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NEW STRATEGIES TO PREVENT FETAL AND NEONATAL COMPLICATIONS IN RHESUS D IMMUNIZATION

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To my maternal grandmother Karin who gave life to nine living children
and to my paternal grandmother Ulla who was the thirteenth living child of
my great grandmother Emilia. They were all hardworking women.

ABSTRACT

The general purpose of this thesis was to investigate if fetal and neonatal complications due to RhD immunization in the mother could be prevented by 1) reducing procedure-related complications in intrauterine blood transfusions and by 2) reducing the incidence of RhD immunization by providing routine antenatal anti-D prophylaxis during pregnancy selectively to non-immunized RhD negative women with RhD positive fetuses.

Paper I was a retrospective study including 284 intrauterine transfusions in 84 women 1990-2010. Perinatal survival was 91.8 %. Complications occurred in 4.9 % of procedures of which 1.4 % were fatal. Procedure-related complications were significantly more common when transfusions were performed in a free loop of the umbilical cord compared to the intrahepatic part of the umbilical vein (OR 5.4, 95% CI: 1.2 to 23.7, P=0.025). There was no significant difference between the intrahepatic route and the placental cord insertion (P=0.83).

Paper II was a pharmacokinetic study on 16 women measuring plasma concentrations of anti-D IgG at predefined time points after administration in gestational weeks 28-30. The half-life was in median 23 days (12.5 - 30.3). At ten weeks after injection, plasma concentrations ranging from 1-4 ng/mL were found in all samples available for analysis. We estimated that 75 % of women would have had detectable anti-D IgG concentrations ≥1 ng/mL at the time of delivery.

In paper III we performed a large prospective cohort study on the diagnostic accuracy of a single-exon noninvasive method to determine fetal *RHD* genotype in the first trimester of pregnancy. Plasma samples from 4118 pregnancies were included in the analysis. Median gestational age for blood sampling was 10 weeks. From eight gestational weeks, sensitivity was 98.9 % (95% CI 98.3 - 99.3) and specificity 98.9 % (95% CI 98.1 - 99.4). From 10 weeks of gestation sensitivity was 99.3 % and from 22 weeks 100 %.

Paper IV was a retrospective study on all (290) RhD immunized pregnant women in Stockholm 1990-2008. Fifty-one % (147/290) of the women were sensitized with their first-born child and 33 % (96/290) with their second born child. At least half of the

women were immunized in the third trimester, which could possibly have been prevented with antenatal prophylaxis. Fifty-six % (144/259) of the neonates in subsequent pregnancies required treatment for hemolytic disease, independently of in which order or pregnancy the women were immunized.

In paper V we performed a prospective cohort study offering anti-D prophylaxis in gestational week 28-30 selectively to all RhD negative pregnant women in Stockholm with an *RHD* positive fetus. Selective prophylaxis was provided in 4590 pregnancies resulting in an incidence of RhD immunization in the study cohort of 0.21 percent (95% CI 0.12 - 0.31) (20/9380). The reference cohort consisted of all RhD pregnant women giving birth in the same region 2004-2008 and the incidence in this group was 0.46 percent (95% CI 0.37 - 0.56) (86/18.546). The risk ratio (RR) for sensitization was 0.46 (95% CI 0.28 - 0.75) with the new program.

This thesis shows that without routine antenatal anti-D prophylaxis, the majority of women become RhD immunized during pregnancy with their first or second child. The risk of hemolytic disease of the fetus and newborn is the same regardless of in which order of pregnancy a woman become immunized and occurs in more than half of subsequent pregnancies. Non-invasive fetal *RHD* genotyping in the first trimester of pregnancy can be performed with high accuracy and enables administration of routine antenatal anti-D prophylaxis (RAADP) selectively to RhD negative women with *RHD* positive fetuses. This reduces the risk of RhD immunization to 0.21 percent. RAADP usually lasts for ten weeks after injection but thereafter concentrations are variable and not all women will have detectable anti-D levels at term and post-term, which might be a cause of residual immunizations. Perinatal survival in pregnancies requiring intrauterine blood transfusion is high, but the risk of procedure-related complications can be further reduced by applying a safer technique and with timely referrals to a specialized center before severe anemia develops.

LIST OF PUBLICATIONS

I. Tiblad E, Kublickas M, Ajne G, Bui TH, Ek S, Karlsson A, Wikman A and Westgren M.
Procedure-related complications and perinatal outcome after intrauterine transfusions in red cell alloimmunization in Stockholm.
Fetal Diagn Ther 2011;30:266-273

II. Tiblad E, Wikman A, Rane A, Jansson Y and Westgren M.
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Acta Obstet Gynecol Scand 2012;91:587-592

III. Taune Wikman A, Tiblad E, Karlsson A, Olsson ML, Westgren M and Reilly M.
Noninvasive single-exon fetal *RHD* determination in a routine screening program in early pregnancy.
Obstet Gynecol 2012;120: 227-34

IV. Tiblad E, Westgren M, Karlsson A and Wikman A.
Consequences of being Rhesus D immunized during pregnancy.
Submitted

V. Tiblad E, Wikman A, Ajne G, Blanck A, Jansson Y, Karlsson A, Nordlander E, Shassti Holländer B and Westgren M.
Targeted routine antenatal anti-D prophylaxis in the prevention of RhD immunization – outcome of a new antenatal screening and prevention program.
Manuscript

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LIST OF ABBREVIATIONS

HDFN	Hemolytic disease of the fetus and newborn
RBC	Red blood cells
RhD	Rhesus D
IgG	Immunoglobulin G
MPS	Mononuclear phagocyte system
FMH	Fetomaternal haemorrhage
IgM	Immunoglobulin M
APC	Antigen presenting cell
IAT	Indirect antiglobulin titer
PSV	Peak systolic velocity
MCA	Middle cerebral artery
MoM	Multiples of the median
DAT	Direct agglutination test
MCV	Mean cell volume
IUT	Intrauterine blood transfusion
PPROM	Preterm premature rupture of membranes
AMIS	Antibody mediated immune suppression
RAADP	Routine antenatal anti-D prophylaxis
TOP	Termination of pregnancy
Cl	Clearance
Vd	Distribution volume
T½	Half-life
PCR	Polymerase chain reaction
Cff DNA	Cell-free fetal DNA
FITC	Fluorescein isothiocyanate

LIS	Laboratory information system
C_{max}	Maximal plasma concentration
Cl_{ast}	Last measured plasma concentration
NNT	Number needed to treat
GA	Gestational age
LMP	Last menstrual period
NGS	Next generation sequencing

1 INTRODUCTION

Hemolytic disease of the fetus and newborn (HDFN) was until the 1960s an important cause of perinatal morbidity and mortality. Today it is a rare, but potentially severe, complication of pregnancy. The most common cause of HDFN is maternal immunization against the red blood cell (RBC) Rhesus D (RhD) antigen. There are many blood group systems with a variety of RBC antigens and among these the RhD antigen is the most immunogenic, i.e. most capable of inducing an antibody response in individuals lacking the antigen. When an RhD negative pregnant woman (lacking the RhD antigen on her RBCs) is exposed to RhD positive (carrying the RhD antigen on the RBCs) fetal red blood cells during pregnancy or delivery this may induce an immune response and the production of anti-D immunoglobulin G (IgG) antibodies in the mother. IgG antibodies cross the placenta from the maternal blood circulation into the fetal blood circulation in the current and all subsequent pregnancies. The binding of these anti-D antibodies to the RhD antigen on the surface of the fetal RBCs will lead to their destruction in the mononuclear phagocyte system (MPS) [1]. This hemolysis can cause fetal and neonatal anemia and increased bilirubin levels in the newborn. In severe cases, this may lead to fetal death due to severe anemia resulting in heart failure and hydrops fetalis. The newborn is at risk for induced prematurity, anemia, cholestasis and hyperbilirubinemia. Severe hyperbilirubinemia may lead to neurological sequele due to deposition of unconjugated bilirubin in brain tissue (kernicterus) if not recognized and adequately treated. Postnatal management includes intense phototherapy and exchange transfusion to reduce hyperbilirubinemia and blood transfusion to treat anemia [2]. These pregnancies demand large resources in terms of monitoring, prenatal therapy and neonatal intensive care.

The prevention and treatment of RhD immunization and HDFN has constituted one of the major achievements of obstetric and neonatal medicine and led to a large reduction in perinatal mortality and neonatal disease. The elucidation of the pathophysiology of the disease and discovery of anti-D prophylaxis, improvement of neonatal care including exchange transfusion and care of premature babies, introduction of fetal blood sampling to asses fetal anemia and antenatal blood transfusions to the fetus and lately non-invasive monitoring of fetal anemia, have all been important factors for the

success. Challenges still remaining are improving the safety of intrauterine blood transfusions, preventing iatrogenic prematurity and further reducing the incidence of RhD immunization.

2 BACKGROUND

2.1 EPIDEMIOLOGY OF RHESUS D IMMUNIZATION IN PREGNANCY AND AFTER DELIVERY

An RhD negative blood group is most common in Caucasian populations (15%) and more rare in African (5-7%) and Asian populations (0-2%) [3]. The prevalence of RhD negative women in Sweden is reported to be 14.6 percent [4]. The majority of RhD immunizations occur as a consequence to fetomaternal haemorrhage during delivery of an RhD positive child. Before the introduction of postnatal anti-D prophylaxis in the 1960s, about 17 percent of RhD negative women delivering an ABO compatible RhD positive baby became RhD immunized during pregnancy or after delivery. Antibodies could be detected within six months after delivery in one half of the women and in the beginning of the subsequent pregnancy in the other half. The corresponding frequency in RhD negative women delivering ABO incompatible RhD positive babies was 2 percent, resulting in an overall risk of 13.2 percent of RhD immunization in RhD negative women delivering RhD positive babies[5]. About 10-20 percent of immunized primigravidas have developed anti-D antibodies already at the time of delivery[6 7].

The prevalence of RhD immunization in the population of Canada was 10/1000 births before 1965[8]. It is likely that the incidence in Sweden was similar. Administration of anti-D after delivery of an RhD positive child, together with anti-D prophylaxis during pregnancy for events that can cause fetomaternal haemorrhage (FMH), reduced the incidence of immunization in RhD negative women worldwide to approximately 1-2 percent [9 10]. Over the last 30 years, the prevalence of RhD immunization in Sweden has been reported to be about 1 percent[4 11 12]. Residual immunization occurs due to failure of administration of prophylaxis at risk events during pregnancy or after delivery. In a few cases, the FMH at delivery will be greater than covered by the standard dose of anti-D IgG provided. However, the most common reason for residual immunization is silent FMH during pregnancy, most often in the third trimester [13].

It is difficult to assess the incidence of perinatal mortality due to red cell immunization. It has been estimated that perinatal mortality in the UK was reduced from 46/100.000 births before 1969 to 5-6/100.000 births in 2004[14 15]. In France, RhD immunization has

been reported to cause perinatal death in 2-5/100.00 births[9]. In Sweden the incidence of perinatal mortality due to RhD immunization and HDFN is unknown.

2.2 PATHOPHYSIOLOGY AND IMMUNOLOGICAL MECHANISMS

The Rh blood group system is the most complex known. Genes for the Rh blood group system are inherited from both parents and are co-dominantly expressed in the offspring. Most RhD negative individuals in all populations have a homozygous deletion of the *RHD* gene, but there are many *RHD* gene variants and the prevalence differs between different ethnic populations [16-18]. Some of these variant genes do not produce an immunogenic RBC RhD antigen while others do. In a large study on multiethnic obstetric and transfusion patients, the prevalence of mutated *RHD* alleles was 0.95 percent [19]. If the fetus inherits the *RHD* gene from the father (approximately 60% do) RhD immunization can occur due to fetomaternal haemorrhage with passage of fetal red blood cells into the maternal circulation during pregnancy or after delivery.

Figure 1. The Rhesus gene and the most common variants.
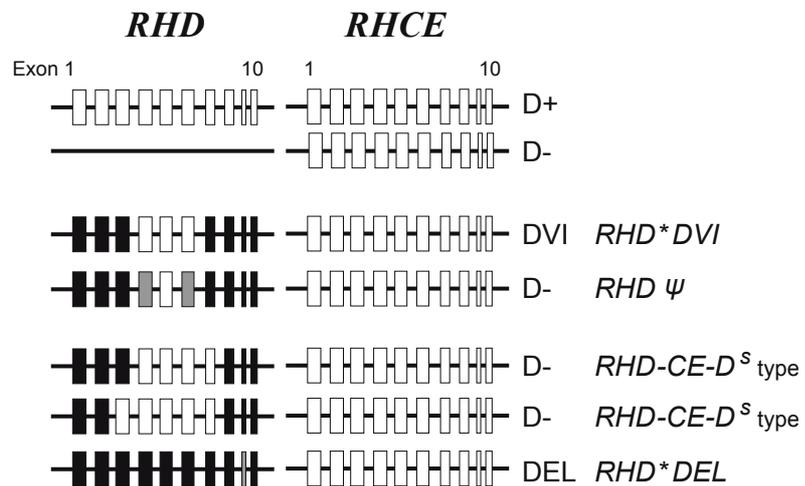
Black: RHD exons; white: RHCE exons; gray: mutated exons.

Size and frequency of fetomaternal hemorrhage, ABO compatibility and individual responsiveness of the immune system all matters whether an RhD negative woman will become sensitized or not. It is estimated that a minimum volume of 0.1-0.5 ml fetal blood is required to induce a primary immune response. Thereafter it is enough with very small booster volumes (<0.1 ml) of FMH to stimulate the production of antibodies[20-22]. A large hemorrhage can be enough to stimulate both a primary and secondary response. In some individuals it is probably enough with very small repeated hemorrhages to induce a primary and secondary immune response [23-25]. The RhD antigen is well developed by 7 weeks gestation[26]. FMH in the first trimester can occur and likely cause sensitization in some women [13]. Twenty to 30 percent of individuals are non-responders, which means that exposure to even large volumes of RhD positive RBCs are not equal to sensitization [23 24]. In addition, ABO incompatibility (about 20% of pregnancies) provides partial protection since fetal RBCs getting into the maternal circulation will be rapidly cleared by pre-existing IgM antibodies to the ABO system, before the maternal immune system will recognize the foreign RhD antigen. ABO incompatibility protects against a primary, but not a secondary response [25].

It has been shown that, after infusion of RhD positive RBCs into RhD negative volunteers, it takes 1 to 5 months before detectable anti-D antibodies appear in the circulation even when large volumes of packed RBCs are infused (500ml) [23 27]. This is explained by the fact that macrophages do not have receptors for blood group antigens and the life span of red blood cells are 90 to 120 days [28]. In the absence of red cell antibodies, foreign RBC will circulate until they become apoptotic or senescent. They will then be removed in the spleen and if enough RhD peptides are processed and presented by antigen-presenting cells (APC) this can cause an inflammatory immune response [29]. The APCs activate RhD specific helper T cells, which in turn will stimulate RhD specific B cells to proliferate and differentiate into antibody secreting plasma cells. Following an immune response, immunologic memory is established (memory T and B cells) and the response to next exposure will be rapid with antibodies increasing within a week [3 30]. The antigen density on the RBCs plays a role and some types of RhD positive red blood cells (cDE antigens) are known to be more immunogenic than others. It has also been shown experimentally that the levels of anti-D in immunized individuals correlate with the number of RhD peptides that will stimulate T-cell proliferation [31]. IgG antibodies produced are cross the placenta to the fetal circulation and bind to the RhD epitopes on fetal RBCs, causing their destruction in the fetal MPS

5

system, mostly in the spleen. IgG is actively transported over the placental interface. Very small amounts are found in the fetus in the first trimester, but thereafter the levels increase constantly with the most rapid increase in the third trimester. At term, the concentrations of IgG in general is higher in cord blood that in maternal blood [32].

2.2.1 Fetomaternal hemorrhage during pregnancy and delivery

The phenomenon of fetomaternal hemorhage has been known for a long time [33-35]. Small amounts of fetal red blood cells pass into the maternal circulation as part of normal physiology during pregnancy and at delivery. The size and frequency of these small fetomaternal transfusions or hemorrhages increase with gestation and are the greatest at delivery. Explanations for this have been proposed to be the increasing placental surface and increase of uterine and fetal activity [36]. In addition, the fetoplacental blood volume during the third trimester and at term is much larger than in the first and second trimesters [37]. Detectable FMH has been reported to occur in 3-54 percent of pregnancies in the first trimester, 12-63 percent in the second trimester and in 40-70 percent in the third trimester [25 38-40]. The volumes are usually small, 0.01–0.6 ml, and do not affect the fetus.

