmune thrombocytopenia: a randomized controlled trial. *Obstet Gynecol* 2007;110:249–55.

110 Bussel JB, Berkowitz RL, Hung C, Kolb EA, Wissert M, Primiani A et al. Intracranial hemorrhage in alloimmune thrombocytopenia: stratified management to prevent recurrence in the subsequent affected fetus. *Am J Obstet Gynecol* 2010;203:135–14.

111 Kamphuis MM, Paridaans N, Porcelijn L, de Haas M, van der Schoot CE, Brand A et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG* 2010;117:1335–43.

112 Pacheco LD, Berkowitz RL, Moise KJJ, Bussel JB, McFarland JG, Saade GR. Fetal and neonatal alloimmune thrombocytopenia: a management algorithm based on risk stratification. *Obstet Gynecol* 2011;118:1157–63.

113 Norbury G, Norbury CJ. Non-invasive prenatal diagnosis of single gene disorders: how close are we? *Semin Fetal Neonatal Med* 2008;13:76–83.

114 Gonzalez-Gonzalez MC, Trujillo MJ, Rodriguez de Alba M, Garcia-Hoyos M, Lorda-Sanchez I, Diaz-Recasens J et al. Huntington disease-unaffected fetus diagnosed from maternal plasma using QF-PCR. *Prenat Diagn* 2003;23:232–4.

115 Bustamante-Aragones A, Trujillo-Tiebas MJ, Gallego-Merlo J, Rodriguez de Alba M, Gonzalez-Gonzalez C, Cantalapiedra D et al. Prenatal diagnosis of Huntington disease in maternal plasma: direct and indirect study. *Eur J Neurol* 2008;15:1338–44.

116 Saito H, Sekizawa A, Morimoto T, Suzuki M, Yanaihara T. Prenatal DNA diagnosis of a single-gene disorder from maternal plasma. *Lancet* 2000;356:1170.

117 Li Y, Page-Christiaens GCML, Gille JJP, Holzgreve W, Hahn S. Non-invasive prenatal detection of achondroplasia in size-fractionated cell-free DNA by MALDI-TOF MS assay. *Prenat Diagn* 2007;27:11–7.

118 Lim JH, Kim MJ, Kim SY, Kim HO, Song MJ, Kim MH et al. Non-invasive prenatal detection of achondroplasia using circulating fetal DNA in maternal plasma. *J Assist Reprod Genet* 2011;28:167–72.

119 Chitty LS, Griffin DR, Meaney C, Barrett A, Khalil A, Pajkrt E et al. New aids for the non-invasive prenatal diagnosis of achondroplasia: dysmorphic features, charts of fetal size and molecular confirmation using cell-free fetal DNA in maternal plasma. *Ultrasound Obstet Gynecol* 2011;37:283–9.

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

121 Ho SSY, Chong SSC, Koay ESC, Ponnusamy S, Chiu L, Chan YH et al. Noninvasive prenatal exclusion of haemoglobin Bart's using foetal DNA from maternal plasma. *Prenat Diagn* 2010;30:65–73.

122 Yan TZ, Mo QH, Cai R, Chen X, Zhang CM, Liu YH et al. Reliable detection of paternal SNPs within deletion breakpoints for non-invasive prenatal exclusion of homozygous alpha-thalassemia in maternal plasma. *PLoS One* 2011;6:e24779.

123 Nasis O, Thompson S, Hong T, Sherwood M, Radcliffe S, Jackson L et al. Improvement in sensitivity of allele-specific PCR facilitates reliable noninvasive prenatal detection of cystic fibrosis. *Clin Chem* 2004;50:694–701.

124 Bustamante-Aragones A, Gallego-Merlo J, Trujillo-Tiebas MJ, de Alba MR, Gonzalez-Gonzalez C, Glover G et al. New strategy for the prenatal detection/exclusion of paternal cystic fibrosis mutations in maternal plasma. *J Cyst Fibros* 2008;7:505–10.

125 Chiu RWK, Lau TK, Leung TN, Chow KCK, Chui DHK, Lo YMD. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *Lancet* 2002;360:998–1000.

126 Li Y, Di Naro E, Vitucci A, Zimmermann B, Holzgreve W, Hahn S. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. *JAMA* 2005;293:843–9.

127 Galbiati S, Brisci A, Lalatta F, Seia M, Makrigiorgos GM, Ferrari M et al. Full COLD-PCR protocol for noninvasive prenatal diagnosis of genetic diseases. *Clin Chem* 2011;57:136–8.

128 Lun FMF, Tsui NBY, Chan KCA, Leung TY, Lau TK, Charoenkwan P et al. Noninvasive prenatal diagnosis of monogenic diseases by digital size selection and relative mutation dosage on DNA in maternal plasma. *Proc Natl Acad Sci U S A* 2008;105:19920–5.

129 Tsui NBY, Kadir RA, Chan KCA, Chi C, Mellars G, Tuddenham EG et al. Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA. *Blood* 2011;117:3684–91.

130 Tong YK, Ding C, Chiu RWK, Gerovassili A, Chim SSC, Leung TY et al. Noninvasive prenatal detection of fetal trisomy 18 by epigenetic allelic ratio analysis in maternal plasma: Theoretical and empirical considerations. *Clin Chem* 2006;52:2194–202.

131 Lo YMD, Tsui NBY, Chiu RWK, Lau TK, Leung TN, Heung MMS et al. Plasma placental RNA allelic ratio permits noninvasive prenatal chromosomal aneuploidy detection. *Nat Med* 2007;13:218–23.

132 Lo YMD, Lun FMF, Chan KCA, Tsui NBY, Chong KC, Lau TK et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. *Proc Natl Acad Sci U S A* 2007;104:13116–21.

133 Go ATJI, Visser A, Mulders MAM, Blankenstein MA, van Vugt JMG, Oudejans CBM. 44 single-nucleotide polymorphisms expressed by placental RNA: assessment for use in noninvasive prenatal diagnosis of trisomy 21. *Clin Chem* 2007;53:2223–4.

134 Chim SSC, Jin S, Lee TYH, Lun FMF, Lee WS, Chan LYS et al. Systematic search for placental DNA-methylation markers on chromosome 21: toward a maternal plasma-based epigenetic test for fetal trisomy 21. *Clin Chem* 2008;54:500–11.

135 Fan HC, Blumenfeld YJ, El-Sayed YY, Chueh J, Quake SR. Microfluidic digital PCR enables rapid prenatal diagnosis of fetal aneuploidy. *Am J Obstet Gynecol* 2009;200:543–7.

136 Tong YK, Jin S, Chiu RWK, Ding C, Chan KCA, Leung TY et al. Noninvasive prenatal detection of trisomy 21 by an epigenetic-genetic chromosome-dosage approach. *Clin Chem* 2010;56:90–8.

137 Lim JH, Kim SY, Park SY, Lee SY, Kim MJ, Han YJ et al. Non-invasive epigenetic detection of fetal trisomy 21 in first trimester maternal plasma. *PLoS One* 2011;6:e27709.

138 Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, Leung TY et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. *Proc Natl Acad Sci U S A* 2008;105:20458–63.

139 Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. *Proc Natl Acad Sci U S A* 2008;105:16266–71.

140 Chiu RWK, Akolekar R, Zheng YWL, Leung TY, Sun H, Chan KCA et al. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: large scale validity study. *BMJ* 2011;342:c7401.

141 Ehrich M, Deciu C, Zwiefelhofer T, Tynan JA, Cagasan L, Tim R et al. Noninvasive detection of fetal trisomy 21 by sequencing of DNA in maternal blood: a study in a clinical setting. *Am J Obstet Gynecol* 2011;204:205–11.

142 Leung TN, Zhang J, Lau TK, Chan LY, Lo YM. Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia. *Clin Chem* 2001;47:137–9.

143 Sekizawa A, Jimbo M, Saito H, Iwasaki M, Sugito Y, Yukimoto Y et al. Increased cell-free fetal DNA in plasma of two women with invasive placenta. *Clin Chem* 2002;48:353–4.

144 Caramelli E, Rizzo N, Concu M, Simonazzi G, Carinci P, Bondavalli C et al. Cell-free fetal DNA concentration in plasma of patients with abnormal uterine artery Doppler waveform and intrauterine growth restriction - a pilot study. *Prenat Diagn* 2003;23:367–71.

145 Lazar L, Rigo JJ, Nagy B, Balogh K, Mako V, Cervenak L et al. Relationship of circulating cell-free DNA levels to cell-free fetal DNA levels, clinical characteristics and laboratory parameters in preeclampsia. *BMC Med Genet* 2009;10:120.

146 Alberry MS, Maddocks DG, Hadi MA, Metawi H, Hunt LP, Abdel-Fattah SA et al. Quantification of cell free fetal DNA in maternal plasma in normal pregnancies and in pregnancies with placental dysfunction. *Am J Obstet Gynecol* 2009;200:98–6.

147 Farina A, Sekizawa A, Sugito Y, Iwasaki M, Jimbo M, Saito H et al. Fetal DNA in maternal plasma as a screening variable for preeclampsia. A preliminary nonparametric analysis of detection rate in low-risk nonsymptomatic patients. *Prenat Diagn* 2004;24:83–6.

148 Vora NL, Johnson KL, Lambert-Messerlian G, Tighiouart H, Peter I, Urato AC et al. Relationships between cell-free DNA and serum analytes in the first and second trimesters of pregnancy. *Obstet Gynecol* 2010;116:673–8.

149 Gerovassili A, Nicolaides KH, Thein SL, Rees DC. Cell-free DNA levels in pregnancies at risk of sickle-cell disease and significant ethnic variation. *Br J Haematol* 2006;135:738–41.

150 Wataganara T, Peter I, Messerlian GM, Borgatta L, Bianchi DW. Inverse correlation between maternal weight and second trimester circulating cell-free fetal DNA levels. *Obstet Gynecol* 2004;104:545–50.

151 Hahn S, Rusterholz C, Hosli I, Lapaire O. Cell-free nucleic acids as potential markers for preeclampsia. *Placenta* 2011;32 Suppl:S17–S20.

152 Zhong XY, Laivuori H, Livingston JC, Ylikorkala O, Sibai BM, Holzgreve W et al. Elevation of both maternal and fetal extracellular circulating deoxyribonucleic acid concentrations in the plasma of pregnant women with preeclampsia. *Am J Obstet Gynecol* 2001;184:414–9.

153 Swinkels DW, de Kok JB, Hendriks JCM, Wiegerinck E, Zusterzeel PLM, Steegers EAP. Hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome as a complication of preeclampsia in pregnant women increases the amount of cell-free fetal and maternal DNA in maternal plasma and serum. *Clin Chem* 2002;48:650–3.

Chapter 2

Reliability of fetal sex determination using maternal plasma

P.G. Scheffer[a,b]
C.E. van der Schoot[a]
G.C.M.L. Page-Christiaens[b]
B. Bossers[c]
F. van Erp[d]
M. de Haas[a,c]

[a] Department of Experimental Immunohaematology, Sanquin Research Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Division of Perinatology and Gynaecology, University Medical Centre Utrecht, Utrecht, The Netherlands
[c] Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands
[d] Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Obstet Gynecol 2010;115:117–26

Abstract

Objective To determine the diagnostic accuracy of noninvasive fetal sex determination in maternal plasma.

Methods All consecutive patients for whom fetal sex determination in maternal plasma was performed in our laboratory from 2003 up to 2009 were included in the study. Real-time polymerase chain reaction was performed for the *SRY* gene and multicopy DYS14 marker sequence. A stringent diagnostic algorithm was applied. In the case of a positive result for both Y chromosome-specific assays, a male-bearing pregnancy was reported. In the case of a negative result, the presence of fetal DNA was ascertained through the use of 24 biallelic insertion/deletion polymorphisms or paternally inherited blood group antigens. Only if the presence of fetal DNA was confirmed was a female-bearing pregnancy reported. Results were compared with the pregnancy outcomes.

Results A total of 201 women was tested. The median gestational age was 9 0/7 weeks (interquartile range 8 0/7 to 10 0/7 weeks). In 189 of 201 cases (94%), a test result was issued; in 10 cases, the presence of fetal DNA could not be confirmed; in two cases, an early miscarriage was observed. Pregnancy outcome was obtained in 197 cases, including 105 male-bearing and 81 female-bearing pregnancies and 11 miscarriages. Sensitivity and specificity of the test were 100% (95% confidence intervals 96.6–100% and 95.6–100%, respectively). In all 10 cases in which the presence of fetal DNA could not be confirmed, a female was born.

Conclusion Noninvasive fetal sex determination in maternal plasma is highly reliable and clinically applicable.

Introduction

Knowledge of the fetal sex at an early gestational age is relevant for pregnant women carrying an X-linked chromosomal abnormality or for those at risk of being pregnant with a child with congenital adrenal hyperplasia (CAH). In CAH, it determines whether dexamethasone treatment, started immediately after pregnancy was established to reduce virilisation of the female external genitalia in affected females, needs to be continued or not. Traditionally, early fetal sex determination has been performed by using invasive techniques, such as chorionic villus sampling or amniocentesis. These procedures, however, still carry a risk of miscarriage around 1%[1] and cannot be performed until 11 weeks of gestation. Reliable determination of fetal sex by means of ultrasonography cannot be done in the first trimester, because development of the external genitalia is not complete.[2]

The discovery of cell-free fetal DNA in plasma of pregnant women at the end of the last century offered a new source of material for prenatal diagnosis.[3] Cell-free fetal DNA originates from apoptotic syncytiotrophoblasts from the placenta.[4] It can be detected in the maternal circulation from as early as 5 weeks of gestation,[5] and it is cleared within several hours after birth.[6] Its chief feature is that it can be obtained noninvasively. With regard to fetal sex determination, it would allow for definitive sex determination earlier in pregnancy, sparing most female fetuses from unnecessary invasive testing because of X-linked disorders or guiding clinical management in other cases, such as CAH.

Polymerase chain reaction (PCR)-based detection and identification of fetal DNA in maternal plasma is at present only possible for those sequences absent from the maternal genome (e.g., sequences on the Y chromosome in male-bearing pregnancies).[7] If amplification of the target sequence is not detected, the fetus is assumed not to carry that particular allele. However, undetectable low concentrations of fetal DNA can cause false-negative results. Several approaches have been explored to exclude these false-negative results,[8-10] but the search for a universal fetal DNA identifier is still ongoing.[11,12]

Already, noninvasive prenatal diagnosis by means of cell-free fetal DNA in maternal plasma has found its way into clinical practice. Numerous groups have reported on fetal rhesus (Rh) D genotyping in D-negative mothers,[13-15] as well as on other blood groups.[16] Reports on diagnosing single gene disorders[17,18] are promising. Several reports on fetal sex determination in maternal plasma have been published. Use of both the single copy *SRY* gene sequence[19-21] and the multicopy DYS14 marker sequence of the *TSPY* gene on the Y chromosome have been reported.[22,23] Although results are encouraging, diagnostic accuracy varies between protocols and methods used, with sensitivity and specificity ranging from 31% to 100%.[24,25] Moreover, most data have been obtained in a research setting, rather than from actual clinical practice (for an overview, see Wright and Burton[7]).

As a national reference laboratory, we have been offering noninvasive fetal sex determination for clinical purposes since the beginning of 2003 using a stringent diagnostic algorithm

with the inclusion of fetal DNA identifiers. As part of our protocol, we collected the outcome data of all tested pregnancies. The aim of this present study was to analyse the results for noninvasive fetal sex determination in maternal plasma, performed in a clinical setting over a 6-year period, and to determine its diagnostic accuracy.

Methods

All consecutive patients for whom fetal sex determination in maternal plasma was performed in our laboratory from 2003 up to 2009 were included in the study. Tests were performed at the request of clinical geneticists or gynaecologists throughout The Netherlands for different indications. Gestational age and indication for the test were listed on the application form by the requesting physician. We advised a minimal gestational age of 7 weeks for CAH and 9 weeks for all other indications. Before blood sampling, ultrasonography was recommended to establish vitality of a singleton pregnancy and to check for the presence of an empty second sac.

Ethylenediaminetetraacetic acid anticoagulated blood was drawn from both the mother (30 mL) and, if possible, the reporting father (10 mL) and was processed within 48 hours.[21] Both parental samples were typed serologically for Rh D, C/c, E/e and for K/k to identify paternal blood group antigens that could potentially serve as a marker to confirm the presence of fetal DNA.

DNA was extracted in duplicate from 2×2 mL plasma using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany), following the 'Blood or Body Fluids Protocol' recommended by the manufacturer. Volumes of the used reagents were increased proportionately to accommodate the 2-mL sample size. Adsorbed DNA was eluted with 60 µL of water.

Real-time PCR analysis was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman chemistry. This technique allows for the amplification of a specific sequence of DNA. If the target sequence is present, exponential DNA amplification causes a proportional increase in a reporter dye fluorescence. The number of amplification cycles required to reach a fixed threshold signal intensity is termed the cycle threshold. Extracted DNA was analysed for *SRY* and from 2005 on also for DYS14 to increase sensitivity. Also, part of the *albumin* gene was amplified to measure the quantity of total cell-free DNA in the sample. Primer and probe sequences were as previously described.[5,23] The singleplex reactions were set up in a volume of 50 µL, using 25 µL of Taqman Universal PCR Master Mix (Applied Biosystems) and 9 µL of extracted DNA for the *SRY* and DYS14 PCR and 3 µL for the *albumin* PCR. Primers and probes were used at final concentrations of 900 and 150 nM for *SRY* and 300 and 100 nM for DYS14 and the *albumin* gene.

For both isolations, PCR for *SRY* was performed in triplicate, whereas PCR for DYS14 was performed in duplicate. As a positive control, 1 mL of pooled plasma from 120 pregnant

women (at 28–30 weeks of gestation) was simultaneously tested. One millilitre of plasma from a nonpregnant female donor served as a negative control. To recognise contamination, a no-template control, containing DNA-free water, was also included. Each replicate was judged for amplification according to previously defined cycle threshold values (Table 1). The results were interpreted using the scoring model as outlined in Table 1. Interpretation of the combined results for DYS14 and *SRY* led to a positive (i.e., male), negative (i.e., female), or inconclusive test result (Table 2). Because, as a result of its multicopy sequence, the DYS14 assay is far more sensitive (with a 10-fold lower detection limit),[23] a positive result for *SRY* with a negative result for DYS14 was considered a technical failure.

Table 1. Scoring model for *SRY* and DYS14 polymerase chain reaction

	Replicate			Conclusion PCR assay	Isolation 1	Isolation 2	Result PCR (*SRY* or DYS14)
	1	2	3				
SRY PCR	+	+	+	Positive	Positive	Positive	Positive
	+	+	–	Positive			
	+	–	–	Inconclusive	Positive	Inconclusive	Inconclusive (*SRY*)
	–	–	–	Negative			Positive (DYS14)
					Positive	Negative	Technical failure*
DYS14 PCR	+	+		Positive			
	+	±		Positive	Inconclusive	Inconclusive	Inconclusive
	+	–		Inconclusive			
	±	±		Inconclusive	Negative	Inconclusive	Negative
	±	–		Inconclusive			
	–	–		Negative	Negative	Negative	Negative

PCR, polymerase chain reaction; Ct, cycle threshold value.
SRY PCR: positive if Ct is less than 42; negative if Ct is 42 or more. DYS14 PCR: positive if Ct is less than 35; negative if Ct is 38 or more; inconclusive if Ct is at least 35 but less than 38.
*Repeat test using same sample.

In the case of a negative result for *SRY* and DYS14, suggesting a female-bearing pregnancy, the presence of fetal DNA was ascertained by testing for paternal sequences absent from the maternal genome. If serologic testing of the parents indicated that a paternal blood group antigen (i.e., Rh D, C/c, E or K) could serve as a potential fetal marker, real-time quantitative PCR for the relevant sequence was performed in maternal plasma.[16,21] When no blood group polymorphism was found to be suitable, leukocyte-derived DNA from both parents was screened for 24 biallelic insertion/deletion polymorphisms (indels) by multiplex PCR. Primer and probe sequences of 18 indels had been described earlier for quantitative assessment of haematopoietic chimerism after bone marrow transplantation.[26] In addition to this set of markers, we added six indels found online[27] (for primer and probe sequences, see Table 3).

Those alleles heterozygously or homozygously present on the paternal genome and absent from the maternal genome could potentially serve as a marker to confirm the presence of fetal DNA. Targeting these potential markers and again *SRY* as a control, we performed a second singleplex PCR, using freshly isolated DNA from the same maternal plasma sample. By dilution series, it was previously shown that the sensitivities of the indel markers and *SRY* PCR are similar.[9] If amplification was seen, the presence of fetal DNA was confirmed, and a female-bearing pregnancy was reported. In case no paternal blood was available, maternal genomic DNA was screened for all indels. Alleles absent from the maternal genome were then tested for in maternal plasma. Only when we were able to confirm the presence of fetal DNA, a negative result for *SRY* and DYS14 was issued and we concluded that the fetus was female. If the presence of fetal DNA could not be confirmed, the overall test result was inconclusive.

Table 2. Diagnostic algorithm for combined results of DYS14 and *SRY* polymerase chain reaction

Result DYS14 PCR	Result *SRY* PCR	Result test	Further proceedings
Positive	Positive	Positive	Report male
	Inconclusive	Positive, but low amount of fetal DNA	Report male
	Negative	Inconclusive; too low amount of fetal DNA; suspicion of miscarriage	Report "likely male" and request new sample
Inconclusive	Positive	Technical failure	Repeat test*
	Inconclusive	Inconclusive; too low amount of fetal DNA; suspicion of miscarriage	Report "likely male" and request new sample
	Negative	Inconclusive; too low amount of fetal DNA; suspicion of miscarriage	Report "likely male" and request new sample
Negative	Positive	Technical failure	Repeat test*
	Inconclusive	Technical failure	Repeat test*
	Negative	Negative	Confirm presence of fetal DNA; report female

PCR, polymerase chain reaction.
*Repeat test using same sample.

As part of test performance quality control, when reporting the result to the requesting physician, we requested to receive follow-up of the fetal sex, whether determined by karyotyping, by ultrasonography, or after birth, with the patients' informed consent. After collecting all pregnancy outcomes, descriptive statistics were generated using Prism 4 software (GraphPad Software, San Diego, CA, USA). The Fisher exact test (two-sided) was used to determine sensitivity and specificity with 95% confidence intervals (CIs).

Table 3. Primer and probe sequences for biallelic insertion/deletions polymorphisms (indels)

Marker name	Position	5' Primer 3'	Taqman (FAM/VIC-TAMRA) probe
S01a	F	GGT ACC GGG TCT CCA CAT GA	VIC-CTG GGC CAG AAT CTT GGT CCT CAC A-TAMRA
	R*	GGG AAA GTC ACT CAC CCA AGG	
S01b	F	GTA CCG GGT CTC CAC CAG G	
S03	F	CTT TTG CTT TCT GTT TCT TAA GGG C	VIC-CAT ACG TGC ACA GGG TCC CCG AGT-TAMRA
	R	TCA ATC TTT GGG CAG GTT GAA	
S04a	F*	CTG GTG CCC ACA GTT ACG CT	FAM-TCC TGG CAG TGT GGT CCC TCC AGA A-TAMRA
	R	AAG GAT GCG TGA CTG CTA TGG	
S04b	R	AGG ATG CGT GAC TGC TCC TC	
S05a	F	AAA GTA GAC ACG GCC AGA CTT AGG	VIC-CCC TGG ACA CTG AAA ACA GGC AAT CCT-TAMRA
	R*	CAT CCC CAC ATA CGG AAA AGA	
S05b	F	AGT TAA AGT AGA CAC GGC CTC CC	
S06	F	CAG TCA CCC CGT GAA GTC CT	FAM-CCC ATC CAT CTT CCC TAC CAG ACC AGG-TAMRA
	R	TTT CCC CCA TCT GCC TAT TG	
S07a	F	TGG TAT TGG CTT TAA AAT ACT GGG	FAM-TCC TCA CTT CTC CAC CCC TAG TTA AAC AG-TAMRA
	R	TGT ACC CAA AAC TCA GCT GCA	
S07b	F	GGT ATT GGC TTT AAA ATA CTC AAC C	
	R	CAG CTG CAA CAG TTA TCA ACG TT	
S08a	F	CTG GAT GCC TCA CTG ATC CA	VIC-CTC CCA ACC CCC ATT TCT GCC TG-TAMRA
	R*	TGG GAA GGA TGC ATA TGA TCT G	
S08b	F	GCT GGA TGC CTC ACT GAT GTT	
S09a	F*	GGG CAC CCG TGT GAG TTT T	FAM-TGG AGG ATT TCT CCC CTG CTT CAG ACA G-TAMRA
	R	TCA GCT TGT CTG CTT TCT GGA A	
S09b	R	CAG CTT GTC TGC TTT CTG CTG	
S10a	F	GCC ACA AGA GAC TCA G	FAM-CAG TGT CCC ACT CAA GTA CTC CTT TGG A-TAMRA
	R*	TGG CTT CCT TGA GGT GGA AT	
S10b	F	TTA GAG CCA CAA GAG ACA ACC AG	
S11a	F	TAG GAT TCA ACC CTG GAA GC	VIC-CAA GGC TTC CTC AAT TCT CCA CCC TTC C-TAMRA
	R*	CCA GCA TGC ACC TGA CTA ACA	
S11b	F	CCC TGG ATC GCC GTG AA	
MID668a	F	GGA CGG GAC TTT CCA ATA CTA TAT GTC AG	FAM-CAA GGA AAA AGC ATT TCA AAT GAG GCT ACT AGG A-TAMRA
	R*	AAG GCT ACT TGG ATC AAA TTG ACT CT	
MID668b	F	ACG GGA CTT TCC AAT ACT ATA TGT AAT ACA A	
MID836a	F	GGC TTA TAT CAA ATG TCC TTA TGA ATT TAC A	FAM-TCA TTT TTA TAG TCT GTT CGG CAC TTG AAG TAC TCC T-TAMRA
	R*	ACA TCC CAG AGA GTT ACT ATT CCA TTA TC	
MID836b	F	GCT TAT ATC AAA TGT CCT TAT GAA TGC TTA	
MID402	F	CAT TGA AAA ATA ATG AAA ATT CTC AGA AAG	FAM-AAT GGC AAT TTA CCA AGA CAT AAT TTA GTG AGA AGA ATG G-TAMRA
	R	GCT GTC CTC TAT TAA GCC AAA CAC A	
MID847	F	CCT TTG CAA CTC ACC AGG TTC T	FAM-CAT CCG GGC TGG CAA TTC CTG AT-TAMRA
	R	CAA CTC CCA AAC AGA CTT TGT ATC TC	

F, forward primer; R, reverse primer. *Common primer.

Markers S01a-S11b derived from Alizadeh et al.[26] Markers MID668a/b, MID836a/b, MID402 and MID847 from online data file from Marshfield Medical Research Foundation[27]; criteria were: an insertion/deletion sequence consisting of four or more nucleotides, not located on the Y-chromosome and an even distribution of allele frequency in the European population.

Results

From 2003 up to 2009, 201 pregnant women were tested (Figure 1). The median gestational age at time of blood sampling was 9 0/7 weeks (interquartile range 8 0/7 to 10 0/7 weeks). In one case, testing was performed before the 7th week of gestation (at 5 5/7 weeks) and in 19 cases, testing was performed after the 12th week of gestation (range 12–31 weeks). The majority of tests (*n* = 156; 78%) was performed for X-linked conditions, including 51 (25%) for Duchenne/Becker muscular dystrophy and 31 (15%) for haemophilia. In 39 cases (19%), tests were performed for CAH. Five tests (2%) were performed for other than the aforementioned indications. These included a fetal intraabdominal mass on ultrasonography with an ovary cyst in the differential diagnosis (at 16 weeks of gestation), genital ambiguity on ultrasonography (at 31 weeks), intended use of antiandrogenic medication (spironolactone) because of a maternal apparent mineral corticosteroid excess and very high blood pressures (at 13 weeks), maternal carrier of breast cancer gene (at 8 weeks), and an extremely elevated level of maternal testosterone (12 weeks). In one case, the indication for testing was not traceable anymore.

