# Prevention of Haemolytic Disease of the Fetus and Newborn with Reference to Anti-D

by

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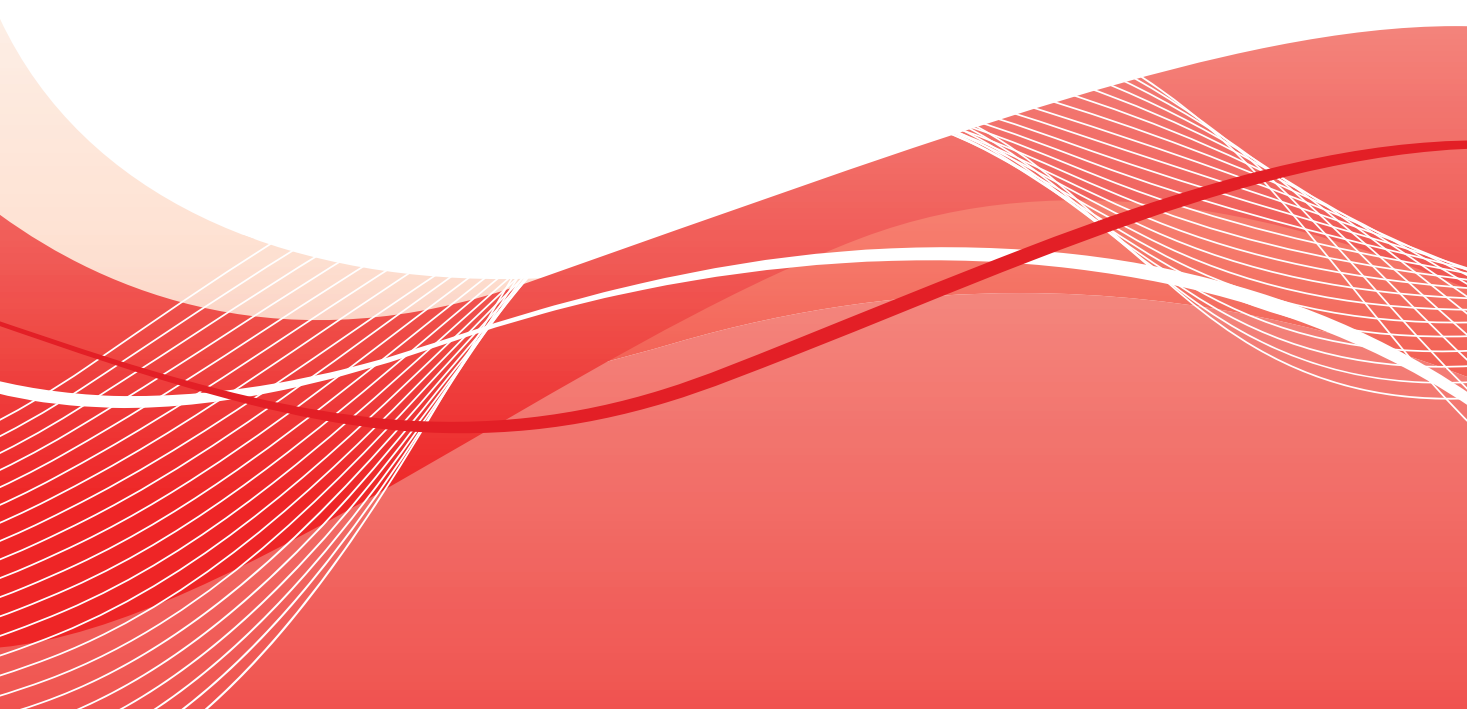

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## Abstract

**Aim:** This study was undertaken to assess the frequency of all immunization in a retrospective review cohort of RhD-negative and RhD-positive pregnant women in a region of Saudi Arabia and to assess the severity of hemolytic disease of the fetus and newborn (HDFN). The results were compared with figures from international literature to evaluate possible measures, such as more awareness of hemolytic disease of the fetus and newborn, to improve the care to pregnant women and their babies.

**Method:** This is a retrospective study at maternity and children hospital; and at the regional laboratory and blood bank, Dammam from January, 2012 to December, 2013. Laboratory and clinical data on ABO, RhD, Rh phenotype, K status, red cell antibodies, and if applicable: red cell antibody titers, antigen typing of the father and disease severity, including provided treatment to the child were gathered.

**Contents:** The chapters in this master thesis deal with Hemolytic Disease of the Fetus and Newborn (HDFN) with a reference to Rhesus incompatibility involving anti-D. The first chapter provides the introduction of the disease, its background, incidence of RhD hemolytic disease of the fetus and newborn, and its pathophysiology. The second chapter defines context of the thesis which includes maternal immunization, antenatal laboratory testing of mother and follow-up, laboratory testing of newborn, paternal testing, antibody screening protocol, and monitoring and evaluation of alloimmunized women. Chapter three describes about the general objection like raising the awareness of RhD-HDFN in the population, and specific objective to determine the prevalence of RhD negativity and frequency of alloimmunization of pregnant women in the region, important investigations carried out to assess the severity of the disease and management of the affected babies. Chapter four describes about the material and methods used in the study. Chapter five describes the results of the investigations carried out.

Out of 1179 pregnant women investigated, blood group O had the highest prevalence at 568 (48.18%), with RhD-positive at 512 (90.16%) and RhD-negative at 56 (9.84%). Group A was seen next in prevalence at 333 (28.24%), with RhD-positive at 304 (91.29%) and RhD-negative at 29 (8.71%). Group B was third in prevalence at 226 (19.16%), with RhD-positive at 203 (89.82%) and RhD-negative at 23 (10.18%). Group AB was last in prevalence at 52 (4.40%), with RhD-positive at 48 (92.30%) and RhD negative at 4 (7.70%). RhD-positivity was seen in 1067 (90.51%) women and RhD-negativity in 112 women (9.49%), which is low when compared to 15.10% of English/European population. Antibody screening of 1179 pregnant women showed 34 (2.88%) positive antibody screen. 16/112(14.24%) were identified in RhD-negative women, and 18/1067(1.68%) in RhD-positive women. Among 34 positive antibodies screened women, 30 (88.23%) had Rh associated antibodies, where as only 4 (11.77%) had non-Rh associated antibodies. In case of RhD-negative women 9/112(8.03%) showed anti-D due to active immunization, and 2/112 (1.78%) had anti-D due to passive anti-D Ig prophylaxis, whereas 2/112 (1.78%) had anti-C+D, and 3/112(2.67%) had non-Rh associated antibodies. This gives overall prevalence of anti-D-8.03% among RhD-negative pregnant women, which is quite high when compared to the 1-2% in developed countries, and that is mainly associated with the moderate to severe HDFN. Since the study population was small, it was difficult to conclude on the significance of the observed difference in the severity of the disease when compared to developed countries. However, it is likely RhD immunization can be better prevented by introduction of antenatal anti-D prophylaxis. Antibody identification among RhD-positive pregnant women was low 18/1067(1.68%) as compared to 16/112(14.24%) among RhD-negative women. Other Rh associated alloantibodies identified were; 8 anti-c (26.66% of Rh associated, and 23.52% of total), 7 anti-E (23.33% of Rh associated, and 20.58% of total), 2 anti-C+D (6.66% Rh associated, and 5.88% of total), 1 anti-E+C (3.33% of Rh associated, and 2.94% of total), and 1 anti-e (3.33% of Rh associated and 2.94% total). Out of 4 non-Rh associated antibodies there were 1 each identified as anti-K, anti-Jka, anti-Fyb and anti-Lea. Chapter six is on the discussion of the results obtained and analysis of the relations between these results with the objectives defined, and finally. Chapter seven is about the personnel conclusion on the project analyzed and personnel experience related to the project.