Fetomaternal haemorrhage can also be provoked. Reported risk factors vary between studies and are in summary: twin pregnancies, external cephalic version, severe abdominal trauma, manual removal of the placenta, placental abruption, preeclampsia, placental tumors, amniocentesis, stillbirth and cesarean section [28 41-48]. Large FMH > 30 ml of fetal blood is rare and the incidence is approximately 3/3.000 births [28]. In 55-80% of cases of large hemorrhage a clinical explanation is missing and the majority occurs before labor [28 49]. The pathophysiology of large hemorrhages is likely different from small ones occurring in normal pregnancies and remains unknown.

It is difficult to accurately determine the amount and frequency of FMH. Different studies report varying results depending on the size of the study sample, the frequency of testing, laboratory methods used and formulas applied for the calculation. Most studies have used the Kleihauer-Betke test, which is known to suffer from poor reproducibility and insufficient sensitivity and specificity [50-52]. Flow cytometry for quantification of fetal RBCs has been shown to be more accurate, efficient and less operator dependent. A large study summarizing data on FMH after delivery in 20.000

pregnancies found that in 96 percent of women the FMH was less than 1 ml fetal blood and in 98 percent less than 2 ml [28]. A recent prospective study evaluating FMH using flow cytometry after delivery in normal vaginal delivery and cesarean section in 3457 women found that in 99.7 percent of women the FMH at delivery was ≤ 10 ml fetal blood [53].

2.3 MANAGEMENT OF RHD IMMUNIZED PREGNANCIES

The goal in managing red cell immunized pregnancies is to be able to accurately predict fetal anemia and optimize timing of invasive interventions and delivery. All pregnant women should undergo testing for blood group typing and red cell antibodies at the booking visit. A second antibody screen is most often performed in pregnancy week 25-35, at least in RhD negative women. In women found to be RhD immunized, blood group serology is performed on the father of the baby and estimation of heterozygosity (55%) or homozygosity if he is RhD positive. If he is heterozygous there is a 50 percent chance that the fetus has inherited the paternal gene and is RhD positive as well. In these cases, noninvasive diagnosis of fetal *RHD* genotype from maternal plasma should be carried out if available [54-56]. Invasive prenatal testing for *RHD* genotype is not to recommend due to the risk of both fetal loss and of boosting the antibody production in the mother by provoking a FMH [56,57]. If the fetus is RhD negative there is no risk of hemolytic disease. If the fetus is RhD positive close monitoring of the pregnancy is necessary.

The risk assessment in each immunized pregnancy is based on the individual obstetric history and previous pregnancies affected by HDFN. Maternal titer of anti-D antibodies measured by the indirect antiglobulin titer (IAT) method is the first step in evaluating the degree of immunization. Titers are followed every two to four weeks depending on the levels and an increase of two titersteps is regarded as significant. Titer results can vary between laboratories and hence each laboratory has to assess their own cut-off value for when further investigations are recommended [58]. Titers have to be interpreted with caution since there is a considerable overlap between the antibody titers in women whose fetuses are severely affected and only mildly affected, especially after the first immunized pregnancy [59,60]. Flow cytometry quantitation of anti-D antibody concentration in addition to titers increases the predictive value for severity of HDFN compared to titers only [60,61]. Anti-D concentration < 4 IU/ml or < 0.7 μg/ml (5 IU = 1

μg) is rarely associated with severe HDFN, whereas concentration > 3 μg/ml is interpreted as high risk of severe hemolytic disease[1][62].

When titers and anti-D concentrations indicate risk of fetal anemia Doppler interrogation of the peak systolic velocity (PSV) in the fetal middle cerebral artery (MCA) is useful. Mari and coworkers published a convincing study on the good predictive value of MCA PSV[63]. Using this method, fetal anemia can be diagnosed in a noninvasive and risk-free way and this method has become the gold standard in the evaluation of fetuses at risk of HDFN [64]. MCA PSV measurement is reliable in gestational age 18 to 35 weeks and should be repeated every one to two weeks when fetal anemia is suspected [65]. Peak systolic velocities above 1.5 multiples of the median (MoM) indicate moderate to severe fetal anemia with a false positive rate of 12 percent. Results < 1.5 MoM are considered reassuring but measurements are operator dependent and have to be performed in a proper way. When MCA PSV Doppler indicate fetal anemia, this can be confirmed with ultrasound-guided cordocentesis. Fetal blood sampling allows assessment of fetal hematocrit and hemoglobin, blood group serology, direct agglutination test (DAT), mean cell volume (MCV), reticulocyte count and bilirubin. Since the procedure is associated with a risk of fetal demise in 1-2 percent it should only be performed when there is a high suspicion of clinical significant fetal anemia and donor blood should be available for transfusion at the same procedure if necessary [65][66].

Timing of delivery depends on severity of immunization. In RhD immunization induction of labor is usually advised in 37-38 weeks of gestation [56][65][67]. Prematurity should be avoided if possible, since this adds to the neonatal morbidity and need for neonatal care. Prematurity increases the risk of hyperbilirubinemia and kernicterus since the fetal liver in a preterm baby is even less capable of conjugating bilirubin than in a term baby.

2.3.1 Intrauterine fetal blood transfusion

The purpose of intrauterine fetal blood transfusions (IUT) is to prevent fetal demise and to maintain the pregnancy until term or close to term to avoid additional morbidity. The first intrauterine fetal blood transfusion was performed intraperitoneally by Liley in 1963[68]. Subsequently, an intravascular technique was developed by visualizing the fetal

vessels fetoscopically[69]. The ultrasound guided approach still used today was introduced by Bang in 1982[70]. Since then, thousands of ultrasound-guided intrauterine fetal blood transfusions have been performed worldwide and the techniques and safety have improved. Survival rate after IUT has been reported to be 84 to 97 percent and the risk of fatal complications has been reported to vary between 0.8 to 2.5 percent per procedure. [65 66 71-75].

To access the fetal circulation the umbilical vein is punctured. A recent Cochrane review stated that there are too few randomized controlled trials to determine the optimal procedural technique to use when performing IUT [76]. Today, the umbilical vein either at its insertion into the placenta or in the intrahepatic portion in the fetal abdomen is used depending on fetal and placental position. The intrahepatic approach has been shown to be associated with improved fetal survival compared to transfusion in a free loop of the cord[77]. Advantages with the intrahepatic approach are that an umbilical artery cannot be punctured and extravasated blood can be absorbed from the peritoneal cavity. The advantage with transfusion in the placental cord insertion is that penetration of the fetus with the needle is avoided. Intraperitoneal transfusion is still in use in very early gestational ages and if access to the intravascular space is difficult to achieve[78 79]. Fetal paralysis is recommended independently of insertion site to avoid procedure complications due to fetal movements [73].

The volume of packed red blood cells to transfuse will depend on the estimated fetoplacental blood volume, fetal hematocrit and hematocrit of the donor RBC unit in order to achieve a hematocrit of 40-50% after transfusion [37 80-82]. Larger volumes can be transfused antenatally compared to postnatally due to the large vascular capacity of the placenta[83]. After two to three transfusions, fetal red cell production is suppressed and the fetoplacental blood volume will consist of adult donor RBCs. The maternal hemolytic anti-D antibodies will not destroy the transfused RhD negative RBCs. Since the adult donor RBCs used for transfusion have different properties from fetal, MCA Doppler is no longer reliable in predicting fetal anemia [84 85]. It has been suggested that after one transfusion a cut-off of 1.69 MoM is more accurate in predicting severe anemia. After two IUTs, the timing of next transfusion is calculated by an estimated decline in the hematocrit with about 1 percent per day [86 87].

All invasive procedures carry an inherent risk of preterm premature rupture of membranes (PPROM), preterm labor and infection. In addition, the presence of hydrops fetalis, arterial puncture, transfusion in a free loop of the umbilical cord, refraining from fetal paralysis and advanced gestational age are all factors that have been shown to be associated with an increased risk of procedure complications and a lower survival rate [72 73 75]. Severe thrombocytopenia, which occurs more often in severely anemic fetuses and hydropic fetuses, is associated with a higher risk of perinatal mortality as well [88 89].

2.3.2 Management of pregnancies complicated by RhD immunization in Stockholm

The vast majority of pregnant women in our country are early bookers at the maternity care centers in the first trimester of pregnancy. Maternity care is free of charge. Before September 2009, the red cell antibody screening program during pregnancy in Stockholm included testing in the first trimester and in RhD negative women additional testing around week 25 and 37 of gestation. In nulliparae, antibody testing in the second trimester (week 25) was not included in the routine program. Since September 2009, screening for red cell antibodies is performed in the first trimester of pregnancy in all women and in RhD negative women additional testing is recommended around 25 weeks of gestation. All women with red cell antibodies are referred to the Karolinska University Hospital for further management. In women with red cell antibodies, the blood type of the partner is determined. Antibody levels are analyzed by routine blood group serology titration in gel using indirect antiglobulin technique. A titer of >1/64 or an increase of two titersteps are considered critical. Monitoring of antibody levels is initiated no later than in pregnancy week 16-18. Titers are measured every four weeks if ≤ 1/32 and every second week if > 1/32. Antibody quantitation is performed by flow cytometry if the titer is ≥1/128[90]. When obstetric history and antibody levels indicate risk for fetal anemia, the pregnancy is monitored weekly with Doppler assessment of the MCA PSV [63 64]. Induction of labour is advised in 36 - 37 weeks of gestation. At birth, cord blood is sampled for serology and DAT as well as for measurement of hemoglobin, hematocrit and bilirubin of the newborn.

2.3.3 Protocol for intrauterine blood transfusions in Stockholm

Management according to MCA Doppler interrogation was introduced in 2000 and values > 1.5 MoM of the MCA PSV is used as the threshold for fetal intervention. Prior to the introduction of MCA, the decision of invasiveness was mostly based on obstetric history and/or significant increased antibody levels and it still is in very early cases of severe HDFN. Under aseptic conditions and ultrasound guidance, a 20 gauge needle is inserted either into the umbilical vein in the fetal liver or at the placental cord insertion site, using a free hand technique. Until the mid 1990s a free loop of the cord was often punctured, but this technique was later abandoned. In all cases where transfusion includes penetration of the fetal skin with the needle, fetal analgesia and paralysis are applied (alfentanil 0.015 mg/kg and suxamethonium 2.5-5 mg/kg or atracurium besylate 0.15 mg/kg) [91]. Antibiotics or tocolysis is not used. Staff from the Department of Transfusion Medicine is available during the procedure and provides a bedside blood count analysed by a hematology instrument within minutes from the first blood sampling. Transfusions are made with fresh (less than five days), leukocyte-reduced, irradiated blood group O red blood cell units negative for the antibody corresponding antigens and further matched for the maternal RBC phenotype (Rhesus, Kell, Duffy). RBC units are centrifuged and resuspended in saline to a hematocrit of 75-90%. An estimated blood volume of 100 ml per 1000 g fetal weight is anticipated and the transfusion volume is based on the concept described by Nicolaides et al[37 80-82]. If possible, a final blood sample is taken at the end of the procedure to determine the hemoglobin and hematocrit values after transfusion. Depending on the degree of anemia, the next transfusion will be carried out within 2-3 weeks, estimated from MCA PSV or calculated from the expected decline in hematocrit. After the procedure, all cases with a gestational age more than 28 weeks are monitored with fetal heart tracing for an hour before discharge.

2.4 PREVENTION OF RHD IMMUNIZATION

2.4.1 Anti-D prophylaxis

In 1900, von Dungern showed that immunization to an antigen can be prevented by the presence of passive antibodies to the antigen[92]. Sixty year later, it was experimentally shown that the same was true for prevention of RhD immunization[93]. During the 1960s,

clinical experiments evaluating the preventive effect of polyclonal anti-D immunoglobulin administered after exposure to RhD positive RBCs in RhD negative male volunteers were performed[94-96]. Shortly thereafter, studies were carried out in RhD negative women given birth to RhD positive babies. Immunization could be prevented if anti-D immunoglobulin was administered within 72 hours after delivery[97-99]. Many studies on postnatal anti-D prophylaxis have been performed since then and six randomized controlled trials have been evaluated in a Cochrane review. This review confirmed that postnatal prophylaxis significantly reduces the incidence of RhD immunization detected in the first trimester in a subsequent pregnancy with a risk ratio (RR) of 0.12[100]. The reduction was seen regardless of the ABO status of the mother and baby. The report stated that there is no clear evidence on the optimal dose of anti-D IgG for postpartum prophylaxis.

The exact mechanisms by which passive anti-D antibodies prevent immunization remain largely unknown but involve antibody-mediated immune suppression (AMIS) and reduce the immunogenicity of RhD positive RBCs. AMIS is the concept that the antibody response against an antigen can be prevented by the passive administration of antibodies against the same antigen . Masking of the RhD antigens on the RBCs are not an explanation since only 5 to10 percent of the epitopes are covered by administrated anti-D antibodies [30]. One possible explanation is that anti-D IgG bound to fetal RBCs in the maternal circulation induce rapid clearance of these cells through a tolerogenic pathway in the spleen, avoiding processing by antigen presenting cells and activation of an inflammatory immune response [29]. It is also hypothesized that activation and antibody production by RhD-reactive B-cells is suppressed by negative feedback mechanisms when cell-bound IgG and antigen bind to the B-cell receptor. Only naïve B-cells can be inhibited by polyclonal anti-D, not memory B-cells, which explains why only a primary response can be prevented with prophylaxis. T-cell activation is not inhibited by polyclonal anti-D IgG [30].

In order to prevent immunization, prophylaxis must be administered before sensitization has taken place and in sufficient dose. It has been shown experimentally that 10 μg anti-D IgG is enough to protect against exposure of 1 ml of RhD positive fetal blood or 0.5 ml of fetal RBCs (fetal hematocrit is approximately 50%)[23 24 101 102]. There is a dose-response relationship and 10 μg/ml fetal blood will be suppressive of a primary immune response regardless of the volume of FMH. In the original dose

studies from the 1960s, RhD positive adult RBCs were infused intravenously to male volunteers.

There is no universal policy on the dose to administer during pregnancy or after delivery, but there is evidence to support that the minimum recommended dose is 50 μg before 20 weeks of gestation and at least 100 μg from 20 weeks [14][102]. Postnatal prophylaxis should consist of at least 100 μg within 72 hours of delivery of an RhD positive baby to have optimal effect. Postnatal doses used vary between countries from 100-300 μg [9][14][103]. If 100 μg is used it is recommended to be combined with routine post delivery testing for FMH exceeding the amount of anti-D IgG administrated. This is not considered as necessary when a dose of 200-300 μg is used, since FMH greater than 20 ml is very uncommon [38][53].

The dose used for routine antenatal anti-D prophylaxis (RAADP) varies as well from a single injection of 200-300 μg at 28-30 weeks of gestation to 100 μg as a repeated injection at 28 and 34 weeks of gestation. There are no good quality studies comparing the efficacy of different dosing and timing regimens of RAADP. Administration of polyclonal anti-D antibodies during pregnancy does not cause hemolytic anemia or hyperbilirubinemia in the fetus or neonate[5][104]. Cord blood serology can be DAT positive at birth due to still detectable levels of passive anti-D antibodies from prophylaxis, but these neonates are not at risk of hemolytic disease and this laboratory result alone should not lead to admittance to a neonatal care unit or prolonged stay at the hospital.

Current anti-D preparations are prepared from high-titer anti-D plasma from hyperimmunized RhD negative human donors and exist in a limited supply globally. The donors are immunized by exposure to RhD positive red blood cells and exposure has to be repeated to boost the immune system. Both the donors of RBCs and plasma are rigorously tested for infectious diseases and the plasma undergoes several steps to ensure viral safety. A total guarantee of unknown pathogens cannot be made and there have been a theoretical concern about the transmission of variant Creutzfeldt-Jacobs disease [1]. There are ethical concerns regarding exposing plasma donors to foreign RBCs and using human plasma derived products in pregnancy exposing at least two individuals. Most countries are not self-supplying in anti-D plasma. In Sweden, the anti-D plasma is imported from the United States. Efforts have been made in

developing monoclonal or recombinant anti-D antibodies to replace the polyclonal anti-D from human donors. Clinical trials have been performed but so far these antibodies have not been as effective as polyclonal antibodies from human donors in preventing sensitization[105-108].