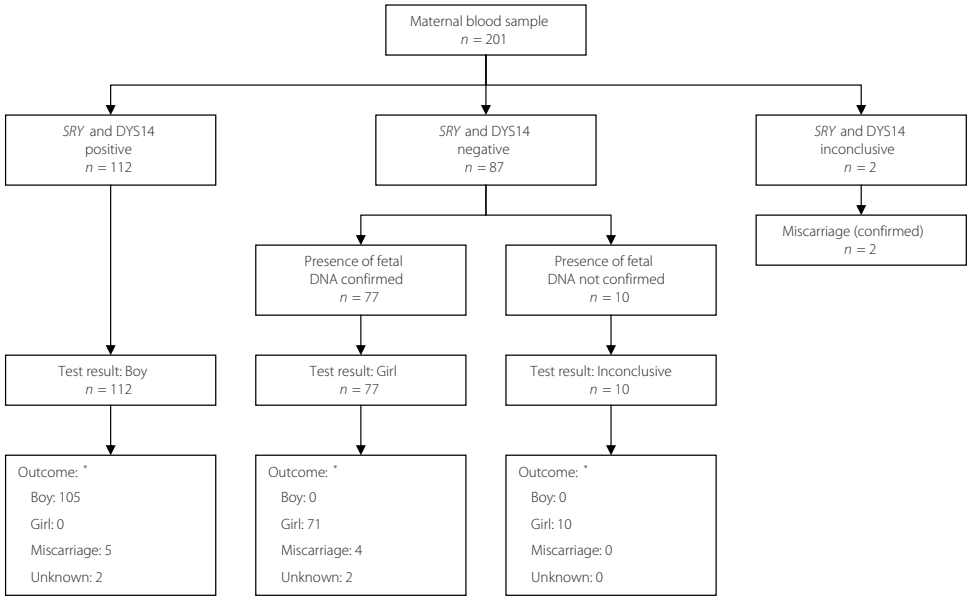

Figure 1. Test results and outcomes for pregnant women tested from 2003 up to 2009.
*As determined by karyotyping, ultrasonography, or after birth.

In the first 48 cases (from 2003 until 2005), only a PCR for *SRY* was performed. On the 153 samples thereafter, PCR for both *SRY* and DYS14 was performed. Results were conclusive on first testing in 193 of 201 samples. PCR had to be repeated in eight cases, using the same blood sample (*n* = 5) or requiring a second sample (*n* = 3). These latter three cases were all

from the first cohort, when only *SRY* was targeted.

Pregnancy outcome and fetal sex, as determined by karyotyping, by ultrasonography, or after birth, were ascertained in 197 cases (98%; Figure 1). *SRY* and DYS14 PCR was positive in 105 of 105 plasma samples of women bearing a male fetus, resulting in a 100% sensitivity (95% CI 96.6–100%). In all 81 plasma samples of women bearing a female fetus, PCR for *SRY* and DYS14 was negative, resulting in a 100% specificity (95% CI 95.6–100%). A total of 11 women miscarried.

Y chromosome-specific sequences were found in 112 samples, 83 samples (74%) scoring 6/6 positive replicates for *SRY* and all but one sample scoring 4/4 replicates positive for DYS14 (Table 4). As plotted in Figure 2, the mean cycle threshold value for *SRY* in male-bearing pregnancies was 37.83 (standard deviation [SD] 1.41). The mean cycle threshold value for DYS14 in male-bearing pregnancies was 32.27 (SD 1.04).

Table 4. Results for *SRY* and DYS14 polymerase chain reaction*

SRY	6/6 (*n*) (Mean Ct)	5/6 (*n*) (Mean Ct)	4/6 (*n*) (Mean Ct)	3/6 (*n*) (Mean Ct)	2/6 (*n*) (Mean Ct)	1/6 (*n*) (Mean Ct)	0/6 (*n*) (Mean Ct)
DYS14							
4/4 (*n*) (Mean Ct)	64 (*SRY* 37.66) (DYS14 32.03) Male	18 (*SRY* 38.72) (DYS14 33.06) Male	3 (*SRY* 39.91) (DYS14 33.33) Male	0	1 (*SRY* 41.31) (DYS14 35.05) Inconclusive (miscarriage)	1 (*SRY* 39.72) (DYS14 36.13) Inconclusive (miscarriage)	0
3/4 (*n*) (Mean Ct)	1 (*SRY* 36.80) (DYS14 33.37) Male	0	0	0	0	0	0
2/4 (*n*) (Mean Ct)	0	0	0	0	0	0	0
1/4 (*n*) (Mean Ct)	0	0	0	0	0	0	0
0/4 (*n*) (Mean Ct)	0	0	0	0	0	3 (*SRY* 39.67) (DYS14 40.69)‡ Female	62 (*SRY* N/A) (DYS14 39.96)‡ Female
Not done#	18 (*SRY* 37.42) Male	8 (*SRY* 38.27) Male	0	0	0	0	22 (*SRY* N/A) Female

Ct, cycle threshold value; N/A, not applicable.

*Number of positive replicates and mean Ct ($n = 201$).

#From 2003 until 2005, only polymerase chain reaction for *SRY* was performed ($n = 48$).

‡Mean Ct values for DYS14 with Ct ≥38.00 and <50.00, not including undetermined Ct values (i.e. Ct ≥50).

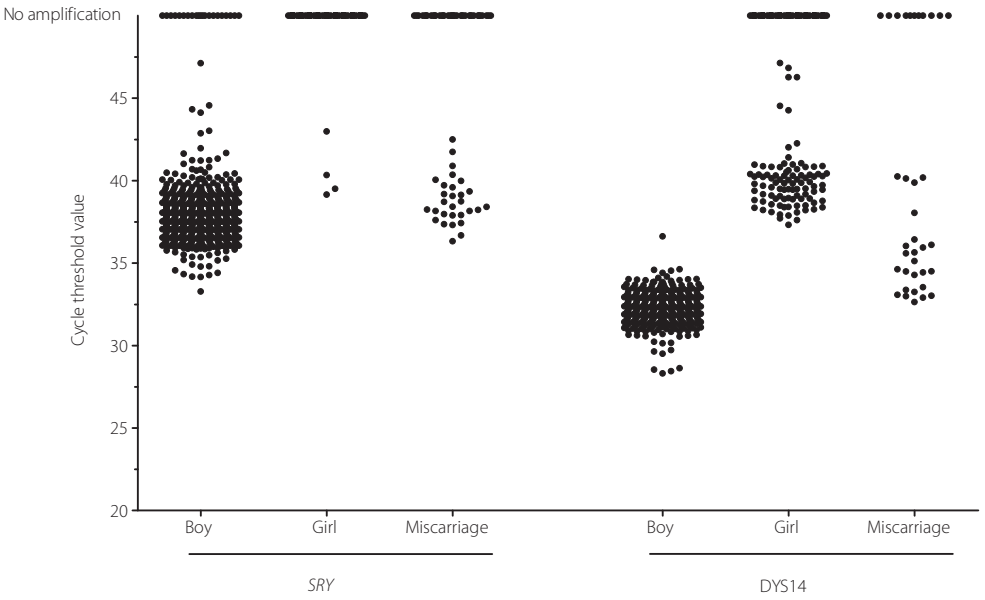

Figure 2. Cycle threshold values for *SRY* and DYS14 for reported outcomes (n = 197).

Eighty-seven samples were negative for Y chromosome-specific sequences. Although no positive replicates for DYS14 were found, amplification was observed in 35% of the replicates from women pregnant with a girl (mean cycle threshold value 40.00, SD 1.87; Figure 2).

For two samples, the combined *SRY* and DYS14 PCR result was inconclusive. These samples had relatively high (greater than 35 and less than 38) cycle threshold values for DYS14, with only 1/6 and 2/6 positive replicates for *SRY* found, pointing to the presence of very low amounts of fetal DNA. When a second blood sample was requested, a miscarriage was reported in both cases.

In 9 of 87 cases in which no Y chromosome-specific sequences were detected, a paternal blood group antigen could successfully be used to confirm the presence of fetal DNA. In the remaining 78 cases, indel markers were used (Figure 3). In four cases, no paternal blood was obtained, but maternal DNA was nevertheless screened for all indels available in the panel. Results for screening for informative indel alleles are shown in Table 5. On average, three (SD 1) possibly informative alleles were found per pregnancy. Using indel markers, we were able to confirm the presence of fetal DNA in 68 of 78 cases (87%). In five cases, only negative results were found, with the informative indel alleles only heterozygously present in the father. In three cases, the result was inconclusive because nonspecific amplification with maternal, leukocyte-derived genomic DNA was also obtained. In all 10 cases in which the presence of fetal DNA could not be confirmed, a female was born.

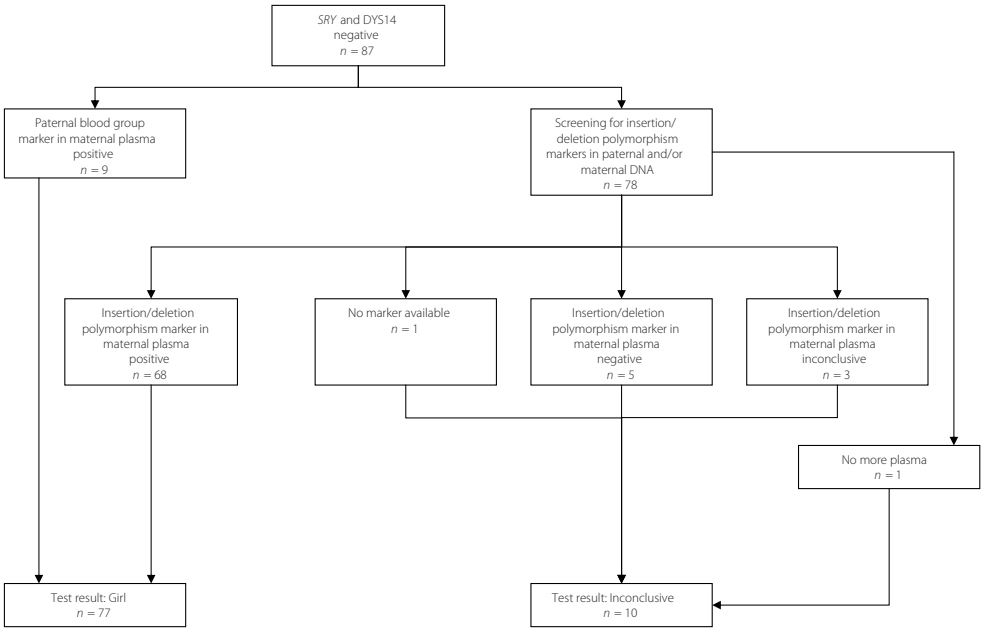

Figure 3. Confirmation of the presence of fetal DNA for samples negative for Y chromosome-specific sequences.

Overall, a test result was issued in 94% (189 of 201). In general, test results were reported to the requesting physician within 2 (in the case of a male) to 4 (in the case of a female) working days after blood sampling. Invasive testing could be avoided in 64 of 156 pregnancies (41%) tested because of X-linked disorders (36% excluding haemophilia). Invasive testing could also be avoided in the patient carrying the breast cancer gene, because a male-bearing pregnancy was predicted. For CAH, the duration of high doses steroids treatment could be reduced by several weeks in 27 of 39 pregnancies (69%). In the patient with apparent mineral corticosteroid excess, the fetus seemed to be male and the second-choice treatment of prednisone and alpha-methyldopa was prescribed. A healthy boy was born. In the case with the fetal intraabdominal mass on ultrasonography, a female bearing-pregnancy was predicted, not allowing narrowing of the differential diagnosis at that time. A girl with a cloacal malformation was born.

Discussion

This study demonstrates that fetal sex determination in maternal plasma is highly reliable. We have shown noninvasive fetal sex determination performed in a clinical setting to be accurate. Through the application of two different PCR assays on two separate DNA isolations, as well as through confirmation of the presence of fetal DNA in the case of negative PCR results, we

Table 5. Number of informative alleles per pregnancy with at least one homozygously positive allele or only heterozygously positive alleles in the father ($n = 74$)

Number of informative alleles	Number of pregnancies			
	At least one homozygously positive allele	Only heterozygously positive alleles	No positive alleles	Total
No informative allele	N/A	N/A	1	1
One informative allele	1	4	N/A	5
Two informative alleles	9	10	N/A	19
Three informative alleles	13	7	N/A	20
Four or more informative alleles	21	8	N/A	29
Total (%)	44 (60)	29 (39)	1 (1)	74 (100)

N/A, not applicable.

were able to report fully conclusive results and guide clinical management in 189 of 201 cases. Moreover, no false-negative or false-positive results were found.

Combining both PCR results for the Y-linked sequences *SRY* and DYS14 and using a stringent algorithm, we were able to issue a result on first testing in all but five cases (3%). With 3/4 positive replicates for the DYS14 PCR, we were confident to report a male pregnancy with only 4/6 positive replicates for *SRY*. In the earlier stages of our protocol, when only PCR for *SRY* was performed, a similar *SRY* result would have prompted us to repeat the test or even request a new sample. Indeed, several groups targeting only one Y chromosome-specific sequence reported a higher frequency of repeat testing.[28,29]

The DYS14 assay targets a multicopy sequence and therefore has a higher sensitivity than *SRY*.[23] Because four of five repeated tests were performed because of discrepant results between the *SRY* assays (data not shown), testing for only the DYS14 sequence could be considered. Although this has been suggested by Zimmermann et al.[23] and Picchiassi et al.,[29] we do not favour this approach. In our opinion, the added value of the *SRY* assay is that it causes the overall test result to be less vulnerable to false-positive results due to, e.g., contamination. Furthermore, the relatively high frequency of amplification signals obtained with the DYS14 assay in female-bearing pregnancies (see also Figure 2), albeit at high cycle threshold values, is still difficult to explain. Because no amplification signals of *SRY* are seen in the plasma of women pregnant with a girl, the *SRY* assay increases the specificity of the test as a whole.

It has been shown that fetal DNA is present in maternal plasma in anembryonic pregnancies[4] and even before fetal circulation is established.[30] Therefore, we cannot exclude the possibility that a vanishing (male) twin could cause false-positive PCR results. We did not encounter this in the current study. We do, however, recommend ultrasonography before blood sampling, with explicit attention for the presence of a second gestational sac.

No false-negative results were found in our study. We presume this can be attributed to the fact that we used 2 mL of plasma to extract DNA and the addition of 9 microlitres of eluted DNA to the reaction volume. Other groups that used much smaller volumes of plasma and extracted DNA reported a higher frequency of repeat testing,[20] reported more false-negative results,[29,31] or were not able to issue results before the 10th week of gestation.[32]

Because female fetuses are not detected directly, but only inferred by a negative result for Y chromosome-specific sequences, it remains of the utmost importance to confirm the presence of fetal DNA when a negative result for *SRY* and DYS14 is found. We were able to confirm the presence of fetal DNA in 87% of samples tested for biallelic insertion/deletion polymorphisms. Some authors have questioned the use of indel markers,[33] addressing the fact that it does not represent a true internal control, its labour-intensive character and the lack of informativeness unless a large number of polymorphisms are used. Performed by experienced technicians, screening and repeat testing for the 24 indel markers took no longer than 1 working day. Although we show that paternal markers are clinically applicable, a truly universal fetal marker independent of paternally inherited sequences would overcome the aforementioned objections. Epigenetic differences between maternal and fetal DNA are currently being explored. Promising results have been published on sequences within the tumour suppressor genes *maspin* and *RASSF1A*, methylated differently in mother and child.[11,12] However, in all 10 cases with an inconclusive test result due to failure to confirm the presence of fetal DNA, a female-bearing pregnancy was reported. This proves the robustness of the *SRY* and DYS14 assays and we therefore recommend the following: If the presence of fetal DNA cannot be confirmed and the indication for fetal sexing is an X-linked disease not affecting the development of the external genitalia, a female-bearing pregnancy can be reported, to be confirmed by ultrasonography in the second trimester. When the indication is, e.g., CAH or androgen insensitivity syndrome, fetal sex cannot be determined ultrasonographically and invasive testing is to be offered in the case of inconclusive results. With our approach, there is no need for ultrasonography if the presence of fetal DNA is confirmed.

Noninvasive fetal sex determination is a clinical reality. There is no longer a need for invasive procedures to determine fetal sex. Fetal sex determination in maternal plasma allows for early knowledge of the fetal sex, adding to timely clinical management. It can reduce the need for invasive procedures in pregnant women carrying an X-linked chromosomal abnormality up to 50%, decreasing the risks for iatrogenic damage.

Acknowledgements

The development and validation of the polymerase chain reaction protocols were performed within the European Commission for Special Non-invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence (LSHB-CT-2004–503243).

The authors thank Lutgarde Govaerts, clinical geneticist (Erasmus Medical Centre, Rotterdam), Eva Pajkrt, gynecologist (Academic Medical Centre, Amsterdam), Maaike Vreeburg, clinical geneticist (Maastricht University Medical Centre, Maastricht), Jiddeke van de Kamp, clinical geneticist (VU University Medical Centre, Amsterdam), Nicolette den Hollander, clinical geneticist, and Jennie Verdoes, research nurse (Leiden University Medical Centre, Leiden) and Katelijne Bouman, clinical geneticist (University Medical Centre Groningen, Groningen), for the clinical implementation of the protocol.

References

1 Mujezinovic F, Alfirevic Z. Procedure-related complications of amniocentesis and chorionic villous sampling: a systematic review [published erratum appears in Obstet Gynecol 2008; 111:779]. *Obstet Gynecol* 2007;110:687–94.

2 Odeh M, Granin V, Kais M, Ophir E, Bornstein J. Sonographic fetal sex determination. *Obstet Gynecol Surv* 2009;64:50–7.

3 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

4 Alberry M, Maddocks D, Jones M, Abdel Hadi M, Abdel-Fattah S, Avent N, et al. Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenat Diagn* 2007;27:415–8.

5 Rijnders RJP, van der Luijt RB, Peters EDJ, Goeree JK, van der Schoot CE, Ploos van Amstel JK, et al. Earliest gestational age for fetal sexing in cell-free maternal plasma. *Prenat Diagn* 2003;23:1042–4.

6 Lo YM, Zhang J, Leung TN, Lau TK, Chang AM, Hjelm NM. Rapid clearance of fetal DNA from maternal plasma. *Am J Hum Genet* 1999;64:218–24.

7 Wright CF, Burton H. The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. *Hum Reprod Update* 2009;15:139–51.

8 Pertl B, Sekizawa A, Samura O, Orescovic I, Rahaim PT, Bianchi DW. Detection of male and female fetal DNA in maternal plasma by multiplex fluorescent polymerase chain reaction amplification of short tandem repeats. *Hum Genet* 2000;106:45–9.

9 Page-Christiaens GCML, Bossers B, van der Schoot CE, de Haas M. Use of bi-allelic insertion/deletion polymorphisms as a positive control for fetal genotyping in maternal blood: first clinical experience. *Ann N Y Acad Sci* 2006;1075:123–9.

10 Dhallan R, Guo X, Emche S, Damewood M, Bayliss P, Cronin M, et al. A non-invasive test for prenatal diagnosis based on fetal DNA present in maternal blood: a preliminary study. *Lancet* 2007;369:474–81.

11 Chim SSC, Tong YK, Chiu RWK, Lau TK, Leung TN, Chan LYS, et al. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc Natl Acad Sci U S A* 2005;102:14753–8.

12 Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN, et al. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.

13 Bianchi DW, Avent ND, Costa JM, van der Schoot CE. Noninvasive prenatal diagnosis of fetal Rhesus D: ready for Prime(r) Time. *Obstet Gynecol* 2005;106:841–4.

14 Geifman-Holtzman O, Grotegut CA, Gaughan JP. Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood: a meta-analysis. *Am J Obstet Gynecol* 2006;195:1163–73.

15 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* 2008;336:816–8.

16 Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c, and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion* 2007;47:2126–33.

17 Chiu RWK, Lau TK, Leung TN, Chow KCK, Chui DHK, Lo YMD. Prenatal exclusion of beta thalassaemia major by examination of maternal plasma. *Lancet* 2002;360:998–1000.

18 Li Y, Page-Christiaens GCML, Gille JJP, Holzgreve W, Hahn S. Non-invasive prenatal detection of achondroplasia in size-fractionated cell-free DNA by MALDI-TOF MS assay. *Prenat Diagn* 2007;27:11–7.

19 Bartha JL, Finning K, Soothill PW. Fetal sex determination from maternal blood at 6 weeks of gestation when at risk for 21-hydroxylase deficiency. *Obstet Gynecol* 2003;101(pt 2):1135–6.

20 Hromadnikova I, Houbova B, Hridelova D, Voslarova S, Kofer J, Komrska V, et al. Replicate real-time PCR testing of DNA in maternal plasma increases the sensitivity of noninvasive fetal sex determination. *Prenat Diagn* 2003;23:235–8.

21 Rijnders RJP, Christiaens GCML, Bossers B, van der Smagt JJ, van der Schoot CE, de Haas M. Clinical applications of cell-free fetal DNA from maternal plasma. *Obstet Gynecol* 2004;103:157–64.

22 Honda H, Miharu N, Ohashi Y, Samura O, Kinutani M, Hara T, et al. Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. *Hum Genet* 2002;110:75–9.

23 Zimmermann B, El-Sheikhah A, Nicolaides K, Holzgreve W, Hahn S. Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma. *Clin Chem* 2005;51:1598–604.

24 Johnson KL, Dukes KA, Vidaver J, LeShane ES, Ramirez I, Weber WD, et al. Interlaboratory comparison of fetal male DNA detection from common maternal plasma samples by real-time PCR. *Clin Chem* 2004;50:516–21.

25 Finning KM, Chitty LS. Non-invasive fetal sex determination: impact on clinical practice. *Semin Fetal Neonatal Med* 2008;13:69–75.

26 Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C, et al. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. *Blood* 2002;99:4618–25.

27 Center for Medical Genetics, Marshfield Medical Research Foundation. Mammalian Genotyping Service. Available at: http://www.marshfieldclinic.org/mgs/. Retrieved spring 2006.

28 Chitty LS, Daniels G, Finning K. Prospective Register of Outcomes of Free-Fetal DNA Testing (PROOF): results of the first year's audit. *J Med Genet* 2007;44(suppl 1)S28.

29 Picchiassi E, Coata G, Fanetti A, Centra M, Pennacchi L, Di Renzo GC. The best approach for early prediction of fetal gender by using free fetal DNA from maternal plasma. *Prenat Diagn* 2008;28:525–30.

30 Guibert J, Benachi A, Grebille AG, Ernault P, Zorn JR, Costa JM. Kinetics of *SRY* gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique. *Hum Reprod* 2003;18:1733–6.

31 Sekizawa A, Kondo T, Iwasaki M, Watanabe A, Jimbo M, Saito H, et al. Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem* 2001;47: 1856–8.

32 Hyett JA, Gardener G, Stojilkovic-Mikic T, Finning KM, Martin PG, Rodeck CH, et al. Reduction in diagnostic and therapeutic interventions by non-invasive determination of fetal sex in early pregnancy. *Prenat Diagn* 2005;25:1111–6.

33 Daniels G, Finning K, Martin P, Massey E. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* 2009;29:101–7.

Chapter 3

Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a seven-year clinical experience

P.G. Scheffer[a,b]
C.E. van der Schoot[a]
G.C.M.L. Page-Christiaens[b]
M. de Haas[a,c]

[a] Department of Experimental Immunohaematology, Sanquin Research Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Division of Perinatology and Gynaecology, University Medical Centre Utrecht, Utrecht, The Netherlands
[c] Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands

BJOG 2011;118:1340–8

Abstract

Objective To evaluate the diagnostic performance of noninvasive fetal blood group genotyping.

Design Descriptive analysis.

Setting Dutch national reference laboratory for pregnancies complicated by alloimmunisation.

Population All consecutive alloimmunised pregnant women for whom fetal blood group genotyping of rhesus D, c, E or of K in maternal plasma was performed from 2003 up to 2010.

Methods The test results of each individual assay were collected. Real-time polymerase chain reaction was performed for *RHD* exon 5 and *RHD* exon 7, or the specific allele of the *RHCE* or *KEL* gene. A stringent diagnostic algorithm was applied. In the case of a negative result, the presence of fetal DNA was ascertained by the analysis of the Y chromosome-specific *SRY* gene or other paternal genetic markers. Results were compared with available serology after birth or genotyping results of amniotic fluid cells.

Main outcome measures Percentage of conclusive test results and diagnostic accuracy.

Results A total of 362 tests was performed (D: $n = 168$; c: $n = 49$; E: $n = 85$; K: $n = 60$). The median gestational age was 17 weeks (range 7–38 weeks). In 351 women (97%), a test result was issued: in seven samples, the presence of fetal DNA could not be confirmed; in two samples, nonspecific amplification in the K assay led to an inconclusive result; in two samples, a maternal silent *RHD* gene prevented fetal *RHD* genotyping. No false-positive or false-negative results were found among those women for whom cord blood serology or genotyping results of amniotic fluid cells were available ($n = 212$).

Conclusions Noninvasive fetal blood group genotyping is accurate and applicable in a clinical diagnostic setting.

Introduction

Haemolytic disease of the fetus and newborn (HDFN) is caused by maternal alloantibodies directed against fetal red cell surface antigens that the mother herself lacks. The D antigen of the rhesus (Rh) blood group system is the most frequently involved antigen in HDFN and despite the widespread use of prophylactic antenatal and postpartum anti-D immunoglobulin, RhD alloimmunisation is still a significant cause of fetal and neonatal morbidity and mortality.[1,2] In addition, alloimmunisation to the c antigen of the Rh blood group system and the K antigen of the Kell blood group system can cause severe HDFN.[3,4] Antibodies against the C and E antigens of the Rh system or against antigens of other blood group systems rarely lead to clinical manifestations.[5]

In alloimmunised pregnant women, knowledge of the fetal antigen status is beneficial to tailor pregnancy management.[1] In general, blood group antigens are biallelic co-dominant systems and if the father is heterozygously positive for a certain blood group antigen there is a 50% chance that the fetus does not carry the risk antigen. In these pregnancies, there is no risk of HDFN and no further follow-up is needed. If, however, the fetus does inherit the implicated antigen, careful monitoring for fetal anaemia with serial assessment of maternal antibody titres and activity, fetal Doppler ultrasound measurements of the peak systolic velocity in the middle cerebral artery, and, ultimately, intrauterine fetal blood sampling may be indicated. Traditionally, fetal blood group genotyping has been performed through amniocentesis. This invasive procedure carries a small risk of miscarriage[6] and could potentially enhance maternal sensitisation.[7] The discovery of cell-free fetal DNA in the plasma of pregnant women at the end of the twentieth century presented a noninvasive, and therefore safe, method to determine the fetal blood group genotype.[8,9] Since then, numerous groups have reported on fetal *RHD* genotyping in D-negative mothers with close to 100% accuracy.[10,11]

Although a small number of laboratories across Europe now offer this test to alloimmunised pregnant women diagnostically,[12] most studies have been performed with samples from nonimmunised D-negative pregnant women, to evaluate the use of this test to restrict antenatal anti-D immunoglobulin prophylaxis.[13-16] Moreover, most data have been obtained in a research setting rather than in a clinical setting and lacked a control for the presence of fetal DNA in case negative results were obtained.[17] Few studies have reported on noninvasive genotyping of fetal *c*, *E* and *K*.[18-20]

As a national reference laboratory, we have been offering noninvasive fetal blood group genotyping of rhesus D, c, E and of K in maternal plasma for alloimmunised pregnant women since the beginning of 2003 using a stringent diagnostic algorithm with the inclusion of fetal DNA identifiers to exclude false-negative results. The aim of the present study was to evaluate the diagnostic performance of these noninvasive fetal blood group genotyping tests, performed in a clinical setting over a 7-year period.

Methods

Sanquin Diagnostic Services is the national reference laboratory for pregnancies complicated by alloimmunisation in The Netherlands.

For this study, we collected the test data of all consecutive alloimmunised pregnant women for whom fetal blood group genotyping in maternal plasma was performed in our laboratory from 2003 up to 2010. Fetal D typing was offered from 2003, fetal K typing from 2006, and typing for c and E from 2007. Tests were offered in all alloimmunised pregnancies in which the father was heterozygously positive for the target antigen and was strongly advised if the antibody titre was ≥16 (≥2 for anti-K) or if the anti-body-dependent cell-mediated cytotoxicity test result was ≥30%.[21] Tests were performed at the request of midwives or gynaecologists throughout The Netherlands. We advised a minimum gestational age of 9 weeks for fetal D, c and E typing, and 12 weeks for fetal K typing, because of the lower sensitivity of the K assay.