**Keywords:** RhD-HDFN; Alloimmunization; Hydrops fetalis; Anti-D Immunoglobulin; Phototherapy; Exchange blood transfusion

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## Abbreviations

HDFN: Hemolytic Disease of the Fetus and Newborn

RhD: Rhesus D

ISBT: International Society of Blood Transfusion

TF: Transcription Factors

FMT: Fetomaternal Transfusion

IgG: immunoglobulin G

AGT: Antiglobulin Test

IAT: Indirect Antiglobulin Test

LISS: Low Ionic Strength Saline

PEG: Polyethylene Glycol

RT: Room Temperature

AHG: Anti-Human Globulin

DTT: Dithiothreitol

MCA: Middle Cerebral Artery

MCH: Maternity and Children Hospital

DAT: Direct Antiglobulin Test

IAT: Indirect Antiglobulin Test

IUD: Intra Uterine Death

# Chapter 1

## Introduction

Hemolytic disease of the fetus and newborn (HDFN) is one of the severe complications of pregnancy. Until the 1960s it was an important cause of perinatal morbidity and mortality. Although nowadays it is rare in most of the developed countries, it still remains a potentially severe complication of pregnancy in many developing and economically poor countries. The most common cause of HDFN is maternal immunization against the red blood cell Rhesus D (RhD) antigen. There are as many as 35 blood group systems identified by international society of blood transfusion (ISBT) in addition to that Transcription factors influencing antigen expression (TF); e.g. GATA1 & KLF1 have be identified. There are many RBC antigens with majority of them are present in various blood group systems, some are categorized by the ISBT in blood group systems, some in blood group collections in 200 series, some as high frequency antigens in 901 series and some as low frequency antigens in 700 series. The RhD antigen is part of the RH blood system and is one of the most immunogenic, i.e. mostly capable of inducing an antibody response in individuals lacking the antigen. When an RhD negative pregnant woman is exposed to RhD positive fetal red blood cells during pregnancy or around delivery (60% cases) due to fetomaternal transfusion (FMT); this may induce an immune response and the production of anti-D immunoglobulin G (IgG) antibodies in the mother. Feto maternal transfusions mostly occur spontaneously and generally do not cause symptoms. Some causes that increase the chances of FMT are abortion (both induced and spontaneous), caesarean section, a manual removal of placenta, blunt abdominal trauma, or amniocentesis. The transport of IgG from mother to fetus is mediated by an IgG-Fc receptor named FcRn. The binding of anti-D antibodies to the RhD antigen on the surface of the fetal RBCs will lead to their destruction by macrophages in the spleen [1,2]. HDFN due to RhD is not usually seen in the first pregnancy unless there is the history of previous RhD positive blood transfusion.

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 fetal hydrops. 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, known as kernicterus if not recognized and adequately treated. Postnatal management includes intense phototherapy and exchange transfusion to reduce hyperbilirubinemia and blood transfusion to treat anemia [3]. Alloimmunized pregnant women need proper monitoring, prenatal therapy and if their babies are affected they need 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 pathophysiology of the affected newborn 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. There are still some challenges remaining as how to improve the safety of intrauterine blood transfusions, and how to prevent iatrogenic prematurity and further reducing the incidence of RhD immunization. The blood bank and transfusion service play critical roles in supporting the diagnosis and treatment of these conditions, including the appropriate provision of prophylaxis with commercially available and relatively expensive anti-D Ig. In this thesis the frequency and severity of alloimmunization was reviewed among a two-year cohort of pregnant women in Dammam, Saudi Arabia to evaluate whether measures are needed to be taken to improve prevention of alloimmunization or to improve the care of pregnant women.

## Background

### Historical overview

In most of the developed world the incidence of HDFN due to maternal RhD immunization has decreased due to postnatal immunoprophylaxis of anti-D immunoglobulin from 14 to 1-2 %, and with subsequent antenatal immunoprophylaxis it has gone down to 0.1% [4]. However in developing and economically poor countries, anti-D still remains to be one of the common antibodies found in pregnant women. Besides the anti-D alloantibody, cases of moderate to severe HDFN attributed to other alloantibodies have been described from some Asian countries as well [5,6].

## Incidence of RhD-HDFN

Prevalence of RhD immunization in the Saudi Arabia population is low as 2%. This low incidence is mostly due the early detection of Rh negativity of the mothers before or as early as possible during pregnancy [7]. It is mainly due to the prophylactic administration of anti-D after delivery of an RhD positive child, together with anti-D prophylaxis during pregnancy for events that can cause FMT. It has reduced the incidence of immunization in RhD negative women worldwide to approximately 1-2 % [8]. Residual immunization occurs due to failure of administration of prophylaxis at risk events during pregnancy or after delivery. In a few cases, the FMT 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 FMT during pregnancy, most often in the third trimester [9]. 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 [10,11]. In France, RhD immunization has been reported to cause perinatal death in 2-5/100.00 births [9]. In Saudi Arabia the incidence of perinatal mortality due to RhD immunization and HDFN is unknown.

## Pathophysiology of RhD-HDFN

Hemolytic disease of the fetus and newborn (HDFN) is the destruction of fetal and newborn red cells by maternal alloantibodies specific for inherited paternal red cell alloantigen(s). The maternal IgG antibody crosses the placenta into the fetal circulation where its F(ab) binds to the corresponding fetal red cell antigen, leading to the destruction of these antibody-coated red cells in fetal spleen after adhering to Fc receptors of macrophages. The process is known as extravascular hemolysis. Some red cell antibodies mainly those which are directed against antigens expressed glycophorin A or the Kell glycoprotein are not associated with extravascular hemolysis of the fetal red cells but also bind to and destruct the erythroid progenitor cells and thus suppress erythropoiesis [1]. Both chronic hemolysis and chronic suppression of the erythropoiesis finally result in fetal anemia.

The fetal marrow initially responds to fetal anemia by increasing erythropoiesis and releases many of the newly produced red cells into the circulation prematurely as nucleated precursors, leading to the term “erythroblastosis fetalis.” With worsening anemia, erythropoiesis expands to the liver and spleen, causing their enlargement (Hepatosplenomegaly) and portal hypertension. This results in decrease in fetal movements and as the fetal anemia progresses and leads to cardiomegaly, a hyperdynamic circulation, cardiac decompensation. A resulting decrease in liver production of albumin leads to reduced plasma colloid osmotic pressure, generalized edema, ascites, and effusions known as “fetal hydrops. Untreated, fetal hydrops, with its associated high-output cardiovascular failure, can lead to fetal death in the majority of cases or asphyxia. At this stage the fetal anemia can be corrected by intrauterine transfusion in order to prevent fetal hydrops and asphyxia at birth. Severe disease can occur as early as 18 to 20 weeks’ gestation, and severity usually increases in subsequent pregnancies. Fetal red cell destruction produces a large amount of hemoglobin, which is transformed into the yellow coloring agent bilirubin. This bilirubin needs to be conjugated to be secreted, and its conjugation is performed by the liver, but the fetal and also the neonatal liver is still too immature to perform this at a sufficient level. Prior to birth, bilirubin is mainly transferred to the mother’s circulation and subsequently where it is conjugated by the maternal liver for excretion. After birth, newborn bilirubin levels may raise rapidly, resulting in coloring of the skin and yellow sclera, which is also visible at the mucous membranes of the mouth of the child (jaundice). At this stage phototherapy results in photo-oxidation of bilirubin in the skin, this converts bilirubin into a water-soluble substance that can be secreted by the kidneys. Severe hyperbilirubinemia may result in irreversible damage to the central nervous system, known as ‘kernicterus’ [12], caused by bilirubin deposition in the basal ganglia and brain stem nuclei. Children who survive with kernicterus develop a severe form of athetoid cerebral palsy, hearing problems and psychomotor handicaps. Development of kernicterus can be prevented by (Figure 1) timely starting therapy with phototherapy or exchange transfusions to lower bilirubin levels.

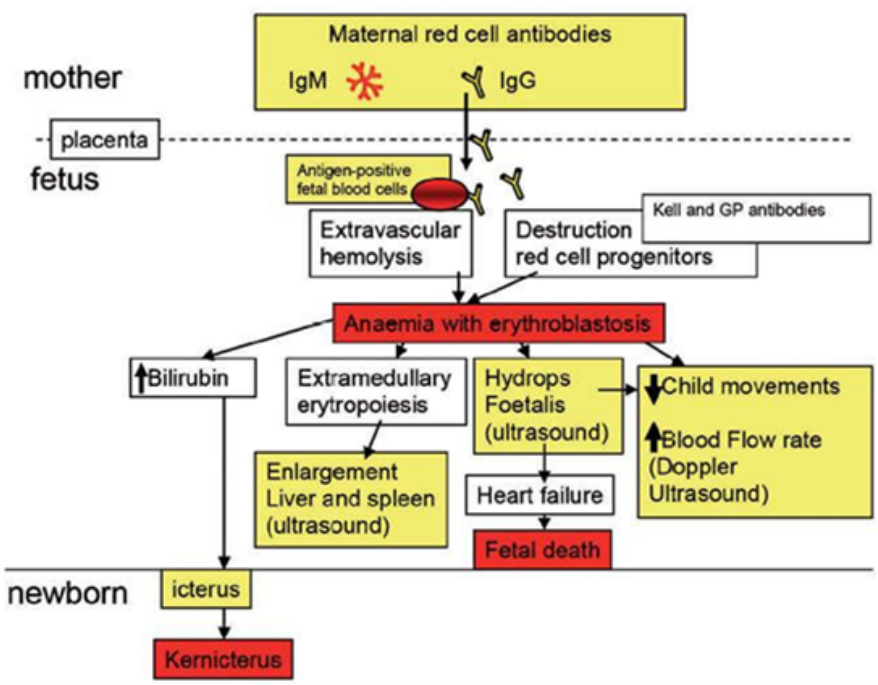


**Figure 1**: Schematic representation of Pathophysiology of HDFN.

# Chapter 2

## Context

### Maternal immunization

Complex genetic factors influence the ability of individuals to respond to red cell antigens. RhD is the most potent immunogenic blood group antigen and, as little as 0.1 to 1 mL of RhD positive red cells can stimulate antibody production. Immunization is reduced when the mother is ABO incompatible with the fetus, probably because of shortened fetal red cell life span. Besides the immunization against a blood group antigen, the severity of HDFN is determined by the immunoglobulin subclass of the developed antibody, amount of antibody in the fetus, biological activity of the of the antibody and the number of antigenic sites on the fetal red cells. RhD-HDFN is still regarded as relatively frequently occurring problem in many developing countries possibly due to inadequate prenatal care or due to the failure of prophylactic administration of anti-D Ig. Clinical severity of the disease shows a considerable variation. It is generally believed that the women who is RhD-positive cannot have her fetus or newborn affected with RhD-HDFN, but rare cases of anti-D isoimmunization have been described in pregnant women who are initially phenotyped as RhD-positive, but who are actually carrying an RhD variant most common of weak D group (Partial-DVI) [13]. It is very important in the developing countries to have a proper prenatal screening program so that HDFN can be detected and treated as early as possible. The design of the screening programme may differ from country to country or even differ in different centers of the same country. The aim of the programme should be to identify those pregnant ladies whose fetus is at risk of severe HDFN that can be treated by intrauterine transfusion or by inducing preterm delivery.