2.4.2 Anti-D prophylaxis protocol in Sweden

Currently, there are no national guidelines for anti-D prophylaxis in Sweden. General postnatal anti-D prophylaxis was introduced in Sweden by the National Board of Health and Welfare in 1969. A dose of 200-300 μg anti-D IgG within 72 hours of delivery was recommended [109]. Screening for fetomaternal hemorrhage larger than covered by the standard dose of anti-D IgG after delivery is not routinely performed in our country. Since 1975 prophylaxis is also provided after termination of pregnancy (TOP) or miscarriage[110]. The recommendations are to administer prophylaxis at miscarriages from 12 weeks of gestation and at medically induced abortion from 9 weeks of gestation [111]. After surgical TOP, prophylaxis is always recommended. Other situations during pregnancy when anti-D prophylaxis is advised are after intrauterine procedures such as amniocentesis, chorionic villus sampling, cordocentesis, fetoscopy and shunting as well as after external cephalic version. Prophylaxis should also be considered after abdominal trauma and antepartum hemorrhage after the first trimester of pregnancy. There are no data on failure rate in providing prophylaxis when indicated.

Routine antenatal anti-D prophylaxis has not been introduced in Sweden. The first study on routine antenatal anti-D prophylaxis in our country was carried out in 1984 and showed significant reduction in RhD immunizations[112]. Introduction of RAADP in gestational week 28-30 was advocated in the Swedish Medical Journal by authorities already in 1998 -2000, supported by scientific evidence [110 113-115]. Why this proposal was not implemented and the results from the 1984 study not taken into account are unclear. It might depend on the fact that in 1997 the National Board of Health and Welfare decided not to publish national guidelines but leave that to the medical associations. One can speculate that with the relatively small population of Sweden, there are few cases of RhD immunization in each hospital and limited awareness among obstetricians and maternal-fetal medicine specialist of the risk of immunization during pregnancy due to silent FMH.

2.4.3   The effect of routine antenatal anti-D prophylaxis

After the introduction of postnatal prophylaxis, it became clear that the most important reason for residual sensitization was silent FMH during pregnancy, most often in the third trimester of pregnancy [5][13]. Bowman calculated that immunization during pregnancy accounts for 14 percent of all cases of RhD immunization[107]. The first clinical trials on routine antenatal prophylaxis were performed in Canada and published in 1978[5][116]. The incidence of RhD immunization in the population studied was reduced from 1.8 percent to 0.14 percent. Since then, several studies have been published on the effectiveness of routine antenatal anti-D prophylaxis in the third trimester of pregnancy and RAADP has been introduced in many countries. In the studies published, the control groups consisted of women who had received anti-D prophylaxis at routine indications during pregnancy and after delivery and the study populations of women who in addition received RAADP. Incidence in both control groups and study populations varied between the studies. A meta-analysis in which the authors pooled the results from 11 studies concluded that the incidence in the control groups was approximately 1.4 percent (95% CI 1.2 – 1.6) and in the study population 0.5 percent (95% CI 0.4 – 0.6) [117]. However, many of the studies were of poor quality and afflicted by problems in study design, assessment of sensitization and many participants were lost to follow-up. Recently a bias-adjusted meta-analysis was published including all data from ten previous published studies on RAADP [5][13][112][116][118-121]. The authors adjusted the analysis for differences in study quality and design to reduce the risk of biased results. They concluded that there is strong evidence for the effectiveness of RAADP in preventing immunization in pregnant RhD negative women. The pooled odds ratio for sensitization with RAAPD was estimated as 0.31 (95% CI 0.17 – 0.56) [122]. In addition, the Dutch national RAADP program introduced in 1998 was recently evaluated. The incidence of RhD immunization in para-1 who had received RAADP in their first pregnancy was investigated and compared with the incidence in para-1 before the program was started. The incidence was reduced from 0.67 (95% CI 0.50-0.84) to 0.31 (95% CI 0.21-0.41) percent.

An in-depth health technology assessment including a cost-effectiveness analysis was performed in the UK prior to and five years after the introduction of RAADP. It was concluded that antenatal prophylaxis to all RhD negative women was cost-effective. An

economical evaluation in the Netherland estimated that the introduction of selective RAADP would be cost-effective in the Dutch setting [123-126].

A drawback with providing RAADP to all RhD negative women is that approximately 40 percent will carry an RhD negative fetus and these women are not at risk for sensitization. Until recently, there was no risk-free method available for determination of the RhD type of the fetus. However, the possibility of prenatal noninvasive genotyping of fetal *RHD* in maternal plasma offers a way to select the women who will benefit from prophylaxis. Two countries have introduced this method nationally in combination with selective RAADP only to women with *RHD* positive fetuses, Denmark in 2010 and the Netherlands in 2011 [127].

2.4.4 Pharmacokinetics of antenatal anti-D immunoglobulin

Plasma concentration of anti-D IgG after administration will depend on the bioavailability, i.e. uptake from the injection site, and distribution volume (Vd). Most preparations of anti-D IgG are made for intramuscular injection. Clearance (Cl) from the intravascular compartment depends on distribution volume and half-life (T½): $Cl=(Vd \times 0.693)/ T\frac{1}{2}$ [128]. Duration of protective levels of anti-D IgG will depend on individual clearance and eventually consumption due to fetomaternal hemorrhage. Immunoglobulin G is cleared by the MPS.

There are several different preparations and brands of polyclonal anti-D immunoglobulin and their use vary between countries. In Sweden, two different preparations are registered: Rhesonativ® 125 μg and 250 μg and Rhophylac® 300 μg Studies have been published on the pharmacokinetics of plasma anti-D after antenatal administration of different doses both using a single dose and two dose protocol [116 129-132]. The studies report duration of detectable levels (1-2 ng/mL) of anti-D until delivery (11-12 weeks after injection) in 30 – 60 percent of women and mean half-life varying between 17 to 24 days. In the last years, laboratory methods using flow cytometry enable reliable quantitation of antibody levels at lower plasma concentrations[90].

2.5 NONINVASIVE DETERMINATION OF FETAL *RHD* GENOTYPE IN CELL-FREE FETAL DNA IN MATERNAL PLASMA

In the past, the only way to analyze the fetal RhD status in an RhD immunized pregnant woman, when the father of the baby was heterozygous for the RhD gene, was by invasive prenatal testing. Polymerase chain reaction (PCR) analysis was performed on the fetal cells [133]. Because of the risks of the procedure and the risk of boosting the antibody response in the mother, efforts were made to determine the fetal RhD status using fetal cells in maternal blood to be able to avoid invasive fetal testing [134-136]. Since fetal cells are present in very small numbers in maternal circulation, the isolation of the cells is technically challenging, time consuming and expensive. The first report confirming the presence of cell-free fetal DNA (cff DNA) in maternal plasma was published in 1997 [137]. The investigators showed that they could detect sequences from the Y chromosome in cell-free DNA from plasma and serum in women pregnant with a male fetus. One year thereafter, it was demonstrated that the fetal *RHD* genotype could be determined in the plasma of RhD negative women [138] [139]. Cell-free fetal DNA is a better source for noninvasive prenatal diagnosis than fetal cells in maternal circulation, since it is present in much higher concentrations and is easier to isolate. Another advantage is that, compared to fetal cells in the maternal circulation, cell-free fetal DNA is cleared from the maternal circulation within hours of delivery, excluding the possibility of detecting genetic material from a previous pregnancy [140]. So far there are no optimal genetic or epigenetic markers for fetal DNA to distinguish it from cell-free maternal DNA. This has limited the detection to paternally inherited genes or de novo mutations not present in the maternal DNA. Fetal DNA can be enriched by size selection, since the fetal oligonucleotides are smaller than the maternal. This approach is so far not suitable for screening purposes [141] [142].

The main source of cff DNA in maternal circulation is the trophoblast [143-145]. Evidence to support this are i) cell-free fetal DNA in maternal circulation can be detected from 5 weeks of pregnancy before fetoplacental circulation is established [146-148], ii) it can be detected in maternal plasma in anembryonic pregnancies [149], iii) confined placental mosaicism is reflected in cffDNA [150] [151], iiii) the epigenetic signature in cff DNA is the same as in placental tissue [152] [153]. Cell-free fetal DNA is released into the maternal circulation as part of the ongoing remodeling of the placenta, from apoptotic trophoblast cells, and consists of small oligonucleotides (< 150 bp) [154] [155]. The fraction

of fetal DNA of the total amount of cell-free DNA in maternal plasma varies from 3-20 percent and the absolute concentration increases with gestation [156] [157]. Most of the cell-free DNA in plasma is of maternal origin and the fetal fraction varies both within and between pregnancies [158-161]. The fetal fraction of cell-free DNA is higher in plasma than in serum, making plasma the best source for non-invasive prenatal diagnosis [157] [162].

Noninvasive determination of fetal *RHD* genotype in cff DNA in maternal plasma was first introduced in pregnancies at risk of HDFN and many studies have been published that confirm the safety aspects of the method [54] [55] [163] [164]. Recent studies have focused on fetal *RHD* testing in non-immunized women and demonstrate results with high accuracy[126] [127] [165] [166]. The maternal blood samples in these studies were mostly collected in the second or third trimester of pregnancy. Only few studies have evaluated noninvasive *RHD* determination in the first trimester of pregnancy and these studies included small study samples and were not performed in a routine setting[167-169].

3 AIMS

The general purpose of this thesis was to investigate if fetal and neonatal complications due to RhD immunization in the mother could be prevented by 1) reducing procedure-related complications in intrauterine blood transfusions 2) reducing the incidence of RhD immunization by providing routine antenatal anti-D prophylaxis during pregnancy selectively to non-immunized RhD negative women with RhD positive fetuses.

The pre-specified hypotheses were:

1. It is possible to isolate fetal DNA in maternal plasma and determine the fetal RhD blood group. Is the test reliable enough to be used and trusted routinely in the clinical setting?
2. By treating RhD negative mothers carrying RhD positive fetuses with antenatal anti-D prophylaxis in pregnancy week 29, we speculate that it is possible to reduce the number of immunized mothers with at least 50 percent.

The specific aims were:

To describe the perinatal outcome after intrauterine blood transfusion and to identify areas that can be improved in order to further reduce perinatal morbidity and mortality related to the procedure (paper I).

To determine the pharmacokinetic profile and duration of detectable plasma levels of anti-D immunoglobulin after administration of 250 μg in 28-30 weeks of pregnancy in RhD negative women (paper II).

To develop a simple and robust assay suitable for fetal *RHD* screening in the first trimester of pregnancy and to estimate the sensitivity and specificity of the test after its implementation in an unselected pregnant population (paper III).

To investigate in which pregnancy and when immunization occurred in a cohort of RhD immunized women to whom routine antenatal anti-D prophylaxis was not provided and to describe the consequences of RhD immunization for the index pregnancy and for subsequent pregnancies (paper IV).

To estimate the incidence of RhD immunization in our population after introduction of routine antenatal anti-D prophylaxis selectively to women with *RHD* positive fetuses (paper V).

4 SUBJECTS AND METHODS

4.1 PAPER I

4.1.1 Study population and design

We performed a retrospective study including all pregnancies treated with intrauterine blood transfusion due to red cell immunization at our hospital from June 1990 to June 2010. The Karolinska University Hospital is the tertiary referral center for all pregnancies complicated by red cell immunization in the Stockholm region and receives referrals for IUTs from other regions of Sweden, covering approximately 40 percent of all cases in the country during the period studied. Primary outcome variables were fetal and neonatal survival, procedure-related complications and gestational age at delivery. Procedure-related complications were defined as bradycardia leading to immediate delivery, intrauterine fetal death within a week from the last procedure, chorioamnionitis and/or premature preterm rupture of membranes.

Data on obstetric history, invasive procedures, pregnancy outcome, autopsies, antibody type and titers were retrieved from medical records, local databases and transfusion medicine registers and entered into a standardized web-based register (www.gravimm.se). Data on cases before April 2008 were entered retrospectively and thereafter prospectively. Neonatal data was imported from the Swedish Quality Register on Neonatal Intensive Care (www.pnq.se) and from medical records.

4.1.2 Statistical methods

Logistic regression modelling was used to estimate the risk of a procedure complication to occur. First, a univariate logistic regression analysis was performed for each of the selected predictor variables. All variables with $P \leq 0.2$ in the univariate analysis were included in the final multivariate logistic regression model. Analysis of variables associated with the risk of a procedure complication to occur was done both per procedure and per pregnancy.

4.2 PAPER II

4.2.1 Study population and design

The aim of this study was to define the pharmacokinetic profile and duration of detectable levels of anti-D after antenatal administration of 250 μg (1250 IU) of anti-D IgG. We performed a prospective observational study on a subgroup of women participating in the large prospective cohort study of RhD negative pregnant women offered selective routine antenatal anti-D prophylaxis (paper V). Eligible participants were healthy women without a previous history of pregnancy complications, with a singleton pregnancy and estimated due date confirmed by ultrasound examination in the second trimester. Exclusion criteria were anti-D prophylaxis received earlier in the current pregnancy or presence of anti-D antibodies at the time of inclusion or earlier. The anti-D IgG was administrated as a single intramuscular injection between 28 to 30 weeks of pregnancy. The plasma concentration was followed by serial blood samples and quantitation using flow cytometry at pre-defined time points: 0, 3, 10±2, 14±2, 28±2, 42±2, 56±2, 63±2, 70±2, 77±2 and 84±2 days after injection. The plasma from each woman was incubated with enzyme treated RhD positive red blood cells (cDe/cde) and then with fluorescein isothiocyanate (FITC)- conjugated anti-human IgG. Mean fluorescence intensity was compared against a standard curve based on an international reference anti-D (NIBSC 73/515). Enzyme treatment of the red blood cells was performed in order to increase the sensitivity of the method and the lowest detectable concentration of anti-D IgG was 1 ng/mL.

4.2.2 Statistical methods and pharmacokinetic analysis

Time versus concentration profiles for anti-D IgG levels were generated graphically from the concentration values at the predefined time points. Number of samples collected at each time point was reported in a separate table. Individual half-life was estimated from all available data points, except when the number of data points was too low or when extra anti-D prophylaxis was given during the study period for obstetric reasons. Plasma samples taken after administration of extra anti-D prophylaxis were excluded from the pharmacokinetic analysis. The half-lives and elimination constant (k) values were calculated by linear regression analysis. Pharmacokinetic parameters from the set of evaluable data were presented by medians and range or means and standard

deviations. The last concentration measured (C_{last}) was included in the statistics if it was collected no more than two weeks prior to a term delivery.

4.3 PAPER III

4.3.1 Study population and design

To estimate the diagnostic accuracy of the noninvasive test for fetal *RHD* genotype, we performed a prospective population based cohort study starting in September 2009. All RhD negative pregnant women in Stockholm county were invited to participate and the pregnancies included in the study were all with an antenatal fetal *RHD* genotype result and pregnancy outcome by 1st of May 2011. At the booking visit at the maternity care center, two EDTA anti-coagulated blood samples were taken as part of routine blood sampling. One sample was used for standard ABO RhD blood typing and RBC antibody screening and the other was used for noninvasive fetal *RHD* genotyping in women who typed RhD negative. Plasma were separated with centrifugation and frozen in two 1 mL aliquots until DNA extraction and PCR analysis. All samples were analysed centrally at the Department of Immunology and Transfusion Medicine. The PCR analysis was designed for the multiplex detection of exon 4 of the *RHD* gene and the glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH* which is a house keeping gene, i.e. always expressed by the cells) in cell-free DNA. *GAPDH* was used as an internal control to confirm the presence and concentration of DNA in the samples. An *RHD* positive and *RHD* negative control was included in all analyses but we used no control for fetal DNA. To ensure that a too high concentration of maternal DNA did not hamper the detection of fetal DNA, the total DNA content in each sample was estimated by comparing the obtained *GAPDH* cycle threshold (ct) value in the sample with the *GAPDH* ct value in the positive control with a known DNA concentration. Strict criteria for the ct values to confirm required DNA concentrations in the samples were applied (*GAPDH* ct 27-35).