Ethylenediaminetetraacetic acid anticoagulated blood was drawn from both the mother (30 mL) and, if possible, from the reporting father (10 mL) and was sent to our laboratory. Maternal blood samples were centrifuged at 1200 × *g* for 10 minutes within 48 hours of sampling. The plasma fraction was again centrifuged at 2400 × *g* for 20 minutes and the supernatant was collected and stored at –20°C until further processing.[22] In the case of fetal K typing, the blood samples were sent by express courier and processed within 8 hours to prevent the increase of the proportion of maternal DNA caused by lysis of nucleated blood cells in the tube that could hamper the specificity of the assay. Both parental samples were typed serologically for D, C/c, E/e and K/k to identify paternal blood group antigens that could potentially serve as a genetic control marker to confirm the presence of fetal DNA.

DNA was extracted in duplicate from 2×2 mL plasma using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Hilden, Germany), following the 'Blood or Body Fluids Protocol' recommended by the manufacturer. Volumes of the used reagents were increased proportionately to accommodate the 2-mL sample size. Adsorbed DNA was eluted with 60 µL of water.

Real-time polymerase chain reaction (PCR) analysis was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman chemistry. For *RHD* detection, *RHD* exon 5[23] and *RHD* exon 7[22] were analysed by duplex PCR. Both PCRs are positive when an intact *RHD* gene is present (Figure 1) but no product of *RHD* exon 5 is generated when a nonfunctional *RHD* pseudogene or an *RHD-CE-D*^{s} gene is present, both genes commonly found in people from African descent.[24,25] *RHD* exon 7 PCR is positive in almost all *RHD* genes, but not in *RHD-CE-D*^{s}. The use of both of these targets prevents false-positive results in fetuses carrying only an *RHD* pseudogene or *RHD-CE-D*^{s} gene, and, at the same time, allows for fetal *RHD* typing in D-negative mothers carrying these variant *RHD* genes.[26] For detection of the *c* and *E* alleles of the *RHCE* gene, allele-specific primers from Finning et al.[18] were used. These primer sets show a high specificity with DNA levels up to 50 ng (unpublished results). For detection of the *K* allele of the *KEL* gene, we used an in-house-

developed allele-specific primer. To prevent mispriming of the *K* allele-specific primer on the antithetic *k* allele, we designed a *k* allele-specific peptide nucleic acid (PNA) probe. Clamping of this PNA probe to the *k* allele prevents nonspecific amplification, ensuring allele-specificity of the reaction. To minimise the nonspecific amplification of maternal cell-free DNA and to achieve optimal PNA clamping, the level of maternal cell-free DNA was aimed to be as low as possible. Therefore, plasma was separated preferentially within 8 hours of blood sampling in the case of fetal K typing. As a control marker to confirm the presence of fetal DNA, PCR analysis of the Y-chromosome-specific *SRY* gene was also performed (only applicable in male-bearing pregnancies).[27] Part of the *albumin* gene was amplified as a control for DNA isolation.[28] All primer and probe sequences are listed in Table S1. The reactions were set up in a volume of 50 µL, using 25 µL Taqman Universal PCR Master Mix (Applied Biosystems) and 10 µL extracted DNA (for the *albumin* PCR, 3 µL extracted DNA was used in a 25-µL reaction volume). Primers and probes were used at final concentrations of 300 and 100 nM (900 and 150 nM for *SRY*, respectively). The final concentration of the *k*-specific PNA probe was 2 nM. Cycling conditions for all PCRs were 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of denaturation for 15 seconds at 95°C and primer annealing and elongation for 1 minute at 60°C.

For each of the two DNA isolations undertaken on each sample, PCR for the specific blood group antigen was performed in triplicate. Each replicate was judged for amplification according to previously defined cycle threshold (Ct) values (see Table S2). Interpretation of the combined results of both isolations led to a positive (i.e. antigen-positive fetus) or negative (i.e. antigen-negative fetus) test result. In the case of discrepant results between the two isolations, the test result was inconclusive. Discrepant results between *RHD* exon 5 and *RHD* exon 7 PCR (i.e. more than 2 Ct value difference) or Ct values outside the expected range for fetal DNA (i.e. <34) called for further serological and molecular parental *RHD* analysis. Because of potential nonspecific amplification of maternal DNA in the *K* PCR (inherent to the gene/assay), Ct values >43 led to an inconclusive result. Also, because of the lower sensitivity of the K assay, when a negative *K* genotyping result was obtained before 18 weeks of gestation, repeat testing at 18 weeks was recommended.

The PCRs for *SRY* and *albumin* were performed in duplicate. *SRY* was scored positive when the Ct value was <42. For the *albumin* PCR, a standard of genomic DNA was used (15000, 1500, 150 pg) to quantify the amount of total cell-free DNA in the sample.

In the case of a negative result for a specific blood group antigen and a positive result for *SRY* (thereby proving the presence of fetal DNA in the sample), a blood group antigen-negative fetus was reported. In the case of a negative result for a specific blood group antigen and a negative result for *SRY*, the presence of fetal DNA was ascertained through the use of a set of 24 biallelic insertion/deletion polymorphisms or another discrepant paternally inherited blood group antigen (for a detailed description, see Scheffer et al.[29]). Only when we were

able to confirm the presence of fetal DNA (one or more paternal markers positive in maternal plasma) was a negative result for the specific blood group antigen issued. If the presence of fetal DNA could not be confirmed, the overall test was inconclusive.

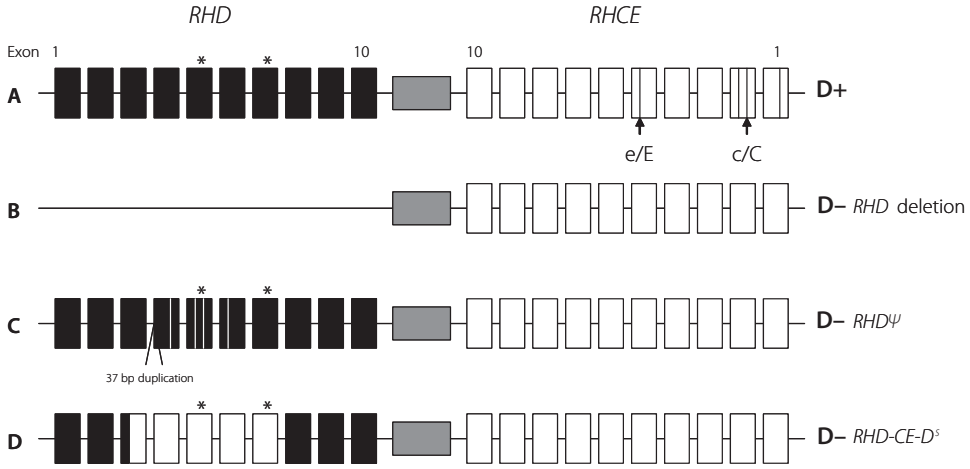

Figure 1. Diagrammatic representation of the *RHD* and *RHCE* genes in four haplotypes. (A) Normal *RHD* gene leading to a D-positive (D+) phenotype. (B) Complete deletion/absence of the *RHD* gene leading to a D-negative (D–) phenotype. (C) Mutations (white bands) in exons 4, 5, and 6 of the *RHDΨ* leading to a D– phenotype. (D) *RHCE*-derived exons (white boxes) in the *RHD-CE-D*^{s} hybrid gene leading to a D– phenotype. Asterisks in the *RHD* gene denote the sites used for polymerase chain reaction amplification (exons 5 and 7). Black bands in the *RHCE* gene represent the single nucleotide polymorphisms (SNPs) encoding either E or e and C or c. Black boxes, *RHD* exons; white boxes, *RHCE* exons; *RHDΨ*, *RHD* pseudogene.

As part of continuous test performance quality control, upon reporting the test result to the physician, we requested that we receive follow-up of the neonatal blood group phenotype, in the case that cord blood serology was performed. In the case of subsequent amniocentesis (e.g. because of an inconclusive PCR result in maternal plasma), we requested that we receive the fetal blood group genotyping result. If no follow-up was received, the requesting physician was contacted by telephone after the expected date of birth to ask whether the blood group phenotype or genotype had been determined.

After collecting all results, descriptive statistics were generated using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). The Fisher exact test (two-sided) was used to determine sensitivity and specificity with 95% confidence intervals (95% CI).

Results

From 2003 up to 2010, 362 tests were performed for a total of 346 alloimmunised pregnant women (Table 1). Sixteen women had both anti-c and anti-E alloantibodies and testing for both fetal blood group antigens was performed. The median gestational age at the time of blood sampling was 17 weeks (range 7–38 weeks) for all assays. Five women carried twins; two of these had anti-D alloantibodies, one had anti-E alloantibodies and two had both anti-c and anti-E alloantibodies. In 2009, when testing for all four blood group antigens was available, the majority of tests was performed for fetal E typing (39%), followed by typing for D (29%), c (17%) and K (15%). Most tests were requested when serology still indicated the absence of a clinical risk (i.e. antibody titres <16 and antibody-dependent cell-mediated cytotoxicity test results <30% for anti-D, anti-c, and anti-E; see Table S3).

Table 1. Number of fetal blood group genotyping tests performed from 2003 up to 2010

Test	Year							
	2003	2004	2005	2006	2007	2008	2009	Total
D	14	10	20	33	20	39	32	168
c	–	–	–	–	9	21	19	49
E	–	–	–	–	6	36	43	85
K	–	–	–	3	16	24	17	60
Total	14	10	20	36	51	120	111	362

Fetal *RHD* typing was performed in 168 women. Conclusive results were obtained in 161 (96%). A positive test result was issued for 113 samples. In the majority of these (n = 104), both *RHD* exon 5 and exon 7 PCR were positive, with Ct values within the expected range for fetal DNA (between 34 and 39). In six other women (Table 2; cases 1–6), an abnormally high level of amplification of *RHD* exon 7 (Ct values between 29 and 32) indicated amplification of a maternal *RHD* allele. Maternal *RHD* analysis confirmed the presence of a nonfunctional *RHD* pseudogene. In three further women (cases 7–9), a discrepancy between the level of amplification of fetal *RHD* exon 5 and *RHD* exon 7 was observed. Serological and molecular *RHD* typing of the father revealed the presence of an *RHD* variant gene (*DIVa*, *DAU5*, and *DNU*, respectively), that apparently was inherited by the fetus (leading to, respectively, a negative *RHD* exon 7, a negative *RHD* exon 5, and a weaker *RHD* exon 7 PCR result).

In 51 pregnancies, both *RHD* exon 5 and exon 7 PCR were negative, pointing to a D-negative fetus. In two further pregnancies with a negative *RHD* exon 5 PCR result (Table 2; cases 10 and 11), an abnormally high level of amplification of *RHD* exon 7 exposed a maternal *RHD* pseudogene, which was confirmed after maternal *RHD* analysis. Of these 53 pregnancies, the presence of fetal DNA could be confirmed in 48 and a D-negative fetus was reported. In the other five, the presence of fetal DNA could not be confirmed and the test result was reported as inconclusive.

In two more cases of fetal *RHD* typing (Table 2; cases 12 and 13), no test result could be issued because of a high level of amplification of both *RHD* exon 5 and *RHD* exon 7. Sequencing of the *RHD* gene in maternal genomic DNA demonstrated the presence of a so-called silent *RHD* gene, in which a mutation in the gene leads to the loss of function. Subsequent genotyping of amniotic fluid cells showed a D-positive and a D-negative child, respectively.

Table 2. Results for maternal and paternal *RHD* analysis performed because of atypical fetal *RHD* exon 5 and/or *RHD* exon 7 polymerase chain reaction results

Case	*RHD* PCR plasma		Maternal *RHD* analysis (genotype)	Paternal *RHD* analysis (genotype)	Conclusion fetal RhD status
	Exon 5 Ct	Exon 7 Ct			
1	37	32	*RHDΨ/d*	N/A	D positive
2	39	29	*RHDΨ/d*	N/A	D positive
3	35	30	*RHDΨ/d*	*RHD/DAU*	D positive
4	38	31	*RHDΨ/d*	N/A	D positive
5	38	32	*RHDΨ/d*	*RHD/RHD*	D positive
6	37	30	*RHDΨ/RHD-CE-D^s*	*RHD/DIII type 5*	D positive
7	38	Und	*d/d*	*DIVa/d*	D positive
8	Und	36	*d/d*	*RHD/DAU5*	D positive
9	35	39	*d/d*	*RHD/DNU*	D positive
10	Und	31	*RHDΨ/d*	*RHDΨ/DAU*	D negative
11	Und	31	*RHDΨ/RHD-CE-D^s*	N/A	D negative
12	30	30	*RHD(343delC)/d*	N/A	Inconclusive
13	31	31	*RHD(IVS1+1G>A)/d*	*d/d*	Inconclusive

Ct, cycle threshold value; *d*, deletion/complete absence of *RHD* gene; N/A, not available; PCR, polymerase chain reaction; *RHD*, normal *RHD* gene; *RHDΨ*, *RHD* pseudogene; Und, undetermined (no amplification).
RHDΨ, RHD-CE-D^s, RHD(343delC) and *RHD(IVS1+1G>A)*: *RHD* variant genes leading to a D-negative phenotype.
DAU, DIII type 5, DIVa, DAU5 and DNU: *RHD* variant genes leading to a D-positive phenotype.

Fetal *c* typing was performed in 49 pregnancies. Conclusive results were obtained in all. The PCR was positive in 30 pregnancies and negative in 19.

Fetal *E* typing was performed in 85 pregnancies. All test results were conclusive. The PCR was positive in 52 pregnancies and negative in 33.

Fetal *K* typing was performed in 60 pregnancies. Conclusive results were obtained in 56 (93%). A positive test result was issued in 24 pregnancies; a negative test result in 32. In two pregnancies, negative for *K*, the presence of fetal DNA could not be confirmed and the test result was reported as inconclusive. In two other pregnancies, very high (>43) Ct values were obtained and we could not discriminate between the presence of a very low amount of fetal DNA and nonspecific amplification of maternal DNA, leading to an inconclusive test result. Both of these samples were drawn at 16 weeks of gestation. Repeat testing at 17 weeks in one woman showed similar Ct values and the test result remained inconclusive. A K-negative child was born. In the other woman, no second maternal blood sample was

obtained because of an intrauterine fetal demise. Cord blood serology showed that the child was K-positive. Of 17 pregnancies negative for *K* before 18 weeks of gestation, repeat testing was performed in seven. All were still negative the second time.

In 71 of the 139 pregnancies (51%) in which the PCR result for the specific blood group antigen was negative, the presence of fetal DNA could be confirmed by a positive result for *SRY* (Figure 2). In 61 other pregnancies, biallelic insertion/deletion polymorphism markers (*n* = 50; 36%) or another paternally inherited blood group antigen (*n* = 11; 8%) could be used. In seven samples (5%), the presence of fetal DNA could not be confirmed and the test results were reported as inconclusive (five for fetal *RHD* typing and two for fetal K typing, as described above).

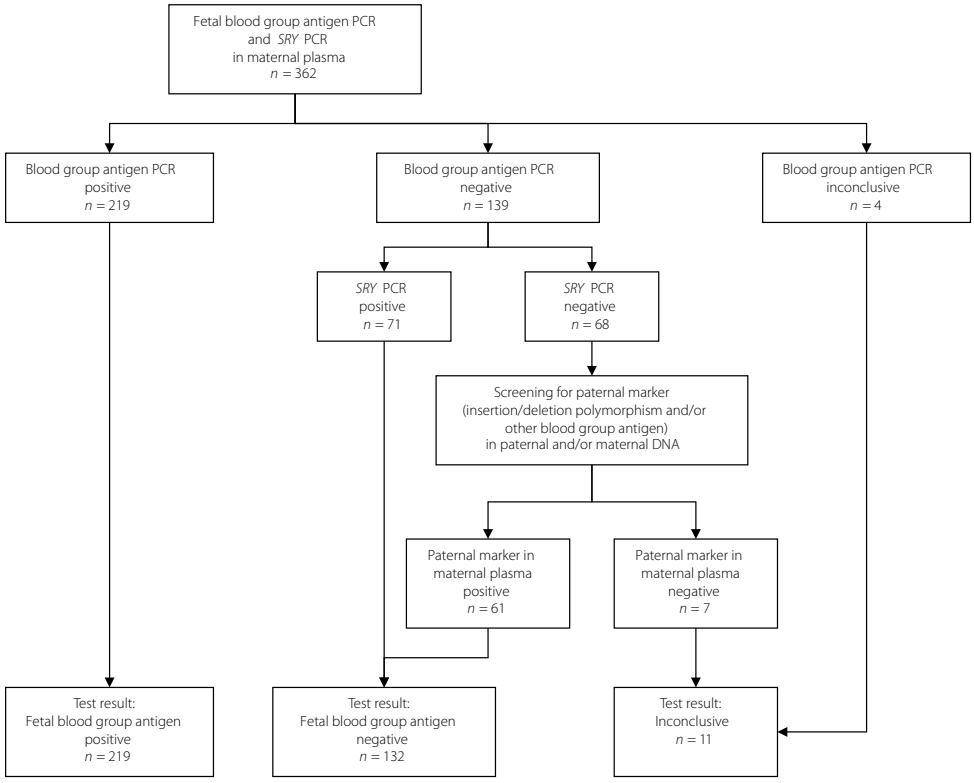

Figure 2. Test results for alloimmunised pregnant women tested from 2003 up to 2010.

Overall, a test result was issued in 97% (351 of 362). In general, test results were reported to the requesting physician within two (in the case of an antigen-positive fetus) to four (in the case of an antigen-negative fetus) working days after blood sampling (data not shown).

Cord blood serology (*n* = 204) or genotyping results of amniotic fluid cells (*n* = 8) was available

in 212 of the 362 cases (59%; Table 3). Fetal *RHD* typing was positive in 96 of 96 plasma samples from women reportedly giving birth to a D-positive child, resulting in a 100% sensitivity (95% CI 96.2–100%). In all 37 plasma samples from women reportedly giving birth to a D-negative child, fetal *RHD* typing was negative, resulting in a 100% specificity (95% CI 90.5–100%). Cord blood serology for c and E was performed in 19 and 21 women, respectively. No discrepancies with maternal plasma PCR results were found. Results for fetal *K* typing could be confirmed in 30 pregnancies. No false-positive or false-negative results were found.

Table 3. Maternal plasma polymerase chain reaction results vs. cord blood serology or genotyping results of amniotic fluid cells (*n* = 212)

Assay	Newborn phenotype or fetal genotype*	*n*	Maternal plasma PCR result		
			Positive	Negative	Inconclusive
D	D positive	97	96	0	1
	D negative	42	0	37	5
c	c positive	14	14	0	0
	c negative	5	0	5	0
E	E positive	16	16	0	0
	E negative	5	0	5	0
K	K positive	18	17	0	1
	K negative	15	0	13	2

*As determined by cord blood serology or by genotyping of amniotic fluid cells.

In three of the five women carrying twins, postnatal cord blood serology was performed. In the first, the test result for *RHD* was positive and a D-positive girl and D-negative boy were born. In the second, the test result for *RHD* was negative and two D-negative boys were born. In the third, test results for *c* and *E* were positive and both boys born had a c-positive and E-positive phenotype.

Of the seven pregnancies with an inconclusive test result because of failure to confirm the presence of fetal DNA, amniocentesis was performed in two, showing a D-negative and a K-negative fetus, respectively. Postnatal cord blood serology in an additional three women showed all three infants to be D-negative. In the remaining two women, cord blood serology was not performed. In all seven pregnancies, a girl was born.

Discussion

In this study we have evaluated the diagnostic performance of noninvasive fetal blood group genotyping performed over a 7-year period in a national reference laboratory in The Netherlands. We have shown noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised women to be accurate and applicable in a clinical diagnostic setting.

Through the application of a stringent diagnostic algorithm on two separate DNA isolations and the confirmation of the presence of fetal DNA in the case of negative PCR results, we were able to report fully conclusive results in 351 of 362 tests performed. Moreover, as far as we could ascertain, no false-positive or false-negative results were found.

All test results were used by the referring physician to guide the clinical and laboratory management of the alloimmunised pregnancy. In our series, 126 women (including six with both anti-c and anti-E antibodies) could be reassured that their fetus was not at risk of haemolytic disease and extensive monitoring for fetal anaemia during pregnancy was not required. Two hundred and nine women (including ten with both anti-c and anti-E antibodies) were informed that their fetus was at risk of haemolytic disease and the appropriate pregnancy management could be arranged. None of these women had to undergo an invasive procedure to determine the fetal antigen status. Women whose test results were inconclusive (n = 11) were monitored as at risk of HDFN, at least until genotyping of amniotic fluid cells, if performed, proved otherwise.

The antigens of the Rh system are encoded by *RHD* and *RHCE*, two almost homologous genes located on chromosome 1 (Figure 1).[30] In the Caucasian population, homozygous deletion of the *RHD* gene is the predominant cause of the D-negative phenotype. In contrast, 82% of D-negative black Africans do not have a homozygous deletion of *RHD*, but carry one or two *RHD* variant genes: the *RHD* pseudogene[24] or the *RHD-CE-D*^{s} hybrid gene.[25] Neither produces any epitopes of D. When genotyping for fetal *RHD* in a multiethnic population, such as the Dutch, it is important that false-positive results do not result from the presence of these relatively common variant genes. Through the analysis of both exon 5 and exon 7 of *RHD* we avoided such false results and were able to predict the fetal D status in eight women in whom an *RHD* pseudogene variant gene was present in the mother (two also carrying *RHD-CE-D*^{s}), whether or not inherited by the fetus. At the same time, combining both exon 5 and exon 7 PCR results, more rare *RHD* genes producing variant D antigens could be detected, preventing false-negative results in three women. In one of the largest validation studies published on noninvasive fetal *RHD* genotyping, Rouillac-Le Sciellour et al.[31] amplified *RHD* exon 7 and exon 10 in 893 maternal plasma samples. They had to exclude 26 D-negative women carrying an *RHD* pseudogene and five carrying *RHD-CE-D*^{s}, unable to predict the fetal phenotype with the combination of these two targets. Chinen et al.[32], tested 102 D-negative women in a Brazilian population and reported two false-positive results in women carrying an *RHD* pseudogene, using an exon 7–exon 10 approach. Other groups using these two targets did reach 100% accuracy,[33-35] but study populations were presumably all-white.

Noninvasive fetal *RHD* genotyping is not only a valuable tool in the management of RhD-alloimmunised pregnancies, but also allows antenatal anti-D immunoglobulin prophylaxis to be reserved for only those nonimmunised D-negative pregnant women that carry a D-positive fetus.[13-16] In such a fetal *RHD* screening setting, typing would be performed upon

automated DNA extractions to facilitate high-throughput screening, with an accepted certain loss of sensitivity and specificity at a more advanced gestational age and with a primary aim to avoid false-negative results, whereas false-positive results would be less important.[36-37]

No false-positive or false-negative results were reported for the allele-specific assays for *c* and *E* used in our protocol. Similar conclusions were published by Finning et al.[18], who used the same set of primers and reached 100% accuracy in 46 samples from pregnant women with anti-E alloantibodies at a more advanced gestational age (mean 23 weeks) and 44 samples from pregnant women with anti-c alloantibodies (mean 26 weeks), with an initial three inconclusive results in the latter. Adhering to their protocols, Gutensohn et al.[20] confirmed their findings in 87 and 100 samples from nonimmunised c-negative and E-negative pregnant women, respectively.

Using conventional allele-specific primers on a real-time PCR platform, detection of the fetal *K* allele can be hampered by nonspecific amplification of the maternal *k* allele[18] and only a few laboratories therefore offer this test diagnostically.[12] To increase the specificity of the assay we used a PNA probe, preventing mispriming of the *K*-allele-specific primer. Only in two of the 60 samples tested for *K* did nonspecific amplification prevent a conclusive result. In a publication by Finning et al.[18] in which the authors introduced locked nucleic acids in the *K*-specific primer to increase specificity, three inconclusive results and one false-negative result were reported out of 70 samples tested for *K*. Because locked nucleic acids reduce the sensitivity of the assay, the authors recommended retesting at 28 weeks of samples with a *K*-negative result tested before 28 weeks of gestation. This is relatively late given the fact that K alloimmunisation can lead to severe fetal anaemia already early in pregnancy.[3] With our approach, the fetal K status can be reliably predicted before 18 weeks of gestation.

To ensure a low-as-possible maternal DNA concentration in the sample, blood samples drawn for fetal K typing are processed within 8 hours of sampling. Recently, a new type of blood collection tube, Cell-free DNA BCT (Streck Inc., La Vista, NE, USA), was shown to minimise post-sampling maternal cell-free DNA background for up to 14 days after sampling.[38] Use of these tubes might allow for a longer period of time before processing, obviating the need for courier transport.

Because a fetal antigen-negative phenotype is not detected directly, but only inferred by a negative result for the antigen-specific PCR, we regard the confirmation of the presence of fetal DNA in such a case to be of the utmost importance. We were able to confirm the presence of fetal DNA by other paternally inherited DNA sequences (i.e. *SRY*, an insertion/deletion polymorphism or other blood group antigen) in 95% (132 of 139) of women with a negative PCR result for a specific blood group antigen. Although this percentage may be clinically acceptable, the ideal fetal marker would be universally applicable (in 100% of women) and independent of paternally inherited sequences. The *RASSF1A* gene, methylated differently between mother and child, has been proposed by Chan et al.[39], but in our hands

the use of this methylation marker has not yet been proven specific and sensitive enough for routine application (unpublished results). Recently however, an Australian group published a description of its successful use in confirming the presence of fetal DNA in 16 of 16 samples negative for *RHD* and *SRY* in a study with 140 samples from D-negative pregnant women.[40] As other methylation markers are emerging,[41] their use as a universal fetal marker may be implemented in diagnostic protocols soon.

A limitation of our study is the fact that test results could be compared with cord blood serology or genotyping results of amniotic fluid cells in only 59% of cases. Therefore, accuracy statements should only be made based on these cases. As our study is an evaluation of data obtained in a clinical diagnostic setting, cord blood serology results were collected in a retrospective manner. It was left to the physician's clinical judgement at the time of birth whether or not to have cord blood serology performed. We presume that infants for whom cord blood serology was not performed did not have any clinical signs of anaemia. This is supported by the finding that no false-negative results were found in those women in whom cord blood serology was performed. Although we cannot fully exclude false-positive results in those women for whom cord blood serology was not performed, no false-positive results were observed in those antigen-positive-predicted women in which it was (65% for all assays combined).

Conclusion

Noninvasive fetal blood group genotyping is a clinical reality. There is no longer a need for invasive procedures to determine the fetal D, c, E or K antigen status. In alloimmunised pregnant women, noninvasive fetal blood group genotyping provides an easy and safe method to determine whether a fetus is at risk of haemolytic disease, preventing extensive laboratory and clinical monitoring in antigen-negative cases.

Acknowledgements

The development and validation of the *RHD* polymerase chain reaction protocols have been performed within the European Commission for Special Non-invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence (LSHB-CT-2004-503243).

We greatly acknowledge Aicha Ait Soussan (Sanquin Research Amsterdam, Amsterdam) and Bernadette Bossers (Sanquin Diagnostic Services, Amsterdam) for technical support. We thank Diana van den Akker, Peter Ligthart and Lianne Schuitemaker (Sanquin Diagnostics Amsterdam) and Jennie Verdoes (Leiden University Medical Centre, Leiden) for help with retrieving cord blood serology results.

References

1 Moise KJJ. Management of rhesus alloimmunization in pregnancy. *Obstet Gynecol* 2008;112:164–76.

2 Koelewijn JM, de Haas M, Vrijkotte TGM, van der Schoot CE, Bonsel GJ. Risk factors for RhD immunisation despite antenatal and postnatal anti-D prophylaxis. *BJOG* 2009;116:1307–14.

3 van Wamelen DJ, Klumper FJ, de Haas M, Meerman RH, van Kamp IL, Oepkes D. Obstetric history and antibody titer in estimating severity of Kell alloimmunization in pregnancy. *Obstet Gynecol* 2007;109:1093–8.

4 Koelewijn JM, Vrijkotte TGM, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in The Netherlands. *Transfusion* 2008;48:941–52.

5 Moise KJ. Fetal anemia due to non-Rhesus-D red-cell alloimmunization. *Semin Fetal Neonatal Med* 2008;13:207–14.

6 Mujezinovic F, Alfirevic Z. Procedure-related complications of amniocentesis and chorionic villous sampling: a systematic review [published erratum appears in *Obstet Gynecol* 2008;111:779]. *Obstet Gynecol* 2007;110:687–94.