## Laboratory Screening

### Maternal testing

**ABO and RhD typing:** Each pregnant lady should be tested for ABO and RhD typing in order to identify RhD-negative woman. It is advisable to use monoclonal IgM anti-D as RhD typing reagent to avoid RhD-VI detection. Also, the antiglobulin test (AGT) for weak D should not be used to avoid labeling a weak D female as Rh-positive because such ladies may require anti-D Ig prophylaxis to prevent RhD alloimmunization.

### Antibody screening and identification

**Indirect antiglobulin test (IAT):** It should be performed using reagent screening red cells in saline, or in order to reduce the incubation period low ionic strength saline (LISS) or polyethylene glycol (PEG) is quite often used to detect clinically significant antibodies, column agglutination method and solid phase are also available and quite suitable. A validation method that detects IgG antibodies reactive at 37°C must be used. Hence the immediate spin phase and room temperature (RT) incubation, and the use of poly specific antihuman globulin (AHG) reagent must be omitted. Immunoglobulin class is established by treating maternal serum with Dithiothreitol (DTT). Antibody screening and identification gives the clue to the potential risk for HDFN.

### Follow-up tests

Although nowadays red cell antibody screening is very sensitive, however in early pregnancy antibody titer may be too low to be detected Further testing of the RhD negative pregnant women should be performed at least between 28 to 34 weeks of gestation prior to the administration of prophylaxis anti-D Ig to detect late formation of anti-D. Also in RhD immunized women, recurrent testing is very important to monitor the antibody strength, detect any additional antibody if present and determine an appropriate time for intervention. During follow-up any change in titer by two or more dilution is indicative of a significant change. Previously frozen serum samples must be tested in parallel so that the change in titer is not due difference in technique [14]. Some laboratories establish critical titers below which hydrops fetalis is not anticipated, usually below 1/16-1/32, with close monitoring if >1/8. Following administration of prophylactic anti-D Ig, anti D can be detected by IAT for up to about eight to twelve weeks. Immune anti-D becomes detectable approximately four weeks after exposure to D positive cells and reaches a peak after six-eight weeks [15], while prophylactic anti-D levels fall with time, immune anti-D levels usually remain stable or rise. Despite these differences, it is said that distinction between prophylactic and immune anti-D may not be easy. However, identification of immune or prophylactic anti-D can be made easily, by serial determination of antibody titers along with the review of patient's medical history. The Kleihauer-Betke test is a sensitive cytochemical test to identify fetal cells in maternal circulation and serves to identify subjects where additional dose of anti-D Ig is required.

**Infant testing:** The most important serologic test for the diagnosis of RhD-HDFN is the direct antiglobulin test (DAT) with anti-IgG reagent. A positive DAT indicates sensitization of fetal red cells and is in itself not diagnostic of HDFN. The DAT results must be interpreted in the clinical context. Where the infant has a positive DAT with suggestive clinical findings; a red cell elute confirms the antibody specificity, and presence of the corresponding antigen on cord cells confirms the diagnosis of HDFN. In RhD hemolytic disease, the DAT may be strongly positive without clinical signs of the disease. The DAT strength does not correlate with the severity of the hemolysis and a positive DAT may invalidate the results of RhD typing (blocked D) [16]. For the resolution of the 'blocked D', elution technique must be performed. Elution will also be necessary when the diagnosis of HDFN is in doubt, as in rare cases of ABO incompatibility with a negative DAT.

**Paternal test:** Husbands ABO and Rh phenotype provides information to predict the likelihood of fetus to carry the

relevant red cell antigen. The diagnosis of RhD-HDFN is confirmed if the RhD antigen is detected on the baby's cord blood cells [17].

## Antenatal Screening Protocol

Antenatal screening practices vary amongst countries. In most developed countries, the increase in relative proportion of non-RhD alloantibodies has led to the implementation of regular antenatal screening protocols [18]. Countries like Netherlands, Sweden have national antenatal screening programs in place for over two decades [19]. However, in most developing countries including Indian subcontinent, antenatal screening is generally limited to RhD-negative women only. A recent report from India stressed the need for screening RhD positive women as well, after they described alloantibodies in two RhD-positive women [5]. In general the antenatal screening protocol recommends testing of all pregnant women, RhD positive or negative for, ABO and RhD type along with a red cell antibody screen at about the twelfth week of gestation [20]. Protocols used as follow-up of alloimmunized women are different but can be as follows: If a clinically significant antibody is detected, monthly tests till 32 weeks and two weekly tests till term are indicated. If no alloantibody is detected in the first antenatal visit, screening should be repeated at 28-32 weeks. After this, no further testing is necessary if the antibody screens remain negative [21]. For RhD-negative women receiving anti-D Ig, sampling must be done prior to the injection.

## Monitoring and Evaluation of Immunized Women

For mild HDFN (IAT‹1/8) with uneventful obstetric history monthly anti-D titration and Ultrasonography should be adequate. When the anti-D titration remains 1/16, fewer than 5% of the fetuses will require neonatal exchange transfusion. Repeated measurement of bilirubin levels in amniotic fluid taken after amniocentesis to judge the level of hemolysis in the fetus is required as obsolete, since fetal anemia can be judged non-invasively by Doppler Ultrasound [22] (Figure 2). Many developed nations have national antenatal screening programs such as, the Dutch screening program in Netherlands and a similar screening program in Sweden. However in developing countries, anti D continues to be a common alloantibody found in pregnant women. Failure to administer anti-D Ig or antenatal sensitization prior to its administration is the likely cause. Inadequate prenatal care due to unawareness and financial constraints further compound the problem. Besides anti-D antibody, ABO incompatibility and other alloantibodies have been reported to cause severe HDFN in many Asian countries. In addition many Asian countries have recently identified alloantibodies other than anti-D as a cause of moderate-severe hemolytic disease [23] In Saudi Arabia a moderately severe HDFN due to combined anti-E and anti-c was reported. [24], what is more concerning is that, some of these have been described in RhD-positive women. Besides, reports have also highlighted that ABO incompatibility is not always benign and may require active management. Hence, keeping in view all of the above facts, universal antenatal screening in all pregnant women needs to be initiated irrespective of their RhD status in their first trimester as in Saudi Arabia, since RhD-positive women are like RhD-negative women able to form alloantibodies that can cause severe HDFN, such as anti-c or anti-K. A close follow up throughout pregnancy is required to detect irregular antibodies. Although universal screening seems justified, the cost and infrastructure required would be immense. Developing countries and under resourced nations need to consider universal antenatal screening and frame guidelines accordingly. A frequency of RhD-negative blood group in the regional laboratory and blood bank, Dammam (capital city in the eastern province of Saudi Arabia) was conducted in 1997 which showed RhD-negative frequency of 9.18%. The gene frequency for RhD was calculated to be 0.7. Rh phenotypes present in the decreasing order of frequency were R1r, R1R1, R0r, R2r, rr, R1R2, R2R2, R1Rz, r'r, RzRz, R2Rz, r"r, r'r". Out of 7093 voluntary donors, screened 6442 (90.82%) were RhD positive and 651 were RhD- negative (9.18%). Frequency of RhD-negative phenotype has been reported and compared with English/European population [25].

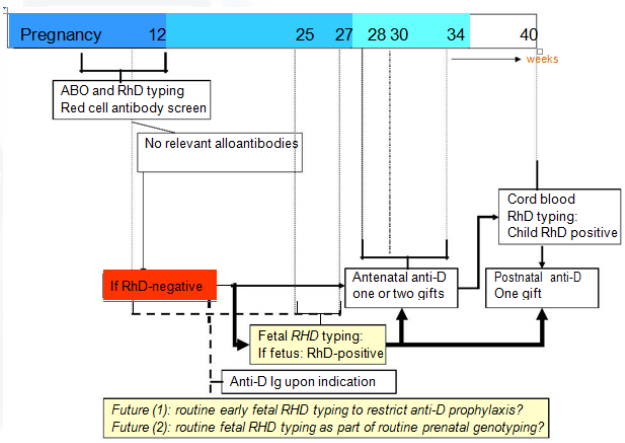


**Figure 2**: Timing of different steps in prenatal screening for detection and prevention of alloimmunisation in pregnancy, as currently performed.