Cord blood serology results at birth were used as reference standard to assess the diagnostic accuracy of the antenatal noninvasive fetal *RHD* genotyping.

Since transport times from the maternity care centers vary, we evaluated the effect of storage time on the quality of the analysis before start of the main study. Samples taken

in early pregnancy in 25 women were stored as whole blood at room temperature for a) < 24 hours b) 36-48 hours c) 60-72 hours before centrifugation and plasma separation.

4.3.2 Statistical methods

The pregnancies included in the study were described with percentages for categorical variables and medians and ranges for continuous variables. Sensitivity and specificity of noninvasive fetal *RHD* genotyping were calculated from the results of fetal *RHD* determination compared with positive and negative cord blood serology results. Confidence intervals were estimated using the exact binomial probabilities. Sensitivity was then recalculated excluding samples collected earlier than successive cut-off values for gestational age and presented graphically. All inconclusive and missing samples were reported in detail in tables and a flow diagram.

4.4 PAPER IV

4.4.1 Study population and design

Paper IV is a retrospective cohort study on all RhD immunized pregnant women in Stockholm from 1990 to 2008. During this period RAADP was not provided. Primary outcome variables were timing of RhD sensitization and frequency of HDFN. Secondary outcome variables were perinatal outcome including fetal or neonatal mortality, prematurity due to HDFN and number of subsequent children following the pregnancy when sensitization occurred (index pregnancy). The women were divided into three groups depending on parity. Group 1 consisted of women immunized with the first born child, group 2 of women immunized with the second born child and group 3 of women immunized with the third or later born child (range 3rd to 7th child). Women who had previously given birth to an RhD positive child and had detectable anti-D antibodies in the first trimester in the following pregnancy, were considered to have been sensitized after delivery of the previous child. In women who seroconverted during pregnancy we used the date of the first positive antibody test to define in which trimester they were sensitized. In each case, the date of the first positive anti-D antibody test was confirmed with the transfusion medicine laboratory information system (LIS). HDFN was defined as the need for treatment with IUT, exchange transfusion or phototherapy. Prematurity due to HDFN was defined as delivery < 37 gestational weeks in combination with the diagnosis of HDFN as defined above.

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All RhD immunized women in the cohort were identified using the register of the Department of Immunology and Transfusion Medicine. Data on intrauterine blood transfusions and exchange transfusions were obtained from the same source. Results from RBC antibody tests were obtained from the transfusion medicine LIS. Data on obstetric and perinatal outcome, pregnancy interventions and postnatal treatment was retrieved from medical records, local databases at the Department of Obstetrics and Gynecology and from the National Perinatal Quality Register for neonatal care (www.pnq.se). All data were entered into the quality register for red cell immunization during pregnancy (www.gravimm.se).

4.4.2 Statistical methods

Comparisons between groups were assessed with the Fisher´s exact test. Statistical significance was set to $P < 0.05$. Missing data was excluded in the analyses and indicated in the tables by the total number of individual data points included for each variable.

4.5 PAPER V

4.5.1 Study population and design

In paper V, we performed a prospective observational cohort study with historic controls. All RhD negative pregnant women in Stockholm county from September 1st 2009 to December 31st 2011, without anti-D antibodies in the 1st trimester of pregnancy, were eligible. The study cohort was defined as women with an *RHD* positive fetus who had received routine antenatal anti-D prophylaxis and who delivered between January 1st 2010 and March 31st 2012. The reference cohort consisted of all RhD negative women giving birth in the same region 2004 to 2008.

Management of RhD negative women in Stockholm during the study period is described in Figure 2. Noninvasive fetal *RHD* genotyping was performed mostly in the first trimester of pregnancy, as described in paper III. A single injection of 250 to 300 µg anti-D IgG was administered intramuscularly in gestational week 28 to 30 to women with *RHD* positive fetuses. A plasma sample was taken and frozen before the injection. This sample was analysed only in women with a negative second trimester antibody

screening test who subsequently had a positive test at delivery, to determine whether they were already sensitized at the time of RAADP or not. Extra anti-D prophylaxis was also administered at events during pregnancy with increased risk of FMH as well as after delivery. Women who declined to participate in the study or did not receive RAADP for other reasons received routine care with antibody screening in gestational week 25 and 37.

Figure 2. Study protocol for RhD negative pregnant women without anti-D antibodies at first trimester antibody screening.
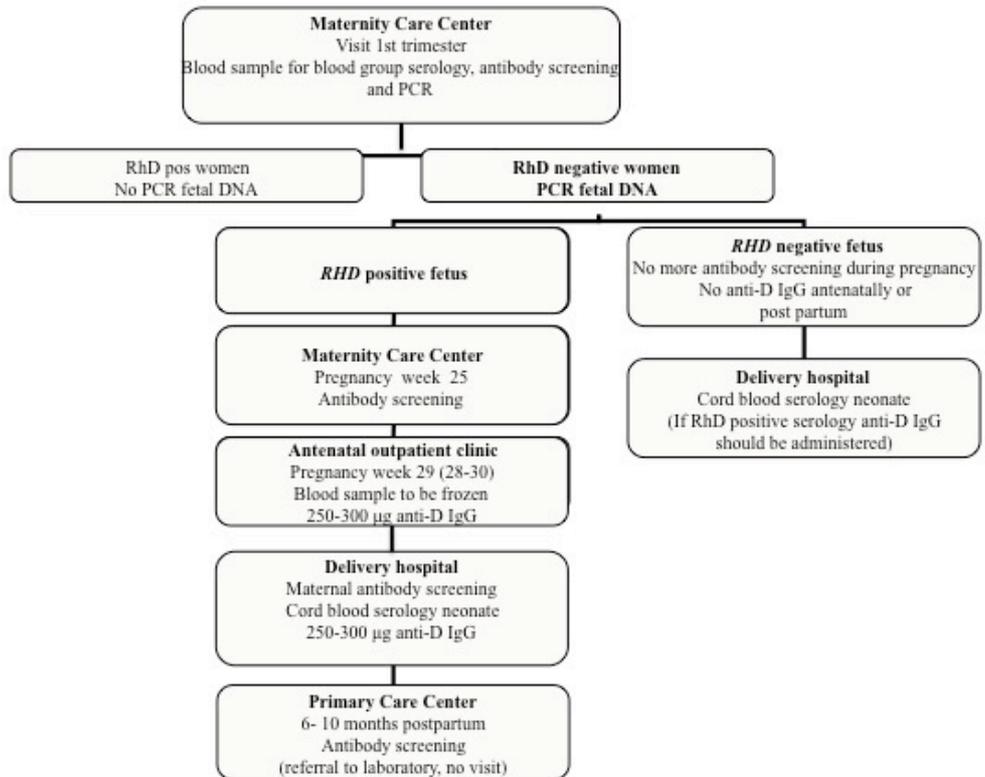
Main outcome measure was the incidence of RhD immunization developing during or after pregnancy in the two cohorts. All pregnancies with an *RHD* positive genotype were entered into a study database and follow-up data were recorded prospectively. Results from antibody screenings and cord blood serology were retrieved from the LIS

at the Department of Immunology and Transfusion Medicine. Data on obstetric and neonatal outcome were retrieved from electronic medical records. New RhD immunizations during the study period were defined as women who seroconverted after the first trimester of pregnancy or postpartum. Statistics on RhD negative deliveries during the study period and reference period were retrieved from the regional delivery database (Obstetrix) and from the Swedish Medical Birth Register. The incidence of new cases in the reference cohort was determined by searching the regional quality register (www.gravimm.se) for all RhD immunized women who seroconverted during pregnancy or after a delivery during the five-year period. The date of the first anti-D positive plasma sample was confirmed against the transfusion medicine LIS. All women in the reference cohort with anti-D antibodies after delivering abroad, due to anti-D prophylaxis or unclear cases were excluded.

4.5.2 Study size

The birth rate in Stockholm has varied between 25.000 to 27.000 births per year. A power analysis was performed before the start of the study to determine the necessary study size. We hypothesised a reduction of RhD immunization from 1 to 0.5 percent. Using a two-sided test for binomial distribution at a significance level of 0.05 and 80 percent power, it was estimated that 4540 RhD negative women were required in each cohort.

4.5.3 Statistical methods

An intention-to-treat analysis was applied to the estimation of reduction of incidence of immunizations. All new cases during the study period were included in the analysis even if the woman had not received RAADP. Frequencies and absolute numbers of missing data are reported in tables and figures. Incidence was calculated by dividing all new cases in each period by the total number of RhD negative pregnant giving birth during each respective period. We used summary statistics to describe the study cohort and a Chi-square test to compare the incidence in the two cohorts.

4.6 ETHICAL CONSIDERATIONS

All five studies in this thesis have been approved by the Regional Ethics Committee. Approval numbers: paper I (2010/1996-31/4), paper II (2010/862-31/1), paper III (2009/479-31/4), paper IV (2010/1996-31/4), paper V (2009/479-31/4).

In paper II and V all women received oral and written information and gave written consent to receive antenatal anti-D immunoglobulin and participate in the studies. The Medical Products Agency decided a permission not to be required for the study in paper II since the specific brand of anti-D used was already registered and in use in Sweden.

5 RESULTS

5.1 HIGH PERINATAL SURVIVAL AFTER INTRAUTERINE BLOOD TRANSFUSIONS BUT PROCEDURE-RELATED COMPLICATIONS CAN BE FURTHER REDUCED.

A total of 284 intrauterine transfusions were performed in 84 pregnancies in 72 women. There was one set of twins resulting in 85 fetuses. Overall survival was 91.8 percent (78/85). Complications occurred in 4.9 percent (14/284) of procedures of which 1.4 percent (4/284) were fatal. Hydrops fetalis was present in ten cases. The most common red cell immunization requiring intrauterine treatment was RhD immunization that occurred in 83 percent of the women (60/72). Of these women, at least 28 percent (17/60) had been immunized during their first pregnancy. Ten of the 72 women had previously experienced intrauterine fetal deaths.

The mean number of transfusions per pregnancy was 3.4 with a median of 4 (range: 1-10). Of the 284 transfusions, 46 percent (130/284) were performed in the intrahepatic part of the umbilical vein, 25 percent (71/284) in the placental cord insertion, 16 percent (44/284) in a free loop of the umbilical cord and in 12 percent (35/284) it was not possible to establish the insertion site from the medical records. Intraperitoneal transfusion was performed at four occasions due to technical problems with achieving intravascular access. Procedure-related complications were significantly more common when transfusions were performed in a free loop of the umbilical cord compared to the intrahepatic part of the umbilical vein (OR 5.4, 95% CI: 1.2 - 23.7, P=0.025). There was no significant difference between the intrahepatic route and the placental cord insertion (P=0.83). The most common complications were technical problems when needling the fetal vessels, bradycardia during the procedure or abnormal fetal heart tracing after the procedure. When analysing factors associated with a procedure-related complication to occur per pregnancy, only gestational age at first IUT was significantly associated with an increased risk (OR 0.8, 95% CI: 0.6 - 0.9, P=0.019). Four cases of fetal death were due to procedure complications. Two out of four presented with hydrops fetalis at the first IUT and one was performed < 22 gestational weeks. Another three perinatal deaths occurred as a result of HDFN or as a complication to postnatal treatment. Two of these fetuses suffered from hydrops fetalis at the time of referral, of which one presented as early as 20 weeks of gestation and was considered not possible

to rescue with IUT. In the other case, the mother developed mirror syndrome and the baby died postnatally after delivery at 30 weeks of gestation. The third case had three successful IUTs, but the baby died of heart failure during an exchange transfusion on the first day of life.

Of the live-born children the median gestational age at delivery was 36 weeks (range: 28-40) and 24 percent (19/80) were born before 34 weeks of gestation. Median duration of neonatal care was eight days (range: 0 - 64). All but two neonates required phototherapy and 61 percent (49/80) were treated with exchange transfusion. Other complications during the neonatal period were related to prematurity, the most common being pulmonary adaptation disorders. Six neonates developed infant respiratory distress syndrome needing endotracheal ventilation.

5.2 DURATION OF ANTI-D IMMUNOGLOBULIN G UP TO TEN WEEKS AFTER ANTENATAL ADMINISTRATION OF 250 MICROGRAMS ANTI-D PROPHYLAXIS.

Sixteen women received 250 µg anti-D IgG at a median gestational age of 29+3 (range (28+2 - 30+4). A total of 124/134 (92%) possible samples were collected and analysed. 116/124 (94%) were collected at the predefined time points, another seven samples within three days and one within four days of the predefined time points. No samples were available on day 84 because all women had delivered by then. Maximal plasma concentration (C_{max}) was usually seen at three to ten days after injection with a median value of 25 ng/mL (range: 9 - 58 ng/mL). The half-life ($t_{\frac{1}{2}}$) varied in an expected way between individuals with a median of 23 days (range: 12.5 - 30.3 days). We found detectable levels ≥1 ng/mL of anti-D IgG within two weeks of delivery in 11 of 12 women (C_{last}).

Figure 3 illustrates the mean concentrations with standard deviations for all samples analysed at each time point. Only four women delivered after 40 weeks of gestation and of these only two could be included in the analysis since one had received extra anti-D prophylaxis due to external cephalic version and the values of another woman showed a very divergent pattern and were excluded. At ten weeks (70 days) after injection, four

plasma samples from uncomplicated pregnancies were available for analysis. All had detectable levels of anti-D ranging from 1-4 ng/mL.

Figure 3. Mean plasma concentrations of anti-D IgG days after intramuscular injection (ng/mL +/- SD).
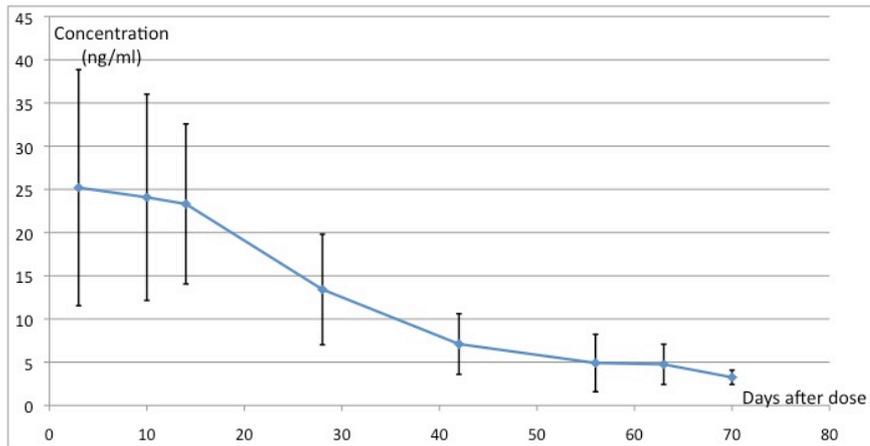
The data points represent different number of subjects due to missing values towards the end of the study period: Days 3 (n=15), 10 (n=14), 14 (n=14), 28 (n=12), 42 (n=10), 56 (n=11), 63 (n=8), and 70 (n=4), respectively.

If extrapolating the individual pharmacokinetic linear curves in the 12 women with a value for C_{last}, 75 percent (9/12) would have had anti-D IgG concentrations ≥ 1 ng/mL at the time of delivery.

5.3   RELIABLE FETAL *RHD* DETERMINATION IN EARLY PREGNANCY WITH A SINGLE-EXON SCREENING STRATEGY.

During the study period, antenatal samples from 4118 pregnancies were received and included in the analysis. Median gestational age for blood sampling was 10 (range: 3-40) gestational weeks. The analysis of effect of storage time on the samples showed that valid analysis could be performed after one to three days of storage with comparable ct values and reliable results. The majority of samples in the main study was separated and frozen within two days and DNA extraction and PCR analysis was done within one week. Characteristics and results of the 4118 study samples are described in Table 1.