7 Tabor A, Bang J, Norgaard-Pedersen B. Feto-maternal haemorrhage associated with genetic amniocentesis: results of a randomized trial. *Br J Obstet Gynaecol* 1987;94:528–34.

8 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

9 Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* 1998;352:1196.

10 Geifman-Holtzman O, Grotegut CA, Gaughan JP. Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood – a meta-analysis. *Am J Obstet Gynecol* 2006;195:1163–73.

11 Daniels G, Finning K, Martin P, Massey E. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* 2009;29:101–7.

12 Daniels G, van der Schoot CE, Gassner C, Olsson ML. Report of the third international workshop on molecular blood group genotyping. *Vox Sang* 2009;96:337–43.

13 van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Page-Christiaens GCML, de Haas M. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006;13:53–7.

14 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* 2008;336:816–8.

15 Minon JM, Gerard C, Senterre JM, Schaaps JP, Foidart JM. Routine fetal RHD genotyping with maternal plasma: a four-year experience in Belgium. *Transfusion* 2008;48:373–81.

16 Muller SP, Bartels I, Stein W, Emons G, Gutensohn K, Kohler M, et al. The determination of the fetal D status from maternal plasma for decision making on Rh prophylaxis is feasible. *Transfusion* 2008;48:2292–301.

17 Freeman K, Szczepura A, Osipenko L. Non-invasive fetal RHD genotyping tests: a systematic review of the quality of reporting of diagnostic accuracy in published studies. *Eur J Obstet Gynecol Reprod Biol* 2009;142:91–8.

18 Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c, and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion* 2007;47:2126–33.

19 Geifman-Holtzman O, Grotegut CA, Gaughan JP, Holtzman EJ, Floro C, Hernandez E. Noninvasive fetal RhCE genotyping from maternal blood. *BJOG* 2009;116:144–51.

20 Gutensohn K, Muller SP, Thomann K, Stein W, Suren A, Kortge-Jung S, et al. Diagnostic accuracy of noninvasive polymerase chain reaction testing for the determination of fetal rhesus C, c and E status in early pregnancy. *BJOG* 2010;117:722–9.

21 Oepkes D, van Kamp IL, Simon MJ, Mesman J, Overbeeke MA, Kanhai HH. Clinical value of an antibody-dependent cell-mediated cytotoxicity assay in the management of Rh D alloimmunization. *Am J Obstet Gynecol* 2001;184:1015–20.

22 Rijnders RJP, Christiaens GCML, Bossers B, van der Smagt JJ, van der Schoot CE, de Haas M. Clinical applications of cell-free fetal DNA from maternal plasma. *Obstet Gynecol* 2004;103:157–64.

23 Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002;42:1079–85.

24 Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D-negative blood group phenotype. *Blood* 2000;95:12–8.

25 Faas BH, Beckers EA, Wildoer P, Ligthart PC, Overbeeke MA, Zondervan HA, et al. Molecular background of VS and weak C expression in blacks. *Transfusion* 1997;37:38–44.

26 Grootkerk-Tax MGHM, Soussan AA, de Haas M, Maaskant-van Wijk PA, van der Schoot CE. Evaluation of prenatal RHD typing strategies on cell-free fetal DNA from maternal plasma. *Transfusion* 2006;46:2142–8.

27 Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768–75.

28 Pongers-Willemse MJ, Verhagen OJ, Tibbe GJ, Wijkhuijs AJ, de Haas V, Roovers E, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. *Leukemia* 1998;12:2006–14.

29 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, Bossers B, van Erp F, de Haas M. Reliability of fetal sex determination using maternal plasma. *Obstet Gynecol* 2010;115:117–26.

30 Avent ND, Reid ME. The Rh blood group system: a review. *Blood* 2000;95:375–87.

31 Rouillac-Le Sciellour C, Puillandre P, Gillot R, Baulard C, Metral S, Le Van Kim C, et al. Large-scale pre-diagnosis study of fetal RHD genotyping by PCR on plasma DNA from RhD-negative pregnant women. *Mol Diagn* 2004;8:23–31.

32 Chinen PA, Nardozza LM, Martinhago CD, Camano L, Daher S, Pares DB, et al. Noninvasive determination of fetal Rh blood group, D antigen status by cell-free DNA analysis in maternal plasma: experience in a Brazilian population. *Am J Perinatol* 2010;27:759–62.

33 Brojer E, Zupanska B, Guz K, Orzinska A, Kalinska A. Noninvasive determination of fetal RHD status by examination of cell-free DNA in maternal plasma. *Transfusion* 2005;45:1473–80.

34 Clausen FB, Krog GR, Rieneck K, Nielsen LK, Lundquist R, Finning K, et al. Reliable test for prenatal prediction of fetal RhD type using maternal plasma from RhD negative women. *Prenat Diagn* 2005;25:1040–4.

35 Gautier E, Benachi A, Giovangrandi Y, Ernault P, Olivi M, Gaillon T, et al. Fetal RhD genotyping by maternal serum analysis: a two-year experience. *Am J Obstet Gynecol* 2005;192:666–9.

36 Clausen FB, Krog GR, Rieneck K, Rasmark EEF, Dziegiel MH. Evaluation of two real-time multiplex PCR screening assays detecting fetal RHD in plasma from RhD negative women to ascertain the requirement for antenatal RhD prophylaxis. *Fetal Diagn Ther* 2011;29:155–63.

37 Tynan J, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. *Am J Obstet Gynecol* 2011;204:251–6.

38 Fernando MR, Chen K, Norton S, Krzyzanowski G, Bourne D, Hunsley B, et al. A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. *Prenat Diagn* 2010;30:418–24.

39 Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN, et al. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.

40 Hyland CA, Gardener GJ, Davies H, Ahvenainen M, Flower RL, Irwin D, et al. Evaluation of non-invasive prenatal RHD genotyping of the fetus. *Med J Aust* 2009;191:21–5.

41 Nygren AO, Dean J, Jensen TJ, Kruse S, Kwong W, van den Boom D, et al. Quantification of fetal DNA by use of methylation-based DNA discrimination. *Clin Chem* 2010;56:1627–35.

Supporting information

Chapter 3

Table S1. Primer and probe sequences for fetal D, c, E and K typing

Name	Sequence 5' to 3'*	Allele
RHDex5_F	CGC CCT CTT CTT GTG GAT G	*D, CE*
RHDex5_R	GAA CAC GGC ATT CTT CCT TTC	*D*
RHDex5_P	VIC-TCT GGC CAA GTT TCA ACT CTG CTC TGC T-TAMRA	*D*
RHDex7_F	GGG TGT TGT AAC CGA GTG CTG	*D*
RHDex7_R	CCG GCT CCG ACG GTA TC	*D*
RHDex7_P	FAM-CCC ACA GCT CCA TCA TGG GCT ACA A-TAMRA	*D*
RHc_F	TGG GCT TCC TCA CCT CAA A	*c*
RHc_R	TGA TGA CCA CCT TCC CAG G	*c*
RHc_P	FAM-CAA TCC TGC TGG ACG GCT TCC TGA-TAMRA	*D, CE*
RHE_F	TGG CCA CGT GTC AAC TCT C	*E*
RHE_R	CTG TCA CCA CAC TGA CTG CTA G	*CE*
RHE_P	FAM-CCA AAG GAA GAA TGC CAT GTT CAA CAC CTA C-TAMRA	*CE*
K1_F	AGC CCC CTC TCT CTC CTT TA	*K, k*
K1_R	CTG ACT CAT CAG AAG TCT CAG GA	*K*
K1_P	FAM-CAG AGA TGC GCC AGC CTC CAA-TAMRA	*K, k*
K1_PNA_probe	CAG CGT TCG G	*k*
SRY_F	TGG CGA TTA AGT CAA ATT CGC	*SRY*
SRY_R	CCC CCT AGT ACC CTG ACA ATG TAT T	*SRY*
SRY_P	FAM-AGC AGT AGA GCA GTC AGG GAG GCA GA-TAMRA	*SRY*
Alb_F	TGA AAC ATA CGT TCC CAA AGA GTT T	*Albumin*
Alb_R	CTC TCC TTC TCA GAA AGT GTG CAT AT	*Albumin*
Alb_P	FAM-TGC TGA AAC ATT CAC CTT CCA TGC AGA-TAMRA	*Albumin*

F, forward primer; R, reverse primer; P, probe.
*see Methods section for the appropriate references.

Table S2. Scoring model for fetal *RHD* exon 5, *RHD* exon 7, *c*, *E* and *K* polymerase chain reaction

	Isolation 1			Isolation 2			Test result	Further proceedings
Replicate	1	2	3	1	2	3		
	+	+	+	+	+	+	Positive	Report positive fetal blood group antigen
	+	+	+	+	+	–	Positive	Report positive fetal blood group antigen
	+	+	–	+	+	–	Positive	Report positive fetal blood group antigen
	+	+	+	+	–	–	Inconclusive	Repeat test with same sample
	+	+	+	–	–	–	Inconclusive	Repeat test with same sample
	+	+	–	–	–	–	Inconclusive	Repeat test with same sample
	+	+	–	+	–	–	Inconclusive	Repeat test with new sample
	+	–	–	+	–	–	Inconclusive	Repeat test with same sample
	+	–	–	–	–	–	Negative	Confirm presence of fetal DNA; report negative fetal blood group antigen
	–	–	–	–	–	–	Negative	Confirm presence of fetal DNA; report negative fetal blood group antigen

PCR, polymerase chain reaction; Ct, cycle threshold value.
Replicates are scored positive (+) if Ct is 34 or more and negative (–) if no amplification is observed. Ct's less than 34 in the *RHD* exon 5 and/or *RHD* exon 7 PCR suggest amplification of maternal DNA and are scored as inconclusive. Ct's more than 43 in the *K* PCR are scored as inconclusive because of potential nonspecific amplification of the maternal *k* allele.

Table S3. Antibody-dependent cell-mediated cytotoxicity test results and antibody titres before fetal blood group genotyping in maternal plasma (year 2009)

Test			ADCC <30%									ADCC ≥30%								Antibodies in previous pregnancy‡	Total
	Titre*	N/A#	–	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:4	1:32	1:64	1:128	1:256	1:512	1:1000	1:2000		
D			6	2	1	2	4	3	2	1			1		4	2				4	32
c		8	5	2	1		1							1	1						19
E		16	11	6	2	1	1	1	1	2	1				1						43
K		1		1	1	1	1	1	1	1		1		1	2	1	1	1	1	1	17

N/A, not applicable; ADCC, antibody-dependent cell-mediated cytotoxicity test.
*As determined by indirect antiglobulin test.
#Antibodies present, but ADCC test not performed and titration negative.
‡Fetal genotyping requested because of the presence of clinically relevant (i.e. titre ≥16) antibodies in previous pregnancy.

Chapter 4

Noninvasive fetal genotyping of human platelet antigen-1a

P.G. Scheffer[a,b]
A. Ait Soussan[a]
O.J.H.M. Verhagen[a]
G.C.M.L. Page-Christiaens[b]
D. Oepkes[c]
M. de Haas[a]
C.E. van der Schoot[a]

[a] Department of Experimental Immunohaematology, Sanquin Research Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Division of Perinatology and Gynaecology, University Medical Centre Utrecht, Utrecht, The Netherlands
[c] Department of Obstetrics, Leiden University Medical Centre, Leiden, The Netherlands

BJOG 2011;118:1392–5

Abstract

We describe a reliable noninvasive fetal human platelet antigen (HPA)-1a genotyping assay on a real-time polymerase chain reaction (PCR) platform using cell-free fetal DNA isolated from maternal blood. Nonspecific amplification of maternal cell-free DNA is overcome by pre-PCR digestion of the cell-free DNA with the *Msp1* restriction enzyme. Noninvasive fetal HPA-1a genotyping offers a safe method for alloimmunised pregnant women to determine whether their fetus is at risk of fetal or neonatal alloimmune thrombocytopenia (FNAIT) and whether interventions to prevent intracranial haemorrhage are required. The availability of this test is relevant to the ongoing debate on screening pregnancies for HPA-1a-mediated FNAIT.

Introduction

Fetal or neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies directed against paternally inherited antigens present on fetal platelets. In 80% of cases of FNAIT, antibodies are directed against the human platelet antigen (HPA)-1a.[1] FNAIT complicates about 0.1% of all pregnancies and is associated with major fetal and neonatal morbidity and mortality. The most feared complication is an intracranial haemorrhage (ICH), occurring in approximately 10–20% of cases. The majority of these bleedings occurs *in utero*. Antenatal management of FNAIT, ultimately aimed at preventing ICH, may consist of weekly administration of intravenous immunoglobulins, with or without corticosteroids, to the mother, or fetal blood sampling with platelet transfusions.[2] In most centres, elective caesarean section is the preferred mode of delivery in pregnancies at risk of FNAIT. In the absence of screening programmes, the disease is only diagnosed after the birth of a symptomatic neonate, that is, fetal or neonatal bleeding, or occasionally by chance in the case of neonatal blood tests for other reasons. Consequently, antenatal treatment is currently provided only in pregnancies subsequent to the birth of a previously affected child.[3]

In HPA-1a-alloimmunised pregnant women, knowledge of the fetal HPA-1a status determines the pregnancy management. Only if the fetus is HPA-1a positive is there a risk of FNAIT, and the pregnancy is managed as such. In about 30% of cases, there is a 50% chance that the fetus will inherit a paternal HPA-1b allele, and there is no risk of FNAIT. Currently, when the father is heterozygously HPA-1a/1b positive, the fetal HPA genotype is determined by amniocentesis. This invasive procedure carries a small risk of miscarriage and can potentially cause the boosting of antibody formation.

In 1997, Lo et al.[4] reported the presence of cell-free fetal DNA in maternal plasma and serum, and its implications for noninvasive prenatal diagnosis. Since then, polymerase chain reaction (PCR)-based noninvasive genotyping of paternally inherited fetal alleles not present in the maternal genome, for example, *RHD* genotyping in D-negative mothers and the detection of Y chromosome-specific sequences for fetal sexing, has found its way into clinical practice.[5] However, testing for the presence or absence of fetal alleles that only differed slightly from their maternal counterpart, for example, only one nucleotide, appeared to be more difficult and, for certain alleles, remained severely hampered by nonspecific amplification of the overwhelmingly present maternal cell-free DNA. For this reason, a reliable noninvasive fetal HPA-1a genotyping assay has not yet been described.

In this communication, we report a noninvasive fetal HPA-1a genotyping assay in which the nonspecific amplification of maternal cell-free DNA is overcome by pre-PCR digestion of the cell-free DNA with the *Msp1* restriction enzyme. This enzyme specifically recognises the maternal HPA-1b allele DNA sequence, whilst leaving the fetal HPA-1a allele DNA sequence intact (Figure 1).

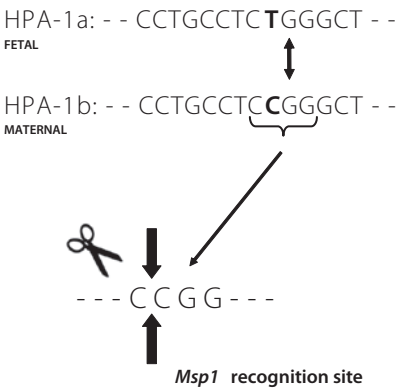

Figure 1. *Msp1* digestion. The HPA-1a allele differs one single nucleotide (196 T>C) from the HPA-1b allele. The T>C substitution in the HPA-1b allele creates an *Msp1* endonuclease recognition site. Pre-PCR processing of cell-free DNA with *Msp1* will digest the HPA-1b allele, while leaving the fetal HPA-1a allele intact.

Methods

Sample collection

Frozen plasma and serum samples from pregnant women with anti-HPA-1a antibodies were collected at the Leiden University Medical Centre (national referral hospital for pregnancies at risk of FNAIT) and Sanquin Diagnostic Services (national reference laboratory) under Medical Ethics Committee approval. All blood samples had been drawn from women who had undergone a previously affected pregnancy and for whom amniocentesis had been performed in the index pregnancy to determine the fetal HPA-1a status. Informed consent was obtained in all cases.

DNA extraction and *Msp1* digestion

DNA was extracted from 1 mL of plasma or serum with the QIAamp DSP Virus kit (Qiagen Inc., Hilden, Germany) using a modified spin-column method. Briefly, 1 mL of lysis buffer was added to 100 µL of protease and 1 mL of plasma or serum and incubated at 56°C for 20 minutes. After mixing with ethanol (96%), the mixture was applied to the QIAamp column and centrifuged at 6000 × *g* for 1 minute. The columns were washed twice and incubated with an open lid at 56°C for 5 minutes. DNA was eluted with 60 µL of water. Forty-four microlitres of extracted DNA were digested with 20 units of the *Msp1* restriction enzyme (New England BioLabs Inc., Ipswich, MA, USA) at 37°C for 2 hours, during which time the HPA-1b allele was digested, followed by 20 minutes of enzyme heat inactivation at 80°C. Genomic DNA from an HPA-1b/1b donor (5 ng) and from an HPA-1a/1b donor (500 pg) was digested at the same time to serve as negative and positive controls, respectively.

PCR analysis

Real-time PCR analysis was performed with the StepOne-Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Taqman chemistry. DNA was analysed for HPA-1a using an allele-specific reverse primer. To reduce primer annealing to the (remaining) nontarget HPA-1b allele, the third nucleotide at the 3' end of the allele-specific primer was mismatched. Primer and probe sequences were as follows: forward primer, 5'-GTAGAGA GTCGCCATAGCTCTGATT-3'; reverse primer (allele-specific), 5'-CACAGCGAGGTGAGCACA-3'; probe, 5'-FAMCCTGTAAGACAGGAGCCCAAAGAGAAGTC-TAMRA-3'. Part of the *albumin* gene was amplified as a control for DNA isolation.[5]

The singleplex reactions were set up in a volume of 25 µL, using 12.5 µL of Taqman Universal PCR Master Mix (Applied Biosystems) and 10 µL of extracted DNA (3 µL for the *albumin* PCR). Primers and probes were used at final concentrations of 300 and 100 nM. Cycling conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of denaturation for 15 seconds at 95°C and primer annealing and elongation for 1 minute at 60°C.

DNA was analysed for HPA-1a in triplicate. Test results were considered to be valid only if amplification with the digested DNA from the HPA-1b/1b control donor was not observed. All test results were compared with the genotyping results of amniotic fluid cells.

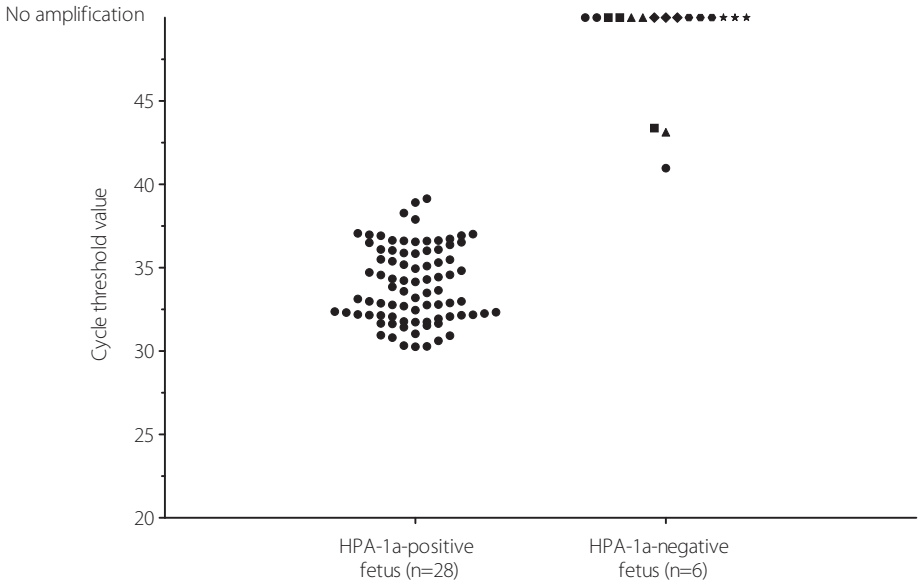

Figure 2. Cycle threshold (Ct) values for human platelet antigen (HPA)-1a-positive and HPA-1a-negative fetuses. The Ct values of all three replicates per sample are shown. The different symbols for the HPA-1a-negative fetuses represent the six different cases.

Results

A total of 34 samples was collected (plasma: *n* = 28; serum: *n* = 6). Twenty-eight women carried an HPA-1a-positive fetus and six women carried an HPA-1a-negative fetus. The median gestational age at the time of blood sampling was 32 weeks (range 12–39 weeks). All 28 samples from women carrying an HPA-1a-positive fetus showed positive PCR results in three of the three replicates, with a mean cycle threshold value (Ct) of 34.0 (±2.2 SD) (Figure 2). In the six samples from women carrying an HPA-1a-negative fetus, no amplification (*n* = 3) or amplification in one of the three replicates (*n* = 3) was observed (Ct values of 41.0, 43.1 and 43.4, respectively).

Discussion

We have developed a reliable noninvasive fetal HPA-1a genotyping assay on a real-time PCR platform. False-positive results caused by nonspecific amplification of maternal cell-free DNA were completely excluded by sequence-specific enzyme digestion. At the same time, the sensitivity of the assay was retained, as no false-negative results were observed.

The HPA-1a/b polymorphism results from a single nucleotide change (196T>C) in the *ITGB3* gene, leading to a phenotypic leucine to proline amino acid change in the glycoprotein. This subtle difference in DNA sequence between the HPA-1a and HPA-1b alleles has thus far prevented the development of a reliable fetal HPA-1a genotyping assay using cell-free fetal DNA isolated from maternal blood. As cell-free fetal DNA represents only a small fraction (3–10%) of the *total* cell-free DNA in the maternal blood, the maternally derived cell-free DNA molecules greatly outnumber the fetal derived cell-free DNA molecules. Previous attempts to develop a reliable noninvasive fetal HPA-1a genotyping assay in our laboratory were hampered by mispriming of the HPA-1a-specific primer to the overwhelmingly present maternal HPA-1b allele, resulting in false-positive or inconclusive test results (data not shown). The use of extra mismatches in the HPA-1a-specific primer, the incorporation of locked nucleic acids in the HPA-1a-specific primer, and the use of a peptide nucleic acid probe (blocking the HPA-1b allele) all resulted in invalid assays.

The thymine to cytosine substitution (T>C) in the HPA-1b allele creates a DNA sequence recognition site (5'-CCGG-3') for the *Msp1* restriction enzyme (Figure 1). Therefore, pre-PCR processing of the cell-free DNA with *Msp1* will digest the maternally derived HPA-1b alleles, whilst leaving the eventually present fetal HPA-1a alleles unimpaired. Indeed, positive PCR results were obtained in all replicates of the samples from women carrying an HPA-1a-positive fetus, whereas no amplification signals were observed in 15 of the 18 replicates of the six samples from women carrying an HPA-1a-negative fetus (Figure 2). In the three other replicates, Ct values of more than 41 were observed, pointing to nonspecific amplification of the remaining HPA-1b molecules. These single replicates could clearly be distinguished from true-positive amplification (three of three replicates with Ct values of <39), and thus did not

influence correct assignment.

In our study, blood samples ranging from as early as 12 weeks of gestation to the late third trimester were tested. Although we have shown that fetal HPA-1a genotyping using cell-free fetal DNA isolated from maternal blood can be performed in the first trimester, the potential risk of false-negative results caused by low fetal DNA concentrations can be avoided by performing the test later in pregnancy (e.g. early in the second trimester), as the amount of cell-free fetal DNA in the maternal circulation gradually increases during pregnancy.

Noninvasive fetal HPA-1a genotyping offers a safe and relatively easy method for HPA-1a-alloimmunised pregnant women with a heterozygous partner to determine whether or not their fetus is at risk of FNAIT and whether antenatal interventions to prevent ICH are required. It allows for the determination of the fetal HPA-1a status without exposing the mother or fetus to the risks associated with invasive procedures, which are currently performed in up to 30% of alloimmunised pregnancies because of a heterozygous father. In view of the current focus on the noninvasive antenatal management of FNAIT, in which fetal blood sampling and intrauterine platelet transfusions are minimised or eliminated completely,[2] a noninvasive test to establish the fetal risk is clearly called for. Knowledge of the fetal HPA-1a status may further contribute to the avoidance of unnecessary caesarean sections in HPA-1a-alloimmunised pregnant women with a heterozygous partner.

Noninvasive fetal HPA-1a genotyping could also play an important role in FNAIT screening programmes. FNAIT is mainly caused by HPA-1a antibodies and, in the European population, the prevalence of HPA-1a negativity is about 2%.[3] In analogy with red cell antibody screening programmes, screening pregnancies for HPA-1a-mediated FNAIT has been advocated by several groups.[3,6] Drawbacks to its introduction have been the lack of consensus on how to identify the pregnancies at risk and on the optimal antenatal management of screen-positive cases. Nonetheless, in such an FNAIT screening setting, noninvasive fetal HPA-1a genotyping would help to optimise the selection of pregnancies at risk without the need for paternal zygosity testing or invasive procedures to determine the fetal HPA-1a status.

The assay described in this communication is applicable only to pregnancies complicated by alloimmunisation to HPA-1a, involving 80% of FNAIT cases. Noninvasive assays to determine the fetal status of other clinically relevant, but rarer, HPAs, such as HPA-5b or HPA-15b, have not yet been developed.

Conclusion

We have developed a reliable noninvasive fetal HPA-1a genotyping assay that offers a safe method for HPA-1a-alloimmunised pregnant women to determine whether their fetus is at risk of FNAIT and whether antenatal treatment should be initiated. The availability of this test adds an important perspective to the ongoing debate on whether or not to screen pregnancies for HPA-1a-mediated FNAIT.

References

1 Knight M, Pierce M, Allen D, Kurinczuk JJ, Spark P, Roberts DJ, et al. The incidence and outcomes of fetomaternal alloimmune thrombocytopenia: a UK national study using three data sources. *Br J Haematol* 2011;152:460–8.

2 Mechoulan A, Kaplan C, Muller JY, Branger B, Philippe HJ, Oury JF, et al. Fetal alloimmune thrombocytopenia: is less invasive antenatal management safe? *J Matern Fetal Neonatal Med* 2011;24:564–7.

3 Kamphuis MM, Paridaans N, Porcelijn L, de Haas M, van der Schoot CE, Brand A, et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG* 2010;117:1335–43.

4 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

5 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, Bossers B, van Erp F, de Haas M. Reliability of fetal sex determination using maternal plasma. *Obstet Gynecol* 2010;115:117–26.

6 Tiller H, Killie MK, Skogen B, Oian P, Husebekk A. Neonatal alloimmune thrombocytopenia in Norway: poor detection rate with nonscreening versus a general screening programme. *BJOG* 2009;116:594–8.

Chapter 5

The controversy about controls for fetal blood group genotyping by cell-free fetal DNA in maternal plasma

P.G. Scheffer[a,b]
M. de Haas[a,c]
C.E. van der Schoot[a]

[a] Department of Experimental Immunohaematology, Sanquin Research Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Division of Perinatology and Gynaecology, University Medical Centre Utrecht, Utrecht, The Netherlands
[c] Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands

Curr Opin Hematol 2011;18:467–73

Abstract

Purpose of review Fetal blood group genotyping using cell-free fetal DNA from maternal plasma is routinely performed in alloimmunised women and has been introduced for targeted antenatal anti-D prophylaxis. The necessity to control for extraction of fetal DNA in these tests is questioned by many. This review describes the various types of controls for preventing false-negative results and discusses their value.

Recent findings Polymorphic markers like short tandem repeats, insertion/deletion polymorphisms or single nucleotide polymorphisms can be used as a fetal DNA control in pregnancies in which a Y-chromosome marker is not applicable, but workload is considerable and more than 99% coverage is only reached on platforms allowing high level of multiplexing. Recently, the universal fetal marker *RASSF1A* has been introduced, enabling the demonstration of fetal DNA after methylation-sensitive digestion of maternal DNA. The analysis of recently published noninvasive fetal blood group genotyping studies showed that false-negative results were only encountered in studies lacking a control for the presence of fetal DNA, albeit at a low frequency of 0.1–0.2%.

Summary Because of the potentially severe consequences of false-negative results in alloimmunised women, a blood group antigen-negative result should in these cases only be issued if fetal DNA is demonstrated. However, the low frequency of false-negative results makes it acceptable to perform screening studies without a fetal marker.