Table1 Data for the RhD-negative frequencies has been taken from male voluntary donors but are not seen significantly different from female pregnant women (Table2). As for as blood transfusion policies are concerned in Saudi Arabia Rh and Kell phenotype is specially taken care of, and it is the policy to transfuse same Rh and Kell (K) phenotype blood to the patients except in rare Rh phenotype. In case same Rh phenotype blood is not available concerned physician has to be informed before the blood is issued for transfusion and documented. Red cell antigen K frequency is almost same as seen in Caucasian population i.e. almost 91% of the individuals are negative to K antigen. Although the frequency of RhD-negativity is less as compared to the

frequencies in Western Europe and north America/Canada, but the number of pregnancies is high among the Saudi population. So due to the high chance an RhD-negative Saudi women is repeatedly carrying an RhD positive child, the prevalence of RhD immunization may be relatively high in Saudi population. Also other red cell antibodies may be formed. Therefore, antenatal screening of all pregnant women up to the age of 50 has been started in Saudi Arabia for the last three years before 12-weeks of their gestation. These women are tested for ABO and Rh typing (DCcEe), and Kell antigen. Antibody screen is also performed for all these women, and if negative for antibody screen no further antibody screen is done for RhD positive women, but for D, C and K negative women antibody screen is repeated at 27$^{th}$ week of their pregnancy. All RhD negative women are given prophylactic anti-D Ig at 28-30 weeks of pregnancy if their repeat antibody screen is negative and also within 72 hours after delivery if they give birth to RhD positive baby. For immunized women their antibody titer is determined to evaluate critical titer which is required to be carefully monitored throughout their pregnancy, and also their fetal status is required to be monitored by MCA color Doppler studies. Because fetal anemia results in increased cardiac output, noninvasive color Doppler Ultrasonography is nowadays successfully used to monitor the severity of HDFN. Since it being a non-invasive approach, it has almost replaced invasive amniotic bilirubin sampling. The fetal middle cerebral artery (MCA) peak blood flow velocity has been shown to correlate with the severity of the anemia and to be diagnostically equivalent to amniocentesis, without the any risks. Recent studies have found good correlation between MCA peak velocity, fetal hemoglobin, and ΔOD 450 readings, and also in pregnant women with anti-K antibodies, amniotic fluid analysis does not correlate well with the degree of fetal anemia. Therefore in these patients ultrasonography is generally preferred. However other invasive procedures like amniocentesis is not being done, and also molecular studies of fetal genotyping is not being reported to be done yet in most of the hospitals in Saudi Arabia.

**Table 1**: Frequency of RhD-negative phenotype.

| Fisher-Race Heplo Type | Wiener Heplo Type | Saudis | English/ European |
|---|---|---|---|
| ce | rr | 8.19% | 15.10% |
| Cce | r'r | 0.82% | 0.76% |
| cEe | r''r | 0.11% | 0.96% |
| CcEe | r'r'' | 0% | 0.02% |
| cE | r''r'' | 0% | 0.01% |
| Cc | r'r' | 0% | 0.01% |
| Total | | 9.18% | 16.82% |

**Table 2**: ABO and RhD prevalence.

| | O | A | B | AB | Total |
|---|---|---|---|---|---|
| RhD-Positive | 512 | 304 | 203 | 48 | 1067 |
| Percentage | 90.16 | 91.29 | 89.82 | 92.3 | 90.51 |
| RhD-Negative | 56 | 29 | 23 | 4 | 112 |
| Percentage | 9.84 | 8.71 | 10.18 | 7.7 | 9.49 |
| Total | 568 | 333 | 226 | 52 | 1179 |
| Percentage | 48.18 | 28.24 | 19.16 | 4.42 | 100 |

# Chapter 3

## General Objective

In the Eastern Province of Saudi Arabia, RhD alloimmunization is occurring. The objective of this study is to investigate to whether raising the awareness of RhD alloimmunization in RhD-negative women is necessary to improve the prevention and management of RhD-HDFN through the introduction of nationwide prevention programs, such programs have proved to be effective and successful in reducing the rate of antibodies development, hence reducing the rate of maternal sensitization. Once HDFN was a major obstetric problem that had a large impact on fetal and neonatal morbidity and mortality, but today, without an appropriate programme, almost half of the untreated HDFN will result in death or severe brain damage. In developing countries, especially those lacking an efficient prophylactics programme, this causes an important public health problem. In fact, it has been estimated that more than 50 thousand fetuses could be affected by this condition every year. But with the established use of post-natal anti-D prophylaxis for RhD-negative women, together with its increasing use for routine antenatal prophylaxis, the incidence of Rh-D sensitization has dramatically fallen. Since 15 % of the Caucasian population in Europe and North America is RhD negative, and with the sensitization against other red cell antigens such as RhC, Rhc, RhE, Rhe, and Kell, this pathology could still affect a large number of pregnancies every year, with significant health and financial implications. On the other hand, in developed countries if the fetus is affected by HDFN, survival rates can exceed 90 percent if anemia is diagnosed and treated with intrauterine blood transfusions in a timely manner, which is usually not possible in developing countries where fetal medicine is not being effectively practiced? In developed countries women with rising red cell antibody titer are usually referred to tertiary fetal medicine units for specialized management. The main challenge facing fetal medicine specialists today is not the skill required for invasive therapy, but rather the non-invasive monitoring of the disease so that its progress can be predicted to guide the need and timing of intrauterine transfusions to minimize unnecessary invasive testing. Although, HDFN can be life threatening but in the case of anti-D it is a disease that can be prevented with the prophylactic administration of anti-D Ig. The serious consequence of HDFN can be lessened by early laboratory diagnosis and treatment. In the eastern province of Saudi Arabia prophylactic administration of anti-D Ig has been given routinely during the both pre and postnatal period of the pregnant women for the last three years, so the main objective of this thesis is to estimate how far it has helped to prevent the prevalence of RhD-HDFN in this region.

## Specific Objective

**a.** To determine the prevalence of RhD-negativity among pregnant women in the Easter Province of Saudi Arabia who attended Maternity and Children hospital (MCH), Dammam, for the period of two years from January, 2012 to December, 2013 after evaluating their ABO and Rh type.

**b.** To determine the prevalence of alloimmunization among these pregnant women at MCH, and at the Regional Laboratory and Blood Bank, Dammam.

**c.** To estimate the risk of HDFN caused by maternal immunization, by determining the antibody titers, and typing the father and baby of these pregnant women for ABO, Rh, and Kell antigen

**d.** To assess the severity of HDFN caused by maternal immunization by reviewing the treatment given to affected babies with HDFN.

# Chapter 4

## Material and Methods

This is a retrospective study from the maternity and children hospital (MCH), Dammam, between January 2012 to December 2013 where all the pregnant women were tested for ABO and RhD typing and antibody screening. MCH has a policy to test all the pregnant women in their 1st and 3rd trimester for antibody detection, and if positive, blood samples of these pregnant women are sent to the regional laboratory and blood bank, Dammam, for antibody identification and titration, and in case exchange blood transfusion was required for supply of correspondent antigen negative blood. The study at MCH involved 1179 samples from pregnant women over the period of two years. Data were acquired by standardized methodology using blood sample forms during antenatal care. Samples were collected by trained phlebotomists and sent to the MCH blood bank where ABO/ Rh typing were done using DiaMed Gel Technique, Cressier, Switzerland. Antibody identification was done by using three screening cells from the same company at 37°C by indirect antiglobulin test (IAT). The prevalence of ABO/Rh Typing is shown in Table 1. Out of these 1179 pregnant women only 34 patient samples which were positive by initial antibody screen at MCH were referred to the Regional Laboratory and blood bank, Dammam (3 mL in EDTA, and 5 mL in plain vial-for each patient) for antibody identification and titration. In the Regional Laboratory and blood bank the study involved red cell antigen tying by using microplate with dried monoclonal ready to use antibodies for ABO grouping and reverse grouping and also for the determination of Rh and Kell phenotype from Tango Optima/ BIO-RAD . Other red cell phenotyping was done by using test cell reagents by gel technique from BIO-RAD. Antibody detection was done by using 3-cell screening panel and identification by 10-cell panel testing cells using DiaMed/ BIO-RAD gel technique at 37 C by IAT.

ABO and Rh typing of these samples were determined first and then these samples were screened for antibody detection and identification. Samples tested positive for any red cell alloantibody were further tested for antibody titration by serial dilutions of the serum against selected cells from DiaMed/ BIO-RAD. The results were expressed as the reciprocal of the of the highest serum dilution that causes macroscopically apparent agglutination. Titration of alloantibodies identified ranged from 1:1 to 1:256. Alloimmunized women's files were reviewed for their medical and obstetric history, which included their number of pregnancies, previous history of any blood transfusion or immunization, and or any previous history of HDFN and its management (e.g. exchange blood transfusion and or phototherapy to the affected baby).