Table 1. Characteristics of study samples (n= 4118)

Trimester of analysis	
1st trimester (weeks 3-12)	3108 (75.5%)
2nd trimester (weeks 13-28)	774 (18.8%)
3rd trimester (weeks 29 and over)	177 (4.3%)
Unknown	59 (1.4%)
Fetal RhD after first sample (n=4118)	
Positive	2401 (58.3%)
Negative	1552 (37.7%)
Inconclusive	165 (4.0%)
Fetal RhD after second sample in 147/165 (n=4100)	
Positive	2485 (60.7%)
Negative	1601 (39.1%)
Inconclusive	14 (0.34%)
[of which maternal gene]	[14]
Blood group serology	
Positive	2297 (55.8%)
Negative	1355 (32.9%)
Missing	466 (11.3%)
Miscarriage/abortion	260
Moved	72
Delivered elsewhere	47
Stillbirth	4
Home delivery	3
Not done or unknown	80

In 211 (5.1%) cases a reanalysis had to be done on the second aliquot of frozen plasma due to inconclusive results. After reanalysis of the second aliquot 165 (4%) of tests remained inconclusive. A new blood sample was obtained from 147/165 and analyzed. In 2485 (60.3%) of cases the fetal genotype was determined as *RHD* positive and in 1601 (38.8%) as *RHD* negative and 32 (0.8%) remained inconclusive. In 14/32 the test results were inconclusive due to a maternal *RHD* gene variant and in 18/32 we did not receive a second sample, mostly due to miscarriage or termination of pregnancy (n=13). In the inconclusive cases due to a maternal gene variant, fetal *RHD* was reported as undetermined and anti-D prophylaxis was recommended at standard indications. Cord blood serology was missing in 466 pregnancies, which resulted in 3652 serology results from which the diagnostic accuracy of the noninvasive fetal

genotyping test could be estimated. The reasons for missing cord blood samples are described in Table 1.

Sensitivity and specificity of the test and false negative and positive results are presented in Table 2. Overall sensitivity (n= 3652) was 97.6 percent (95% CI 96.9 - 98.2) and specificity 98.9 percent (95% CI 98.2 - 99.4). When excluding samples obtained before 8 gestational weeks, sensitivity increased to 98.9 percent (95% CI 98.3 - 99.3) and specificity remained at 98.9 percent (95% CI 98.1 - 99.4). Sensitivity increased with gestational age at blood sampling, as described in Figure 4. From 22 weeks of gestational age, sensitivity was 100 percent.

Figure 4. Sensitivity of non-invasive fetal *RHD* genotyping in relation to gestational age.
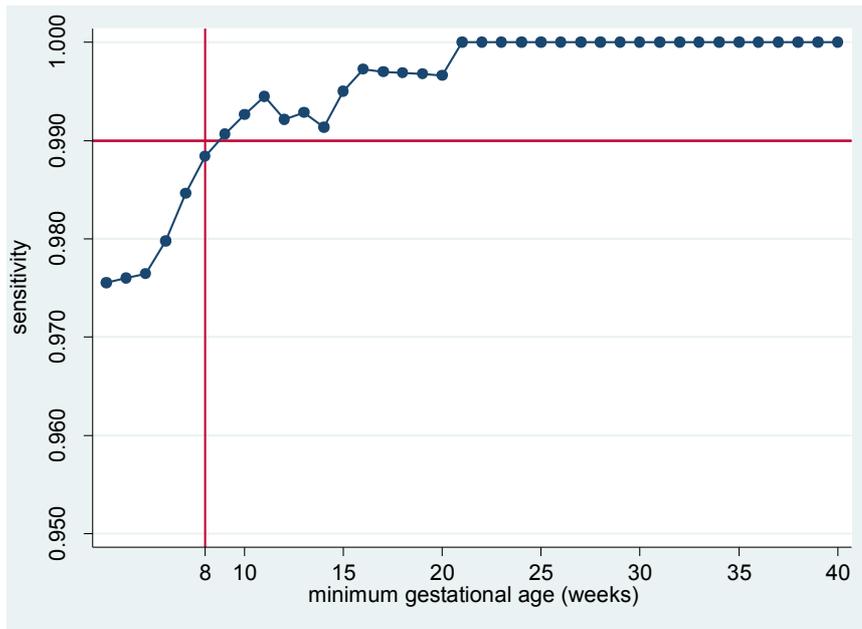
From 8 gestational weeks sensitivity was 98.9%, from 10 weeks 99.3% and from 22 weeks 100%.

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Table 2. Performance of fetal *RHD* genotyping compared to cord blood serology as reference standard.

All Samples	Number	Percent [95% CI]
Serology positive (n=2297)		
Fetal *RHD*:		
Positive	2236	
False Negative	55	97.3
Inconclusive (with maternal gene)	5	2.4 [1.8, 3.1]
Inconclusive (2nd sample not received)	1	0.02
Serology negative (n=1355)		
Fetal *RHD*:		
False Positive	15	
Negative	1331	1.1 [.6, 1.8]
Inconclusive (with maternal gene)	8	98.2
Inconclusive (2nd sample not received)	1	0.06
Samples from gestational week 8 onwards		
Serology positive (n=2073)		
Fetal *RHD*:		
Positive	2045	98.6
False Negative	23	1.1 [.7, 1.7]
Inconclusive (with maternal gene)	4	
Inconclusive (2nd sample not received)	1	
Serology negative (n=1218)		
Fetal *RHD*:		
False Positive	14	1.1 [.6, 1.9]
Negative	1196	98.1
Inconclusive (with maternal gene)	7	
Inconclusive (2nd sample not received)	1	

5.4 MOST WOMEN BECOME IMMUNIZED DURING PREGNANCY WITH THEIR FIRST OR SECOND CHILD AND MORE THAN HALF OF ALL SUBSEQUENT CHILDREN SUFFER FROM HEMOLYTIC DISEASE OF THE FETUS AND NEWBORN.

A total of 290 RhD immunized pregnant women were included in the study. Fifty-one percent (147/290) of the women were immunized with their first-born child and 33 percent (96/290) with their second born child. The majority of women, 73 percent (121/290), seroconverted during pregnancy, in the second or third trimester, and 21 percent (61/290) after delivery. Details on outcome of the index pregnancy and timing of immunization are described in Table 3 and 4.

Table 3. Order of pregnancy (index pregnancy) in which the women were RhD sensitized (n=290).

	Group 1 1st born child n = 147	Group 2 2nd born child n = 96	Group 3 3rd -7th born child n= 47	Total
Number sensitized during ongoing pregnancy	96/147 (65%)	84/96 (88%)	32/47 (68%)	212 (73%)
Anti-D detection in 1st trimester	6	0	0	6 (2%)
Anti-D detection in 2nd trimester	10 (7%)	23 (24%)	5 (11%)	38 (13%)
Anti-D detection in 3rd trimester	62 (42%)	46 (48%)	24 (51%)	132 (46%)
Unclear if anti-D detected in 2nd or 3rd trimester	24(16%)	15 (16%)	3 (6%)	42 (14%)
Sensitized after delivery	41/147 (28%)	12/96 (12%)	8/47 (17%)	61 (21%)
Unclear when sensitized	4	0	7	11 (4%)

Thirty-six of 147 (24%) women who were immunized during pregnancy with their first-born child had a previous history of at least one first trimester TOP or miscarriage.

In eight cases, we could not rule out that this was the sensitizing event. Two of these mothers had detectable anti-D antibodies in the first trimester and two women had seroconverted in week 16 of pregnancy. In four women, it was unclear when exactly anti-D antibodies were detected. We could not exclude they were sensitized before they became pregnant with their first child. The remaining 28 cases seroconverted during ongoing pregnancy, in the 2nd or 3rd trimester, proven by a previous negative antibody test in first trimester. In addition, two women had anti-D antibodies in the first trimester in their first pregnancy. One had a history of previous blood transfusion and the other was pregnant after in vitro fertilization.

Table 4. Perinatal outcome of index pregnancy and frequency of hemolytic disease of the fetus and neonate in relation to in which pregnancy the women were immunized.

	Group 1 1st born child n = 147	Group 2 2nd born child n = 96	Group 3 3rd -7th born child n= 47	Total	Comparison between groups
Prematurity due to HDFN	7/147 (5%)	4/96 (4%)	2/44 (5%)	13/287 (4%)	P=1.00
DAT positive neonate	65/89 (73%)	65/78 (83%)	25/34 (74%)	155/201 (77%)	Not calculated
IUT	0/144 (0%)	2/95 (2%)	3/45 (7%)	5/284 (2%)	P=0.01
Exchange transfusion	12/127 (9%)	6/75 (8%)	1/35 (3%)	19/237 (8%)	P=0.54
Phototherapy	31/92 (34%)	36/71 (51%)	8/28 (29%)	75/191 (39%)	P=0.04
Intrauterine fetal demise	4/147 (3%)	0/96 (0%)	2/45 (4%)*	6/288 (2%)	P=0.11
Neonatal death	1/147#	0/96	0/45	1/288 (0.3%)	P=1.00

*HDFN: hemolytic disease of the fetus and neonate, DAT: direct agglutination test, IUT:intrauterine blood transfusion, *: one intrauterine fetal demise after IUT, the other due to abruptio placentae.*

Most women had at least one more child after the index pregnancy: 89 percent (131/147) in group 1, 48 percent (46/96) in group 2 and 38 percent (18/47) in group 3. All together, the 290 women in the cohort had 267 children after their index pregnancy. Perinatal outcome of these children is presented in Table 5. Fifty-six percent (144/259) of the neonates in subsequent pregnancies required treatment for HDFN, defined as need for IUT and/or exchange transfusion and/or phototherapy.

Overall, induced prematurity less than 37 gestational weeks was common, but premature delivery before 34+0 weeks due to HDFN only occurred in 2 percent (12/557) of the pregnancies. Perinatal mortality due to HDFN or as a complication to intrauterine blood transfusion or postnatal exchange transfusion was 1.6 percent (9/557).

Table 5. Cumulative frequency of hemolytic disease, intrauterine blood transfusions, preterm birth due to hemolytic disease of the fetus and newborn and neonatal treatment in all pregnancies following the index pregnancy.

	Group 1 1st born child	Group 2 2nd born child	Group 3 3rd -7th born child	Total	Comparison between groups
Total no of children born after index pregnancy	181	62	24	267	
Developed other red cell antibodies in addition to anti-D	50 (34%)	17 (18%)	10 (21%)	77/290£ (27%)	Not calculated
RhD pos children	127/176 (72%)	50/59 (85%)	18/24 (75%)	195/259 (75%)	Not calculated
HDFN#	98/177 (55%)	34/58* (59%)	12/24 (50%)	144/259* (56%)	P=0.66
Prematurity due to HDFN	52/181 (29%)	12/62* (19%)	4/23 (17%)	68/266* (26%)	P=0.35
IUT	40/178 (22%)	9/62* (14%)	5/24 (21%)	54/264* (20%)	P=0.62
Exchange transfusion	47/163 (29%)	10/54* (18%)	5/23 (22%)	62/240* (26%)	P=0.44
Phototherapy	88/166 (53%)	30/55* (55%)	11/23 (48%)	129/244* (53%)	P=0.81
Intrauterine fetal demise	5/180 (3%)¶	2/62 (3%)°	0/24 (0%)	7/266 (3%)	P=1.00
Neonatal death	1/180 (0.6%)§	1/62 (2%)	1/24 (4%)§	3/266 (1%)	P=0.14

HDFN: hemolytic disease of the fetus and neonate; IUT: intrauterine blood transfusion

£: presented as cumulative frequency in the total of 290 RhD immunized women

#: Frequency of hemolytic disease of the fetus and newborn defined as treated antenatally with intrauterine blood transfusions and/or postnatally with exchange transfusions and/or phototherapy

*:1 case with severe Kell alloimmunization not included

¶: all due to HDFN, 3/5 as a complication to IUT or cordocentesis

°: one due to complication after IUT, one intrapartum demise due to extreme prematurity

§: neonatal death as complication to an exchange transfusion

5.5 ROUTINE ANTENATAL ANTI-D PROPHYLAXIS SELECTIVELY TO WOMEN WITH *RHD* POSITIVE FETUSES HALVES THE RISK OF RHD IMMUNIZATION.

Between January 1st 2010 and March 31st 2012, 9380 RhD negative women gave birth in Stockholm. Noninvasive fetal genotyping was performed in 8374 pregnancies. 3270 fetuses were determined to be *RHD* negative (39%) and 5104 (61%) *RHD* positive. 4521 women participating with 4590 *RHD* positive pregnancies received RAADP. Data on all included and excluded women and outcomes are described in Figure 5. The mean and median gestation age for receiving RAADP was 29.3 and 29 respectively.

Figure 5. Study participants and outcome

RhD negative women giving birth 1st january 2010 to 31st March 2012
9380

RHD genotyped
8374

RHD neg genotype
3270

RHD pos genotype
5104

Did not receive RAADP
514

Received RAADP
4590

RhD immunized 1st trimester
25

Other reason
145

Anti-D seroconversion before RAADP
5

Declined participation
79

Miscarriage/TOP
260

Unknown outcome
105

IUFD/stillborn
15

born Live
4544

Premature delivery before RAADP
19

Moved from region before RAADP
53

Late booking or received RAADP abroad
18

Unknown
55

4590 pregnancies resulted in 4559 births. In 105 pregnancies the outcome was unknown due to move out of the region (103 women) or women withdrawing from the study after receiving RAADP (2 women).

Twenty new cases of RhD immunization have been confirmed during the study period and follow-up, resulting in an incidence of 0.21 percent (95% CI 0.12 - 0.31) (20/9380). Of these new cases, three women had not received RAADP and fetal genotyping had not been performed. This happened in the beginning of the study period when the compliance to the protocol was incomplete. In the reference cohort, the incidence of RhD immunization was 0.46 percent (95% CI 0.37 - 0.56) (86/18546), which results in a risk ratio (RR) for sensitization of 0.46 (95% CI 0.28 - 0.75) with the new program. The absolute risk difference was 0.25 percent, which corresponds to a number needed to treat (NNT) of 400. The risk reduction was statistically significant (P=0.0013). Data on new cases of immunizations during the study period are described in Table 6.

Table 6. Details on women RhD immunized during the study period.

No	Gravida	Para	GA* RAADP#	1st pos antibody test GA	GA delivery	Comment
1	3	1	29	1st trimester subsequent pregnancy	38+6	Immunised after delivery. Neg screen at delivery.
2	6	1	31	At delivery	38+6	Immunised 3rd trimester. Frozen sample not available. Neg screen at 25 weeks GA.
3	1	0	30	30	41+0	Immunised before RAADP, Frozen sample positive.
4	2	1	29	29	40+4	Immunised before RAADP. Frozen sample positive.
5	4	2	28	10 months postpartum	39+0	Immunised after delivery. Neg screen at delivery.
6	7	2	Not received	26	36+0	Immunised before RAADP
7	2	1	Not received	25	37+0	Immunised before RAADP
8	1	0	Not received	24	36+4	Immunised before RAADP
9	1	0	31	At delivery	42+2	Immunised 3rd trimester. Frozen sample not available. Neg screen at 26 weeks GA.
10	1	0	29	1st trimester subsequent pregnancy	39+2	Unclear if immunised during third trimester or after delivery. Missing screen at delivery. Frozen sample negative.
11	2	1	29	29	39+6	Immunised before RAADP. Frozen sample positive.
12	3	2	Not received	24	Unknown	Immunised before RAADP. Moved from region.
13	2	0	32	32	37+2	Immunised before RAADP. Frozen sample positive.
14	3	1	30	30	41+5	Immunised before RAADP. Frozen sample positive.
15	2	1	29	At delivery	41+1	Immunised 3rd trimester. Frozen sample negative.
16	5	3	29	25	37+0	Immunised before RAADP
17	1	0	Not received	25	34+0	Immunised before RAADP. Severe HFDN requiring multiple intrauterine transfusions. Neonate with

						congenital cytomegalovirus infection.
18	2	1	Not received	28	38+0	Fetal *RHD* genotyping not performed. Missed at beginning of study.
19	2	1	Not received	36	37+5	Fetal *RHD* genotyping not performed. Missed at beginning of study.
20	4	1	Not received	1st trimester subsequent pregnancy	42	Fetal *RHD* genotyping not performed. Missed at beginning of study.

* GA:gestational age; # RAAPD: routine antenatal anti-D prophylaxis

Fifty-five percent (11/20) of the women were already sensitized before being scheduled for anti-D prophylaxis. Three women were sensitized in spite of receiving RAADP and had seroconverted at delivery. In two of these, the frozen sample was not available and the possibility of sensitization before prophylaxis cannot be ruled out. Two women had a negative antibody test at delivery but anti-D was detected at 10 months postpartum or in the first trimester in a subsequent pregnancy. Another woman had antibodies in the first trimester in the subsequent pregnancy. The antibody screen at delivery was missing in her case and we could not clearly define if she had been sensitized in the third trimester or after delivery.