Introduction

Noninvasive fetal blood group genotyping using cell-free fetal DNA isolated from maternal plasma is considered an important tool in the identification and subsequent management of pregnancies at risk of hemolytic disease of the fetus and newborn (HDFN).[1] Over the past decade, noninvasive fetal blood group genotyping tests have been introduced in many laboratories.[2] Nowadays, noninvasive fetal genotyping of the most important blood group antigens responsible for HDFN, that is, the D, C, c, and E antigens of the Rh blood group system and the K antigen of the Kell blood group system is offered as a routine clinical service for alloimmunised pregnant women in many European countries.[3] In addition, in nonimmunised D-negative pregnant women, noninvasive fetal *RHD* genotyping allows the use of antenatal anti-D prophylaxis to be restricted to only those women carrying a D-positive fetus (around 60%). Following large-scale feasibility studies,[4-6] nationwide fetal *RHD* screening programmes have been introduced in Denmark and The Netherlands.[7]

The determination of a fetal blood group genotype by using cell-free fetal DNA isolated from maternal plasma is based upon the detection of a paternally inherited fetal blood group allele not present in the maternal genome.[8] Because cell-free fetal DNA represents only a small fraction (3–6%) of the total cell-free DNA in the maternal plasma and is present in extremely low concentrations,[9] highly efficient DNA extraction and sensitive detection technology is required.[10]

Whereas a blood group antigen-positive phenotype can be readily predicted on the basis of a positive signal, a blood group antigen-negative phenotype is merely inferred by the absence of a signal. As well as such a result being indicative of a negative phenotype (true negative), it could also arise from the absence of fetal DNA in the detection system (false negative).

In this review we will focus on the 'positive controls' for fetal blood group genotyping by cell-free DNA in maternal plasma, as the controls for detecting contamination ('negative controls') are not different from those in other genotyping assays. Yet it should be mentioned that because the amount of cell-free fetal DNA is near the limit of sensitivity and a positive signal, therefore, might resemble nonspecific amplification; most laboratories choose to perform the assay in triplicate to discriminate between false-positive results (only one of three replicates positive) and true-positive results (more than two of three replicates positive) and/or to perform two independent DNA extractions, of which, the results should be in accordance.

Positive controls can be divided into those showing the reliability of the assay on a series of plasma samples and those controlling the performance of the assay within a certain sample. Ideally, the latter controls are performed within the same reaction as the blood group assay. Several types of positive controls can be recognised: controls for total DNA extraction, controls for fetal DNA extraction, and controls for polymerase chain reaction (PCR) performance. We will first review the various types of controls and finally discuss the value of including such controls in noninvasive fetal blood group assays for different indications. Noninvasive fetal

blood group genotyping in alloimmunised women demands the use of a wider set of controls than fetal *RHD* genotyping in D-negative women to target anti-D immunoprophylaxis.

Controls for total (fetal and maternal) DNA extraction from maternal plasma

In principle, any genomic DNA sequence can be used to detect total cell-free DNA extracted from maternal plasma (Table 1).[11-26] Because the cell-free DNA concentration in plasma is highly influenced by lysis of maternal nucleated cells during storage of the blood,[27] the relevance of including such a control is debatable. However, measurement of the total amount of extracted cell-free DNA can also be used as quality parameter for the plasma sample, as it is indicative for lysis of blood cells, and high maternal cell-free DNA concentrations can lead to false-negative results because of impaired amplification of fetal alleles[6] or false-positive results in assays with limited specificity such as for K or HPA-1a.[13,28,29]

Maternal plasma can also be spiked with a fixed amount of DNA that is not present in the human genome. In the Free DNA Fetal Kit RhD (Institut de Biotechnologies Jacques-Boy, Reims, France), maize DNA is provided for this purpose.[30] Amplification of this exogenous DNA added to the plasma provides a control for both adequate DNA extraction and PCR amplification.

Controls for fetal DNA extraction from maternal plasma

The most important control to prevent false-negative results is the control on extraction of sufficient amount of cell-free fetal DNA. For more general assays laboratories can make use of genetic reference materials established by the WHO to control the performance of their test, but this is more difficult for noninvasive fetal genotyping. The unique-size characteristic of cell-free fetal DNA makes only plasma from pregnant women suitable as a reference sample. But obviously, sufficient plasma cannot be obtained from an individual pregnant woman to produce such a standard. To mimic cell-free fetal DNA as close as possible, freeze-dried plasma of a D-positive man diluted into plasma of a D-negative woman has been used to prepare an international WHO reference standard (material 07/222) for the detection of *RHD* in plasma (Metcalfe P, Rigsby P, Tait E, Urbaniak S, unpublished observation). The International Society of Blood Transfusion endorsed this standard, and the minimum sensitivity was set at a dilution of 1:2. Laboratories can use this standard to calibrate their in-house standards. The in-house standard we use in our laboratory to monitor the performance of our assay during time consists of pooled plasmas from D-negative women pregnant with a D-positive child, diluted into plasma from women carrying D-negative children.[31]

Table 1. Published studies on fetal blood group genotyping in maternal plasma (January 2009 – June 2011)

Author	Technique	No. samples tested*	Gestation (weeks)	Tested plasma equivalent per replicate (mL)#	No. replicates	Target gene/allele	Total cell-free DNA control	Fetal cell-free DNA control	Sensitivity (%)	False-negative results
Akolekar et al.[11]	Real-time PCR	591	11–14	N/A	3	*RHD*	*CCR5*	None	98.2	6
Bombard et al.[12]	Mass spectrometry	236	11–13	0.33	1	*RHD*	*TGIF*	*DBY*, *SRY*, *TTTY2*‡	97.2	4
Scheffer et al.[13]	Real-time PCR	212	7–38	0.33	3	*RHD*, *c*, *E*, *K*	*albumin*	*SRY*, biallelic polymorphisms	100	0
Gutensohn et al.[14]	Real-time PCR	181¥	12–28	N/A	2	*C*, *c*, *E*	*β-globin*	None	100	0
Grill et al.[15]	Mass spectrometry	178	N/A	0.04	2	*RHD*	None	None	96.1	5
Tynan et al.[16]	Mass spectrometry	150	<32	0.36	1	*RHD*	*albumin*, *AMG*	*SRY*‡	100	0
Hyland et al.[17]	Real-time PCR	140	12–40	0.07	4	*RHD*	*CCR5*	*SRY*, *RASSF1A*	100	0
Achargui et al.[18]	Conventional PCR	120	10–40	0.13	1	*RHD*	None	None	98.8	1
Chinen et al.[19]	Real-time PCR	102	7–36	0.08	3	*RHD*	*β-globin*	None	100	0
Cardo et al.[20]	Real-time PCR	100	9–13	N/A	3	*RHD*	*β-globin*	None	100	0
Clausen et al.[21]	Real-time PCR	97§	6–37	0.04	3	*RHD*	*GAPDH*	None	98.7	1
Sedrak et al.[22]	Real-time PCR	90	7–24	N/A	2	*RHD*	*β-globin*	None	96.7	2
Amaral et al.[23]	Real-time PCR	88	11–39	0.07	3	*RHD*	*CCR5*	*SRY*‡	100	0
Tounta et al.[24]	Multiplex fluorescent PCR	84	7–24	0.16	2	*RHD*	None	*SRY*, *RASSF1A*	100	0
Wang et al.[25]	Real-time PCR	78	14–40	0.16	3	*RHD*	*β-globin*	*SRY*, STR's	100	0
Mohammed et al.[26]	Real-time PCR	21	20–39	0.08–0.17	2	*RHD*	*β-globin*	None	92.3	1

AMG, amelogenin gene-XY chromosomes; *CCR5*, C-C chemokine receptor type 5; *DBY*, DEAD-box RNA helicase Y; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; N/A, not available; *RASSF1A*, Ras association domain family 1 isoform A; *SRY*, Sex-determining region Y; *TTTY2*, Testis-specific testis transcript Y-linked 2; STR, short tandem repeat. Studies are sorted according to sample size.

*Based on available neonatal serologic outcome.

#As calculated by the formula: (*DNA input PCR / elute*) x *plasma volume*.

‡In these studies testing for Y chromosome-specific sequences was not used to confirm the presence of fetal DNA in the case of a negative result for *RHD*.

¥The authors tested two different methods of primer/probe sets. Results of the most accurate 'method 2' are shown.

§Ninety-seven plasma samples obtained from 61 blood samples collected from 38 D-negative pregnant women, respectively. The authors tested five different assays: monoplex exon 5, monoplex exon 7, duplex exon7/10, triplex exon 7/10, and triplex exon 5. Results of the most accurate duplex assay are shown.

However, to control for the presence of sufficient fetal DNA in a certain plasma sample, a true fetal marker is needed. The most widely used fetal DNA marker is a Y chromosome-specific sequence, such as *SRY* (Table 1),[12,13,16,17,23-25] applicable in about 50% of the pregnancies. Testing for this sequence can be performed in parallel with the blood group antigen PCR[13,25] or as a multiplex within the same reaction.[12,16,17,23,24] In pregnancies with female fetuses, detection of paternally inherited polymorphisms not present in the maternal genome can be used to confirm the presence of fetal DNA. For example, testing for another discrepant blood group antigen between mother and child can be used, if applicable.[13] In order to use other polymorphic DNA markers, the maternal (and ideally paternal) polymorphic status needs to be assessed by genotyping of genomic DNA. Because of their extensive variability short tandem repeat (STR) loci are attractive fetal markers and have been successfully applied in four samples by Wang et al.[25] Also other groups have reported the use of STRs for detection of cell-free fetal DNA, albeit not as a control assay.[32,33] However, the sensitivity of STR-based PCR assays is low due to competition for amplification with excess of maternal STR loci. The use of a panel of 14 biallelic insertion/deletion polymorphisms (indels) allowed us to confirm the presence of fetal DNA in up to 90% of cases negative for the target blood group antigen (n = 68) or *SRY* (n = 78) by real-time PCR.[13,31] Smaller-sized studies (32 and 16 cases negative for *RHD* and *SRY*, respectively) were equally successful in detection fetal DNA sequences using indels.[34,35] However, this approach requires the availability of a large set of indels and has not been validated in different ethnic groups yet. Moreover, the number of systems required to cover more than 90% mother/fetus couples increases exponentially. Using a panel of only eight indels, two independent laboratories in the United Kingdom found the approach not informative in more than 60% of cases, and this approach is no longer pursued by them.[36] Multiplexing more systems is more easily reached on a mass spectrometry platform.[12,15,16] Tynan et al.[37] presented a universal multiplexed single nucleotide polymorphism (SNP) genotyping method on this platform. By multiplexed genotyping of 92 panethnic SNPs, a more than 0.99 probability of detecting at least four informative loci per sample was achieved. In conclusion, although it is possible to confirm the presence of fetal DNA using polymorphic markers in most cases, either the workload is considerable (real-time PCR) or the platform is rarely available (mass spectrometry). So, there is a clear need for sex- and polymorphism-independent fetal DNA markers.

In 2006, Chan et al.[38] described the *RASSF1A* gene as a universal fetal marker. The promoter of this gene is methylated in fetal (placental) DNA, but unmethylated in maternal (leukocyte derived) DNA. Pre-PCR processing of cell-free DNA with a methylation-sensitive restriction enzyme, results in digestion of maternal *RASSF1A* promoter sequences, while leaving the fetal sequences intact. Hyland et al.[17] reported the successful confirmation of the presence of fetal DNA using *RASSF1A* in 16 of 16 samples negative for *RHD* and *SRY*. More recently, Tounta et al.[24] designed a multiplex fluorescent PCR that allowed simultaneous amplification of *RHD* exons

7 and 10, *SRY*, *RASSF1A* and *β-actin* confirming the presence of fetal DNA in all 14 samples negative for *RHD* and *SRY*. In two cases that were considered inconclusive, incomplete digestion was observed. Li et al.[39] showed no false-positive results for *RASSF1A* in 20 plasma samples from nonpregnant women with positive detection in 85 plasma samples from pregnant women. Zejskova et al.[40] showed similar results. Recently, Nygren et al.[41] identified *SOX14* and *TBX3* as two additional universal fetal markers for cell-free DNA from plasma. The drawback of methylation-based assays is the risk of incomplete digestion and including an extra digestion control is therefore required. Incomplete digestion might be overcome by adding an exonuclease in the reaction mixture to eliminate single stranded DNA that would escape digestion.[41] An alternative approach could be bisulphite conversion of cell-free DNA, but because of presumed degradation of DNA this has not been applied for cell-free fetal DNA yet.

Control PCRs used to confirm the presence of fetal DNA should have a sensitivity equal to that of the blood group antigen PCR and should be highly fetal-specific to avoid false-positive amplification. Most of the cell-free fetal DNA is less than 200 bp in length.[42] Therefore, the target used as fetal identifier should be minimal of similar size as the product generated in the fetal blood group assay.

In future, next generation sequencing on cell-free DNA might be implemented to screen for aneuploidies or inherited diseases.[43] The fetal blood groups can be deduced from those sequencing data.[44] Because the absence of fetal DNA will be readily recognised, there will be no risk for false-negative results.

PCR controls

The inclusion of a standard (preferably calibrated to the WHO standard) can control the sensitivity of the PCR assay. Theoretically, PCR inhibitors might be present in an individual plasma sample, which can be detected by adding a commercially available internal positive control (IPC) to the PCR mix. No data on the presence of inhibitors in DNA extractions from plasma are available in the literature. We included an IPC in a series of 78 samples and did not encounter inhibition in any of the maternal plasmas (unpublished data), so presumably this control is not needed.

Value of controls

To evaluate the clinical value of different positive controls we reviewed the literature. Table 1 shows the most-recently published studies on diagnostic noninvasive fetal blood group genotyping (for articles reviewing earlier studies, see Daniels et al.[2] and Legler et al.[45]). In Table 2, the large-scale screening studies are listed.

Table 2. Large-scale fetal *RHD* screening studies

Author	No. samples tested	Gestation (weeks, median)	Tested plasma equivalent per replicate (mL)*	No. replicates	*RHD* exons	Cell-free DNA control	Sensitivity (%)	False-negative results
Van der Schoot et al.[4]	1257	30	0.25	3	7	None	99.6	3
Müller et al.[5]	1022#	25	0.19	2	5, 7	*β-globin*	99.7	2
Finning et al.[6]	1869	28	0.07	3	5, 7	*CCR5*	99.7	3
Clausen et al.[7]	2312‡	25	0.08–0.17	3–4	5, 7; 7, 10; or 5, 10	*CCR5* or *GAPDH* or *SOD*	99.9	2

CCR5, C-C chemokine receptor type 5; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *SOD*, superoxide dismutase gene.
*As calculated by the formula: (*DNA input PCR / elute*) x *plasma volume*.
#The authors tested two DNA extraction methods; spin-column (QIAamp DSP Virus Kit; Qiagen Inc., Hilden, Germany) versus magnetic tips (Chemagen MSM1; Chemagen, Baesweiler, Germany). Results of the most accurate spin-column method are shown.
‡Evaluation of the first six months of routine antenatal *RHD* screening in Denmark. Data compiled from five different regions.

Although in the vast majority of studies a control for total DNA extraction was used, only two studies reported the exclusion of samples because of the absence of DNA in five of 591 samples[11] and two of 236 samples.[12] In all other studies, in which a control for total DNA extraction was used (comprising 1259 diagnostic and 5203 screening samples), in all but one sample[6] sufficient DNA was isolated from plasma. So, the value of a total DNA extraction control to prevent false-negative results seems to be limited. In contrast, this control is useful for preventing false-positive results in women carrying a variant *RHD* gene.[5,11,13] To prevent false-negative results, the inclusion of a positive control for fetal DNA extraction is much more relevant. As shown in Table 1, most laboratories do not include such a control. Although retesting on a second plasma sample collected at least 2 weeks later in case of a negative result has been recommended by some groups,[30,46] such a strategy adds considerably to the workload without a confirmation that indeed sufficient fetal DNA was present. From Table 1 it can be seen that all 20 false-negative results were obtained among the 1954 samples from studies lacking the inclusion of a true fetal control,[11,12,14-16,18-23,26] whereas no false-negative results were observed in the 514 samples from studies having a fetal marker.[13,17,24,25] Although it is tempting to speculate that the false-negative results were due to lack of fetal DNA and would have been prevented by including a fetal marker, this cannot be definitively concluded, as the studies do not report on the outcome of inconclusive cases in which no fetal DNA was demonstrated.

However, also results of high-throughput noninvasive *RHD* screening studies, in which no fetal control was included, indicate that false-negative results are inevitable, and all four studies experienced a similar false-negative rate of about 0.1–0.2% (Table 2).

It is known that early in pregnancy the fetal cell-free DNA concentration is lower,[9] and indeed

more false-negative results were reported in studies on diagnostic samples obtained in the first trimester.[11,12] Later in pregnancy, the cell-free fetal DNA concentration is, on average, 150 genome equivalents/ml,[41] which ensures a sufficient yield of fetal DNA if at least 500 mL of plasma is used for extraction. However, studies in which fetal DNA is quantified show that cell-free fetal DNA concentration varies about two log between women.[9,27,41] Therefore, in some women also later in pregnancy insufficient fetal DNA might be obtained and the combined data reported so far suggest that this is the case in 0.2% of the pregnancies.

Conclusion

The necessity of including various types of controls in a noninvasive fetal blood group genotyping assay depends on the use of the assay result. If the fetal blood group genotype is determined because of maternal alloimmunisation, the assay is used to determine whether the fetus is at risk of HDFN and careful monitoring for fetal anemia and intrauterine treatment is required.[1] Moreover, the assay is used to reassure the woman that her pregnancy is not at risk and that extensive clinical monitoring can be omitted. In these cases, the assay is usually performed early in pregnancy, at which time cell-free fetal DNA concentration is lower. In our opinion, when fetal genotyping is performed because of maternal alloimmunisation, the presence of fetal DNA should be confirmed by one of the above-described technical approaches. If the assay is used for screening of pregnancies to guide anti-D immunoglobulin prophylaxis, the assay is performed later in pregnancy and the risk of a false-negative result, presumably around 0.2%, might be considered as acceptable, and only controls for monitoring the performance of the assay during time are indispensable. In such screening assays, the decision to also include positive controls for fetal DNA extraction will depend on the associated extra costs in relation to the effects.[47,48]

References

1 Illanes S, Soothill P. Management of red cell alloimmunisation in pregnancy: the non-invasive monitoring of the disease. *Prenat Diagn* 2010;30:668–73.

2 Daniels G, Finning K, Martin P, Massey E. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* 2009;29:101–7.

3 Daniels G, van der Schoot CE, Gassner C, Olsson ML. Report of the third international workshop on molecular blood group genotyping. *Vox Sang* 2009;96:337–43.

4 van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Page-Christiaens GCML, de Haas M. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006;13:53–7.

5 Muller SP, Bartels I, Stein W, Emons G, Gutensohn K, Kohler M, et al. The determination of the fetal D status from maternal plasma for decision making on Rh prophylaxis is feasible. *Transfusion* 2008;48:2292–301.

6 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* 2008;336:816–8.

7 Clausen FB, Christiansen M, Steffensen R, Jorgensen S, Nielsen C, Jakobsen M, et al. Report of the first nationally implemented clinical routine screening for fetal RHD in RhD negative pregnant women to ascertain the requirement for antenatal RhD prophylaxis. Transfusion 2011.

8 Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* 1998;352:1196.

9 Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768–75.

10 Legler TJ, Liu Z, Mavrou A, Finning K, Hromadnikova I, Galbiati S, et al. Workshop report on the extraction of foetal DNA from maternal plasma. *Prenat Diagn* 2007;27:824–9.

11 Akolekar R, Finning K, Kuppusamy R, Daniels G, Nicolaides KH. Fetal RHD genotyping in maternal plasma at 11-13 weeks of gestation. *Fetal Diagn Ther* 2011;29:301–6.

12 Bombard A, Akolekar R, Farkas D, Vanagtmael A, Aquino F, Oeth P, et al. Fetal RHD genotype detection from circulating cell-free fetal DNA in maternal plasma in non-sensitized RhD negative women. *Prenat Diagn* 2011;31:802–8.

13 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, de Haas M. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience. *BJOG* 2011;118:1340–8.

14 Gutensohn K, Muller SP, Thomann K, Stein W, Suren A, Korte-Jung S, et al. Diagnostic accuracy of noninvasive polymerase chain reaction testing for the determination of fetal rhesus C, c and E status in early pregnancy. *BJOG* 2010;117:722–9.

15 Grill S, Banzola I, Li Y, Rekhviashvili T, Legler TJ, Muller SP, et al. High throughput non-invasive determination of foetal Rhesus D status using automated extraction of cell-free foetal DNA in maternal plasma and mass spectrometry. *Arch Gynecol Obstet* 2009;279:533–7.

16 Tynan JA, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. *Am J Obstet Gynecol* 2011;204:251–6.

17 Hyland CA, Gardener GJ, Davies H, Ahvenainen M, Flower RL, Irwin D, et al. Evaluation of non-invasive prenatal RHD genotyping of the fetus. *Med J Aust* 2009;191:21–5.

18 Achargui S, Tijane M, Benchemsi N. Fetal RHD genotyping by PCR using plasma from D negative pregnant women. *Transfus Clin Biol* 2011;18:13–9.

19 Chinen PA, Nardozza LM, Martinhago CD, Camano L, Daher S, Pares DB, et al. Noninvasive determination of fetal Rh blood group, D antigen status by cell-free DNA analysis in maternal plasma: experience in a Brazilian population. *Am J Perinatol* 2010;27:759–62.

20 Cardo L, Garcia BP, Alvarez FV. Non-invasive fetal RHD genotyping in the first trimester of pregnancy. *Clin Chem Lab Med* 2010;48:1121–6.

21 Clausen FB, Krog GR, Rieneck K, Rasmark EEF, Dziegiel MH. Evaluation of two real-time multiplex PCR screening assays detecting fetal RHD in plasma from RhD negative women to ascertain the requirement for antenatal RhD prophylaxis. *Fetal Diagn Ther* 2011;29:155–63.

22 Sedrak M, Hashad D, Adel H, Azzam A, Elbeltagy N. Use of free fetal DNA in prenatal noninvasive detection of fetal RhD status and fetal gender by molecular analysis of maternal plasma. *Genet Test Mol Biomarkers* 2011;15:627–31.

23 Amaral DRT, Credidio DC, Pellegrino JJ, Castilho L. Fetal RHD genotyping by analysis of maternal plasma in a mixed population. *J Clin Lab Anal* 2011;25:100–4.

24 Tounta G, Vrettou C, Kolialexi A, Papantoniou N, Destouni A, Tsangaris GT, et al. A multiplex PCR for non-invasive fetal RHD genotyping using cell-free fetal DNA. *In Vivo* 2011;25:411–7.

25 Wang XD, Wang BL, Ye SL, Liao YQ, Wang LF, He ZM. Non-invasive foetal RHD genotyping via real-time PCR of foetal DNA from Chinese RhD-negative maternal plasma. *Eur J Clin Invest* 2009;39:607–17.

26 Mohammed N, Kakal F, Somani M, Zafar W. Non-invasive prenatal determination of fetal RhD genotyping from maternal plasma: a preliminary study in Pakistan. *J Coll Physicians Surg Pak* 2010;20:246–9.

27 Fernando MR, Chen K, Norton S, Krzyzanowski G, Bourne D, Hunsley B, et al. A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. *Prenat Diagn* 2010;30:418–24.

28 Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c, and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion* 2007;47:2126–33.

29 Scheffer PG, Ait Soussan A, Verhagen OJHM, Page-Christiaens GCML, Oepkes D, de Haas M, van der Schoot CE. Noninvasive fetal genotyping of human platelet antigen-1a. *BJOG* 2011;118:1392–5.

30 Rouillac-Le Sciellour C, Serazin V, Brossard Y, Oudin O, Le Van Kim C, Colin Y, et al. Noninvasive fetal RHD genotyping from maternal plasma. Use of a new developed free DNA fetal kit RhD. *Transfus Clin Biol* 2007;14:572–7.

31 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, Bossers B, van Erp F, de Haas M. Reliability of fetal sex determination using maternal plasma. *Obstet Gynecol* 2010;115:117–26.

32 Davanos N, Spathas DH. Relative quantitation of cell-free fetal DNA in maternal plasma using autosomal DNA markers. *Clin Chim Acta* 2011;412:1539–43.

33 Tang DI, Li Y, Zhou X, Li X, Zheng F. Multiplex fluorescent PCR for noninvasive prenatal detection of fetal-derived paternally inherited diseases using circulatory fetal DNA in maternal plasma. *Eur J Obstet Gynecol Reprod Biol* 2009;144:35–9.

34 Brojer E, Zupanska B, Guz K, Orzinska A, Kalinska A. Noninvasive determination of fetal RHD status by examination of cell-free DNA in maternal plasma. *Transfusion* 2005;45:1473–80.

35 Zhou L, Thorson JA, Nugent C, Davenport RD, Butch SH, Judd WJ. Noninvasive prenatal RHD genotyping by real-time polymerase chain reaction using plasma from D-negative pregnant women. *Am J Obstet Gynecol* 2005;193:1966–71.

36 Hill M, Finning K, Martin P, Hogg J, Meaney C, Norbury G, et al. Non-invasive prenatal determination of fetal sex: translating research into clinical practice. *Clin Genet* 2011;80:68–75.

37 Tynan JA, Mahboubi P, Cagasan LL, van den Boom D, Ehrich M, Oeth P. Restriction enzyme-mediated enhanced detection of circulating cell-free fetal DNA in maternal plasma. *J Mol Diagn* 2011;13:382–9.

38 Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN, et al. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.

39 Li Y, Jazzaz JA, Kellner LH, Brown SA. Incorporation of fetal DNA detection assay in a noninvasive RhD diagnostic test. *Prenat Diagn* 2010;30:1010–12.

40 Zejskova L, Jancuskova T, Kotlabova K, Doucha J, Hromadnikova I. Feasibility of fetal-derived hypermethylated RASSF1A sequence quantification in maternal plasma: next step toward reliable noninvasive prenatal diagnostics. *Exp Mol Pathol* 2010;89:241–7.

41 Nygren AO, Dean J, Jensen TJ, Kruse S, Kwong W, van den Boom D. Quantification of fetal DNA by use of methylation-based DNA discrimination. *Clin Chem* 2010;56:1627–35.

42 Sikora A, Zimmermann BG, Rusterholz C, Birri D, Kolla V, Lapaire O, et al. Detection of increased amounts of cell-free fetal DNA with short PCR amplicons. *Clin Chem* 2010;56:136–8.

43 Hahn S, Lapaire O, Tercanli S, Kolla V, Hosli I. Determination of fetal chromosome aberrations from fetal DNA in maternal blood: has the challenge finally been met? *Expert Rev Mol Med* 2011;13:e16.

44 Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, Lun FMF, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. *Sci Transl Med* 2010;2:61ra91.

45 Legler TJ, Muller SP, Haverkamp A, Grill S, Hahn S. Prenatal RhD testing: a review of studies published from 2006 to 2008. *Transfus Med Hemother* 2009;36:189–98.

46 Avent ND. RHD genotyping from maternal plasma: guidelines and technical challenges. *Methods Mol Biol* 2008;444:185–201.

47 Minon JM, Gerard C, Schaaps JP, Foidart JM. Rh D foeto-maternal alloimmunization prophylaxis with anti-D immunoglobulins reviewed in the era of foetal RHD genotyping. *Acta Clin Belg* 2009;64:195–202.

48 Szczepura A, Osipenko L, Freeman K. A new fetal RHD genotyping test: costs and benefits of mass testing to target antenatal anti-D prophylaxis in England and Wales. *BMC Pregnancy Childbirth* 2011;11:5.