# Chapter 5

## Results

Table 2 represents the blood group frequencies among these 1179 pregnant women. Blood group O had the highest prevalence at 568 (48.18%) out of the total, with RhD positive at 512 (90.16%) and RhD-negative at 56 (9.84%). Group A was seen next in prevalence at 333 (28.24%), with RhD-positive at 304 (91.29%) and RhD-negative at 29 (8.71%). Group B was third in prevalence at 226 (19.16%), with RhD-positive at 203 (89.82%) and RhD-negative at 23 (10.18%). Group AB was last in prevalence at 52 (4.40%), with RhD-positive at 48 (92.30%) and RhD negative at 4 (7.70%). RhD-positivity was seen in 1067 (90.51%) women and RhD-negativity in 112 women (9.49%).

### Antibody detection/Identification

Table 3-7 represents the clinical data of alloimmunized pregnant women, their obstetric history, their ABO/Rh and Kell typing, their antibody/ies identification and titration, their baby's father and baby ABO/Rh and Kell typing, and the HDFN effect on their baby's and its management. Out of the screening of 1179 pregnant women, only 34 (2.88%) showed positive antibody screen. Out of them 16/112(14.24%) were identified in RhD-negative women, and 18/1067(1.68%) in RhD-positive women. Out of these 34 positive antibodies screened women, 30(88.23%) had Rh associated antibodies, where as only 4(11.77%) had non-Rh associated antibodies. Among all screened RhD negative women 9/112(8.03%) showed anti-D due to active immunization, and 2/112(1.78%) the anti-D was due t o passive anti-D Ig prophylaxis, where as 2/112(1.78%) had anti-C+D, and 3/112(2.67%) had non-Rh associate d antibodies. This gives overall prevalence of 8.03% anti-D antibody among RhD-negative pregnant women. Antibody identification among RhD positive pregnant women was low 18/1067(1.68%) as compared to 16/112(14.24%) among RhD negative women.

**Table 3:** The clinical data of alloimmunized pregnant women with anti-D.

| | No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Age | 28 | 38 | 35 | 20 | 41 | 40 | 35 | 24 | 31 | 33 | 31 |
| | ABO | O | O | A | O | AB | B | A | O | B | B | O |
| Mother With anti-D | Rh & K phenotype | rr, K- | rr, K- | rr, K- | rr, K- | rr, K- | rr, K- | r'r, K- | rr, K- | rr,K+ | rr, K- | r'r, K- |
| | Ab. Titer | 1:32 | 1:4 | 1:32 | 1:16 | 1:8 | 1:256 | 1: 1 | 1: 1 | 1: 8 | 1:256 | 1:16 |
| | Obs. History | G2P 1 | G6P 5 | G5P 4 | G3P1+ 0 | G2P 1 | Recurre nt fetal loss | Primi | Primi | G2P1 | Previous H/ O transfusion | unknown |
| Father | ABO | A | A | O | B | A | B | A | O | O | B | O |
| | Rh & K phenotype | R1r, K- | R1r, K- | R1R 1, K- | R1r, K- | R1R 1, K- | R1R1, K- | R1r, K- | R1r,K- | R1r, K- | R1R1,K- | R1r,K- |
| Baby | ABO | A | O | A | O | A | - | A | O | O | B | O |
| | Rh & K phenotype | R1r, K- | rr, K- | R1r, K- | R1r,K- | R1r, K- | | rr,K- | rr, K- | R1r, K- | R1r, K- | R1r, K- |
| | HDFN | Yes | No | Yes | Yes | Yes | IUD | No | No | Yes | Yes | Yes |
| | Management | PT & EBT | Nil | PT & EBT | PT & EBT | PT | - | Nil | Nil | PT | EBT | PT |

**Table 4**: The clinical data of alloimmunized pregnant women with anti-C.

| | No | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|---|
| | Age | 32 | 30 | 41 | 27 | 36 | 31 | 48 | 29 |
| | ABO | O | A | O | O | A | B | O | O |
| Mother With anti-c | Rh & K phenotype | R1R1,K- | R1R1,K- | R1R1,K- | R1R1,K- | R1R1,K- | R1R1, K- | R1R1,K- | R1R1,K- |
| | Ab. Titer | 1:2 | 1:4 | 1:2 | 1:2 | 1:4 | 1:1 | 1:4 | 1:4 |
| | Obs. History | MP | MP | MP | G2G1 | MP | G3P2 | MP | G4P3 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Father | ABO | O | O | A | O | A | B | O | O |
| | Rh & K phenotype | R1r, K- | R1r, K- | R1R1, K- | R1r, K- | R1R 1, K- | R1R1, K- | R1r, K- | R1r,K- |
| Baby | ABO | A | O | A | O | A | - | A | O |
| | Rh & K phenotype | R1r, K- | rr, K- | R1R2, K- | R1r,K- | R1R2, K- | R1r, K- | R1r, K- | rr, K- |
| | HDFN | No | Mild | No | No | Moderate | No | No | Mild |
| | Management | Nil | PT | Nil | Nil | PT | Nil | Nil | PT |

**Table 5**: The clinical data of alloimmunized pregnant women with anti-E.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Mother With anti-E | **No** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| | **Age** | **40** | **36** | **41** | **28** | **30** | **39** | **27** |
| | **ABO** | **O** | **A** | **O** | **O** | **A** | **B** | **O** |
| | Rh & K phenotype | R1R1,K- | R1R1,K- | R1r,K- | R1r,K- | R1r,K- | R1R1, K- | R1r,K- |
| | Ab. Titer | 1:1 | 1:4 | 1:1 | 1:4 | 1:1 | 1:2 | 1:4 |
| | Obs. History | G5G4 | Two abortion | G4P3 | Blood Transfusion | G4P3+1 | G6P5 | Blood Transfusion |
| Father | ABO | O | O | O | O | O | O | O |
| | Rh & K phenotype | R1R2, K+ | R1R2, K- | R2r, K- | R1R2, K- | R2r, K- | R2r, K- | R1R2, K- |
| Baby | ABO | O | O | O | O | O | - | O |
| | Rh & K phenotype | R1R2, K- | R1R2, K- | R2r, K- | R2r,K- | R2r, K- | R1R2, K- | R1R2, K- |
| | HDFN | No | Mild | No | Mild | No | No | Mild |
| | Management | None | EBT | None | PT | None | None | PT |

**Table 6**: The clinical data of alloimmunized pregnant women with other Rh antibodies.

| | | | | | |
|---|---|---|---|---|---|
| Mother with other Rh antibodies | **No** | **1** | **2** | **3** | **4** |
| | **Age** | **28** | **42** | **33** | **27** |
| | **ABO** | **A** | **0** | **O** | **A** |
| | Rh & K phenotype | rr, K- | rr, K- | R1R1, K- | R2R2, K+ |
| | Antibody | Anti-C+D | Anti-C+D | Anti-E+c | Anti-e |
| | Antibody titer | 1:01 | 1:02 | 1:02 | 1:01 |
| | Obs. History | G3P2 | MP | G3P2 | G2P1 |
| Father | ABO | O | O | B | A |
| | Rh & K phenotype | R1R1, K- | R1r, K- | R1R2, K- | R1r, K- |
| Baby | ABO | A | O | O | A |
| | Rh & K phenotype | R1r, K- | rr, K- | R1R1, K- | R1R2, K- |
| | HDFN | No | No | No | No |
| | Management | Nil | Nil | Nil | Nil |

**Table 7**: The clinical data of alloimmunized pregnant women with other red cell antibodies.

| | No | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| | Age | 44 | 22 | 38 | 31 |
| | ABO | B | O | O | O |
| Mother with other red cell antibodies | Rh, K% correspondent antigen phenotype | rr, K- | R1r, K- Jk (a-b-) | rr, K- Le(a-b-) | rr, K- Fy (a-b-) |
| | Antibody | Anti-K | Anti-Jka | Anti-Lea | Anti-Fyb |
| | Antibody titer | 1:08 | 1:02 | 1:01 | 1:01 |
| | Obs. History | G3P2 | MP | G3P2 | G2P1 |
| Father | ABO | O | AB | A | O |
| | Rh, K & other correspondent phenotype | R1r, K+ | R1r, K- Jk (a+b-) | R1R1, K- Le (a+b+) | R1r, K- F y(a-b+) |
| Baby | ABO | A | O | O | A |
| | Rh, K & other correspondent phenotype | R1r, K- | rr, K- Jk (a-b-) | R1r, K- | R1r, K- Fy(a-b-) |
| | HDFN | No | No | No | No |
| | Management | Nil | Nil | Nil | Nil |