Frequencies of available antibody screening results at the follow-up time points were 88 percent in the second trimester, 74 percent at delivery, 57 percent post partum and 85 percent at least at delivery or post partum. Postpartum samples are continuously being followed-up.

6 DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Paper I

Paper I was a retrospective cohort study collecting data from medical records, local databases and registers. From 2008 and onwards data on immunized pregnancies and outcomes as well as ultrasound evaluations and intrauterine transfusions have been registered prospectively into a standardized web-based quality register (www.gravimm.se). We searched different sources of information both in the Department of Obstetrics and Gynecology and Department of Immunology and Transfusion medicine and we estimate the number of missed cases to be minimal, not influencing the results of the study. The retrospective design afflicts the study with certain limitations. Since the operators reported on their own complications in conjunction with the procedures, there is an obvious risk of record bias and misclassification. We tried to handle this problem by evaluating the procedures from all different sources, but it is obvious that the original report by the operator in some cases lack adequate objectivity and detail.

The study cohort was moderate in size with relatively few events (14 procedure complications). To analyze variables potentially associated with the risk of procedure complications, we applied a multivariate logistic regression model. Possible predictor variables that could influence the risk of procedure complications were first assessed in a univariate analysis and variables with $p \leq 0.2$ were included in the model. This gave two predictor variables (year of treatment, route of IUT) for the model when analyzing risk of complication per procedure and four variables (hydrops, GA at 1st IUT, Hb at 1st IUT, year of treatment) for the model when analyzing the risk of a procedure complication to occur per pregnancy. This resulted in few events per variable and wide confidence intervals for some of the odds ratios. This affected specifically the estimation of the risk of a complication to occur per procedure in relation to route of IUT, since the procedures were divided into four groups depending on insertion site of the needle. Logistic regression is a large sample method and usually the recommendation is to have at least ten outcome events per predictor variable for the model to be reliable [170 171]. If the cohort would have been larger and with more

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procedure complications, it might be that other predictor variables had been significantly associated with an increased risk of procedure-related complications in the logistic regression analysis. To reduce the number of predictor variables in the multivariate analysis, we used stepwise modelling (selecting only variables with P ≤ 0.2 in the univariate model). It is valid to do when predicting risks, but is not to recommend when investigating the cause of an outcome since it is not a good method for choosing potential confounders [172].

6.1.2 Paper II

The most important limitation with this study was the small sample size which resulted in very few evaluable plasma samples at term and post term since most women were delivered by then. This hampered the analysis of how many women would be protected at 11 and 12 weeks after administration of prophylaxis. In the study design, we did not plan for a plasma sample to be taken at the time of delivery, which in retrospect would have provided valuable information. However, the number of plasma concentration values at each time point enabled an estimation of the individual variation in half-life, which in combination with distribution volume determines the individual clearance of anti-D IgG. The range in individual half-life values supports the conclusion that not all women will have measurable plasma levels of anti-D IgG at term and post term if RAADP is administrated in 28-30 weeks of gestation.

6.1.3 Paper III

This was a prospective study with a large study sample including the majority of pregnant RhD negative women in Stockholm during the defined period. When reporting the results of the study, we followed the STARD protocol for studies on diagnostic accuracy (http://www.stard-statement.org). The aim of the STARD initiative is to improve the accuracy and completeness of reporting of studies on diagnostic accuracy. None of the fetal genotyping results or cord blood serology results was excluded in the final analysis and all inconclusive and missing samples were reported. Hence no important selection bias was introduced in the study. The genotyping results were read and reported to the maternity care centers before the outcome of the pregnancy was known. Cord blood serology was performed by staff not aware of the genotyping results, which means that the interpretation of both the antenatal and postnatal test was blinded. Since most of the samples were taken in the first trimester,

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before first or second trimester ultrasound dating of the pregnancy usually had been done, we had to rely on last menstrual period (LMP) for estimation of gestational age. This introduced a risk of misclassification of the gestational age of some samples since the dating according to LMP may not always be correct and since a first trimester miscarriage may not yet have been detected. For pregnancies that resulted in the birth of a baby, gestational age at blood sampling is likely to be equally under- and overestimated according to LMP and not influence the validity of the results.

Together, this guarantees a high internal validity of the study. Regarding external validity (generalizability) one can speculate that the sensitivity and specificity of the test can vary slighty between different populations since the concentration of cff DNA in maternal plasma varies with ethnicity and body weight and since the prevalence of *RHD* gene variants differs with ethnicity [159 161]. In Stockholm county, 27.6 percent of the population has a first or second generation immigrant background: European Union 10.4%, Asia and Middle East 8.2%, Europe outside the European Union 3.2%, Africa 2.8%, South America 2.0%, North America 0.7%, unknown 0.3% [173]. For screening purposes, we believe the external validity of the study is high.

6.1.4 Paper IV

This was a retrospective cohort study with its limitations regarding reliability of collected data and our findings have to be interpreted with caution. We searched the transfusion medicine registers for all women followed due to anti-D antibodies during the period. All individual medical records were reviewed. For some variables there was a considerable number of missing data, which was not included in the statistical analysis comparing difference in frequencies between the groups. The Fisher´s exact test was applied instead of a Chi-Square test since there were very few events for some variables.

The strengths of our study include centralized care and registers of all pregnancies with red cell immunization in our region. Detection and classification of when immunization occurred depend on screening frequency. We chose to be conservative in the classification of when the women were sensitized, the date of first positive anti-D titer was confirmed with the transfusion medicine LIS and if there was uncertainty the latest confirmed date was used. If there was uncertainty with which child a woman was

sensitized we classified her into the later group. This means that some women could have been sensitized earlier than described in our study. Since we were interested in practical implications for new antenatal prevention programs, we used parity instead of pregnancy when defining the three groups of RhD immunized women. Even though the reporting of previous pregnancies is generally good in Sweden, information in medical records about miscarriages and terminations might be less reliable. Because of this, there is a possibility that a miscarriage or TOP between children could have caused immunization in a few cases. For nulliparae, however, we analyzed all cases with previous miscarriages or TOP and in 5.4 percent (8/147) we could not rule out that this had been the sensitizing event.

The generalizability of our results is limited due to differences in anti-D prophylaxis guidelines and obstetric practise between countries. For Sweden, our results are considered to be valid. During the 19 years period studied, some obstetric practises have changed as well as perinatal management of HDFN. Recommendations for anti-D prophylaxis have remained unchanged. We did not investigate the effect of different time periods on timing of immunization and outcome. In addition, there is no data on failure to administer anti-D prophylaxis when indicated in our country. Even though maternity care is free of charge and the vast majority of women attend maternity care and deliver in a hospital, it is likely that postnatal prophylaxis and prophylaxis during pregnancy is forgotten in some instances. Considering the Swedish health care system, the failure rate is likely to be lower than reported from other countries [174].

6.1.5 Paper V

We performed a prospective community-based cohort study aiming at evaluating the outcome of a new prevention program in a routine setting. The aim was to include all RhD negative women in our population, but during the first six months of the study period recruitment to the program was slow, which explains the discrepancy between the number of RhD negative women who gave birth during the study period and the number of women who underwent noninvasive screening for fetal genotype. The compliance of women and health care professionals with the new protocol has increased with time. The use of an intention-to-treat analysis including all cases of new RhD immunizations (even those who did not receive RAADP) during the study period

means that our study provides a good indication of the expected effectiveness of this new program in our population.

The strengths of our study include a large study sample, prospective collection of data, central management of all cases of red cell immunization in our region and reliable registers of these pregnancies. We used two time points, both at delivery and six to ten months postpartum, to find women who seroconverted. There was loss to follow-up regarding plasma samples at all time points, but in 85 percent of cases at least one plasma sample at delivery or post partum was available for analysis of the presence of anti-D antibodies. In the reference cohort, antibody screening was performed only at 37 weeks of pregnancy and not at delivery or postpartum. Not all women in the reference cohort had a subsequent pregnancy in which antibodies from sensitization late in the third trimester or at delivery in the previous pregnancy would be found. We believe the detection rate of new RhD immunization in the two cohorts to be comparable. We expect a few more cases to appear in the study cohort on long-term follow-up. New cases of RhD immunization may also be detected in the future in women in the reference cohort who will have another child.

There is a possibility of increased awareness of RhD status of mother and child during the study period with an increased likelihood to receive anti-D prophylaxis at other possible sensitizing event during pregnancy. We cannot exclude that this was a confounder and contributed to the reduced incidence in the study cohort. In our experience, it is also possible that some caregivers omit extra prophylaxis when indicated if the woman recently received RAADP.

The incidence of RhD immunization in the reference cohort was 0.46 and hence lower than expected when designing the study. The prevalence of RhD immunization during the five-year period 2004-2008 was 0.7 percent. Since the study sample was large enough this did not affect the power of the study. The low frequencies compared to previous reports might be due to improved obstetric management of RhD negative women in situations with risk for FMH and also improved compliance to anti-D prophylaxis recommendations over the years. Most reports on the prevalence of RhD immunization with a postnatal prophylaxis program were published in the 1970s and 1980s and reports already in the 1990s show a lower prevalence compared to the earlier studies [117 124]. When calculating the incidence in the reference group, all cases with

anti-D antibodies after delivering abroad, due to anti-D prophylaxis or unclear cases were excluded. Such cases are probably not excluded in studies reporting the prevalence of RhD immunization from investigations of the presence of antibodies only [4]. The incidence of RhD immunization in the Dutch population before introduction of routine antenatal anti-D prophylaxis in 1998 was reported to be 0.5 percent, which is similar to our findings [175].

We chose an observational cohort study to assess the effectiveness of the new intervention and not a randomized controlled study. In our opinion, there is already strong evidence for the effectiveness of RAADP in preventing sensitization to the RhD antigen[122 123 125 175 176]. A randomized study in which RAADP is administered to all RhD negative women in one arm, and RAADP is offered selectively to women with an *RHD* incompatible fetus in the other arm would have been complicated to carry out in our setting and it is questionable whether such a study would have been successful or would have provided more reliable outcome data. The use of a historical control group has its limitations. However, obstetric and transfusion practice and laboratory methods have remained essentially unchanged during the reference and study periods. We believe no important selection bias or confounders were introduced in our study. The generalizability of our results is considered to be good.

When reporting the study we adhered to the STROBE guidelines (http://www.strobe-statement.org). The aim of the STROBE statement is to strengthen the reporting of observational studies in epidemiology.

6.2 FINDINGS AND INTERPRETATIONS

In paper I we confirm that RhD immunization is still the most common cause of severe HDFN requiring intensive antenatal monitoring and treatment in Sweden. When there is fetal anemia, antenatal treatment with intrauterine transfusions is efficient and results in a high perinatal survival rate. Survival rate after IUT has been reported to be 84 to 97 percent and the risk of fatal complications has been reported to vary between 0.8 to 2.5 percent per procedure. [65 66 71-75]. Similar to previous studies, we found complications to occur more often when needling a free loop of the umbilical cord, but no difference in complications rates between puncturing the intrahepatic part of the umbilical vein or at

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the site of the placental cord insertion[71 73]. A common symptom was fetal bradycardia, which is known to occur when an umbilical artery is punctured instead of the vein or when the vessel is perforated leading to fetal bleeding. Needling problems was one of the most common causes of complications. We were not able to analyze the influence of operator experience regarding complication risks, but it has been shown that to perform safe IUTs requires a certain learning curve and number of procedures per year to maintain the skills [177]. It might be that some complications could have been avoided if more experienced staff had been at hand or if the procedure had been postponed until the fetus was in a more favorable position. Many procedure-related complications occurred late in gestation. This is similar to findings from the Dutch national center when evaluating 740 IUTs it was found that advancing gestational age was correlated with procedure complications [73]. It might be due to an increased risk when needling the umbilical vein in late gestation but also to a lower operator threshold to perform an emergency delivery in the third trimester of pregnancy if fetal bradycardia is observed[73]. We also found that a procedure complication was more likely to occur when IUTs had to be started early in pregnancy. This is usually necessary in cases of very severe HDFN that will require many procedures during the pregnancy, even though the total number of IUTs per pregnancy was not significantly correlated with the risk of complications in our analysis. Other factors, shown to be associated with an increased risk of complications in other studies, are procedures before 22 weeks of gestation and the presence of hydrops fetalis [72 178 179]. Of the seven perinatal deaths that occurred in our cohort, four presented with hydrops fetalis. Two of those required the first IUT already in the 20th week of gestation. In early onset severe RhD immunization, the fetus can be severely anemic with Hb < 50 g/L without signs of hydrops [179]. In these cases, an early risk assessment (< 16 weeks) evaluating the obstetric history and antibody concentrations is important. Intraperitoneal transfusions can be considered before intravascular access can be safely achieved [78]. Intravenous immunoglobulin (IVIG) treatment started early in the second trimester to the mother can be considered as well, but so far there are no randomized controlled studies proving the efficiency of IVIG.

We suggest that complications due to intrauterine blood transfusions as well as perinatal mortality and morbidity in severe hemolytic disease of the fetus and newborn can be further reduced by:

- starting treatment before severe anemia and hydrops fetalis develops. This requires close monitoring of high-risk pregnancies with MCA PSV Doppler and timely referrals to a specialized center before sonographic signs of hydrops appears. Evaluation of the obstetric history and antibody concentrations are important as well in detecting high-risk cases that will benefit from early referral.
- puncturing the intrahepatic part of the umbilical vein or the cord root in the placenta when performing cordocentesis
- always being prepared to transfuse directly if needed when performing cordocentesis
- centralizing invasive treatment since a high level of expertise is required in severe and early cases and the procedures should be performed by a few experienced operators
- maintaining the pregnancy until term (37 weeks) to avoid additional morbidity due to prematurity

Interestingly, 10/72 women in paper I had a history of intrauterine fetal death which was likely the sensitizing event. This finding marks the importance of evaluating FMH in cases of intrauterine fetal death and of providing anti-D prophylaxis as soon as possible after diagnosis in RhD negative women.

In paper II, we were interested in the duration of measurable prophylactic levels of anti-D at term. There were enough data points for analysis until ten weeks after administration of anti-D prophylaxis but at 11 weeks it was only possible to analyze one sample and at 12 weeks after injection all women were delivered. Concentrations of anti-D ten weeks after injection varied from 1-4 ng/mL. If extrapolating the individual pharmacokinetic linear curves in the 12 women with a value for C_{last}, 75 percent (9/12) would have had anti-D IgG concentrations ≥ 1 ng/mL at the time of delivery. Seven of these 12 women delivered < 11 weeks after injection and five women at about 11 weeks after injection. Our findings are in line with previous studies reporting individual variations in clearance of anti-D IgG and in plasma concentrations at delivery. In these studies, detectable levels of anti-D after injection at 28 weeks of gestation were found in 30-60 percent of women 11-12 weeks later [116 129 131]. Clearance depends on the half-life and distribution volume. Our study showed an individual

variation in half-life between women from 12.5 to 30.3 days, which supports the conclusion that not all women will have protective levels of anti-D IgG at the time of delivery. Uptake from muscular compartments may vary as well. Eventually consumption of anti-D in case of larger fetomaternal hemorrhage will affect the remaining amount and duration of anti-D IgG. Lower maximal plasma concentrations was seen in obese women and this has also been reported by other authors [131]. However, body weight does not seem to influence the duration of anti-D in plasma, which means protection against sensitization is expected to be the same independently of body weight.

In theory, a two-dose protocol (100 μg in 28 and again in 34 weeks of gestation), which is used in the UK, provides higher plasma concentrations at term compared to a single dose and less anti-D is used to achieve protection. This could not be confirmed in a recent study evaluating that protocol [131]. Only 30 percent of women had plasma concentrations of 2 ng/mL or greater at 40 weeks of gestation. Compliance to a second dose may be low and in that case the protection in late pregnancy will be less [120].