Chapter 6

A nation-wide fetal *RHD* screening programme for targeted antenatal and postnatal anti-D immunoglobulin prophylaxis: first-three-month analysis

P.G. Scheffer[a,b]
F.F. Thurik[a,b]
B. Veldhuisen[a,c]
R. Jonker[c]
M. de Haas[a,c]
C.E. van der Schoot[a]

[a] Department of Experimental Immunohaematology, Sanquin Research Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Division of Perinatology and Gynaecology, University Medical Centre Utrecht, Utrecht, The Netherlands
[c] Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, The Netherlands

Introduction

Rh D blood group incompatibility between a D-negative pregnant woman and her fetus is the most common cause of haemolytic disease of the fetus and newborn (HDFN), a condition characterised by fetal anaemia, hydrops and intrauterine demise, and neonatal hyperbilirubinaemia and eventually kernicterus.[1] The disease results from maternal immunoglobulin G class anti-D alloantibodies crossing the placenta and, consequently, the destruction of D-positive fetal and/or neonatal erythrocytes.[2] The maternal D alloimmunisation is triggered by fetomaternal haemorrhage (FMH) during pregnancy and/or delivery of a D-positive child.[3] Routine postnatal administration of anti-D immunoglobulin following the birth of a D-positive child – introduced in the 1960s – has been shown to substantially decrease maternal alloimmunisation detected in a subsequent pregnancy from 15% to 1.6%.[4] In the more recent years, routine antenatal anti-D prophylaxis (RAAPD), in which anti-D immunoglobulin is administered between week 28 and 34 of gestation, has become the standard care for D-negative pregnant women in many developed countries.[5] In combination with postnatal prophylaxis, this practice has led to a further reduction of maternal immunisations of more than 50%.[6,7] In The Netherlands, RAAPD was introduced in 1998 and consists of one single dose of 200 µg (1,000 IU) anti-D immunoglobulin administered in the 30th week of gestation.
In a Caucasian population, around 38% of D-negative pregnant women carries a D-negative fetus[8] and, thus, receives antenatal anti-D immunoprophylaxis unnecessarily. This imposes a cost burden on health services and an unnecessary demand on a short-in-supply drug that is produced from the blood of hyperimmunised volunteer donors and carries a small but nonnegligible risk of transmitting unknown or unrecognised viruses or prions.[9]
Soon after the discovery that cell-free fetal DNA molecules are present in the plasma and serum of pregnant women,[10] noninvasive genotyping of the fetal *RHD* gene in the plasma from D-negative pregnant women was established.[11,12] Nowadays, noninvasive fetal *RHD* genotyping in D-alloimmunised pregnant women is offered as a routine clinical service in many European laboratories with a reported 100% diagnostic accuracy.[13,14] Some commercial laboratories in the United States also provide testing.[15,16] Standardised methodology is available and international external quality assurance schemes are in place.[17,18] Advances in the understanding of the genetic background of the Rh system have allowed for strategically designed assays that recognise variations of the *RHD* gene, preventing both false-positive and false-negative results.[19,20] Over the last few years, several high-throughput fetal *RHD* genotyping studies using automated DNA extraction and robotic liquid handling have been conducted, reaching sensitivities of 99.7% to 99.9%.[21-23] The results of these studies have shown that large-scale fetal *RHD* genotyping is feasible, allowing for targeted antenatal immunoprophylaxis in nonimmunised D-negative pregnant women. In such a fetal *RHD* screening setting, the primary objective is to prevent false-negative results, as undetected D-positive fetuses can lead to maternal immunisation. In 2010, a clinical routine screening

programme for fetal *RHD* in D-negative pregnant women to ascertain the requirement for antenatal anti-D immunoprophylaxis was implemented on a national level in Denmark.[24]
Following a government-supported feasibility and cost-effectiveness study[25] and based on the advice of the Health Council of The Netherlands, the Dutch minister of Health, Welfare and Sport decided in 2010 to implement fetal *RHD* screening in the national prenatal screening programme. This meant that in all nonimmunised D-negative pregnant women in The Netherlands the fetal D status would be determined in the plasma from the pregnant woman at 27 weeks of gestation and that both antenatal anti-D immunoprophylaxis in the 30th week of gestation and postnatal prophylaxis would be administered to only those women for whom a D-positive child was predicted. The basic assumption of the fetal *RHD* screening programme was that the new programme should not significantly change the prevalence of pregnancies complicated by D alloimmunisation. To evaluate whether fetal *RHD* screening could indeed be performed as a centralised test for The Netherlands given the logistical demands, the minister decided that during the first year of the screening programme postnatal cord blood serology would still be continued, however also in a centralised setting. This was based on the advice of the National Institute for Public Health and the Environment (RIVM), the director of the prenatal screening programme in The Netherlands. This enabled the monitoring of the performance of the fetal *RHD* screening programme, since serological outcomes could be compared with the fetal *RHD* screening results. Based on the estimated percentage of false-negative results for cord blood serology,[6,26] the critical limit of false-negative results for the fetal *RHD* screening programme was set at 0.25%.
On July 1st 2011, the new policy has come into operation. Here, we present a first-three-month analysis of the fetal *RHD* screening programme in The Netherlands.

Methods

The Dutch fetal *RHD* screening programme is organised by the National Institute for Public Health and the Environment (RIVM) by order of the minister of Health, Welfare and Sport and paid by government resources. The fetal *RHD* screening is part of the antenatal Screening programme for Infectious diseases and Erythrocyte immunisation (PSIE), offered to all pregnant women early in pregnancy, at their first antenatal visit, preferably before 12 weeks of gestation. All fetal *RHD* screening tests are performed at Sanquin Diagnostic Services.

Blood samples

Fetal *RHD* screening and repeated red cell antibody screening was offered in week 27 of pregnancy to all nonimmunised D-negative pregnant women, as determined by RhD typing and antibody screening at their first visit. Nine millilitres of ethylenediaminetetraacetic acid anticoagulated blood was drawn at 27 weeks by the woman's care giver (i.e. midwife, general

practitioner or gynaecologist) and sent by regular surface mail or Sanquin private courier service to our laboratory. Upon arrival, blood collection tubes were labelled and kept at room temperature until further processing. Blood up to five days after sampling was accepted for testing.

Serological typing and antibody screening

ABO and RhD typing and antibody screening of the pregnant woman was performed with the automated WA-Diana system (DiaMed GmbH, Cressier, Switzerland) or AutoVue Innova system (Ortho Clinical Diagnostics, Raritan, NJ, USA) using the appropriate manufacturer's protocols. RhD typing was performed with a monoclonal anti-D reagent (LHM 59/20 [LDM3] + 175-2) that does not recognise the DVI phenotype. Aberrant serology results (≤2+-reactivity) were further investigated manually using in-house available reagents and molecular typing.

Automated plasma separation and DNA extraction

Plasma was collected prior to any other handling of the sample. Blood samples were centrifuged at 1200 × *g* for 10 minutes and 2.5 mL of plasma was robotically dispensed into 5-mL tubes with a Xiril robotic workstation (Xiril AG, Hombrechtikon, Switzerland). The remains of the blood sample were used for antibody screening and maternal serology (see above). The plasma fraction was centrifuged at 2400 × *g* for 20 minutes and, subsequently, 1 mL of plasma was dispensed into two deep-well 96-well plates each with the Xiril robot. One plate was stored at –20°C as a backup; the other was presented to the MagNa Pure 96 Instrument (Roche Holding AG, Basel, Switzerland) for automated DNA extraction. DNA was extracted from 1 mL of plasma using the DNA and Viral NA Large Volume Kit (Roche) following the manufacturer's instructions. DNA was eluted with 50 µL of elution buffer. In each run of 48 samples, 1 mL of plasma from a preformed plasma pool from several D-negative pregnant women was included as a standard.

Real-time polymerase chain reaction analysis

Duplex real-time polymerase chain reaction (PCR) analysis for *RHD* exon 5 and *RHD* exon 7 was performed as previously described.[19] In this assay, the *RHD* exon 5 PCR is negative when only an *RHD* pseudogene (*RHDΨ*) is present. The reactions were set up in a volume of 25.35 µL. A mix of 10 µL of TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), primers and probes for *RHD* exon 5 (VIC-labelled) and *RHD* exon 7 (FAM-labelled), and 15 µL of extracted DNA was robotically dispensed (Xiril) into a 96-well PCR plate. Primers and probes were used at final concentrations of 300 and 100 nM, respectively. PCR was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems). Conditions were 20 seconds at 95°C, followed by 50 cycles of 1 second at 95°C and 20 seconds at 60°C. Cycle threshold (Ct) values were collected at fixed thresholds of 0.05. Only runs in which the Ct values of the

internal standard were within the preset range of 33.0 to 40.0 in two of three replicates for both *RHD* exon 5 and *RHD* exon 7 were considered valid. When these criteria were not met, DNA extraction and PCR were repeated with the backup samples.

For each sample, the assay was performed in triplicate. Samples were scored by computer software. A replicate was scored positive if the Ct value was <40.0. Samples in which the total number of positive replicates of both PCRs was two or three were flagged and their amplification plots were individually reviewed. Replicates with so-called creeping amplification curves were ignored. After this manual correction of the raw data, samples with three or more positive replicates were scored as positive. In samples in which only one of the two exons was amplified (2 or 3 positive replicates), a fetal *RHD* variant gene was suspected. Mean Ct values <30 for either one of the two PCRs were considered to be possibly due to amplification of a maternal *RHD* variant gene. To fine-tune the scoring algorithm, samples indicating the presence of a fetal or maternal *RHD* variant gene were rescued for further detailed serological and molecular analysis. Only samples in which both exons were scored negative were reported as an 'RhD negative' fetal screening result and administration of anti-D immunoglobulin administration was advised as 'not necessary'. In any other case, the fetal screening result was reported as 'RhD positive' and administration of anti-D immunoglobulin was recommended.

Reports were generated electronically and sent to the pregnant woman's care giver and 200 µg (1,000 IU) of anti-D immunoglobulin was administered in the 30th week of gestation and within 48 hours after birth only in the case of an 'RhD positive' fetal screening result.

Postnatal cord blood serology

As part of the national evaluation of the fetal *RHD* screening programme, postnatal cord blood samples were sent to our laboratory to determine the newborn's RhD serology. Cord blood serology was performed with the WA-Diana system (DiaMed GmbH) using two monoclonal anti-D reagents, LHM 59/20 (LDM3) + 175-2 and ESD-1M + 175-2. The latter recognises the DVI phenotype, which is immunogenic for a D-negative mother. Serology tests were performed all days, except on Sunday and Bank Holidays. All cord blood serology results were compared with the fetal *RHD* screening result. Putative false-negative fetal *RHD* screening results were immediately reported to the obstetric care giver for timely postnatal anti-D administration.

Molecular characterization of RHD variant genes

All maternal or newborn samples in which an *RHD* variant gene was suspected, were subjected to an *RHD* multiplex ligation-dependent probe amplification (MLPA) assay on genomic (buffy coat) DNA. In this assay, the far majority of *RHD* variant genes is recognised and identified, as well as the hemi- or homozygotic presence of the *RHD* gene. In the case the MLPA results indicated the presence of a normal *RHD* gene, all exons and promoter region

were D-specifically amplified and sequenced as previously described (L. Haer-Wigman et al., submitted).

Data collection and analysis

For the present analysis, we collected the data of all fetal *RHD* screening tests performed in the first 3 months of the national screening programme (July 1st to September 30th 2011). Cord blood serology results were collected until December 31st 2011, at which time the majority of the screened pregnant women was expected to have given birth. After collecting all data, descriptive statistics were generated using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). The chi-square test (two-sided) was used to determine sensitivity and specificity with 95% confidence intervals (95% CI). To compare the Ct values of different groups we applied the Mann-Whitney U test.

Results

From July 1st to September 30th 2011, blood samples from 7052 pregnant women were sent to our laboratory for fetal screening. Serological RhD typing of the maternal red cells showed that 111 women (1.6%) were in fact D positive, leaving a total of 6941 samples for fetal screening. The red cells of nine of these samples showed weak (≤2+) reactivity.

An 'RhD positive' fetal screening result was reported in 4259 cases (61.4%) and an ' negative' fetal screening result was reported in 2682 cases (38.6%). In 4176 samples (60.2%), both exon 5 and exon 7 PCR were positive, with Ct values within the expected range for fetal DNA (i.e. >30; see Figure 1). Median Ct values for exon 5 and exon 7 were 34.5 (2.5th–97.5th percentile, 32.5–37.1) and 35.0 (2.5th–97.5th percentile, 33.0–37.7), respectively. In 4014 of these samples (96.1%), all three replicates of both exon 5 and exon 7 were positive. In 109 samples (2.6%), a single negative replicate was observed (either for exon 5 or exon 7). The Ct values of the positive replicates of these 109 samples were significantly higher than the Ct values of the positive samples in which no negative replicate was observed (Mann-Whitney U test, P=<0.0001; see Figure 2), suggesting that the failure to obtain six positive replicates was due to a lower amount of fetal DNA.In 39 further samples (0.9%), two negative replicates were observed and in 12 samples (0.3%), three negative replicates (exon 5 and exon 7 combined) were observed; in these samples the Ct values of the positive replicates were also significantly higher (P=<0.0001). In the remaining two positive samples, respectively four and five negative replicates were observed, but these samples were nevertheless reported as 'RhD positive', as per supervisor's discretion at the time.

In 21 samples (0.3%), a fetal *RHD* variant gene was suspected, because of amplification of only one of the two exons: eight samples showing amplification of only *RHD* exon 5 (median Ct value, 35.7); 13 samples showing amplification of only *RHD* exon 7 (median Ct value, 34.8). In all these cases, an 'RhD positive' screening result was issued.

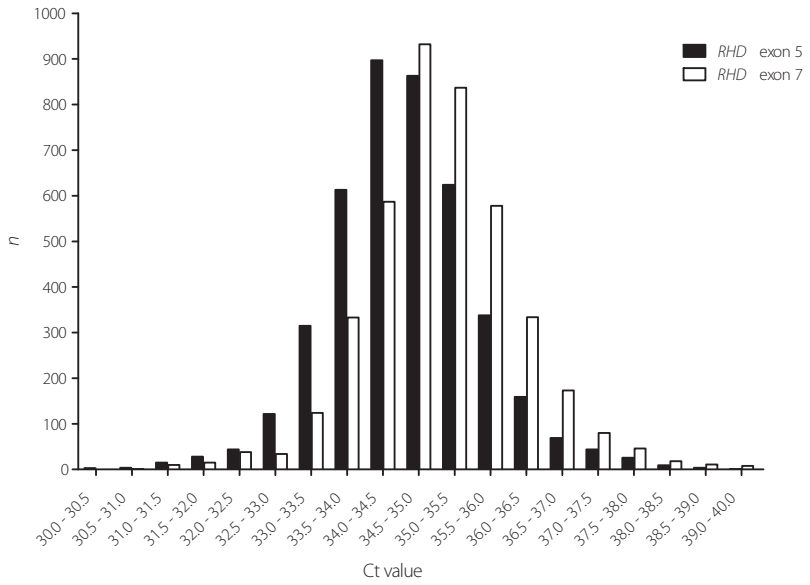

Figure 1. Distribution of the mean Ct values of samples with a positive *RHD* exon 5 and *RHD* exon 7 PCR result within the expected range (Ct > 30) for fetal DNA (*n* = 4176).

In 62 samples (0.9%), a maternal *RHD* variant gene was suspected, because of a relatively high level of amplification (Ct values <30) of both *RHD* exon 5 and *RHD* exon 7 (*n* = 25) or *RHD* exon 7 only (*n* = 37) and/or aberrant red cell serology results (*n* = 9, as mentioned above). Median Ct values were 28.3 (2.5th–97.5th percentile, 22.1–30.8) and 28.7 (2.5th–97.5th percentile, 22.1–30.5) for *RHD* exon 5 and *RHD* exon 7, respectively. Detailed serological and molecular analysis of 59 of these samples showed that indeed all these women carried an *RHD* variant gene (Table 1). Forty-five women (76.3%) were at risk of immunisation because they were carrying a D-negative *RHD* gene (*n* = 25) or an *RHD* gene encoding partial D (*n* = 20). Fourteen women (23.7%) were found not to be at risk of immunisation since they were carrying an *RHD* gene encoding weak D (*n* = 9) or DEL (*n* = 5). Unfortunately, of the remaining three samples in which a maternal *RHD* variant gene was suspected, the maternal red cells and buffy coat had not been rescued for further analysis. In all 62 cases, an 'RhD positive' screening result was issued.

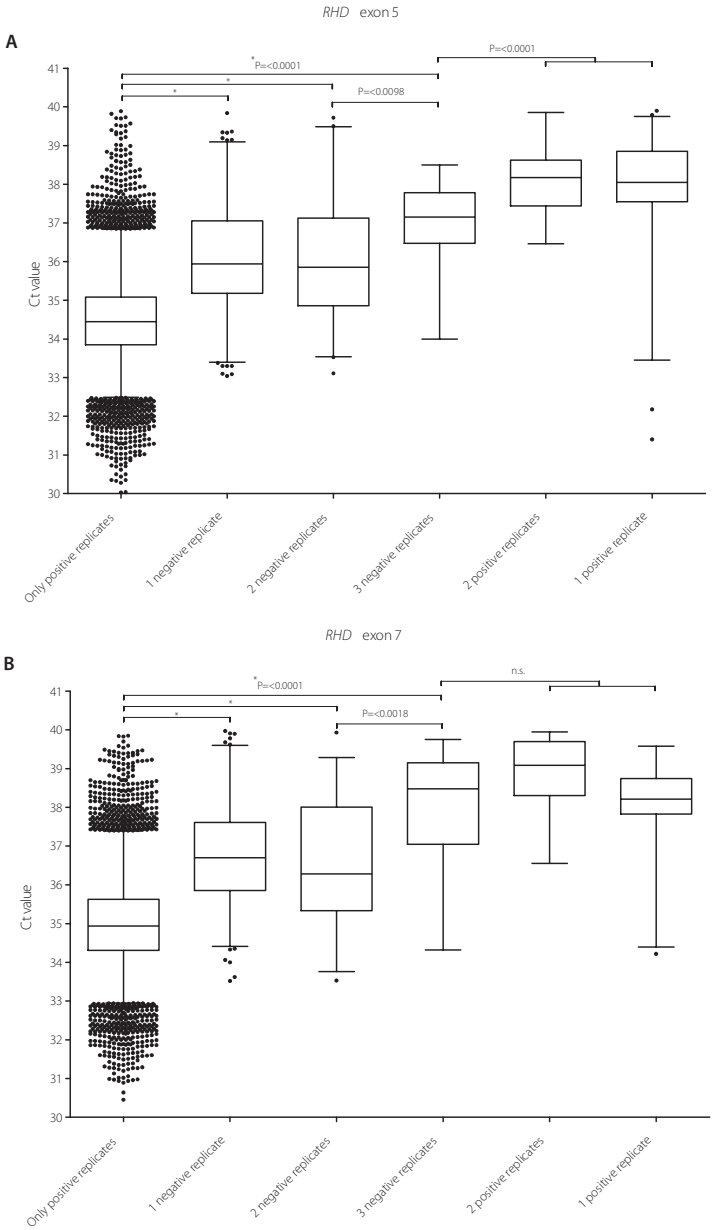

Figure 2. Ct values and number of positive/negative replicates. (A) Ct values for *RHD* exon 5 PCR. Only positive replicates: median Ct value, 34.5 (2.5th–97.5th percentile, 32.5–36.8); 1 negative replicate: 35.9 (33.5–38.9); 2 negative replicates: 35.9 (33.7–39.4); 3 negative replicates: 37.2 (34.5–38.4); 2 positive replicates: 38.2 (36.5–39.6); 1 positive replicate: 38.1 (35.5–39.7). (B) Ct values for *RHD* exon 7 PCR. Only positive replicates: 34.9 (33.0–37.4); 1 negative replicate: 36.7 (34.5–39.5); 2 negative replicates: 36.3 (33.9–39.1); 3 negative replicates: 38.5 (35.0–39.7); 2 positive replicates: 39.1 (36.7–39.9); 1 positive replicate: 38.2 (35.5–39.6). Boxes represent 25th and 75th percentiles; lines in boxes represent median values; horizontal lines outside boxes represent 2.5th and 97.5th percentiles; dots represent outliers.

Table 1. Maternal *RHD* variant genes (*n* = 59)

RHD allele	*n*	*RHD* exon 5 PCR	*RHD* exon 7 PCR	D phenotype	Maternal risk of immunisation
RHDΨ	21	–	+	Negative	Yes
DVI type 1/type 2	14*	–	+	Partial D‡	Yes
DFR	5	+	+	Partial D¥	Yes
Unknown *RHD* (negative phenotype)#	3	+	+	Negative	Yes
RHD 424-426delATG	1	+	+	Negative	Yes
DAR	1	+	+	Partial D¶	Yes
Weak D type 2	7	+	+	Weak DΩ	No
Weak D type 1	1	+	+	Weak D‡	No
Weak D type 4.0/8.0	1	+	+	Weak D‡	No
RHD 1227G>A	2	+	+	DEL‡	No
RHD IVS3+1G>A	1	+	+	DEL‡	No
RHD 87insT	1	+	+	DEL‡	No
Unknown *RHD* (DEL phenotype)#	1	+	+	DEL‡	No

*_DVI type 1_ (*n* = 1); _DVI type 2_ (*n* = 13)
#No mutations shown with MLPA, sequencing analysis to be performed.
‡D negative with routine serology.
¥Weak (≤2+) reactivity (*n* = 3) or D negative (*n* = 2) with routine serology.
¶Weak (≤2+) reactivity with routine serology.
ΩWeak (≤2+) reactivity (*n* = 5) or D negative (*n* = 2) with routine serology.

In 2682 samples (38.6%), both *RHD* exon 5 and *RHD* exon 7 PCR were negative. In 173 of these samples (6.5%), a single positive replicate was observed (Figure 2). In 18 further samples (0.7%), two positive replicates were observed. The Ct values for *RHD* exon 5 of these positive replicates were significantly different from the Ct values of the positive samples in which three negative replicates were observed (Figure 2). Postnatal cord blood serology results were available from 5164 deliveries (74.4%; Table 2). The distribution in D positive and D negative typing results was 59.9% and 40.1%. There was a 98.9% concurrence (5105/5164) between the reported fetal *RHD* screening result and postnatal cord blood serology result.

There were three 'false-negative' results (0.06%; 3/5164). In all three cases, no positive replicates for *RHD* exon 5 or *RHD* exon 7 had been observed. For each of these three cases, the *RHD* exon 5 and *RHD* exon 7 PCR were repeated (as singleplex) after manual DNA extraction from the stored frozen plasma. In two cases, both PCRs were now positive with Ct values ≥37.0. In retrospect, one of these samples was run in a PCR test showing a suboptimal fluorescent signal, however, sensitivity had been concluded to be reached based on the Ct values obtained with the internal standards. In one case, *RHD* exon 5 and *RHD* exon 7 PCR remained negative upon repeat testing and a simultaneously performed *RASSF1A* PCR (modified from Chan et

al.[27]) did not indicate the presence of fetal DNA. Genetic fingerprinting analysis excluded the possibility of sample mixup.[28]

There were 56 'false-positive' results (1.1% [56/5164]; summarised in Table S1). In 21 of these cases, an 'RhD positive' fetal screening result had been reported to the clinic, but a maternal (*n* = 15; Table S1, cases 1–15) or fetal RhD variant (*n* = 6; cases 16–21) was already suspected at the time of testing. Detailed serological and molecular analysis of the maternal and cord blood samples could explain the positive plasma PCR results in 18 of these 21 cases. In three of the six cases in which a fetal *RHD* variant was suspected, the molecular *RHD* analysis of the cord blood sample had not yet been performed at time of this report (cases 19–21). In three of the 15 mothers carrying a *RHD* variant gene leading to a DEL phenotype, the fetus inherited this particular variant (cases 10–12) and, thus, the D-negative cord blood serology result was, in fact, incorrect. In 35 of the 56 'false-positive' cases, there had been no indication of a fetal or maternal *RHD* variant. Detailed serological and/or molecular RhD analysis of the cord blood sample had been performed in 30 of these 35 cases at time of this report; showing eight newborns carrying weak D (*n* = 7) or DEL (*n* = 1) *RHD* genes (cases 49–56). Thus, these cases were, in fact, false-negative with routine serology. In 22 further cases, MLPA analysis revealed the complete absence of the *RHD* gene in the newborn (cases 22–32, 35–42, and 46–48). Five of these cases belonged to the small group of 'RhD positive' samples in which two or more negative replicates had been observed, including the only sample in which five negative replicates had been observed and an 'RhD positive' screening result had been reported. In the other 17 cases in which the complete absence of the *RHD* gene in the newborn was observed, only positive (*n* = 11) or only one negative (*n* = 6) replicates had been observed (cases 22–32 and 35–40).

Compared with routine postnatal cord blood serology results sensitivity and specificity of the fetal *RHD* screening were 99.9% (95% CI 99.7–100%) and 97.3% (95% CI 96.5%–98.0%), respectively (Table 2). Taking into account the 11 false-negative cord blood serology results that were correctly predicted 'RhD positive' by PCR, specificity of the fetal *RHD* screening was 97.8% (95% CI 97.1%–98.4%).

Table 2. Fetal *RHD* screening results and postnatal cord blood serology

Fetal *RHD* screening result	Postnatal cord blood serology result		Total
	D positive	D negative	
'RhD positive'	3088[#]	56 (45)[*]	3144
'RhD negative'	3	2017[‡]	2020
Total	3091	2073	5164

[*]Fifty-six 'false-positive' fetal *RHD* screening results compared with routine cord blood serology. Detailed serological and molecular RhD analysis of 51 of these 56 cord blood samples revealed that in 11 cases the serology result was false negative, however.
[#]Including four twin pregnancies, included as single cases.
[‡]Including one twin pregnancy, included as a single case.

In the eight cases in which a fetal *RHD* variant gene was suspected because of amplification of only *RHD* exon 5, cord blood samples were available in seven. One of these samples was serologically D-negative (discussed above as one of the false-positive results and shown in Table S1 [case 21]). The other six cord blood samples were serologically D positive. MLPA was performed on three; two 'normal' *RHD* genes and one *DIII type 4* or *DIVa-2* were found. In both two cases in which MLPA showed the presence of a 'normal' *RHD* gene, amplification of *RHD* exon 7 had also been observed in 1 of 3 replicates (Ct values 37.2 and 40.0, respectively).
In the 13 cases in which a fetal *RHD* variant gene was suspected because of amplification of only *RHD* exon 7, cord blood samples were available in 12. Five cord blood samples were serologically D negative and at least three of these newborns were indeed carrying an *RHDΨ* (MLPA), belonging to the above described group of false-positive results as samples suspected for a fetal variant (Table S1, cases 16–18). Seven cord blood samples were serologically D positive. MLPA had been performed on two; one showed a *DV type 7*, the other a *DAU-5*.

Discussion

We present a first-three-month analysis of the nation-wide fetal *RHD* screening programme in The Netherlands. The programme was introduced to restrict prophylactic antenatal and postnatal anti-D immunoglobulin administration to only those D-negative pregnant women that carry a D-positive child. We report a sensitivity of 99.9% (95% CI 99.7–100%) for the prediction of the fetal D phenotype by determining the fetal *RHD* genotype from cell-free fetal DNA isolated from maternal plasma. Blood sample processing and PCR setup were fully automated to allow the throughput of the around 100 samples each working day. Of the 6941 D-negative pregnant women tested in the first 3 months of the fetal *RHD* screening programme, 2682 (38.6%) were predicted to carry a D-negative child and anti-D immunoglobulin was not administered antenatally and postnatally. According to the cord blood serology results available from 5164 newborns (74.4%), there were only three false-negative fetal *RHD* screening results (0.06%).
The fetal D phenotype was predicted from the results of a duplex *RHD* exon 5–*RHD* exon 7 real-time quantitative PCR on cell-free fetal DNA isolated from maternal plasma. Based on the number of positive and negative PCR replicates a clear distinction between an 'RhD positive' and an 'RhD negative' screening result could be made (Figure 2). The far majority of samples showed only positive or only negative replicates. Only in 18 samples (0.3%), two positive replicates were observed and these were reported as 'RhD negative'. Since no false-negative results were found for all 12 of these 18 samples for which cord blood serology results were available, this seems to be the right approach. Whereas in previous studies inconclusive results due to an 'intermediate number' of positive replicates were encountered,[22,24] we observed only a very small group of 12 samples (0.2%) in which only three positive replicates were observed

and we reported these samples as 'RhD positive'. This seems to be the right approach since serology results of eight of the ten available cord bloods of these samples indicated the birth of a D-positive child. These samples probably have very low fetal DNA concentrations that are near the detection limit of the assay. False-positive results due to positive replicates caused by nonspecific amplification in this small group of samples are inevitable and these were indeed observed in the other two cases with known cord blood serology.