The other Rh associated alloantibodies identified were; 8 anti-c (26.66% of Rh associated, and 23.52 % of total), 7 anti-E (23.33% of Rh a sociated, and 20.58% of total), 2 anti-C+D (6.66% Rh associated, and 5.88% of total), 1 anti-E+c (3.33% of Rh associated, and 2.94% of total), and 1 anti-e (3.33% of Rh associated and 2.94% total). Babies affected with HDFN were from the pregnancies in which the mother had antibody titer ranging from 1:8 to 1: 256. Out of 8 women whose babies were affected with HD FN, 6 had anti-D titer ranging from 1:8 to 1:32, all of these babies received treatment with phototherapy and exchange blood transfusion, whereas 3 received only phototherapy according t their bilirubin level. In other 2 women with high anti-D titer of 1:256, 1 of the baby received several exchange blood transfusions whereas the other woman had fetal loss due to intra-uterine death (IUD). In the group of RhD-positive women, only one case with severe HDFN was noted, due to anti-E, last measured titer 1:4, treated with exchange transfusion and five other cases with mild disease treated with photo therapy (3x anti-c and 2x anti-E). Out of 4 non-Rh associated antibodies there were 1 each identified as anti-K, anti-Jka, anti-Fyb and anti-Lea. Age of the pregnant women with positive anti-D screen ranged from 24 to 41 years, their obstetric history was variable from primigravida to multiparous, 1 woman had a history of previous abortion, 1 had history of blood transfusion and the other 1 had unknown history. 9 of these women were rr phenotype and 2 were r'r phenotype. HDFN was present in 8 out of 11 babies, 3 women were not affected because their babies were RhD negative with rr phenotype and father's were R1r phenotype. 2 out of these 3 women were primigravida in their third trimester with low anti-D titer of 1:1, due to the passive immunization of prophylactic anti-D Ig, whereas 1 woman was multiparous with ant-D titer of 1: 4, but her baby was not affected with HDFN because of rr phenotype. Babies affected with HDFN had antibody titer ranging from 1:8 to 1: 256. It was not known whether these immunized women were given anti-D Ig prophylaxis during their earlier pregnancies or not. Out of 8 women whose babies were affected with HDFN, 6 had anti-D titer ranging from 1:8 to 1:32, and 3 of these babies received treatment with phototherapy and exchange blood transfusion, whereas 3 received only phototherapy. In other 2 women with high anti-D titer of 1:256, 1 of the baby received several exchange blood transfusions whereas the other woman had fetal loss due to intrauterine death (IUD).

Anti-c was identified in 8 women, all of these women were RhD-positive with R1R1 phenotype, their age ranged from 27 to 48 years and all had multiparous obstetric history. The anti-c titer ranged from 1:1 to 1:4. Fathers' phenotype was rr in 2, R1r in 4, R1R2 in 2. Since all these men were possessing c antigen and these women were multiparous and negative to c antigen, the maternal immunization could be developed after fetomaternal transfusion from their previous pregnancies. Babies of 5 women had R1r phenotype(c antigen positive), but only 3 had HDFN with 2 mildly and 1 moderately affected. These 3 babies received treatment with phototherapy. Other 3 babies had R1R1 phenotype (c antigen negative) and they were not affected with HDFN. Anti-E was identified in 7 women, all of these women were RhD-positive, 4 had R1r phenotype

and 3 had R1R1 phenotype. Their age ranged from 27 to 40 years. 4 of these women had multiparous obstetric history, 2 had previous history of blood transfusion and 1 had previous history of abortions. Anti-E titer ranged from 1:1 to 1:4. Father's phenotype was R1R2 in 4 and R2r in 3. Since all these men were positive to E antigen, and all these 7 women were negative to E antigen with multiparous obstetric history, the maternal immunization was likely to be of fetomaternal origin as there was no history of previous transfusion in these women, usually transfusion associated immunization is low in pregnant women in Saudi Arabia as compared to western countries and pregnancies are the main cause of immunization. 4 babies were of R1R2 phenotype and 3 were of R2r phenotype. Although, babies of all these 7 women were positive for E antigen, HDFN was mildly seen in only 3 of them and treated by phototherapy.

Anti-C+D was identified in 2 women, both of these women were RhD-negative with rr phenotype. Both had multiparous obstetric history, one was 28 and other 42 years old. Fathers were of R1R1 and R1r and babies were of R1r and rr phenotype respectively. Antibody titer was low ranging from 1:1 to 1:2. At antenatal stage it is always necessary to differentiate anti-D from anti-G (anti-CD) in order to decide for prophylactic anti-D Ig administration in case the anti-D is not present. However, since the antibody titer was low, and after reviewing patient's files anti-D was possibly due to anti-D Ig administration given to them during their third trimester. None of the baby was affected with HDFN. Anti E+c was identified in a 33 year old woman. She was RhD-positive with R1R1 phenotype. Baby's father was R1R2 and baby was R1R1 phenotype. Anti-E with anti-c was confirmed with rare phenotype RzRz cells. Baby was not affected with HDFN. Anti-e was identified in a 27 year old woman with obstetric history of G2P1. She was RhD positive with R2R2 phenotype. Baby's father was R1r and baby R1R2 phenotype. R2R2 phenotype is rare in almost all populations. Baby was not affected with HDFN. Among non-Rh associated antibodies anti-K was seen in one multiparous woman with K negative phenotype. Baby's father was K positive phenotype, so the immunization was either due her previous pregnancies or more possibly due to previous transfusion. Her baby was K negative phenotype, as out of 8-9% of individuals who are K positive almost 98% of them are heterozygous (Kk) for Kell antigen. Although the antibody titer was clinically significant 1:8, but baby being K negative was not affected with HDFN. In addition to anti-K, other non-Rh associated antibodies like, anti-Jka, Lea, and Fyb were also identified in each one of these 3 pregnant woman, as shown in Table 7. But as far as HDFN is concerned these antibodies are not clinically considered significant.

The fellow-up was seen in only 3 (33.33%) of the pregnant women out of 9 who had active anti-D antibody identified in their 1$^{st}$ trimester. Other 2 of the pregnant women had passive anti-D identified in their 3$^{rd}$ trimester due to prophylactic administration of anti-D Ig. None of the pregnant women in this study had any previous history immunization recorded. History of blood transfusion was also recorded in 3 of the pregnant women, 2 with anti-E and 1 with anti-K.

# Chapter 6

## Discussion

The current study is describing the pattern of distribution of ABO and Rh blood group in the pregnant women in the eastern province of Saudi Arabia showing the highest prevalence of O group (48.18%), followed by A (28.24%), B (19.16%) and AB (4.42%). The highest prevalence of O group in this region is not unique as almost similar frequencies have been shown in other studies in this region of Saudi Arabia. [7], indicating that blood group O is the most prevalent followed by A, B and AB subsequently, and thus blood group AB is the least frequent blood group among pregnant women in Saudi Arabia. Therefore the chance of ABO incompatibility occurring among the babies of O group mothers is taken into consideration more often especially if the father is not of O blood group. The prevalence of RhD-positive blood group was 90.51% and the RhD-negative was 9.49% in pregnant women at MCH, Dammam in the Eastern region of Saudi Arabia These findings are in concordance with what was reported in the in the Tabouk regions and Madina Munawara by Ozoylu et al. [26], where RhD-positivity was reported as 90.5% and RhD-negativity as 9.5% [25]. In the Western region where Rh positivity was 92.5% and Rh-negativity was 7.5% by Bondagji NS [27] In the central region RhD-positivity is reported to be 91.5% and RhD- negativity 8.5% by Talib et al. [28], but it was and marginally different from the one report by Al-Ibrahim et al. [29] with a prevalence of 85.9% for RhD-positivity and 14.1% RhD- negativity [28]. Table 8 RhD-negative prevalence in different populations varies by race and ethnicity. In this study the prevalence is 9.49%, which is less than Caucasians population (15%), but higher when compared to 5-8% of African American and 1-2% of Asians and Native American [30]. Local population based studies from Saudi Arabia on male and female voluntary blood donors showed that the prevalence of RhD negative blood group in the eastern region of Saudi Arabia were 9.18% in Table 1 [25], and in southwest Saudi Arabia 7.2% [31]. The estimated prevalence of RhD negativity among pregnant women was not known in Saudi Arabia, but on the basis of my two year retrospective study on pregnant women in the maternity and children hospital, Dammam, in the eastern province of Saudi Arabia, it shows that this is not statistically different from publications in the literature i.e. 9.46% in Table 2. The recognition of the prevalence of RhD-negativity in this region that will allow different health authorities to estimate the costs and the success of prevention programme for RhD alloimmunization on evidence based facts. RhD alloimmunization is a serious preventable disease that develops in RhD-negative pregnant women and carries major impact in the prenatal outcome including major morbidity and mortality [32]. In recent years, major advances in the prevention and treatment of RhD alloimmunization have developed. The development and implementation of antenatal RhD immune globulin prophylaxis has led to a significant reduction in the frequency of maternal alloimmunization with anti-D antibodies [33,34].