It is not clear how much anti-D is required to prevent sensitization in an individual pregnant woman. Only a small proportion of women (0.90 %) will have an FMH during gestational weeks 30-39 that will exceed 1 mL of fetal blood[13]. Of these, about 20-30 percent will be non-responders to a possible sensitizing event [101]. Theoretically, a plasma concentration of 1 ng/mL corresponds to a total amount of residual anti-D IgG of 10 μg, which is believed to be enough to neutralize 1 mL of fetal blood[101 102 13 131]. Pregnant women differ from non-pregnant women and men in distribution volumes and metabolism of drugs [180-182]. The original dose studies on anti-D prophylaxis, forming the basis for recommendations, were carried out in male prison volunteers. Apart from differences in distribution volumes and metabolism, the immune system in a pregnant woman is "reprogrammed" towards active tolerance to paternal antigens and the route of antigen delivery is different as well [183 184]. Translation of what corresponds to a protective plasma level in pregnant women from these original dose studies cannot easily be done.

In paper III we estimated the diagnostic accuracy of a new method to determine fetal *RHD* genotype from cell-free DNA in maternal plasma in early pregnancy. This study is so far the largest published study on non-invasive fetal *RHD* testing. We studied the

performance of the test in an unselected pregnant population and the majority of the samples were taken and analyzed in the first trimester of pregnancy. We show that the test has a high diagnostic accuracy and can be used and trusted in a routine clinical setting for screening purposes. The frequencies of reanalyses and inconclusive results were low. Most of the samples taken before 8 weeks of gestation were correctly diagnosed, but we found the false negative rate to be too high. After this was recognized (August 2010), blood samples taken before 8 weeks of pregnancy were not analyzed and the guidelines to the maternity care centers were changed. A new blood sample was requested if the first one was taken too early.

The purpose of our work was to develop a test suitable for screening in a routine clinical setting, considering practicality, cost and safety. We used only exon 4 in our analysis, which differs from the recommendations from the SAFE network (The Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network) [185]. They recommend a combination of exon 5 and 7 to detect the pseudogene (*RHD*φ) present in many individuals with African descent[17 186]. Our test was designed specifically not to detect the pseudogene. We believe it is desirable not to detect a maternal or fetal pseudogene in a screening assay with the purpose to provide anti-D prophylaxis to women with risk of RhD immunization. An individual with the pseudogene does not produce an RhD antigen that can induce an immune response and will be typed RhD negative with serology. A woman with the pseudogene will benefit from prophylaxis if she carries an *RHD* positive fetus.

The false negative rate was higher than in recent large-scale *RHD* screening studies performing the test after 25 gestational weeks. This is expected since the concentration of fetal DNA is lower in maternal plasma in early pregnancy[157 187]. From 22 weeks of gestation there were no false negative results and sensitivity was 100 percent, which proves that the single-exon strategy is as reliable as other approaches using different combinations of exons[55 126 127 165 166]. Among samples defined as false positive compared to cord blood serology, we suspect some be due to a variant *RHD* gene in the child (and hence true positives), not detected by routine serology methods. The prevalence of *RHD* gene variants in the Swedish population is not known and these false positive samples will be analyzed further with genomic typing. The majority of the first set of inconclusive samples, where a new blood sample needed to be requested, was due to too high levels of maternal DNA in the sample after long storage and

transport times. We showed that storage times up to three days gave reliable results. Others have reported that transport times up to five days can be accepted in the analysis of second trimester samples[188]. But when the analysis is performed in the first trimester a too high level of maternal DNA increases the risk of missing a weak fetal *RHD* signal since the concentration of fetal DNA is much lower. All inconclusive results after a second plasma sample were confirmed to be due to a maternal *RHD* gene variant.

The advantage with fetal *RHD* genotyping in the first trimester of pregnancy is not only the possibility to selectively provide RAADP in the beginning of the third trimester to women with and *RHD* positive fetus, avoiding exposure of women who will not benefit from the prophylaxis. Unnecessary anti-D prophylaxis can also be avoided earlier in pregnancy, after miscarriages and terminations of pregnancy as well as after invasive prenatal testing (i.e. amniocentesis and chorionic villus sampling). Approximately 40 percent of the women will carry a compatible *RHD* negative fetus and need not to be followed with repeat antibody screening test during the pregnancy. Knowing the fetal *RHD* genotype already in the first trimester also enables future strategies to prevent early RhD sensitization in the second trimester of pregnancy.

The risk of false negative results can be further reduced by using a control to confirm the presence of fetal DNA in the samples[152]. But so far no optimal control for fetal DNA has been identified. To add a fetal DNA control to the analysis adds workload, extra cost and potentially more reanalyses, which makes it of limited value to use in a PCR based screening test where the false negative rate is low to begin with[142]. However, in the near future next generation sequencing (NGS) will be available in the clinic. These efficient methods enable fast sequencing of many fetal DNA sequences from cell-free DNA in maternal plasma. It is likely that NGS will replace PCR based analyses and include *RHD* determination. This technique will also allow correct determination of *RHD* gene variants as well as including for example analysis of polymorphic markers that makes it possible to distinguish fetal from maternal DNA.

In paper IV we found that half of the RhD immunized women in the cohort became immunized with their first-born child and one third with their second born child. Forty-six percent developed anti-D antibodies in the third trimester. The results of this study confirm the assumption that at least half of the cases could have been prevented by RAADP in the beginning of the third trimester of pregnancy. In addition, it is possible

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that some of the women classified as immunized after delivery may have been sensitized close to term or post term, but not detected at that time since the last routine antibody screening test was done around 37 weeks.

In the index pregnancies, the frequency of severe HDFN (i.e. requiring IUT or exchange transfusion) was low but more than a third of the neonates required phototherapy and hence an extended stay in the hospital. The frequency of phototherapy appeared higher in the group of women immunized with their second child. It might reflect that a larger proportion of women in this group seroconverted already in the second trimester, but this finding has to be interpreted with caution since data was missing in many cases. There was also a difference regarding IUT in the index pregnancies between the groups. This might reflect a lower threshold for intervention in multiparous women, but the absolute numbers were small.

Although primiparous women of natural reason will give birth to more children after the index pregnancy than women immunized in later pregnancies, the severity of the disease seems to be similar. In subsequent pregnancies, more than half (56%) of the neonates suffered from HDFN requiring treatment and 25 percent needed IUT and/or exchange transfusion. There was no difference between the groups. In recent studies from both France and the Netherlands, a similar risk of 20-25 percent of developing severe HDFN was found, once RhD immunization had occurred [175] [189]. In an older Swedish study (1980-1991) the frequency of HDFN and severe HDFN was comparable, 46 and 29 percent respectively [12]. Paper I and IV confirm that HDFN is still a cause of perinatal morbidity and mortality. The risk of additional morbidity and length of stay in the hospital increases with prematurity. Paper IV showed that overall prematurity < 34 weeks was as low as 2 percent, but it was as high as 24 percent in the subgroup requiring IUT, as shown in paper I.

In both paper IV and V, 10-25% of RhD immunized women was immunized in the second trimester of pregnancy in spite of a negative antibody test in the first trimester. Our findings confirm the original incidence studies in Canada from the 1960s and 1970s. The Canadian studies showed that, before introduction of postnatal anti-D prophylaxis, 10-20 percent of RhD negative primigravidas became immunized during ongoing pregnancy and eight percent of these women developed anti-D antibodies before 29 weeks of gestation. In 16 percent, anti-D antibodies were present already in

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gestational week 29-34, indicating that sensitization occurred before the third trimester of pregnancy [5][6]. Our findings are also in line with the results from a study on the Dutch RhD prevention program, in which the incidence of immunizations occurring between 12 to 30 weeks of pregnancy was 0.24 percent before introduction of RAADP and 0.25 percent after [175]. It might be that these women represent a subgroup with more aggressive immune response with a higher risk for severe HDFN in their offspring. This is a hypothesis and has to be confirmed in future studies. Early immunization also increases the exposure time to hemolytic antibodies for the fetus and risk of HDFN in the index pregnancy requiring postnatal treatment. Since the overall aim with anti-D prophylaxis is to prevent HDFN and considering the possibility to provide prophylaxis selectively to women with RhD incompatible fetuses, it might be reasonable to provide anti-D prophylaxis routinely in the beginning of the second trimester as well, at least in nulliparae. The cost-effectiveness of such a strategy needs to be evaluated. This decision will also depend on the availability and cost of hyperimmunized D plasma and the possible replacement by monoclonal or recombinant anti-D IgG [190].

In paper V we show that first trimester noninvasive antenatal screening for fetal *RHD* combined with routine antenatal anti-D prophylaxis given in 28-30 weeks of pregnancy selectively to women with *RHD* positive fetuses significantly reduce the incidence of RhD immunizations. The risk reduction is comparable to what has been reported in studies evaluating the outcome of giving RAADP to all RhD negative women [122][123][175] [176]. The use of the selective approach means that women not at risk for sensitization avoid unnecessary exposure to anti-D immunoglobulin and health care resources may be more wisely used. The NNT to prevent one sensitization was 400, which means that noninvasive *RHD* screening would have to be performed in 400 pregnancies (leading to a delivery), but only about 60 percent (NNT 240) of these would need RAADP. However, the NNT to prevent one case of severe HDFN in the subsequent pregnancy (occurs in about 25%) will be higher and depends on how many more children each woman will have. In the cohort reported in paper IV, 89 percent of women immunized with their first child had at least a second child. The same proportion of women immunized with their second and third or later child, was 48 and 38 percent, respectively.

Screening of fetal red blood cells in maternal blood after delivery to assess the magnitude of FMH, which is recommended in some countries, is not routinely

performed in Sweden [14][103]. In a few women the standard dose of 250-300 μg will not be sufficiently protective in case of a large FMH. This is estimated to cause immunization in only 0.08 percent of RhD negative women at risk [7]. Risk factors for RhD immunization in spite of antenatal and postnatal prophylaxis include assisted delivery and cesarean section, postmaturity, pregnancy-related red blood cell transfusion and younger age [191]. However, in as many as 43 percent of women no risk factor is identified and to find women with large FMH routine post delivery screening for fetal RBCs would need to be carried out. One of the two women confirmed to have been sensitized at delivery in our study cohort had undergone an elective caesarean section.

The effect of RAADP depends not only on the size and frequency of silent FMH during the third trimester, but also on the time interval between administration and delivery, as shown in paper II. Two of three women immunized in spite of RAADP in our study cohort in paper V delivered after 41 weeks of gestation (11 and 12 weeks after injection of RAADP). The risk and size of fetomaternal hemorrhages during pregnancy increase with gestation and are the greatest close to term and post term [123]. The results in paper II, IV and V indicate that a proportion of women will be at risk of becoming sensitized at term and post term if not delivered, even if RAADP in the beginning of the third trimester is administered. The problem of too low residual anti-D concentration in post term pregnancies (12 weeks after injection) was recognized by Bowman in Canada already in the 1980s [7]. Others have come to similar conclusions [132][192]. In a study on 407 women receiving the two dose prophylaxis (100 μg) and 157 women the one dose (300 μg) showed that 39 percent of women in the former group and 78 percent in the latter had no detectable anti-D at delivery [193]. However, the authors used a higher cut-off level of detectable anti-D concentrations than we did in our study. The dose recommendations for RAADP is based on the mean half-life of anti-D IgG of approximately 23 days, which in most cases should result in residual protective levels 12 weeks after injection. However, a considerable number of women have a faster clearance of anti-D IgG due to shorter half-lives [129][131]. In our study 4/16 women had a half-life of less than 16 days. Providing RAADP in 30 weeks of gestation, as in the Netherlands, would not solve the problem with pregnancies continuing after 40 weeks. In the view of the theoretical risk of transmission of infectious diseases associated with anti-D prophylaxis it is important that if given the dose should be enough to prevent immunization.

We propose it should be considered to give the next injection of anti-D prophylaxis in 38-39 weeks of pregnancy, to women not delivered. Provided a high enough dose is administered at 38 -39 weeks, the anti-D IgG will last long enough to protect after delivery and can replace the standard post partum prophylaxis in most instances, as a postnatal dose of 100 μg is usually sufficient [53 101 102]. Whether a repeat dose at 38-39 weeks of gestation can safely replace the postnatal administration requires further investigations. The American Congress of Obstetricians and Gynecologist´s guidelines states that if delivery occurs within three weeks of a standard antenatal administration of anti-D, the postnatal dose made be withheld in the absence of excessive fetomaternal haemorrhage. It could also be considered to give a higher dose than 300 μg at the 28 weeks injection. However, this would require larger amounts of anti-D.

Should we strive to reduce RhD immunizations further when the incidence with existing recommendations is low, most cases are detected in time with antibody screening programs and the antenatal and neonatal management is successful in most cases? In general, prevention is to prefer before treatment and the most important way in which perinatal mortality and morbidity from HDFN can be reduced further is to prevent maternal RhD immunization. With the possibility to give prophylaxis selectively to women with RhD incompatible fetuses the cost of providing RAADP to all women independently of parity will be reduced and the supply of anti-D IgG will be more wisely used. Is it worth the cost? The British National Institute of Clinical Excellence estimated it to be cost-effective to introduce non-selective RAAPD to all RhD negative pregnant women in 2002 as well as in a follow-up study five years after its introduction. However, the results of such an analysis will depend on different populations and health care settings, the cost and sensitivity of the genotyping and the cost of the administration of RAADP. Selective RAADP is most likely cost-effective in the first RhD incompatible pregnancy, but whether it is in subsequent pregnancies remains an open question and has to be addressed in future studies.

7 CONCLUSIONS

RhD immunization is still the most common cause for severe hemolytic disease of the fetus and newborn requiring intrauterine treatment.

Perinatal survival in pregnancies requiring intrauterine blood transfusion is high. The risk of procedure-related complications can be further reduced if avoiding cordocentesis in a free loop of the umbilical cord. Perinatal mortality may be reduced with timely referrals to a specialized center before severe anemia or hydrops fetalis develops.

Routine antenatal anti-D prophylaxis in 28-30 weeks of pregnancy usually lasts for ten weeks after injection but thereafter concentrations are variable and not all women will have detectable anti-D levels at term and post-term.

Without routine antenatal anti-D prophylaxis, the majority of women become RhD immunized during pregnancy with their first or second child, most often in the third trimester. The risk of hemolytic disease of the fetus and newborn is the same regardless of in which order of pregnancy a woman become immunized and occurs in more than half of subsequent pregnancies.

Non-invasive fetal *RHD* screening in the first trimester of pregnancy, using a single-exon PCR assay, can be performed with a high diagnostic accuracy.

Routine antenatal anti-D prophylaxis in gestational weeks 28 - 30 selectively to RhD negative women with *RHD* positive fetuses significantly reduces the risk of RhD immunization to 0.21 percent.

8 CLINICAL IMPLICATIONS

The results of this thesis lead to some clinical implications and suggestions.

First, the results of paper I lead to the conclusion that the management of intrauterine blood transfusions should be centralized to a specialized center with a high as possible expertise in performing the procedure. In 2012 the National Board of Health and Welfare decided that all cases requiring intrauterine transfusions in Sweden should be centralized to one national center, starting 1 of January 2013. Timely referrals will be necessary to further reduce the risk of perinatal mortality and morbidity in severe cases.

It is evident from this thesis that even if studying cases of RhD immunization in Stockholm during a time period as long as 20 years, it is hard to assemble a study size large enough to allow reliable statistical analysis. The introduction of selective RAADP will lead to a further reduction in the prevalence of RhD immunization in our country. National collaboration in clinical significant immunizations and HDFN as well as a national register could be a way to address this problem and maintain competence and enable future development and research.

To further reduce the incidence of RhD immunization in our country and to use anti-D IgG efficiently, we propose national introduction of first trimester non-invasive screening for fetal *RHD* combined with selective routine antenatal anti-D prophylaxis in 28 weeks of gestation. A study on cost-effectiveness of selective RAADP is underway and planned to be published in 2013.