False-negative results are the main concern in fetal *RHD* screening assays, because anti-D immunoprophylaxis would unjustly be withheld from the D-negative pregnant woman, with a resulting risk of immunisation and potential morbidity and mortality from HDFN in subsequent pregnancies. We observed false-negative results in only three cases (0.06%) according to the 5164 available cord blood samples tested at Sanquin. In theory, false-negative results arise from the absence of (sufficient) DNA in the detection system, either due to DNA extraction failure or low fetal DNA concentrations in the plasma sample, or from PCR failure or sample mix-up. Since two of the three samples with a reported false-negative result were positive upon repeat testing of frozen plasma, the initial false-negative result could have been caused by DNA extraction failure or insufficient PCR amplification. Indeed, one of these samples was run in a PCR test with lower amplification signals albeit with Ct values of the internal standards within the preset range. As a quality parameter, we have now added the performance of the PCR run based on achieved ΔRn (the magnitude of the fluorescence signal generated during the PCR). The third false-negative sample remained negative upon repeat testing and since labelling errors were excluded, it seems most likely that the amount of fetal DNA in the sample was below the detection limit of the assay. Indeed, in this sample we could not confirm the presence of fetal DNA by performing a methylation-sensitive PCR for the universal fetal DNA marker *RASSF1A*. We show a slightly lower percentage of false-negative results than has been reported in previously conducted large-scale fetal *RHD* screening feasibility studies (0.06% vs. around 0.2%).[21-23] This might be explained by the standardised handling and analysis of samples within the screening programme as compared to the less standardised handling of samples within a research setting. Recently, Clausen et al.,[24] reporting the compiled data from five Danish health care regions on the Danish nation-wide fetal *RHD* screening programme, similarly showed false-negative results in 0.09% of cases (2/2312).

There was a relatively large group of false-positive results (n = 56; 1.1%). This outcome was expected since we aimed to avoid false-negative results and therefore applied a conservative approach for the prediction of D-negative fetuses. In the feasibility study by Finning et al.,[22] an even larger group of false-positive results was observed. They concluded that routine application of fetal *RHD* screening to target anti-D prophylaxis would result in the unnecessary administration of antenatal immunoprophylaxis in about 2.1% of cases due to false-positive (14/1869) or inconclusive (25/1869) test results. In the Danish report on the implementation of fetal *RHD* screening,[24] antenatal immunoprophylaxis was unnecessary recommended in

1.7% of cases as a result of inconclusive (33/2312) or false-positive (6/2312) results.
The prediction of D positivity from an *RHD* genotyping result is complicated by the presence of *RHD* variant genes. Sometimes these *RHD* variant genes, either positive in both *RHD* exon 5 and *RHD* exon 7 PCR, or only positive in *RHD* exon 7 PCR, result in a D-negative phenotype. We decided to issue an 'RhD positive' fetal screening result in all cases in which the PCR results indicated the presence of a maternal or fetal *RHD* variant gene, rather than an inconclusive result, since in all these cases antenatal immunoprophylaxis was advised. However, based on the results of our analysis it might be possible to modify the algorithm in cases in which only one maternal *RHD* exon is amplified.
In cases in which the pregnant woman carries an *RHD* variant gene resulting in the amplification of both *RHD* exon 5 and *RHD* exon 7 at a maternal level, a prediction of the fetal D phenotype is not possible. Routine elucidation of the molecular background of the *RHD* variation in these women as part of the screening programme could be performed as some of these women might actually not be at risk of immunisation (those women carrying weak D or DEL alleles). However, this would delay and complicate the programme and these cases are relatively rare. It was therefore decided to advise administration of immunoprophylaxis in these cases without further molecular analysis of the maternal *RHD* gene. For the evaluation study we rescued maternal DNA of samples with Ct values <30 for both *RHD* exon 5 and *RHD* exon 7. Indeed, the presence of a maternal *RHD* variant gene was observed in all 24 samples tested. However, results obtained with the nine serologically D-positive women with aberrant serology incidentally also showed Ct values >30 and <31.5 (data not shown). Moreover, the analysis of a group of 488 D-positive pregnant women (Figure S1) revealed that 4.5% of those women had Ct values >30 and <31.5. From these data we conclude that with a cut-off of a Ct value of 30 we underestimate the prevalence of maternal *RHD* variants among the screened D-negative pregnant women. Thus, if routine maternal *RHD* genotyping would be applied to reveal whether amplification signals are derived from the mother or fetus, and to elucidate the maternal *RHD* genotype, a cut-off of 31.5 should be chosen. For the current algorithm for the prediction of the fetal D phenotype the cut-off is not relevant, since all positive amplifications will be reported as an 'RhD positive' screening result.
If a pregnant woman carries an *RHD* variant gene resulting in amplification of only one *RHD* exon, the fetal D phenotype might be concluded from the amplification analysis of the other exon, which can be done since amplification of fetal DNA in general generates higher Ct values (less DNA) than amplification of maternal DNA. *RHD* genes positive for only *RHD* exon 7 are rather common and were observed in 0.5% of cases (n = 37, of which 35 were further analysed). These cases were found to be due to the presence of the so-called *RHDΨ* (0.3%) and the *DVI* (0.2%) variant gene in the mother. Women carrying these variants can become immunised, so prediction of the fetal D phenotype is relevant. Around 66% of D-negative African Blacks carries an *RHDΨ*, an inactive *RHD* gene containing a 37-base pair duplication

plus a nonsense mutation.[29] The prevalence of the DVI phenotype (partial D) in Caucasians is 0.02%.[30] Evidently, a pregnant woman with a DVI phenotype will not be immunised when carrying a DVI fetus.

Of the 21 women carrying an *RHDΨ*, amplification of *RHD* exon 5 at a fetal level was observed in 16 samples. Cord blood serology was available in 15 of these cases, all showing a D-positive child (Table 3). No amplification of *RHD* exon 5 was observed in five samples and the birth of a D-negative child was confirmed in all. Of the 14 women with a DVI phenotype, amplification of *RHD* exon 5 at a fetal level was observed in seven cases. Cord blood serology showed a D-positive child in all. No amplification of *RHD* exon 5 was observed in the other seven samples; as expected, in half of these cases the newborn had a DVI phenotype ($n = 3$) and the other half were D-negative ($n = 4$). From Table 3 it can be concluded that in samples from women with an *RHD* exon 7-positive *RHD* variant gene, prediction of the fetal D phenotype can be based on the result of the *RHD* exon 5 PCR. Only in the very rare occasion that a mother with an *RHDΨ* is carrying a DVI fetus or a fetus with another (rarely occurring) *RHD* variant negative in the *RHD* exon 5 PCR, immunisation may occur from the false-negative prediction. If the typing algorithm would be adjusted and all samples in which *RHD* exon 7 is amplified with a Ct value <30 and no amplification of *RHD* exon 5 is observed, are scored as 'RhD negative', this would have reduced the false positivity rate to 0.85%.

Table 3. Relevance of maternal *RHDΨ* and DVI variants

		Anti-D immunoprophylaxis indicated?	
Maternal RhD variant	*n* (available postnatal cord blood serology)	Yes (*RHD* exon 5 PCR positive in maternal plasma)	No (*RHD* exon 5 PCR negative in maternal plasma)
RHDΨ	20	15 (15)	5 (5)[#]
DVI	14	7 (7)	7 (7)[*]

[#]Fetus D negative.
[*]Fetus D negative ($n = 4$) or DVI ($n = 3$).

Samples showing amplification of only *RHD* exon 5 at a maternal level are not likely to be encountered in a fetal *RHD* screening setting, because these women would be phenotypically D-positive. Indeed, we did not encounter any.

Samples showing amplification of only *RHD* exon 5 at a fetal level (suggesting the presence of a fetal *RHD* variant gene) should be regarded as D positive. Samples showing amplification of only *RHD* exon 7 at a fetal level may reflect the presence of a fetal *RHDΨ*, however, since this can not be definitely concluded, these samples should always be reported as 'RhD positive'.

Finally, we recognised a group of 17 false-positive samples with amplification levels within the normal fetal range for both *RHD* exon 5 and *RHD* exon 7. In theory, placental mosaicism,[31] or maternal *RHD* chimerism[32] could be an explanation for these false-positive results.

However, the frequency of these conditions is expected to be low. Another explanation is sample contamination or mislabelling. Maternal *RHD* chimerism, sample contamination and mislabelling will be further investigated.

In conclusion, the present report shows the high reliability of the *RHD* screening programme, preventing unnecessary anti-D immunoglobulin administration to D-negative women carrying a D-negative child. Because the negative predictive value of this fully automated assay is 99.9% (95% CI 99.6–100 %), this first analysis indicates that, in a centralised setting, it is indeed possible to guide both antenatal and postnatal anti-D immunoprophylaxis by fetal *RHD* screening in maternal blood obtained at 27 week of gestation and thus to stop postnatal cord blood serology testing. However, this analysis is based on the cord blood samples received by Sanquin only. A longer period of nation-wide evaluation of the fetal *RHD* screening programme, including all (also locally typed) cord blood serology results obtained in a one-year time period, will provide insight in the robustness of the fetal *RHD* screening programme.

References

1 Urbaniak SJ, Greiss MA. RhD haemolytic disease of the fetus and the newborn. *Blood Rev* 2000;14:44–61.

2 Levine P, Katzin EM, Burnham L. Isoimmunization in pregnancy. *JAMA* 1941;116:825–7.

3 Chown B. Anaemia from bleeding of the fetus into the mother's circulation. *Lancet* 1954;1:1213–5.

4 Crowther C, Middleton P. Anti-D administration after childbirth for preventing Rhesus alloimmunisation. *Cochrane Database Syst Rev* 2000;CD000021.

5 Engelfriet CP, Reesink HW, Judd WJ, Ulander VM, Kuosmanen M, Koskinen S et al. Current status of immunoprophylaxis with anti-D immunoglobin. *Vox Sang* 2003;85:328–37.

6 Koelewijn JM, de Haas M, Vrijkotte TGM, Bonsel GJ, van der Schoot CE. One single dose of 200 microg of antenatal RhIG halves the risk of anti-D immunization and hemolytic disease of the fetus and newborn in the next pregnancy. *Transfusion* 2008;48:1721–9.

7 Pilgrim H, Lloyd-Jones M, Rees A. Routine antenatal anti-D prophylaxis for RhD-negative women: a systematic review and economic evaluation. *Health Technol Assess* 2009;13:iii, ix–iii,103.

8 Race RR, Mourant AE. The Rh chromosomes in England. *Blood* 1948;3:689–95.

9 Power JP, Lawlor E, Davidson F, Yap PL, Kenny-Walsh E, Whelton MJ et al. Hepatitis C viraemia in recipients of Irish intravenous anti-D immunoglobulin. *Lancet* 1994;344:1166–7.

10 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

11 Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. Detection of fetal RHD–specific sequences in maternal plasma. *Lancet* 1998;352:1196.

12 Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998;339:1734–8.

13 Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002;42:1079–85.

14 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, de Haas M. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience. *BJOG* 2011;118:1340–8.

15 Sequenom Center for Molecular Medicine. SensiGene Fetal RHD Genotyping. www.sequenomcmm.com

16 Lenetix Inc. RHD Genotyping. www.lenetix.com

17 Legler TJ, Liu Z, Mavrou A, Finning K, Hromadnikova I, Galbiati S et al. Workshop report on the extraction of foetal DNA from maternal plasma. *Prenat Diagn* 2007;27:824–9.

18 Daniels G, van der Schoot CE, Olsson ML. Report of the fourth International Workshop on molecular blood group genotyping. *Vox Sang* 2011;101:327–32.

19 Grootkerk-Tax MGHM, Soussan AA, de Haas M, Maaskant–van Wijk PA, van der Schoot CE. Evaluation of prenatal RHD typing strategies on cell-free fetal DNA from maternal plasma. *Transfusion* 2006;46:2142–8.

20 Avent ND. RHD genotyping from maternal plasma: guidelines and technical challenges. *Methods Mol Biol* 2008;444:185–201.

21 van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Page-Christiaens GCML, de Haas M. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006;13:53–7.

22 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* 2008;336:816–8.

23 Muller SP, Bartels I, Stein W, Emons G, Gutensohn K, Kohler M et al. The determination of the fetal D status from maternal plasma for decision making on Rh prophylaxis is feasible. *Transfusion* 2008;48:2292–301.

24 Clausen F, Christiansen M, Steffensen R, Jorgensen S, Nielsen C, Jakobsen M et al. Report of the first nationally implemented clinical routine screening for fetal RHD in D– pregnant women to ascertain the requirement for antenatal RhD prophylaxis. *Transfusion* 2011.

25 van der Schoot CE, Bleker OP, Bonsel GJ, Christiaens GCML, Kanhai HH. Prenatal fetal RhD blood group typing to enhance efficiency of antenatal anti-D immunoprophylaxis in RhD–negative pregnant women. ZonMw 2005;945-01-010.

26 Legler T, Muller S, Haverkamp A, Grill S, Hahn S. Prenatal RhD Testing: A Review of Studies Published from 2006 to 2008. *Transfus Med Hemother* 2009;36:189–98.

27 Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN et al. Hypermethylated RASSF1A in maternal plasma: A universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.

28 Jeffreys AJ, Wilson V, Thein SL. Individual-specific 'fingerprints' of human DNA. *Nature* 1985;316:76–9.

29 Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D–negative blood group phenotype. *Blood* 2000;95:12–8.

30 Wagner FF, Kasulke D, Kerowgan M, Flegel WA. Frequencies of the blood groups ABO, Rhesus, D category VI, Kell, and of clinically relevant high-frequency antigens in south–western Germany. *Infusionsther Transfusionsmed* 1995;22:285–90.

31 Flori E, Doray B, Gautier E, Kohler M, Ernault P, Flori J et al. Circulating cell-free fetal DNA in maternal serum appears to originate from cyto- and syncytio-trophoblastic cells. Case report. *Hum Reprod* 2004;19:723–4.

32 Salaru NN, Lay WH. Rh blood group mosaicism in a healthy elderly woman. *Vox Sang* 1985;48:362–5.

Supporting information

Chapter 6

Table S1. Comprehensive serological and molecular data of all 56 cases with an 'RhD positive' fetal screening result and a D-negative routine cord blood serology result

Case	*RHD* exon 5			*RHD* exon 7			Detailed maternal serology	MLPA maternal DNA	Detailed cord blood serology	MLPA cord blood
1	**50.0**	**50.0**	**50.0**	*28.4*	*28.4*	*28.2*	DVI	DVI type1 /d cc Ee	N/A	
2	**50.0**	**50.0**	**50.0**	*28.0*	*28.0*	*28.0*	DVI	DVI type2 /d Cc ee	neg	
3	**50.0**	**50.0**	**50.0**	*27.2*	*27.5*	*27.6*	DVI	DVI type2 /d Cc ee	neg	
4	36.5	**50.0**	**50.0**	*29.3*	*29.3*	*29.3*	DVI	DVI type2 /d Cc ee	neg	
5	**50.0**	**50.0**	**50.0**	*27.5*	*27.5*	*27.8*	negative	Dpseudo /d cc ee	neg	
6	**50.0**	**50.0**	**50.0**	*30.4*	*30.4*	*30.2*	negative	Dpseudo /d cc ee	neg	Dpseudo /d cc ee
7	**41.3**	**43.7**	**50.0**	*30.3*	*30.3*	*30.3*	negative	Dpseudo /d cc ee	neg	Dpseudo /d cc ee
8	**50.0**	**50.0**	**50.0**	*29.4*	*29.9*	*29.3*	negative	Dpseudo /r's cc ee	neg	
9	**50.0**	**50.0**	**50.0**	*29.7*	*29.9*	*29.4*	negative	Dpseudo /r's cc ee	N/A	
10	*28.9*	*28.6*	*28.7*	*29.1*	*29.0*	*29.0*	abs/elution positive	Del 1227G>A /d Cc ee	abs/elution positive	Del /Del 1227G>A Cc ee
11	*29.7*	*29.9*	*29.8*	*30.0*	*30.2*	*29.9*	abs/elution positive	Dd Cc ee --> *RHD* 87insT	abs/elution positive	
12	*24.5*	*24.3*	*24.3*	*24.5*	*24.5*	*24.3*	abs/elution positive	Weak D type 4.0/8.0 (homoz) cc ee	abs/elution positive	
13	*28.5*	*29.0*	*28.3*	*29.2*	*29.8*	*28.9*	weak	Weak D type2 /d Cc Ee	N/A	
14	*28.2*	*28.3*	*28.1*	*28.7*	*28.7*	*28.9*	negative	Dd Cc ee	neg	
15	*24.5*	*24.3*	*24.5*	*24.5*	*24.5*	*24.7*	negative	Dd Cc ee -->*RHD* 424-426delATG	neg	dd cc ee
16	**50.0**	**50.0**	**50.0**	35.5	34.5	34.1			neg	Dpseudo /d cc ee
17	**50.0**	**50.0**	**50.0**	33.1	33.6	33.1			N/A	Dpseudo /d cc ee
18	**50.0**	**50.0**	**50.0**	34.5	35.1	34.7			neg	Dpseudo /r's cc ee
19	**50.0**	**50.0**	**50.0**	39.0	39.4	**50.0**			neg	
20	**50.0**	**50.0**	**50.0**	38.1	38.3	**50.0**			neg	
21	**41.5**	39.7	39.3	**50.0**	**50.0**	**50.0**			neg	
22	36.7	36.9	35.6	36.8	36.6	36.8			neg	dd cc ee
23	36.1	36.8	37.4	37.3	39.1	37.5			neg	dd cc ee
24	34.9	36.5	35.5	36.4	39.4	35.8			neg	dd cc ee
25	34.1	35.0	35.0	34.0	34.2	34.2			neg	dd cc ee
26	34.3	34.0	34.6	35.1	34.8	35.0			neg	dd cc ee
27	34.9	36.7	35.7	35.7	36.4	36.0			neg	dd cc ee
28	34.6	35.9	35.0	36.5	35.5	36.7			neg	dd cc ee
29	38.8	36.5	39.3	38.2	38.0	37.3			neg	dd cc ee
30	34.5	34.3	35.0	36.8	38.2	36.4			neg	dd cc ee
31	36.0	36.6	37.0	36.6	37.1	39.2			neg	dd cc ee
32	35.5	35.2	34.8	35.6	35.2	35.2			neg	dd cc ee
33	33.7	34.4	33.9	34.9	34.9	35.2			N/A	
34	34.8	34.1	34.4	34.8	34.9	35.3			N/A	

Table S1. (Continued)

Case	*RHD* exon 5			*RHD* exon 7			Detailed maternal serology	MLPA maternal DNA	Detailed cord blood serology	MLPA cord blood
35	37.8	37.5	37.3	36.9	36.9	**50.0**			neg	dd cc ee
36	(42.4)	37.2	38.3	37.3	36.3	**50.0**			neg	dd cc ee
37	37.0	37.7	**50.0**	37.0	37.5	37.9			neg	dd cc ee
38	38.7	37.6	**50.0**	38.2	39.0	38.1			neg	dd cc ee
39	35.4	35.6	**50.0**	37.3	37.4	36.5			neg	dd cc ee
40	37.2	36.8	**50.0**	37.0	37.1	37.3			N/A	dd cc ee
41	37.2	36.9	**50.0**	37.0	38.2	**50.0**			neg	dd cc ee
42	39.1	**50.0**	**50.0**	38.0	37.8	38.0			neg	dd cc ee
43	37.3	38.5	**50.0**	39.0	39.2	**50.0**			neg	
44	38.0	39.0	**50.0**	37.4	39.0	**50.0**			neg	
45	35.9	37.5	**50.0**	36.9	38.2	**50.0**			N/A	
46	36.7	36.9	**50.0**	39.7	**50.0**	**50.0**			neg	dd CC ee
47	38.1	**50.0**	**50.0**	36.9	38.8	**50.0**			neg	dd cc ee
48	**40.4**	36.1	**50.0**	**40.3**	**50.0**	**50.0**			N/A	dd cc ee
49	35.3	35.1	35.1	37.0	36.4	36.0			weak	Weak D type2 /d cc Ee
50	34.4	35.5	35.5	35.1	37.2	36.1			weak	Weak D type2 /d cc Ee
51	36.6	33.7	36.0	**50.0**	38.8	36.4			weak	Weak D type2 /d cc Ee
52	35.4	35.4	35.0	35.8	36.2	35.4			weak	Weak D type2 /d cc Ee
53	33.3	33.3	33.1	33.7	34.2	34.2			weak	Weak D type2 /d cc Ee
54	34.5	33.6	33.7	34.8	34.5	35.2			weak	Weak D type2 /d cc Ee
55	32.0	31.7	31.8	32.0	32.2	31.9			Weak	Weak D type2 /d cc Ee
56	35.9	36.4	35.2	35.9	37.1	37.4			abs/elution positive	Dd cc Ee

MLPA, multiplex ligation-dependant probe amplification.
Cycle threshold values of the fetal *RHD* screening assay are given in the first columns.

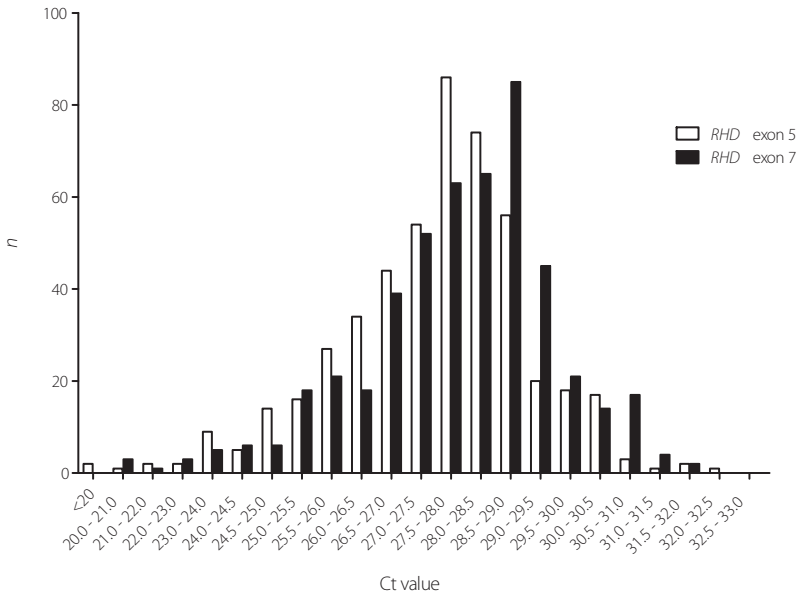

Figure S1. Distribution of the mean Ct values of samples from D-positive pregnant women at 27 weeks of gestation (n = 488). Median Ct values for *RHD* exon 5 and *RHD* exon 7 are 27.7 (2.5th–97.5th percentile, 23.7–30.4) and 28.1 (2.5th–97.5th percentile, 24.0–30.8), respectively. Data obtained from maternal blood samples sent in for fetal *RHD* screening, but upon serological typing of red cells were found to be from D-positive pregnant women (July 1st – December 31st 2011).

Chapter 7

Summary and concluding remarks

The discovery of cell-free fetal DNA in maternal plasma and serum opened up new possibilities for noninvasive prenatal genetic diagnosis.[1] In the maternal circulation, cell-free fetal DNA is present within an overwhelming background of maternal cell-free DNA.
Because of this coexistence with maternal DNA sequences, current clinical applications of cell-free fetal DNA rely on the detection of unique fetal sequences not shared by the maternal genome, i.e. those that the fetus has inherited from its father or mutations that have arisen *de novo*. Although a vast amount of literature on the clinical applications of noninvasive fetal genotyping of paternally inherited alleles had been published, most data were obtained in a research setting and the performance of these tests in actual clinical practice was not known. In this thesis we focused on the performance of noninvasive fetal genotyping of paternally inherited alleles in everyday clinical practice. The results of our studies are summarised below.

In **chapter 2** we analysed the test results of 201 consecutive pregnant women for whom noninvasive fetal sex determination was performed in our laboratory over a 6-year period. Tests were performed for medical indications at the request of clinical geneticists or gynaecologists throughout The Netherlands. We showed that noninvasive fetal sex determination in a clinical setting is highly reliable. In our laboratory, two different Y chromosome-specific markers were applied. The DYS14 marker showed high sensitivity with occasional amplification in female-bearing pregnancies. The *SRY* marker was less sensitive but increased the specificity of the test as a whole. The combination of these two markers resulted in a 100% diagnostic accuracy. In a recently published meta-analysis on more than 6500 pregnancies, no significant difference between the individual test performance of DYS14 or *SRY* was shown, but our approach was not evaluated.[2] We further showed that conclusive results could be obtained as early as 7 weeks of gestation. This is in line with our previous findings that cell-free fetal DNA can be detected in the maternal circulation as early as 5 weeks of gestation[3] and suggests that, if clinically indicated, noninvasive fetal sex determination could be performed at this early gestational age, though likely with a higher risk of inconclusive results, because of still relatively low cell-free fetal DNA concentrations. In the above mentioned meta-analysis, the authors concluded that the overall performance of noninvasive fetal sex determination in maternal blood is poor prior to 7 weeks.[2] Knowledge of the fetal sex before 7 weeks of gestation is only medically relevant for pregnant women at risk of being pregnant with a child with congenital adrenal hyperplasia. In carriers of X-linked conditions requesting invasive prenatal diagnosis, noninvasive fetal sex determination at 10 weeks allows timely scheduling of chorionic villous sampling from 11 weeks onward.

In **chapter 3** we analysed the test results of 362 consecutive red cell alloimmunised pregnant women for whom noninvasive fetal blood group genotyping of Rh D, c, E, or of K was performed in our laboratory over a 7-year period. In The Netherlands, all pregnant women

are screened for the presence of red cell antibodies at first booking. If antibodies are found, knowledge of the fetal blood group antigen status is beneficial to determine whether a fetus is at risk of haemolytic disease and whether prenatal monitoring of antibody titres and activity and parameters for fetal anaemia is required. Real-time polymerase chain reaction on cell-free DNA isolated from maternal plasma was performed for *RHD* exon 5 and *RHD* exon 7, or the specific allele of the *RHCE* gene or *KEL* gene. In the latter assay, a peptide nucleic acid (PNA) probe was used to prevent mispriming of the *K* allele-specific primer on the antithetic *k* allele, known to cause nonspecific amplification. In contrast to the approach of a British group who introduced locked nucleic acids (LNAs) in the *K* allele-specific primer to enhance assay specificity,[4] our PNA probe did not reduce the overall sensitivity of the assay. For all assays, no false-positive or false-negative results were found when compared with the 212 available cord blood serology or amniotic fluid cells genotyping results. All test results were used by the pregnant woman's care giver to guide the clinical and laboratory management of the alloimmunised pregnancy. Noninvasive fetal blood group genotyping is increasingly offered as a clinical service in laboratories worldwide[5] and should now be considered part of standard pregnancy care for red cell alloimmunised pregnant women.[6] Blood collection tubes that minimise post-sampling maternal cell-free DNA background can be used for sending samples to reference laboratories.[7]

The results of **chapters 2 and 3** demonstrate that the performance of noninvasive fetal genotyping of paternally inherited alleles in everyday clinical practice is good. All assays described were based on real-time PCR. An alternative platform could be mass spectrometry (MS).[8] The advantage of MS is its multiplexing capacity, allowing for the simultaneous interrogation of multiple fetal loci. This might be relevant for the *RHD* locus to predict the fetal D phenotype, since assays can be designed that recognise all possible *RHD* variants. Furthermore, this technique enables the inclusion of a panel of polymorphic markers as a control for the presence of fetal DNA, which is much more convenient than our current approach for which extensive testing of the mother and, ideally also the reported father, is needed to select a suitable marker. However, the sophisticated and expensive MS equipment is currently not readily available to the majority of diagnostic laboratories, making real-time PCR the current method of choice.