RhD-negative subtypes depending on the presence or absence of the RhD antigen on red blood cells. However, this nomenclature is not technically correct because the Rh blood system consists of the C, c, D, E, and e antigens (there is no d antigen). In this study the overall alloimmunization rate is shown to be 2.88%, so majority of the women 97.12 % were not immunized. The majority of the alloimmunization was seen against Rh antigens 88.23% against the 11.77% in non-Rh antigens. Rh associated antibodies were seen in both RhD-positives as well in RhD-negative pregnant women. Out of 34 pregnant women identified with alloantibodies, 31 (91.17%) had a single antibody whereas only 3 (8.83%) had developed 2 antibodies. However in case of 16 RhD-negative immunized pregnant women 9/112 had anti-D antibody (8.03%) and 2/112 had anti-C+D (1.78%). The overall immunization against RhD antigen alone was only 8.03%, as out of 112 RhD-negative women only 9 had developed anti-D, which is quite high when compared to 1-2% in most of the developed countries, and is further reported to have decreased to 0.2-0.3% in countries that has successfully implement the routine antenatal anti-D prophylaxis (RAADP). This shows that RhD alloimmunization in this region of Saudi Arabia is high and has not been completely eliminated. The recognized causes for continued emergence of sensitized pregnancies include both failure to administer prophylactic anti-D Ig in accordance with published guidelines and sensitization in early gestation before routine third trimester antenatal anti-D prophylaxis [35]. Failure to administer anti-D Ig either prenatal or postnatal seems to be the main cause of RhD immunization in these pregnant women in this study. Therefore it is very important to improve the programme for prevention of RhD immunization.

It has been reported that the development of screening and prevention program may reduce the rate of alloimmunization by a 90% [36]. The implementation of Rhesus prevention programs in the developed countries was based on understanding the estimated risks of Rhesus alloimmunization by determining the prevalence of sensitization among pregnant women in their local population. Unfortunately, there is a major deficiency in our knowledge regarding the rate of alloimmunization among pregnant women in Saudi Arabia. In this current study, 34 pregnant were tested positive for antibodies during pregnancy, with alloimmunization rate of 2.88 % of the total number of women during the study period, and since the prevalence of RhD-negativity is low as compared to West and European countries, out of 112 RhD-negative pregnant women 9 had developed anti-D. This figure alertly exceeds the reported figures in the developed countries that applied the Rhesus prevention programs and followed the proper guidelines which are not being practiced in most of the developing countries. As seen in this study, it is 9/112 (8.03%) when compared to 1-2% in the developed countries [37]. It may be that a strict follow-up of the guidelines will reduce the RhD immunized cases. Furthermore, in this relatively small cohort of followed RhD-positive pregnant women, we

observe with one case with ant-E mediated severe and – cases either anti-c or anti-E mediated HDFN, which was necessary to be treated with phototherapy. Screening of red cell antibodies in these cases led to timely treatment of newborns. The prevention program should be supported and organized by a nationwide screening program, utilizing anti-D immunoglobulin at its appropriate time and dosages as described in the literature. Informative national data base on RhD-negative pregnant women is an important applicable tool in implementing of such programmes and has to be developed hand in hand with the prevention and screening programs. Traditionally, the literature describe the outcome among RhD-alloimmunized pregnancies to the following categories, mild to moderate hemolytic anemia, and hyperbilirubinemia occurs in 25-30% and hydrops fetalis occurs in 25% [38]. The current study showed that out of 16 RhD- negative immunized women 9/16 (56.25%) had anti-D. 2/9 (22.22%) with mild to moderate HDFN and were managed with phototherapy, whereas 7/9 (77.77%) had a moderate to severe HDFN, 6 of them were managed by phototherapy and exchange blood transfusion, and 1 by several exchange transfusions. Phototherapy has been proven to be effective by drastically decreasing the necessity of exchange blood transfusion as shown in this study where out of 30 babies with Rh associated antibodies 11(36.66%) were managed with phototherapy. Unfortunately, one woman who had history of recurrent fetal loss suffered a perinatal mortality with intrauterine death at 21$^{st}$ week of gestation, that could be explained by the lack of organized screening and prevention programs in addition to the late reporting of the patients to specialized centers with facilities to perform intrauterine intravascular blood transfusion.

There is no local experience to compare these results with, as the literature is lacking the description of local experience in such an important fetal and neonatal disease. Further studies are needed to explore the experience and the outcome of Rhesus alloimmunization in different regions of Saudi Arabia. This study shows that the prevalence of non-anti-D antibodies among the pregnant women seems to be comparable to the studies in western population, although due to the lower frequencies of these antibodies a larger cohort should be studied. Out of 34 pregnant women with positive antibody screen, 11 of whom had anti-D identified (9 in their 1st trimester and 2 in their 3$^{rd}$ trimester due passive immunization of anti-D Ig), so only 3 out of 9 had repeat antibody titration requested and there was no significant increase in titer recorded. This shows out of 34 only 3(8.82%) were requested for repeat of antibody titration despite the fact that they showed positive antibodies testing; in addition 6 (66.66%) pregnant women out of 9 with active anti-D had lost the follow up. These obstacles can be improved by designated part of the prevention programs for patients counseling regarding their clinical problem and the introduction of clinical protocols for screening and prevention of Rhesus alloimmunization to all care giver in the field of Obstetrics & Gynecology and Family Medicine particularly the junior staff including interns and residents [39]. In Netherlands a case control study of 42 Rhesus alloimmunized women concluded that Rhesus alloimmunization may be further reduced by strict compliance to guidelines which support the above mentioned recommendations [40].

## Implications for the policy

For a good clinical practice, the policy of testing ABO/RhD, red cell alloantibodies detection/identification and titration of all pregnant women should continue as a routine process. The justification for this is mainly to prevent hemolytic disease of the fetus and newborn by identifying pregnant women early to prevent rather than treat the condition. Although evidence base is limited, there is an antenatal population who are able to benefit from a test through early detection of blood type followed by appropriate management. The benefits of a systematic screening programme have not been assessed in Saudi Arabia. However, experiences in other countries, in particular the Netherlands, have shown that screening programmes have identified the extent of the problem and helped monitor outcomes. Once it has been established that the pregnant women is negative to RhD antigen, and in case her husband is positive to RhD antigen. The father of the fetus could be homozygous DD or heterozygous Dd for RhD antigen, in case the father is heterozygous then the chance is 50% that the baby would be RhD-positive, otherwise baby has to be-RhD positive and that becomes the risk factor for RhD-negative pregnant woman to D immunization. In that case best prevention is getting immunization with anti-D immunoglobulin. A high tittered anti-D immunoglobulin is commercially available for the use in preventing alloimmunization to D antigen. Dosage differs from countries to countries e.g. in UK the dose is 100µg (500 IU) on 28$^{th}$ and 34$^{th}$ week and at postpartum, in Netherlands 200µg (1000 IU) at 30$^{th}$ weeks and at postpartum, in Spain it is 300µg (1500 IU) at 28$^{th}$ week and at postpartum, in Germany 300µg (1500 IU) at 28$^{th}$ week and at postpartum, in Denmark it is 250-300µg (1250-1500 IU) at 29$^{th}$ week and at postpartum, In Canada it is 300µg (1500 IU) at 28$^{th}$ week or 100-120µg (500IU-600 IU) at 28$^{th}$ and 34$^{th}$ week and at postpartum. In US and Saudi Arabia it is 300µg (1500 IU) at 28$^{th}$ week which is based on the fact that 92% of the women who develop anti-D during pregnancy do so at or after 28$^{th}$ weeks [35] and at postpartum.

## Screening and Dosage for anti-D Immunoglobulin

**Antepartum Administration:** In a pregnancy where the mother is RhD-negative and the father is RhD-positive, the fetus may be RhD-positive and the mother may be at risk for D alloimmunization. Such females are candidates for the prophylaxis anti-D Ig to prevent alloimmunization.

The following females are not candidates for anti-D Ig: D-negative female whose baby is known to be RhD-negative, RhD-negative female previously immunized to RhD, and any RhD-positive female. The decision

to perform weak D testing depends upon policy of the facility. Women with red cells that are clearly positive on the weak D test should be considered RhD-positive and should not receive anti-D Ig, although rarely a positive weak D test can be caused by a partial RhD antigen. Very rare cases of HDFN have been reported in babies born to women with partial D antigen with allo-anti-D. In women at risk for D alloimmunization, the American College of Obstetricians and Gynecologists (ACOG) recommends initial anti-D Ig administration at 28 weeks' gestation based on the observation that 92% of women who develop anti-D during pregnancy do so at or after 28 weeks [35]. Indications for administration of additional doses of anti-D Ig during pregnancy include invasive procedures such as amniocentesis and cordocentesis, manipulations such as version, and abortion. In many countries the cost-benefit ratio the prophylactic administration of antenatal anti-D Ig has been questioned [11]. The reduction of number of the cases with severe HDFN is evident with administration of antepartum anti-D Ig, but not as important when compared to postpartum administration. Since the cost of anti-D Ig is relatively high and when administered to all RhD-negative pregnant women, most of them may receive it unnecessary if carrying RhD-negative child. Nowadays in some developed countries non-invasive fetal RhD genotyping is routinely performed to address this issue. In Denmark and Netherlands fetal RhD genotyping is performed to target antenatal anti-D Ig prophylaxis, even in Netherlands RhD genotyping has made it possible to discontinue RhD cord blood typing, and since January 2013, the administration of both antenatal and postnatal anti-D Ig is based on the result of the fetal genotyping assay. Cord blood typing in Netherlands is only restricted to twin pregnancies and in case of inconclusive fetal typing results.