In addition we suggest an optimized screening and prevention program:

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┌─────────────────────────────────────────────────────────────┐
│                      10 weeks GA                             │
│        Blood group typing, red cell antibody screening,     │
│      non-invasive screening fetal RHD in RhD negative women │
└─────────────────────────────────────────────────────────────┘

┌──────────────────────────────┐    ┌──────────────────────────────┐
│      Fetal RHD positive       │    │      Fetal RHD negative       │
│  Antibody test at 25 weeks GA │    │      No more antibody tests    │
│      or just before RAADP     │    │ No anti-D prophylaxis during   │
│                               │    │  pregnancy or after delivery   │
└──────────────────────────────┘    └──────────────────────────────┘

┌──────────────────────────────┐
│         28 weeks GA           │
│     250-300 µg anti-D IgG     │
└──────────────────────────────┘

┌──────────────────────────────┐
│  Delivery (or 38-39 weeks GA) │
│     250-300 µg anti-D IgG     │
└──────────────────────────────┘
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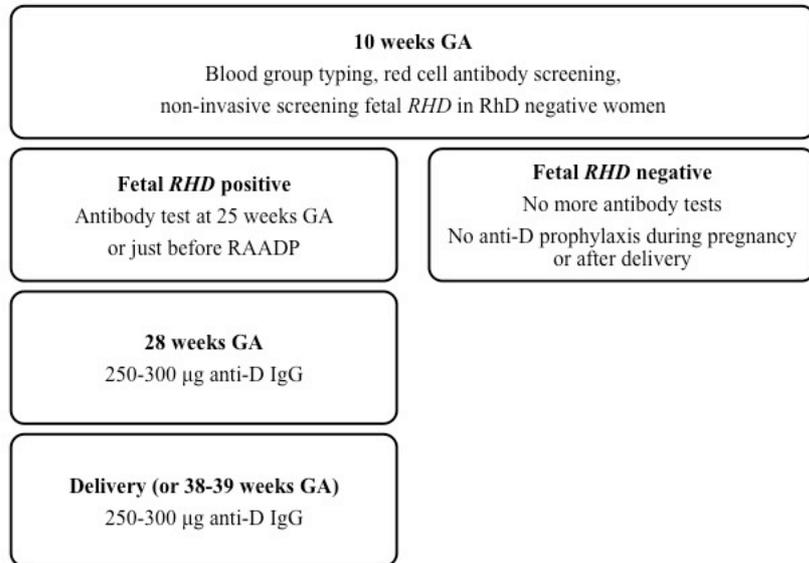
Most clinical significant RhD immunizations will be detected in the first or second trimester of pregnancy. In women who seroconvert during the third trimester of pregnancy, severe hemolytic disease of the fetus and neonate is very rare. In addition, after administration of RAADP it can be difficult to discriminate between passive anti-D IgG due to prophylaxis from immune anti-D. Since the *RHD* genotype of the fetus will be known already during pregnancy, cord blood serology will not be necessary to determine which women should receive prophylaxis. DAT testing and blood group serology in the newborn is advised only on clinical indications.

9 SUGGESTIONS FOR FUTURE RESEARCH

The long-term results of the selective RAADP program in terms of reduction in the incidence of severe hemolytic disease of the fetus and newborn requiring intrauterine blood transfusion and intensive neonatal care will be evaluated in the future. There is also a need for studies on the long-term outcome in children treated with intrauterine transfusions.

A cost-effectiveness study on selective RAADP remains to be done. It is underway and planned to be published in 2013. If routine second trimester prevention is to be considered as well, the clinical and economical effect of this has to be investigated.

The failure rate in providing anti-D prophylaxis when indicated in our country is not known. Studies on this issue and actions to improve compliance could further reduce the incidence of RhD immunization.

If a repeat dose of anti-D prophylaxis in gestational age 38-39 weeks is to replace the postnatal administration, this requires detailed studies on the anti-D levels at delivery and follow-up studies on RhD negative women.

Perinatal loss rate due to red cell immunization and the incidence of cerebral palsy and other major neurological sequele due to HDFN in our country are not known and would be interesting to study.

10 SVENSK SAMMANFATTNING

10.1 BAKGRUND

Ca 15 procent av den svenska befolkningen har en RhD negativ blodgrupp. Under graviditet kan detta orsaka problem i de fall en RhD negativ kvinna bär ett RhD positivt foster. Läckage av fostrets röda blodkroppar till mammans cirkulation (fetomaternell blödning) kan aktivera hennes immunförsvar till att bilda antikroppar mot fostrets röda blodkroppar (immunisering). Antikropparna leder till att fostrets röda blodkroppar bryts ner (hemolys) och orsakar efter hand blodbrist. I allvarliga fall leder detta till hjärtsvikt och ansamling av vätska i fostret (hydrops fetalis) och slutligen fosterdöd. I de fall allvarlig blodbrist uppstår redan under graviditeten kan fostret behandlas med ultraljudsledda blodtranfusioner för att minska risken för fosterdöd. Hos det nyfödda barnet leder den ökade nedbrytningen av röda blodkroppar också till blodbrist men framförallt till att en nedbrytningsprodukt – bilirubin – ansamlas och kan orsaka neurologiska skador hos barnet. Den nyfödde behöver ofta behandling med intensiv ljusbehandling och utbytestransfusion samt ofta också vanliga blodtransfusioner.

Risken för att RhD immunisering ska inträffa kan minskas genom att ge Rh profylax till modern vid situationer under graviditeten som innebär en ökad risk för fetomaternell blödning. Denna risk är som störst i samband med förlossning och genom att införa Rh profylax efter förlossning i början av 1970-talet minskade risken för RhD immunisering från 14 till 1-2 procent. Rh profylax ges också under graviditet vid situationer med ökad risk för fetomaternell blödning, såsom kirurgisk abort, sent missfall, fostervattenprovtagning och yttre vändning. Rh profylax består av anti-D antikroppar utvunna ur plasman från RhD immuniserade blodgivare. Den exakta verkningsmekanismen är okänd, men profylaxen förhindrar att moderns immunförsvar aktiveras mot fostrets röda blodkroppar. Med ovanstående profylaxrutin så uppstår majoriteten av nya immuniseringar under pågående graviditet, oftast till följd av tyst fetomaternell blödning i tredje trimestern. För att förhindra detta så ger man rutinmässigt i många länder ytterligare en spruta Rh profylax i början av tredje trimestern till samtliga RhD negativa gravida. Studier har visat att denna preventiva åtgärd ytterligare minskar incidensen RhD immunisering till 0.2-0.3 procent. Nackdelen med att ge Rh profylax till samtliga RhD negativa gravida är att ca 40 procent bär ett RhD negativt foster och är inte utsatta för risk att bilda antikroppar.

Denna grupp får förebyggande behandling i onödan. Upptäckten av cell-fritt DNA från fostret i en gravid kvinnas blod har lett till möjligheten att med molekylärbiologisk teknik riskfritt bestämma fostrets RhD genotyp redan under graviditeten. Genom att använda denna metod kan Rh profylax under graviditeten ges selekterad endast till de kvinnor som har nytta av den.

10.2 SYFTE

Det övergripande syftet med denna avhandling var att undersöka om nya fall av RhD immunisering kan reduceras genom att rutinmässigt erbjuda riktad Rh profylax under graviditeten till kvinnor med RhD positivt foster samt om komplikationer vid blodtransfusioner till fostret kan förhindras. I delarbete I undersökte vi komplikationsfrekvensen och perinatal dödlighet och sjuklighet vid blodtransfusioner till foster med hemolytisk sjukdom till följd av immunisering hos modern. Vi försökte också hitta faktorer som var relaterade till ökad risk för komplikationer i samband med ingreppet. I delarbete II studerade vi hur länge profylaktiska anti-D antikroppar finns kvar i den gravida kvinnans cirkulation efter injektion i graviditetsvecka 28-30. Syftet med delarbete III var att utvärdera den diagnostiska säkerheten hos ett nytt test för att bestämma fostrets RhD genotyp i ett blodprov från mamman. I delarbete IV ville vi beskriva i vilken graviditet och när under graviditeten RhD immuniserade kvinnor i Stockholm 1990-2008 blivit immuniserade och konsekvenserna i form av perinatal sjuklighet och död hos de barn de fick. Slutligen utvärderade vi i delarbete V möjligheten att minska incidensen av RhD immunisering hos gravida kvinnor genom att erbjuda riktad rutinmässig Rh profylax i början av tredje trimestern till de kvinnor som bär ett inkompatibelt RhD positivt foster.

10.3 DELARBETE I

I denna retrospektiva studie inkluderades samtliga fall av blodtransfusion till foster, på grund av immunisering, som utförts i Stockholm 1990-2010. Medicinska data hämtades från journaler samt lokala databaser och register. Totalt 284 transfusioner genomfördes i 84 graviditeter. Total perinatal mortalitet var 8,2% och av de levande födda barnen var graviditetsålder vid födelsen i median 36 veckor, varav 24% föddes före graviditetsvecka 34. Alla utom två nyfödda barn behövde ljusbehandling och 61% behandlades dessutom med utbytestransfusioner. Komplikationer inträffade vid 4,9% av ingreppen och 1,4% ledde till dödlig utgång. Efter logistisk regressionsanalys visade

det sig att transfusion i fri navelsträngsslynga samt behov av att starta transfusioner tidigt i graviditeten var korrelerat till komplikationsrisk. Majoriteten av fallen (83%) av svår fetal hemolytisk sjukdom berodde på RhD immunisering hos modern.

10.4 DELARBETE II

Delarbete II var en prospektiv observationsstudie som inkluderade 16 gravida kvinnor vilka redan ingick i en större kohortstudie i Stockholm (delarbete V). Kvinnorna erhöll 250 µg (1250 IU) anti-D IgG intramuskulärt i graviditetsvecka 28-30. Plasmakoncentration anti-D IgG bestämdes genom blodprover som togs med förutbestämda intervall efter injektionen och analyserades med flödescytometri. Halveringstiden räknades ut med linjär regressionanalys. Maximal plasmakoncentration uppmättes mellan dag 3-10 efter injektion och var i medianvärde 25 ng/ml (9-58 ng/ml). Halveringstiden beräknades till i median 23 dagar (12.5-30.3 dagar). Mätbara anti-D IgG nivåer fanns hos 11/12 kvinnor inom två veckor före förlossning. Av de fyra kvinnor som ej var förlösta 10 veckor efter injektion så hade samtliga mätbar plasmakoncentration mellan 1-4 ng/mL. Studiens resultat belyste att om rutinmässig Rh profylax ges i graviditetsvecka 28-30 har sannolikt inte alla kvinnor fullt skydd i fullgången graviditetstid och särskilt inte de kvinnor som går över tiden.

10.5 DELARBETE III

Studien var en prospektiv kohortstudie som startade 1 september 2009 i Stockholm. I blodprover som rutinmässigt tas på gravida vid inskrivning vid barnmorskemottagning analyserades fetal *RHD* genotyp i blodprover från samtliga RhD negativa kvinnor. Cell-fritt DNA isolerades från proverna och analyserades med avseende på förekomst av *RHD* genen med RT-PCR. Som referensmetod för att utvärdera tillförlitligheten hos testet användes blodgruppsserologi utfört på navelsträngsblod vid födelsen. 4118 kvinnor vars graviditetsutfall var känt per 1 maj 2011 inkluderades i analysen. Mediangestationsålder för provtagning var 10 graviditetsveckor och 75,5 procent av proverna var tagna i första trimestern. Efter exklusion av blodprover tagna mycket tidigt i graviditeten, före graviditetsvecka 8, var sensitiviteten respektive specificiteten båda 98,9% för metoden. Från graviditetsvecka 10 var sensitiviteten 99.3 procent och från vecka 22 100%.

10.6 DELARBETE IV

Delarbete IV var en retrospektiv studie som inkluderade samtliga RhD immuniserade gravida kvinnor i Stockholm 1990-2008. Under denna period fick RhD negativa kvinnor inte rutinmässigt antenatal Rh profylax, 290 kvinnor identifierades genom sökning i Blodentralen Karolinskas register. Medicinska data hämtades från journaler, databaser och lokala register. Av kvinnorna blev hälften (51%) immuniserade i samband med sitt första barn och en tredjedel (33%) i samband med sitt andra barn. Majoriteten (73%) utvecklade anti-D antikroppar under pågående graviditet, oftast i tredje trimestern. Mer än en tredjedel (39%) av barnen vid den första immuniserade graviditeten behövde behandling för hemolytisk sjukdom, oftast endast ljusbehandling. I efterföljande graviditeter behövde 56 procent av barnen behandling för hemolytisk sjukdom,: 53 procent ljusbehandling, 26 procent utbytestransfusioner efter födelsen och 20 procent blodtransfusioner till fostret under graviditeten. Risken för hemolytisk sjukdom i efterföljande graviditeter var densamma oavsett i vilken graviditet kvinnan blev immuniserad. Den totala perinatala mortaliteten i gruppen till följd av hemolytisk sjukdom eller som komplikation till behandling var 1.6 procent.

10.7 DELARBETE V

Denna studie var en prospektiv kohortstudie inkluderande alla RhD negativa gravida kvinnor i Stockholm, som inte var RhD immuniserade i början av graviditeten, från 1 september 2009 till och med 31 december 2011. Genom studien som utgör delarbete III genotypades alla foster till RhD negativa kvinnor och de som bar ett RhD positivt foster erbjöds en extra spruta Rh profylax i graviditetvecka 28-30. Studiekohorten definierades som de kvinnor som fått antenatal Rh profylax och födde barn mellan 1 januari 2010 och 31 mars 2012. Jämförelsegrupp utgjordes av samtliga RhD negativa gravida i Stockholm 2004-2008. Nya fall av RhD immunisering definierades som kvinnor som utvecklade anti-D antikroppar under graviditeten eller efter förlossning. Statistik på antal RhD negativa förlossningar under de två perioderna hämtades från den regionala förlossningsdatabasen (Obstetrix, studieperioden) samt från Medicinska Födelseregistret (2004-2008). Incidensen i jämförelsegruppen bestämdes genom att söka i det regionala kvalitetesregistret för graviditetsimmunisering (www.gravimm.se) efter alla kvinnor som immuniserats under en graviditet eller efter en förlossning 2004-2008. Datum för första positiva antikroppsprov verifierades med Blodcentralens laboratoriedatabas.

9380 RhD negativa kvinnor födde barn i Stockholm från 1 januari 2010 till och med 31 mars 2012. Icke-invasiv fetal genotyping genomfördes i 8374 gravidieter, varav 3270 foster var RhD negativa (39%) och 5104 (61%) RhD positiva. I 4590 av dessa graviditeter administrerades antental Rh profylax. Under studieperioden och uppföljningstiden har 20 nya fall av RhD immunisering upptäckts. Det resulterar i en incidens på 0.21 percent (95% CI 0.12 - 0.31) (20/9380). I jämförelsegruppen var incidensen 0.46 percent (95% CI 0.37 - 0.56) (86/18.546), vilket resulterar i ett risk ratio (RR) för RhD immunisering på 0.46 (95% CI 0.28 to 0.75). Den absoluta skillnaden i risk var 0.25 procent och NNT (number needed to treat) 400. Riskreduktionen var statistiskt signifikant (P=0.0013).

10.8 SLUTSATSER

- RhD immunisering är fortfarande den vanligaste orsaken till allvarlig hemolytisk sjukdom hos foster och nyfödda och till att blodtransfusion till foster krävs under graviditeten.

- Den perinatala överlevnaden efter blodtransfusion till foster är hög. Komplikationsfrekvensen relaterad till ingreppet kan minskas genom att undvika att sticka i fri navelsträngsslynga och genom att i tid remittera riskgraviditeter till ett specialiserat centrum innan dess att uttalad hemolytisk sjukdom uppstår hos fostret.

- Anti-D profylax given i graviditetsvecka 28-30 räcker i minst tio veckor efter injektion för de flesta kvinnor. Därefter varierar mätbara koncentrationer mellan kvinnor och inte alla kommer att ha tillräcklig mängd kvar för skydd i fullgången tid och vid överburenhet.

- Utan rutinmässig Rh profylax i tredje trimestern blir de allra flesta kvinnor immuniserade med sitt första eller andra barn och under pågående graviditet. Konsekvenserna för efterföljande barn är desamma oavsett i vilken graviditet kvinnan blivit immuniserad och hälften behöver behandling för hemolytisk sjukdom.

- Icke-invasiv bestämning av fostrets RhD genotyp i första trimestern har hög tillförlitlighet jämfört med blodgruppering av navelsträngsblod vid födelsen.

- Rutinmässig riktad Rh profylax i graviditetsvecka 28-30 till RhD negativa kvinnor med RhD positivt foster minskar risken för RhD immunisering till 0,21 procent.

Resultaten i denna avhandling kan bidra till att minska komplikationsfrekvensen för de graviditeter som kräver behandling med blodtransfusion till fostret samt till att optimera program för prevention av RhD immunisering under graviditet.

11 ACKNOWLEDGEMENTS

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