The development of a noninvasive fetal platelet genotyping assay of human platelet antigen (HPA)-1a was described in **chapter 4**. Whereas nonspecific amplification of maternal cell-free DNA could be overcome by using a PNA probe in the fetal *K* assay (chapter 3), we now took to a pre-PCR digestion of the cell-free DNA with an endonuclease. Digestion of the antithetic HPA-1b alleles effectively increased the assay's specificity. This approach could also be useful for other assays in which the maternal allele gives rise to nonspecific amplification. Drawback of the use of an endonuclease in noninvasive fetal genotyping assays is the risk of incomplete

digestion and therefore controls for digestion are required. This adds considerably to the workload. Noninvasive fetal HPA-1a genotyping offers a safe method for HPA-1a alloimmunised pregnant women with a heterozygous partner to determine whether their fetus is at risk of fetal or neonatal alloimmune thrombocytopenia; avoiding the risk of boostering antibody formation inherent to invasive procedures. It allows for expensive or potentially harmful antenatal interventions such as intravenous immunoglobulins or diagnostic cordocentesis to be restricted to only fetuses at risk. Thirty percent of individuals is HPA-1a/b heterozygous, whereas only 2% is HPA-1b/1b homozygous. This means that around 30% of women with anti-HPA-1a antibodies will have a heterozygous partner and therefore can carry a HPA-1a negative child. The availability of this test further adds an important perspective to the ongoing debate on whether or not to implement screening programmes to prevent HPA-1a-mediated FNAIT.[9]

In **chapter 5** we reviewed the literature on the positive controls in noninvasive fetal blood group genotyping assays. Because these assays have been found to be generally robust, much controversy about the need of such controls existed. Our review showed that in noninvasive fetal blood group genotyping assays false-negative results are obtained in 0.1–0.2% and that they are only encountered in studies lacking a control for the presence of fetal DNA. We therefore advocated the use of positive controls if testing is performed because of red cell alloimmunisation. Similarly, in assays for fetal sex determination, which are performed very early in pregnancy when fetal DNA levels are still relatively low, a control to confirm the presence of fetal DNA is informative. However, in all 15 cases described in chapters 2 and 3 of this thesis in which no result was issued because the presence of fetal DNA could not be confirmed and with known pregnancy outcome, the newborn was found to be 'negative' (i.e. female or a blood group antigen negative phenotype). Therefore, one might still question the absolute need for positive controls in noninvasive fetal genotyping assays that rely on the detection of unique fetal sequences. If testing is performed for screening purposes to ascertain the requirement of anti-D immunoglobulin prophylaxis in D-negative pregnant women, a control for the presence of fetal DNA can be omitted.

The analysis of the first 3 months of the fetal *RHD* screening programme for the guidance of antenatal and postnatal anti-D immunoglobulin prophylaxis was described in **chapter 6**. From July 1st to September 30th 2011, a total of 6941 women at risk of D immunisation was tested. Based on the fetal *RHD* screening result the (unnecessary) administration of antenatal immunoprophylaxis was prevented in 2682 women (38.6%). Of nearly 75% of newborns, a cord blood sample was sent to Sanquin for serological analysis, enabling the comparison of the predicted phenotype and the true Rh D status of the newborn. Moreover, the availability of the cord blood and the back-up sample of the maternal plasma allowed us to analyse all discrepant results. This chapter is the interim report of an evaluation study which will

continue for another 9 months. The results are highly promising and justify the continuation of the programme. Our main concern was that the fetal *RHD* screening programme would lead to an increase in immunisations because of false-negative results. As a fact, the cell-free fetal DNA concentration is low in a maternal blood sample and in a considerable number of samples near to the limit of detection. The large variation in pre-laboratory handling of blood samples, as is to be expected in a nation-wide programme, might easily lead to a loss of sensitivity. Our major conclusion is therefore that in the current setting it indeed seems to be safe to administer not only antenatal but also postnatal prophylaxis based on the fetal *RHD* screening result, since we observed only three false-negative results (0.06%), resulting in a negative predictive value of 99.9% (95% CI 99.6%–100%). Thorough evaluation of these false-negative results has already led to a slight modification of the quality parameters of the assay, so possibly the false-negative rate might even become lower when the complete results of the first evaluation year are available. What is more, in 11 cases (0.2%) we observed a false-negative cord blood serology result. In absence of the screening programme, postnatal anti-D immunoprophylaxis would have been withheld in these cases. So, our preliminary conclusion is that the fetal *RHD* screening programme might even lead to less immunisations. However, it should be noted that the present analysis is based on a selection of about 75% of pregnancies in which the cord blood was centrally analysed at Sanquin. The percentage of predicted D-negative newborns in this group (39.1%) was found to be somewhat higher than the percentage of 'RhD negative' screening results in the other one-fourth of pregnancies (37.3%). Presumably, obstetric care-givers send in cord blood for confirmation of 'RhD negative' screening results more often, which seems logical. This illustrates the importance of aiming for a complete follow-up and analysis of cord blood samples of all tested pregnancies.

Discrimination between 'RhD positive' and 'RhD negative' results was almost universally easy. The far majority of samples showed either six positive or six negative replicates. There was only a small group of 12 samples (0.17%) which were scored 'RhD positive' based on borderline PCR results (i.e. three positive replicates). But still, the far majority of these women were carrying D-positive fetuses, so the weak PCR signals are mainly due to the extremely low concentrations of fetal DNA in these samples. Further research on why this subset of women has such low cell-free fetal DNA concentrations is warranted. In a British feasibility study,[10] 1.7% of the samples were inconclusive because of borderline PCR results. About half of them were from women carrying D-positive fetuses. This suggests that their approach has led to a slightly less sensitive assay. Since their PCR assay was identical to ours, the fact that they only used 560 µL of plasma for DNA isolation could be a reason. Indeed, the number of false-negative results they encountered (0.2%) was higher, but because in their study cord blood was not available for further analysis no definite conclusions can be drawn.

As expected, in a considerable number of pregnancies (1.2%), fetal *RHD* screening was complicated by the presence of variant *RHD* genes. Since all these variant genes will be

characterised during the evaluation study, a reliable estimate on the frequency of these genes in the reproductive Dutch population will be obtained for the first time. This will provide data to predict the fetal D status also in women carrying certain variant *RHD* genes, such as the *RHD* pseudogene or the DVI variant. In these women, it might be considered to guide the antenatal immunoprophylaxis based on the fetal *RHD* screening result, but to advise cord blood serology for PCR-negative cases.

In conclusion, the results presented in this thesis indicate that cell-free fetal DNA is a reliably analyte for prenatal genetic diagnosis in everyday clinical practice. Already, invasive procedures solely to determine the fetal sex or red cell antigen status belong to the past. Large-scale nation-wide prenatal screening programmes for the prediction of the fetal Rh D blood group phenotype using cell-free fetal DNA are in place. Yet, the full diagnostic potential of cell-free fetal DNA in maternal plasma remains to be realised. Current advances in single molecule counting techniques allow for the detection and identification of DNA sequences beyond those that are paternally inherited, bringing noninvasive diagnosis and screening for a variety of genetic conditions, including chromosomal aneuploidies, to clinical use in the near future.[11]

References

1 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.

2 Devaney SA, Palomaki GE, Scott JA, Bianchi DW. Noninvasive fetal sex determination using cell-free fetal DNA: a systematic review and meta-analysis. *JAMA* 2011;306:627–36.

3 Rijnders RJP, van der Luijt RB, Peters EDJ, Goeree JK, van der Schoot CE, Ploos van Amstel JK et al. Earliest gestational age for fetal sexing in cell-free maternal plasma. *Prenat Diagn* 2003;23:1042–4.

4 Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c, and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion* 2007;47:2126–33.

5 Daniels G, van der Schoot CE, Olsson ML. Report of the fourth International Workshop on molecular blood group genotyping. *Vox Sang* 2011;101:327–32.

6 Illanes S, Soothill P. Management of red cell alloimmunisation in pregnancy: the non-invasive monitoring of the disease. *Prenat Diagn* 2010;30:668–73.

7 Fernando MR, Chen K, Norton S, Krzyzanowski G, Bourne D, Hunsley B et al. A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. *Prenat Diagn* 2010;30:418–24.

8 Zhong X, Holzgreve W. MALDI-TOF MS in Prenatal Genomics. *Transfus Med Hemother* 2009;36:263–72.

9 Kamphuis MM, Paridaans N, Porcelijn L, de Haas M, van der Schoot CE, Brand A et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. *BJOG* 2010;117:1335–43.

10 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. Effect of high throughput RHD typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study. *BMJ* 2008;336:816–8.

11 van den Oever J, Balkassmi S, Verweij E, van Iterson M, Adama van Scheltema P, Oepkes D et al. Single molecule sequencing of free DNA from maternal plasma for noninvasive trisomy 21 detection. *Clin Chem* 2012.

Nederlandse samenvatting

In dit proefschrift worden de resultaten beschreven van onderzoek naar de klinische toepassingen van cel-vrij foetaal DNA geïsoleerd uit moederlijk bloed. Cel-vrij foetaal DNA is afkomstig van apoptotische (door gereguleerde celdood gestorven) syncytiotrophoblastcellen van de placenta en kan reeds vanaf vijf weken zwangerschap worden gedetecteerd in het bloed van de zwangere vrouw. Omdat de genetische opmaak (het DNA-profiel) van de placenta in de regel een weerspiegeling is van die van de ongeboren foetus (beide ontstaan uit dezelfde bevruchte eicel), biedt cel-vrij foetaal DNA de mogelijkheid de genetische opmaak van de foetus te achterhalen door middel van een eenvoudige bloedafname bij de moeder. Deze vorm van zogenaamde *niet-invasieve* foetale genotypering – in tegenstelling tot *invasieve* foetale genotypering, zoals een vruchtwaterpunctie of vlokkentest, die elk een klein (ca. 1%) risico op een miskraam dragen – wordt echter gecompliceerd door het feit dat het cel-vrij foetaal DNA slechts in gefragmenteerde stukjes aanwezig is in de moederlijke circulatie en bovendien slechts ongeveer 5% van al het cel-vrij DNA in het moederlijke bloed betreft. De overige 95% van het cel-vrij DNA is afkomstig van de moeder zelf. Isolatie van cel-vrij DNA uit moederlijk bloed levert dus een mengsel van foetaal en maternaal DNA op. Enkel die gedetecteerde DNA-sequenties in dit mengsel waarvan bekend is dat de moeder deze zelf niet bezit, kunnen dus met zekerheid aan de ongeboren foetus worden toegeschreven. Dit zijn per definitie DNA-sequenties die de foetus van zijn vader heeft overgeërfd of mutaties die spontaan zijn ontstaan.
In de afgelopen jaren is een aanzienlijk aantal publicaties over de niet-invasieve foetale genotypering van paternaal overgeërfde allelen (een allel: elk van de verschillende vormen van een gen) verschenen. Echter, de meeste resultaten werden gegenereerd in onderzoekslaboratoria en het was onbekend hoe deze tests in de dagelijkse klinische praktijk presteerden. In dit proefschrift onderzochten wij de *performance* van de niet-invasieve foetale genotypering van paternaal overgeërfde allelen in de dagelijkse klinische praktijk.

In **hoofdstuk 1** worden de biologische karakteristieken van cel-vrij foetaal DNA beschreven en passeren enkele (potentiële) klinische toepassingen de revue.

In **hoofdstuk 2** wordt de diagnostische nauwkeurigheid van de niet-invasieve foetale geslachtstypering beschreven. Wij analyseerden de testresultaten van alle opeenvolgende zwangere vrouwen bij wie een niet-invasieve foetale geslachtstypering werd verricht in ons laboratorium gedurende een periode van zes jaar. De test werd alléén verricht op medische indicatie en enkel op aanvraag van een klinisch geneticus of gynaecoloog. Cel-vrij DNA geïsoleerd uit maternaal plasma werd getest op twee Y-chromosomale markers, *SRY* en DYS14, middels een real-time polymerasekettingreactie (PCR). Slechts indien een positief PCR-resultaat voor beide markers werd gevonden, rapporteerden wij de zwangerschap van een jongetje. Wanneer het PCR-resultaat voor beide markers negatief was, werd met

andere paternaal overgeërfde genetische markers de aanwezigheid van foetaal DNA in het bloedmonster vastgesteld. Alléén wanneer de aanwezigheid van foetaal DNA kon worden bevestigd, rapporteerden wij de zwangerschap van een meisje. Alle testuitslagen werden vergeleken met het geslacht van het kind na de geboorte of tijdens echografie later in de zwangerschap.
In totaal werden 201 vrouwen getest. Het merendeel van de tests werd verricht vanwege dragerschap van een X-gebonden aandoening (waarbij enkel jongetjes aangedaan kunnen zijn). De mediane zwangerschapduur ten tijde van de test was 9+0/7 weken (interkwartiel 8+0/7 tot 10+0/7 weken). In 94% van de gevallen (189 van 201) kon een testresultaat worden verkregen; in tien gevallen kon de aanwezigheid van foetaal DNA niet worden bevestigd en in twee gevallen duidden de PCR-resultaten op een miskraam. In 105 gevallen werd de geboorte van een jongetje teruggerapporteerd. In al deze gevallen waren de PCR-resultaten voor *SRY* en DYS14 positief. In 81 gevallen werd de geboorte van een meisje teruggerapporteerd. In al deze gevallen waren de PCR-resultaten voor *SRY* en DYS14 negatief (inclusief de tien gevallen waarin de aanwezigheid van foetaal DNA in het monster niet kon worden bevestigd). De sensitiviteit en specificiteit van de test was 100% (met respectievelijke 95%-betrouwbaarheidsintervallen van 96,6–100% en 95,6–100%). Wij concludeerden dat niet-invasieve foetale geslachtstypering in maternaal plasma zeer betrouwbaar is en klinisch toepasbaar.

In **hoofdstuk 3** wordt de diagnostische nauwkeurigheid van de niet-invasieve foetale bloedgroepgenotypering beschreven. Het vaststellen van de foetale bloedgroepantigeenstatus tijdens de zwangerschap is relevant indien de zwangere vrouw antistoffen tegen het desbetreffende bloedgroepantigeen draagt. Wij analyseerden de testresultaten van alle opeenvolgende zwangere vrouwen bij wie een niet-invasieve foetale bloedgroepgenotypering werd verricht in ons laboratorium gedurende een periode van zeven jaar. De tests werden verricht voor de D-, c- en E-antigenen van het rhesusbloedgroepsysteem en voor het K-antigeen van het Kellbloedgroepsysteem. Cel-vrij DNA geïsoleerd uit maternaal plasma werd middels een voor elk van de afzonderlijke bloedgroepantigenen ontwikkelde real-time PCR geanalyseerd. In het geval van een negatief PCR-resultaat voor het desbetreffende bloedgroepantigeen, werd met de Y-chromosomale marker *SRY* (enkel toepasbaar in zwangerschappen met een mannelijke foetus) of met andere paternaal overgeërfde genetische markers de aanwezigheid van foetaal DNA in het bloedmonster vastgesteld. Alle testuitslagen werden vergeleken met de navelstrengserologie na de bevalling.
In totaal werden 362 tests verricht (D: $n = 168$; c: $n = 49$; E: $n = 85$; K: $n = 60$). De mediane zwangerschapsduur was 17 weken (variatiebreedte 7 tot 38 weken). In 97% van de gevallen (351 van 362) kon een testresultaat worden verkregen; in zeven gevallen kon de aanwezigheid

van foetaal DNA niet worden bevestigd, in twee gevallen leidde aspecifieke amplificatie in de *K*-PCR tot een ongeldig resultaat en in twee gevallen werd in de PCR voor rhesus D een moederlijke rhesus D DNA-sequentie geamplificeerd. Navelstrengserologie werd verkregen in 212 gevallen; er werden geen fout-positieve of fout-negatieve testresultaten gevonden. Wij concludeerden dat niet-invasieve foetale bloedgroepgenotypering nauwkeurig is en toepasbaar in de klinisch-diagnostische praktijk.

De ontwikkeling van een niet-invasieve foetale genotyperingstest voor het humane bloedplaatjesantigeen-1a (HPA-1a) wordt beschreven in **hoofdstuk 4**. Het vaststellen van de foetale HPA-1a-status tijdens de zwangerschap is relevant indien de zwangere vrouw antistoffen tegen HPA-1a draagt. In dat geval kunnen die antistoffen leiden tot afbraak van foetale HPA-1a-positieve bloedplaatjes met ernstige complicaties zoals hersenbloedingen tot gevolg. De ontwikkeling van een niet-invasieve foetale HPA-1a-genotyperingstest wordt bemoeilijkt door de overweldigende achtergrond van moederlijk DNA en het gegeven dat het HPA-1b-allel (waar de zwangere vrouw homozygoot voor is) slecht één enkele nucleotide verschilt van het HPA-1a-allel. Dit leidt tot aspecifieke amplificatie in de real-time PCR. Ten einde aspecifieke amplificatie van het (maternale) HPA-1b-allel te voorkomen maakten wij gebruik van een enzym (*Msp1*) dat specifiek de DNA-sequentie van het HPA-1b-allel "knipt", maar het HPA-1a-allel ongemoeid laat. Wij toonden aan dat pre-PCR enzymdigestie van cel-vrij DNA met *Msp1* aspecifieke amplificatie voorkwam in de bloedmonsters van zes HPA-1a-negatieve vrouwen die zwanger waren van een HPA-1a-negatieve foetus, terwijl in alle bloedmonsters van 28 HPA-1a-negatieve vrouwen die zwanger waren van een HPA-1a-positieve foetus positieve PCR-resultaten werden verkregen.

Hoofdstuk 5 geeft een overzicht van de verschillende positieve controles voor niet-invasieve foetale genotyperingstests die gebaseerd zijn op de detectie van paternaal overgeërfde allelen. Omdat een negatief PCR resultaat geen garantie geeft dat er daadwerkelijk cel-vrij foetaal DNA aanwezig is in het detectiesysteem, moet een dergelijke uitslag met zorg worden geïnterpreteerd. Niet-invasieve foetale genotyperingstests zijn over het algemeen robuust gebleken en verschillende laboratoria kiezen ervoor bij een negatief PCR-resultaat geen controletest voor de aanwezigheid van foetaal DNA te verrichten. Wij concludeerden dat wanneer een niet-invasieve foetale bloedgroepgenotypering wordt verricht vanwege de aanwezigheid van antistoffen bij de zwangere vrouw de aanwezigheid van foetaal DNA in het bloedmonster altijd moet worden bevestigd. Indien de foetale bloedgroepgenotypering wordt verricht in het kader van *screening* kan het overwogen worden een controle voor de aanwezigheid van foetaal DNA achterwege te laten.

Een analyse van de eerste drie maanden van het landelijke foetale *RHD* screeningsprogramma

wordt beschreven in **hoofdstuk 6**. Dit programma heeft tot doel een gerichter anti-D-immunoprophylaxebeleid te kunnen voeren, waarbij alleen die (rhesus) D-negatieve zwangere vrouwen die een D-positief kind dragen ante- en postnataal anti-D toegediend krijgen, in plaats van alle D-negatieve zwangere vrouwen. Wij analyseerden de testresultaten van alle D-negatieve zwangere vrouwen bij wie in de 27ste week van de zwangerschap een foetale *RHD* screening werd verricht in maternaal plasma en van wie wij postnataal navelstrengbloed ontvingen ter bepaling van het foetale phenotype. Slechts in drie van de 5164 gevallen (0.06%) werd een fout-negatief screeningsresultaat gevonden. Deze vrouwen ontvingen onterecht antenataal geen anti-D. Anderzijds werden bij navelstrengserologie 11 fout-negatieve resultaten gevonden; vrouwen die zonder het screeningsprogramma postpartum anti-D onterecht onthouden zou zijn. Uit deze interim-analyse concludeerden wij dat het foetale *RHD* screeningsprogramma veilig is en dat in deze eerste drie maanden toediening van anti-D bij in ieder geval 2017 vrouwen terecht is voorkomen.

De resultaten van de onderzoeken beschreven in dit proefschrift laten zien dat niet-invasieve foetale genotypering van paternaal overgeërfde allelen zeer betrouwbaar is in de alledaagse klinisch-diagnostische praktijk. Invasieve procedures enkel om het foetale geslacht of bloedgroep te bepalen behoren inmiddels tot het verleden. Foetale rhesus D screeningsprogramma's zijn de realiteit. Cel-vrij foetaal DNA heeft de toekomst.

Dankwoord

Op de omslag van dit proefschrift staat slechts één naam. Dit proefschrift was echter niet tot stand gekomen zonder de hulp en inspanningen van velen. Veel dank ben ik verschuldigd aan allen die direct of indirect hebben bijgedragen aan dit proefschrift, in het bijzonder:

Prof. dr. C.E. van der Schoot, beste Ellen. Ik had mij geen prettiger promotor kunnen wensen! Je slagvaardige begeleiding leidde in *no-time* tot de publicatie van de eerste artikelen en de afronding van mijn proefschrift binnen drie jaar. Ik heb genoten van onze samenwerking, waarin ook altijd ruimte was voor niet-werkgerelateerde zaken en die hopelijk ook na afronding mijn promotie nog lang zal voortduren. Heel veel dank.

Prof. dr. G.H.A. Visser, beste Gerard. Jij schiep het kader waarbinnen ik mijn promotieonderzoek kon verrichten. Het vertrouwen dat je in mij – als onervaren onderzoeker met slechts een vleugje *kliniek* – stelde, heb ik als zeer waardevol ervaren. Jouw immer positieve opvattingen over het project tijdens onze sporadische ontmoetingen waren een steun in de rug en een extra stimulans de klus in drie jaar te klaren. Veel dank.

Dr. M. de Haas, beste Masja. Nog voordat ik officieel was aangenomen, ontving ik al een mailtje van je waarin je mij een werkplek met computer bij jou op de kamer aanbood. Méér welkom kan een beginnende promovendus zich niet voelen! Wanneer ik (wetenschappelijk) weer eens vastliep, trok jij mij met één of twee – al dan niet geschreven – zinnen weer vlot. Wat heb ik veel van je geleerd. Ontzettend bedankt.

Dr. G.C.M.L. Page-Christiaens, beste Lieve. Dat ik straks een gepromoveerde arts ben, heb ik aan jou te danken. Enigszins zoekende na mijn eerste baan als arts-assistent gynaecologie/verloskunde betrok je mij bij dit project en samen gingen we bij de hoogleraren langs. Heel veel dank voor je niet-aflatende inspanningen, steun en begeleiding de afgelopen jaren. Woorden schieten te kort.

Prof. dr. E.P.J.G. Cuppen, prof. dr. A. Franx en prof. dr. V.V.A.M. Knoers wil ik bedanken voor het zitting willen nemen in de beoordelingscommissie.
Prof. D.W. Bianchi, thank you very much for you willingness to serve as a member on the assessment committee. I am honoured by your presence at the public defence of this thesis.

Prof. dr. D. Oepkes, beste Dick. Niet enkel dank voor je bereidheid zitting te willen nemen in de beoordelingscommissie, maar ook dank voor de prettige samenwerking die geleid heeft tot hoofdstuk 4 van dit proefschrift. Jouw idee om het als abstract voor de *SGI* in te sturen was een schot in de roos! De ontwikkelingen rond *NITRO* zal ik op de voet blijven volgen.

Aicha en Bernadette, zonder jullie had ik het op het lab nooit gered. Jullie brachten mij de fijne kneepjes van de plasma-DNA-isolatie en real-time PCR bij en eenmaal zelfstandig in het lab kon ik altijd op jullie terugvallen. Veel dank dat jullie van deze dokter ook een klein beetje laborant hebben gemaakt.

Lonneke, superkamergenote, wat hebben we een plezier gehad! Dank voor heel veel mooie momenten met een lach en soms een spreekwoordelijke traan. Natuurlijk sta jij straks als paranimf naast mij!

Fellow PhD students from IHE (old and new): Annemieke, Esther, Helga, Kim, Lussy, Marijke, Sabrina, Sofieke, Rick, Tamara and Janine, thanks for being such nice colleagues. I could not have wished for better ones! Marion, Franca, Suilan, Remco en Arthur, onmisbaar voor de gezelligheid. *Newbies* from HEP, thank you for making our wing an even warmer nest (while adding to the science!).

Gestur, Onno en Emile, dank voor de enerverende lunches en (pseudo-)wetenschappelijke discussies. Ik ben vaak aan het denken gezet. Carlijn en Daphne, altijd geïnteresseerd. Dank daarvoor. Barbera, dank voor al je hulp op het post-PCR-lab en gezelligheid op de kamer.

Florentine, zonder jou geen hoofdstuk 6! Wat was het fijn dat je ons kwam versterken de laatste maanden. En wat leuk dat je nu – voorlopig – het stokje overneemt! Bedankt!

Collega's van Immunocytologie, dank voor jullie hulp en gezelligheid op het lab. Lily in het bijzonder.

Anita en Mo, geen vraag was jullie teveel, geen minuut duurde jullie te lang. Heel veel dank.

Collega's van Erytrocytenserologie, zeer bedankt voor al het administratieve uitzoekwerk. Dit proefschrift is ook jullie werk. Daan, doen we nog eens een rondje Loosdrecht?

Rachel. Hong Kong. *Need I say more?* Dank dat ook jij mijn tijd bij Sanquin zo gezellig maakte.

Claudia Folman en Hans Zaaijer dank ik als leden van mijn OIO-begeleidingscommissie.

Oud-collega's en gynaecologen uit het Diakonessenhuis in Utrecht wil ik ook graag bedanken. Bij jullie werd mijn keuze voor de gynaecologie en verloskunde definitief, al had ik dat zelf nog niet zo één-twee-drie door… Maarten en Nico, dank voor jullie destijds buitengewone interesse.

Spelers en staf van de VSC-zaterdagselectie, bedankt voor vele jaren ultieme ontspanning: trappen tegen een bal. En een goede derde helft…

Bram, Daan, David, Floris, Joris, Marnix, Ralph, Rik, Robbie, Shu-ta, Teun en Thomas: op weekend, kerstavond, wintersport of lustrum, met jullie speel ik altijd een *flawless victory*!

Raphaël, bedankt voor de koffie en goede discussies (heb jij er ooit één gewonnen?). *No span!*

Chris, Ingrid, Judith en Sjoerd. Al zien we elkaar weinig, ik koester onze (geneeskundige) band. Dank voor jullie aanhoudende interesse.

Micha (buur *for life!*), Gerben en Signe, uit het oog is niet altijd uit het hart. Rosalie, doen we nog eens een *waltz*?

Ofke, lieve *McB*, wat kan ik van onze vriendschap genieten! (wij zijn dan ook beiden Iers...). Hoe te gaaf dat jij straks naast mij staat. Met jou als paranimf wordt het sowieso top! Dank je wel.

Wouter en Gerdy, Margot en Simone, de *koude kant* is allang niet koud meer. Dank voor jullie warm onthaal.

Lieve familie in Hilversum en Amsterdam, waarbij het zo fijn "thuis" komen is, *merci pour tout*. Laura, Daphne en Axel, lieve nichtjes en neef, blijf origineel!

Lieve zus, Hester, allang geen klein zusje meer. Wat ben ik trots op jou! Voorlopig geen logeerpartijen op de Schinkelkade meer; dan moet onze razendsnelle humor maar per sms…

Lieve ouders, aan wie ik zovéél te danken heb. Jullie hebben mij altijd gestimuleerd mezelf te blijven ontwikkelen, waardoor ik ben wie ik nu ben. Dank voor jullie onvoorwaardelijke liefde en steun.

Emily, *lief*, met jou samen is alles makkelijk! Vast ook ons volgende hoofdstuk…

Curriculum Vitae

Peter Gerhard Scheffer werd op zondag 15 februari 1981 geboren in Utrecht waar hij opgroeide als oudste in een gezin van twee kinderen. Na het behalen van zijn gymnasiumdiploma aan het St. Bonifatiuscollege in Utrecht reisde hij gedurende een jaar door Australië, Nieuw-Zeeland en Indonesië. In het hieropvolgende jaar behaalde hij zijn propedeuse rechtsgeleerdheid aan de Universiteit Utrecht. In 2001 werd hij ingeloot voor de studie geneeskunde aan diezelfde universiteit. Naast zijn reguliere co-schappen liep hij onder andere keuzeco-schappen perinatologie en medische genetica en een co-schap heelkunde in Cork, Ierland.
In 2007 behaalde hij zijn artsenbul waarna hij gedurende ruim een jaar als arts-assistent niet-in opleiding tot specialist op de afdeling Gynaecologie/Verloskunde van het Diakonessenhuis in Utrecht werkte (opleider dr. P.C. Scholten). In maart 2009 begon hij met promotieonderzoek op de afdeling Experimentele Immunohaematologie van Sanquin Research Amsterdam onder begeleiding van prof. dr. C.E. van der Schoot en prof. dr. G.H.A. Visser. De resultaten van dit onderzoek worden beschreven in dit proefschrift.
In maart 2012 is hij begonnen met de opleiding tot gynaecoloog in het St. Elisabeth Ziekenhuis in Tilburg (opleider dr. P.F. Boekkooi).