## Postpartum Administration

The RhD-negative mother who is not immunized to RhD should also receive an appropriate dose of anti-D Ig after delivery of RhD-positive baby, and it should given as soon as possible after delivery, preferably within 72 hours. For that cord blood from babies born to RhD-negative mothers should be tested for the D antigen. If the baby is RhD-negative, no further anti-D Ig is necessary. However, if the infant tests RhD-positive, the mother should have a postpartum blood sample screened for FMT in order to determine the appropriate anti-D Ig dose. The rosette test is a sensitive method to detect FMT of approximately 10 mL or more. The maternal sample is incubated with anti-D, and then indicator D-positive red cells are added. The indicator red cells will form agglutinates (rosettes) around the fetal D-positive red cells. The fetal cells must be RhD-positive and the mother RhD-negative for the test to be valid. The rosette test may be falsely positive if the mother is positive for the weak RhD phenotype, and falsely negative if the baby is weak RhD. If the rosette testing is negative, a dose of 300μg (1500 IU) of anti-D Ig is given in Saudi Arabia, which is considered to be sufficient to prevent immunization after delivery. It is expected that this dose suppresses immunization by 15 mL red cells or 30 mL whole blood. It is important to note that the presence of residual anti-D from antepartum anti-D Ig does not indicate ongoing protection. A positive rosette test indicates the occurrence of a large FMT. While it has been estimated that only 0.3% of pregnancies are complicated by FMT of greater than 30 mL, a large FMT is an important and a preventable cause of failed immunoprophylaxis [41]. It is mandatory for all the transfusion services to adopt a policy for anti-D Ig prophylaxis of Rh-negative pregnant women identified to be at risk for immunization. Thus, in patients with a positive rosette test, a quantitative test such as the Kleihauer-Betke (acid/elution) or alternative approaches such as flow cytometry (which is the second most commonly used method for this purpose) [42] must be performed to accurately calculate the dose of anti-D Ig. The principle of the Kleihauer-Betke test is the resistance of fetal hemoglobin to acid treatment. A thin smear of maternal blood is made on a slide, treated with acid, rinsed, counterstained, and read microscopically. The maternal cells will appear as ghosts and the fetal cells will be pink. The fetal cells and maternal cells are counted separately for a total of 2000 cells.

The following formula is used to calculate the fetal bleeding: Fetal cells x maternal blood volume (mL) /Total cells counted = Fetal hemorrhage (mL)

Example: 6 cells/2000 cells × 5000 mL = 15 mL fetal whole blood

Because a single 300μg vial of anti-D Ig will suppress alloimmunization by 30 mL of fetal blood, and in the present example the calculated fetal bleeding was 15 mL, the number of anti-D Ig vials can be calculated as 15 mL/30 mL/ vial = 0.5 vial. Because of the inherent wide estimate of the test, if the calculated dose to the right of the decimal point is ≥0.5 vial, round up to the next whole number and add one vial; if <0.5, round down and add one vial. In the above example, the dose to be given is two vials. As postpartum anti-D Ig prophylaxis is recommended to be given within 72 hours of delivery. If prophylaxis is delayed, the likelihood of preventing alloimmunization is decreased. Nonetheless, the ACOG recommends that treatment still be administered, even if delayed past 72 hours, because some studies have found partial protection has occurred as late as 13 days after exposure and, possibly, as late as 28 days [43]. Furthermore, if the D type of the baby is unknown or undetermined (e.g., stillborn), ant-D Ig should also be administered. Depending on the preparation, anti-D Ig can be given by intramuscular (IM) or intravenous (IV) injection. Care must be taken that the IM-only preparation is not given by IV injection, as complement activation could occur. Multiple IM doses should be given at different sites or at different times within 72 hours. Alternatively, multiple doses of the IV preparation may be administered according to instructions in the package insert.

## Serology and Mechanism

Administration of anti-D Ig during pregnancy may produce a positive antibody screen in the mother, as seen in 2 of

the cases in this study but the titer is rarely greater than 2 and thus poses no risk to the fetus. Occasionally, the DAT may be positive in the baby without any evidence of hemolysis. About 10% (20-30µg) of the 28-week gestation dose will be present at delivery (half-life of IgG is 25 days), and can be detected and identified as anti-D. This anti-D should not be interpreted as active immunization, and the postpartum anti-D Ig dose should be given if the baby is RhD-positive. Antibody titers in the mother do not correlate with the effectiveness of the anti-D Ig or the amount of FMT. Anti-D can be detected in the maternal circulation for as long as 6 months. If it becomes necessary to distinguish passively administered anti-D from the anti-D formed by alloimmunization, transfusion service staff can take advantage of the fact that anti-D Ig is entirely IgG, on the other hand active immunization produces an antibody response with an IgM component. Thus, anti-D produced by the mother can often be detected in saline phase and completely or partially inactivated by 2 mercaptoethanol or DTT treatments, whereas anti-D Ig cannot. Additionally, passively acquired anti-D rarely achieves a titer above 4. The mechanism of action of anti-D Ig has not been completely elucidated. Current hypotheses suggest that Rh Ig-coated red cells may be removed by the reticuloendothelial system before stimulating an anti-D immune response. Additionally, suppression and feed- back mechanisms may be involved because the amount of antibody known to prevent immunization is much less than that needed to bind to all D antigen sites on fetal red cells.

# Chapter 7

## Personal Conclusion

All the pregnant women should be offered testing for ABO blood group and RhD status in early pregnancy. It is recommended that routine antenatal anti-D Ig prophylaxis is offered to all non-sensitized pregnant women who are RhD-negative (Technology appraisal guidance, June 2008). Women should be screened for atypical red-cell alloantibodies in early pregnancy and again at 28 weeks, regardless of their RhD status. Pregnant women with clinically significant atypical red-cell alloantibodies should be offered referral to a specialist centre for further investigation and advice on subsequent antenatal management. If a pregnant woman is RhD-negative, consideration should be given to offering partner testing to determine whether the administration of anti-D Ig prophylaxis is necessary. Although, blood group O is the most common blood group in the studied population followed by A, B and AB subsequently. The RhD negative blood group is 9.49% among pregnant women in the eastern province of Saudi Arabia. The prevalence of RhD alloimmunization in the present study is higher than figures reported in many developed countries that not only adopted and Rhesus prevention programmes but strictly followed the proper guidelines to prevent RhD alloimmunization in pregnancy, which still represent an avoidable direct cause of high perinatal morbidity and mortality in the developing countries. The adherence to strict guidelines for the development of a nationwide program is the best strategy in the management of such perinatal disease in developing countries. While developed countries view cost-effective preventive options as inexpensive, these same options represent expensive alternatives in third world countries. In terms of cost and cost-effectiveness, the option of providing RhD prophylaxis to first births appears to be the most financially feasible and efficient option. The annual requirements for anti-D Ig under this option are 3-4 times less than the requirements under the other options. This option also offers the greatest effect on reduction in Rhesus sensitization per dose of Rh immunoglobulin administered. These recommendations apply especially to countries such as India and those of Africa where negative prevalence rates range between 5% and 8%.

Non-invasive fetal RhD genotyping can be used to target antenatal anti-D Ig prophylaxis to RhD-negative pregnant women carrying RhD-positive children. Reliability of fetal RhD genotyping is high. Inconclusive test results are generated because of the presence of RHD sequences in the pregnant woman. More research is needed to conclude on the optimal timing of fetal RhD genotyping (e.g. early in pregnancy). Nowadays fetal RhD genotyping is now routinely performed in Denmark and Netherlands to target antenatal anti-D Ig prophylaxis. Although the cost of anti-D Ig prophylaxis differs from country to country, Cost-benefit ratios are influenced by costs of tests, which are largely influenced by economy of scale. The earlier in pregnancy fetal RhD genotyping reliably can be performed, the more one will benefit. The necessary investment in equipment, knowledge and logistics, for example centralized testing, make it difficult to implement this technology in developing countries. It may be the final step in a screening programme. In conclusion, my study shows that more awareness on the relatively high prevalence of anti-D-sensitized pregnancies in the Damman region, and the consequences in the number of babies with severe HDFN, may stimulate the adherence to the guidelines of antibody screening and anti-D prophylaxis and finally reduce the occurrence of severe HDFN.

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